Metabolic Activation of Dacarbazine by Human Cytochromes P450: The Role of CYP1A1, CYP1A2, and CYP2E1[1]

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ABSTRACT

Dacarbazine (DTIC), a widely used anticancer agent, is inactive until metabolized in the liver by cytochromes P450 to form the reactive *N*-demethylated species 5-[3-hydroxymethyl-3-methyl-triazen-l-yl]-imidazole-4-carboxamide (HMMTIC) and 5-[3-methyl-triazen-1-yl]-imidazole-4-carboxamide (MTIC). The modest activity of DTIC in the treatment of cancer patients has been attributed in part to lower activity of cytochromes P450 (P450) in humans when compared with rodents. Importantly, the particular P450 isoforms involved in the activation pathway have not been reported. We now report that the DTIC *N*-demethylation involved in MTIC formation by human liver microsomes is catalyzed by CYP1A1, CYP1A2, and CYP2E1. The most potent inhibitors of DTIC *N*-demethylation were α-naphthoflavone (CYP1A1 and CYP1A2), quercetin (CYP1A2), chlorzoxazone (CYP1A2 and CYP2E1), and di-sulfiram (CYP2E1). Antihuman CYP1A2 antiserum also inhibited DTIC *N*-demethylation. DTIC *N*-demethylation in a panel of 10 human liver microsome preparations was correlated with the catalytic activities for CYP1A2 (ethoxyresorufin *O*-deethylation and caffeine N^3-demethylation) in the absence of α-naphthoflavone and with the catalytic activities for CYP2E1 (chlorzoxazone 6-hydroxylations) in the presence of α-naphthoflavone. DTIC metabolism was catalyzed by recombinant human CYP1A1, CYP1A2, and CYP2E1. The K_m (V_{max}) values for metabolism of DTIC by recombinant human CYP1A1 and CYP1A2 were 595 μM (0.684 nmol/min/mg protein) and 659 μM (1.74 nmol/min/mg protein), respectively. The CYP2E1 K_m value exceeded 2.8 mM. Thus, we conclude that (*a*) CYP1A2 is the predominant P450 that catalyzes DTIC hepatic metabolism; (*b*) CYP2E1 contributes to hepatic DTIC metabolism at higher substrate concentrations; and (*c*) CYP1A1 catalyzes extrahepatic metabolism of DTIC.

INTRODUCTION

DTIC[4] is a DNA-methylating agent with a broad spectrum of antitumor activity in mouse tumor models. Clinical activity of this agent in human malignancies is restricted to melanoma, Hodgkin's disease, and sarcoma. DTIC is used in a curative regimen with Adriamycin, bleomycin, and vincristine (ABVD) for Hodgkin's disease and is the most active single agent used to treat advanced melanoma and soft tissue sarcoma (1).

The DNA methylation produced by DTIC is dependent upon oxidative *N*-demethylation by P450s (2, 3). Early investigators identified formaldehyde and AIC as products of DTIC *N*-demethylation by mouse and rat liver microsomal preparations (2, 3). The proposed cascade of DTIC metabolism and formation of the reactive methylating molecule is illustrated in Fig. 1. The initial product of P450-catalyzed oxidation of DTIC, the carbinolamine HMMTIC, produces MTIC after elimination of formaldehyde. Rapid decomposition of MTIC yields the major plasma and urine metabolite AIC and the reactive species methane diazohydroxide, which produces molecular nitrogen and a methyl cation believed to be the methylating species. The importance of this pathway was confirmed *in vivo* by the recovery of $^{14}CO_2$ in expired breath and HMMTIC, AIC, and [^{14}C]methylguanine in urine after an i.p. dose of [^{14}C-methyl]DTIC (4). In human pharmacokinetic studies, metabolism was the predominant pathway for DTIC clearance. Plasma exposure to AIC was equivalent to that of DTIC, whereas urinary recovery of DTIC and AIC was low and highly variable.

The P450s comprise a superfamily of enzymes that catalyze the metabolism of many drugs including several anticancer agents (5) A single isozyme is often predominant in the metabolism of a given drug (6). The expression of P450 is often highly variable among individuals because of genetic, physiological, and environmental factors (6). Metabolism by P450s may be inhibited or induced by concomitant drug treatment. The consequences of individual differences in P450 isozyme expression and drug interactions caused by coadministered drugs on DTIC antitumor activity and plasma clearance are presently unclear, and the limited published pharmacokinetic data (7, 8) is insufficient to evaluate population variability of DTIC metabolism.

Rodent P450 isoforms may have different activity and substrate specificity when compared with their human homologues (9), thereby contributing to species-specific differences

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[4] The abbreviations used are: DTIC, dacarbazine; MTIC, 5-[3-methyl-triazen-1-yl]-imidazole-4-carboxamide; HMMTIC, 5-[3-hydroxymethyl-3-methyl-triazen-l-yl]-imidazole-4-carboxamide; AIC, aminoimidazole carboxamide; HPLC, high-pressure liquid chromatography; P450, cytochrome P450.

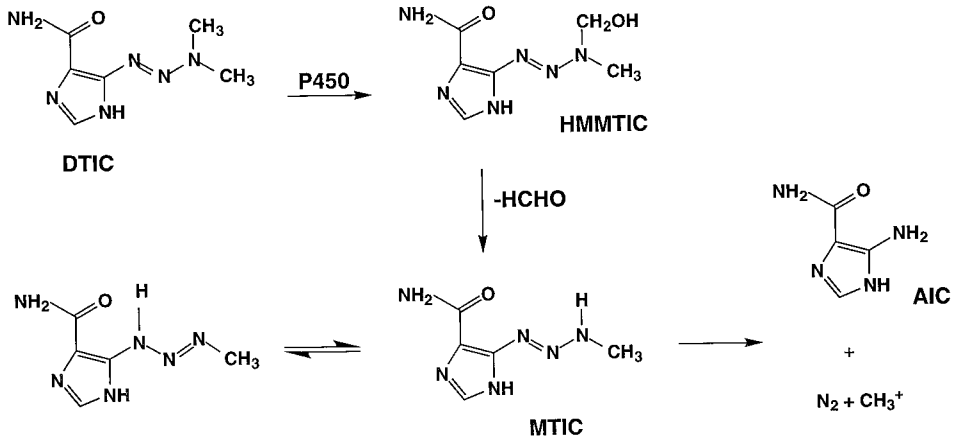

Fig. 1 Pathway of DTIC metabolism.

in drug metabolism. The limited antitumor activity of DTIC in humans compared with mice has been attributed to reduced metabolism by human P450s as compared with rodent liver P450s (10). This hypothesis, however, has been difficult to confirm because of poor stability of the active metabolite, MTIC.

Efforts to improve DTIC activity have focused on the development of analogues that improve the delivery of the methylating metabolites. For example, the recent clinical development of temozolomide—an analogue with demonstrated activity against metastatic melanoma (11) and high-grade astrocytoma (12)—was based on the presumption that greater and more reproducible exposure to MTIC could be achieved by the chemical hydrolysis of temozolomide at physiological pH rather than by the hepatic metabolism of DTIC (13). These efforts do not, however, provide any guidance in how to better use DTIC in the treatment of those neoplasms for which this agent has demonstrated activity.

In the present study, we characterized the human *in vitro* metabolism of DTIC by delineating the specific P450 isoforms responsible for MTIC formation in the liver. This data provides a more complete understanding of DTIC metabolic activation and of the basis for differences in DTIC activity in animal models and humans. This data may also contribute to improvements in the therapeutic effects of DTIC by optimizing metabolic activation in patients.

MATERIALS AND METHODS

Chemicals and Reagents. DTIC and paclitaxel were obtained from the Pharmaceutical Resources Branch, National Cancer Institute (Bethesda, MD). SKF525A was a generous gift from SmithKline Beecham (Philadelphia, PA). Glucose-6-phosphate, glucose-6-phosphate dehydrogenase, and NADP were obtained from Boehringer Mannheim, (Indianapolis, IN). AIC, 6-methyl-nicotinamide, metyrapone, α-naphthoflavone, coumarin, tolbutamide, quinidine, disulfiram, erythromycin, quercetin, chlorzoxazone, phenacetin, sulfaphenazole, and caffeine were obtained from Sigma Chemical Co. (St. Louis, MO). All of the other reagents and HPLC solvents were the highest grade available and were used as received (*e.g.*, heptane sulfonic acid, triethylamine, phosphoric acid, methanol, DMSO, hydrochloric acid, KH_2PO_4, K_2HPO_4, Tris, and $MgCl_2$).

Human Liver Microsomal Fractions. Human liver microsomal fractions were provided by Jerry M. Collins (Center for Drug Evaluation and Research, United States Food and Drug Administration, Rockville, MD). Human liver samples (HL5, HL6, HL7, and HL8), medically unsuitable for liver transplantation, were acquired under the auspices of the Washington Regional Transplant Consortium (Washington, DC). A description of the preparation and characterization of the liver microsomal fractions has been published previously (14).

Incubation Conditions. Microsomal suspensions were incubated in amber glass vials maintained at 37°C in a shaker bath. Each 1-ml incubation mixture contained human liver microsomes (1 mg of protein), $NADP^+$ (0.4 mM), glucose-6-phosphate (25 mM), glucose-6-phosphate dehydrogenase (0.7 units/ml), magnesium chloride (5 mM), and Tris (50 mM) or potassium phosphate (100 mM) buffer adjusted to pH 7.4. Control incubation mixtures contained boiled microsomes or active microsomes with a nitrogen or CO/O_2 atmosphere. The incubation mixtures were preincubated for 2 min before the initiation of the reaction on the addition of 500 μM DTIC. At the end of the incubation period, reactions were terminated by the addition of 2 ml of ice-cold methanol that contained 50 ng 6-methylnicotinamide as an internal standard for HPLC analysis. The aqueous methanol supernatants obtained after centrifugation (10,000 × *g* for 2 min) were concentrated to dryness under vacuum and reconstituted in HPLC mobile phase for analysis. Chemical inhibitors were dissolved in various solvents (water, methanol, or DMSO). Appropriate controls containing drug vehicles only were also performed in parallel incubations.

Correlation of DTIC Metabolism with Marker Activities of Selected Human P450 Forms. A Hepatoscreen Test kit that contained 10 human liver microsome preparations was obtained from Human Biologicals, Inc. (Phoenix, AZ). Microsomal suspensions were prepared in the reaction buffer to achieve a final protein concentration of 1 mg/ml and were incubated with DTIC (500 μM) for a 30-min reaction period as described above.

Metabolite Formation by cDNA-expressed Human P450 Enzymes. Microsomal suspensions from the B-lymphoblastoid cell line AHH-1 $TK^{+/-}$ expressing cDNA constructs for CYP1A1, CYP1A2, CYP2B6, CYP2C8, CYP2C9a, CYP2C9c, CYP2E1, and CYP3A4 were obtained from Gentest Corporation (Woburn, MA). After dilution with the reaction buffer to achieve a final protein concentration of 1 mg/ml, microsomal suspensions were incubated with DTIC (500 μM) for reaction periods of 30 min and 120 min. Control reactions included microsomal preparation from cells that contained the expression vector alone. Because documentation provided by Gentest noted that common organic solvents such as methanol, ethanol, DMSO, and so forth, inhibited some P450 forms, DTIC stock solutions were prepared in 0.01 N HCl.

In experiments to determine the kinetics of DTIC *N*-demethylation by CYP1A1, CYP1A2, and CYP2E1, each 100-μl incubation mixture contained DTIC (200–2000 μM) human liver microsomes (0.05 mg protein), and the cofactor mixture described in "Incubation Conditions." Reactions were terminated after 45-min incubation.

Immunoinhibition. Antihuman CYP1A1 and CYP1A2 antiserum (Gentest Corporation, Woburn, MA) was preincubated with human liver microsomes on ice for 30 min before adding reaction buffer and DTIC (500 μM). Incubations were carried out for 30 min as described above.

Sample Preparation and HPLC Analysis. After termination of the reactions by protein precipitation with methanol, the supernatants obtained by centrifugation were dried under vacuum, reconstituted in mobile phase, and kept at room temperature for no less than 20 min before HPLC to ensure conversion of HMMTIC and MTIC to AIC.

Separations were achieved by a column-switching technique in which an Apex I octadecyl precolumn (Jones Chromatography, 3 cm × 4.6 mm; 5 μm) was installed in a 10-port Valco switching valve in place of a sample loop and an Apex I (Jones Chromatography, Littleton, CO) octadecyl analytical column (25 cm × 4.6 mm; 5 μm) was installed between the switching valve solvent exit port and the UV absorbance detector. This configuration permitted rapid analysis because late-eluting peaks could be passed to waste before elution onto the analytical column. The mobile phase consisted of 90% 10 mM heptane sulfonic acid, 50 mM phosphoric acid (pH 2.0) with 0.1% triethylamine and 10% methanol. The flow rate, injection volume, and UV absorbance wavelength were 1 ml/min, 40 μl, and 280 nm, respectively.

RESULTS

Metabolism of DTIC by Human Liver Microsomes. *N*-Demethylation of DTIC was monitored by the formation of AIC, the decomposition product of the initial oxidative metabolite HMMTIC. Reverse-phase HPLC with a mobile phase containing heptane sulfonic acid permitted separation of AIC, the internal standard 6-methyl-nicotinamide and other components of the incubation mixture. A column-switching technique prevented nonpolar materials from eluting onto the analytical column, eliminated interferences, and allowed reduced run times.

The P450-dependent *N*-demethylation of DTIC and concomitant formation of AIC by human liver microsomes was confirmed by several observations. AIC formation was dependent on the presence of NADPH and oxygen in the incubation mixtures, inhibited by replacing the atmosphere in the reaction vessel with N_2 and absent from incubation mixtures prepared with boiled microsomal suspensions (data not shown).

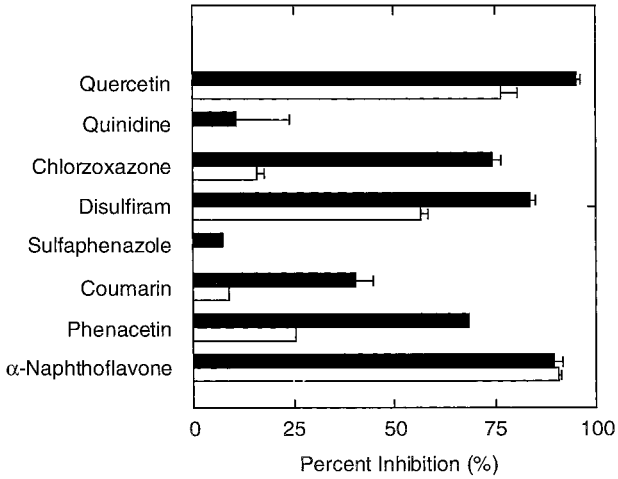

Fig. 2 Inhibition of DTIC metabolism by selective chemical inhibitors of various P450 subfamilies. DTIC was incubated with human liver microsomes in the presence of 20 μM (*open bar*) and 200 μM (*filled bar*) of the inhibitor. *Error bars*, the SD of the mean values obtained from three experiments.

The inhibition of DTIC *N*-demethylation in HL8 liver microsomes by chemical inhibitors of P450 enzymes is summarized in Fig. 2. The most potent inhibitors of AIC formation were α-naphthoflavone, quercetin, phenacetin, chlorzoxazone, and disulfiram, which suggests CYP1A, CYP2E1, and CYP3A enzymes contributed to DTIC metabolism. α-Naphthoflavone, quercetin, phenacetin, and chlorzoxazone were also potent inhibitors of DTIC metabolism by three other liver microsome preparations (data not shown). With the exception of coumarin (CYP2A), other P450 inhibitors-selective (erythromycin, tolbutamide, sulfaphenazole, and quinidine) did not reduce DTIC metabolism (data not shown). Concentration-dependent inhibition by quercetin, chlorzoxazone, and disulfiram was observed over the range 20–200 μM, whereas inhibition by α-naphthoflavone was essentially complete (>85%) at concentrations above 0.2 μM (Fig. 3*A*). The predominant role of CYP1A2 in human liver microsomes was confirmed by incubation with antihuman CYP1A2 antibody, which inhibited DTIC metabolism in four human liver microsome by greater than 75% (Fig. 3*B*).

Correlation of DTIC Metabolism with Marker Activities of Selected Human P450 Forms. DTIC metabolism was studied in a panel of 10 human liver microsomes with defined catalytic activities for major P450-selective substrates. The median ($n = 10$) rate of NADPH-dependent AIC formation from DTIC by human liver microsomes was 76 pmol/min/mg protein (range, 34–463 pmol/min/mg protein). Among the nine marker activities examined (Table 1), the highest correlation coefficients ($r > 0.94$) were found for the relationship between AIC formation and the CYP1A2 markers caffeine N^3-demethylation and 7-ethoxyresorufin *O*-dealkylation (Fig. 4). The correlation

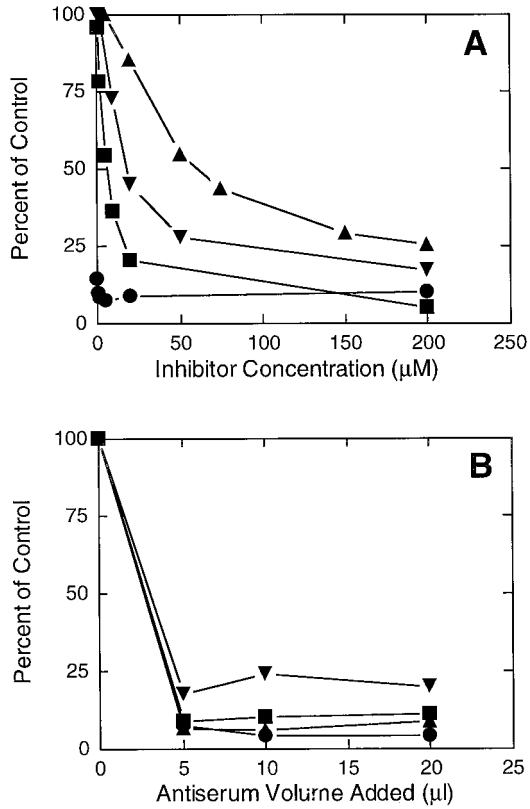

Fig. 3 Inhibition of DTIC metabolism in human liver microsomes. *A*, HL8 microsomes were incubated with DTIC in the presence of the chemical inhibitors α-naphthoflavone (●), disulfiram (▼), quercetin (■), and chlorzoxazone (▲). *B*, HL5 (●), HL6 (▼), HL7 (■) and HL8 (▲) microsomes were incubated with DTIC after 30 min preincubation with antihuman CYP1A2 antiserum.

coefficients for the relationships between AIC formation and marker activities representing other P450s (CYP2A, CYP2C, CYP2D, CYP2E, CYP3A, and CYP4A) were much lower ($r <$ 0.40). Incubation of DTIC with microsomal suspensions in the presence of 2 μM α-naphthoflavone reduced DTIC metabolism by 38–96% and abolished the correlations with the CYP1A2 marker activities. This residual DTIC metabolism was correlated with chlorzoxazone 6-hydroxylation, tolbutamide methylhydroxylation, and testosterone 6β-hydroxylation (data not shown).

Metabolism by cDNA-expressed human P450s. Recombinant human P450 isoforms were used to verify individual P450s that catalyze DTIC metabolism. DTIC metabolism was catalyzed by cDNA-expressed CYP1A1, CYP1A2, and CYP2E1 but not by CYP2C9 or CYP3A4. The amount of product formed was linear with time, and the catalytic activity of CYP1A1 (3.62 ± 0.75 pmol AIC formed/pmol P450/min) was 3-fold greater than that of CYP1A2 (1.26 ± 0.24 pmol AIC formed/pmol P450/min) and 16-fold greater than that of CYP2E1 (0.22 ± 0.04 pmol AIC formed/pmol P450/min). α-Naphthoflavone, quercetin, chlorzoxazone, and phenacetin-inhibited CYP1A1- and CYP1A2-catalyzed DTIC metabolism. Chlorzoxazone and phenacetin were selective for CYP1A2-catalyzed DTIC metabolism (data not shown).

Table 1 Correlation of DTIC *N*-demethylation with catalytic activities of human P450-selective substrates

DTIC *N*-demethylation was measured by AIC formation in a panel of 10 human liver microsomes. Triplicate determinations were made for each microsomal preparation. The correlation coefficient (r) was determined from a graph of each P450 catalytic activity *versus* the mean AIC formation value (pmol/min/mg microsomal protein). Values represent the mean of three experiments.

	P450 Form	r
7-Ethoxyresorufin *O*-dealkylation	1A2	0.946
Caffeine N^3-demethylation	1A2	0.977
Coumarin 7-hydroxylation	2A6	0.396
Tolbutamide methylhydroxylation	2C9	0.140
5-Mephenytoin 4′-hydroxylation	2C19	0.022
Dextromethorphan *O*-demethylation	2D6	0.300
Chlorzoxazone 6-hydroxylation	2E1	0.177
Testosterone 6β-hydroxylation	3A4	0.155
Lauric acid 12-hydroxylation	4A1	0.188

The kinetics of DTIC *N*-demethylation were defined with individual cDNA-expressed enzymes because saturable enzyme kinetics were not observed in human microsomes. The K_m and V_{max} values for CYP1A1 were 595 ± 111 μM and 10.2 ± 2.1 pmol product/pmol P450/min, respectively. The K_m and V_{max} values for CYP1A2 were 659 ± 88 μM and 13.8 ± 2.4 pmol product/pmol P450/min, respectively. Saturable kinetics were not observed for cDNA-expressed CYP2E1 even when the enzyme concentration was reduced 4-fold from 0.5 to 0.125 mg/ml microsomal protein. Analysis of the CYP2E1 kinetic data over the range studied suggests the K_m values exceeded 2.8 mM. Higher concentrations were not examined because of the limited aqueous solubility of DTIC.

DISCUSSION

We found that human CYP1A1, CYP1A2, and CYP2E1 catalyzed *in vitro* metabolism of DTIC, based on the collective results of chemical- and immuno-inhibition studies, correlations with marker substrates of P450 isoform activities in a human liver panel, and metabolism by cDNA expression systems. The combined data from several *in vitro* approaches were used to infer the predominant catalytic activity because the results of individual approaches can lead to erroneous conclusions.

The principle P450 isoform responsible for the *in vitro* metabolism of DTIC by human liver microsomes is CYP1A2. With the exception of coumarin, each of the chemicals that inhibited DTIC metabolism were inhibitors of CYP1A2. α-Naphthoflavone (15) and phenacetin (16) are selective inhibitors of CYP1A subfamily isozymes. The CYP3A subfamily inhibitor quercetin (17) and CYP2E1 inhibitors disulfiram (18) and chlorzoxazone (19) also inhibit CYP1A subfamily isozymes (15, 19, 20). A complementary study of the DTIC metabolism in a panel of human liver microsomes found a correlation only with catalytic activities for CYP1A2 substrates ethoxyresorufin and caffeine. The key role of CYP1A2 in human liver microsomal metabolism of DTIC was confirmed by nearly complete inhibition by an antihuman CYP1A2 antibody.

In contrast to CYP1A2, CYP2E1 most likely plays a secondary role based on lower affinity and lower expression in liver tissue. DTIC metabolism in a panel of human liver microsomes

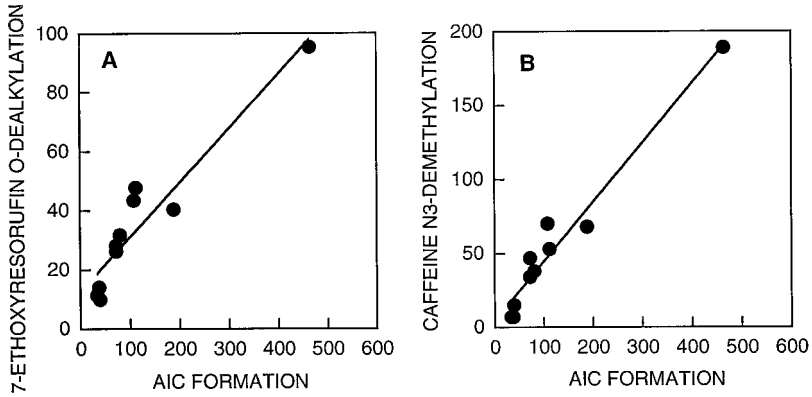

Fig. 4 Correlation of DTIC metabolism with (*A*) 7-ethoxyresorufin *O*-dealkylation (r = 0.946), and (*B*) caffeine N^3-demethylation (r = 0.977) in a panel of 10 human liver microsome preparations. Enzyme activity data are expressed as pmol product formed/mg protein/min. *Values*, the mean of three experiments.

was correlated with the catalytic activity for CYP2E1 only when the CYP1A2 catalytic activity was inhibited by α-naphthoflavone. In addition, CYP2E1 (7%) accounts for a lower fraction of the total liver cytochrome P450 content than CYP1A2 (13%; Ref. 21). Although residual DTIC metabolism in α-naphthoflavone containing incubations was also correlated with testosterone 6β-hydroxylation and tolbutamide methylhydroxylation, it is unlikely that CYP3A4 and CYP2C9, respectively, are involved in DTIC metabolism. Of the CYP3A4-selective inhibitors examined, only quercetin, which is cross-reactive with CYP1A2, reduced DTIC metabolism in human liver microsomes. This inhibitor also reduced cDNA-expressed CYP1A1- and CYP1A2-catalyzed DTIC metabolism. The CYP2C9-selective inhibitor tolbutamide did not inhibit DTIC metabolism. Finally, cDNA-expressed CYP2C9 and CYP3A4 did not metabolize DTIC.

The catalytic activity of CYP1A1 is interesting because this enzyme is found in extrahepatic tissue. Using highly selective antibodies against CYP1A1 and CYP1A2, we confirmed observations of other investigators (6, 22–24) that CYP1A1 is not found in the liver to any appreciable extent.[5] Although detectable levels of CYP1A1 mRNA have been found in human liver (22), protein content is low or undetectable even in individuals exposed to substances that induce CYP1A1 in other tissues (6, 23, 24). Because CYP1A1 is also expressed in some tumors, activation of DTIC to DNA-methylating metabolites may occur within tumors.

There is substantial variability in P450 expression among individuals because of genetic, physiological, and environmental factors (23). A 40-fold range in CYP1A2 protein concentration and mRNA expression has been reported in human liver microsome panels (24). The DTIC catalytic activity in the liver panel used in these investigations varied over a 13-fold range. Consequently, the variability of DTIC antitumor activity and toxicity may be associated with the variability of CYP1A2 expression. The potential clinical impact of DTIC activation by CYP1A2 may be investigated by exploring the relationship between DTIC pharmacokinetics and CYP1A2 phenotype, using caffeine as a probe drug (25).

The antitumor activity and toxicity of DTIC may be altered by concomitant medications. Because DTIC has limited activity as a single agent, it has been combined with other antitumor agents to improve response (26–28). Drug interactions between DTIC and drugs most often used in combination with DTIC, including doxorubicin, cyclophosphamide, ifosphamide, and vincristine (29, 30), are unlikely because none are known inhibitors or inducers of CYP1A2 or CYP2E1. An interaction between DTIC and O^6-benzylguanine, an inhibitor of the DNA repair enzyme AGT (31), is possible because O^6-benzylguanine is also a CYP1A2 substrate (32). Anticonvulsants that induce CYP3A4 and are commonly prescribed in the treatment of brain tumors would not be expected to exhibit interactions with DTIC (12). The CYP1A inducer omeprazole (33, 34) and the CYP2E1 inducer isoniazid (6) may induce the DTIC metabolism and potentiate its activity or toxicity.

It is unlikely that the restricted spectrum of DTIC activity against human tumors compared with rodent tumors is due to lower metabolic activation in humans. In fact, DTIC metabolism by humans is most likely equivalent, if not greater than the metabolism by rodents. The kinetics of DTIC metabolism for mouse liver microsomes (K_m = 0.25–1.33 mM and V_{max} = 29 pmol/min/mg protein; Ref. 2) are equivalent to those for CYP1A2, which was the high-affinity enzyme in human liver microsomes (K_m = 0.6 mM). Recently, Yamagata *et al.* (35) reported that CYP1A subfamily enzymes contribute to DTIC metabolic activation by rat liver microsomes. CYP1A subfamily enzymes comprise 13% of the total amount of P450 in humans but <3% of the P450 in the rat (9).

In summary, we identified three human P450s that catalyze the metabolism of DTIC. The principle hepatic P450 that catalyzes DTIC metabolism is CYP1A2, but CYP2E1 may participate under circumstances in which CYP1A2 expression is low. CYP1A1, primarily an extrahepatic P450, also catalyzes DTIC metabolism and may be important to the activation of DTIC at its site of action. Differences in P450 expression may account for the variability in DTIC metabolism in patients.

[5] Unpublished data.

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