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Development, validation, and use of a novel [2-carbon-13] uracil breath test to detect dihydropyrimidine dehydrogenase deficiency.

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DEVELOPMENT, VALIDATION, AND USE OF A NOVEL [2-^{13}C] URACIL BREATH TEST TO DETECT DIHYDROPYRIMIDINE DEHYDROGENASE DEFICIENCY

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LORI K. MATTISON

ABSTRACT

Dihydropyrimidine dehydrogenase (DPD) deficiency is an autosomal codominantly inherited pharmacogenetic syndrome associated with severe, unanticipated, 5-fluorouracil (5-FU) toxicity in cancer patients. As the initial and rate-limiting enzyme of the pyrimidine catabolic pathway, DPD degrades more than 80% of an administered dose of 5-FU. When DPD deficient cancer patients are administered 5-FU, dangerously increased 5-FU AUC, exposure, and toxicity may occur. DPD deficiency is present in 3-5% of the general population.

Given the frequency of DPD deficiency and the severity of DPD-mediated 5-FU toxicity, development of a diagnostic test to rapidly screen cancer patients for DPD deficiency prior to 5-FU administration is critical. Unfortunately, due to a lack of characterization of the majority of the 30 reported *DPYD* (gene that encodes DPD) sequence variations, development of a clinically useful genotypic test is not feasible. Therefore the development of a rapid phenotypic test, an oral [2-^{13}C] uracil breath test (UraBT), was the primary objective of this dissertation research. The major findings of this research are the following: 1) the amino acid sequence of the DPD enzyme is conserved among mammals and invertebrates, suggesting that a comparative genetic approach may be used to prioritize *DPYD* sequence variations for future functional characterization; 2) DPD deficient individuals may be identified by reduced breath $^{13}CO_2$ concentrations and altered breath $^{13}CO_2$ kinetics following oral administration of [2-^{13}C] uracil in a UraBT;

3) UraBT sensitivity and specificity is 100 and 96%; 4) DPD deficient subjects have altered plasma [2-^{13}C] uracil and [2-^{13}C] dihydrouracil pharmacokinetics; 5) PBMC DPD activity and [2-^{13}C] uracil and [2-^{13}C] dihydrouracil pharmacokinetics significantly correlate with the UraBT DOB_{50}; 6) the UraBT is a rapid assay which may be used to screen previously unexamined populations, such as Indians, for DPD deficiency; and 7) the African American population, particularly African American women, may have significantly reduced pyrimidine catabolism as demonstrated by reduced UraBT DOB_{50} values and PBMC DPD activity, which may increase the risk of 5-FU toxicity. These data support the future evaluation and use of the UraBT as a screening assay to identify DPD deficiency in cancer patients prior to 5-FU administration.

DEDICATION

To my mother, Kathleen, for all the hardships she endured and sacrifices she made as a single parent. Attainment of my Ph.D. is as much her accomplishment as it is my own.

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LIST OF ABBREVIATIONS

λ_z	terminal elimination rate constant
5-FU	5-fluorouracil
ANC	absolute neutrophil count
AUC	area under the curve
AUC_τ	area under the concentration-time curve from time 0 to last measured time
bp	base pair
cDNA	complementary DNA
CL/F	apparent clearance
C_{max}	maximum concentration
DHPLC	denaturing high-performance liquid chromatography
DNA	deoxyribonucleic acid
DPD	dihydropyrimidine dehydrogenase enzyme
DPYD	dihydropyrimidine dehydrogenase gene
DOB	delta over baseline; concentration of $^{13}CO_2$ in breath
DOB_{50}	concentration of breath $^{13}CO_2$ 50 minutes after [2-^{13}C]uracil administration
DUUR	ratio of plasma dihydrouracil AUC to uracil AUC
F	forward primer
FAD	flavin adenine dinucleotide
FBAL	α-Fluoro-β-alanine

Fe-S	iron-sulfur
FMN	flavin mononucleotide
FNR	false negative rate
FPR	false positive rate
FUH_2	5,6-dihydrofluorouracil
FUPA	5-fluoro-ureidopropionic acid
HPLC	high performance liquid chromatography
IRB	institutional review board
mRNA	messenger RNA
NAD	nicotinamide adenine dinucleotide, reduced form
NADPH	β-nicotinamide adenine dinucleotide phosphate, reduced form
PBMC	peripheral blood mononuclear cell
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PDR	cumulative percentage of [2-^{13}C] uracil dose recovered in breath as $^{13}CO_2$
R	reverse primer
r_p	Pearson's correlation coefficient
RACE	rapid amplification of cDNA ends
RNA	ribonucleic acid
SNP	single nucleotide polymorphism
$t_{1/2}$	elimination half-life
T_{max}	time to maximum concentration

TP	thymidylate phosphorylase
TS	thymidylate synthase
UraBT	[2-^{13}C] uracil breath test
V_z/F	terminal apparent distribution volume

INTRODUCTION

The long-term goal of my dissertation research has been the development of an assay to detect cancer patients at risk of developing 5-fluorouracil (5-FU) toxicity resulting from dihydropyrimidine dehydrogenase (DPD) deficiency. 5-FU and its prodrugs (e.g. capecitabine) are prescribed to treat gastrointestinal malignancies [1, 2]. Like many other chemotherapeutic agents, 5-FU demonstrates a narrow therapeutic index (i.e. the difference between toxic and efficacious dose is small) [3, 4]. A recent clinical study has observed that approximately 34% of colorectal cancer patients develop severe 5-FU associated toxicities at standard doses [5]. Pharmacokinetic/pharmacodynamic studies have linked 5-FU toxicity to decreased plasma 5-FU clearance and increased plasma 5-FU half-life and area under the curve (AUC) [6-8]. Yet, prediction of 5-FU associated toxicities is difficult due to the wide inter-patient variability in 5-FU pharmacokinetics.

The DPD enzyme (EC 1.3.1.2) has an important role in modulating 5-FU pharmacokinetics. As the initial and rate-limiting enzyme of pyrimidine catabolism, DPD determines the amount of 5-FU available for anabolism (cytotoxicity) by degrading more than 80% of an administered dose of 5-FU to 5-fluoro-dihydrouracil [9-11]. Marked (6-fold) inter-patient variability has been observed in the catabolic activity of this polymorphic enzyme [12] . Pharmacokinetic studies have demonstrated a linear positive correlation between DPD enzyme activity and 5-FU clearance and inverse correlations between DPD enzyme activity and 5-FU AUC and half-life [7, 13-15]. Cancer patients with DPD deficiency (reduced DPD enzyme activity) experience severe 5-FU toxicity resulting from

reduced drug catabolism as demonstrated by decreased 5-FU clearance and increased 5-FU AUC compared to cancer patients with DPD enzyme activity in the normal range [8, 16-18]. Toxicities presenting in DPD deficient cancer patients subsequent to 5-FU administration include mucositis, stomatitis, granulocytopenia, diarrhea, neuropathy, and death [19-21]. Approximately 43-64% of cancer patients who present with severe 5-FU toxicity are DPD deficient [19, 22].

DPD deficiency is an autosomal codominantly inherited pharmacogenetic syndrome [23]. To date more than 30 sequence variations have been observed in the *DPYD* gene (the gene that encodes the mRNA which in turn codes for the DPD enzyme), leading to multiple genotypes and three phenotypes characterized by normal DPD enzyme activity, partial DPD deficiency, and profound DPD deficiency [12, 21]. Population studies (performed in predominantly Caucasian populations) demonstrated that approximately 3-5% of the population is partially DPD deficient while 0.1% of the population is profoundly DPD deficient [12, 24-26].

The frequency of DPD deficiency in the general population suggests that routine screening for DPD deficiency should be performed prior to 5-FU administration to cancer patients. Hence, many potentially useful genotypic and phenotypic methods have been developed to assess DPD [27-32]. However, development of a rapid, simple, sensitive and specific genotypic test has been limited by the heterogeneity of the *DPYD* gene [1, 33]. These barriers have been a stimulus for the development of phenotypic tests to assess DPD enzyme activity.

Numerous phenotypic methods have been used to assess DPD enzyme activity. Several thin-layer chromatographic, antibody, high-performance liquid chromatography

(HPLC) and mass spectrometry methods have been used to measure plasma and urine pyrimidine (uracil and thymine) concentrations [34-37]. Although these methods can detect individuals with complete DPD deficiency, they often fail to identify individuals with partial DPD deficiency [6]. To date, the most widely accepted phenotypic method to assess DPD enzyme activity is the DPD radioassay. This procedure measures the conversion of [6-^{14}C] 5-FU to [6-^{14}C] FUH_2 following isolation of a cytosolic protein fraction from peripheral blood mononuclear cells (PBMCs) or other tissues [32]. However, this assay has several disadvantages that limit its use to primarily clinical research laboratories. Specifically, the PBMC DPD radioassay requires special equipment and training, and generates radioactive waste. Furthermore, the assay is time-consuming (> 8 hrs) and labor-intensive. Therefore, it is difficult to assess DPD enzyme activity of cancer patients prior to treatment with 5-FU or one of its derivatives.

The objective of this dissertation research is to improve 5-FU chemotherapy by developing an assay to rapidly detect cancer patients at risk for DPD-mediated 5-FU toxicity. The majority of this dissertation consists of five published manuscripts and one manuscript currently in review, five of six on which I am the first author.

The first manuscript (published in Pharmacogenomics in 2002) reviews the background and recent advances of pharmacogenetic tests for DPD assessment. This manuscript introduces 1) 5-FU metabolism and mechanisms of action; 2) the role of DPD in 5-FU metabolism; 3) biochemical analysis of the human DPD enzyme; 4) localization and genotypic analysis of the human *DPYD* gene; 5) DPD pharmacogenetics; 6) limitations of *DPYD* genotypic assays; 7) limitations of DPD phenotypic assays; and 8) limitations for use of DPD as a prognostic marker of tumor 5-FU response and patient survival. This

manuscript provides sufficient background and context to critically evaluate the importance of this dissertation research (the development of an assay to rapidly detect DPD deficiency in cancer patients) to patient care.

The second manuscript (published in Pharmacogenetics in 2002) utilizes a comparative genetic approach to prioritize known *DPYD* sequence variations for genotypic studies and functional characterization. This approach examines whether known *DPYD* sequence variations in the coding region of the *DPYD* gene occur in evolutionarily conserved domains. This manuscript establishes that 1) the translated mouse DPD cDNA demonstrates high sequence identity to the reported DPD amino acid sequences from rat, pig, cow, and human; 2) homologous amino acid sequences are present in *Drosophila melanogaster* and *Caenorhabditis elegans*; 3) DPD functional domains (i.e. uracil, FAD, and NADPH binding sites and iron sulfur motifs) show evolutionary conservation; 4) eight *DPYD* sequence variations occur in conserved domains (*DPYD**3, *DPYD**4, *DPYD**7, *DPYD**8, *DPYD**10, *DPYD**11, M166V, and D949V); and 5) four *DPYD* sequence variations occurred in non-conserved regions (*DPYD**5, *DPYD**6, *DPYD**9A, and *DPYD**12). These data suggest that the conserved loci of the eight *DPYD* sequence variants may be important to enzyme structure or function; mutations occurring at these sites may result in reduced DPD enzyme activity. This conclusion is further supported by clinical observations of reduced DPD enzyme activity from subjects with *DPYD**7, *DPYD**8, *DPYD**10, *DPYD**11 and D949V sequence variations and expression studies of *DPYD**8 and *DPYD**10 sequence variations.

The third manuscript (published in Clinical Cancer Research in 2004) describes the development and optimization of a phenotypic test, an oral UraBT, to rapidly detect DPD deficiency. This manuscript establishes that 1) DPD deficient subjects have significantly lower breath $^{13}CO_2$ concentrations following [2-^{13}C] uracil administration compared to normal subjects; 2) DPD deficient subjects have a significantly lower percentage of a [2-^{13}C] uracil dose recovered in breath as $^{13}CO_2$ (PDR) compared to normal subjects; 3) breath $^{13}CO_2$ concentrations occurring 50 minutes subsequent to [2-^{13}C] uracil administration (DOB_{50}) best classify subjects as normal or DPD deficient; 4) the UraBT demonstrates no significant inter-assay variability; 5) the UraBT demonstrates no significant intra-assay variability; and 6) no significant changes in the $^{13}CO_2$ concentrations of breath bags occur after storage for up to 210 days at room temperature. These data suggest that it may be possible to develop the UraBT into a rapid, single time-point (50 minute) diagnostic assay which could be applied to screen cancer patients for DPD deficiency prior to administration of 5-FU chemotherapy. The stability of the $^{13}CO_2$ in breath bags suggests that sample analysis does not have to occur on-site. Instead, cancer patients may be screened at remote hospitals and clinics with subsequent sample analysis performed by a reference laboratory.

The fourth manuscript (published in Clinical Cancer Research in 2006) provides a pharmacokinetic validation of the UraBT by characterizing relationships present among UraBT-associated breath $^{13}CO_2$ metabolite formation, plasma [2-^{13}C] dihydrouracil formation, plasma [2-^{13}C] uracil clearance, and PBMC DPD enzyme activity from normal and DPD deficient subjects. This manuscript establishes that 1) partially and profoundly DPD deficient subjects have reduced elimination of orally administered [2-^{13}C] uracil

compared to normal subjects; 2) partially and profoundly DPD deficient subjects have reduced DPD-mediated-formation of [2-^{13}C] dihydrouracil compared to normal subjects; 3) partially and profoundly DPD deficient subjects have reduced UraBT $^{13}CO_2$ concentration-time profiles compared to normal subjects; 4) PBMC DPD enzyme activity is significantly correlated with several pharmacokinetic variables of [2-^{13}C] uracil elimination; 5) PBMC DPD enzyme activity is significantly correlated with several pharmacokinetic variables of DPD-mediated [2-^{13}C] dihydrouracil formation; 6) UraBT DOB_{50} concentrations are significantly correlated with several pharmacokinetic variables of [2-^{13}C] uracil elimination; 7) UraBT DOB_{50} concentrations are significantly correlated with several pharmacokinetic variables of DPD-mediated [2-^{13}C] dihydrouracil formation; and 8) UraBT DOB_{50} concentrations are significantly correlated to PBMC DPD enzyme activity. These data suggest that the reduced breath $^{13}CO_2$ concentrations observed from DPD deficient subjects result from reduced *in vivo* DPD-mediated catabolism of orally administered [2-^{13}C] uracil. The significant correlations observed among the UraBT DOB_{50} concentrations, plasma [2-^{13}C] dihydrouracil pharmacokinetics, plasma [2-^{13}C] uracil pharmacokinetics, and PBMC DPD enzyme activity provide further evidence that the UraBT may be useful for the assessment of *in vivo* DPD enzyme activity.

The fifth manuscript (published in Cancer Chemotherapy and Pharmacology in 2006) is a pilot study conducted to evaluate the feasibility and performance of the UraBT prior to commencement of a large population study. A small population (n=13) of Indian subjects were rapidly screened for DPD deficiency subsequent to identification of DPD deficiency in an Indian cancer patient with 5-FU toxicity. This manuscript establishes that 1) DPD deficiency, previously unrecognized in the Indian population, is present; 2)

Indian subjects with normal DPD enzyme activity demonstrated UraBT $^{13}CO_2$ concentrations and indices (DOB_{50}, C_{max}, T_{max}, and PDR) similar to those observed from the 50 normal subjects examined during UraBT development; and 3) the DPD deficient Indian subject demonstrated altered UraBT $^{13}CO_2$ concentrations and indices (DOB_{50}, C_{max}, T_{max}, and PDR) similar to those observed from the seven partially DPD deficient subjects examined during UraBT development. These results suggest that the UraBT may not only be applied to examine cancer patients, but may also be applied to examine racial and ethnic groups for DPD deficiency to identify "at risk" populations.

The sixth manuscript (submitted to Clinical Cancer Research in 2006) uses the UraBT to screen African Americans for DPD deficiency. African American colorectal cancer patients have been observed to have increased 5-FU-associated toxicity (leucopenia and anemia) and decreased overall survival compared to Caucasian patients. One potential source for these disparities may be differences in 5-FU catabolism resulting from racial differences in DPD enzyme activity. This manuscript establishes that 1) African American subjects have significantly lower PBMC DPD enzyme activity compared to Caucasian subjects; 2) the prevalence of DPD deficiency was 3-fold greater in the African American population (8.0%) compared to the Caucasian population (2.8%); 3) African American women had a significantly lower mean PBMC DPD enzyme activity compared to African American men and Caucasian women and men; 4) African American women were observed to have the highest prevalence of DPD deficiency (12.3%) compared to African American men (4.0%), Caucasian women (3.5%), and Caucasian men (1.9%); and 5) African American subjects had a significantly lower DOB_{50} concentrations and PDR values compared to Caucasian controls. These results suggest that Afri-

can Americans may be predisposed to severe 5-FU-associated toxicities resulting from lower mean DPD enzyme activity and an increased incidence of DPD deficiency.

These studies may be useful for the design and development of large clinical studies to evaluate the relationship present between the UraBT, DPD enzyme activity, and 5-FU toxicity. It is anticipated that clinical application of the UraBT will improve patient care by minimizing DPD-mediated 5-FU toxicity.

IMPLICATIONS OF DIHYDROPYRIMIDINE DEHYDROGENASE ON 5-FLUOROURACIL PHARMACOGENETICS AND PHARMACOGENOMICS

by

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ABSTRACT

A prominent example of the potential application of pharmacogenomics and pharmacogenetics to oncology is the study of dihydropyrimidine dehydrogenase (DPD) in 5-fluorouracil (5-FU) metabolism. 5-FU is currently one of the most widely administered chemotherapeutic agents used for the treatment of epithelial cancers. DPD is the rate-limiting enzyme in the catabolism and clearance of 5-FU. The observation of a familial linkage of DPD deficiency from a patient exhibiting 5-FU toxicity suggested a possible molecular basis for variations in 5-FU metabolism. Molecular studies have suggested there is a relationship between allelic variants in the *DPYD* gene (the gene that encodes DPD) and a deficiency in DPD activity, providing a potential pharmacogenetic basis for 5-FU toxicity. In the last decade, studies have correlated tumoral DPD activity with 5-FU response, suggesting it may be a useful pharmacogenomic marker of patient response to 5-FU based chemotherapy. This article reviews the basis and discusses the challenges of pharmacogenetic and pharmacogenomic testing of DPD for the determination of 5-FU efficacy and toxicity.

INTRODUCTION

The antimetabolite, 5-fluorouracil (5-FU), is a one of the most commonly used antineoplastic drugs prescribed to treat colon, breast and skin cancers [1]. Initial studies demonstrated that the anticancer effect of 5-FU is mediated by anabolism, which is determined by the rate of catabolism or clearance of the drug. In recent years, 5-FU toxicity and efficacy has been demonstrated to be related to the activity of dihydropyrimidine dehydrogenase (DPD) (EC 1.3.1.2), the initial and rate-limiting enzyme in the catabolism of 5-FU

[2]. Recent studies have suggested DPD deficiency may be caused by allelic variants within the *DPYD* gene [3]. To date, > 21 reported variants (mutations and polymorphisms) have been described and ongoing studies are continuing to examine the relationship between *DPYD* genotypes and DPD phenotypes [4-6]. Other studies have demonstrated an association between DPD levels and tumor response to 5-FU [7, 8], suggesting DPD levels may be a potentially valuable pharmacogenomic marker of 5-FU efficacy. This review summarizes the background and recent advances in the development of pharmacogenetic and pharmacogenomic tests for DPD assessment.

THE ROLE OF DPD IN 5-FU METABOLISM

Synthesized over 40 years ago, Heidelberger *et al.* designed 5-FU to exploit the increased avidity of tumors for uracil [9]. As a uracil analog, 5-FU was found to exert its antitumor effects mainly through the inhibition of thymidylate synthase (TS), disrupting intracellular nucleotide pools necessary for DNA synthesis. Other possible sites of action include incorporation into RNA with the resultant disruption of RNA synthesis following its anabolism and incorporation into DNA resulting in fragmentation [2].

Biochemical analysis of the DPD enzyme began with its characterization in rat and pig liver [10, 11]. In 1992, Lu *et al.* purified human DPD enzyme to homogeneity, thus allowing a more extensive molecular and biochemical characterization [12]. DPD was found to be a homodimeric enzyme (consisting of two 100 kDa subunits) encoded by a 150 Kb gene (*DPYD*) on chromosome 1p22 [13]. Subsequent studies have shown that the *DPYD* gene has 23 exons that are translated into a 1025 amino acid protein containing FMN, NADPH and flavin adenine nucleotide (FAD) binding sites and four iron sulfur

motifs [14, 15]. Harris *et al.* showed DPD activity to follow a circadian rhythm with highest and lowest peaks occurring at 1 a.m. and 1 p.m. respectively [16]. However, the regulatory determinants of DPD activity have not been well understood. Both positive and negative regulatory elements in the *DPYD* promoter have been identified [17] but their role in DPD regulation has not been well characterized.

The significance of DPD in 5-FU metabolism emerged from observations demonstrating the inherited nature of DPD deficiency in patients with severe 5-FU toxicity. In 1985, Tuchman *et al.* observed pyrimidinemia and pyrimidinuria in the family of a patient with severe fluorouracil toxicity, suggesting a hereditary basis for inadequate 5-FU metabolism [18]. A major link was established when our laboratory correlated this imbalance to deficiencies in DPD activity in the family of a patient with severe 5-FU toxicity and pyrimidinemia [19].

Biochemical characterization of DPD in the catabolism of 5-FU has helped to provide a basis for these observations. The catabolic pathway was found to convert 5-FU to 5-fluoro-β-alanine in three steps mediated by DPD, dihydropyrimidinase, and β-alanine synthase enzymes respectively (Figure 1) [2]. The products of this pathway were excreted in the urine [20]. As the initial and rate-limiting enzyme of catabolism, DPD therefore was a major determinant of 5-FU clearance. Pharmacokinetic studies determined that ~ 85% of administered 5-FU doses were catabolized, with 1-3% of the drug anabolized, suggesting that catabolism had a major role in determining 5-FU clearance [2, 20]. Hence, deficiencies in DPD activity reducing 5-FU catabolism resulted in increased drug exposure and 5-FU toxicity.

THE PHARMACOGENETICS OF DPD DEFICIENCY

Many studies now suggest that, in a high proportion of individuals, 5-FU toxicity can be attributed to reduced DPD activity. In cancer patients with 5-FU toxicity, Van Kuilenburg *et al.* found 59% with a reduced DPD activity [21], while Johnson *et al.* observed a frequency of 43% [22]. Furthermore, two studies examining DPD activity in unselected patient populations demonstrated that approximately 3-5% of the general population is at risk of 5-FU toxicity [7, 23]. These results suggest that a small yet significant proportion of the population may benefit from measurement of DPD activity before 5-FU treatment.

This possibility has prompted the development of a number of optimized protocols for measuring DPD activity [24, 25]. These assays have been used retrospectively to confirm DPD deficiency in a number of patients demonstrating 5-FU toxicity [26], although some of the limitations of the assays need to be resolved before they may be of any clinical utility prior to treatment.

One limitation has been the contention over the use of peripheral blood mononuclear cells (PBMCs) as a source for measuring DPD activity. While studies suggest that the liver is the primary site of 5-FU catabolism [2] and hence the most suitable tissue for measuring DPD activity, its poor accessibility for assay has meant that many protocols have relied on PBMCs as a surrogate source for DPD measurement. However, the correlations between PBMC and liver DPD activity is significant but weak ($r = 0.56$, $p = 0.002$) [27]. Furthermore, studies investigating the association between PBMC DPD activity and 5-FU clearance have reported good [28], weak [7], and no correlation [29].

Additionally, Etienne *et al.* observed no association between PBMC DPD activity and the development of 5-FU toxicity [7].

A second limitation has been the circadian regulation of DPD activity. As discussed earlier, studies have shown 2-fold differences in DPD levels within the same individual as a result of circadian regulation [16], making sampling time an important issue in determining DPD activity. A third difficulty has been the large tissue requirement, long analysis time, and radioactivity of many current activity assays, making them poorly amenable to routine laboratories. These issues remain to be resolved.

In the last decade, the discovery of a linkage between *DPYD* allelic variants and reduced DPD activity suggested that pharmacogenetic testing for DPD deficiency may be possible. In 1995, Meinsma *et al.* demonstrated a familial linkage of an allelic variant in the *DPYD* gene from a patient with DPD deficiency, thus establishing a correlation between the *DPYD* variant and reduced activity [3]. This variant consisted of a G→A mutation in an exon/intron splice site leading to the omission of exon 14, presumably resulting in a catalytically inactive enzyme. This mutation (now designated *DPYD**2*A* [5]) has since been independently correlated with DPD deficiency in other case studies and pedigree analyses [2, 21, 26, 30-32].

To assess the potential of detecting the *DPYD**2*A* variant as a pharmacogenetic marker of 5-FU toxicity, a number of population studies have been conducted. In these studies, *DPYD**2*A* variants have been found in 4/332 (1.2%) [33], 6/250 (1.2%) [34], and 8/851 (0.94%) [35] individuals, suggesting that ~1 in 100 individuals may benefit from *DPYD* variant detection to identify 5-FU toxicity prior to its administration.

Currently, however, the value of *DPYD* variant detection to identify individuals prone to 5-FU toxicity remains to be determined. Studies on populations of individuals deficient in DPD activity and patients with 5-FU toxicity detected *DPYD***2A* variants in only 14/22 (64%) [36] and 6/25 (24%) [35] cases respectively, suggesting that other factors may be involved in these phenotypes. Indeed, since identification of the *DPYD***2A* allelic variant, > 21 others have been identified (Table 1). To address the increasing number of reported allelic variants, nomenclature system has been devised [5]. However, the significance of these variants currently remains unclear. In many cases, the variant allele frequencies and their effects on DPD activity remain to be evaluated. Furthermore, in the cases where they have been investigated, there has been little consensus, particularly in the determination of the functional effects of the variants.

The reasons for these discrepancies remain unclear, but some methodological and biological factors can be identified. Differences in analytical sensitivity and population selection may have contributed to variations in allele frequency between studies. The different end points for functional activity used between studies may be a significant source of disparity between observed findings. While some studies have relied on clinical end points of 5-FU toxicity or DPD deficiency, others have evaluated functional activity of variants in artificial expression systems. Other possible explanations offered have included the compound heterozygosity of variants and the lack of comprehensive sequence, haplotype and pedigree analysis [37], and the unknown influence of epigenetic regulatory events such as DPD autoregulation [38], allelic regulation [6], and ubiquitination [26] between individual cases. Clearly, these issues will need to be resolved before the pharmacogenetic potential of *DPYD* variants can be clarified.

THE PHARMACOGENOMIC ROLE DPD IN 5-FU EFFICACY

The role of DPD in catabolism suggested that it may also be a significant determinant of 5-FU efficacy in tumors. This prompted investigators to examine whether tumor DPD activity may influence tumor response to 5-FU. In 1994, Beck *et al.* provided support for this hypothesis when they observed a correlation between low DPD activity and increased 5-FU sensitivity in cancer cell lines [39]. This was followed by Etienne *et al.* who confirmed this association in tumors resected from head and neck cancer patients [40]. These studies have suggested that DPD activity could be used as a pharmacogenomic marker of tumoral 5-FU response.

In recent years, the use of DPD as a prognostic indicator of 5-FU response has been investigated using a new generation of assays. The large tissue requirement and laborious nature of DPD enzyme activity assays have resulted in studies investigating the feasibility of antibody and polymerase chain reaction (PCR)-based methods for measuring DPD protein and mRNA levels as surrogates for DPD enzyme activity. Results showing correlation between DPD activity, immunohistochemical score [41], protein [42] and mRNA levels [43, 44] in colorectal tissue have demonstrated the validity of these methods.

Results from these new methods for assessing DPD have demonstrated associations between DPD levels and tumor response. In an examination of five colorectal cancer cell lines, Nita *et al.* demonstrated an association between DPD protein levels and 5-FU response [45]. Other studies correlated DPD mRNA levels with response in gastrointestinal cell lines [46], human xenografts [47], and gastric tumors [48].

However, the association between DPD mRNA and protein levels and patient survival is currently unclear. In a recent immunohistochemical study of 119 breast cancer patients, Horiguchi *et al.* observed a significant correlation between high DPD protein levels and poor patient survival after treatment with 5-FU or 5-FU derivatives [49]. However, other studies investigating protein levels in non-small cell lung [50], gastric [51], liver, and colorectal [52] cancers and DPD mRNA levels in gastric cancer [53] found no significant association.

Recently, studies have suggested that the prognostic index of DPD levels may be improved by its combined assessment with levels of other 5-FU metabolic enzymes. Fujiwaki *et al.* demonstrated a high thymidylate phosphorylase (TP):DPD ratio was an independent marker of poor survival in ovarian cancer patients [54]. Studies conducted by Salonga and others have shown that, while 11 of 22 nonresponding patients could be identified based on DPD mRNA levels alone, all 22 could be discriminated by assessing DPD, TP and TS mRNA levels together [8].

EXPERT OPINION

Numerous studies have now proven the principle that DPD can potentially be a useful pharmacogenetic and pharmacogenomic marker of 5-FU toxicity and efficacy. Biochemical and pharmacokinetic studies have characterized the important role DPD has in 5-FU catabolism and determining its bioavailability. Pedigree studies have demonstrated a familial linkage between 5-FU toxicity and DPD deficiency as well as sequence variants in the *DPYD* gene. Translational studies have shown correlations between DPD levels and tumor response and patient survival. However, a number of issues pertaining

to DPD measurement and controversial findings need to be resolved before it may be widespread clinical use.

New approaches for measuring enzyme activity, currently the most reliable method of identifying DPD deficiency, need to be developed to make this assay amenable for use before the onset of toxicity in patients receiving 5-FU chemotherapy. Sampling issues and use of radioisotopes need to be eliminated and these new assays must be tested prospectively. Lastly, a rapid, easy-to-use assay is desirable.

While the detection of allelic variants may provide a useful pharmacogenetic test, the current discrepancies in the characterization need to be resolved (Table 1). This includes achieving a consensus on the incidence of these variants and the relationship between genotype and the DPD deficient phenotype.

Although many studies have correlated DPD levels with 5-FU response, the poor correlation with patient survival needs further investigation. Elucidation of the factors (including the role of other genes involved in drug metabolism) responsible for this disparity will help to provide a better understanding of the true value of determining DPD levels. The new generation assays are of potential clinical interest as they promise to provide high-throughput, inexpensive alternatives for assessing DPD mRNA and protein levels. However, the reliability and feasibility of these assays remain to be rigorously evaluated.

Another important consideration that needs to be further explored is the regulation of DPD activity. Understanding the mechanism through which this occurs will help the optimization of therapeutic strategies.

Finally, the issue of standardization needs to be addressed. Any assays that show clinical promise need to be incorporated into standardized protocols and independently validated in prospective trials.

OUTLOOK

DPD has the potential of becoming one of the leading examples of the application of pharmacogenetics and pharmacogenomics in oncology. Further studies of DPD pharmacogenetics and pharmacogenomics may help to clarify the role of DPD as a marker for chemotherapeutic application of 5-FU based drugs. The resolution of current issues may occur through the characterization of the entire *DPYD* gene, particularly the identification of allelic variants in the introns and promoter. This will lead to a better understanding of its relationship to DPD deficiency and the development of pharmacogenetic tests to identify patients prone to 5-FU toxicity. Elucidation of the factors affecting DPD regulation and its relationship to patient prognosis will lead to the development of standardized protocols for improved patient stratification and 5-FU efficacy, providing a more individualized approach in the application of 5-FU based therapies.

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Highlights

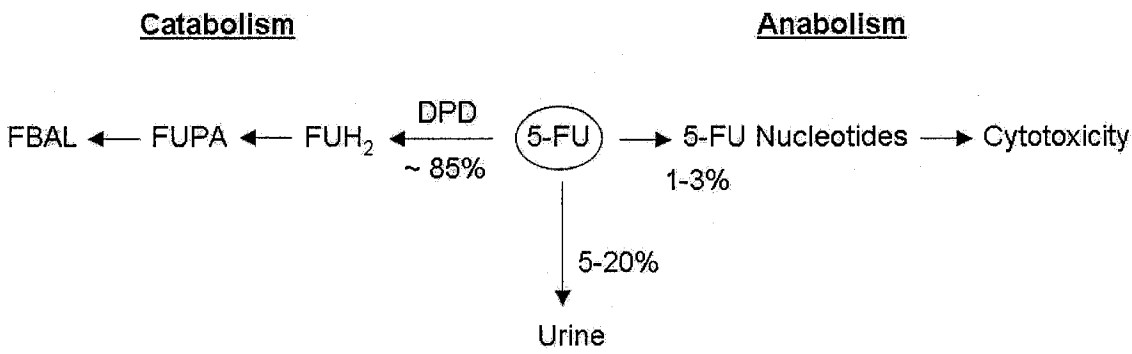
- 5-FU is currently one of the most widely administered chemotherapeutic agents used for the treatment of breast, colon, and skin cancers.
- The efficacy of 5-FU is determined by anabolism of the drug.
- DPD is the rate-limiting enzyme of the catabolic pathway and has a major role in determining 5-FU anabolism, degradation and clearance.
- 5-FU toxicity has been attributed to deficiencies in DPD activity.
- Reduced DPD activity may be a result of allelic variation in the *DPYD* gene, suggesting a pharmacogenetic test for DPD deficiency may be of clinical benefit.
- Over 21 *DPYD* allelic variants have now been identified, however the relationship between these variants and alterations in DPD activity remains unclear.
- Future characterization of *DPYD* variants and determination of other factors involved in *DPYD* gene regulation and DPD activity may clarify the role of DPD as a potential pharmacogenetic marker of 5-FU toxicity.
- Studies have correlated tumoral DPD levels with 5-FU efficacy, suggesting that it may be a potentially useful pharmacogenomic of response to 5-FU-based therapy. However, before DPD may be considered to be an independent marker for tumor response and patient survival, additional studies must be performed.
- The clinical utility of a new generation of assays for quantitating DPD are currently being investigated.

Table 1 Known *DPYD* Allelic Variants and Their Reported Relationship to DPD Activity

Allelic variant	Exon	Nucleotide	Amino acid	Allelic frequency (%) [Ref.]	Reported effect on DPD activity; [Ref.] Reduced	Normal	Unclear
*DPYD**2A*	14	Intron 14 G1A	Exon 14 skipping	0.7 [31], 1.3 [6]	[3,21,26, 30-32]	[6]	
*DPYD**2B*	13/14	Intron 14 G1A+A1627G	I543V/ Truncated protein				
*DPYD**3	14	1897delC	Truncated protein		[55]		
*DPYD**4	13	G1601A	S534N	0.8 [56]		[31]	[6, 56]
*DPYD**5A*	13	A1627G	I543V	23 [6], 28 [56]	[21]	[56]	
*DPYD**5B*	13/Intron 13	A1627G + Intron 13 C39T	I543V + Intron 13 C39T	23 [6]			[6]
*DPYD**6*	18	G2194A	V732I	5.8 [56], 9.5 [6]	[21]	[6, 56]	
*DPYD**7*	4	295-298 delT-CAT	Truncated protein		[57]		
*DPYD**8*	7	C703T	R235W		[57]		
*DPYD**9A*	2	T85C	C29R	3.7 [58], 33.8 [6]	[21, 59]	[6, 37]	
*DPYD**9B*	2/21	T85C+ G2657A	C29R+ R886H				[59]
*DPYD**10*	23	G2983T	V995F		[60]		
*DPYD**11*	10	G1003T	V335L		[61]		
*DPYD**12*	2/11	G62A+ G1156T	R21Q+ E386Ter		[61] (E386Ter)	[61] (R21Q)	
*DPYD**13*	13	T1679G	I560S	1.35 [6]	[6, 37]		
F632F	14	T1896C	F632F	9.8 [58]			[58]
H25R	2	A74G	H25R	0.46 [58]			[58]
L572V	13	C1714G	L572V	0.46 [58]			[58]
M166V	6	A496G	M166V		[21]	[37]	
812delT	8	812delT	NA	0.46 [58]			[58]
D949V	22	A2846T	D949V		[21]		
Intron 10 T-15C	Intron 10	T-15C	NA	21.6 [6]		[6]	
Intron 13 G40A	Intron 13	G40A	NA	29.7 [6]			[6]

DPD: Dihydropyrimidine Dehydrogenase

Fig. 1. The role of DPD in 5-FU metabolism. The diagram illustrates the distribution of 5-FU to its metabolic pathways. DPD: Dihydropyrimidine dehydrogenase; FBAL: 5-Fluoro-β-alanine; FU: Fluorouracil; FUH_2: Dihydrofluorouracil; FUPA: 5-Fluoroureidopropionic acid.



SPECIFIC AIMS

The overall objective of this dissertation research was to improve 5-FU chemotherapy by developing an assay which could be used to rapidly screen cancer patients for DPD deficiency. The following specific aims were examined:

1. Utilize a comparative genetic approach to prioritize *DPYD* sequence variations (occurring within the coding region of the gene) for functional characterization and inclusion in genotypic tests by identifying variations that occur in evolutionary conserved regions:

    a. Clone and sequence the complete mouse liver DPD cDNA;

    b. Align the translated mouse liver DPD cDNA to the reported DPD amino acid sequences from pig, rat, human and cow;

    c. Identify homologous amino acid sequences from *C. elegans* and *D. melanogaster*;

    d. Perform a multiple sequence alignment of human and nonhuman DPD amino acid sequences;

    e. Determine the identity and homology between human and nonhuman sequences;

    f. Determine the identity and homology of DPD substrate and cofactor interaction sites between human and non-human sequences;

    g. Identify *DPYD* sequence variations occurring in highly conserved domains of the DPD amino acid.

2. Develop and optimize a [2-^{13}C] uracil breath to phenotypically detect DPD deficiency:

    a. Recruit subjects with normal DPD enzyme activity or known DPD deficiency;

b. Perform dose optimization studies with fixed and weight-adjusted doses of [2-^{13}C] uracil to determine the dose which provides maximal separation between the $^{13}CO_2$ breath profiles from normal and DPD deficient subjects;

c. Determine the UraBT duration which allows inspection of the elimination phase of the $^{13}CO_2$ breath profiles from DPD deficient subjects;

d. Determine UraBT inter-assay and intra-assay variability;

e. Recruit subjects with unknown DPD enzyme activity;

f. Phenotypically assess PBMC DPD enzyme activity by radioassay;

g. Genotypically assess the *DPYD* gene of DPD deficient subjects by DHPLC;

h. Characterize $^{13}CO_2$ breath profiles from normal and DPD deficient subjects;

i. Compare UraBT indices (C_{max}, T_{max}, AUC, and PDR) from normal and DPD deficient subjects;

j. Identify a UraBT parameter to correctly classify normal and DPD deficient subjects;

k. Calculate UraBT sensitivity and specificity using the radioassay as the standard;

l. Determine $^{13}CO_2$ retention by breath bags.

3. Validate the UraBT by comparing to other phenotypic methods to assess DPD enzyme activity:

a. Determine plasma [2-^{13}C] uracil and [2-^{13}C] dihydrouracil concentrations from blood samples collected from subjects while they are performing the UraBT;

b. Compare plasma [2-^{13}C] uracil and [2-^{13}C] dihydrouracil pharmacokinetics from subjects with normal DPD enzyme activity and partial and profound DPD deficiency;

c. Correlate UraBT DOB_{50} concentrations to markers of [2-^{13}C] uracil catabolism (e.g. plasma [2-^{13}C] uracil clearance and [2-^{13}C] dihydrouracil appearance);

d. Correlate PBMC DPD enzyme activity to markers of [2-^{13}C] uracil catabolism (e.g. plasma [2-^{13}C] uracil clearance and [2-^{13}C] dihydrouracil appearance);

e. Correlate PBMC DPD enzyme activity to DOB_{50} concentrations.

4. Examine the feasibility and performance of the UraBT by screening Indians for DPD deficiency:

   a. Rapidly screen Indian subjects for DPD deficiency using the UraBT;

   b. Validate the UraBT DOB_{50} classification by determining the PBMC DPD enzyme activity of all subjects;

   c. Use DHPLC to screen the *DPYD* gene of DPD deficient subjects for the *DPYD***2A* sequence variation.

5. Evaluate pyrimidine catabolism in the African American and Caucasian populations:

   a. Characterize the distribution of PBMC DPD enzyme activity from African Americans and Caucasians;

   b. Determine the prevalence of DPD deficiency in each population based on the PBMC radioassay;

   c. Characterize the distribution of the UraBT DOB_{50} from African Americans and Caucasians and the percent dose of [2-^{13}C] uracil catabolized to $^{13}CO_2$.

A COMPARATIVE ANALYSIS OF TRANSLATED DIHYDROPYRIMIDINE DEHYDROGENASE cDNA; CONSERVATION OF FUNCTIONAL DOMAINS AND RELEVANCE TO GENETIC POLYMORPHISMS

by

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ABSTRACT

A pharmacogenetic syndrome caused by molecular defects in the dihydropyrimidine dehydrogenase gene (*DPYD*) results in partial to complete loss of dihydropyrimidine dehydrogenase (DPD) enzyme activity with patients exhibiting life-threatening toxicity following administration of routine doses of 5-fluorouracil. To date, more than 19 reported mutations have been putatively associated with DPD deficiency with 16 occurring within the open reading frame of the cDNA. The purpose of this study was to examine the conservation of functional domains (including the uracil, flavine adenine dinucleotide and NADPH binding sites) across three phyla (Chordata, Arthropoda, and Nematoda) and the conservation of regions corresponding to the previously reported mutations. Comparative analysis of the uracil and NADPH binding sites in mammals and invertebrates demonstrated 100% amino acid identity between mammals and *Drosophila melanogaster*. *Caenorhabditis elegans* demonstrated 89% and 88% identity in these domains, respectively. The mammalian sequences demonstrated 100% identity in two iron sulphur motifs (amino acids 953-964 and 986-997) with significant conservation in *D. melanogaster* (92% and 92% identity, respectively) and *C. elegans* (100% and 92% identity, respectively). Comparative amino acid analysis revealed nonconservation in the loci of four *DPYD* mutations [*DPYD**12 (R21Q), *DPYD**5 (I543V), *DPYD**6 (V732I), *DPYD**9A* (C29R)]. Seven mutations occurred in highly conserved regions [M166V, *DPYD**8 (R235W), *DPYD**11 (V335I), *DPYD**4 (S534N), *DPYD**9B* (R886H), D949V, *DPYD**10 (V995F)]. In summary, this comparative analysis identified conserved regions which may be critical to enzyme structure and/or function. The conservation of loci where *DPYD* mutations occur was also examined to evaluate their functional significance

on DPD enzyme activity. These data should prove useful in the evaluation of newly discovered mutations in the *DPYD* gene.

INTRODUCTION

Dihydropyrimidine dehydrogenase (EC 1.3.1.2, dihydrouracil dehydrogenase, dihydrothymine dehydrogenase, DPD) is the initial and rate-limiting enzyme responsible for the reduction of uracil and thymine to 5,6-dihydrouracil and 5,6-dihydrothymine, respectively [1,2]. Pharmacokinetic studies have demonstrated that DPD catabolizes more than 85% of an administered dose of the antineoplastic drug, 5-fluorouracil (5-FU) to the inactive metabolite, 5,6-dihydro-5-fluorouracil [3]. Although early studies focused on the anabolic pathway (responsible for the cytotoxicity of 5-FU), the catabolic pathway has been shown to determine the amount of 5-FU available for anabolism [4]. The importance of the catabolic pathway in 5-FU toxicity has been dramatically demonstrated in Japan where 16 deaths occurred following concurrent administration of 5-FU and the antiviral drug sorivudine [5], a potent inhibitor of the DPD enzyme [6, 7].

The toxicity observed in the Japanese patients has also been observed in cancer patients with a metabolic defect that significantly decreases DPD activity (DPD deficiency) [8-10]. In humans, this pharmacogenetic syndrome (which results from molecular defects in the *DPYD* gene) can result in a range of enzymatic deficiency that spans from partial to profound (complete) loss of enzyme activity with approximately 3-5% of the "normal" population demonstrating partial deficiency [11]. The clinical significance of DPD deficiency was recently examined in 103 cancer patients who developed grade IV toxicity after receiving standard 5-FU chemotherapy [10]. Examination of DPD enzyme

activity in these patients revealed a large over-representation (43%) of DPD deficient individuals [10].

In humans, the *DPYD* gene is located on chromosome 1p22 and consists of 23 exons which encode a 1025 amino acid protein containing uracil, flavine mononucleotide (FMN), NADPH, and flavine adenine dinucleotide (FAD) binding sites, along with four iron sulphur motifs [12-17]. To date, more than 19 mutations have been reported to be associated with DPD deficiency, with 16 occurring in the open reading frame of the DPD cDNA [18-20]. While several of these reported mutations result in significant changes in the translated DPD protein (e.g. *DPYD**2*A* resulting in a deletion of exon 14, and *DPYD**3 resulting in an altered reading frame), 14 reported mutations result in single amino acid substitutions where the functional significance remains unclear [19].

In this study, we examine the conservation of the DPD functional domains through sequence analysis of five mammals (human, mouse, rat, cow and pig) and two invertebrates (*Drosophila melanogaster* and *Caenohabditis elegans*). The loci where *DPYD* mutations occur were also examined in these species to gain insight into their conservation and functional significance.

MATERIALS AND METHODS

Sequence Analysis

DPD nucleotide and amino acid sequences were aligned using Assembly Align and MacVector 4.5 Sequence Analysis software (Scientific Imaging Systems, New Haven, Connecticut, USA). Conserved domains ranging from 12 to 43 amino acids were used in NCBI BLAST (http://www.ncbi.nlm.nih.gov) and Blocks

(http://www.blocks.fhcrc.org) searches of GenBank to identify additional homologous sequences in nonmammalian species. The mouse DPD cDNA was used to identify homologous sequences in the mouse dbEST data base (http://www.ncbi.nlm.nih.gov). DNAMAN software (Lynnon Biosoft, Vaudreuil, Quebec, Canada) was utilized to perform a multiple alignment from which a homology tree was constructed and two sequence alignments to establish percentage identity and homology between human and nonhuman sequences [21]. These sequences were then examined for *O*-glycosylation sites (NetOGlyc 2.0, http://www.cbs.dtu.dk/servicies/NetOGlyc).

Cloning the Murine DPD cDNA

The murine DPD cDNA was cloned and sequenced using methods previously described by our laboratory [22]. Briefly, murine total liver RNA was extracted from a snap frozen murine liver using TRIzol (Life Technologies, Grand Island, New York, USA) and primed with random hexamers using a First Strand cDNA Synthesis kit (Pharmacia, Piscataway, New Jersey, USA) according to the manufacturer's instructions. Polymerase chain reaction (PCR) primers were designed from conserved domains shared between porcine, bovine, rat and human DPD cDNA sequences (Table 1). The entire murine DPD cDNA was cloned in four overlapping fragments. The 5′ untranslated region of the cDNA was obtained by rapid amplification of cDNA ends (RACE) using Marathon Ready cDNA following protocols supplied by the manufacturer (Clontech, Palo Alto, California, USA). The 3′ untranslated region of the mouse was amplified using a murine DPD specific forward primer obtained directly from sequence data and oligo (dT) from the First Strand Synthesis kit (Pharmacia, Piscataway, New Jersey, USA). PCR products were

subcloned into the Invitrogen pCR 2.1 vector (Carlsbad, California, USA). DNA prepared from positive clones was analyzed for insert size by digestions with *Eco*RI and sequenced three times using an ABI Prism 377 Sequencer (PE Applied Biosystems, Foster City, California, USA).

RESULTS

Murine DPD cDNA

The murine DPD cDNA is 3364 nucleotides in length and contains a 101 bp 5′ untranslated region with the initiating ATG occurring at nucleotides 102-104. The open reading frame is 3079 bp which encodes a 1025 amino acid protein. The murine DPD cDNA contains a 186 nucleotide 3′ untranslated region with a termination codon (TAA) occurring at bp 3177-3179. Examination of the mouse dbEST database identified 50 partial clones which varied in size from 19-814 bp.

Identification and Comparison of Homologous Amino Acid Domains

Alignment of mammalian species revealed several conserved domains (e.g. the uracil binding site occurring at amino acids 661-678). These were subsequently used in Blast and Blocks searches of GenBank to identify homologous sequences in other genomes. Sequences from two invertebrate species (*C. elegans* and *D. melanogaster*) were shown to be homologous to human DPD. Sequence analysis demonstrated that the translated human sequence had the greatest identity to the pig (93% identity and 96% homology) and cow (92% identity, 96% homology), with less identity to mouse (89% identity, 94% homology) and rat (89% identity, 94% homology). Analysis of invertebrate se-

quences revealed a 1034 amino acid overlap between *Drosophila* and human sequences which demonstrated 65% identity and 93% homology, while *C. elegans* and human sequences demonstrated 65% identity and 79% homology in a 1031 amino acid overlap. Figure 1 depicts a homology tree that was constructed from a multi-sequence alignment of the seven species.

Utilizing the data generated with human, cow, rat and pig, putative catalytic domains were evaluated (Fig. 2). The structure of the human *DPYD* gene [15] was utilized to delineate exon boundaries.

Conservation of DPD Functional Domains

Uracil/5-FU Binding Site

Sequence analysis of the uracil binding motif revealed that mammals and *Drosophila* exhibited 100% amino acid identity (Fig. 2). *C. elegans* exhibited 89% identity with two conserved amino acid changes (A703I and R717K; these position numbers correspond to amino acids 664 and 678 in the mammalian sequence) (Fig. 2).

Five additional 5-FU interaction sites (N609, N668, S670, N736, and T737) demonstrated 100% identity among the seven species. The four amino acids (T575, I613, M642, and N668), whose side chains form the pocket to accommodate the fluorine atom of 5-FU, exhibited 100% identity among the seven species.

FMN Interaction Site

The sequences of the seven species were examined to assess the conservation of the 332 amino acid FMN binding motif (corresponding to amino acids 527-858 in the

mammalian sequences). This analysis revealed that the human FMN binding region had the highest homology to porcine (97%) followed by bovine (96%), murine (96%), rat (95%) and *C. elegans* (84%) sequences. In addition the *Drosophila* and human sequences demonstrated 85% homology in a 332 amino acid region containing three gaps.

Within the FMN binding region are two lyseine residues (574 and 709) that interact with the isoalloxazine ring of the FMN cofactor. These two amino acids demonstrated 100% identity among the seven examined species.

FAD Binding Site

Sequence analysis of the FAD binding motif revealed that *C. elegans*, rat, bovine, porcine, and murine sequences have 91% identity with the human FAD binding motif (Fig. 2). The human FAD binding motif is the only sequence that has an alanine (A) at position 474. The four remaining mammalian species (mouse, rat, cow, and pig) each contain a proline (P) at position 474, while *C. elegans* and *Drosophila* (amino acid 513 in the *C. elegans* sequence and amino acid 472 in the *Drosophila* sequence) each contain a lysine (K) at this position (Fig. 2). The FAD binding motif of *Drosophila*, when compared with the human FAD binding motif, showed five amino acid changes demonstrating 54% identity (Fig. 2). Examination of the crystalline structure of the porcine DPD enzyme revealed additional amino acids that directly interact with the FAD cofactor (amino acids 129, 198, 218, 219, 226, 235, 261, 481 and 489). Analysis of these amino acids revealed that amino acids 198, 218, 235, 481, and 489 demonstrated 100% identity among all seven species (Fig. 2). The valine (V) present at amino acid 129 demonstrated 100% identity between the five mammals and *Drosophila* (position 131 in the *Drosophila*

sequence). However, *C. elegans* had a conserved valine (V) to isoleucine (I) amino acid change at this position (position 169 in the *C. elegans* sequence) (Fig. 2). Examination of the mammalian sequences at position 219 and *C. elegans* (position 257 in the *C. elegans* sequence) demonstrated 100% identity. *Drosophila* had a conserved lysine (K) to arginine (R) amino acid change at this position (position 216 in the *Drosophila* sequence) (Fig. 2). The leucine (L) at position 226 (mammalian sequence) demonstrated 100% identity in *C. elegans*, *Drosophila*, human, mouse, rat and pig. However, the bovine sequence had a conserved leucine (L) to isoleucine (I) amino acid change at this position (Fig. 2). The leucine (L) present at position 261 (mammalian sequence) demonstrated 100% identity in *C. elegans*, *Drosophila*, human, mouse, bovine, and pig. However, the rat sequence had a conserved (L) to isoleucine (I) amino acid change in this position (Fig. 2).

NADPH Binding Site

At the NADPH binding motif, the mammalian species and *Drosophila* demonstrated 100% identity. The *C. elegans* sequence demonstrated 88% identity, 100% homology, due to two conserved amino acid changes [isoleucine (I) to valine (V) and phenylalanine (F) to methionine (M)] at positions 375 and 384 (Fig. 2). The crystalline structure of the porcine DPD enzyme revealed additional amino acids that directly interact with the NADPH cofactor (amino acids 364, 365, 371, 438, and 481) which demonstrated 100% amino acid identity among mammals and invertebrates.

Iron Sulphur Motif

The coordinating amino acids of the first *N*-terminus Fe-S motif (amino acids 79, 82, 87, and 140) demonstrated 100% identity among mammals and invertebrates. The coordinating amino acids of the second *N*-terminus Fe-S motif [three cysteine residues (amino acid 91, 130, and 136) and one glutamine (amino acid 156)] also demonstrated 100% identity across the seven species.

The third iron sulphur motif (amino acids 953-964 in the mammalian sequences, 989-1000 in the *C. elegans* sequence and 953-964 in the *Drosophila* sequence) demonstrated 100% identity among mammals and *C. elegans* (Fig. 2) while *Drosophila* demonstrated 92% identity with one nonconserved asparagine (N) to alanine (A) amino acid substitution at position 964 (in the *Drosophila* sequence) (Fig. 2). The coordinating cysteines of the third Fe-S motif (cysteines 953, 956, 959, and 996) demonstrated 100% identity across the seven species.

At the fourth iron-sulphur motif (amino acids 986-997 in the mammalian and *Drosophila* sequences and amino acids 1023-1034 in the *C. elegans* sequence), the mammals exhibited 100% identity (Fig. 2). However, one nonconserved amino acid change [leucine (L) to tyrosine (Y)] occurred in *C. elegans* (at position 1030 in *C. elegans* sequence) resulting in 92% identity. *Drosophila* sequence contained one conserved leucine (L) to valine (V) change (at position 993 in the *Drosophila* sequence) resulting in 92% identity and 100% homology (Fig. 2). Cysteines 963, 986, 989, and 992 coordinate the fourth Fe-S motif. These four amino acids demonstrated 100% amino acid identity among the seven species.

Analysis of the *DPYD** Mutations

The amino acid sequences of the seven species were analyzed to identify the degree of conservation in regions that correspond to the loci of the human *DPYD* mutations (Table 2; Fig. 2). All seven species exhibited 100% identity in the loci of seven *DPYD* mutations [*DPYD**4 (S534N), *DPYD**8 (R235W), *DPYD**10 (V995), *DPYD**11 (V335L), *M166V* and *D949V*]. *DPYD**9*B* contains two mutations C29R together with R886H. The first mutation (C29R) demonstrated nonconservation between species while the R886H mutation demonstrated 100% identity among human, murine, rat, swine, and invertebrate sequences (Table 2; Fig. 2).

*DPYD**12 contains two mutations R21Q together with a change resulting in a stop codon at amino acid 386. The first mutation (R21Q) demonstrated 100% identity between mammals and *Drosophila* while *C. elegans* contained a conserved change from arginine (R) to lysine (K) (Table 2; Fig. 2). However, *Drosophila* demonstrated a conserved valine (V) to glycine (G) change at amino acid 732 (Table 2; Fig. 2).

The locus of *DPYD**5, I543V was nonconserved among the seven species. Human and porcine sequences each contained an isoleucine (I) at this locus while both invertebrates contained a glutamic acid (E), and both rodents each contained a proline (P). The bovine sequence contained a threonine (T) at this locus. In the region corresponding to the locus of *DPYD**9*A*, C29R the human locus contained a cysteine (C), the rodent and bovine sequences each contained an arginine (R), and both invertebrates contained a valine (V) (Table 2; Fig. 2).

Predicted Post-Translational Modifications of the Seven Homologous Sequences

The sequences of the seven species underwent analysis to identify potential *O*-glycosylation sites. One potential *O*-glycosylation site (corresponding to amino acid 550 in the mammalian sequence, amino acid 545 in the *Drosophila* sequence and amino acid 589 in the *C. elegans* sequence) was conserved among the seven species. A second potential *O*-glycosylation site was conserved among mammals and *C. elegans* (corresponding to amino acids 595 and 634, respectively). Two additional sites (threonine 471 and 981) were conserved among the mammalian species; however, this conservation did not extend to the invertebrate sequences (Table 3).

DISCUSSION

In this study alignment of human [14], cow [22], mouse, rat [23], and pig [14, 17] amino acid sequences permitted the comparison of functional and catalytic domains of the DPD enzyme. These analysis extend earlier studies from our laboratory and others examining the kinetic and biochemical properties of DPD purified from human, bovine, rat, and pig liver [12, 24-27]. In addition, the conservation of regions corresponding to loci associated with DPD deficiency was examined.

Earlier studies had utilized radioactive substrate to identify the uracil binding motif in tryptic digests of purified bovine liver DPD [26]. Examination of this motif demonstrated 100% identity among the five mammalian species. The high degree of conservation of this region permitted additional Blast and Blocks searches to be performed which, ultimately, identified homologous sequences in two invertebrate species (*Drosophila* and *C. elegans*). Further examination of this region demonstrated 100% homology among the

seven species. Of interest is a highly conserved 21 amino acid region that occurs immediately after the uracil binding motif. Although the high homology suggests that this region is critical for uracil binding or FMN interaction, detailed kinetic analysis remains to be performed in order to elucidate whether mutations occurring in this region may interfere with substrate binding. Recent studies examining the crystalline structure of the recombinant porcine DPD revealed additional amino acids involved in substrate binding (amino acids 609, 668, 670, 736, and 737) [17] which demonstrated 100% conservation among the seven species.

Kinetic studies of the purified enzyme demonstrated that DPD required FAD as a cofactor [12, 16, 25, 26]. The human and porcine FAD binding motif [14] was identified by homology to the FAD binding regions (TXXXXVFAXGD, where X represents any amino acid) of several FAD reductases [28]. At this binding motif, five mammalian species (human, mouse, rat, pig, and cow) and *C. elegans* demonstrated 92% identity (100% homology), while *Drosophila* demonstrated only 54% identity (72% homology). Of interest is the significant amount of conservation the five mammalian and the *C. elegans* sequences exhibit within the TXXXX region of the FAD binding motif (100% and 80% homology in the mammalian species and *C. elegans*, respectively). However, *Drosophila* demonstrated only 40% homology in this region. Further examination of the TXXXX region using site-directed mutagenesis needs to be performed to clarify its role on FAD binding and enzyme activity.

Biochemical studies demonstrated that DPD required FMN as a cofactor [12,16,25,26]. The FMN binding domain for the DPD enzyme was initially identified through homology to the FMN binding region of dihydroorotate dehydrogenase from

Lactococcus lactis [29]. This 332 amino acid domain demonstrated regions of variable conservation among the seven species. However, recent studies have localized the FMN binding region to amino acids K574 and K709 [17] with both demonstrating 100% identity among the seven examined species. This significant degree of conservation suggests that these amino acids may have a critical role in enzyme function through acceptance of electrons after reduction of FAD [17]. To assess the effects of K574 and K709 on enzyme function, detailed kinetic studies using mutated DPD would need to be performed.

Iron and sulphur were present in the purified DPD enzyme [12, 25]. Based upon homology to the previously described Fe-S motifs of an iron sulphur protein from bovine mitochondrial complex I [30], two Fe-S motifs were identified in the *C*-terminus of the porcine and human DPD enzyme [14]. Examination of the two *C*-terminus Fe-S motifs revealed significant conservation in both the mammalian and invertebrate sequences. Additional studies later identified the presence of two novel Fe-S motifs in the *N*-terminus of the DPD enzyme that are coordinated by amino acids 79, 82, 87, and 140 in the first motif, and amino acids 91, 130, 136, and 156 in the second motif [16, 17, 29]. The coordinating cysteines of both motifs demonstrate 100% identity among the seven species suggesting these amino acids may be redox active, having a critical role in electron transport.

Several recent reports examining DPD deficiency have focused on the identification of mutations in the DPD cDNA in individuals demonstrating unanticipated 5-FU toxicity. Due to differences in evolutionary pressure, amino acids located in regions critical for DPD structure and/or catalytic function would be expected to be conserved. Significant conservation of the loci corresponding to *DPYD**4 (S534N), *DPYD**6 (V732I), *DPYD**8 (R235W), *DPYD*9B* (R886H), *DPYD* *10 (V995F), *DPYD**11 (V335I),

*DPYD**12 (R21Q), *M166V*, and *D949V* was demonstrated among mammals and invertebrates. These data suggest a critical role for these amino acids in the maintenance of DPD structure and/or function. Assessment of the I543V amino acid substitution present in *DPYD**2*B*, *DPYD**5, and *DPYD**5*B*, revealed a lack of conservation among mammals and invertebrates. The amino acids present at the loci of *DPYD**9*A* (C29R) also demonstrated a lack of conservation, suggesting that amino acids occurring in these two regions may not be critical to DPD structure and/or function. This observation is supported, in part, by the identification of the *DPYD**9*A* (C29R) mutation in individuals with normal DPD enzyme activity (data not shown).

Analysis of the amino acid sequence of the seven species demonstrated that several *DPYD* mutations occur near the two *N*-terminus iron sulphur motifs including: *DPYD**12 (R21Q), *DPYD**9*A*/9*B* (C29R) and *M166V*. Two mutations [*D949V* and *DPYD**10 (V995F)] occur near or within the two *C*-terminus Fe-S motifs, respectively. Mutations occurring in the regions of the Fe-S motifs may disrupt electron transport from FAD to FMN and thus influence DPD enzyme activity. Several of the mutations occurring in this region were previously reported to result in lowered DPD enzyme activity (*DPYD**9*A*, *DPYD**9*B*, and *DPYD**10) [18, 31, 32]. However, the effects of other mutations (*DPYD**12, *M166V*, and *D949V*) on DPD enzyme activity remain to be determined.

Further examination also demonstrated that two previously reported mutations (*DPYD**4 and *DPYD**5) occur within exon 13 (which encodes for the first FMN interaction site) (Fig. 2). Since previous reports suggest that these mutations do not have an appreciable effect on enzyme activity [18, 33-35], they may occur too far upstream (40 and 31 amino acids, respectively) to affect FMN binding in this region.

Sequence analysis also demonstrated that one mutation (*DPYD**8) occurs at a conserved locus that is responsible for interaction with FAD. Studies utilizing human recombinant DPD demonstrated that this mutation results in loss of enzyme activity [36].

Further examination of uracil/5-FU interaction sites with respect to proximity to *DPYD* mutants demonstrated that one mutation (*DPYD**6) occurs at a conserved locus that is four amino acids upstream from a uracil/5-FU interaction site that occurs at amino acid 736. A previous study of this mutation has demonstrated that this mutation has no effect on enzyme activity [18, 33-35].

A recent report examining regulation of *DPYD* gene suggests that there are critical regulatory elements located downstream of the transcription start site [37]. Further, a 6-fold variation in DPD enzyme activity in healthy and cancer patient populations [11] as well as a circadian variation in human [38] and rat species [39] suggest that the DPD enzyme is highly regulated. One possible mechanism by which this may occur is through the regulation of DPD mRNA levels. Several studies have demonstrated a correlation between DPD mRNA levels and enzyme activity which suggests rapid mRNA and protein turnover [9, 40, 41]. Numerous AU rich domains (which have been shown to affect mRNA stability) [42] were identified in the DPD 3′ untranslated region of all five mammalian species (data not shown). Further studies measuring mRNA half-life in these species should clarify the role of mRNA stability in determining DPD enzyme activity.

In summary, we compared the conservation of functional domains critical to DPD enzyme structure and/or function in five mammalian and two invertebrate species. In addition, the conservation of loci where previously reported mutations have been described was examined. Alignment of these sequences allowed us to determine the proximity of

these mutations to catalytic domains. These analyses were used to assess which mutations were relevant in DPD deficiency. As new *DPYD* mutations continue to be identified, this approach should prove useful in further evaluation of their functional significance.

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Table 1 Sequence of Primers Used for PCR Amplification of the Mouse DPD cDNA

Primer Designation	Primer Sequence (5′ to 3′ Orientation)	Fragment Length
DPD-1F	ACTCACTATAGGGCTCGAGCGGC	1181 bp
DPD-1R	AGAGTGACAGGCGCACATTCC	
DPD-2F	TGGCTACAGAGCTGCATTTATTGG	1563 bp
DPD-2R	CCACTTTCAGCTGAGTCAATTCC	
DPD-3F	ATCGCAAGAGCAGCAAAGGAA	742 bp
DPD-3R	GCCAGAGTCATTACAGGTCATGTA	
DPD-4F	AGCAAAATCAGACAGAAAGATCA	631 bp
DPD-4R	AACTGGAAGAATTCGCGGCCGCAGGAAT$_{18}$	

The four pairs of primers used to clone the mouse DPD cDNA. F, forward primers; R, reverse primers.

Table 2 Previously Defined *DPYD* Mutations [18,19]

Reported Mutations	Human	Mouse	Rat	Cow	Pig	*Drosophila*	*C. elegans*
*DPYD*2B*[a] (*DPYD* 2A* + I543V)	N/A	N/A	N/A	N/A	N/A	N/A	N/A
*DPYD**3 termination at 633	P	P	P	P	P	P	P
*DPYD*4* S534N	S	S	S	S	S	S	S
*DPYD*5*[a] I543V	I	P*	P*	T	I	E**	E**
*DPYD*5B*[a] I543V + intron 13 C39T	N/A	N/A	N/A	N/A	N/A	N/A	N/A
*DPYD*6* V732I	V	V	V	V	V	G*	V
*DPYD*7* truncated protein	S	S	S	S	S	S	S
*DPYD*8* R235W	R	R	R	R	R	R	R
*DPYD*9A* C29R	C	R**	R**	R**	H**	V**	V**
*DPYD*9B* C29R + R886H	R	R	R	C**	R	R	R
*DPYD*10* V995F	V	V	V	V	V	V	V
*DPYD*11* V335L	V	V	V	V	V	V	V
*DPYD*12* R21Q	R	R	R	R	R	R	K*
*DPYD*12* (386)	E	E	E	E	E	E	E
M166V	M	M	M	M	M	M	M
D949V	D	D	D	D	D	D	D

[a]The conservation of the I543V mutation present in *DPYD*2B*, *DPYD*5* and *DPYD*5B* was assessed. The human *DPYD* mutations, and their respective amino acid changes, are listed in column 1. The remaining columns note the amino acids present in the regions corresponding to the loci of human *DPYD* mutants in the sequence of specified mammal or invertebrate. *Conserved amino acid change, **nonconserved amino acid change.

Table 3 Potential *O*-Glycosylation Sites in the DPD Protein

Species	*O*-Glycosylation Site[a]
Mammals	471, 550, 595, 981
Drosophila melanogaster	545
Caenorhabditis elegans	589, 634

Amino acids that may be *O*-glycosylated in human, rat, mouse, bovine, pig, *Drosophila* and *C. elegans*. [a]The software used to determine *O*-glycosylation sites was previously reported to identify 83% of glycosylated and 90% of non-glycosylated serine and threonine residues from a previously known and verified mucin *O*-glycosylation site [43].

Fig. 1. Homology tree of the five mammalian DPD amino acid sequences and two homologous sequences from invertebrates. Human, murine, rat, bovine, and porcine translated cDNA sequences were aligned and conserved regions were identified. These regions were then used in NCBI Blast and Blocks searches to identify homologous sequences in *Drosophila* and *C. elegans*. These seven sequences then underwent multisequence alignment to establish the percentage identity and construct a homology tree. The percentages shown at each branch-point represent the percentage identity between the specified species and the remaining homologs.

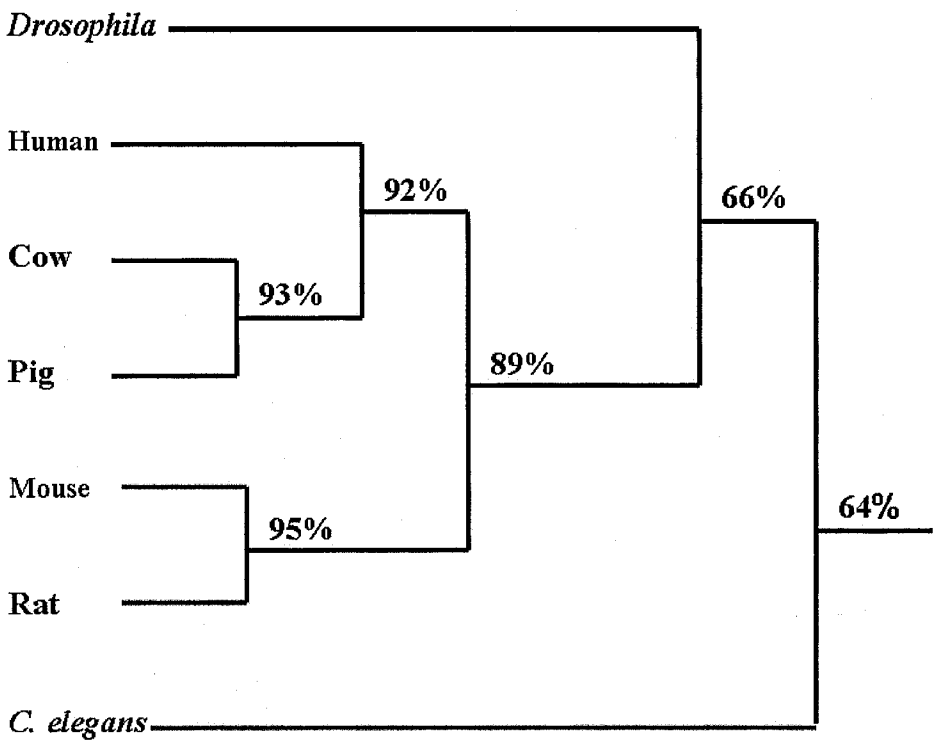
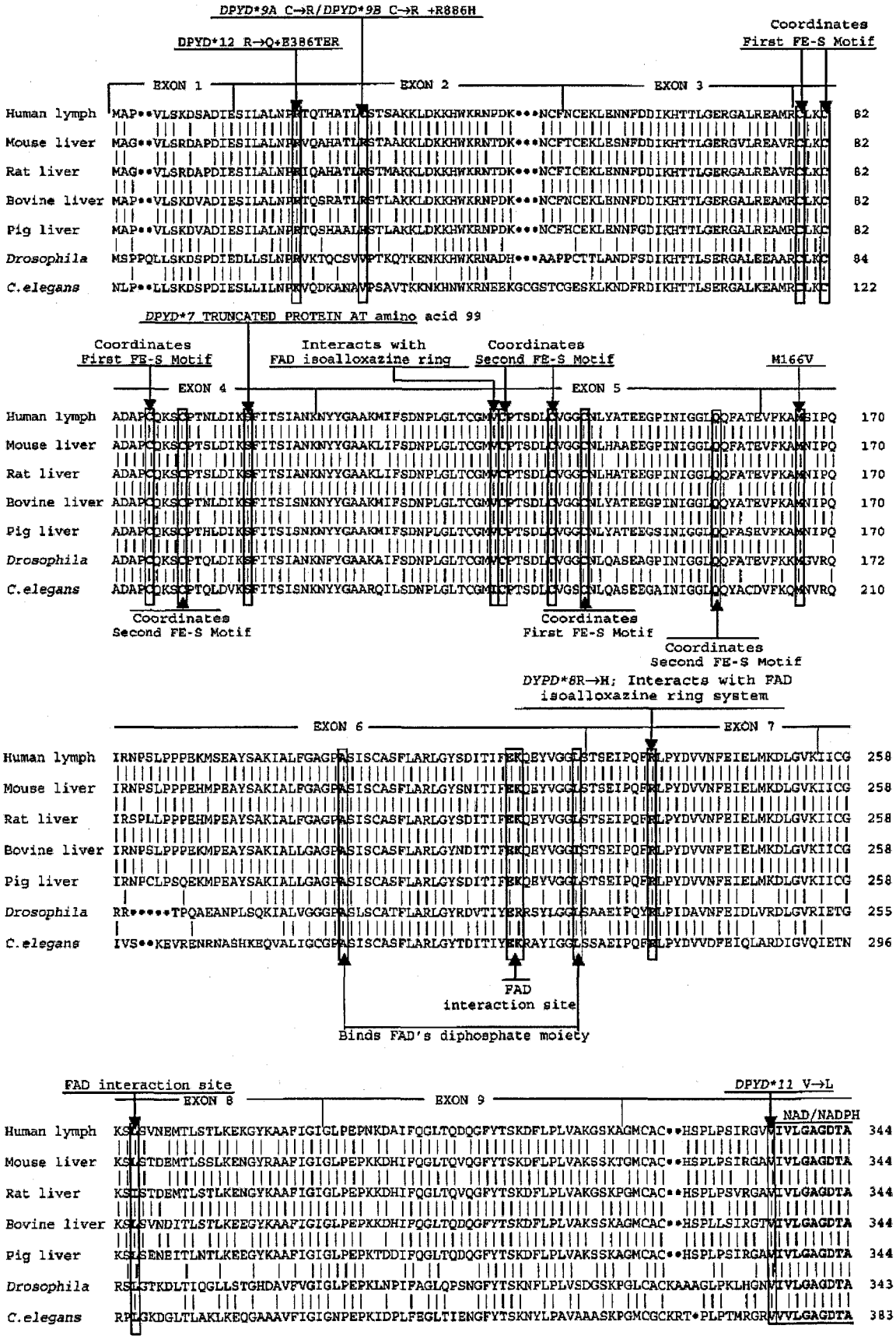
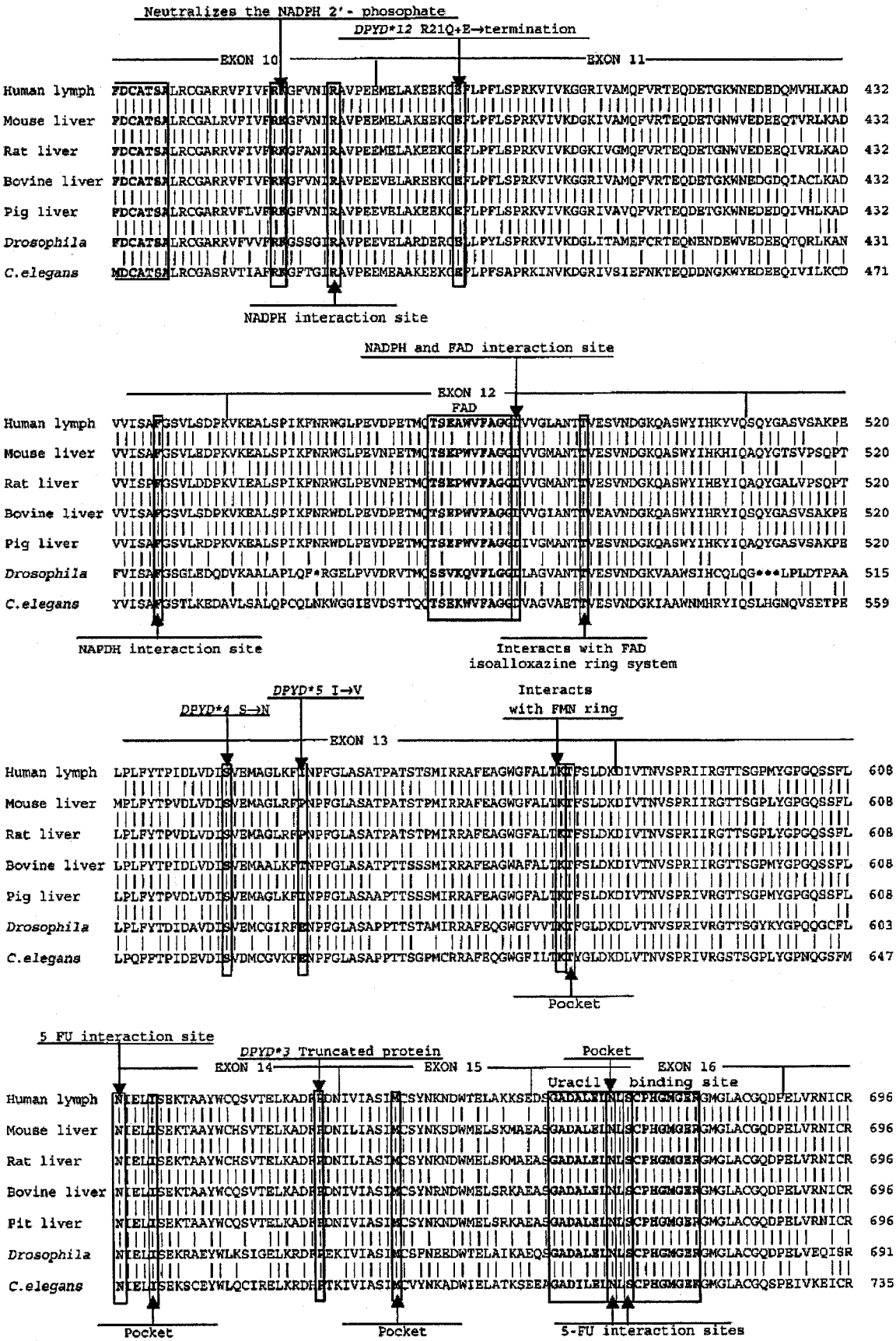
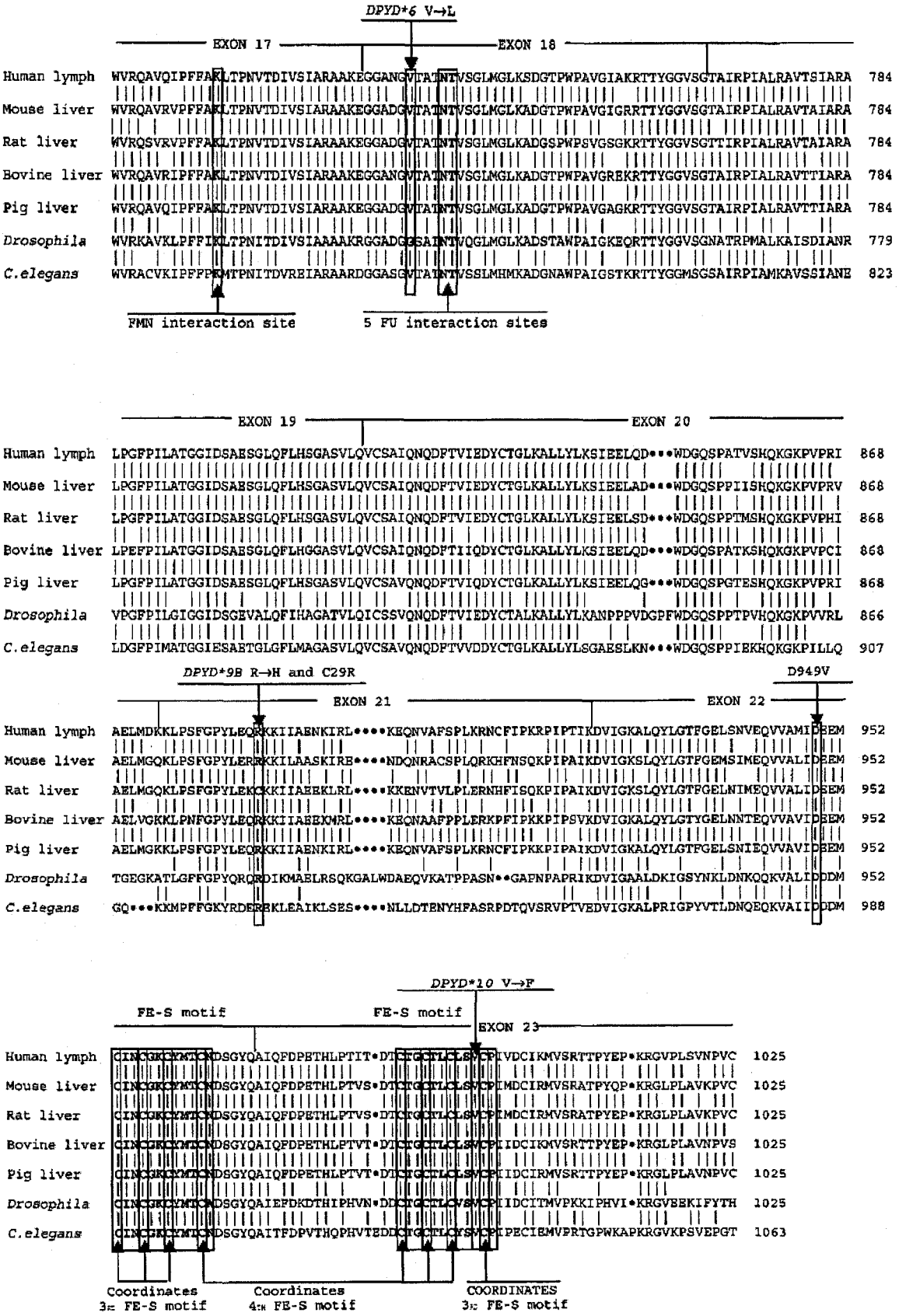


Fig. 2.

Amino acid alignment of the seven species, delineation of catalytic regions, and regions corresponding to the *DPYD* mutations. The amino acid sequences of the seven species underwent multisequence alignment. The *C*-terminus of the *Drosophila* sequence contains an unaligned region of 6 amino acids (data not shown). The *N*-terminus of the *C. elegans* sequences contains an unaligned region of 37 amino acids (data not shown) and the *C*-terminus contains an unaligned region of 21 amino acids (data not shown). Exon boundaries were delineated based upon the structure of the *DPYD* gene. Catalytic binding motifs for uracil, FAD, NADPH and Fe-S motifs are indicated (boxed and labelled). Additional substrate and cofactor interaction sites based on the recent crystal studies of the recombinant porcine are also defined (boxed and labelled). Regions that correspond to human *DPYD* mutant loci are also indicated (boxed and labelled).







RAPID IDENTIFICATION OF DIHYDROPYRIMIDINE DEHYDROGENASE DEFICIENCY BY USING A NOVEL [2-^{13}C] URACIL BREATH TEST

by

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ABSTRACT

Purpose: Dihydropyrimidine dehydrogenase (DPD)-deficient cancer patients have been shown to develop severe toxicity after administration of 5-fluorouracil. Routine determination of DPD activity is limited by time-consuming and labor-intensive methods. The purpose of this study was to develop a simple and rapid [2-^{13}C] uracil breath test, which could be applied in most clinical settings to detect DPD-deficient cancer patients.

Experimental Design: Fifty-eight individuals (50 "normal," 7 partially, and 1 profoundly DPD-deficient) ingested an aqueous solution of [2-^{13}C] uracil (6 mg/kg). $^{13}CO_2$ levels were determined in exhaled breath at various time intervals up to 180 min using IR spectroscopy (UBiT-IR_{300}). DPD enzyme activity and *DPYD* genotype were determined by radioassay and denaturing high-performance liquid chromatography, respectively.

Results: The mean (±SE) C_{max}, T_{max}, δ over baseline values at 50 minutes (DOB_{50}) and cumulative percentage dose recovered (PDR) for normal, partially and profoundly DPD-deficient individuals were 186.4 ± 3.9, 117.1 ± 9.8, and 3.6 DOB, 52 ± 2, 100 ± 18.4, and 120 min, 174.1 ± 4.6, 89.6 ± 11.6 and 0.9 DOB_{50}, and 53.8 ± 1.0, 36.9 ± 2.4, and < 1 PDR, respectively. The differences between the normal and DPD-deficient individuals were highly significant (all *P*s <0.001).

Conclusions: We demonstrated statistically significant differences in the [2-^{13}C] uracil breath test indices (C_{max}, T_{max}, DOB_{50} and PDR) among healthy and DPD-deficient individuals. These data suggest that a single time-point determination (50 min)

could rapidly identify DPD-deficient individuals with a less costly and time-consuming method that is applicable for most hospitals or physicians' offices.

INTRODUCTION

Dihydropyrimidine dehydrogenase (DPD) deficiency is an autosomal codominantly inherited pharmacogenetic syndrome with a variable phenotype that ranges from partial to complete loss of DPD enzyme activity (1, 2). The clinical impact of DPD deficiency has been dramatically demonstrated by studies showing that 43–60% of the patients with severe toxicity (including death) after administration of standard doses of 5-fluorouracil (5-FU) are partially or profoundly DPD deficient (3, 4). Population studies have shown the prevalence of partial and profound DPD deficiency to be 3–5% and 0.1% in the general population, respectively (5-7).

Several methods including high-performance liquid chromatography, mass spectrometry, thin layer chromatography, and denaturing high-performance liquid chromatography (DHPLC) have been developed to identify DPD deficiency in cancer patients (8-11). Unfortunately, these methods remain too complex and time consuming for routine clinical use and are unavailable in most treatment facilities. The availability of a method to accurately determine exhaled ^{13}C in breath offers a novel approach for the detection of DPD deficiency through the administration of [2-^{13}C] uracil. Recent studies have successfully used a similar approach to diagnose *Helicobacter pylori* infection in a Food and Drug Administration-approved ^{13}C-urea breath test and to examine metabolic abnormalities in carbohydrate, lipid and amino acid metabolism (12-16).

In the present study, we describe the development of a rapid, simple, and noninvasive [2-^{13}C] uracil (UraBT), which can be performed in most cancer-treatment facilities or physicians' offices. After oral administration of [2-^{13}C] uracil, $^{13}CO_2$ in exhaled breath was measured and indices [C_{max}, T_{max}, δ over baseline values at 50 min (DOB_{50}), and cumulative percentage of ^{13}C dose recovered in breath (PDR)] were assessed for their ability to discriminate between healthy and DPD-deficient individuals.

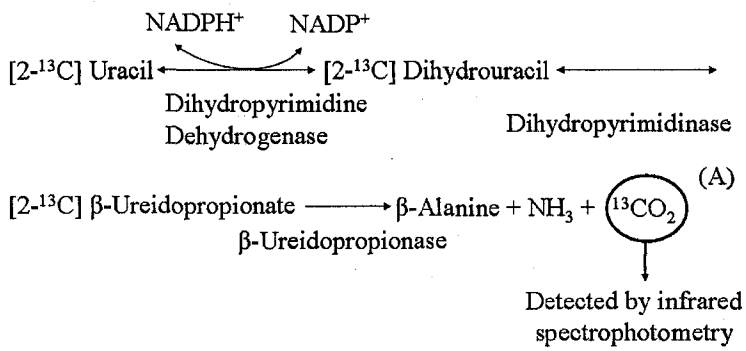
MATERIALS AND METHODS

Principle of the UraBT

The principle of the UraBT is based on metabolism of [2-^{13}C] uracil by the enzymes of the pyrimidine-catabolic pathway to produce $^{13}CO_2$ (see below, Eq. A). In DPD-deficient individuals, reduced [2-^{13}C] uracil catabolism would be expected to result in decreased exhaled $^{13}CO_2$ levels.

$^{13}CO_2$ and $^{12}CO_2$ in exhaled breath samples is measured byIR spectrometry using the UBiT-IR_{300} (Meretek Diagnostics, Lafayette, CO). The amount of $^{13}CO_2$ present in breath samples is expressed as a δ over baseline ratio that represents a change in the $^{13}CO_2$ / $^{12}CO_2$ ratio of breath samples collected before and after [2-^{13}C] uracil ingestion (see Eq. B; Ref. 17).

The amount of [2-^{13}C] uracil metabolized and released into the breath as $^{13}CO_2$ was determined for each time point using the equation described by Amarri *et al* (18). These results were expressed as PDR. The UBiT-IR_{300} instrument used to calculate the $^{13}CO_2$ / $^{12}CO_2$ ratio is discussed in detail elsewhere (17).



$$DOB = \left[\frac{^{13}CO_2}{^{12}CO_2}\right]_{\text{post-dose sample}} - \left[\frac{^{13}CO_2}{^{12}CO_2}\right]_{\text{pre-dose sample}} \quad \text{(B)}$$

Study Design

Informed consent was obtained from each volunteer who participated in this institutional review board-approved study at the University of Alabama at Birmingham. Exclusion criteria for healthy individuals included respiratory or metabolic disorders.

Preliminary studies in four healthy individuals [one man and three women; mean age, 32 years (range: 22–48 years)] and three partially DPD-deficient individuals (one man and two women, ages 27, 23, and 31, respectively) evaluated the dose of [2-^{13}C] uracil and time course that could provide maximal separation of breath patterns between these groups. Doses of [2-^{13}C] uracil included fixed doses of 100, 200, or 300 mg as well as doses adjusted to body weight (1, 3, 6, or 12 mg/kg) examined from 0 to 180 min after ingestion.

After dose optimization and determination of the time frame to be examined, subsequent studies used a 6 mg/kg dose of [2-^{13}C] uracil evaluated over 180 min. The UraBT

indices (C_{max}, T_{max}, DOB_{50} and PDR) were determined in 50 healthy individuals [19 men and 32 women; mean age, 30 years (range, 19–70 years)] and seven partially DPD deficient individuals [six men and one woman; mean age, 35 years (range, 23–59 years)] and one profoundly DPD-deficient individual (one woman; age, 58 years). Two of the partially DPD-deficient individuals who participated in the initial dose escalation studies also participated in this phase of the study. In addition, all individuals participating in this study were phenotypically characterized by a DPD radioassay and genotypically characterized for known sequence variations associated with DPD deficiency by DHPLC with confirmation by sequence analysis as described below.

Uracil Breath Test

Following an overnight fast, volunteers started the protocol at approximately 8:00 a.m. All volunteers were weighed and baseline breath samples were collected in 1.2-liter aluminum-lined bags (Otsuka Pharmaceuticals, Tokushima, Japan). Volunteers then ingested an aqueous solution (over a period of 15 s) containing 6 mg/kg bodyweight of [2-^{13}C] uracil (99.9 %; Cambridge Isotope Laboratories Inc., Andover, MA). This was followed by the collection of 21 breath samples over 180 min (obtained every 5 min for 30 min and then every 10 min thereafter). The $^{13}CO_2$ / $^{12}CO_2$ ratio of each breath sample was determined by IR spectroscopy using the UBiT-IR_{300} instrument and data analysis performed as described above.

DPD Radioassay

Sixty ml of blood was drawn from a volunteer's peripheral vein at approximately 12 p.m. on the same day as their UraBT to limit variation resulting from the circadian rhythm in DPD enzyme activity (19). DPD activity in peripheral blood mononuclear cells (PBMC) was determined using a radioassay described previously (8). Individuals with PBMC DPD activity ≤ 0.18 nmol/min/mg protein were considered to be DPD-deficient (5).

DHPLC Analysis of the *DPYD* Gene

DHPLC analysis was used to genotype the coding region of the *DPYD* gene of "normal" and DPD-deficient individuals as described previously (11). All *DPYD* sequence variants identified by DHPLC were confirmed by DNA sequencing using a dideoxynucleotide chain termination method (Big Dye Kit; Applied Biosystems, Foster City, CA) and capillary electrophoresis on an ABI 310 Automated DNA Sequencer (Applied Biosystems).

Discrimination of "Normal" and DPD-Deficient Individuals

Statistical comparisons between the 50 healthy and eight DPD-deficient individuals (seven partially and one profoundly deficient) for each of the UraBT indices (C_{max}, T_{max}, DOB_{50} and PDR) were made using the two-sample *t*-test and the signed rank Wilcoxon test. The performance of the UraBT as a potential diagnostic test was evaluated through statistical classification procedures using linear discriminant functions (a mathematical rule for categorizing subjects as DPD deficient or healthy based upon their breath

patterns). The accuracy of the resulting classification was assessed through the observed false positive rate (FPR = proportion of healthy individuals incorrectly categorized as DPD-deficient) and the observed false negative rate (FNR = proportion of DPD-deficient individuals incorrectly categorized as healthy). The sensitivity and specificity of the UraBT were defined as the percentage of healthy and DPD-deficient individuals that were correctly classified [(1-FPR) x 100% and (1-FNR) x 100%, respectively]. Additionally, the performance of this classification scheme was further evaluated using cross-validation.

Assay Variability

Interassay variability was examined using repeated measures ANOVA to find any significant differences between breath test profiles within seven individuals (four healthy and three partially DPD-deficient) who repeated the UraBT from 1 to 8 months after their initial examination. Additionally, equivalence between the individuals' different UraBT profiles was examined using a bioequivalence test as described by Phillips (20) and Diletti *et al.* (21). Intra-assay variability was examined among four healthy individuals (five repetitions), analyzed using repeated measures ANOVA, and summarized by coefficient of variation.

Assessment of Breath Collection Bag Integrity with Time

The integrity of the breath collection bags was assessed following storage at room temperature for up to 210 days. One hundred and thirty-two samples from 24 volunteers underwent duplicate analysis 90 (n=30), 120 (n=32), 150 (n=20), 180 (n=25) or 210

(n=25) days after their initial examination. Integrity was analyzed using repeated measures ANOVA and summarized by coefficient of variation.

Statistical Analysis

All statistical summaries and analyses as described above were produced in SAS® Version 8.2, using procedures such as MEANS, GLM, MIXED, NLIN, NPAR1WAY and DISCRIM.

RESULTS

Preliminary UraBT Studies

Preliminary studies demonstrated that the administration of [2-^{13}C] uracil dose adjusted to kilogram (body weight) generated less variable $^{13}CO_2$ breath patterns and indices (C_{max}, T_{max}, DOB_{50}, and PDR) than single-fixed doses of 100, 200 or 300 mg. The variability in breath patterns between the largest (107 kg) and smallest (50 kg) volunteers (Volunteers A and D, respectively) when a fixed 300-mg dose of [2-^{13}C] uracil is administered is shown in Fig. 1*A*. The reduction in variability following standardization of the dose of [2-^{13}C] uracil to 6 mg/kg is shown in Fig. 1*B*. Similar results were also observed in the breath patterns from the other volunteers who received both fixed and weight-adjusted doses (data not shown). Preliminary studies also demonstrated that the optimal dose of [2-^{13}C] uracil that was needed to achieve maximal separation between the breath patterns of healthy and partially DPD-deficient volunteers was 6 mg/kg (data not shown). Time course studies from 0 to 180 min demonstrated that the elimination phase of $^{13}CO_2$

breath patterns from normal and DPD-deficient individuals could be examined within 180 min (data not shown).

Discrimination of Normal and DPD-Deficient Volunteers Using the UraBT

The $^{13}CO_2$ breath patterns and PDR (mean ± SE) from normal, partially, and profoundly DPD-deficient volunteers are shown in Figs. 2 and 3, respectively. Highly significant differences in the UraBT indices (C_{max}, T_{max}, DOB_{50} and PDR) were observed between normal and DPD-deficient individuals (all *P*s < 0.001; Table 1) .

Multiple linear discriminant functions were computed to determine which UraBT indices best classified DPD-deficient individuals. The discriminant function fitted on the DOB_{50} was demonstrated to be the optimal classification rule over all other time points. Subjects having a $DOB_{50} < 128.9$ were classified as DPD deficient and those with a DOB_{50} value ≥ 128.9 were classified as normal. Using these criteria, the UraBT demonstrated an observed 100% sensitivity (with all of the DPD-deficient individuals correctly identified as DPD deficient) and 96% specificity (with 48 of 50 individuals correctly classified as normal).

Inter- and Intra-Assay Variability

Assessment of the interassay variability of the UraBT demonstrated the results were reproducible with no significant differences observed between original breath patterns and those obtained from a second UraBT repeated several months later. Bioequivalence was significantly demonstrated at a 5% level of significance. All intraassay coefficient of variations were less than 5%.

Integrity of Breath Collection Bags

$^{13}CO_2$ content in breath collection bags stored at room temperature was assessed 90, 120, 150, 180, or 210 days after their initial examination. No significant differences were observed between the $^{13}CO_2$ content of sample bags before and after storage. $^{13}CO_2$ levels of breath samples before and after storage were highly correlated ($R^2 > 99\%$; Fig. 4).

DPD Enzyme Activity by Radioassay

The DPD enzyme activity of all individuals enrolled in this study was determined. The mean (± SE) PBMC DPD activity of the 50 "normal" individuals was 0.30 ± 0.01 nmol/min/mg protein (range, 0.19 to 0.44). The mean PBMC DPD activity of the seven partially deficient individuals was 0.10 ± 0.02 nmol/min/mg protein (range, 0.03 to 0.17). The PBMC DPD activity of the profoundly deficient individual was undetectable.

DPYD Genotype

No sequence variants previously associated with DPD deficiency were identified in the *DPYD* gene of the "normal" individuals (data not shown). Table 2 summarizes the sequence variations identified in the partial and profoundly DPD deficient individuals (D-1 through D-8). D-1, D-2, D-3, and D-4, demonstrated a heterozygous *DPYD**2A* (IVS14+1G>A) genotype and a partially DPD-deficient phenotype. D-3 also demonstrated an additional heterozygous sequence variant in exon 19 (2329G>T, A777S). D-6 demonstrated a homozygous *DPYD**2A* (IVS14+1G>A) genotype and a profoundly DPD- deficient phenotype (no detectable DPD enzyme activity). D-7 demonstrated three

heterozygous sequence variations [*DPYD**13 (1679T >G, I560S), *DPYD*9A* (85 T>C, C29R) and 496 A>G, (M166V)] and a partially DPD-deficient phenotype. D-5 and D-8 had no known sequence variants previously associated with DPD deficiency within the coding region of the *DPYD* gene, although a partially DPD-deficient phenotype was demonstrated.

DISCUSSION

In the United States, adverse drug reactions (ADR) have been shown to account for over 100,000 fatalities/year and remain the fourth leading cause of death after heart disease, cancer, and stroke (22). It is estimated that 31-34% of the 2 million patients who receive 5-FU/year exhibit severe toxicity (including death) and that approximately 50% of these patients with adverse drug reactions secondary to 5-FU have reduced DPD activity or no DPD activity (3, 23–25). Although the frequency and lethality of DPD deficiency distinguishes this syndrome from other pharmacogenetic disorders (*i.e.* thiopurine methyltransferase deficiency), the clinical diagnosis of DPD deficiency remains difficult because the appearance of life-threatening toxicity is typically the first symptom of this pharmacogenetic syndrome. Despite over 10 years of research, clinically feasible assays to identify DPD-deficient individuals are not yet available in cancer treatment facilities.

In the current study, a facile, non-invasive UraBT was developed and optimized to discriminate between healthy, partially or profoundly DPD deficient individuals. This approach exploits the difference in [2-^{13}C] uracil catabolism between normal and DPD-deficient individuals. Previous pharmacokinetic studies from our laboratory in a profoundly DPD-deficient patient demonstrated reduced 5-FU catabolism with prolonged

elimination half-life compared to the "normal" population (26). Additional studies by our laboratory using eniluracil (a potent DPD inhibitor) showed similar results (27). Taken collectively, these data suggested that the reduced catabolism of [2-^{13}C] uracil in DPD deficient individuals should result in a subsequent decline in $^{13}CO_2$ levels in breath.

Preliminary studies of the UraBT demonstrated that an administered dose of 6 mg/kg [2-^{13}C] uracil generated less variable breath indices (C_{max}, T_{max}) than single-fixed doses of 100, 200 or 300 mg (Fig. 1) and that the elimination phase of $^{13}CO_2$ in normal and DPD-deficient individuals could be examined within 180 min. Administration of 6 mg/kg of [2-^{13}C] uracil to normal, partially and profoundly DPD-deficient individuals showed a significantly reduced C_{max}, DOB_{50}, and PDR and a significantly increased T_{max} in deficient individuals compared to normal individuals (Figs. 2 and 3; Table 1). The maximal $^{13}CO_2$ δ over baseline differences in breath pattern indices were observed at 50 min comparing normal and DPD-deficient individuals ($p < 0.001$). Applying a cutoff value of 128.9 DOB_{50} min, all eight DPD-deficient individuals were correctly identified (100 % sensitivity). In addition, 48 of 50 normal individuals were also correctly identified (96% specificity). These data suggest that one time point (50 min) may be used to discriminate between normal and DPD-deficient patients, however, larger population studies are required to clearly establish the optimal time point where δ over baseline cut-off values would provide maximal separation.

To determine whether exhaled breath samples could be stored and shipped to distant sites for analysis, the integrity of the breath collection bags were examined for changes in $^{13}CO_2$ levels after storage at room temperature for up to 210 days. As shown in Fig. 4, no significant changes were observed in the $^{13}CO_2$ levels. Taken collectively,

the ability to use one time point to discriminate between normal and DPD-deficient individuals and the stability of the breath samples suggests that the UraBT offers the first potentially useful diagnostic assay for the identification of DPD-deficient individuals.

Interestingly, there was no strong correlation between PBMC DPD activity and any of the UraBT indices. This is likely because determining DPD enzyme activity from a single tissue (PBMC) cannot be directly compared to the systemic metabolism of [2-^{13}C] uracil by multiple organs (i.e. liver, colon, lung, brain, spleen, and PBMC) which are known to have various levels of DPD (28, 29).

All DPD deficient individuals examined in this study were genotyped to identify the molecular basis of their DPD deficiency (Table 2). Individuals D-1 through D-4 (with a partially DPD-deficient phenotype) demonstrated a heterozygous *DPYD**2*A* genotype, whereas D-6 (with a profoundly DPD deficient phenotype) demonstrated a homozygous *DPYD**2*A* genotype. D-3 demonstrated a heterozygous sequence variation in exon 19 (2329G>T, A777S) which has been observed previously in a partially DPD-deficient patient (30). D-7 (with a partially DPD-deficient phenotype) demonstrated a heterozygous *DPYD**13, *DPYD**9*A* and M166V genotype. Both *DPYD**2*A* and *DPYD**13 have been confirmed by our laboratory and others to result in DPD deficiency, while *DPYD**9*A* and M166V have been observed in individuals with "normal" activity (31–33). Interestingly, D-5 and D-8 were identified as DPD deficient (using both the radioassay and UraBT), however no known sequence variants were identified in the coding region of the *DPYD* gene. The molecular basis for DPD deficiency in these individuals remains unknown and may be due to sequence variations occurring in an untranslated

region of the *DPYD* gene (including the promoter, 5' untranslated region, 3' untranslated region or intronic regions).

Previous studies have suggested that approximately 50% of cancer patients with severe 5-FU toxicity are DPD deficient; however, the etiology of 5-FU toxicity in the remaining patients is unclear. Increased 5-FU toxicity has also been linked to deficiencies in the dihydropyrimidinase and β-ureidopropionase enzymes and alterations in the thymidylate synthase promoter (34–37). Future studies will examine whether similar methodologies may be used to identify patients with these other pyrimidine-catabolic disorders.

In summary, this study demonstrates that the UraBT can rapidly discriminate between normal, partially, and profoundly DPD-deficient individuals (within 50 min) and offers a useful screening method that can be applied in most clinical settings (*e.g.*, hospitals and physicians' offices) to identify DPD-deficient individuals before 5-FU chemotherapy. Furthermore, this novel approach may be used to collect samples at remote locations with subsequent analysis at a centralized reference laboratory

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Table 1 Uracil Breath Test Indices

Volunteers[a]	C_{max} (DOB)[b]	T_{max} (minutes)[b]	DOB_{50} (DOB)[b]	Percentage dose recovered[b]
Normal (n = 50)	186.4 ± 3.9	52.0 ± 2.0	174.1 ± 4.6	53.8 ± 1.0
Partially DPD[c] deficient (n = 7)	117.1 ± 9.8	100 ± 18.4	89.6 ± 11.6	36.9 ± 2.4
Profoundly DPD deficient (n = 1)	3.6	120	0.9	< 1

[a] Fifty normal and eight (seven partially and one profoundly) DPD-deficient individuals performed the [2-^{13}C] uracil breath test as described in "Materials and Methods."
[b] Highly significant differences in [2-^{13}C] uracil breath test indices were observed between normal and DPD-deficient individuals (all Ps < 0.001).
The data shown are expressed as a mean ±SE.
[c]DOB_{50}, δ over baseline 50 min following [2-^{13}C] uracil ingestion; DOB, δ over baseline; DPD, dihydropyrimidine dehydrogenase.

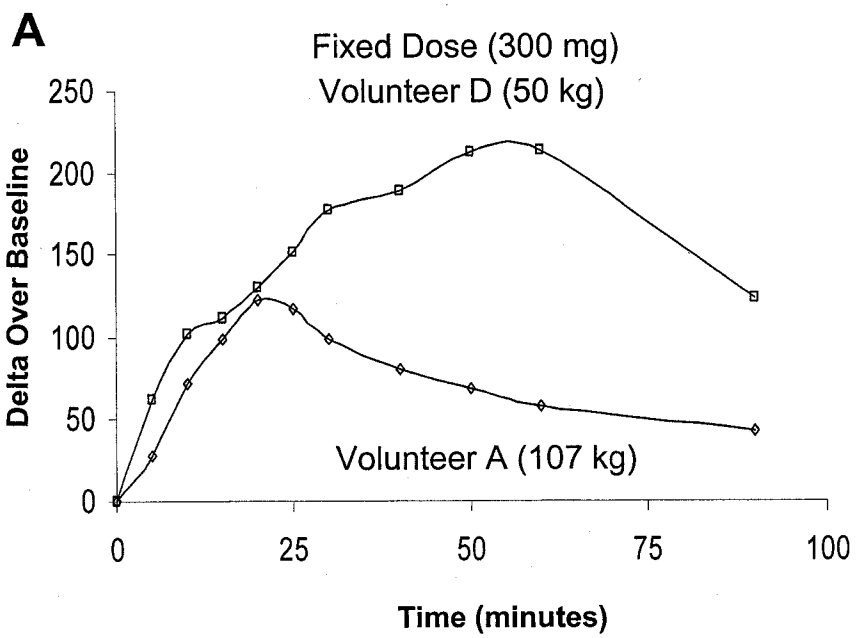
Table 2 Sequence Variants Identified in the Coding Region of the *DPYD* gene in DPD Deficient Volunteers

Location of *DPYD* sequence variant	D-1[a]	D-2	D-3	D-4	D-5	D-6	D-7[a]	D-8
Exon 2							DPYD*9A 85T>C C29R	
Exon 6							496A>G M166V	
Exon 13							DPYD*13 1679T>G I560S	
Exon 14	DPYD*2A IVS14+IG>A	DPYD*2A IVS14+IG>A	DPYD*2A IVS14+IG>A	DPYD*2A IVS14+IG>A		DPYD*2A IVS14+IG>A		
Exon 19			2329G>T A777S					
DPD enzyme activity (nmol/min/mg)	0.07	0.17	0.06	0.14	0.08	Undetectable	0.03	0.17

[a]Data from Johnson *et al*

Fig.1.

Examination of variability of $^{13}CO_2$ breath patterns after administration of a fixed dose (300 mg) and a weight-adjusted dose (6 mg/kg) of [2-^{13}C] uracil. Shown are the $^{13}CO_2$ breath patterns from two healthy volunteers [volunteers A (◇) and D (□)] after administration of a fixed 300-mg dose (*A*) and a weight-adjusted (6 mg/kg) dose (*B*) of [2-^{13}C] uracil. Less variability was observed in breath patterns when the dose of [2-^{13}C] uracil was standardized to weight (6 mg/kg). In the fixed dose studies (*A*), the dose adjusted to weight for largest (107 kg) individual (volunteer A) corresponded to 2.8 mg/kg, whereas the dose adjusted to weight for smallest (50 kg) individual (volunteer D) corresponded to 6 mg/kg.



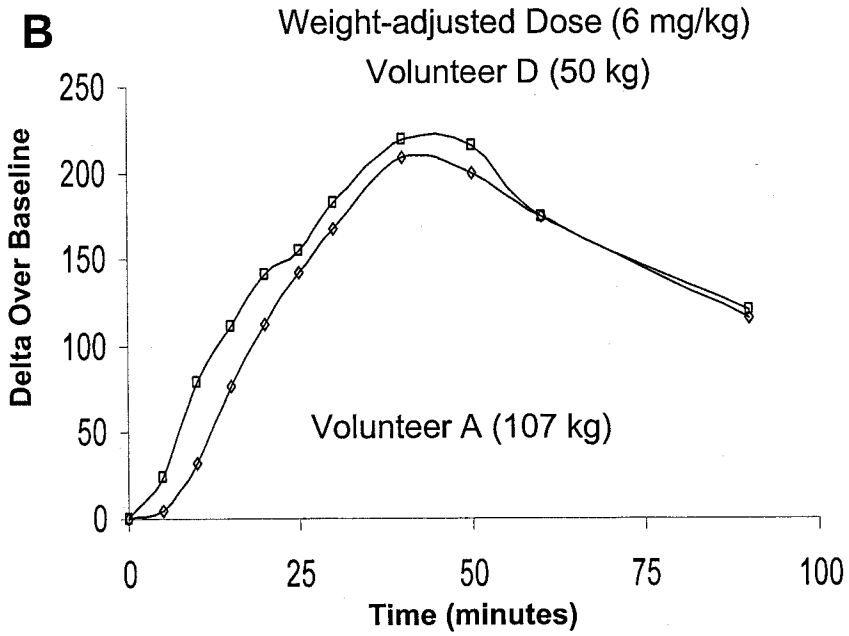
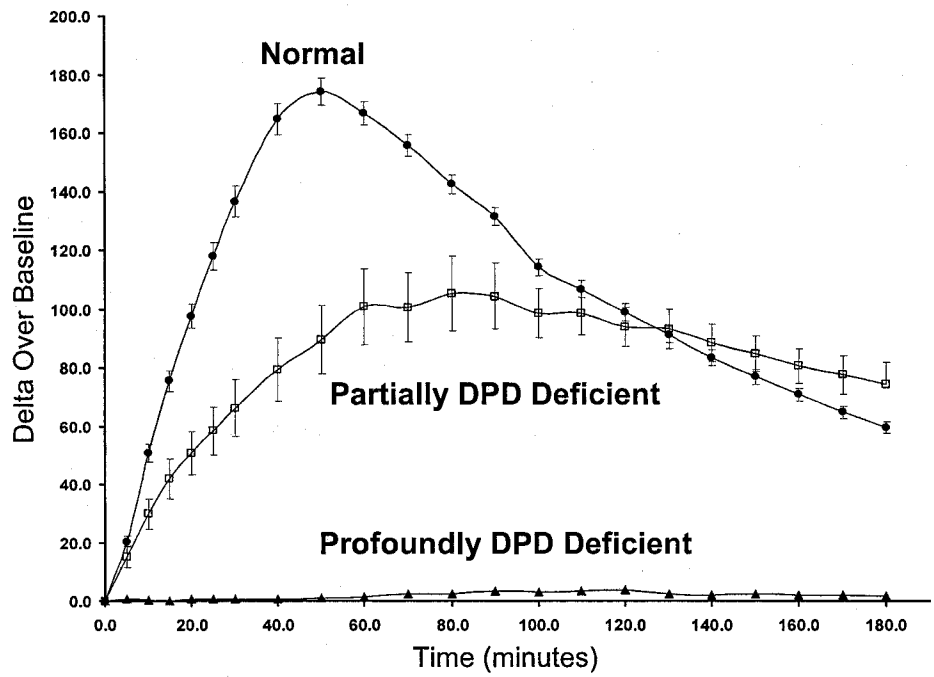


Fig. 2.

$^{13}CO_2$ breath patterns from 50 normal and 8 DPD-deficient volunteers. Normal (●), partially (□), and profoundly (▲) DPD-deficient individuals ingested a 6 mg/kg oral solution of [2-^{13}C] uracil. Breath samples were collected for 180 min after ingestion and the amount of ^{13}C label in breath (expressed as δ over baseline) was determined for each time point (mean ± SE).

Fig. 3.

Percentage of ^{13}C dose recovered from the breath of 50 normal and 8 DPD-deficient volunteers. Normal (●), partially (□), and profoundly (▲) DPD-deficient individuals ingested a 6 mg/kg oral solution of [2-^{13}C] uracil. Breath samples were collected for 180 min after ingestion, and the percentage of ^{13}C dose recovered in breath was determined for each time point (mean ± SE).



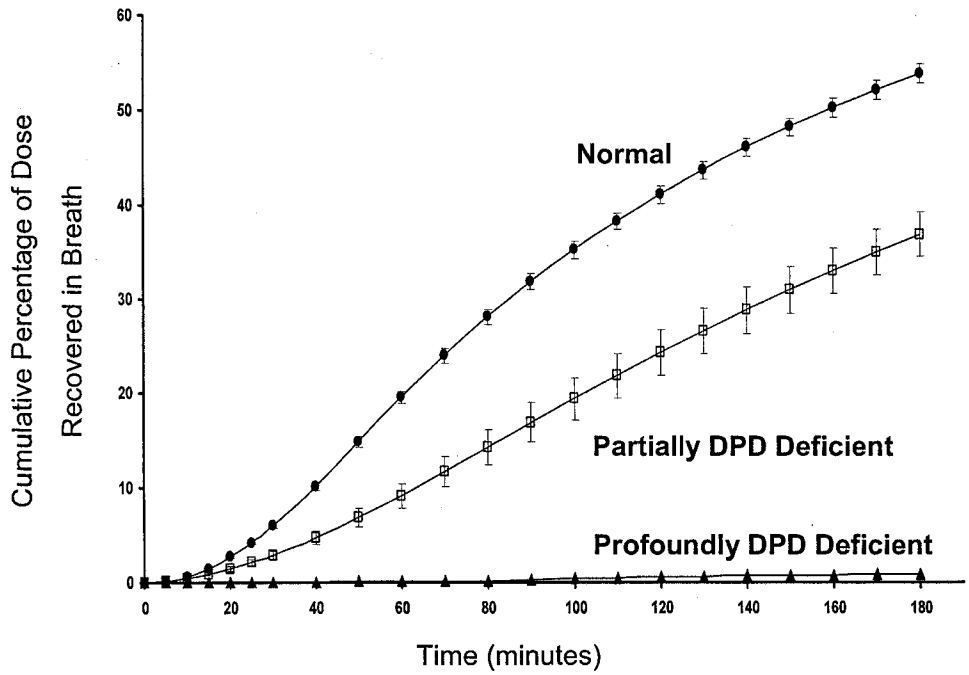
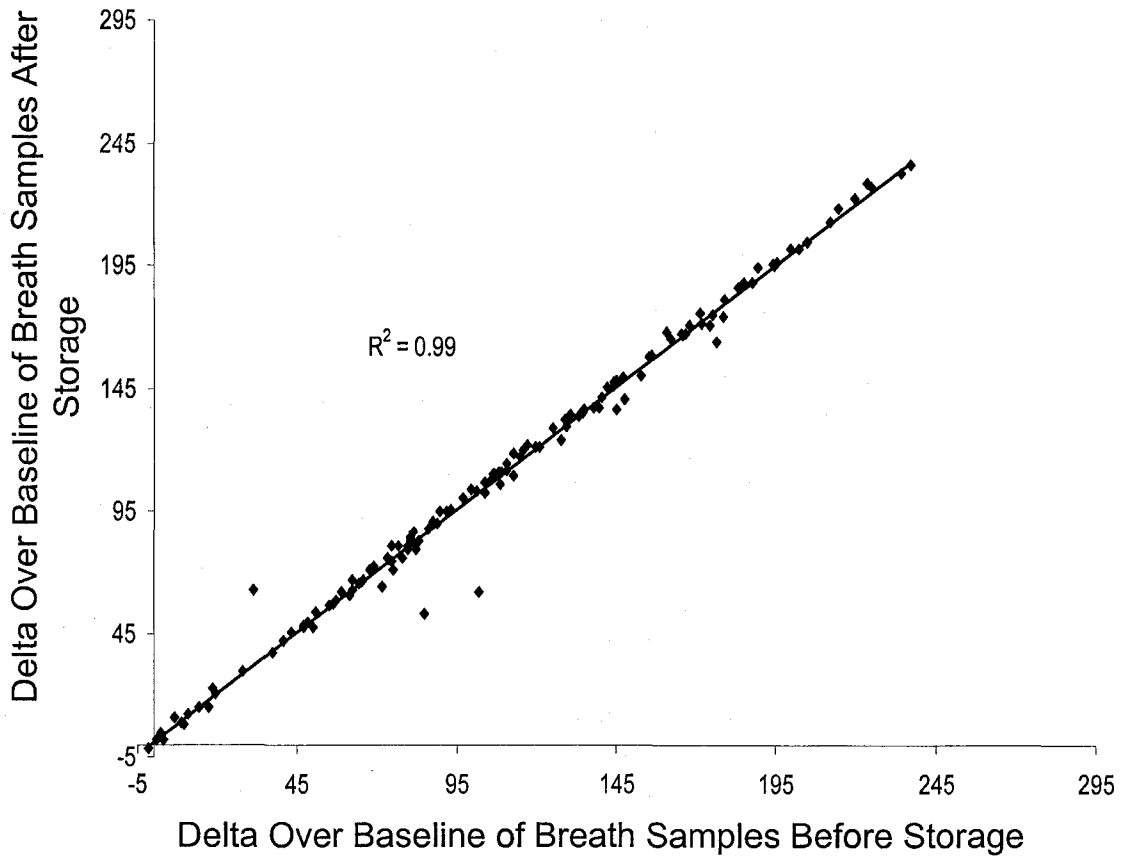


Fig. 4. Integrity of breath collection bags. One hundred thirty-two breath samples underwent duplicate analysis after storage at room temperature for 90 (n = 30), 120 (n = 32), 150 (n = 20), 180 (n = 25), or 210 (n = 25) days after their initial examination to establish the ability of breath collection bags to retain $^{13}CO_2$.



THE URACIL BREATH TEST IN THE ASSESSMENT OF DIHYDROPYRIMIDINE DEHYDROGENASE ACTIVITY: PHARMACOKINETIC RELATIONSHIP BETWEEN EXPIRED $^{13}CO_2$ AND PLASMA [2-^{13}C] DIHYDROURACIL

by

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ABSTRACT

Purpose: Dihydropyrimidine dehydrogenase (DPD) deficiency is critical in the predisposition to 5-fluorouracil dose-related toxicity. We recently characterized the phenotypic [2-^{13}C] uracil breath test (UraBT) with 96% specificity and 100% sensitivity for identification of DPD deficiency. In the present study, we characterize the relationships among UraBT-associated breath $^{13}CO_2$ metabolite formation, plasma [2-^{13}C] dihydrouracil formation, [2-^{13}C] uracil clearance, and DPD activity.

Experimental Design: An aqueous solution of [2-^{13}C] uracil (6 mg/kg) was orally administered to 23 healthy volunteers and 8 cancer patients. Subsequently, breath $^{13}CO_2$ concentrations and plasma [2-^{13}C] dihydrouracil and [2-^{13}C] uracil concentrations were determined over 180 minutes using IR spectroscopy and liquid chromatography-tandem mass spectrometry, respectively. Pharmacokinetic variables were determined using noncompartmental methods. Peripheral blood mononuclear cell (PBMC) DPD activity was measured using the DPD radioassay.

Results: The UraBT identified 19 subjects with normal activity, 11 subjects with partial DPD deficiency, and 1 subject with profound DPD deficiency with PBMC DPD activity within the corresponding previously established ranges. UraBT breath $^{13}CO_2$ DOB_{50} significantly correlated with PBMC DPD activity ($r_p = 0.78$), plasma [2-^{13}C] uracil area under the curve ($r_p = -0.73$), [2-^{13}C] dihydrouracil appearance rate ($r_p = 0.76$), and proportion of [2-^{13}C] uracil metabolized to [2-^{13}C] dihydrouracil ($r_p = 0.77$; all Ps < 0.05).

Conclusions: UraBT breath $^{13}CO_2$ pharmacokinetics parallel plasma [2-^{13}C] uracil and [2-^{13}C] dihydrouracil pharmacokinetics and are an accurate measure of interindividual variation in DPD activity. These pharmacokinetic data further support the future use of

the UraBT as a screening test to identify DPD deficiency before 5-fluorouracil-based therapy.

INTRODUCTION

Dihydropyrimidine dehydrogenase (EC 1.3.1.2, DPD) is the rate-limiting enzyme in uracil and 5-fluorouracil (5-FU) catabolism, converting > 80% of an administered dose of 5-FU to inactive metabolites (1, 2). The initial step of catabolism is mediated by DPD converting 5-FU to 5-dihydrofluorouracil, with subsequent catabolism by dihydropyrimidinase and β-ureidopropionase enzymes to ultimately produce fluoro-β-alanine, ammonia, and CO_2. The latter final metabolic end-products are excreted in the urine and breath (3).

The pharmacogenetic syndrome of complete and partial DPD deficiency is prevalent in ~0.1% and 3% to 5% of the general population, respectively (4). DPD deficiency is a significant pharmacogenetic factor in the predisposition of cancer patients to increased risk of altered 5-FU pharmacokinetics and associated toxicity. Specifically, 60% of patients presenting with severe 5-FU-related hematologic toxicity showed reduced DPD activity (5).

Recent studies have investigated the predictive value of the ratio of plasma dihydrouracil area under the curve (AUC) to uracil AUC (DUUR) for the assessment of DPD activity and potential individualization of 5-FU therapy. Specifically, 5-FU dose optimization may be based on the plasma DUUR observed before 5-FU administration (6). Jiang et al. have also showed that the pre-5-FU treatment DUUR may be a good index of DPD activity (7, 8).

Our laboratory recently reported the rapid noninvasive phenotypic [2-^{13}C] uracil breath test (UraBT) for assessment of DPD activity with 96% specificity and 100% sensitivity (9). Application of the UraBT to a large population of cancer-free subjects (n = 255) showed an observed 86% sensitivity (with 12 of 14 DPD-deficient subjects identified as DPD deficient) and 99% specificity (with 239 of 241 subjects with DPD activity in the reference range identified as normal; ref. 10). To date, however, the clinical relationship between pharmacokinetics of the [2-^{13}C] uracil probe substrate and its metabolites in plasma and breath remains to be elucidated. Based on our initial characterization of the UraBT, we hypothesize that (a) [2-^{13}C] uracil metabolite pharmacokinetic variables in the breath and plasma are reflective of DPD activity and (b) breath $^{13}CO_2$ concentrations as measured through the UraBT correlate with plasma [2-^{13}C] uracil metabolite pharmacokinetics. In the present study, we provide a detailed characterization of the UraBT showing the relationship among breath $^{13}CO_2$ metabolite formation, plasma [2-^{13}C] dihydrouracil formation, [2-^{13}C] uracil clearance, and DPD activity.

MATERIALS AND METHODS

Subjects

Thirty-one subjects (16 men and 15 women; ages 19-70 years) participated in this institutional review board-approved pharmacokinetic examination that was conducted at the General Clinical Research Center at the University of Alabama at Birmingham. Eight subjects were cancer patients who were referred by their oncologist due to known or suspected DPD deficiency. Twenty-three subjects were participants from the University of Alabama at Birmingham campus who volunteered for examination after reading an insti-

tutional review board-approved advertisement placed in the campus newspaper. Due to the rarity of DPD deficiency in the general population, we included six DPD-deficient individuals previously phenotyped [UraBT and DPD peripheral blood mononuclear cell (PBMC) radioassay] and genotyped (denaturing high-performance liquid chromatography analysis of the *DPYD* gene) in the current pharmacokinetic examination (9). Subjects with a history of gastric (i.e., dyspepsia) or respiratory (i.e., asthma) disease were excluded from the study.

DPD Radioassay

PBMC DPD activity was determined for all subjects as described previously (11, 12). To minimize interassay variation in enzyme activity, 60 mL whole blood was collected into heparinized vacutainers at ~12 p.m. on the day of testing and processed within 10 minutes of collection. After Ficoll separation of whole blood, isolated PBMCs were washed with PBS and lysed. The cytosol was collected after cellular debris was removed by centrifugation. The concentration of cytosolic protein was quantified by the Bradford method (13). A reaction mixture containing 250 μg cytosolic protein, NADPH, buffer A, and [6-^{14}C] 5-FU was incubated for 30 minutes. Every 5 minutes, 130 μL aliquots were removed and added to an equal volume of ice-cold ethanol. This mixture was incubated overnight at -80°C, thawed, and filtered to remove protein before high-performance liquid chromatography analysis. [6-^{14}C] 5-FU and [6-^{14}C] FUH_2 were separated and quantified using a previously described reverse-phase high-performance liquid chromatography method (11, 12). The amount of [6-^{14}C] FUH_2 formed at each time point (Y axis) was plotted against time (X axis). Linear regression analysis was used to calculate the equa-

tion of the line and determine the formation rate of [6-^{14}C] FUH_2. DPD enzyme activity was calculated by dividing the formation rate of [6-^{14}C] FUH_2 by the amount of protein used in the reaction mixture (i.e., nmol/min/mg protein). Subjects were considered to be partially DPD deficient by radioassay when their fresh PBMC DPD activity was < 0.18 nmol/min/mg protein (11). Subjects were considered to be profoundly DPD deficient by radioassay when their PBMC DPD activity was undetectable.

Uracil Breath Test

The UraBT principle and detailed methodology has been described previously (9). At ~8 a.m. on the day of testing, fasting subjects were weighed and an aqueous solution containing 6 mg/kg [2-^{13}C] uracil (Cambridge Isotope Laboratories, Inc., Andover, MA) was prepared. Subjects donated three baseline breath samples into 1.2 L breath bags (Otsuka Pharmaceutical, Tokushima, Japan) followed by oral administration of the [2-^{13}C] uracil solution. Post-dose breath samples were collected into 100 mL breath bags (Otsuka Pharmaceutical) during the 180-minute period immediately following [2-^{13}C] uracil administration. IR spectrophotometry (UBiT-IR_{300}, Meretek, Lafayette, CO) was used to measure breath $^{13}CO_2$ concentrations, which were reported in delta over baseline (DOB) notation as described previously (9). Breath profiles were constructed by plotting the concentration of $^{13}CO_2$ in breath at each time point (Y axis) against time (X axis). The percent dose of [2-^{13}C] uracil recovered in the breath as $^{13}CO_2$ (PDR) was calculated as described elsewhere (14). Breath $^{13}CO_2$ maximum plasma concentration (C_{max}), time to C_{max} (T_{max}), and DOB_{50} ($^{13}CO_2$ concentration in breath 50 minutes after [2-^{13}C] uracil administration) were determined by inspection of breath profiles (9). Subjects showing a

DOB_{50} < 128.9 DOB were classified as DPD deficient (9). Subjects showing a $DOB_{50} \geq$ 128.9 DOB were classified as having normal DPD activity (9).

Liquid Chromatography-Tandem Mass Spectrometry Analysis of Plasma [2-^{13}C] Uracil and [2-^{13}C] Dihydrouracil Concentrations

While each subject performed the UraBT, whole blood was simultaneously collected via a heparin lock placed in the participant's arm. A baseline blood sample was collected immediately before oral administration of the [2-^{13}C] uracil solution. Post-dose blood samples were collected into heparinized (green-top) vacutainers at 5, 10, 15, 20, 25, 30, 50, 60, 90, 120, and 180 minutes following [2-^{13}C] uracil administration. Blood was immediately processed after collection and plasma was isolated as follows: 3 mL whole blood was centrifuged at 4°C for 10 minutes at 2200 x g; plasma was immediately pipetted into polypropylene tubes and then stored at -80°C until analysis by liquid chromatography tandem mass spectrometry. Detection and quantification of plasma [2-^{13}C] uracil and [2-^{13}C] dihydrouracil was done following minor modification of a previously described liquid chromatography-tandem mass spectrometry method (15). Briefly, isotope-labeled [$^{13}C_4$,$^{15}N_2$] uracil and [$^{13}C_4$,$^{15}N_2$] dihydrouracil (Cambridge Isotope Laboratories) were used as internal standards. Plasma protein was precipitated by adding 500 μL of a saturated ammonium sulfate solution and 4 mL acetronitrile to 500 μL plasma. Following centrifugation, the organic layer was collected, evaporated, and reconstituted in 200 μL purified water. The mixture was injected into the liquid chromatography tandem mass spectrometry system (TSQ7000, Thermo Finnigan, San Jose, CA). [2-^{13}C] Uracil and [2-^{13}C] dihydrouracil were separated on a Develosil RAPQUEOUS reverse-phase column (5 μm, 2.0 x150 mm; Normura Chemical Co., Ltd., Seto, Japan) in a mobile

phase of 1:99 (v/v) methanol/water. Atmospheric pressure chemical ionization was used to form protonated analytes and fragment them. Selected reaction monitoring was used to detect the fragmentation pattern of parent and daughter ions and quantify the concentrations of [2-^{13}C] uracil and [2-^{13}C] dihydrouracil.

Pharmacokinetic Analysis

Concentration-time profiles of plasma [2-^{13}C] uracil and [2-^{13}C] dihydrouracil were constructed. Noncompartmental methods (WinNonlin version 4.1, Pharsight Corp., Mountain View, CA) were used to determine the pharmacokinetic variables of [2-^{13}C] uracil in plasma, [2-^{13}C] dihydrouracil in plasma, and $^{13}CO_2$ in breath. Calculated pharmacokinetic variables were AUCτ , C_{max}, T_{max}, apparent clearance (CL/F), terminal apparent distribution volume (Vz/F), and elimination half-life ($t_{1/2}$). AUCτ was determined using the trapezoidal rule (16). C_{max} and T_{max} were taken directly from the observed concentration-time data. CL/F was calculated as dose/AUCτ . Vz/F was calculated as dose divided by the product of terminal elimination rate constant, λ_z, and AUCτ . The elimination rate constant was determined by linear regression of the terminal elimination phase concentration-time points; $t_{1/2}$ was calculated as ln(2)/ λ_z.

[2-^{13}C] Dihydrouracil may only be produced in appreciable quantities *in vivo* by the DPD-mediated catabolism of [2-^{13}C] uracil. To assess formation of [2-^{13}C] dihydrouracil (metabolite) from [2-^{13}C] uracil (probe substrate) by DPD, the [2-^{13}C] dihydrouracil appearance rate, amount of [2-^{13}C] dihydrouracil formed, and proportion of [2-^{13}C] uracil metabolized to [2-^{13}C] dihydrouracil were estimated. The [2-^{13}C] dihydrouracil appearance rate in plasma was determined from the slope of the line following

regression analysis of the plasma [2-^{13}C] dihydrouracil concentration-time plot from baseline (t = 0 minute) to C_{max}. The amount of [2-^{13}C] dihydrouracil formed was calculated by multiplying [2-^{13}C] dihydrouracil AUCτ and clearance (17). The proportion of [2-^{13}C] uracil metabolized to [2-^{13}C] dihydrouracil was calculated by dividing the amount of [2-^{13}C] dihydrouracil formed over 180 minutes by the amount of orally administered [2-^{13}C] uracil.

Statistical Analysis

Summary data stratified by DPD activity are presented as mean ± SD. Comparisons of plasma [2-^{13}C] uracil and [2-^{13}C] dihydrouracil concentrations and pharmacokinetic variables between subjects with normal DPD activity and subjects with partial DPD deficiency were assessed by bootstrap *t* tests of hypotheses using PROC MULTTEST in SAS version 9.1. The bootstrap *P*s were compared with the raw *P*s to assess nonnormality of inferences. If the bootstrap *P* was close to the normality-assuming *P*, we concluded that nonnormality was not a concern for the particular variable. For comparisons between the subjects with normal DPD activity and the one subject with profound DPD deficiency, we used the *t* test to perform a single mean comparison to test the mean of subjects with normal DPD activity for each variable against the value for the profoundly DPD-deficient individual. Correlations among UraBT DOB_{50}, PBMC DPD activity, and plasma [2-^{13}C] uracil and [2-^{13}C] dihydrouracil pharmacokinetic variables were evaluated using Pearson's correlation coefficient. For all analyses, $P < 0.05$ was deemed statistically significant.

RESULTS

Determination of PBMC DPD Activity

The DPD enzyme activity was determined for all subjects (mean ± SD). Nineteen subjects showed normal DPD activity (0.27 ± 0.06 nmol/min/mg), 11 subjects showed partial DPD deficiency (0.11 ± 0.05 nmol/min/mg), and 1 subject showed profound deficiency (undetectable DPD activity).

Detection of DPD Deficiency by UraBT

UraBT indices (mean ± SD) obtained in subjects with normal and reduced DPD activity are summarized in Table 1. The UraBT showed 100% agreement with the PBMC radioassay. Subjects with DPD activity in the reference range showed UraBT $DOB_{50} \geq$ 128.9 DOB. All partially and profoundly DPD-deficient subjects showed $DOB_{50} <$ 128.9 DOB. Altered breath $^{13}CO_2$ concentration-time profiles were also observed in all DPD-deficient subjects. Specifically, profoundly and partially DPD-deficient subject(s) showed an increased UraBT T_{max} and reduced UraBT $^{13}CO_2$ C_{max}, DOB_{50}, AUC, and PDR compared with subjects with normal DPD activity (all *P*s < 0.05). UraBT DOB_{50} also showed significant correlation with PBMC DPD activity (Fig. 1A).

Comparison of Plasma [2-^{13}C] Uracil Pharmacokinetics in Subjects with Normal and Reduced PBMC DPD Activity

Plasma [2-^{13}C] uracil pharmacokinetic variables (mean ± SD) obtained from subjects with normal and reduced DPD activity are summarized in Table 2. [2-^{13}C] Uracil was detectable in the plasma of most subjects 5 minutes after oral administration (Fig. 2A). No statistically significant differences were observed in plasma [2-^{13}C] uracil C_{max}

between subjects with normal activity and those with partial or profound DPD deficiency. No significant difference was observed in plasma [2-^{13}C] uracil T_{max} from subjects with normal DPD activity and subjects with partial DPD deficiency. However, a significant difference was observed in plasma [2-^{13}C] uracil T_{max} from the subjects with normal DPD activity and the profoundly DPD-deficient subject.

Reduced [2-^{13}C] uracil catabolism was observed in all DPD deficient subjects (Fig. 2A). Both profoundly and partially DPD-deficient subject(s) showed increased plasma [2-^{13}C] uracil $t_{1/2}$ and AUC and reduced plasma [2-^{13}C] uracil clearance compared with subjects with normal DPD activity (all Ps < 0.05).

PBMC DPD activity was significantly correlated with several pharmacokinetic variables of uracil catabolism. Specifically, PBMC DPD was significantly correlated with plasma [2-^{13}C] uracil clearance (Fig. 1B) and inversely correlated with plasma [2-^{13}C] uracil AUC and $t_{1/2}$ (all Ps < 0.05; Table 3).

The UraBT DOB_{50} were also significantly correlated with several pharmacokinetic variables of uracil catabolism. Specifically, the UraBT DOB_{50} were significantly correlated with plasma [2-^{13}C] uracil clearance and inversely correlated with plasma [2-^{13}C] uracil AUC (Fig. 1C) and $t_{1/2}$ (all Ps < 0.05; Table 3).

Comparison of [2-^{13}C] Dihydrouracil Plasma Pharmacokinetics in Subjects with Normal and Reduced PBMC DPD Activity

Plasma [2-^{13}C] dihydrouracil pharmacokinetic variables (mean ± SD) obtained in subjects with normal and reduced DPD activity are summarized in Table 4. Altered plasma [2-^{13}C] dihydrouracil concentrations were observed in DPD deficient subjects (Fig. 2B). The profoundly deficient subject showed plasma [2-^{13}C] dihydrouracil concentrations beneath the limit of detection; thus, pharmacokinetic variables could not be determined. Partially deficient subjects showed significantly decreased plasma [2-^{13}C] dihydrouracil C_{max} and increased plasma [2-^{13}C] dihydrouracil T_{max} and $t_{1/2}$ compared with subjects with normal DPD activity (all Ps < 0.05). Partially deficient subjects also showed a significant reduction in the proportion of [2-^{13}C] uracil metabolized to [2-^{13}C] dihydrouracil, [2-^{13}C] dihydrouracil appearance rate, amount of [2-^{13}C] dihydrouracil formed, and plasma DUUR (all Ps < 0.05).

[2-^{13}C] Dihydrouracil formation and concentrations were significantly correlated with PBMC DPD activity (Table 3). Specifically, PBMC DPD activity was significantly correlated with the proportion of [2-^{13}C] uracil metabolized to [2-^{13}C] dihydrouracil, [2-^{13}C] dihydrouracil appearance rate, amount of [2-^{13}C] dihydrouracil formed, plasma C_{max}, and plasma DUUR (all Ps < 0.05). PBMC DPD activity was also inversely correlated with plasma [2-^{13}C] dihydrouracil T_{max} (P < 0.05).

[2-^{13}C] Dihydrouracil formation and concentrations were significantly correlated with UraBT DOB_{50} (Table 3). In particular, UraBT DOB_{50} were significantly correlated with the proportion of [2-^{13}C] uracil metabolized to [2-^{13}C] dihydrouracil, [2-^{13}C] dihydrouracil appearance rate, amount of [2-^{13}C] dihydrouracil formed (Fig. 1D), plasma

DUUR, and plasma [2-^{13}C] dihydrouracil C_{max} (all Ps < 0.05). UraBT DOB_{50} were inversely correlated with plasma [2-^{13}C] dihydrouracil T_{max} ($P < 0.05$).

DISCUSSION

Identification of DPD-deficient cancer patients is important in optimizing 5-FU chemotherapy and minimizing life threatening dose-related toxicity. We developed the UraBT, which may be used to screen cancer patients for DPD deficiency before administration of 5-FU (9). The principle of the UraBT was based on earlier metabolic studies that showed uracil and 5-FU are both degraded by the enzymes of the pyrimidine catabolic pathway, with the DPD enzyme having similar affinities for 5-FU and uracil (18–20). These studies provided a basis for use of the nontoxic [2-^{13}C] uracil probe substrate in the UraBT to assess *in vivo* pyrimidine catabolism. Our initial examination of 50 volunteers and 8 DPD-deficient subjects suggested that the UraBT may be a good indicator of DPD activity. In this study, significantly reduced breath $^{13}CO_2$ concentrations (DOB_{50}, C_{max}, AUC, and PDR) were observed from enrolled subjects with DPD deficiency versus those with normal DPD activity. Furthermore, the UraBT detected DPD deficiency with 96% specificity and 100% sensitivity (9). A more recent study of 255 subjects has corroborated our initial findings, with the UraBT showing 99.2% specificity and 85.7% sensitivity for detecting DPD deficiency (10). In the present study, we further validate the UraBT in a population of subjects with normal and reduced DPD activity by comparing breath $^{13}CO_2$ kinetic profiles to plasma [2-^{13}C] uracil and [2-^{13}C] dihydrouracil kinetics.

Examination of plasma [2-^{13}C] uracil concentration-time profiles showed that orally administered [2-^{13}C] uracil was rapidly absorbed and detected in the plasma of

most subjects within 5 minutes of administration. This observation is in agreement with an earlier animal study, which also reported rapid absorption following oral administration of [2-^{13}C] uracil (15).

Following absorption of [2-^{13}C] uracil in subjects with normal DPD activity, the [2-^{13}C] uracil was observed to peak at ~28.9 ± 9.5 minutes. Subsequently, plasma concentrations decreased reflecting both metabolism and elimination as indicated by the appearance of [2-^{13}C] dihydrouracil in the plasma (within 10 minutes) and $^{13}CO_2$ in the breath. Following absorption of [2-^{13}C] uracil in subjects with partial and profound DPD deficiency, significant differences in both metabolism and elimination were noted as indicated by decreased plasma [2-^{13}C] uracil clearance, decreased appearance of [2-^{13}C] dihydrouracil in plasma, and decreased $^{13}CO_2$ concentrations in breath compared with subjects with normal DPD activity. In particular, a significant reduction in the appearance of [2-^{13}C] dihydrouracil in the plasma was observed between partially DPD-deficient subjects and subjects with normal DPD activity, whereas the profoundly DPD-deficient subject showed no detectable plasma [2-^{13}C] dihydrouracil (Fig. 2B). These results suggest that the [2-^{13}C] dihydrouracil appearance rate may be a direct indicator of DPD activity. This conclusion is based on the rationale that DPD-mediated metabolism of [2-^{13}C] uracil to [2-^{13}C] dihydrouracil is the exclusive and singular source of plasma [2-^{13}C] dihydrouracil, with 1 mol [2-^{13}C] uracil being converted to 1 mol [2-^{13}C] dihydrouracil by DPD.

Several previous studies of DPD-deficient cancer patients have reported reduced 5-FU clearance with an increased 5-FU $t_{1/2}$ and AUC after oral and i.v. 5-FU administration (21–24). Our observations with orally administered [2-^{13}C] uracil parallel these find-

ings. Specifically, we observed significantly reduced plasma [2-^{13}C] uracil clearance in partially and profoundly DPD-deficient subjects, which resulted in increased plasma [2-^{13}C] uracil $t_{1/2}$ and AUC compared with subjects with normal DPD activity. Several clinical studies of plasma 5-FU concentrations in cancer patients have also observed inverse correlations between plasma 5-FU concentrations or $t_{1/2}$ and DPD activity as well as positive correlations between 5-FU clearance and DPD activity (25, 26). Our observations with orally administered [2-^{13}C] uracil also parallel these studies. We reported inverse correlations between PBMC DPD activity and both plasma [2-^{13}C] uracil AUC and $t_{1/2}$ as well as a positive correlation between PBMC DPD activity and plasma [2-^{13}C] uracil clearance.

Using [2-^{13}C] uracil, we noted significant correlations between PBMC DPD activity and several [2-^{13}C] dihydrouracil pharmacokinetic variables. In particular, PBMC DPD activity was significantly correlated with plasma [2-^{13}C] dihydrouracil appearance rate, amount of [2-^{13}C] dihydrouracil formed, and [2-^{13}C] dihydrouracil C_{max}. In turn, a significant correlation between DPD-mediated plasma [2-^{13}C] dihydrouracil formation and breath $^{13}CO_2$ formation was observed, suggesting that the UraBT $^{13}CO_2$ kinetic variables are an accurate and sensitive index of systemic DPD activity. This conclusion is supported by the biochemical pathway of uracil catabolism where 1 mol $^{13}CO_2$ is produced for every 1 mol [2-^{13}C] uracil reduced to [2-^{13}C] dihydrouracil by DPD.

Although we observed significant correlations between PBMC DPD activity and [2-^{13}C] uracil clearance as well as between PBMC DPD activity and [2-^{13}C] dihydrouracil formation, not all the variability in these pharmacokinetic variables could be attributed to variability in PBMC DPD activity. In fact, wide variation in DPD activity

levels have been observed throughout various tissues (i.e., PBMC, kidney, colon, and liver), with the primary site of pyrimidine catabolism being the liver. Hence, the $^{13}CO_2$ detected in our assay should be primarily formed in the liver. However, ethical considerations prevented the measurement of hepatic DPD in this human study. An examination of the relationship present between the UraBT and hepatic DPD activity in dogs suggested that systemic DPD activity may be more accurately reflected in breath $^{13}CO_2$ concentrations than PBMC DPD activity (15). Hepatic DPD activity was significantly correlated with systemic DPD-mediated reduction of [2-^{13}C] uracil as measured in breath $^{13}CO_2$ concentrations ($r = 0.9999$; ref. 15). This animal study suggests that hepatic DPD activity should strongly correlate with breath $^{13}CO_2$ formation in humans.

5-FU is characterized by a narrow therapeutic index and significant interpatient variability in its pharmacokinetics, which are both implicated in the wide interpatient variation in efficacy and toxicity (6, 26–29). These observations have led to the development of assays to measure plasma DUUR (or 5-dihydrofluorouracil/5-FU ratio) as a potential index on which 5-FU dose individualization strategies may be based (6, 28, 30). Notably, Jiang et al. suggested the importance of monitoring the formation of dihydrouracil under physiologic conditions, by examining the DUUR, to assess variability in DPD activity and 5-FU pharmacokinetics (7). Our results also parallel their observations. Specifically, we observed a significant correlation between PBMC DPD activity and DUUR. We also observed a significant correlation between UraBT DOB_{50} and DUUR.

In summary, we evaluated the UraBT with respect to PBMC DPD activity and plasma [2-^{13}C] uracil and [2-^{13}C] dihydrouracil concentrations in subjects with normal and reduced DPD activity. In the present study, we showed significant differences in [2-

^{13}C] uracil and [2-^{13}C] dihydrouracil kinetics and UraBT $^{13}CO_2$ concentrations (e.g., DOB_{50}) in subjects with decreased DPD activity versus those with normal DPD activity. The significant correlations between DPD activity and either plasma [2-^{13}C] uracil clearance, [2-^{13}C] dihydrouracil formation, or $^{13}CO_2$ breath concentrations provide further support that the UraBT may be useful for assessment of DPD deficiency before administration of 5-FU.

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Table 1 Comparison of UraBT Indices from Subjects with Normal DPD Activity and Partial and Profound DPD Deficiency

	Normal Activity (n=19)	Partial DPD Deficiency (n=11)	Profound DPD Deficiency (n=1)
DOB_{50} (DOB) *	183.2 ± 31.2†	83.7 ± 25.3†	0.9†
C_{max} (DOB) *	193.8 ± 28.1†	121.8 ± 36.7†	3.6†
T_{max} (min) *	50.5 ± 10.8†	125.4 ± 43.2†	120.0†
AUCτ ($^{0}/_{00}$ min) *	21,597.1 ± 2,634.2†	15,572.9 ± 4,327.4†	348.7†
PDR*	55.7 ± 4.9†	39.8 ± 9.9†	< 1.0†
DPD activity (nmol/min/mg) *	0.27 ± 0.06‡	0.11 ± 0.05‡	Undetectable

NOTE: Data were obtained from 19 subjects with normal DPD activity, 11 subjects with partial DPD deficiency, and 1 subject with profound DPD deficiency following oral administration of [2-^{13}C] uracil (6 mg/kg dose). Data are mean ± SD.
*DOB_{50}, $^{13}CO_2$ concentration in breath (DOB) 50 minutes after [2-^{13}C]uracil administration; C_{max}, maximum concentration of $^{13}CO_2$ in breath; T_{max}, time to C_{max};
AUCτ , area under the $^{13}CO_2$ breath curve; PDR, percent dose of [2-^{13}C] uracil recovered in the breath as $^{13}CO_2$; DPD activity, fresh PBMC DPD enzyme activity.
†$P < 0.05$ for both pairwise comparisons (normal DPDactivity versus partial DPD deficiency and normal DPD activity versus profound DPD deficiency).
‡$P < 0.05$ (normal DPD activity versus partial DPD deficiency).

Table 2 Comparison of Plasma [2-^{13}C] Uracil Pharmacokinetic Variables from Subjects with Normal DPD Activity and Partial and Profound DPD Deficiency

	Normal Activity (n=19)	Partial DPD Deficiency (n=11)	Profound DPD Deficiency (n=1)
[2-^{13}C] Uracil $t_{1/2}$ (min)*	15.9 ± 1.7†	39.3 ± 31.2†	306.6†
[2-^{13}C] Uracil AUCτ (min μg/mL)*	257.5 ± 93.4†	480.3 ± 187.6†	1236.1†
[2-^{13}C] Uracil CL/F (mL/min/kg)*	26.6 ± 10.5†	14.1 ± 6.0†	1.3†
[2-^{13}C] Uracil C_{max} (μg/mL)*	8.45 ± 3.09‡	8.87 ± 3.88‡	10.19‡
[2-^{13}C] Uracil T_{max} (min)	28.9 ± 9.5§‖	33.2 ± 13.8§	60.0‖

NOTE: [2-^{13}C] Uracil (6,g/kg) was orally administered to 19 subjects with normal DPD activity, 11 subjects with partial DPD deficiency, and 1 subject with profound DPD deficiency. Following quantification of plasma [2-^{13}C] uracil concentrations, [2-^{13}C] uracil pharmacokinetic variables were determined. Data are mean ± SD.

*$t_{1/2}$, elimination half-life; AUCτ , area under the plasma [2-^{13}C] uracil concentration-time curve; CL/F, apparent clearance; C_{max}, maximum concentration of [2-^{13}C] uracil in plasma.

†$P < 0.05$ for both pairwise comparisons (normal DPD activity versus partial DPD deficiency and normal DPD activity versus profound DPD deficiency).

‡$P > 0.05$ for both pairwise comparisons (normal DPD activity versus partial DPD deficiency and normal DPD activity versus profound DPD deficiency).

§$P > 0.05$ (normal DPD activity versus partial DPD deficiency).

‖$P < 0.05$ (normal DPD activity versus profound DPD deficiency).

Table 3 Pharmacokinetic Variables of [2-^{13}C] Uracil Catabolism Correlate with DPD Activity and the UraBT

	DPD activity (nmol/min/mg)*	UraBT DOB_{50} (DOB)*
[2-^{13}C] Uracil CL/F (mL/min/kg)	0.67	0.59
[2-^{13}C] Uracil AUCτ (min μg/mL)	-0.72	-0.73
[2-^{13}C] Uracil $t_{1/2}$ (min)	-0.54	-0.59
[2-^{13}C] Uracil metabolized (%)	0.67	0.77
[2-^{13}C] Dihydrouracil appearance rate (μg/mL/min)	0.59	0.76
[2-^{13}C] Dihydrouracil formed (mg)	0.61	0.82
DUUR†	0.67	0.65
[2-^{13}C] Dihydrouracil C_{max} (μg/mL) †	0.43	0.70
[2-^{13}C] Dihydrouracil T_{max} (min)	-0.64	-0.68

*All Pearson correlation coefficients (r_p) are significant (all *P*s < 0.05).
†DUUR, ratio of plasma dihydrouracil AUC/uracil AUC; C_{max}, maximum concentration of [2-^{13}C] dihydrouracil in plasma.

Table 4 Comparison of Plasma [2-^{13}C] Dihydrouracil Pharmacokinetic Variables From Subjects with Normal DPD Activity and Partial and Profound DPD Deficiency

	Normal Activity (n=19)	Partial DPD Deficiency (n=11)	Profound DPD Deficiency* (n=1)
[2-^{13}C] Dihydrouracil C_{max} (μg/mL)	2.00 ± 0.53†	1.36 ± 0.43†	-
[2-^{13}C] Dihydrouracil T_{max} (min)	66.3 ± 22.5†	125.4 ± 29.4†	-
[2-^{13}C] Dihydrouracil $t_{1/2}$ (min)	70.4 ± 24.2†	367.9 ± 354.0†	-
[2-^{13}C] Dihydrouracil formed (mg)	380.0 ± 86.2†	153.7 ± 80.9†	-
[2-^{13}C] Dihydrouracil appearance rate (μg/mL/min)	0.03 ± 0.01†	0.01 ± 0.00†	-
DUUR	0.9 ± 0.4†	0.4 ± 0.3†	-
[2-^{13}C] Uracil metabolized (%)	75.4 ± 9.8†	31.6 ± 19.4†	-

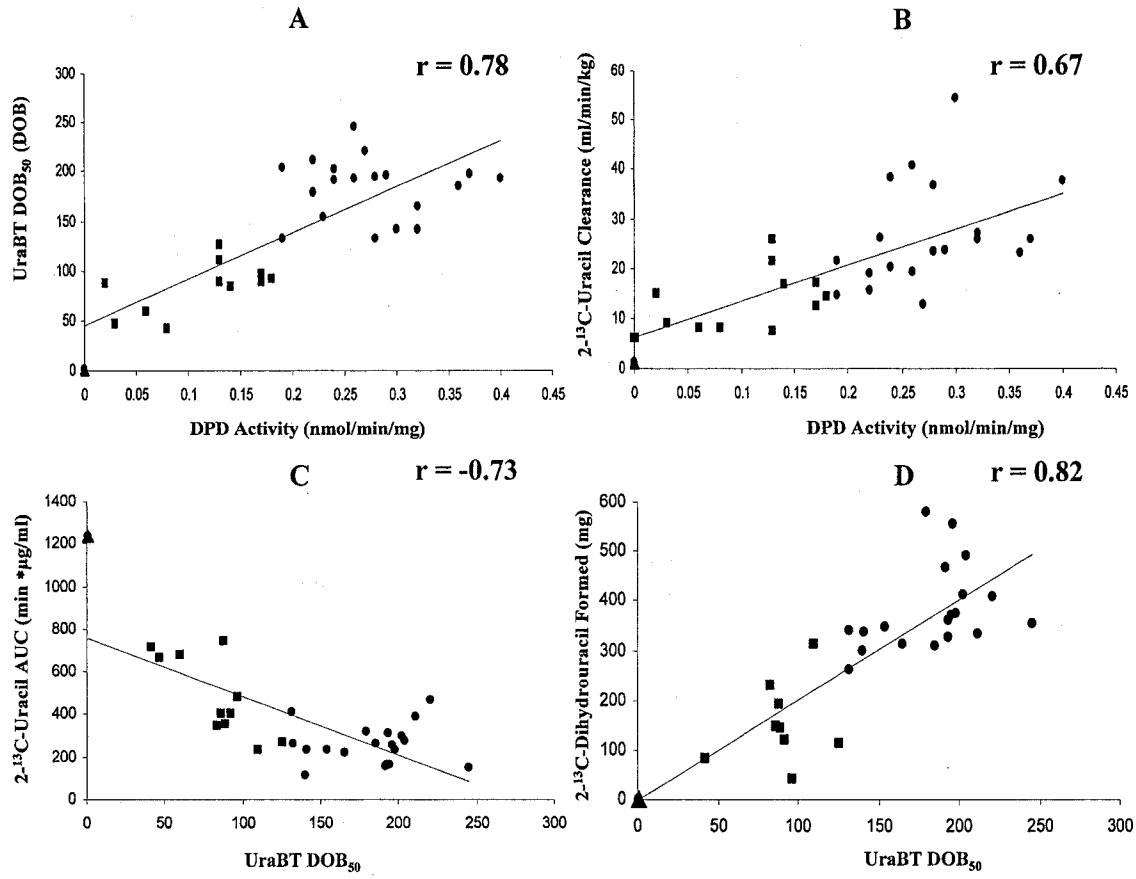
NOTE: [2-^{13}C] Uracil (6 mg/kg)was orally administered to19 subjects with normal DPD activity, 11 subjects with partial DPD deficiency, and 1 subject with profound DPD deficiency. Following quantification of plasma [2-^{13}C] dihydrouracil concentrations, [2-^{13}C] dihydrouracil pharmacokinetic variables were determined. Data are mean ± SD.
*No [2-^{13}C] dihydrouracil was detected in the plasma from the profoundly DPD-deficient subject.
†$P < 0.05$ (normal DPD activity versus partial DPD deficiency).

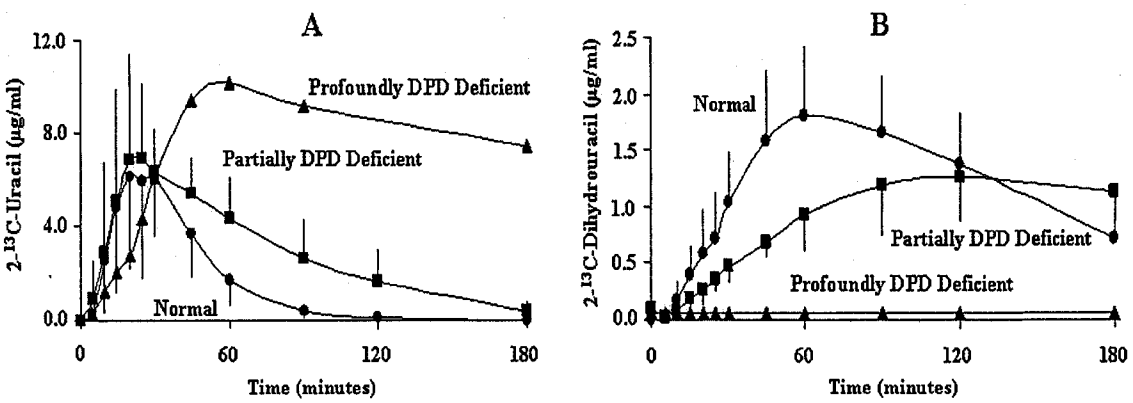
Fig. 1.

Correlation of PBMC DPD activity, the UraBT, [2-^{13}C] uracil clearance, and [2-^{13}C] dihydrouracil formation. Nineteen subjects with normal DPD activity (●), 11 subjects with partial DPD deficiency (■), and 1 subject with profound DPD deficiency (▲) ingested a 6 mg/kg oral solution of [2-^{13}C] uracil and performed the UraBT. DPD activity was determined as described. Plasma samples were collected for 180 minutes after [2-^{13}C] uracil ingestion and the concentration of [2-^{13}C] uracil and [2-^{13}C] dihydrouracil was determined for each plasma sample. [2-^{13}C] Uracil clearance, [2-^{13}C] uracil AUC, and amount of [2-^{13}C] dihydrouracil formed was determined as described. Significant correlation was shown between PBMC DPD activity and UraBT DOB_{50} (*A*; $P < 0.05$), PBMC DPD activity and [2-^{13}C] uracil clearance (*B*; $P < 0.05$), UraBT DOB_{50} and [2-^{13}C] uracil AUC (*C*; $P < 0.05$), and UraBT DOB_{50} and amount of [2-^{13}C] dihydrouracil formed (*D*; $P < 0.05$).

Fig. 2.

Plasma [2-^{13}C] uracil (*A*) and [2-^{13}C] dihydrouracil (*B*) concentrations from subjects with normal DPD activity and partial and profound DPD deficiency. The plasma concentration-time profiles (mean ± SD) of [2-^{13}C] uracil (*A*) and [2-^{13}C] dihydrouracil (*B*) from 19 subjects with normal DPD activity (●), 11 subjects with partial DPD deficiency (■), and 1 subject with profound DPD deficiency (▲) are shown. Points, mean; bars, SD





DIHYDROPYRIMIDINE DEHYDROGENASE DEFICIENCY IN AN INDIAN POPULATION

by

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ABSTRACT

Background: Dihydropyrimidine dehydrogenase (DPD) deficiency is prevalent in 3–5% of the Caucasian population; however, the frequency of this pharmacogenetic syndrome in the Indian population and other racial and ethnic groups remains to be elucidated. *Patients and methods:* We describe an Indian patient who presented to clinic for the treatment of gastric adenocarcinoma with 5-fluorouracil (5-FU) therapy who subsequently was diagnosed with DPD deficiency by using the peripheral blood mononuclear cell (PBMC) DPD radioassay. This observation prompted us to examine the data generated from healthy (cancer-free) Indian subjects who were enrolled in a large population study to determine the sensitivity and specificity of the uracil breath test (UraBT) in the detection of DPD deficiency. Thirteen Indian subjects performed the UraBT. UraBT results were confirmed by PBMC DPD radioassay. *Results:* The Indian cancer patient demonstrated reduced DPD activity (0.11 nmol/min/mg protein) and severe 5-FU toxicities commonly associated with DPD deficiency. Of the 13 Indian subjects [ten men and three women; mean age, 26 years (range: 21– 31 years)] enrolled in the UraBT, 12 Indian subjects demonstrated UraBT breath profiles and PBMC DPD activity within the normal range; one Indian subject demonstrated a reduced breath profile and partial DPD deficiency. *Conclusions:* DPD deficiency is a pharmacogenetic syndrome which is also present in the Indian population. If undiagnosed, the DPD deficiency can lead to death. Future epidemiological studies would be helpful to determine the prevalence of DPD deficiency among racial and ethnic groups, allowing for the optimization of 5-FU chemotherapy.

INTRODUCTION

5-Fluorouracil (5-FU) and its derivatives (e.g., capecitabine) are widely prescribed in the management of gastro-intestinal cancers. Despite widespread use, approximately 31–34% of cancer patients develop severe 5-FU related toxicities [1]. In approximately 61% of these cases, the etiology of 5-FU related toxicities has been linked to reduced activity in the dihydropyrimidine dehydrogenase (DPD) enzyme [2]. As the initial and rate-limiting enzyme of the pyrimidine catabolic pathway, DPD degrades thymine, uracil, and the anticancer drug 5-FU to dihydrothymine, dihydrouracil, and 5-fluoro-dihydrouracil, respectively [3, 4]. Pharmacokinetic studies have suggested reduced DPD activity (DPD deficiency) may reduce 5-FU catabolism resulting in a clinically dangerous increase in 5-FU half-life and severity of 5-FU related toxicities [5, 6].

DPD deficiency is a pharmacogenetic syndrome which manifests primarily as severe life-threatening toxicity subsequent to administration of standard doses of 5-FU [7, 8]. Symptoms frequently observed following the administration of 5-FU include mucositis, granulocytopenia, neuropathy, and death [2, 8, 9]. The prevalence of this autosomal codominantly inherited pharmacogenetic syndrome is approximately 3–5% in the Caucasian population and 8% in the African-American population [10–13]. However, the prevalence in the Indian population has not been determined. We report on an Indian patient with DPD deficiency who developed 5-FU toxicity in the course of his treatment for gastric adenocarcinoma. A literature search suggests that this is the first description of DPD deficiency in an Indian cancer patient. The observation prompted us to further evaluate peripheral blood mononuclear cell (PBMC) DPD activity and uracil breath test

(UraBT) profiles from Indian subjects who were enrolled in a large population study to examine correlation among DPD enzyme activity and the UraBT [14, 15].

PATIENTS AND METHODS

Case Report of an Indian Cancer Patient with Dihydropyrimidine Dehydrogenase Deficiency

A 59-year-old Indian male underwent a distal subtotal gastrectomy with a Bill Roth (B) II reconstruction on 11/17/01. Six of seven lymph nodes were tumor positive with a tumor score of T3N1M0. On February 6, 2002 the patient was started on an adjuvant chemotherapy/radiotherapy based on the McDonald Study for gastric cancer [16]. After completing the initial 5 days of 5-FU/leucovorin therapy for cycle 1, the patient was seen in the oncology clinic with complaints of diarrhea, mouth sores and ulcers associated with bleeding upon minor trauma, and multiple areas of bruising around the area of his central line. His diarrhea was described as loose watery stools, without blood or mucous, with an average of five bowel (range 4–7) movements per day (grade 2). The mucositis had made him unable to tolerate oral intake, causing him to lose eight pounds. He reported significant bruising at the sight of his central line, but denied active bleeding. The patient denied fevers, night sweats, or any other bruising. He also denied peripheral neuropathy, insomnia, and loss of consciousness. He did admit to some dizziness. The physical examination was remarkable for severe mucositis apparent as confluent ulcers encroaching his lips, with notable thrush. The ulcers bled easily upon touching (grade 3). He had a 4–5- cm area of bruising around his porta catheter on the left side of chest with small petechiae around it, without active bleeding (grade 2). Neurologically, the patient's cranial nerves, motor and sensory functions were intact. His laboratory workup including

a complete blood count, fluid balance profile, and liver function tests were within normal limits.

Given the patient's presentation of severe mucositis, diarrhea, and bruising after 1 week of 5-FU chemotherapy, DPD deficiency was a major diagnostic concern. DPD activity was evaluated by radioassay using PBMC as previously described [14]. The patient was given cisplatin 60 mg/m2 with concurrent radiation from 3/4/02 to 4/5/02, which he tolerated well.

Unfortunately, in July of 2002, the patient developed a metastatic small bowel obstruction which was deemed inoperable. After having a discussion with his family, the patient opted for hospice care. The patient died shortly thereafter.

Pilot Study for Detection of Dihydropyrimidine Dehydrogenase Deficiency in the Indian Population

Uracil Breath Test Subjects

Thirteen Indian subjects [ten men and three women; mean age, 26 years (range: 21–31 years)] were recruited as a part of a larger population study at the University of Alabama at Birmingham. Following an explanation of procedures, informed consent was obtained from all subjects prior to initiation of this IRB approved protocol. To be eligible for this study, healthy subjects were at least 19 years old, cancer-free, and had no history of metabolic or respiratory disease.

Rapid Oral UraBT

The UraBT principle and methodology is described in greater elsewhere [15]. To minimize variation resulting from a circadian rhythm in DPD activity [17], the UraBT

protocol started at approximately 8:00 a.m. Fasting subjects were weighed and an aqueous solution containing 6 mg/kg [2-^{13}C] uracil (Cambridge Isotope Laboratories Inc., Andover, MA) was formulated. Subjects donated three baseline breath samples into 1.2-l bags (Otsuka Pharmaceuticals, Tokushima, Japan) prior to administration of the non-radioactive oral solution. Twenty-one post-dose breath samples were collected into 100-ml breath bags (Otsuka Pharmaceuticals, Tokushima, Japan) during the 180-min period immediately following ingestion. Post-dose breath samples were collected every 5 min for the first 30 min and every 10 min thereafter. The concentration of $^{13}CO_2$ in breath, reported in delta over baseline (DOB) notation, was determined by infrared spectrophotometry (Meretek, Lafayette, CO; [18]). These data were graphed [DOB (y axis) versus time (x axis)] and C_{max}, T_{max}, and DOB_{50} ($^{13}CO_2$ concentration in breath 50 min following [2-^{13}C] uracil ingestion) were determined by inspection. The percentage dose of [2-^{13}C] uracil, recovered in the breath as $^{13}CO_2$ (PDR), was calculated as described elsewhere [19]. Subjects were considered to be DPD deficient by UraBT when their DOB_{50} < 128.9 DOB [15].

Peripheral Blood Mononuclear Cell DPD Radioassay

UraBT results were confirmed by DPD radioassay, which is described in greater detail elsewhere [14, 20]. To minimize variation resulting from a circadian rhythm in DPD activity [17], 60 cc of whole blood was drawn from a peripheral vein into heparinized vacutainers at approximately 12:00 p.m. PBMCs were isolated by separation on a ficoll gradient. These cells were washed three times with PBS and lyzed by sonication in an ice bath. The lyzed cells were then centrifuged to remove cellular debris and

the cytosol was collected. The protein concentration of the cytosol was quantitated by a Bradford assay [21]. Two-hundred-fifty micrograms of cytosolic protein was added to a reaction mixture containing NADPH and [6-^{14}C] 5-FU. The reaction mixture was incubated at 37°C for 30 min. One-hundred-twenty-five microliters of aliquots of the reaction mixture were removed every 5 min during the incubation period and added to an equal volume of ice-cold ethanol to terminate the reaction. This mixture was incubated overnight at -80°C, thawed, and then filtered prior to HPLC analysis. Reversed-phase HPLC was used to separate [6-^{14}C] 5-FU from its catabolite, [6-^{14}C] FUH_2. The amount of [6-^{14}C] FUH_2 formed at each time point was quantified and then graphed against time as described elsewhere [14, 20]. From these data, the formation rate of [6-^{14}C] FUH_2 was calculated. DPD enzyme activity was determined by standardizing the formation rate of [6-^{14}C] FUH_2 to the amount protein used in the reaction mixture (i.e., nmol/min/mg protein). Based upon previous population studies by our laboratory, subjects were considered to have DPD enzyme activity within the normal range when their fresh PBMC DPD activity was $\geq$ 0.182 nmol/min/mg protein, partially deficient when their fresh PBMC DPD activity was < 0.182 nmol/min/mg protein but $\geq$ 0.10 nmol/min/mg protein, and profoundly DPD deficient when their fresh PBMC DPD activity was < 0.10 nmol/min/mg protein [14, 20].

*Genotypic Screening for the DPYD*2A Sequence Variant by Denaturing High Performance Liquid Chromatography*

The *DPYD* gene of DPD deficient patients and subjects was evaluated for the *DPYD*2A* sequence variation by using a previously described denaturing high performance liquid chromatography (DHPLC) method [22].

RESULTS

Case Report: Quantification of DPD Activity from an Indian Patient Treated with 5-FU

The fresh PBMC DPD activity was 0.11 nmol/min/mg protein, demonstrating partial DPD deficiency.

Pilot Study: Identification of DPD Deficiency in an Indian Subject by Rapid UraBT

Breath profiles from 12 Indian subjects with DPD activity in the normal range and one Indian subject with partial DPD deficiency are shown in Fig. 1. From this study population, we identified a second partially DPD deficient Indian subject. The Indian subject with partial DPD deficiency demonstrated lower UraBT values compared to Indian controls with DPD activity within the normal range. Specifically, this partially DPD deficient Indian subject demonstrated a lower C_{max}, PDR, and DOB_{50} than Indian subjects with DPD activity within the normal range (Table 1). The partially DPD deficient Indian also demonstrated an increased T_{max} compared to Indian subjects with normal DPD activity (Table 1).

Genotypic Screening of the DPD Deficient Cancer Patient and Volunteer

DHPLC analysis demonstrated that *DPYD**2A* was not present in the *DPYD* gene from either the DPD deficient Indian cancer patient or the DPD deficient Indian volunteer.

DISCUSSION

5-FU and its derivatives are widely prescribed to treat epithelial cancers [23]. Although 5-FU is generally well tolerated at standard doses, approximately 40–60% of cancer patients that develop severe, life-threatening 5-FU toxicities are DPD deficient [24, 25]. The presented Indian patient demonstrated toxicities consistent with 5-FU toxicity. Specifically, we observed mucositis, thrush, and bruising around his central line. He also had complained of diarrhea. The patient's Indian ethnicity was particularly noteworthy, as the given the incidence of DPD deficiency in this population group is not known. We confirmed partial DPD deficiency following measurement of PBMC DPD activity by radioassay.

Most phenotypic assays that are currently available to detect this pharmacogenetic syndrome, including the PBMC radioassay, are too labor-and-time intensive to be routinely used to screen cancer patients prior to 5-FU administration [15]. Recently, we developed and validated an oral UraBT which may potentially be used as a screening method to rapidly detect DPD deficiency in cancer patients prior to 5-FU administration [15]. This *in vivo* assay utilizes [2-^{13}C] uracil, which has a similar substrate affinity for the DPD enzyme as 5-FU [26]. As the [2-^{13}C] uracil substrate is degraded by DPD and other enzymes of the pyrimidine catabolic pathway, the ^{13}C probe is released as $^{13}CO_2$ [15]. The $^{13}CO_2$ present in breath can then be quantified by infrared spectrophotometry [15]. Previously, we demonstrated DPD deficient individuals have an impaired ability to catabolize the [2-^{13}C] uracil, which results in altered $^{13}CO_2$ breath profiles (e.g., significantly lower C_{max}, PDR, and DOB_{50}, and increased T_{max}; reference [15]).

Several genotypic methods have been described to rapidly examine the *DPYD* gene for sequence variations; however, the clinical relevance and application of genotyping will continue to be limited as long as the genotype/phenotype relationship remains unclear. To date, the more than 30 sequence variations in the *DPYD* gene have been described [27]. Yet, only *DPYD**2*A* and *DPYD**13 have been consistently associated with DPD deficiency [28]. Genotypic screening for *DPYD**2*A* may benefit only a limited number of cancer patients due to the low frequency of this variation in the general population (0.94% frequency in the Caucasian population [29]). Furthermore, effect of genetic and epigenetic events on DPD enzyme activity is not well understood. Recently, our laboratory observed aberrant methylation in the *DPYD* promoter of several DPD deficient individuals who demonstrated no sequence variations in their *DPYD* gene [30]. Alternatively, another laboratory has proposed allelic regulation of the *DPYD* gene as a potential mechanism to explain the normal DPD enzyme activity observed from a *DPYD**2*A* heterozygous individual [31]. In the current study, we screened the *DPYD* gene from the DPD deficient Indian cancer patient and the DPD deficient Indian volunteer for the *DPYD**2*A* sequence variation. *DPYD**2*A* was not detected in the *DPYD* gene from either individual. Additional genotypic studies of the *DPYD* gene from these individuals continue to be performed by our laboratory.

Several ethnic studies to phenotypically and/or genotypically evaluate DPD have been conducted in African (Eqyptian, Kenyan, and Ghanaian), Asian (Japanese, Korean, Indian, Pakastani, and Sri Lankan) and European Caucasian (British, German, and Dutch) populations [32–38]. As a part of a larger population study, we examined 13 cancer-free Indians for DPD deficiency using a novel, rapid, and non-invasive UraBT. Indian sub-

jects with DPD activity in the normal range demonstrated UraBT values (C_{max}, T_{max}, DOB_{50}, and PDR) similar to those observed from normal subjects who participated in earlier studies to develop and validate the UraBT [15]. These earlier volunteers were primarily Caucasian or African American. Alternatively, the partially DPD deficient Indian subject demonstrated altered $^{13}CO_2$ breath levels and UraBT indices similar to those previously observed from other partially DPD deficient subjects [15]. Although Indians (N=43) were included in a previous examination of the distribution of DPD activity in Southwest Asians [33], a large population study to examine the frequency of DPD deficiency in Indians is warranted and is now feasible by utilization of UraBT. Furthermore, additional studies of the Indian population should be undertaken to establish whether the genetic basis of DPD deficiency is unique and evaluate the allelic frequency of activity-reducing *DPYD* variants in this population.

Another scintillating feature of this patient with DPD deficiency was the absence of myelosuppression. Indeed, DPD-deficient cancer patients are at risk for developing severe myelosuppression. Raida and colleagues have examined the relationship between myelosuppression and DPD deficiency in a population (n=25) of cancer patients with severe 5-FU toxicity (grades 3–4) [29]. Six of these cancer patients demonstrated either heterozygosity (n=5) or homozygosity (n=1) for the *DPYD**2*A* sequence variation, which is commonly associated with reduced DPD enzyme activity. All six patients demonstrated grade 4 myelosuppression. Our laboratory has also examined the relationship between DPD activity and 5-FU toxicity [24]. We observed that approximately 73% of partially DPD deficient cancer patients (16/22) and 40% of profoundly DPD deficient patients (4/10) experienced moderate to severe granulocytopenia. Interestingly, myelosup-

pression has also been observed in cancer patients who were administered the DPD inactivator and eniluracil [39].

CONCLUSIONS

In summary, we describe DPD deficiency in two Indians: one cancer patient and one healthy volunteer. DPD deficiency is an important pharmacogenetic syndrome to be aware of when considering 5-FU chemotherapy administration. Several 5-FU related toxicities commonly associated with DPD deficiency include mucositis, granulocytopenia, diarrhea, and neuropathy. If undiagnosed, DPD deficiency can lead to death. Treatment consists of stopping 5-FU and is otherwise largely supportive. Further study would be helpful to determine the epidemiological incidence of DPD deficiency among ethnic and racial groups to determine ''at risk'' populations. This issue is of paramount importance, as 5-FU remains the third most commonly used chemotherapy worldwide.

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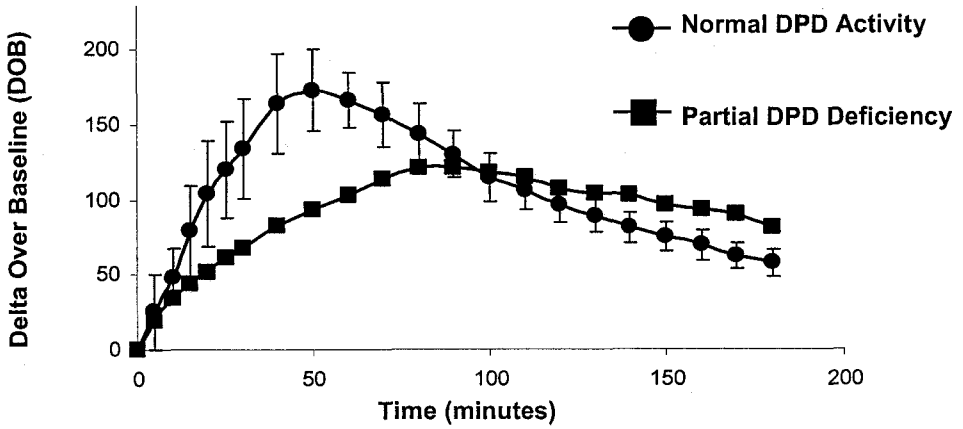
Table 1 Uracil Breath Test and DPD Radioassay Indices from 13 Indians

Volunteers[a]	N	Activity (nmol/min/mg)[b]	DOB_{50} (DOB)[c]	T_{max} (min)[d]	C_{max} (DOB)[c]	Percentage of ^{13}C-uracil dose recovered in breath (%)[c]
Indians with normal DPD activity	12	0.29 ± 0.05	172.4 ± 27.3	50.8 ± 10.8	181.3 ± 24.1	55.1 ± 7.0
Indians with partial DPD deficiency	1	0.17	93.3	80	121.0	47.9

[a]Thirteen cancer-free Indian subjects were examined by [2-^{13}C] uracil breath test and DPD radioassay as described in Patients and Methods.
[b]The peripheral blood mononuclear cell DPD activity is shown. DPD activity within the normal range was observed for 12 Indian subjects. One Indian subject demonstrated partial DPD deficiency (data are shown as mean ± SD).
[c]Lower [2-^{13}C] uracil breath test indices (DOB_{50}, C_{max}, percentage of ^{13}C-uracil recovered in breath) were observed from the partially DPD deficient Indian subject compared to those from Indian subjects with normal DPD activity (data are shown as mean ± SD).
[d]A higher [2-^{13}C] uracil breath test T_{max} was observed from the partially deficient Indian subject compared to Indians with normal DPD activity (data are shown as mean ± SD).

Fig. 1.

The UraBT breath profiles from 12 Indian subjects (mean ± SD) with DPD activity in the normal range (filled circles) and one partially DPD deficient Indian subject are shown (filled squares). All cancer-free subjects ingested a 6 mg/kg solution of [2-^{13}C] uracil. Post-dose breath samples were collected for 180 min after substrate ingestion and the amount of $^{13}CO_2$ in breath [expressed as delta over baseline (DOB)] was determined for each time point.



RACIAL DIFFERENCES IN DIHYDROPYRIMIDINE DEHYDROGENASE ACTIVITY: INCREASED PREVALENCE OF DPD DEFICIENCY IN AFRICAN AMERICANS COMPARED TO CAUCASIANS

by

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ABSTRACT

Purpose: African American colorectal cancer patients have been observed to have increased 5-FU-associated toxicity (leucopenia and anemia) and decreased overall survival compared to Caucasian patients. One potential source for this disparity may be differences in the metabolism of 5-Fluorouracil (5-FU), a chemotherapy drug widely used in both adjuvant and advanced treatment of colorectal cancer as well as other malignancies. Dihydropyrimidine dehydrogenase (DPD), the initial and rate-limiting enzyme of 5-FU catabolism, has previously demonstrated significant (6-fold) inter-patient variability. While distribution of DPD enzyme activity and the frequency of DPD deficiency have been well characterized in the Caucasian population, the distribution of DPD enzyme activity and the frequency of DPD deficiency in the African American population are unknown.

Experimental Design: Healthy African American (n=149) and Caucasian (n=109) volunteers were evaluated for DPD deficiency using both the [2-^{13}C] uracil breath test (UraBT) and peripheral blood mononuclear cell (PBMC) DPD radioassay.

Results: African Americans demonstrated significantly reduced PBMC DPD enzyme activity compared to Caucasians (0.26 ± 0.07 and 0.29 ± 0.07 nmol/min/mg, respectively; p=0.002). The prevalence of DPD deficiency was also 3-fold higher in African Americans compared to Caucasians (8.0 and 2.8% respectively, p =0.07). African American women demonstrated the highest prevalence of DPD deficiency compared to African American men, Caucasian women, and Caucasian men (12.3, 4.0, 3.5 and 1.9%, respectively). **Conclusion:** These results indicate that African Americans, particularly African

American women, have significantly reduced DPD enzyme activity compared to Caucasians which may predispose this population to more 5-FU toxicity.

INTRODUCTION

5-Fluorouracil (5-FU) and its fluoropyrimidine derivatives (e.g. capecitabine) are widely prescribed in oncology practice to treat gastrointestinal malignancies and are often used in the management of breast and head and neck cancer [1-4]. These agents are generally well tolerated; however, 34% of colorectal cancer patients are known to develop severe 5-FU dose-related toxicities [5, 6]. One well-recognized determinant for these observed differences in 5-FU toxicity is the marked (6-fold) variability in the activity of the dihydropyrimidine dehydrogenase enzyme (DPD; EC 1.3.1.2) [7-10]. In particular, it is estimated that 40-60% of cancer patients who present with severe 5-FU toxicity are DPD deficient [11, 12].

Several studies demonstrate the pivotal role of DPD in 5-FU metabolism and response. Earlier biochemical studies showed that DPD, the initial and rate-limiting enzyme of the pyrimidine catabolic pathway, degrades uracil, thymine, and 5-FU to dihydrouracil, dihydrothymine, and 5-fluoro-dihydrouracil respectively [13, 14]. Pharmacokinetic evaluation has further demonstrated that DPD catabolizes more than 80% of an administered dose of 5-FU, thereby determining the amount of 5-FU available for anabolism [8]. Furthermore, data from combined pharmacokinetic/pharmacodynamic studies in cancer patients show that reduced DPD enzyme activity (DPD deficiency) is associated with decreased 5-FU clearance, and increased 5-FU AUC, exposure and toxicity [8, 15, 16].

Population studies by our laboratory and others have shown that approximately 3-5% of the Caucasian population is DPD deficient [2, 10, 17]. While the frequency of this pharmacogenetic syndrome in the general population suggests that routine screening for DPD deficiency should be performed prior to 5-FU administration to cancer patients, the technical complexity of available genotypic and phenotypic assays limit application to retrospective analysis of patients subsequent to the development of 5-FU toxicity [18, 19]. To address this problem, we recently developed a clinically-feasible oral [2-^{13}C] uracil breath test (UraBT) which detected DPD deficiency with 100% sensitivity and 96% specificity in less than 1 hour [19]. More recently, we validated the UraBT by evaluating plasma [2-^{13}C] uracil, [2-^{13}C] dihydrouracil and breath $^{13}CO_2$ pharmacokinetics in subjects with normal DPD enzyme activity as well as DPD deficiency. We showed that UraBT $^{13}CO_2$ concentrations are significantly correlated to DPD enzyme activity and plasma [2-^{13}C] uracil clearance and [2-^{13}C] dihydrouracil formation [20]. These results suggest that the UraBT may be utilized to rapidly assess variability in *in vivo* pyrimidine catabolism.

Several clinical studies have compared toxicity and survival among African American and Caucasian colorectal cancer patients receiving 5-FU regimens. In particular, McCollum and colleagues observed increased toxicity (leucopenia and anemia) in African American colorectal cancer patients compared to Caucasian colorectal cancer patients, while Govindarajan, Alexander, Jessup, and colleagues observed decreased survival in African American colorectal cancer patients compared to Caucasian patients [21-24]. One potential source for this disparity in response may be differences in 5-FU metabolism, particularly 5-FU catabolism, between African Americans and Caucasians. No-

tably, the distribution of DPD enzyme activity and frequency of DPD deficiency in the African American population has not been characterized. A recent study of peripheral blood mononuclear cell (PBMC) DPD enzyme activity from 150 Japanese subjects and our previous study of an Indian cohort using the UraBT suggest that racial differences may be present in the distribution of DPD enzyme activity and frequency of DPD deficiency [1, 25]. In the current study, we utilized the UraBT to screen a population of healthy African American and Caucasian volunteers for DPD deficiency. We examined 1) the distribution of peripheral blood mononuclear cell (PBMC) DPD enzyme activity in African Americans vs. Caucasians; 2) the distribution of the UraBT DOB_{50} in African Americans vs. Caucasians; 3) the frequency of DPD deficiency in African Americans vs. Caucasians; and 4) gender differences in the frequency of DPD deficiency.

SUBJECTS AND METHODS

Subjects

One hundred forty-nine healthy African American volunteers (73 African American women and 76 African American men) and 109 healthy Caucasian volunteers (57 Caucasian women and 52 Caucasian men) participated in this IRB approved protocol at the University of Alabama Hospital's General Clinical Research Center (GCRC, Table 1). Volunteers less than 19 years of age were ineligible for participation. Volunteers were also excluded from the study if they had respiratory, gastric, or metabolic diseases.

Dihydropyrimidine Dehydrogenase (DPD) Radioassay

The DPD radioassay was performed as described in greater detail elsewhere [26]. To minimize variation resulting from a known circadian rhythm in DPD enzyme

activity [27], sixty cc of whole blood was collected at approximately 12:00 p.m. from each subject on the same day as their UraBT. PBMCs were isolated, suspended in Buffer A (35 mM potassium phosphate, 2.5 mM magnesium chloride, and 10 mM 2-mercaptoethanol; pH 7.4), and lysed by sonication. The protein concentration of PBMC cytosol was determined by a Bradford assay [28]. Approximately 250 μg of total protein was added to a reaction mixture containing 200 μM NADPH, Buffer A, 8.2 μM [6-^{14}C] 5-FU (56 mCi/mmol) and incubated at 37°C for 30 minutes. One-hundred-thirty μl aliquots of the reaction mixture were removed every 5 minutes and immediately placed into termination tubes containing an equal volume of ice-cold ethanol. Protein was precipitated by incubating the mixture at -80 °C overnight. The mixture was then thawed and filtered. [6-^{14}C] FUH_2 and [6-^{14}C] 5-FU were separated by reverse phase HPLC and quantified using previously described methods [26]. The amount of [6-^{14}C] FUH_2 formed at each time point (Y axis) was plotted against time (X axis) and the formation rate of [6-^{14}C] FUH_2 was computed. DPD enzyme activity was determined by dividing the [6-^{14}C] FUH_2 formation rate by the amount of total protein added to the reaction mixture. Based upon previous population studies by our laboratory, volunteers were considered to have DPD enzyme activity in the normal range when their fresh PBMC DPD enzyme activity was $\geq$ 0.18 nmol/min/mg protein (95% distribution range), partial DPD deficiency when their fresh PBMC DPD enzyme activity was $<$ 0.18 but $\geq$ 0.10 nmol/min/mg protein (99% distribution range), profound DPD deficiency when their fresh PBMC DPD enzyme activity was $<$ 0.10 but $\geq$ 0.00 nmol/min/mg protein (outside of the lower limit of the 99% distribution range), and complete DPD deficiency when PBMC DPD enzyme activity was undetectable [2, 10, 26].

Uracil Breath Test

The UraBT principle and methodology is described in greater detail elsewhere [19]. To minimize variation resulting from a known circadian rhythm in DPD enzyme activity, the UraBT protocol commenced at 8:00 a.m [27]. Baseline breath samples were collected from overnight fasting volunteers in three 1.2 L bags (Otsuka Pharmaceuticals, Tokushima, Japan). An aqueous solution of 6 mg/kg of [2-^{13}C] uracil (Cambridge Isotope Laboratories, Andover, MA, USA) was ingested and 21 breath samples were collected in 300 ml bags (Otsuka Pharmaceuticals, Tokushima, Japan) over 180 minutes post [2-^{13}C] uracil ingestion. The concentration of $^{13}CO_2$ in post-dose breath samples, reported in delta over baseline (DOB) notation, was measured using IR spectrophotometry (UBiT-IR$_{300}$, Meretek Diagnostics, Lafayette, CO, USA). The breath profile for each subject was constructed by graphing the concentration of $^{13}CO_2$ in breath vs. time (y and x axes respectively). UraBT indices {T_{max}, C_{max}, DOB_{50} (concentration of $^{13}CO_2$ in breath 50 minutes after [2-^{13}C] uracil administration}, and percent of [2-^{13}C] uracil dose recovered in breath as $^{13}CO_2$ over the 180 minutes post [2-^{13}C] uracil ingestion (PDR_{180}) were determined as previously described [19, 29]. Based on our previously published analysis, the UraBT DOB_{50} was determined to optimally identify volunteers with DPD deficiency [19]. UraBT DOB_{50} values < 128.9 DOB categorized volunteers as having DPD deficiency [19]. Alternatively, UraBT DOB_{50} values $\geq$ 128.9 DOB categorized volunteers as having normal DPD enzyme activity [19].

Statistical Analysis

The Kolmogorov-Smirnov test was used to examine whether UraBT DOB_{50}, PDR_{180} and PBMC DPD enzyme activity values were normally distributed. Mean values of continuous outcomes (i.e. PBMC DPD enzyme activity and UraBT DOB_{50}) were computed for each gender and race combination (i.e. Caucasian men, Caucasian women, African American men and African American women). Mean values were compared for each subgroup using generalized linear models with race, gender and the interaction of race and gender covariates as predictors. The least square means and 95% confidence intervals were also computed. The prevalence of DPD deficiency was computed as the proportion of individuals demonstrating reduced PBMC DPD enzyme activity in each race and gender subgroup. The prevalence of DPD deficiency among gender and racial groups was compared using the chi-square or Fishers exact test. The odds ratio and 95% confidence intervals were also computed. All analyses were conducted with SAS Version 9.1. For all analyses a p value of < 0.05 was deemed as statistically significant.

RESULTS

Distribution of PBMC DPD Activity

PBMC DPD enzyme activity from the entire study population (n=258) was normally distributed. The mean (± SD) DPD enzyme activity observed for entire study population (n=258) was 0.27 ± 0.07 nmol/min/mg. Of the 15 volunteers that demonstrated DPD deficiency (< 0.18 nmol/min/mg), 11 volunteers demonstrated partial deficiency and 4 volunteers demonstrated profound DPD deficiency. No volunteers demonstrated complete DPD deficiency.

Distribution of PBMC DPD Activity in the African American and Caucasian Population

The distributions of PBMC DPD enzyme activity observed in healthy African American (n=149) and Caucasian (n=109) volunteers were both normal (Figure 1). The distribution of DPD enzyme activity in the African American population was negatively skewed (with the tail extending into the low range of DPD enzyme activity), whereas the distribution in Caucasian population was positively skewed (with the tail extending into the high range of DPD enzyme activity). However, the skewness of these distributions was not significant (coefficient of skewness: -0.10 and 0.72, respectively).

African American volunteers had significantly lower DPD enzyme activity compared to Caucasian volunteers ($p = 0.002$). The mean (± SD) DPD enzyme activity observed for African Americans and Caucasians was 0.26 ± 0.07 and 0.29 ± 0.07 nmol/min/mg, respectively (Table 1).

Prevalence of DPD Deficiency in the Healthy African American and Caucasian Population

The prevalence of DPD deficiency was 3-fold greater in the African American population compared to the Caucasian population (p=0.07). The prevalence of DPD deficiency in the African American population was 8.0%, with 12 of 149 volunteers demonstrating DPD deficiency. Four of the 12 DPD deficient African American volunteers demonstrated profound DPD deficiency while 8 of the 12 demonstrated partial DPD deficiency. The prevalence of DPD deficiency in the Caucasian population was 2.8%, with 3 of 109 volunteers demonstrating partial DPD deficiency (Table 1).

PBMC DPD Enzyme Activity and Prevalence of DPD Deficiency in African American Women

Stratification of volunteers by gender demonstrated that women had significantly lower DPD enzyme activity compared to men (0.25 ± 0.07 and 0.29 ± 0.07, respectively; $p \leq 0.001$). Further stratification of the volunteers by race and gender demonstrated that African American women had a significantly lower DPD enzyme activity (0.24 ± 0.07 nmol/min/mg) compared to African American men (0.28 ± 0.07 nmol/min/mg), Caucasian women (0.28 ± 0.07 nmol/min/mg), Caucasian men (0.30 ± 0.07 nmol/min/mg; p value ≤ 0.003 for each pair-wise comparisons). African American women were also observed to have the highest prevalence of DPD deficiency (12.3% with nine of 73 volunteers demonstrating DPD deficiency), compared to African American men (4.0% with three of 76 volunteers demonstrating DPD deficiency, p=0.08), Caucasian women (3.5% with two of 57 volunteers demonstrating DPD deficiency, p=0.12), and Caucasian men (1.9% with one of 52 volunteers demonstrating DPD deficiency; p=0.09). Three of the nine DPD deficient African American women demonstrated profound DPD deficiency while the remaining six volunteers demonstrated partial DPD deficiency. One of the three DPD deficient African American men demonstrated profound DPD deficiency while the remaining two demonstrated partial DPD deficiency. All cases of DPD deficiency observed in Caucasian men and women were partial DPD deficiency; no profound DPD deficiency was observed in Caucasians.

UraBT PDR and DOB_{50} Distributions in the Study Population

The PDR_{180} values and UraBT DOB_{50} concentrations from the entire study population (n=258) were both normally distributed. The mean (± SD) PDR_{180} value observed

from entire study population was 53.1 ± 7.4%. The mean (± SD) DOB_{50} concentration observed from the entire study population (n=258) was 174.3 ± 33.5 DOB. Based on the previously established UraBT DOB_{50} cut-point, 19 volunteers (7.4%) were classified as DPD deficient.

Characterization of UraBT PDR and DOB_{50} in Healthy African American and Caucasian Volunteers

PDR_{180} values from African American (n=149) and Caucasian volunteers (n=109) were both normally distributed. The distribution of PDR_{180} values in African Americans and Caucasians were both positively skewed (with the tail extending toward higher PDR_{180} values). However, the skewness of these distributions was not significant (coefficient of skewness: 0.74 and 0.45, respectively).

African American volunteers also demonstrated significantly lower PDR_{180} values compared to Caucasian volunteers ($p = 0.03$). The mean (± SD) PDR_{180} value observed in African Americans and Caucasians was 52.3 ± 7.7 and 54.3 ± 6.9%, respectively (Table 1).

The DOB_{50} distributions observed from African American (n=149) and Caucasian (n=109) volunteers were normally distributed (Figure 2). The DOB_{50} distribution in African Americans was negatively skewed (with the tail extending into the low range of $^{13}CO_2$ breath concentrations), whereas the distribution in Caucasian volunteers was positively skewed (with the tail extending toward higher $^{13}CO_2$ breath concentrations). However, the skewness of these distributions was not significant (coefficient of skewness: -0.23 and 0.19, respectively).

African Americans also demonstrated a significantly lower DOB_{50} concentrations compared to Caucasians ($p = 0.004$). The mean (± SD) DOB_{50} observed in African Americans and Caucasians was 169.1 ± 34.8 and 181.4 ± 31.0 DOB, respectively (Table 1). Of the 19 volunteers who screened positive for DPD deficiency by UraBT, sixteen were African American and three were Caucasian.

DISCUSSION

DPD deficiency predisposes cancer patients to severe, life-threatening 5-FU toxicity. Recently, we developed and optimized the UraBT to rapidly (< 1 hour) screen cancer patients for reduced DPD enzyme activity [19]. Subsequently, we performed a pharmacokinetic validation of the UraBT by characterizing relationships present among UraBT-associated breath $^{13}CO_2$ metabolite formation, plasma [2-^{13}C] dihydrouracil formation, plasma [2-^{13}C] uracil clearance, and PBMC DPD enzyme activity in normal and DPD deficient subjects [20]. More recently, we demonstrated that the UraBT is a rapid method suitable for population studies by screening 13 Indian subjects for DPD deficiency subsequent to our initial identification and characterization of DPD deficiency in an Indian cancer patient with severe 5-FU toxicity [1]. In the current study, we utilized the UraBT to screen for DPD deficiency in a population composed of 258 Caucasian and African American volunteers.

Racial differences, resulting from genetic variability, have been observed in the activity of several drug metabolizing enzymes such as cytochrome P450 2C19 (CYP 2C19), N-acetyltransferase, and thiopurine methyltransferase [30-32]. In the current study, we demonstrated that racial differences are present in DPD enzyme activity. Spe-

cifically, we observed significantly lower PBMC DPD enzyme activity in the African American population compared to the Caucasian population. Others have also observed racial differences in DPD enzyme activity. Sohn *et. al.* observed increased PBMC DPD enzyme activity in Koreans (n=114) compared to the activities that had previously been reported in Caucasians [33]. Comparatively, a large population study of 34,200 Japanese infants did not detect DPD deficiency in any of the enrolled subjects [34].

Our observation of significantly lower PBMC DPD enzyme activity in African American volunteers suggests that African Americans have significantly reduced *in vivo* pyrimidine catabolism compared to Caucasians. Recently, we performed a pharmacokinetic evaluation of the UraBT and demonstrated that the UraBT DOB_{50} is significantly related to markers of [2-^{13}C] uracil degradation (i.e. clearance, half-life, and AUC) and [2-^{13}C] dihydrouracil formation (C_{max}, T_{max}, and rate of appearance) [20]. In the current study, we observed that African Americans had significantly lower UraBT DOB_{50} values compared to Caucasians. Furthermore, African Americans metabolized a significantly lower percentage dose of [2-^{13}C] uracil to $^{13}CO_2$ compared to Caucasians. Taken together, these results suggest that African Americans have significantly lower *in vivo* pyrimidine catabolism compared to Caucasians which may put them at risk for increased DPD-mediated 5-FU toxicity.

A recent study of African American and Caucasian colorectal cancer patients examined whether racial differences in 5-FU toxicity were present [21]. African American patients demonstrated significantly increased leucopenia and anemia compared to Caucasian colorectal cancer patients [21]. Unfortunately, the DPD enzyme activity of the two patient populations was not measured. Additional research examining racial differences

in DPD enzyme activity and occurrence of DPD-mediated 5-FU toxicity in cancer patients are warranted.

Earlier clinical studies observed increased 5-FU toxicity in women compared to men [35, 36]. This led to the hypothesis that women may have lower DPD enzyme activity compared to men. However, subsequent studies were unable to prove or disprove this hypothesis [16, 17, 37-39]. In the current study, we observed significantly lower DPD enzyme activity in women compared to men. Furthermore, stratification by gender and race demonstrated that African American women had the lowest DPD enzyme activity of any other race-gender group ($p \leq 0.003$ for all pairwise comparisons). These results suggest that African American women, in particular, may be at increased risk of DPD-mediated 5-FU toxicity. This is of interest as the *DPYD* gene is located on chromosome 1p22 and has been previously described as having an autosomal codominant inheritance pattern [40, 41].

Recent clinical studies have observed reduced activity in other enzymes of the pyrimidine catabolic pathway. In particular, reduced dihydropyrimidinase (DHPase) and β-ureidopropionase (BUP) activities have been observed in a 5-FU toxic patient and children with neurological abnormalities [42-45]. It may be possible to detect these deficiencies in pyrimidine catabolism using the UraBT. In order for $^{13}CO_2$ be released in breath, the [2-^{13}C] uracil substrate must be catabolized by DPD, DHPase, and BUP. Individuals with reduced DHPase and BUP activities would be expected to have decreased $^{13}CO_2$ breath concentrations compared to individuals with normal pyrimidine catabolism. In the current study, we observed reduced UraBT DOB_{50} concentrations in four subjects with normal DPD enzyme activity. Examination of the entire breath $^{13}CO_2$ concentration-time

profiles from these four subjects demonstrated that two of the four subjects had breath profiles with an early T_{max} (30 and 40 minutes; data not shown) and a C_{max} above the "cut-point" (142.8 and 139.2 DOB; data not shown). These data suggest that normal pyrimidine catabolism is present in these two subjects. However, two of the four subjects demonstrated reduced breath $^{13}CO_2$ concentration-time profiles compared to normal subjects (data not shown). These data suggest that altered pyrimidine catabolism may be present. Genotypic evaluation of the *DPYS* and *BUP* genes from these two subjects are currently being conducted by our laboratory.

In summary, we applied the UraBT to screen for DPD deficiency in a population of African Americans and Caucasians. We demonstrated that the African American population, particularly African American women, had an increased prevalence of DPD deficiency and significantly reduced PBMC DPD enzyme activity and [2-^{13}C] uracil catabolism. These results suggest that African Americans may be at risk for 5-FU toxicity resulting from reduced catabolism. Currently, genotypic studies are being performed by our laboratory to examine the *DPYD* gene of all volunteers with reduced DPD enzyme activity to identify the molecular basis of DPD deficiency. Future studies will prospectively apply the UraBT to identify cancer patients at risk of developing 5-FU toxicity due to reduced DPD enzyme activity.

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Table 1. Characterization of PBMC DPD Activity and UraBT DOB_{50} and PDR in Healthy African American and Caucasian Volunteers

Group	N	DPD Activity* (nmol/min/mg)	Prevalence of DPD Deficiency (%)	DOB_{50} *(DOB)	PDR* (%)
African American	149	0.26 ± 0.07†	8.0	169.1 ± 34.8†	52.3 ± 7.7†
Caucasian	109	0.29 ± 0.07†	2.8	181.4 ± 31.0†	54.3 ± 6.9†

NOTE: Healthy African American (n=149) and Caucasian (n=109) volunteers were examined for DPD deficiency using the UraBT and peripheral blood mononuclear cell DPD radioassay. Data are mean ± SD.

† $P < 0.05$ for all pairwise comparisons (African Americans versus Caucasians).

* DPD activity, fresh PBMC DPD enzyme activity; DOB_{50}, $^{13}CO_2$ concentration in breath (DOB) 50 minutes after [2-^{13}C] uracil administration; PDR, percent dose of [2-^{13}C] uracil recovered in the breath as $^{13}CO_2$.

Fig. 1.

Distribution of PBMC DPD activity in healthy African American and Caucasian volunteers. Shown are the distributions of PBMC DPD activity from healthy African American (n=149; hatched bars) and Caucasian subjects (n=109; white bars). Each distribution was found to be normally distributed by the Kolmogorov-Smirnov test. For reference, individuals with DPD deficiency (PBMC DPD activity $\leq$ 0.18 nmol/min/mg protein) are denoted by red bars. African Americans were found to have significantly lower PBMC DPD activity than Caucasians (p = 0.002). Twelve African Americans were DPD deficient compared to three Caucasians. The prevalence of DPD deficiency among the African American and Caucasian populations was 8.0% and 2.8% respectively.

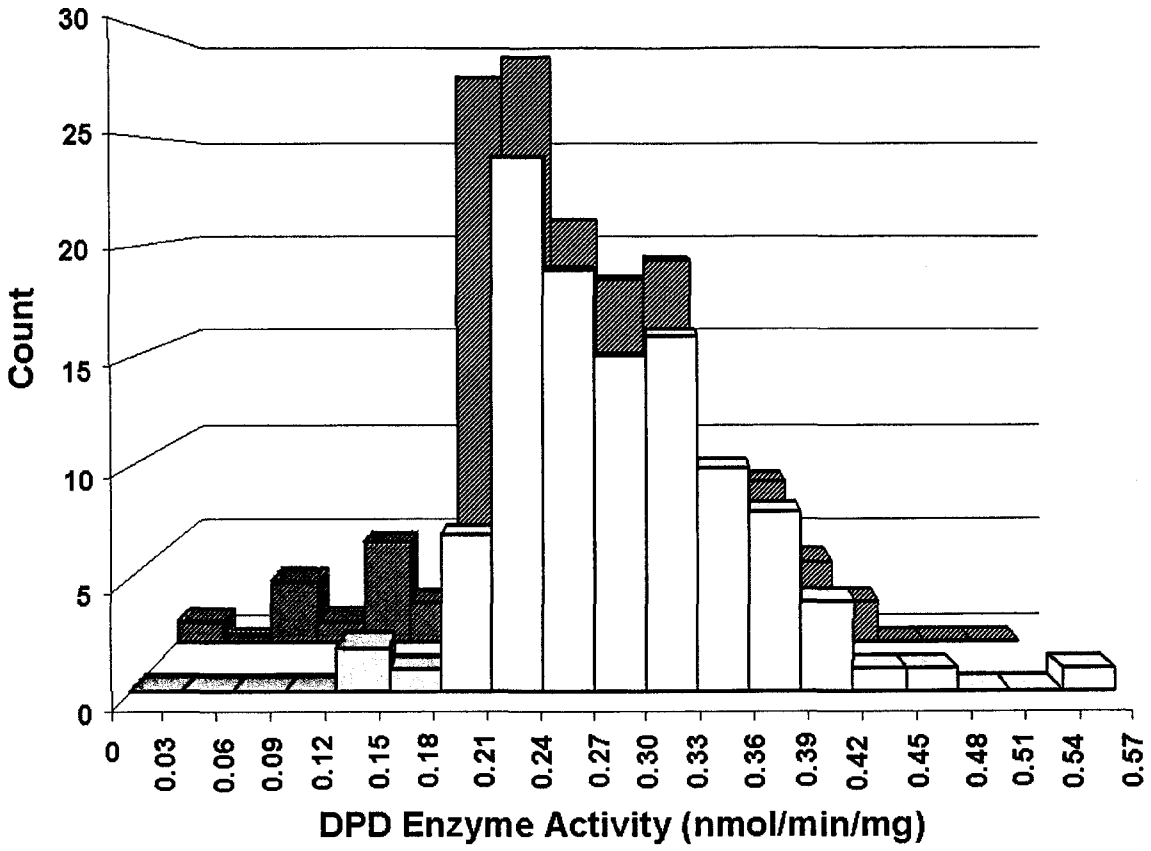
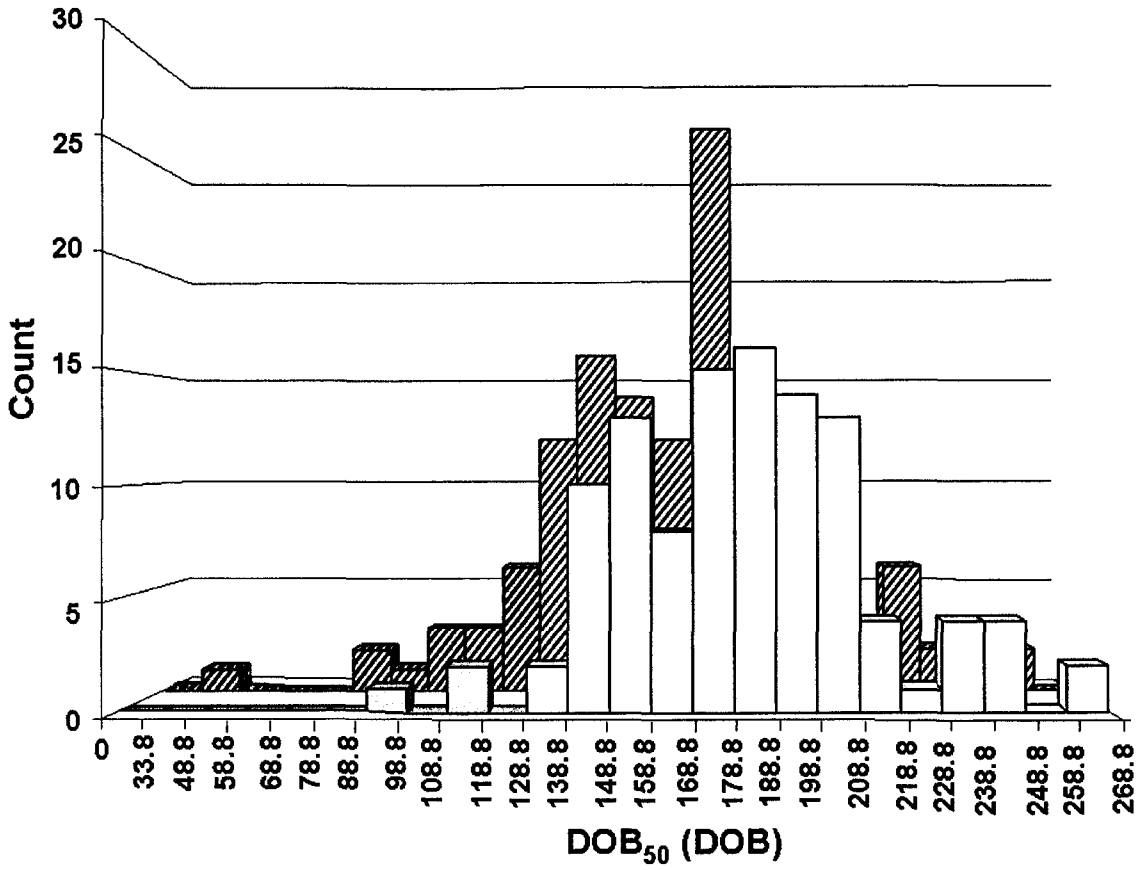


Fig. 2.

Rapid detection of DPD deficiency in healthy African American and Caucasian volunteers. Shown are the UraBT DOB_{50} distributions from healthy African American volunteers (n=149; hatched bars) and Caucasian volunteers (n=109; white bars). Each distribution was found to be normally distributed by the Kolmogorov-Smirnov test. For reference, individuals who screened positive for DPD deficiency (UraBT DOB_{50} < 128.9 DOB) are denoted by red bars. African Americans were found to have significantly lower UraBT DOB_{50} values than Caucasians (p=0.004). Based on the UraBT DOB_{50} cut-point, nineteen participants (7.4% of the study population) screened positive for DPD deficiency.



DISCUSSION

DPD deficiency predisposes cancer patients to severe, life-threatening toxicity subsequent to 5-FU administration [21, 38, 39]. Several genotypic and phenotypic methods have been developed to detect DPD deficiency. However, significant barriers are present which prevent their clinical implementation to hospital laboratories [33, 40]. Therefore, the focus of this dissertation research was to develop a rapid, clinically-feasible assay to detect DPD deficiency. While a genotypic approach was initially investigated, development, validation and use of a phenotypic UraBT was primarily pursued.

Utilization of an Evolutionary Approach to Study the Importance of Sequence Variations in the *DPYD* Gene

Many high throughput, automated methods for detection of single nucleotide polymorphisms (SNPs) are available [41], suggesting that development of a clinically-useful genotypic test to screen cancer patients for DPD deficiency is possible. However, before such assays may be applied, it is necessary to characterize *DPYD* sequence variations to understand their effect on enzyme activity. To date, more than 30 sequence variations have been observed in the *DPYD* gene of healthy human volunteers and 5-FU toxic cancer patients [21]. However, linkage between phenotype and genotype is difficult due to the polymorphic nature of the *DPYD* gene [23]. Presently, four sequence variations have been characterized. *DPYD* *2*A* and *DPYD* *13 were shown to reduce activity following characterization by our laboratory in a three generation pedigree analysis which

compared genotype and phenotype (PBMC DPD enzyme activity) [23]. *DPYD** 8 and *DPYD* *10 were characterized following expression of the wild-type and mutant enzyme [42] in *E. coli*. Characterization of the remaining sequence variations remains to be a priority for the field of DPD pharmacogenetics.

Characterization of *DPYD* sequence variations could be more efficiently performed if we better understood which regions of the DPD enzyme were critical to enzyme activity. Sequence variations, which result in amino acid changes at these critical domains, could then be the first to be evaluated. In the present study, we utilized a comparative genetic approach to assess the conservation of loci where *DPYD* sequence variations occur. To accomplish this goal, we cloned and sequenced the mouse DPD cDNA. Following alignment of the translated sequence to those from human, rat, pig, and cow, we performed BLAST and BLOCKS searches to identify homologous amino acid sequences in *Drosophila* and *C. elegans*. These sequences where then aligned to those from mammals.

The results of this study demonstrated that the DPD enzyme is well conserved from mammalian to invertebrate species. Specifically, the homology observed between human and pig was 96%, human and cow was 96%, human and mouse was 94%, human and rat was 94%, human and *Drosophila* was 93%, and human and *C. elegans* was 79%. Furthermore, amino acids involved in substrate and cofactor binding were also well conserved demonstrating the importance of these loci to enzyme function.

Subsequently, we evaluated the conservation of the loci where *DPYD* sequence variations occur. All seven species exhibited 100% identity in the loci of eight *DPYD* mutations [*DPYD**3, *DPYD**4 (S534N), *DPYD**7, *DPYD**8 (R235W), *DPYD**10

(V995), *DPYD**11 (V335L), M166V and D949V]. Interestingly, an earlier study (which expressed DPD in *E. coli*) showed that *DPYD**8 and *DPYD**10 reduce DPD enzyme activity [42]. Furthermore, *DPYD**7, *DPYD**8, *DPYD**10, *DPYD**11 and D949V have been observed only in subjects with reduced DPD enzyme activity; no normal DPD enzyme activity has been observed in subjects with these sequence variations [33, 42-45]. Taken collectively, these results suggest that *DPYD**7, *DPYD**11 and D949V should be among the first sequence variations to be characterized by our laboratory or others.

Development of a UraBT to Detect DPD Deficiency

The genetic and regulatory complexities of the *DPYD* gene have been significant barriers to the development of a clinically feasible genotypic assay to detect DPD deficiency [1]. Therefore, several phenotypic methods to evaluate DPD have been developed. However, many of these phenotypic assays are too time-consuming or complex to be implemented by most hospital laboratories [21, 33, 40].

The recent Food and Drug Administration approval of a rapid and simple ^{13}C-urea breath test to detect *H. pylori* infection suggested that use of ^{13}C-labeled substrates may be a viable and safe way to measure *in vivo* pyrimidine catabolism [46-49]. Earlier studies of orally administered 5-FU showed that oral 5-FU was well absorbed, which lead us to hypothesize that orally administered uracil would also be well absorbed [50, 51]. Furthermore, previous kinetic studies by our laboratory and others demonstrated that DPD has similar substrate affinities for 5-FU and uracil, which lead us to hypothesize that uracil would also be metabolized with a similar efficiency as 5-FU [9, 52, 53]. Taken collectively, these data were the basis for development of an orally administered UraBT to

assess *in vivo* pyrimidine catabolism. It was hypothesized that individuals with normal DPD enzyme activity would catabolize the orally administered [2-^{13}C] uracil to β-alanine, NH_3 and $^{13}CO_2$ [40]. A portion of the $^{13}CO_2$ present in the body cavity would then be expired into breath [40]. Breath $^{13}CO_2$ concentrations could then be measured by infrared spectrophotometry [54]. DPD deficient individuals, who have reduced [2-^{13}C] uracil catabolism, would have decreased $^{13}CO_2$ breath levels.

Initially, we performed feasibility studies with four normal and three partially DPD deficient subjects. These studies were performed to find the optimum dose and time for the UraBT. Initial studies with 100, 200, and 300 mg doses of [2-^{13}C] uracil demonstrated that dose-proportionality was present. Specifically, individuals with a large body mass demonstrated consistently lower $^{13}CO_2$ breath concentrations compared to the smaller subjects. These data suggested that individuals with a large body mass have a larger central compartment in which the ^{13}C-labeled uracil or metabolites could distribute. Therefore, we normalized the [2-^{13}C] uracil doses to weight. We evaluated several weight-normalized doses (1, 3, 6, and 12 mg/kg). Using a weight-normalized dose of 6 mg/kg, we observed differences in the breath $^{13}CO_2$ concentration-time profiles from normal and DPD deficient subjects. Additionally, the elimination phase of the $^{13}CO_2$ concentration-time profiles were observed from both normal and DPD deficient subjects within the 180 minute period subsequent to oral [2-^{13}C] uracil administration.

We then applied the UraBT to examine a larger study population using the 6 mg/kg dose of [2-^{13}C] uracil. Breath was collected from all subjects for the 180 minute period subsequent to oral [2-^{13}C] uracil administration. A second phenotypic test, the PBMC DPD radioassay, was also used to measure the DPD enzyme activity of each sub-

ject. Individuals with DPD deficiency were genotypically examined by DHPLC to identify the molecular basis for reduced DPD enzyme activity.

In these studies, we observed reduced breath $^{13}CO_2$ concentration-time profiles from DPD deficient subjects. In particular, DPD deficient subjects demonstrated significantly reduced UraBT C_{max}, DOB_{50}, and PDR values compared to normal subjects. Evaluation of the UraBT breath $^{13}CO_2$ concentration-time profiles showed that breath $^{13}CO_2$ concentration-time profiles of normal and DPD deficient subjects were maximally separated 50 minutes subsequent to [2-^{13}C] uracil administration. This permitted categorization of subjects based upon $^{13}CO_2$ concentrations present in the breath 50 minutes following [2-^{13}C] uracil administration (DOB_{50}). Specifically, subjects were classified as "DPD deficient" when breath DOB_{50} values were beneath 128.9 DOB. Alternatively, subjects were classified with "normal" DPD enzyme activity when the breath DOB_{50} values were equal to or greater than 128.9 DOB. Using these criteria, the UraBT demonstrated 100% sensitivity and 96% specificity.

Taken collectively, these data suggest that the observed reduction in breath $^{13}CO_2$ concentration-time profiles and altered UraBT indices from DPD deficient subjects result from a reduction of DPD mediated [2-^{13}C] uracil catabolism. Pharmacokinetic studies were performed to validate these results.

Pharmacokinetic Validation of the UraBT

The significant reduction in breath $^{13}CO_2$ concentrations from DPD deficient subjects suggested that these subjects had reduced *in vivo* catabolism of [2-^{13}C] uracil to [2-^{13}C] dihydrouracil.

To test this hypothesis, we validated the UraBT by comparing breath $^{13}CO_2$ kinetic profiles to plasma [2-^{13}C] uracil and [2-^{13}C] dihydrouracil kinetics in a population of healthy volunteers and cancer patients with normal and reduced DPD enzyme activity. In order to minimize variability in plasma and breath pharmacokinetics resulting from a known circadian variation, the UraBT commenced at approximately 8 a.m [15]. While each subject performed the UraBT, whole blood was simultaneously collected via a heparin lock placed in the participants arm. Pre-dose blood samples were collected prior to [2-^{13}C] uracil administration. Post-dose blood samples were collected over the 180 minute period subsequent to [2-^{13}C] uracil administration. Plasma was isolated from whole blood and [2-^{13}C] uracil and [2-^{13}C] dihydrouracil concentrations were measured by LC-MS-MS [55]. Pharmacokinetic analysis of [2-^{13}C] uracil and [2-^{13}C] dihydrouracil concentration-time profiles were performed using non-compartmental methods. To minimize variability resulting from a known circadian variation in PBMC DPD enzyme activity, DPD enzyme activity was determined for each subject from whole blood collected at approximately 12 p.m [15]. Breath $^{13}CO_2$ concentrations were measured using IR spectrophotometry.

In the present study, we observed reduced *in vivo* [2-^{13}C] uracil elimination in DPD deficient subjects compared to subjects with normal DPD enzyme activity. Specifically, DPD deficient subjects demonstrated significantly reduced [2-^{13}C] uracil clearance and increased $t_{1/2}$ and AUC compared to normal subjects. [2-^{13}C] uracil clearance was significantly correlated to PBMC DPD enzyme activity. These results are in agreement with the results from several clinical studies which examined 5-FU pharmacokinetics in

DPD deficient cancer patients [8, 17, 18, 56]. These data suggest that DPD deficient subjects may have significantly reduced DPD-mediated [2-^{13}C] uracil elimination.

In our study, we also observed altered [2-^{13}C] dihydrouracil pharmacokinetics in DPD deficient subjects compared to normal subjects. Specifically, we observed significantly increased plasma [2-^{13}C] dihydrouracil T_{max} and significantly reduced plasma [2-^{13}C] dihydrouracil C_{max} and DUUR in partially DPD deficient subjects compared to normal subjects. These results are similar to a study of 5-FU and FUH_2 pharmacokinetics a DPD deficient cancer patient with 5-FU toxicity [6]. Furthermore, the DPD mediated conversion of [2-^{13}C] uracil to [2-^{13}C] dihydrouracil, as represented by the DUUR, was significantly correlated to PBMC DPD enzyme activity. The profoundly DPD deficient subject, with no detectable PBMC DPD enzyme activity, demonstrated no DPD-mediated conversion of [2-^{13}C] uracil to [2-^{13}C] dihydrouracil. These data suggest that DPD deficient subjects have significantly reduced DPD-mediated conversion of [2-^{13}C] uracil to [2-^{13}C] dihydrouracil.

In this study, we also observed altered breath $^{13}CO_2$ concentrations in DPD deficient subjects compared to normal subjects. Specifically, we observed significantly reduced DOB_{50} concentrations, C_{max} values, and PDR in DPD deficient subjects compared to normal subjects. DPD deficient subjects also demonstrated significantly increased breath $^{13}CO_2$ T_{max}. Furthermore, significant correlations were observed among PBMC DPD enzyme activity, [2-^{13}C] uracil and [2-^{13}C] dihydrouracil pharmacokinetics and the UraBT. In particular, UraBT DOB_{50} concentrations were significantly correlated to PBMC DPD enzyme activity, [2-^{13}C] uracil clearance, and DUUR. Taken collectively, these data suggest that the reduced breath $^{13}CO_2$ concentrations observed from DPD defi-

cient subjects result from significantly reduced DPD-mediated catabolism of [2-^{13}C] uracil to [2-^{13}C] dihydrouracil.

Use of the UraBT to Screen for DPD Deficiency in an Indian Population

One well-recognized determinant of variability in drug metabolism and response is race [57]. Genetic variability in polymorphic enzymes involved in drug metabolism and elimination may be a potential mechanism for these observed differences. For example, 50% of Caucasians and African Americans, compared to the 10-20% of Asians, demonstrate slow drug acetylation (degradation) by the N-acetyltransferase (NAT2) enzyme placing them at increased risk for toxicity following administration of isoniazid, hydralazine, and dapsone, or sulfasalazine [58].

Racial differences have been also been observed in DPD enzyme activity, with Asians demonstrating increased PBMC DPD enzyme activity and a lower prevalence of DPD deficiency. Specifically, Sohn and colleagues have observed increased PBMC DPD enzyme activity in the Korean population (n=114) compared to those previously reported for the French and North American Caucasian populations [59]. Comparatively, a large population study of 34,200 Japanese infants showed that DPD deficiency was not present in any of the enrolled children [31].

During the course of these studies, we observed partial DPD deficiency in an Indian cancer patient with severe 5-FU toxicity. A literature search demonstrated that DPD deficiency had not been previously described in this population. To investigate whether DPD deficiency was present in other Indians, we applied the UraBT to rapidly screen 13

cancer-free Indian subjects for DPD deficiency in 4 days. UraBT categorization was confirmed by PBMC DPD radioassay.

We observed normal DPD enzyme activity in 12 of the 13 Indian subjects. These normal subjects demonstrated breath $^{13}CO_2$ concentration-time profiles and UraBT indices similar to those observed from normal subjects who previously enrolled in the initial UraBT development and validation studies. We also observed partial DPD deficiency in one Indian subject enrolled in this examination. This subject demonstrated a reduced breath $^{13}CO_2$ concentration-time profile and altered UraBT indices similar to those observed from partially DPD deficient subjects who previously enrolled in the initial UraBT development and validation studies [40, 60]. Prior to this study, DPD deficiency was unrecognized in the Indian population. This study demonstrates that DPD deficiency is present in this population. Large population studies of Indians are warranted to determine the molecular basis and prevalence of this pharmacogenetic syndrome in this population.

Use of the UraBT to Rapidly Screen for DPD Deficiency in a Population of African American and Caucasian Subjects

The speed and diagnostic capability of the UraBT suggested that it is a facile method that could also be applied to screen other populations, aside from cancer patients, to identify "at-risk" groups for DPD deficiency.

Historically women and minorities have been under-represented in clinical research [61]. A recent clinical study of 5-FU efficacy and toxicity in African American colorectal cancer patients showed that African Americans had significantly increased leucopenia compared to Caucasian patients [62]. Leucopenia is a 5-FU associated toxicity that is frequently observed in DPD deficient cancer patients [19, 63, 64]. However, the

investigators of this study did not evaluate DPD enzyme activity. While the distribution of DPD enzyme activity and frequency of DPD deficiency has been extensively described in the Caucasian population, the distribution of DPD enzyme activity and frequency of DPD deficiency in the African American population has not been described. Therefore, we applied the UraBT to screen a population of healthy (cancer-free) African Americans (n=149) and Caucasians (n=109) to evaluate the prevalence of this pharmacogenetic syndrome in the two populations. UraBT categorization of all participants was confirmed by PBMC DPD radioassay.

In this study, we observed significantly lower PBMC DPD enzyme activity in African Americans compared to Caucasians. African American women, in particular, had the lowest PBMC DPD enzyme activity, with significantly lower PBMC DPD enzyme activity compared to African American men, Caucasian women, and Caucasian men. The prevalence of DPD deficiency in African Americans compared to Caucasians was also 3-fold higher (8.0 and 2.8%), however this increased prevalence was not significantly higher ($p < 0.07$). This may be due to the relatively small size of the sample (approximately 100 participants per racial group).

In our study, we also observed that African Americans have significantly lower [2-^{13}C] uracil catabolism compared to Caucasians. Specifically, we observed significantly decreased UraBT DOB_{50} concentrations and PDR in African Americans compared to Caucasians.

Taken collectively, these data suggest that African Americans may be at increased risk from DPD-mediated 5-FU toxicity. Future studies are warranted to 1) confirm the prevalence of DPD deficiency in this minority population; 2) evaluate the relationship

between 5-FU toxicity and DPD enzyme activity in African American colorectal cancer patients; and 3) examine the *DPYD* gene of the African American population to identify novel *DPYD* sequence variations.

In this study, we observed reduced UraBT DOB_{50} concentrations in four subjects with normal DPD enzyme activity. Examination of the entire breath $^{13}CO_2$ concentration-time profiles from these four subjects demonstrated that two of the four subjects had breath profiles with an early T_{max} (30 and 40 minutes; data not shown) and a C_{max} above the "cut-point" (142.8 and 139.2 DOB; data not shown). These data suggest that normal pyrimidine catabolism is present in these two subjects. However, two of the four subjects demonstrated reduced breath $^{13}CO_2$ concentration-time profiles compared to normal subjects (data not shown). [2-^{13}C] Uracil must be catabolized to [2-^{13}C] dihydrouracil and [2-^{13}C] β-ureidopropionate before $^{13}CO_2$ may be released. The presence of normal DPD activity but reduced breath $^{13}CO_2$ concentrations suggest that reduced activity may be present in the other pyrimidine catabolic enzymes. However, measurement of DHPase and BUP activity is not feasible because these enzymes are not expressed in an easily accessible tissue such as PBMCs. Genotypic evaluation of the *DPYS* and *BUP* genes from these two subjects are currently being conducted by our laboratory.

In summary, the studies included in this dissertation have a) utilized an evolutionary approach to prioritize *DPYD* sequence variations for characterization prior to inclusion in genotypic tests; b) developed a rapid, phenotypic test to detect DPD deficiency based on breath $^{13}CO_2$ concentrations following oral administration of a [2-^{13}C] uracil substrate; c) demonstrated that reduced breath $^{13}CO_2$ concentrations observed in DPD

deficient subjects result from reduced *in vivo* catabolism of [2-^{13}C] uracil to [2-^{13}C] dihydrouracil and, ultimately, $^{13}CO_2$; d) showed that the UraBT is a sensitive and rapid method for the detection of DPD deficiency; and e) demonstrated that the African American population, particularly African American women, have significantly decreased DPD enzyme activity compared to the Caucasian population.

SUGGESTIONS FOR FUTURE STUDIES

5-FU has been prescribed for more than 40 years to treat gastrointestinal malignancies [1, 2]. Several clinical studies have shown that DPD has an important role in determining 5-FU response. In particular, cancer patients with DPD deficiency experience severe 5-FU toxicities which may be fatal [8, 20, 22, 38]. Despite more than four decades of research, clinically feasible diagnostic assays to identify DPD deficient cancer patients are not available [1, 21, 33].

In the present studies, we developed and optimized an oral UraBT. We showed that differences in UraBT $^{13}CO_2$ concentrations and indices among normal and DPD deficient subjects result from differences in DPD-mediated [2-^{13}C] uracil catabolism. Evaluation of the diagnostic performance of the UraBT demonstrates that this phenotypic assay is sensitive and specific. Application of the UraBT to screen for DPD deficiency in the African American, Caucasian and Indian populations have shown that the UraBT is a rapid assay. Collectively, these results provide the basis for further clinical development of the UraBT. Most importantly, evaluation of the diagnostic performance of UraBT in a large population of cancer patients is necessary. However, the following studies should be also be performed to provide a more complete understanding of the potential role the UraBT in optimizing 5-FU chemotherapy by reducing DPD-mediated 5-FU toxicity (particularly in instances where other phenotypic and genotypic approaches may not be applied) and perhaps increasing 5-FU efficacy. These studies include 1) evaluation of the diagnostic performance of the UraBT in a large population of cancer patients; 2) evalu-

ation of DPD-mediated pyrimidine catabolism in cancer patients with granulocytopenia; and 3) optimization of 5-FU based chemotherapy, based on the UraBT, to increase efficacy and patient survival.

Diagnostic Performance of the UraBT in Cancer Patients

The majority of studies performed during the course of this dissertation research were performed in healthy (cancer-free) volunteers. However, evaluation of the diagnostic performance and limitations of the UraBT in a population of cancer patients is warranted.

It is necessary to ensure that UraBT $^{13}CO_2$ concentrations or indices in cancer patients are not significantly different from those obtained from healthy subjects. Morphological changes associated with advanced cancer and pathological conditions associated with administration of cytotoxic drugs have been shown to reduce the barrier function of the intestinal tract and alter its permeability [65]. Such changes may affect the absorption of the [2-^{13}C] uracil solution. In turn, this may result in significant alterations to UraBT $^{13}CO_2$ concentrations and indices.

In addition, it is also necessary to establish the effect of liver metastasis on UraBT $^{13}CO_2$ concentrations and indices. Liver tumors may impair liver function. Since pyrimidine catabolism is thought to occur primarily in the liver, this may also affect [2-^{13}C] uracil catabolism [66]. In turn, liver tumors may result in significant alterations to UraBT $^{13}CO_2$ concentrations and indices.

Application of the UraBT to Cancer Patients with Granulocytopenia

Assessment of PBMC DPD enzyme activity in cancer patients is not typically performed until after severe toxicity is observed subsequent to 5-FU administration. Unfortunately, 5-FU toxic cancer patients frequently develop granulocytopenia [20]. Therefore, assessment of PBMC DPD enzyme activity by radioassay is not possible until the absolute neutrophil count (ANC) returns to within the normal range.

Although pyrimidine catabolism occurs primarily in the liver, PBMCs are used as a surrogate tissue for the assessment of DPD enzyme activity due to their accessibility [33, 66]. Furthermore, PBMC DPD enzyme activity has been shown to be significantly correlated to liver DPD enzyme activity [67]. Our data suggest that the UraBT may be used to screen cancer patients with granulocytopenia for DPD deficiency. We observed a significant correlation between UraBT DOB_{50} and PBMC DPD enzyme activity. Based on this observation, future evaluation of the diagnostic performance of the UraBT in cancer patients with granulocytopenia is warranted. To perform such a study, one would initially evaluate 5-FU toxic cancer patients with granulocytopenia by the breath test. After the patients' ANC has returned to within the normal range, PBMC DPD enzyme activity could be assessed by radioassay. The two assays could then be compared to assess the diagnostic performance of the UraBT in this population.

Optimization of 5-FU Chemotherapy Based on UraBT $^{13}CO_2$ Concentrations

Previous studies have demonstrated significant correlation between 5-FU clearance and DPD enzyme activity [13, 68] . While cancer patients with low DPD enzyme activity demonstrate reduced 5-FU clearance and experience severe 5-FU toxicity, cancer

patients with high DPD enzyme activity may have increased 5-FU clearance. These data suggest cancer patients with increased DPD enzyme activity may benefit from higher or more frequent doses of 5-FU. Unfortunately, prior to development of the UraBT, no clinically feasible assay was available to assess DPD enzyme activity. Therefore, 5-FU dose optimization based upon *in vivo* DPD enzyme activity was not feasible.

In the course of this dissertation research, we observed significant correlation between the UraBT, PBMC DPD enzyme activity, and [2-^{13}C] uracil clearance. These data suggest that the UraBT may be potentially applied to optimized 5-FU chemotherapy. In turn, this may increase 5-FU tumor response and survival. To perform such a study, cancer patients would perform the UraBT. Patients that demonstrate significantly higher UraBT PDR or DOB_{50} values may be considered for increased 5-FU doses. Outcomes (i.e. tumor response and increased survival) in cancer patients who have received increased 5-FU chemotherapy could be compared to those who have received the standard of care.

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APPENDIX

INSTITUTIONAL REVIEW BOARD APPROVAL FOR HUMAN USE

OMB No. 0925-0418
Approved for use through 01/31/2001

Protection of Human Subjects
Assurance Identification/Certification/Declaration
(Common Federal Rule)

Policy: Research activities involving human subjects may not be conducted or supported by the Departments and Agencies adopting the Common Rule (56FR28003, June 18, 1991) unless the activities are exempt from or approved in accordance with the common rule. See section 101(b) the common rule for exemptions. Institutions submitting applications or proposals for support must submit certification or appropriate Institutional Review Board (IRB) review and approval to the Department or Agency in accordance with the common rule.

Institutions with an assurance of compliance that covers the research to be conducted on file with the Department, Agency, or the Department of Health and Human Services (HHS) should submit certification of IRB review and approval with each application or proposal unless otherwise advised by the Department or Agency. Institutions which do not have such an assurance must submit an assurance and certification of IRB review and approval within 30 days of a written request from the Department or Agency.

1. Request Type	2. Type of Mechanism	3. Name of Federal Department or Agency and, if known, Application or Proposal Identification No.
☐ ORIGINAL ☐ FOLLOWUP ☐ EXEMPTION	☐ GRANT ☐ CONTRACT ☐ FELLOWSHIP ☐ COOPERATIVE AGREEMENT ☐ OTHER:	

4. Title of Application or Activity	5. Name of Principal Investigator, Program Director, Fellow, or Other
Genetic Polymorphism of Dihydropyrimidine Dehydrogenase (DPD): Identification of DPD Deficiency	DIASIO, ROBERT B

6. Assurance Status of this Project *(Respond to one of the following)*

☐ This Assurance, on file with Department of Health and Human Services, covers this activity:
Assurance Identification no. M-1149 IRB identification no. 02

☐ This Assurance, on file with *(agency/dept)*_____, *covers this activity.*
Assurance Identification no._____ IRB identification no._____ *(if applicable)*

☐ No assurance has been filed for this project. This institution declares that it will provide an Assurance and Certification of IRB review and approval upon request.

☐ Exemption Status: Human subjects are involved, but this activity qualifies for exemption under Section 101(b), paragraph _____.

7. Certification of IRB Review (Respond to one of the following IF you have an Assurance on file)

☒ This activity has been reviewed and approved by the IRB in accordance with the common rule and any other governing regulations or subparts on *(date)* 06-19-02 by: ☒ Full IRB Review or ☐ Expedited Review

☐ This activity contains multiple projects, some of which have not been reviewed. The IRB has granted approval on condition that all projects covered by the common rule will be reviewed and approved before they are initiated and that appropriate further certification will be submitted.

8. Comments	Title
Please note: UAB IRB Protocol Number is F020610007 Protocol subject to Annual continuing review.	Genetic Polymorphism of Dihydropyrimidine Dehydrogenase (DPD): Identification of DPD Deficiency

9. The official signing below certifies that the information provided above is correct and that, as required, future reviews will be performed and certification will be provided.	10. Name and Address of Institution
	University of Alabama at Birmingham 701 20th Street South Birmingham, AL 35294
11. Phone No. *(with area code)* (205) 934-3789	12. Fax No. *(with area code)* (205) 934-1301
13. Name of Official Ferdinand Urthaler, M.D.	14. Title Chairman, IRB
15. Signature Ferdinand Urthaler M.D.	16. Date 06-26-02

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GRADUATE SCHOOL
UNIVERSITY OF ALABAMA AT BIRMINGHAM
DISSERTATION APPROVAL FORM
DOCTOR OF PHILOSOPHY

Name of Candidate Lori Kay Mattison

Graduate Program Pharmacology and Toxicology

Title of Dissertation Development, Validation, and Use of a Novel [2-^{13}C] Uracil Breath Test to Detect Dihydropyrimidine Dehydrogenase Deficiency

I certify that I have read this document and examined the student regarding its content. In my opinion, this dissertation conforms to acceptable standards of scholarly presentation and is adequate in scope and quality, and the attainments of this student are such that she may be recommended for the degree of Doctor of Philosophy.

Dissertation Committee:

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Dean, UAB Graduate School

Date JUN 16 2006