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ORIGINAL ARTICLE

Eduardo Palomino · Brenda Foster · Maya Kempff
Thomas Corbett · Richard Wiegand
Jerome Horwitz · Laurence Baker

Identification and antitumor activity of a reduction product in the murine metabolism of pyrazoloacridine (NSC-366140)

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Abstract *Purpose.* Pyrazoloacridine (PZA) is a newly developed anticancer agent currently undergoing clinical trials. Its mode of action has not been elucidated but the presence in its chemical structure of a 5-nitro functional group and its activity against oxygen-deficient cancerous cells argue in favor of enzymatic nitro reduction as a possible pathway for its antitumor activity. In order to assess the involvement of the nitro functiona lity in PZA activity, as well as to determine other metabolic products, a pharmacological and chemical study of PZA was designed. *Methods.* Urine and stool samples were collected from mice before and after treatment with PZA. Samples were fractionated using chromatographic methods and then evaluated using mass spectrometry (MS). One of the characterized metabolites was synthesized and tested in vitro and in vivo for anticancer activity. *Results.* One major fraction from mouse stool was initially characterized by MS as the 5-aminopyrazoloacridine (5-APZ). This compound was chemically synthesized by catalytic hydrogenation of PZA and stabilized as the hydrochloride salt. 5-APZ was marginally cytotoxic in vitro and was inactive in vivo against a tumor cured by PZA (Panc 03). *Conclusions.* Bioreduction of the nitro group to an amine compound from PZA represents a pathway in the metabolic sequence of PZA. The inactivity of the chemically generated amine product does not provide conclusive evidence that this pathway is not involved in the cytotoxicity of PZA because other intermediates in the nitro reduction pathway may have a role in the activity of PZA. In particular, the hydroxylamine derivative of PZA could give answers to the involvement of this pathway in PZA cytotoxicity.

Key words: PZA · Antitumor activity · Murine metabolism

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E. Palomino
Walker Cancer Research Institute, 110 E. Warren, Detroit, MI 48201, USA

B. Foster (✉) · T. Corbett · R. Wiegand · J. Horwitz · L. Baker[1]
Wayne State University School of Medicine, Department of Internal Medicine, Division of Hematology and Oncology, P.O. Box 02188, Detroit , MI 48202–0188, USA
Tel. 313–745–8029; Fax 313–745–8139

M. Kempff
Wayne State University, Central Instrumentation Facility, Department of Chemistry, 75 Chemistry Bldg., Detroit, MI 48201, USA

Present address
[1]University of Michigan Medical Center 1500 E. Medical Center Drive 3119 Taubman Center. Ann Arbor, MI 48109, USA

Introduction

Considerable interest has attended the development of a family of 9-alkoxy-5-nitro-substituted pyrazoloacridine derivatives. The importance of these molecules as antitumor agents became clear after observations that some of them displayed selectivity in a panel of solid tumor models compared to leukemia [1–3]. One of these compounds, 9-methoxy-*N*,*N*-dimethyl-5-nitropyrazolo [3,4,5-kl] acridine-2(6H) propanamine monomethanosulfonate (NSC 366140 PZA, **1**), was selected for clinical development [4]. However, the mechanism of action and pharmacological fate of PZA are still poorly understood.

A common feature between PZA and other antitumor acridine derivatives, such as nitacrine (9-[3(dimethylamino) propylamino]-1-nitroacridine), is the presence of a readily reduced nitro group substituent. It has been established that the nitro group in nitacrine and analogs makes the molecules selective for hypoxic cells [5]. The role of the nitro group in the

case of PZA has not been established, although activity against hypoxic cells has been demonstrated [2]. Thus, the study of such selectivity in PZA is of potential relevance given the relatively high resistance of hypoxic cells to ionizing radiation and chemotherapy.

Pharmacokinetic studies of PZA in monkeys [6] and mice [7] have shown intra- and interspecies variations with drug exposure. Intraspecies pharmacokinetic variations have been observed in patients during phase I clinical trials [4, 8] but these variations do not appear to be related to major differences in hepatic function. Metabolic variations have been described for some cytotoxic drugs [9, 10] and may explain in part the observed differences in PZA pharmacokinetics in humans. However, a more detailed metabolic profile of PZA is required in order to answer questions concering hypoxic cell selectivity as well as potential metabolic variations.

We chose to study the metabolic pathway of PZA in mice. This report describes the in vivo isolation and chemical synthesis of a previously unreported amine metabolite.

Materials and methods

Chemicals

PZA for animal studies and chemical synthesis was supplied as the solid monomethanosulfonate by the Developmental Therapeutics Program of the National Cancer Institute, Bethesda, Md. For animal studies, the appropriate amount of drug was solubilized prior to treatment in isotonic NaCl or 5% dextrose. All other chemicals were either analytical or HPLC grade and were obtained from J.T. Baker, Phillisburg, N.J. (acetonitrile and acetic acid), Fisher Scientific, Fair Lawn, N.J (HCl), and Aldrich Chemical, Milwaukee, Wis. (ammonium acetate, palladium on carbon). PZA used in the synthesis of the amine derivative was provided by the Pharmaceutical Research Division of Parke-Davis, Ann Arbor, Mich.

Sample Collection

Details of pharmacokinetic studies have been published elsewhere [7]. Briefly, male BDF1 mice (Frederick Cancer Research, Frederick, Md.) were infused in the tail vein with 100 mg/kg of PZA over 1 h. Animals were kept in metabolism cages (Lab Products, Maywood, N.J.) for at least 24 h after infusion for collection of urine and stool. Control samples of urine and stool were collected from the same animals 24 h before treatment. Food and water were dispensed ad libitum to the animals except during the infusion. Collected samples were frozen and stored at $-20^{\circ}C$ until analysis.

Sample preparation and assay

Stool samples were slowly thawed at $0^{\circ}C$ and diluted with deionized water (1 : 9 v/v, wt/v, respectively). PZA was added to stool sample controls in order to assess its retention time and presence as unchanged metabolite [7], and to provide a means of estimating the quantity of the extracted metabolite. After further dilution with chilled methanol, samples were incubated for 10 min at $0^{\circ}C$ and then centrifuged for 10 min at $1000\,g$. Water was added to the supernatant prior to solid phase extraction (SPE) using a 1.0-ml cyano column (J.T.Baker, Phillipsburg, N.J.). PZA and metabolites were eluted from the SPE column using HCl/methanol (1:19 v/v) and then dried at $37^{\circ}C$ under a gentle stream of nitrogen. Samples were resuspended in mobile phase (90% 0.25 *M* ammonium acetate (pH 3.5)/10% acetonitrile) and passed through an analytical column (Cyano Ultrasphere, 0.46×15 cm, Beckman Instruments, Fullerton, Calif.) fitted with a resolve cyano precolumn (Millipore-Waters Chromatography Division, Milford, Mass.) at a flow rate of 1.0 ml/min. The HPLC system consisted of a Waters Maxima Workstation and chromatography program (Waters, Milford, Mass.), a WISP 710 autosampler, two model 510B solvent pumps, and a model 773 detector (Kratos, Westwood, N.J.) set at 460 nm.

Mass Spectrometry

Fast atom bombardment–mass spectrometry (FAB-MS) was carried out on a Kratos MS 50 TC equipped with an ion Tech Limited FAB Gun. The FAB 11NF saddle field was operated with xenon at 6.2 kV. Samples were run in 1 ml thioglycerol at an approximate resolution of 2000 and 10 s/decade scan rate. Electron impact (EI) spectra were taken in a Kratos MS 80 RFA spectrometer with a scan rate of 3 s/decade and a resolution of 1000. High resolution mass spectrometry (HRMS) peak matching was done at resolution of 10 000.

Preparation of 9-methoxy-*N*,*N*-dimethyl-5-aminopyrazolo [3,4,5-kl]-acridine-2(6H)propanamine hydrochloride

A 100 mg sample of PZA methanosulfonate was dissolved in 5 ml 1 *M* NaOH and the free base extracted with a mixture of ethyl acetate/ethanol (4 : 1). The decanted organic phase was filtered through a plug of neutral alumina, collected in a pressure bottle, containing 100 mg 10% Pd/charcoal, and hydrogenated at 20 psi for 1 h in a Parr apparatus. The reduced product was filtered through a plug of neutral alumina into a flask containing 10 ml 1 *M* HCl in methanol. The yellow precipitate formed was filtered to produce 61 mg (75% yield) 9-methoxy-*N*,*N*-dimethyl-5-aminopyrazolo[3,4, 5,kl]acridine-2(6) propanamine hydrochloride (5-APZ), MP $240^{\circ}C$ (dec). Hrms (EI) calculated for $C_{19}H_{23}N_5O$ m/z expected 337.1902, found 337.1910. H-NMR(DMSO-d6, ppm)): 2.20 (m, 2H, H2′), 2.661 (s, 6H, H4′,5′), 3.02 (m, 2H, H3′), 3.741 (s, 3H, Me-O), 6.67 (d, 1H, H7), 6.93 (d, 1H, H8), 7.14 (m, 3H, H3,4,10), 10.251 (s, 2H, NH2), 10.856 (s, 1H, NH).

Anticancer studies

In vitro

Leukemia L1210, colon adenocarcinoma 38 [2], and doxorubicin-resistant mammary adenocarcinoma (Mam 17/Adr) [11] transplantable mouse tumors maintained in the mouse strain of origin, and a rat epithelial cell line (IEC-18) obtained from the American Type Culture Collection (Rockville, Md.) were used in this assay. Leukemia and solid tumor cells, derived directly from the animal, and IEC-18, maintained in tissue culture, were plated in soft agar. After the agar solidified, a 6.5-mm filter paper disk containing the drug was placed on top. Plates were incubated for 6–10 days and the inhibition zone from the edge of the disk was measured using an inverted microscope.

In vivo

Pancreatic ductal adenocarcinoma #03 which is highly responsive to PZA was used [2]. The tumors were implanted bilaterally in

BDF1 mice as 30–60 mg fragments on day 0. The mice were randomized to treatment and control groups (five mice in each). Appropriate amounts of 5-APZ were dissolved in water inmediately prior to treatment. Intravenous treatment was carried out in a manner similar to that described above for sample collection, twice daily, on days 3 and 5. The highest dose tested was 26 mg/kg per injection. Mice were weighed daily until the average weight recovered to pretreatment levels. Tumors were measured twice weekly until the trial was terminated.

Results and discussion

Mass spectrometry

HPLC fractions of mouse stool (sample chromatogram shown in Fig. 1) were collected and their mass spectrogram evaluated. In order to make MS assignments of the collected metabolites, the FAB-MS of authentic PZA was carried out. Its fragmentation, previously unreported, is displayed in Fig. 2. PZA produced in addition to its protonated molecular ion (M +1) at 368 (60%), fragments at m/z 58 (100%), 73 (78%), 91 (52%), 181 (40%), 216 (14%), 323 (16%), and 352 (13%). Its fragmentation involved both the rupture of the amino side chain, producing free (58, 73, 323, and 352) or thioglycerol-complexed (181) fragments, and the breakage of the bond between the phenolic and the monoheteroatomic ring (91, 216, Fig. 2).

Preliminary observations of the stool fraction based on M+1 values and fragmentation patterns, strongly suggested the presence in the stool fraction of a reduced derivative of PZA. The amount of this metabolite was

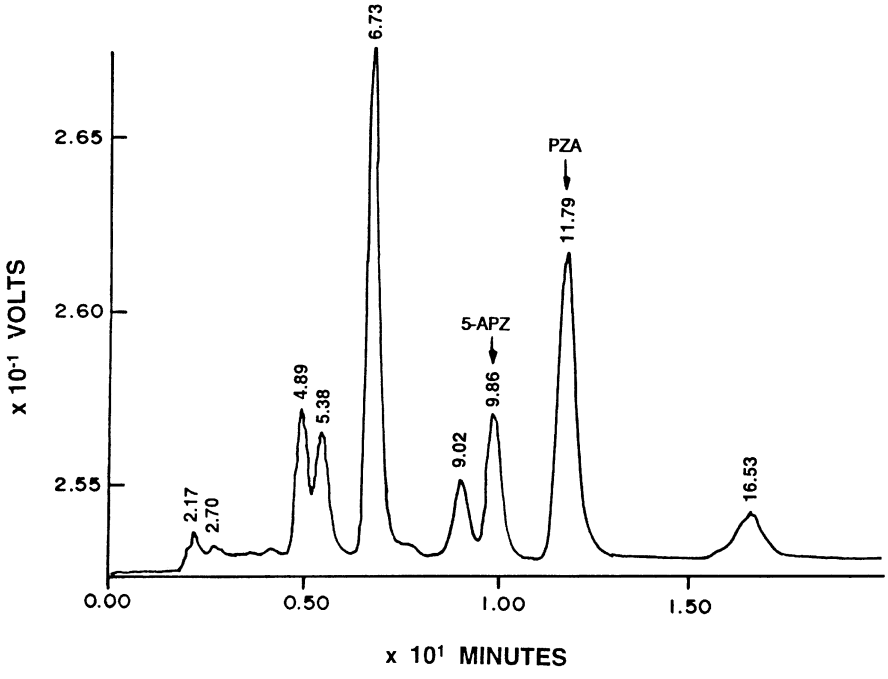

Fig. 1 HPLC chromatogram of stool collected from mice following treatment with PZA

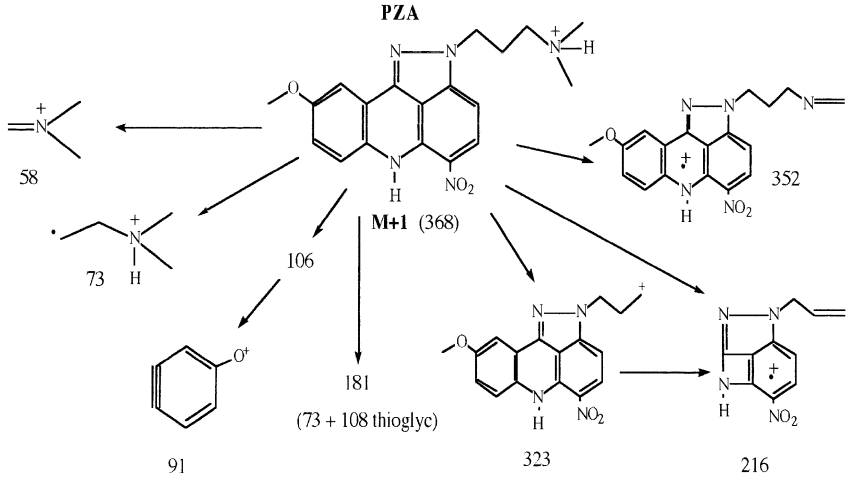

Fig. 2 FAB-MS fragmentation pattern of PZA by mass spectrometry

estimated by HPLC as 0.1% of the administered dose of PZA. MS of this metabolite, tentatively assigned as the 5-amino derivative (5-APZ), gave a molecular ion at 338 (M + 1, 20%) with adducts of thioglycerol at m/z 446 and 551. Three fragments were common with PZA: m/z 73 (50%), 91(100%), and 181(28%), and one was diagnostic at 217 (49%). This latter fragment arose from the protonated molecular ion (338) via methoxybenzyne (106) elimination. The methoxybenzyne fragments further to benzyne phenolate (91).

Confirmation of 5-APZ as a metabolite was established by reduction of the nitro substituent of an authentic sample of PZA via catalytic hydrogenation. Synthetic 5-APZ is stable at room temperature in the solid state but is partially oxidized in aqueous solution. A freshly prepared sample of 5-APZ under FAB-MS gave a strong (100%) M + 1 and a fragmentation pattern identical to that obtained from mice stool. The molecular weight of 5-APZ was confirmed by EI-MS peak match. The EI spectrogram and the FAB-MS fragmentation pattern of synthetic 5-APZ are displayed in Figs. 3 and 4, repectively.

No solid tumor-selective cytotoxicity, as measured by zone units, was observed for 5-APZ in the soft agar disk diffusion assay at 125 mg/disk (Table 1). By contrast, large zones of inhibition were produced for the solid tumors by PZA at 1/25 the level used for 5-APZ. However, degradation of the latter was observed in solution during the long exposure time required under the experimental conditions. For this reason, a direct in vivo evaluation was undertaken.

The 5-APZ derivative, dissolved prior to use, was injected intravenously at a level of 104 mg/kg. A reversible, stupor-like condition occurred in the mice following each injection. Treatment was terminated after day 5 because of a 11% body weight loss, indicating an adequate level of 5-APZ of 26 mg/kg. No meaningful antitumor activity against Pan 03 was observed (T/C in

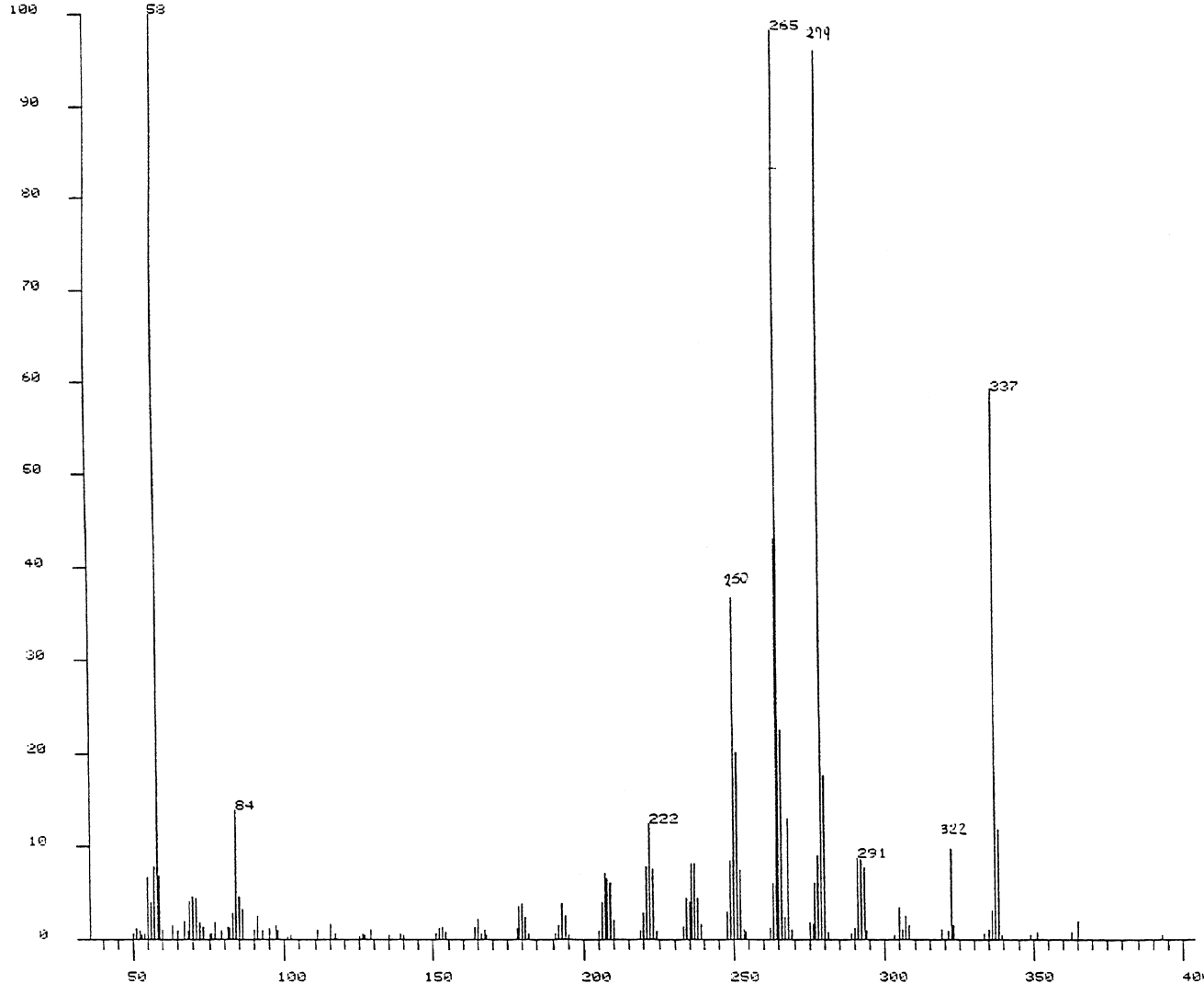

Fig. 3 Mass spectrogram of synthetic APZ under EI

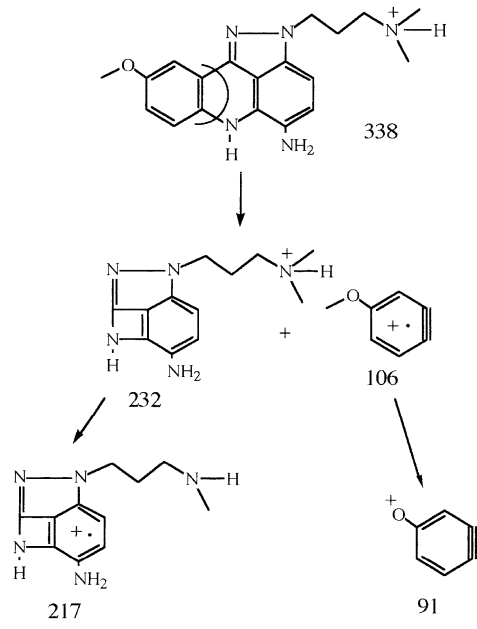

Fig. 4 FAB-MS fragmentation pattern of synthetic 5-amino pyrazoloacridine (5-APZ)

Table 1 Soft agar in vitro assay for 5-APZ and PZA in zu units (200 zu units = 6.5 mm. The larger the zone the greater the cytotoxicity to the tumor)

	L1210	Colon 38	Mam17/ADR	IEC-18
5-APZ (125 mg/disk)	60	90	0	0
PZA (5 mg/disk)	0–50	450–600	0	0

milligrams = 737/1024 = 72%). This same tumor is highly responsive to PZA administered to a total dose of 80 mg/kg [2].

Bioactivation of nitro compounds through the formation of reduction products is a well-established metabolic pathway [12]. The enzymatic reduction of nitroheterocyclic compounds is catalyzed by different flavoproteins and proceeds through a series of electron transfer processes, as has been demonstrated for metronidazole [12,13]. Important intermediates in these steps are the nitroso (R–NO) and the hydroxylamine (R–NHOH) heterocycles (Fig. 5). The amine heterocycle is the final product and is derived from the hydroxylamine via a two-electron transfer.

Metronidazole by itself is a potent antimicrobial agent useful in combating infections by anaerobic bacteria and protozoa [12]. Its activity under hypoxic conditions is derived from the ease of nitro reduction without reoxidation by molecular oxygen (12). Although the mechanism of action of PZA has not been established, it has shown activity under hypoxic conditions [1].

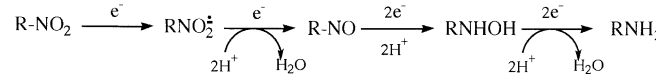

Fig. 5 Schematic representation of the electron transfer processes involved in the reduction of nitroheterocyclic compounds

The identification of the amine derivative of PZA by MS indicates the reduction process as a pathway in PZA metabolism. In this connection it is noteworthy that traces of PZA N-oxide and 9-desmethyl PZA have been recently reported in mouse plasma. The in vitro activity of the latter in A375 human melanoma cells is slightly higher than that of the parent compound [14]. Neither metabolite has yet been detected in the stool samples studied.

The synthetic amine that corresponds to the found metabolite (5-APZ) is oxidized by air in solution at room temperature, the conditions of the in vitro antitumor assay, but is stable indefinitely in the solid state. In vivo studies of 5-APZ showed no antitumor activity. This result is not surprising since in other active nitroheterocycles such as metronidazole, the reduced amine derivative has likewise been found to be devoid of activity [12]. Despite these results, the involvement of nitroreduction intermediates in the antitumor acti vity of PZA is not excluded. For example, the nitroso and the hydroxylamine derivatives of PZA are anticipated intermediates in the formation of the amine and may be involved in the cytotoxic action of PZA. Indeed, the hydroxylamine reduction product of another nitro compound, CB 1954, has been found to be an active cytotoxic metabolite [15].

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