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METABOLIC ACTIVATION OF PROCARBAZINE

EVIDENCE FOR CARBON-CENTERED FREE-RADICAL INTERMEDIATES

BIRANDRA KUMAR SINHA*
Laboratory of Environmental Biophysics, National Institute of Environmental Health Sciences, Research Triangle Park, NC 27709, U.S.A.

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Abstract—The metabolism of procarbazine was studied using spin-trapping techniques. The oxidation of procarbazine, catalyzed by horseradish peroxidase, prostaglandin synthetase [ram seminal vesicle (RSV) microsomes] or rat hepatic microsomal cytochrome P-450, produced carbon-centered free radicals. Cytochrome P-450 also catalyzed this oxidation in the presence of hydrogen peroxide. Horseradish peroxidase activation of procarbazine formed both the methyl radical and the *N*-isopropylbenzylamide radical [$(CH_3)_2CHNHCO(C_6H_4)CH_2\cdot$]. In the presence of RSV or rat hepatic microsomes, mostly the benzyl-type radical was trapped, presumably due to the reactivity of the methyl radical.

Procarbazine, *N*-isopropyl-α-(2-methylhydrazino)-*p*-toluamide, is an effective antitumor agent used in the treatment of Hodgkin's disease [1], brain tumors [2], and malignant lymphoma [3]. It is carcinogenic and induces pulmonary tumors and leukemia in mice [4] and mammary adenocarcinoma in rats [5]. In addition, procarbazine causes depletion of bone marrow and central nervous system depression [6].

The mechanism of action of procarbazine is not well understood and the relationship between procarbazine metabolism and its biochemical actions is unclear. Weinkam and Shiba [7], Dunn *et al.* [8] and Wiebkin and Prough [9] have shown that rat liver microsomal proteins catalyze the oxidative metabolism of procarbazine. The liver microsomal cytochrome P-450-dependent monooxygenase system has been implicated in this oxidation [8, 9]. The metabolism of procarbazine is complex, and a number of metabolites have been identified. These include azo derivatives, the hydrazone and the aldehyde [10]. Terephthalic acid isopropylamide is the major metabolite obtained *in vivo* [11]. In addition, methane [12], formaldehyde [13] and carbon dioxide [14] are formed. Although free-radical reactions have been postulated in the formation of methane and *N*-isopropyl-*p*-toluamide [7, 12], no direct evidence exists for the formation of free-radical intermediates from procarbazine. In this communication, evidence is presented for the formation of these reactive intermediates from procarbazine. Horseradish peroxidase, prostaglandin synthetase and rat liver microsomal proteins all catalyze the formation of free-radical intermediates from procarbazine. The chemical identity of these radicals has also been established with spin-trapping techniques. Since these enzymes are present in mammalian cells, the peroxidative metabolism of procarbazine to free radicals may play a role in the biochemical action of this drug.

* All correspondence should be addressed to: B. K. Sinha, Laboratory of Medicinal Chemistry and Pharmacology, Bldg. 37, Room 5A15, National Cancer Institute, National Institutes of Health, Bethesda, MD 20205.

MATERIALS AND METHODS

Pure procarbazine (NSC 77213) was obtained from the Drug Synthesis and Chemistry Branch, Drug Treatment Program, National Cancer Institute, Bethesda, MD. Horseradish peroxidase (HRP; type VI; RZ = 3.0), superoxide dismutase (SOD), NADPH, glucose-6-phosphate, glucose-6-phosphate dehydrogenase and indomethacin were purchased from the Sigma Chemical Co. (St. Louis, MO). Diethylenetriaminepentaacetic acid (DETAPAC), 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO), and 2-methyl-2-nitrosopropane (MNP) were purchased from the Aldrich Chemical Co. (Milwaukee, WI). DMPO was purified by two distillations before use. Catalase was obtained from the Boehringer-Mannheim Co. (Indianapolis, IN). Arachidonic acid (AA) was obtained from Nuchek Prep Inc. (Elysian, MN) and stored in the dark at −70°.

Ram seminal vesicle (RSV) microsomes were prepared as previously described [15] and the protein concentrations were determined according to the method of Sutherland *et al.* [16] with bovine serum albumin as the standard. The microsomal prostaglandin synthetase activity was determined by measuring AA-dependent oxygen uptake using a Clark-type electrode; RSV microsomes with low enzymatic activity were discarded. Twice washed rat hepatic microsomal fractions were prepared as previously described [17].

Enzymatic activation of procarbazine was carried out in 50 mM phosphate buffer–150 mM NaCl containing 1.0 mM DETAPAC at pH 7.5. A typical reaction mixture contained 1–2 mM procarbazine,

0.25–2 mg/ml protein, and either 100 mM DMPO or 50 mM MNP. The reaction mixtures were either incubated at room temperature (~22°) or at 37° before adding 400 μM H_2O_2, AA or NADPH-generating system (NADPH, glucose-6-phosphate and glucose-6-phosphate dehydrogenase). Where indicated, indomethacin (100–400 μM) or metyrapone (1 mM) was incubated with the reaction mixtures containing microsomes for 10 min before adding AA or an NADPH-generating system.

The electron spin resonance (ESR) spectra were recorded on a Varian E-109 equipped with a TM_{110} cavity at room temperature (~22°). Incubations under anaerobic conditions were carried out by bubbling nitrogen for 15 min, initiating the reactions with H_2O_2, AA or an NADPH-generating system, transferring the mixtures under nitrogen into a flat cell, and recording the spectrum.

RESULTS

Incubation of procarbazine with HRP in the presence of H_2O_2 and DMPO at pH 7.5 resulted in the formation of a carbon-centered DMPO adduct. The ESR spectrum of this DMPO adduct consisted of six lines (Fig. 1A) with the following splitting constants: $a^N = 16.3$ G; $a^H_\beta = 23.5$ G. In addition, an oxygen-centered DMPO adduct with $a^N = 14.0$ G, $a^H_\beta = 12.5$ G, and $a^H_\gamma = 1.5$ G was detected (Fig. 1A). The oxygen-centered DMPO adduct rapidly decayed (half-life ≃ 10 min), and only the carbon-centered adduct remained (Fig. 1B). Procarbazine alone (without any cofactors) did not produce any detectable radical adducts and the formation of these radical adducts from procarbazine required HRP, H_2O_2 and oxygen. The presence of SOD (20 μg/ml) in the incubation mixture had no effect on the radical formation, suggesting that formation and propagation of the radicals was not O_2^- dependent. Furthermore, it also indicates that the oxygen-centered DMPO adduct was not that of DMPO-OOH ($a^N = 14.3$ G, $a^H_\beta = 11.7$ G, $a^H_\gamma = 1.2$ G) [18].

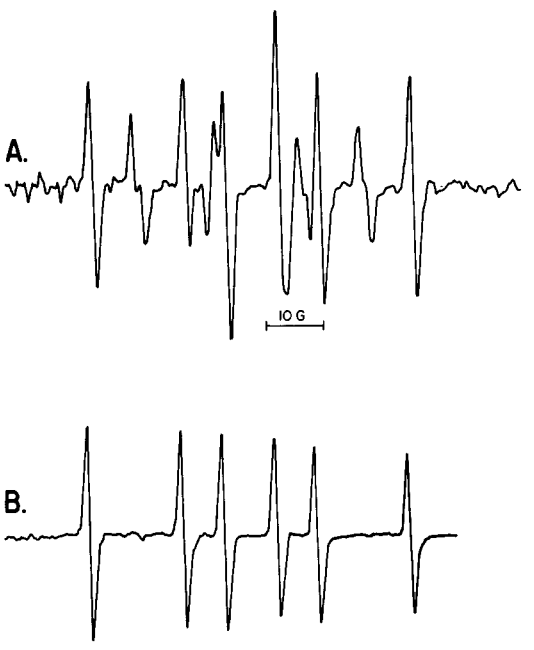

Fig. 1. Electron spin resonance spectrum obtained at 22° from procarbazine (1 mM) during incubation with (A) 1 mg/ml HRP, 400 μM H_2O_2, and 100 mM DMPO in the presence of 1 mM DETAPAC at pH 7.5 and (B) identical to (A) except that the spectrum was recorded after 20 min. The ESR settings: field = 3350 G; field scan = 100 G; modulation frequency = 100 kHz; modulation amplitude = 0.33 G; nominal power = 20 mW; scan time = 8 min and receiver gain was 1.25×10^5 for (A) and 5×10^4 for (B).

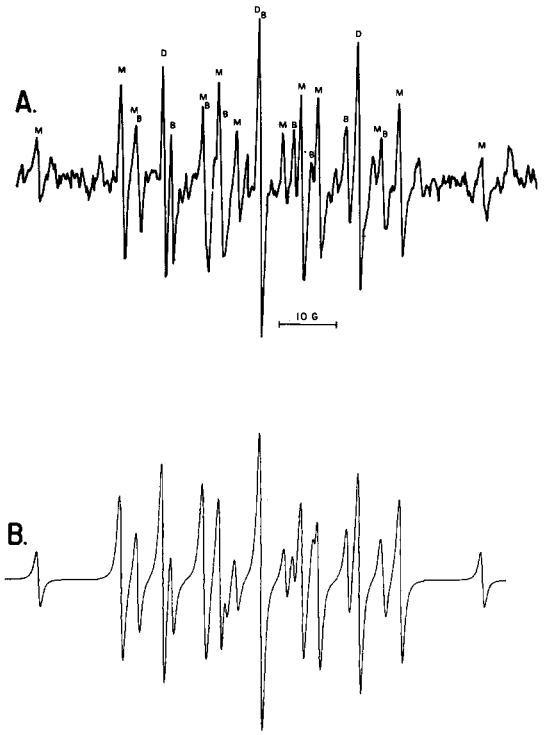

Fig. 2. ESR spectrum (M = methyl; B = benzylic type; and D = DTNB) obtained at 22° from 1 mM procarbazine during incubation with (A) 0.25 mg/ml HRP, 400 μM H_2O_2 and 50 mM MNP, in the presence of 1.0 mM DETAPAC at pH 7.5. The ESR settings were identical to Fig. 1 except that the receiver gain = 1.6×10^5 and the modulation amplitude = 0.66 G. (B) Computer simulation of (A) with $a^N = 17.4$ G; $a^H = 14.5$ G (CH_3-MNP); $a^N = 15.5$ G; $a^H = 6.0$ G ($R(C_6H_4)CH_2$-MNP at the relative concentration of 0.32. Also included in the simulation is the ditertiary butylnitroxide at a relative concentration of 0.39.

The carbon-centered DMPO adduct results from trapping of either $^{\cdot}CH_3$, $R(C_6H_4)CH_2^{\cdot}$ or both, formed during the activation. Since the splitting constants [19] of these DMPO adducts are extremely close (CH_3-DMPO: $a^N = 14.31$ G; $a^H_\beta = 20.52$ G; $PhCH_2$-DMPO: $a^N = 14.6$ G; a^H_β 20.66 G in benzene), it is very difficult to ascertain which of these radicals is trapped with DMPO. Use of MNP as the spin-trap resulted in a complex ESR spectrum (Fig. 2A) from trapping of both the methyl radical (CH_3-MNP: $a^N = 17.8$ G; $a^H = 14.5$ G) and a carbon-centered radical with two equivalent protons, most likely a benzylic-type radical. The splitting constants of this MNP adduct were $a^N = 15.5$ G and $a^H = 6.0$ G. These coupling constants are different from

those published [20] for an unsubstituted benzylic-MNP adduct ($a^N = 16.8$ G; $a^H = 10.9$ G). Substitution, i.e. inductive effects and solvents, are known to affect coupling constants, especially the a^H of radical adducts. The differences in observed coupling constants and those reported may be due to the bulky para-substitution on the benzene ring. Since the trapped-MNP adduct contains two equivalent protons and has a 1:2:1 ESR spectrum, we tentatively assign it to be a benzylic-type ($R(C_6H_4)CH_2\cdot$) radical. The formation of these radicals from procarbazine was time dependent. While the methyl radical was formed rapidly, the $R(C_6H_4)CH_2$ radical appeared more slowly (~20 min). A computer simulation is shown in Fig. 2B. In addition to the CH_3 radical and the $R(C_6H_4)CH_2$ radical, traces of some other radicals including ditertiarybutylnitroxide (DTBN: $a^N = 17.3$ G) were also present. The identity of these other radicals is not known at this time.

When procarbazine was incubated with DMPO and RSV microsomes, a source of prostaglandin synthetase, no spin-adduct was detected. However, when AA was added, a DMPO adduct was immediately formed whose ESR spectrum consisted of six lines with the following splitting constants: $a^N = 16.1$ G, $a^H_\beta = 23.2$ G (Fig. 3A). These splitting constants are similar to those of the carbon-centered DMPO adducts obtained from HRP-catalyzed activation of procarbazine. Use of MNP as the spin trap resulted in an ESR spectrum due to trapping of the $R(C_6H_4)CH_2$ radical with the splitting constants of $a^N = 15.5$ G and $a^H_\beta = 6.0$ G (Fig. 3B), and only traces of methyl radical were detected. In addition, DTBN was also present. Preincubation of RSV

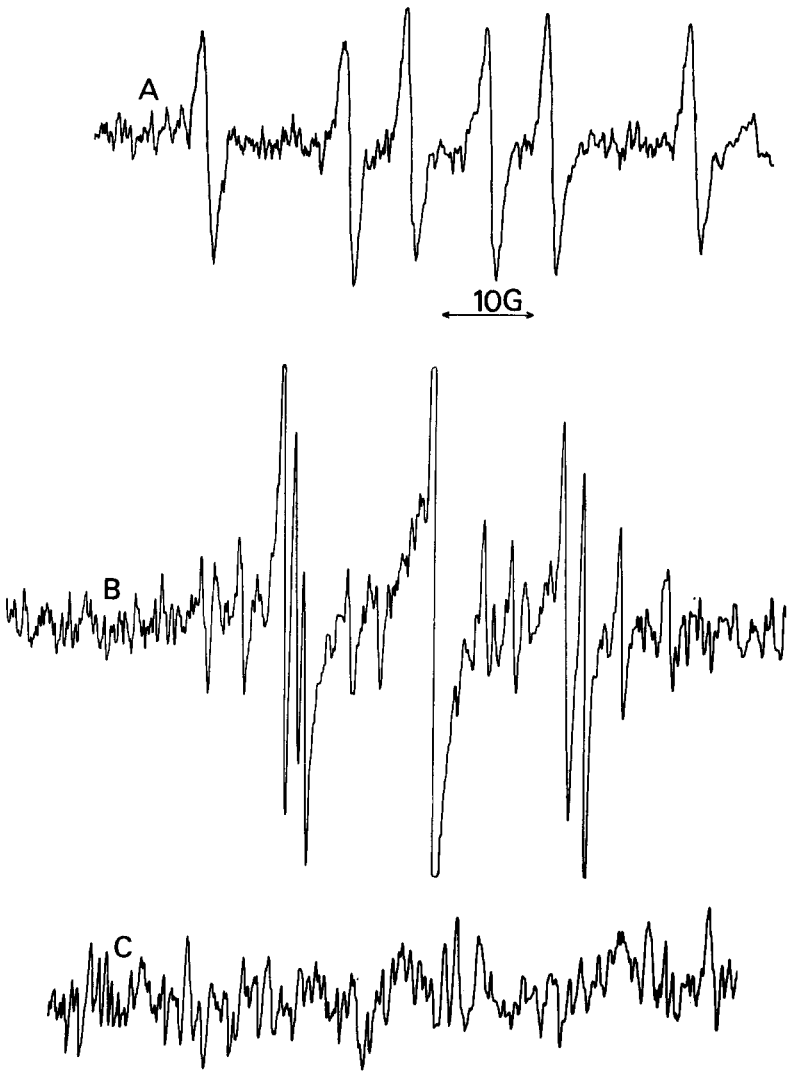

Fig. 3. ESR spectrum obtained at 22° from 1 mM procarbazine during incubation (37°) for 2 min with (A) 1 mg/ml RSV microsomes, 400 μM AA and 100 mM DMPO, in the presence of 1 mM DETAPAC, at pH 7.5, (B) identical to (A) except that 50 mM MNP was used in place of DMPO, and (C) identical to (A) except that RSV microsomes were preincubated with 400 μM indomethacin. The ESR settings were identical to Fig. 1 except that the modulation amplitude was 0.66 G and the receiver gain was 8×10^4 for (A) and 3.2×10^5 for (B) and (C).

microsomes with 400 μM indomethacin, a known inhibitor of prostaglandin synthetase [21], inhibited the radical formation completely (Fig. 3C). Anaerobic conditions also inhibited radical formation.

RSV microsomal prostaglandin synthetase catalyzed radical formation in the presence of H_2O_2 and only a carbon-centered adduct was detected ($a^N = 16.1$ G; $a^H_\beta = 23.2$ G). The splitting constants for this adduct were identical to those obtained in the presence of AA. The presence or absence of oxygen or preincubation with indomethacin had no effect on the radical formation.

When procarbazine was incubated with rat liver microsomal proteins in the presence of DMPO and the NADPH-generating system at 37°, carbon-centered DMPO adducts were formed ($a^N = 16.1$ G; $a^H_\beta = 23.2$ G; Fig. 4A). In the absence of NADPH, no adducts were detected. Although the formation of adducts could be detected with a few minutes of incubation (2–5 min), the maximum adduct concentration was obtained only after 45 min. In addition to the carbon-centered adducts, some OH radical adducts and a nitroxide radical ($a^N = 17.5$ G) were formed. The formation of the nitroxide radical with no β-hydrogen must have been due to oxidation of DMPO. Incubations carried out under anaerobic conditions inhibited adduct formation. The presence of SOD (20 μg/ml) or catalase (20 μg/ml) had no effect on the formation of the carbon-centered adducts; however, they inhibited OH radical formation (Fig. 4B), suggesting that the OH radical was formed from $O_2^{\cdot}/H_2O_2$. The presence of metyrapone (1 mM), an inhibitor of cytochrome P-450, inhibited radical formation from procarbazine. Incubation of procarbazine with heat-denatured microsomal proteins (80°, 30 min) and the NADPH-generating system resulted in formation of OH radicals, and no other radicals were detected.

Rat liver microsomal proteins also catalyzed the formation of carbon-centered DMPO adducts ($a^N = 16.1$ G; $a^H_\beta = 23.2$ G; Fig. 4C) in the presence of H_2O_2 (400 μM). The formation of these adducts was not inhibited by anaerobic conditions. No carbon-centered adducts were detected in the presence of heat-denatured microsomal proteins and H_2O_2. These observations indicate that radical formation by rat liver microsomal proteins was also peroxidase dependent.

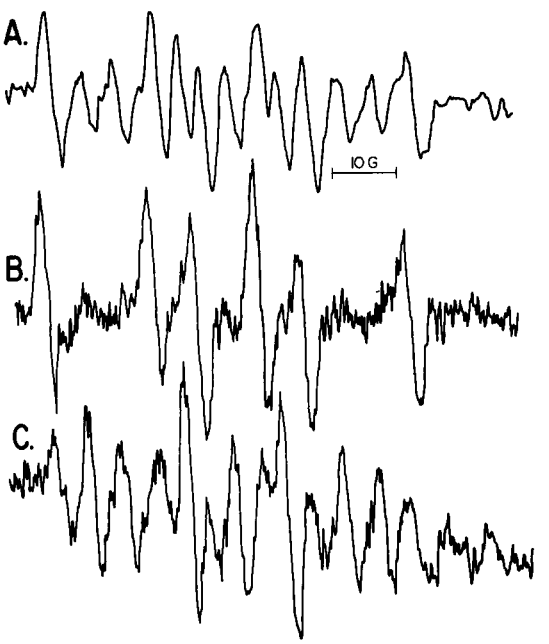

Fig. 4. ESR spectrum obtained at 37° from 2 mM procarbazine during incubation for 45 min with (A) 2 mg/ml rat liver microsomes, 100 mM DMPO and NADPH-generating system at pH 7.5, (B) identical to (A) except that 20 μg/ml SOD (or catalase) was present in the incubation mixtures, and (C) identical to (A) except that H_2O_2 (400 μM) was used in place of the NADPH-generating system. The ESR settings were identical to Fig. 1 except that the receiver gain was 3.2×10^5 and modulation amplitude was 2.6 G.

DISCUSSION

Horseradish peroxidase, prostaglandin synthetase, and rat liver microsomal proteins catalyzed oxidation of procarbazine to free radical intermediates. Procarbazine in the presence of HRP/H_2O_2 formed both the CH_3 radical and the $R(C_6H_4)CH_2$ radical. Based on the detection limit of the instrument and the trapping efficiency of the spin traps, only 1–2% of the procarbazine was converted to these radicals. An oxygen-centered radical was also detected from procarbazine. The splitting constants for this radical were different from those of the DMPO-OOH adduct. Furthermore, SOD had no effect on this radical formation. It appears that the oxygen-centered radical resulted from the reaction of O_2 with one of the carbon-centered radicals to form a hydroperoxy radical. It is of interest to note that no nitrogen-centered radical was detected with HRP/H_2O_2. Misra and Fridovich [22] have postulated the formation of nitrogen-centered radical(s) during the metal-catalyzed oxidation of hydrazine derivatives, and recently such radicals have been detected during enzymatic and metal catalyzed oxidation of hydralazine [23, 24]. Furthermore, the formation of phenyl and ethyl radicals from phenylhydrazine and ethylhydrazine, respectively [25, 26], has been reported. Prostaglandin synthetase also catalyzed the formation of carbon-centered radicals, however; in this case, only traces of methyl radical were detected and the carbon-centered radical, $R(C_6H_4)CH_2\cdot$, predominated. It is very likely that the methyl radical, once formed, reacts rapidly with the microsomal membranes to form a covalent complex or abstracts H· from the lipids. This assumption is reasonable based on reports of the formation of alkylating species from procarbazine with preferential covalent binding of methyl group to proteins [9]. The formation of the radical required oxygen and was inhibited by indomethacin, an inhibitor of the cyclooxygenase [21]. This would suggest that dioxygenation of AA to the cyclic hydroperoxy-endoperoxide, PGG_2, is required. Free radical formation is also peroxidase dependent since it was catalyzed by H_2O_2 and RSV microsomes. This would indicate the involvement of the hydroperoxy-endoperoxide (PGG_2), which is reduced to

hydroxyendoperoxide (PGH_2) [27] during oxidation of procarbazine.

Rat liver microsomal proteins also activated procarbazine to carbon-centered radicals. However, significantly less of these radicals was detected in this system than in the HRP/H_2O_2 system and only $R(C_6H_4)CH_2$ radical was trapped. This was probably due to rapid reaction of the methyl radical with the microsomal proteins or lipids. The formation of the radicals was NADPH and oxygen dependent. Furthermore, heat-denatured microsomal proteins and metyrapone, an inhibitor of cytochrome P-450, inhibited the radical formation. These observations are consistent with the involvement of cytochrome P-450 in oxidation of procarbazine [8, 9]. However, the activation of procarbazine was also peroxidase dependent since H_2O_2 (in place of NADPH) also catalyzed the formation of the radicals. Cytochrome P-450 is known to function as a peroxidase, oxidizing various xenobiotics in the presence of H_2O_2 [28, 29]. The proposed peroxidative activation of procarbazine to free radicals is summarized in Scheme 1.

$$R(C_6H_4)CH_2NHNHCH_3$$

Procarbazine

$$\rightarrow [R(C_6H_4)CH_2\text{-}\dot{N}NHCH_3]$$

$$\rightarrow CH_3\cdot + R(C_6H_4)CH_2\cdot + N_2$$

where $R = (CH_3)_2CHNH—CO—$

In this postulated scheme, the nitrogen-centered radical is an obligatory intermediate for the formation of the methyl and the benzylic radicals. However, formation of other intermediates, e.g. azoprocarbazine, which may also arise from the nitrogen-centered radical, is not excluded.

Although the free-radical metabolism of procarbazine represents a minor metabolic pathway, it may be important in the biological properties of procarbazine. Reactive free-radical intermediates bind to cellular macromolecules [30] and generate oxygen radicals ($O_2^{\cdot}$, $\cdot OH$) which induce lipid peroxidation and cause DNA-strand breaks [31]. Microsomal activation of procarbazine has been shown to generate alkylating species resulting in preferential covalent binding of the methyl group to proteins [9]. It is also possible that the methyl and the $R(C_6H_4)CH_2$ radicals abstract $H\cdot$ from lipids to initiate lipid peroxidation with formation of methan and toluamide derivatives. Furthermore, our preliminary studies with $^{14}CH_3$-procarbazine indicate that the methyl group derived from HRP/H_2O_2 activation of procarbazine binds covalently to DNA. Covalent binding to cellular macromolecules by active species generated during bioactivation of procarbazine may be involved in the toxicity of this drug.

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