Short Communication

Metabolism of dacarbazine by rat liver microsomes: contribution of CYP1A enzymes to dacarbazine N-demethylation

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ABSTRACT:

The N-demethylation of dacarbazine in liver microsomes was significantly increased by treatment of rats with β-naphthoflavone, dexamethasone, or phenobarbital. However, the extent of increase in the N-demethylation observed in β-naphthoflavone-treated rats was much greater than that observed in dexamethasone- or phenobarbital-treated rats. A good correlation between N-demethylation of dacarbazine and O-deethylation of phenacetin was observed when a low concentration of phenacetin was used. Furthermore, the activity of dacarbazine N-demethylase in rat liver microsomes was highly correlated with the amounts of CYP protein immunohistochemically determined with anti-rat CYP1A2 antibodies. In addition, antibodies to rat CYP1A2, and furafylline and α-naphthoflavone, which are known inhibitors of CYP1A enzymes, exhibited inhibitory effects on dacarbazine N-demethylation. These results indicated that CYP1A enzymes may be responsible for N-demethylation of dacarbazine in rat liver microsomes.

5-(3,3-Dimethyl-1-triazeno) imidazole-4-carboxamide (dacarbazine) is an antineoplastic drug which is classified as an alkylating agent (Newell et al., 1987). It is used extensively as a single drug in the treatment of metastatic malignant melanoma and in combination with other drugs for treating renal adenocarcinoma, soft tissue sarcoma, solid tumor, and malignant lymphomas (Lee et al., 1992; Mitchell and Dolan, 1993). In addition, dacarbazine has also shown that 1-aryl-3,3-dialkyltriazenes undergo oxidative metabolism to form 1-aryl-3-monomethyltriazines, and that triazenes including dacarbazine require metabolic activation by the host for antitumor activity (Newell et al., 1987; Audette et al., 1973; Gescher et al., 1981). Aminoimidazole carboxamide, which is a major urinary metabolite of dacarbazine in humans, in humans has been demonstrated to be a product from dacarbazine in liver microsomes (Breithaupt et al., 1982; Skibba and Bryan, 1970; Hill, 1975). Furthermore, it has been suggested that 5-(3-hydroxymethyl-3-methyltriazen-1-yl)-imidazole-4-carboxamide and 5-(3-methyltriazen-2-yl) imidazole-4-carboxamide were dacarbazine metabolites in mice, rats, and humans and that 5-(3-hydroxymethyl-3-methyltriazen-2-yl) imidazole-4-carboxamide decomposes spontaneously to form aminoimidazole carboxamide, with concomitant alkylation of cellular DNA (1, 10–12 Newell et al., 1982; Komori et al., 1983; Kitada et al., 1984). CYP3A4 and CYP2C9 were purified from human liver microsomes according to methods described elsewhere (Shimada et al., 1986; Komori et al., 1988). The antibodies for purified P450 raised to rabbit were carried out according to the method previously reported (Kamataki et al., 1976). NADP, glucose 6-phosphate, and glucose 6-phosphate dehydrogenase were obtained from Oriental Yeast (Tokyo, Japan). Other chemicals used were of the highest grade commercially available.

Materials and Methods

Materials. Dacarbazine was a generous gift from Kyowa Hakko KK. (Tokyo, Japan). CYP1A2 and CYP2B1/2 were purified from liver microsomes of rats treated with 3-methylcholanthrene and phenobarbital by the methods reported previously, respectively (Takasugi et al., 1983; Kitada et al., 1984). CYP3A4 and CYP2C9 were purified from human liver microsomes according to methods described elsewhere (Shimada et al., 1986; Komori et al., 1988). The antibodies for purified P450 were raised to rabbit were carried out according to the method previously reported (Kamataki et al., 1976). NADP, glucose 6-phosphate, and glucose 6-phosphate dehydrogenase were obtained from Oriental Yeast (Tokyo, Japan). Other chemicals used were of the highest grade commercially available.

Animals. Pretreatment of Animals and Preparation of Microsomes. Male Sprague-Dawley rats (8 weeks old), obtained from Takasugi Experimental Animals Co. Ltd. (Saitama, Japan), were used throughout this study. Rats were pretreated intraperitoneally with phenobarbital-Na at doses of 80 mg/kg for 5 days, dexamethasone 50 mg/kg for 3 days, and β-naphtoflavone 40 mg/kg for 3 days. The agents for pretreatment were dissolved in corn oil except for phenobarbital which was dissolved in water. Ethanol was given orally in a solution at a 10% concentration. Rats were given free access to food and water and were killed 24 hr after the last injection. Liver microsomes were prepared by differential centrifugation as described elsewhere (20).

Assays. A typical reaction mixture consisted of 100 mM potassium
phosphate (pH 7.4), 0.1 mM EDTA, NADPH-generating system (0.33 mM NADP, 8 mM glucose 6-phosphate, 0.1 unit of glucose 6-phosphate dehydrogenase, and 6 mM MgCl₂), microsomal protein, and 0.5 mM dacarbazine. Phenacetin was dissolved in 1% methanol at a concentration of 1 mM. The concentration of dacarbazine employed in this study was decided from blood concentration reported when clinically used (Breithaupt et al., 1982; Loo et al., 1976; Buesa and Urrechaga, 1991), and the solubility of the drug in vitro. The reaction was initiated by the addition of the NADPH-generating system which had been preincubated at 37°C for 5 min, and was carried out at 37°C for 20 min with aerobic shaking. N-Demethylase activity of dacarbazine was measured by determination of formaldehyde formed according to the method of Nash (Nash, 1953). Briefly, a yellow color developed with acetylacetone in the presence of ammonium sulfate was measured fluorometrically at 510 nm (excitation was at 410 nm) by the method of Rapoport et al. (1994) with minor modifications. O-Deethylation activities of phenacetin was measured by high performance liquid chromatography according to the method of Loft et al. (1991).

Other Methods. Protein was measured according to the method of Lowry et al. (1951) using bovine serum albumin as the standard. SDS-PAGE and immunoblot analysis were performed according to the method described previously (Laemmli, 1970; Guengerich et al., 1982). The intensity of immunostaining band was measured by means of a Hewlett Packard Scan Jet-c. In the case of immunoblot analysis with anti-CYP1A2 antibodies, CYP1A2 was identified by the mobility on SDS-PAGE. Preparation of IgG was carried out according to the protocol provided by supplier. A statistical significance was analyzed by Student’s t-test.

Results

N-Demethylase activities of dacarbazine in liver microsomes from rats pretreated with various inducers were studied. Although pretreatments of rats with phenobarbital, dexamethasone, and β-naphthoflavone resulted in the increase in N-demethylation of dacarbazine, β-naphthoflavone was much more effective in inducing the N-demethylase activity than were phenobarbital and dexamethasone. Thus, the activities of dacarbazine N-demethylase in liver microsomes from untreated, phenobarbital-, dexamethasone- and β-naphthoflavone-treated rats were 155.2 ± 19.3, 318.5 ± 24.6, 356.7 ± 36.7, and 1331.9 ± 60.5 pmol/min/mg, respectively. In contrast, pretreatment of rats with ethanol did not affect dacarbazine N-demethylation. In addition, the activity of dacarbazine N-demethylase was significantly correlated with that of phenacetin O-deethylation in liver microsomes from ethanol-, phenobarbital-, dexamethasone- and β-naphthoflavone-, and untreated rats when phenacetin was used at the concentration of 10 mM (r = 0.949, p < 0.001). In contrast, no significant correlation between dacarbazine N-demethylation and phenacetin O-deethylation was observed when the activity of phenacetin O-deethylation was measured at the concentration of 100 mM. From these results, it was suggested that CYP1A enzyme may predominantly involve in N-demethylation of dacarbazine in rat liver microsomes, although contribution of P450 enzymes other than CYP1A to the reaction cannot be excluded because N-demethylation of dacarbazine was slightly but significantly increased by pretreatment of rats with phenobarbital and dexamethasone. Fig. 1 shows the correlation of the activity of dacarbazine N-demethylase and the amounts of CYP1A2 and CYP3A immunochromically determined in rat liver microsomes. The amount of CYP1A2 immunochemically determined was positively correlated with the activity of dacarbazine N-demethylase (fig. 1A), indicating that CYP1A2 may, at least, one of the major forms of P450 responsible for dacarbazine N-demethylation in rat livers. On the other hand, the number of proteins that are cross-reactive with antibodies against human CYP3A4 did not correlate with the activity of dacarbazine N-demethylase in rat liver microsomes (fig. 1B). As shown in fig. 2, both furafylline and α-naphthoflavone showed inhibitory effects on N-demethylation of dacarbazine. In contrast, neither cyclosporin A, which is known to be an inhibitor against CYP3A enzymes, nor sulfaphenazole, which is known to be an inhibitor against human...
CYP2C enzymes, affected dacarbazine N-demethylation (fig. 2A). Furthermore, dacarbazine N-demethylation was inhibited by the pretreatment of microsomes with anti-CYP1A2 antibodies but not by pretreatment with anti-CYP3A4, CYP2B1/2, or CYP2C9 antibodies (fig. 2B).

**Discussion**

It has been shown that dacarbazine N-demethylation leading to the formation of monomethyl triazene imidazole carboxamide is an important metabolic pathway for the antineoplastic activity of dacarbazine, since the metabolite liberates a carbonium ion that is responsible for the alkylating property of dacarbazine (Hill, 1975; Spassova and Golovinsky, 1985; Mudipalli et al., 1995). However, the form(s) of P450 responsible for N-demethylation of dacarbazine in rat liver microsomes was not known. Therefore, the present study was designed to clarify the P450 enzyme contribution to proposed metabolic activation of dacarbazine in rat liver microsomes.

Although N-demethylation of dacarbazine was increased by pretreatment of rats with phenobarbital, dexamethasone, and α-naphthoflavone, β-naphthoflavone was the most effective inducer for dacarbazine N-demethylation. Furthermore, increase in activity of N-demethylation in dexamethasone-treated rats was much less than that in the amount of CYP3A enzymes immunoochemically determined. Thus, the increase in the activities in liver microsomes from phenobarbital-, dexamethasone-, and β-naphthoflavon-treated rats were about 2-fold, 2.3-fold, and 8.5-fold, respectively. On the other hand, immunoblot analysis with anti-CYP1A2 antibodies revealed that the amount of CYP1A2 immunoochemically determined in β-naphthoflavone- and dexamethasone-pretreated rats were about 6-fold and 1.7-fold higher than that of controls, respectively. In contrast, the amount of CYP3A enzymes immunoochemically determined with anti-CYP3A4 antibodies was increased by about five times in dexamethasone-pretreated rats but not in β-naphthoflavone-pretreated rats. In addition, dacarbazine N-demethylation activity was significantly correlated with phenacetin O-deethylation measured at a low, but not at a high, concentration of phenacetin. Since O-deethylation of phenacetin by rat liver microsomes has been demonstrated to be biphasic and CYP1A2 has been shown to be a high affinity phenacetin O-deethylase in rat livers (Boobis et al., 1981; Seardic et al., 1990), the correlation between dacarbazine N-demethylation and phenacetin O-deethylation at low concentration was in accord with the results that β-naphthoflavone was the most effective inducer for dacarbazine N-demethylation in rat liver microsomes. These results were also supported by the findings that the activity of dacarbazine N-demethylation was positively correlated with the amount of CYP1A2 immunoochemically determined and was inhibited by anti-CYP1A2 antibodies, whereas neither anti-CYP2B1/2 antibodies nor anti-CYP3A4 antibodies exerted any significant effects on dacarbazine N-demethylation in liver microsomes of β-naphthoflavone-treated rats. Together with these results, it was indicated that although the contribution of CYP2B and CYP3A enzymes to the reaction cannot be excluded at present, CYP1A2 may, at least, be one of the major forms of P450 responsible for dacarbazine N-demethylation in rat liver microsomes.

Since N-demethylation of dacarbazine appears to be the first metabolic pathway for bioactivation of the drug to antineoplastic species, the results presented here indicated that the activity of CYP1A1 and/or 2 may play an important role in cancer chemotherapy using dacarbazine. It has been known that the CYP1A2 isoform is one of the constitutively expressed form of human P450s (Shimada et al., 1994), and that metabolism of phenacetin in vivo is altered by cigarette smoking (Pantuch et al., 1974). Therefore, as in the case of rats, if human CYP1A1 and/or 2 isoform was capable of metabolizing dacarbazine via N-demethylation, it is possible to assume that the level of CYP1A enzymes affects the therapeutic and/or toxic effects of dacarbazine in human. However, the form of P450 that contributes to
dacarbazine N-demethylation in human liver microsomes is not known and is under investigation.

References


