UAB LIBRARIES
The University of Alabama at Birmingham

University of Alabama at Birmingham
UAB Digital Commons

All ETDs from UAB

UAB Theses & Dissertations

1998

cDNA cloning and characterization of the dihydropyrimidine dehydrogenase gene: An enzyme associated with 5-fluorouracil metabolism.

Martin Ray Johnson
University of Alabama at Birmingham

Follow this and additional works at: https://digitalcommons.library.uab.edu/etd-collection

Recommended Citation

Johnson, Martin Ray, "cDNA cloning and characterization of the dihydropyrimidine dehydrogenase gene: An enzyme associated with 5-fluorouracil metabolism." (1998). *All ETDs from UAB*. 6176.
https://digitalcommons.library.uab.edu/etd-collection/6176

This content has been accepted for inclusion by an authorized administrator of the UAB Digital Commons, and is provided as a free open access item. All inquiries regarding this item or the UAB Digital Commons should be directed to the UAB Libraries Office of Scholarly Communication.

INFORMATION TO USERS

This manuscript has been reproduced from the microfilm master. UMI films the text directly from the original or copy submitted. Thus, some thesis and dissertation copies are in typewriter face, while others may be from any type of computer printer.

The quality of this reproduction is dependent upon the quality of the copy submitted. Broken or indistinct print, colored or poor quality illustrations and photographs, print bleedthrough, substandard margins, and improper alignment can adversely affect reproduction.

In the unlikely event that the author did not send UMI a complete manuscript and there are missing pages, these will be noted. Also, if unauthorized copyright material had to be removed, a note will indicate the deletion.

Oversize materials (e.g., maps, drawings, charts) are reproduced by sectioning the original, beginning at the upper left-hand corner and continuing from left to right in equal sections with small overlaps. Each original is also photographed in one exposure and is included in reduced form at the back of the book.

Photographs included in the original manuscript have been reproduced xerographically in this copy. Higher quality 6" x 9" black and white photographic prints are available for any photographs or illustrations appearing in this copy for an additional charge. Contact UMI directly to order.

UMI

A Bell & Howell Information Company
300 North Zeeb Road, Ann Arbor MI 48106-1346 USA
313/761-4700 800/521-0600

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

cDNA CLONING AND CHARACTERIZATION OF THE DIHYDROPYRIMIDINE DEHYDROGENASE GENE: AN ENZYME ASSOCIATED WITH 5-FLUOROURACIL METABOLISM

by

MARTIN RAY JOHNSON

A DISSERTATION

Submitted to the graduate faculty of The University of Alabama at Birmingham, in partial fulfillment of the requirements for the degree of Doctor of Philosophy

BIRMINGHAM, ALABAMA

1998

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

UMI Number: 9828539

UMI Microform 9828539
Copyright 1998, by UMI Company. All rights reserved.

This microform edition is protected against unauthorized
copying under Title 17, United States Code.

UMI
300 North Zeeb Road
Ann Arbor, MI 48103

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

ABSTRACT OF DISSERTATION
GRADUATE SCHOOL, UNIVERSITY OF ALABAMA AT BIRMINGHAM

Degree Ph.D. Program Pharmacology

Name of Candidate Martin Ray Johnson

Committee Chair Robert B. Diasio

Title cDNA Cloning and Characterization of the Dihydropyrimidine Dehydrogenase Gene: An Enzyme Associated With 5-Fluorouracil Metabolism

The anticancer effects of 5-fluorouracil (5-FU) result from anabolism of the drug to its nucleotides, which produce cytotoxicity through inhibition of thymidylate synthase (TS) or incorporation into RNA and/or DNA. In humans, approximately 80% of an administered dose of 5-FU is degraded by dihydropyrimidine dehydrogenase (DPD, EC 1.3.1.2), the initial rate-limiting enzyme in pyrimidine catabolism. Interest in DPD has recently been heightened following identification of cancer patients with an inherited deficiency of DPD. These patients were shown to exhibit severe toxicity (including death) following treatment with 5-FU. The focus of this dissertation research has been to clone and sequence the DPD cDNA from normal and DPD deficient cancer patients. In addition, the structure and organization of the human DPD gene has been determined. The major accomplishments of this dissertation research are listed below.

1. The development of a semiautomated radioassay for measuring DPD activity in human peripheral lymphocytes which has improved our ability to screen patients with DPD deficiency.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

2. Cloning and sequencing the complete cDNA coding for human lymphocyte DPD from a donor with normal DPD activity. Sequence analysis demonstrated an open-reading frame of 3075 bases encoding a 1025 amino acid protein with a predicted molecular weight of 111,392 daltons. These data represent the first complete (including both 5' and 3' untranslated regions) sequence analysis of human DPD cDNA.

3. Cloning and sequencing the complete cDNA coding for human liver DPD from three human liver biopsy samples. Alignment of the lymphocyte DPD cDNA sequence with that of human liver cDNA cloned in our laboratory demonstrated 100% sequence identity, thus demonstrating that there is no difference between human lymphocyte and human liver DPD at the level of mRNA.

4. DPD cDNA was cloned and sequenced from two previously characterized DPD deficient patients. These data revealed a single nucleotide difference A (control) to T (deficient), resulting in a nonconservative amino acid substitution (Asp to Val) at codon 949 in one patient. No sequence difference was identified in the other patient.

5. Last, the structural organization of the DPD gene (intron/exon boundaries) has been determined.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

DEDICATION

To my daughter, Rachael Layne Johnson, for her unquestioning love and support. Her laughter, playful personality, and infinite patience have reminded me of what was important in life.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

ACKNOWLEDGMENTS

The completion of my dissertation and Doctor of Philosophy degree would never have been possible without the support and guidance of my friend and mentor, Dr. Robert B. Diasio. In addition, I would like to express my appreciation to my dissertation committee, which consisted of Drs. Jeff Smith, Robert Leboeuf, William Parker, and Denise Shaw, for their advice, encouragement, and insightful suggestions in the completion of my dissertation. I am especially grateful to Mrs. Kansheng Wang for her constructive suggestions and excellent technical support.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

TABLE OF CONTENTS

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

TABLE OF CONTENTS (Continued)

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

LIST OF TABLES

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

LIST OF FIGURES

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

LIST OF FIGURES (Continued)

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

LIST OF ABBREVIATIONS

BHK	baby hamster kidney
bp	base pair
CMF	cyclophosphamide, methotrexate, and 5-fluorouracil
DHPase	dihydropyrimidinase
DPD	dihydropyrimidine dehydrogenase
dThdPase	thymidine phosphorylase
FBAL	α-fluoro-ß-alanine
FdUMP	5-fluoro-2'-deoxyuridine-5'-monophosphate
FdUDP	5-fluoro-2'-deoxyuridine-5'-diphosphate
FdUrd	5'-fluoro-2'-deoxyuridine
FdUTP	5-fluoro-2'-deoxyuridine-5'-triphosphate
FUH_2	5,6-dihydrofluorouracil
FUMP	5-fluorouridine-5'-monophosphate
FUDP	5-fluorouridine-5'-diphosphate
FUPA	2-fluoro-ß-ureidopropionate
5-FU	5-fluorouracil
FUTP	5-fluorouridine-5'-triphosphate
HPLC	high pressure liquid chromatography
kDa	kilodaltons
K_m	Michaelis-Menten constant
M	molar

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

LIST OF ABBREVIATIONS (Continued)

min	minute
ml	milliliter
mM	millimolar
NADH	nicotinamide adenine dinucleotide (reduced form)
NADPH	nicotinamide adenine dinucleotide phosphate (reduced form)
NMP	nucleoside monophosphate
OPRTase	orotate phosphoribosyltransferase
PAGE	Polyacrylamide gel electrophoresis
PBM	Peripheral blood mononuclear
PBS	phosphate-buffered saline
PRPP	5'-phosphoribosyl-1-pyrophosphate
SDS	sodium dodecyl sulfate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
TK	thymidine kinase
Tris	Tris (hydroxymethyl) aminomethane
TS	thymidylate synthase
UK	uridine kinase
µM	micromolar
UMP	uridine 5'-monophosphate
UrdPase	uridine phosphorylase
ß-UPA	ß-ureidopropionic acid

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

INTRODUCTION

Despite decades of intensive research, cancer remains the second leading cause of death in the United States and in most developed countries. Although there has been substantial progress made in the treatment of cancer, most current types of therapies ultimately fail with about 50% of patients diagnosed with cancer ultimately dying of the disease. One of the major problems in the treatment of cancer is our current inability to predict the outcome of chemotherapy in terms of both host toxicity and tumor response. While one patient will respond well to chemotherapy, another patient diagnosed with the same type of cancer and given the same drug at equivalent doses can completely fail to respond. Finally, some patients treated in an identical manner can demonstrate high levels of host toxicity (including death) with no apparent antitumor response. Recent studies suggest that this variability in pharmacological response may result from individual genetically determined differences in enzyme or receptor function and/or expression (Kobayashi & Ratain, 1993).

Pharmacogenetics may be defined as the study of genetic factors that determine drug pharmacokinetics, toxicity, and efficacy. Determination of the complete molecular basis for both host toxicity and tumor response requires a solid under-

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

standing of the metabolism of the drug, the therapeutic response, and the gene(s) coding for the enzyme(s) involved in drug metabolism. By elucidating the pharmacogenetics related to enzymes in cancer chemotherapy, we will increase our understanding of host and tumor pharmacology, with the ultimate goal of individual optimization of cancer chemotherapy.

Treatment of Cancer

Currently, cancer is treated by surgery, radiation, and chemotherapy. The type of treatment (as well as the expected response) varies depending on the type of cancer and the stage at which the disease was detected (DeVita, Hellman, & Rosenberg, 1989). Although there are several different types of cancer, most share one fundamental feature: the abnormal growth and division of cells which eventually spread through the body (metastasis), invading and interfering with the function of normal organs and tissues. Generally, localized tumors can be effectively treated by surgery and radiation. Unfortunately, metastatic tumors are usually present at the time of diagnosis (although they may be too small to detect), necessitating the administration of chemotherapeutic drugs systemically. The type of chemotherapeutic regimen administered varies depending on the type of cancer and the stage at which it was diagnosed. Adjuvant chemotherapy protocols treat patients who have already undergone surgery in which the primary tumor has been resected. This type of treatment attempts to prevent the progression of metastatic tumors still present

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

in the patient. Neoadjuvant chemotherapy protocols treat patients prior to surgery or irradiation to reduce the bulk of primary tumors (DeVita et al., 1989).

Unfortunately, the drugs currently used for chemotherapy suffer from two serious deficiencies. First, they lack specificity for cancer cells and demonstrate toxicity toward any dividing cell. Cells in the body that undergo rapid division (such as hematopoietic cells, intestinal epithelium, and hair follicle cells) are especially sensitive to these compounds, and the death of these cells accounts for much of the common toxicity observed with chemotherapy (anemia, suppression of the immune system, nausea and vomiting, and hair loss). Second, chemotherapy drugs have an extremely narrow therapeutic index. Thus, the effectiveness of these drugs is limited by their host toxicity, and the ultimate success of chemotherapy is determined by the relative sensitivities of cancer cells and normal cells to the drug being used.

The obvious goal in treatment is to eliminate all the cancer cells while minimizing toxicity to normal cells. However, individual differences in drug metabolism (for both host and tumor cells) result in significant variance in the response to chemotherapy for each specific case. Clinically, the oncologist must carefully regulate the doses of the available chemotherapy drugs to optimize cancer cell death and normal cell survival.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Chemotherapeutic Agents

Current cancer chemotherapy drugs can be divided into five classes, including alkylating agents, antimetabolites, natural products, antibiotics, and miscellaneous agents. The specific pharmacokinetics/pharmacodynamics vary with each drug; however, most of these compounds exert their toxic effects on rapidly dividing cells by interfering with DNA synthesis and/or replication (Chabner & Collins 1990). Since a comprehensive review of each class of chemotherapy drugs is beyond the scope of this dissertation, the remainder of this introduction will focus on 5-fluoropyrimidine antimetabolites, including 5-fluorouracil (5-FU) and 5'-fluoro-2'-deoxyuridine (FdUrd). Emphasis will be placed on 5-FU, a pyrimidine antimetabolite that remains one of the most widely used anticancer drugs (ranking in the top three antineoplastic drugs prescribed in the United States) for the management of several common malignancies, including cancer of the colon, breast, and skin (Scrip's Cancer Chemotherapy Report, 1996).

5-Fluoropyrimidines

5-Fluoropyrimidine drugs, represented by 5-FU and its nucleoside, FdUrd, were introduced as rationally designed antineoplastic agents approximately 40 years ago (Heidelberger et al., 1957). The observation that rat hepatomas utilized more radiolabeled uracil than did nonmalignant tissues presented a possible target for antimetabolite chemotherapy. Pyrimidine derivatives (primarily analogs of ura-

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

cil) were synthesized with physicochemical properties similar to those of the naturally occurring pyrimidines (Figure 1). It was proposed that these analogs (due to their structural similarity to the naturally occurring pyrimidines) could be taken up by cells and metabolized. Once inside the cell, the structural differences between the analog and the natural pyrimidine could possibly interfere with DNA synthesis or replication. Since the genetic material must be duplicated each time a cell divides, it was theorized that these drugs may demonstrate higher toxicity for rapidly dividing cells (such as tumor cells) in comparison to their more slowly growing counterparts.

Over the past 4 decades, fluoropyrimidines have been shown to be remarkably effective in the treatment of human solid tumors, including breast cancer (Chabner & Collins, 1990), gastrointestinal adenocarcinomas (Ensminger et al., 1978), and squamous cell carcinomas of the head and neck (Tennvall et al., 1991). Several pharmacokinetic studies (discussed in detail below) have examined the metabolism of fluoropyrimidines and identified the primary mechanisms responsible for their cytotoxic effects.

Uptake of 5-Fluoropyrimidines

The structural similarity between fluoropyrimidines and their naturally occurring counterparts results in similar uptake and metabolism. 5-FU contains a fluorine atom in place of a hydrogen atom at the fifth position of the pyrimidine

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

ring (Figure 1). This structural difference does not introduce a significant change in the conformation of 5-FU since hydrogen and fluorine are almost the same size (van der waal radii of 1.2Å and 1.35Å, respectively). Ultimately (as will be discussed in detail below), it is the strength of the C-F bond in 5-FU as opposed to the C-H bond in uracil that leads to the cytotoxicity observed following administration of 5-FU.

Early studies suggested that 5-FU rapidly enters cells through passive diffusion (Jacquez, 1962; Kessel & Hall, 1967) with the rate of entry corresponding to the extracellular concentration. Unfortunately, these studies were technically limited since fluxes in concentration could not be assessed over short intervals of time. Subsequent studies using rapid sampling methodology (Wohlhueter, Mclvar, & Plageman, 1980) demonstrated that uracil and 5-FU compete for entry into the cell, suggesting the presence of a carrier mediated transport process. Competitive-inhibition and countertransport studies have demonstrated that 5-FU, uracil, adenine, and hypoxanthine share the same facilitated transport system and that this system is neither temperature nor energy dependent (Domin, Mahony, & Zimmerman, 1993). Last, these studies demonstrated that at low concentrations (pharmacologic conditions), transport is not rate limiting relative to intracellular metabolism and that total intracellular 5-FU concentrations continue to increase due to the formation

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

of nucleotides, which are eventually incorporated into the cell's DNA and RNA.

The nucleosides uridine, deoxyuridine, and FdUrd have also been shown to enter cells through a carrier-mediated process (Bowen, Diasio, & Goldman, 1979; Bowen, White, & Goldman, 1978). These studies demonstrated that intracellular FdUrd reaches equilibrium with extracellular drug within 15 s. Since these nucleosides are rapidly phosphorylated following entry into the cell, rapid sampling methodology was also a critical factor in discriminating between transport and metabolism. These studies demonstrated that at low concentrations (pharmacologic conditions) transport is rapid and not rate limiting relative to phosphorylation.

Metabolism

5-Fluoropyrimidines remain inactive prior to entry into the cell. Following passage across the cell membrane, biotransformation of 5-FU results in either (a) anabolism of the drug into cytotoxic nucleotides, which are ultimately responsible for host and tumor cell death (Chabner & Collins, 1990), or (b) catabolism of the drug through an enzymatic pathway, which ultimately converts 5-FU into biologically inactive metabolites that are excreted in the urine and bile (Daher, Harris, & Diasio, 1990). Approximately 5-20% of the drug is excreted unchanged in the urine (Figure 2).

In humans more than 80% of an administered dose of 5-FU is inactivated and eliminated through the catabolic pathway

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

(Heggie, Sommadossi, Cross, Huster, & Diasio, 1987). However, the cytotoxicity of 5-FU in host and tumor cells only occurs following anabolism to nucleotides. Studies have shown that the amount of 5-FU available for anabolism is determined by the extent of its catabolism (Coustere, Mentre, & Sommadossi, 1991; Heggie et al., 1987). Thus, a delicate balance exists between the enzymatic activation of 5-FU and its catabolic elimination.

Anabolism of 5-FU

As with the uptake of 5-FU, the presence of the fluorine in the pyrimidine ring does not interfere with the entrance of 5-FU into the de novo pyrimidine anabolic pathway. The cytotoxic effects of 5-FU result from the intracellular formation of the nucleotides 5-fluorouridine-5'-triphosphate (FUTP), 5-fluoro-2'-deoxyuridine-5'-triphosphate (FdUTP), and 5-fluoro-2'-deoxyuridine-5'-monophosphate (FdUMP). The three pathways through which 5-FU may be anabolized to form these cytotoxic nucleotides (Figure 3, A, B, & C) appear to occur in most tissues and are particularly active in rapidly dividing cells (Heidelberger, 1965).

In the first pathway (Figure 3A), uridine phosphorylase (UrdPase; EC 2.4.2.3) catalyzes the addition of a ribose to 5-FU, resulting in the formation of fluorouridine (FUrd). This reaction is rapidly followed by phosphorylation of FUrd by uridine kinase (UK; EC 2.7.1.48) to form 5-fluorouridine-5'-monophosphate (FUMP). FUTP is then generated by the se-

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

quential phosphorylation of nucleoside monophosphate kinase (NMP Kinase; EC 2.7.4.4), followed by nucleoside diphosphate kinase (NDP Kinase; EC 2.7.4.6). The cytotoxic effects result from incorporation of FUTP into RNA and the subsequent effects on RNA function and stability (discussed below). 5-fluorouridine-5'-diphosphate (FUDP) may also be metabolized directly to 5-fluoro-2'-deoxyuridine-5'-diphosphate (FdUDP) by ribonucleotide reductase (Figure 3).

The second pathway (Figure 3B) is a condensation reaction of 5-FU with 5'-phosphoribosyl-1-pyrophosphate (PRPP) to form FUMP. This reaction is catalyzed by orotate phosphoribosyltransferase (OPRTase; EC 2.4.2.10) with the hydrolysis of PRPP providing sufficient energy to drive the reaction forward (Reyes & Hall, 1969). The resultant FUMP continues to follow the same phosphorylation reactions that were described above, resulting in the formation of FUTP, which is then incorporated into RNA.

Although the formation of FUMP may proceed by either the sequential, two step reaction catalyzed by UrdPase and UK or by the direct conversion by OPRTase, the dominant pathway in different types of tissues appears to vary. Studies on normal tissues (bone marrow and gastrointestinal mucosa) demonstrated that inhibition of OPRTase decreased toxicity to 5-FU (Houghton & Houghton, 1980, 1983; Schwartz & Handschumacher, 1979). In addition, many tumor cell lines, including leukemias P388, L5178Y, P1534 and sarcoma 180, were shown to use predominantly the OPRTase mediated pathway (Kessel,

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Deacon, Coffey, & Bakamajian, 1972; Reyes & Hall, 1969). Conversely, Novikoff hepatoma, Walker 256 HeLa, and some human colon carcinoma xenografts utilized the UrdPase and UK pathway to activate 5-FU (Benz & Cadman, 1981; Cory, Breland, & Carter, 1979; Houghton & Houghton, et al., 1980, 1983; Schwartz & Handschumacher, 1979). Thus, while one activation pathway for 5-FU may predominate in any given cell line, most cells use both pathways.

The third anabolic pathway (Figure 3C) begins with the addition of deoxyribose-1-phosphate to 5-FU by thymidine phosphorylase (dThdPase; EC 2.4.2.4) to yield FdUrd. This reaction is rapidly followed by phosphorylation of FdUrd into FdUMP by thymidine kinase (TK; EC 2.7.1.75). FdUMP then exerts its cytotoxic effects through inhibition of TS (EC 2.1.1.45) or by phosphorylation (using NMP and NDP kinase) into FdUTP and subsequent incorporation into DNA. Since the three mechanisms of cellular cytotoxicity associated with 5-FU (incorporation into RNA, inhibition of TS, and incorporation into DNA) are primarily responsible for the antitumor effects of 5-FU, they have been extensively studied and are described in detail below.

Incorporation of FUTP Into RNA

Early studies using ^{3}H-labeled 5-FU demonstrated that FUTP competes for incorporation into RNA with the naturally occurring nucleoside UTP (Mandel, 1969). Later studies demonstrated that 5-fluoropyrimidines can be incorporated into all

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

classes of RNA, including ribosomal (Cohen & Glazer, 1985; Herrick & Kufe, 1984), transfer (Ramberg, Ishaq, Rulf, Moeller, & Horowitz, 1978), and messenger (Armstrong, Lewis, Stern, & Cadman, 1986; Dolnick & Pink, 1983). A review of the literature in this area reveals that the specific mechanism of cytotoxicity resulting from incorporation of FUTP into RNA varies greatly depending on the experimental conditions and type of cancer cell line.

In Friend erythroleukemia cells, Novikoff hepatoma and L-1210 cells, cytotoxicity has been shown to correlate with incorporation of 5-FU into preribosomal RNA in the nucleus of the cell. Further, these studies show that this incorporation into preribosomal RNA inhibits the processing of the preribosomal RNA transcript into the lower molecular weight cytoplasmic ribosomal RNA species in a dose and time dependent manner (Herrick & Kufe, 1984; Kanamaru, Kakuta, Sato, Ishioka, & Wakui, 1986; Wilkinson, Tisty, & Hanas, 1975). Recently, a study using L-1210 cells demonstrated that 5-FU also inhibits the methylation of preribosomal RNA and transfer RNA along with the synthesis of messenger RNA (Kanamaru & Wakui, 1988).

Other studies have shown that exposure to 5-FU can affect mRNA processing and translation. Relatively low concentrations of 5-FU have been shown to inhibit polyadenylation, a process which stabilizes RNA (Carrico & Glazer, 1979). Preincubation of K562 erythroleukemia cells with an inhibitor of RNA polymerase II was shown to decrease the

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

incorporation of ^{3}H FUrd into polyadenylated mRNA with a corresponding decrease in cytotoxicity (Heimer & Sartorelli, 1989). Finally, studies by Danenberg, Shea, & Danenberg (1990) have shown that incorporation of FUTP into RNA forms weaker base-pairing between 5-FU and adenine due to partial ionization of 5-FU residues. This results in destabilizing the active conformation of RNA.

Inhibition of TS

The inhibition of TS by FdUMP represents one of the most extensively studied mechanisms causing cytotoxicity in cells following treatment with 5-FU. TS catalyzes the de novo reaction which methylates dUMP (deoxyuridine monophosphate) to dTMP (deoxythymidine monophosphate, thymidylate), obtaining the methyl group for the 5 position of the pyrimidine ring from 5, 10-methylene tetrahydrofolate (Figure 3D). dTMP is the essential precursor of dTTP (thymidine triphosphate), one of the four deoxyribonucleotides necessary for DNA synthesis. The inhibition of TS results in the depletion of intracellular dTMP pools and ultimately leads to cessation of DNA synthesis and cytotoxicity. As noted earlier, the genetic material of each cell must be duplicated each time the cell divides. Since cancer cells divide frequently, they require higher levels of deoxyribonucleotides (including dTTP) and are significantly more sensitive to agents which block nucleotide synthesis.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

To inhibit TS enzyme activity, the 5-FU anabolite FdUMP must form a ternary complex (Figure 4) with 5, 10-methylene tetrahydrofolate and TS (Danenberg & Danenberg, 1978; Santi & McHenry, 1972). A three step mechanism has been proposed for the formation of this ternary complex. First, a sulfhydryl group on a cysteine residue in the catalytic site of TS forms a covalent bond with the sixth position of dUMP (or FdUMP). This results in the activation of the vinylic fifth carbon of the pyrimidine ring, allowing condensation with the methylene group of 5, 10-methylene tetrahydrofolate (Hardy et al., 1987). Second, an electrophilic attack by 5, 10-methylene tetrahydrofolate results in the transfer of a methylene group from 5, 10-methylene tetrahydrofolate to the fifth position of the pyrimidine ring. While this occurs in a similar fashion with either dUMP or FdUMP, the ternary complex that is formed with FdUMP does not proceed past this point (Figure 4). The third reaction involves the reduction of the transferred methylene group to a methyl group by the ß-elimination of a proton from the fifth carbon on the pyrimidine ring. This reaction forms an intermediate which may act as a hydride acceptor in the reducing reaction (Danenberg & Lockshin, 1981). However, the presence of the tightly bound fluorine on the fifth carbon of the pyrimidine ring prevents the formation of this intermediate and "locks" the TS in the ternary complex shown in Figure 4 (Cisneros, Silks, & Dunlap, 1988).

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Cell cytotoxicity may (in theory) be circumvented if circulating levels of thymidine are phosphorylated by TK, providing an alternative route to dTTP synthesis. While intracellular thymidine concentrations may not be sufficient (0.1 to 0.5 μM) to allow this route of escape in most normal and tumor cells, the systemic administration thymidine has been utilized (Takimoto et al., 1996) to decrease 5-FU toxicity in a case where a patient with a pharmacogenetic disease was unable to eliminate 5-FU through the catabolic pathway (discussed in detail below).

The covalent bonding of the ternary complex is extremely stable with a Kd of approximately 10^{-12} M (Lockshin & Danenberg, 1981). However, it has been shown that TS can spontaneously promote the dissociation of FdUMP, resulting in the release of free active TS (Danenberg, 1984; Santi, McHenry, & Sommer, 1974). Thus, FdUMP is not an irreversible inhibitor of TS, and the enzyme is only temporarily inactivated while FdUMP is bound. Several studies have shown that the intracellular concentrations of 5, 10-methylenetetrahydrofolate in human tumors are the limiting factor for complete inhibition of TS in the presence of sufficient FdUMP and TS (Bertino, Levitt, McCullough, & Chabner, 1971; Danenberg & Danenberg, 1978). As the concentration of reduced cofactor increases, the dissociation of FdUMP from the ternary complex decreases (Danenberg & Danenberg, 1978), suggesting that the cytotoxicity of 5-FU may be increased by the addition of a reduced folate. Studies in L-1210 leukemia

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

cells have shown that the ternary complex has a half-life of approximately 2 hr. However, the addition of leucovorin (5-formyltetrahydrofolate) has been shown to increase the half-life of the ternary complex to approximately 35 hr. It has been shown that leucovorin is reduced to 5, 10-methylene-tetrahydrofolate, thus increasing the reduced folate pool and decreasing the dissociation of FdUMP from the stable ternary complex (Bleyer, 1989; Hakala, 1984). This results in a significant increase in the inhibition of TS with a concomitant decrease in intracellular dTTP pools, decreased DNA synthesis, and significant increase in cellular cytotoxicity (Keyomarsi & Moran, 1988).

Early studies with tumor cell lines in vitro confirmed an increase in cytotoxicity when the cells were treated with both 5-FU and leucovorin (Hakala, 1984). These studies motivated several clinical trials which demonstrated consistently higher response rates in patients treated with concomitant administration of 5-FU and leucovorin (Garmont et al., 1988; Laufman, Krzeczowski, & Segal, 1987; Petrelli et al., 1987). This has ultimately resulted in the adoption of a standard 5-FU/leucovorin regimen currently used in the treatment of metastatic colorectal adenocarcinoma (Kemeny, 1996).

Incorporation of FdUTP Into DNA

It is now clear that both 5-FU and uracil can be incorporated into DNA (Caradonna & Cheng, 1980). Following the anabolism of 5-FU into FdUTP (Figure 3C), the "false" nucleo-

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

tide is recognized as a substrate by DNA polymerase and incorporated into DNA instead of dTTP (Ingraham, Tseng, & Goulian, 1980, 1982; Tanaka, Kimura, & Yoshida, 1984). 5-FU incorporation into DNA has been demonstrated in both normal and tumor cells (Danenberg & Lockshin, 1981; Ingraham et al., 1982; Kufe, Major, Egan, & Loh, 1981; Scheutz, Collins, Wallace, & Diasio, 1986) and has been identified as a possible mechanism of cytotoxicity in some cell lines (Kufe, Scott, Fram, & Major, 1983; Schuetz, Wallace, & Diasio, 1984). However, several studies have shown that the contribution of the incorporated 5-FU into DNA, relative to the observed cellular toxicity, varies among different malignant cell lines (Parker, Kennedy, & Klubes, 1987).

Studies in mouse bone marrow cells (Scheutz et al., 1984) and L1210 cells (Kufe et al., 1983) suggest a direct correlation between cytotoxicity and the level of 5-FU incorporation into DNA. Later studies using a 5-FU resistant ovarian cancer cell line demonstrated that the resistant cell line had a 3-fold decrease in the amount of 5-FU incorporated into DNA as compared with the 5-FU sensitive parental ovarian cell line (Chu, Lai, Zinn, & Allegra, 1990). While these studies suggest a relationship between the level of 5-FU incorporation into DNA and cytotoxicity, no correlation could be found in S-49 murine T-lymphoma cells (Parker et al., 1987).

The precise mechanism by which 5-FU incorporation into DNA results in cytotoxicity is not fully understood. Several

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

studies have shown that treatment with 5-FU inhibits DNA elongation and decreases the average length of the DNA chain (Cheng & Nakayama, 1983; Schuetz & Diasio, 1985). Incubation of HCT ileal carcinoma cells with FdUrd resulted in both single and double stranded DNA breaks in a concentration and time dependent manner (Yin & Rustum, 1991).

The inhibition of TS by FdUMP may actually result in increased incorporation of both FdUTP and dUTP into cellular DNA. Since TS catalyzes the formation of dTMP from dUMP, inhibition of the enzyme results in decreased intracellular pools of dTTP with a concurrent increase in FdUMP and dUMP pools (Schuetz et al., 1984). Since both FdUTP and dUTP are substrates for DNA polymerase, as their pool size increases relative to the decreasing pool size of dTTP, there is greater likelihood that either FdUTP or dUTP will be incorporated into DNA.

There are two mechanisms by which DNA damage is prevented. The first mechanism prevents incorporation of FdUTP and dUTP into DNA through the rapid hydrolysis of FdUTP and dUTP into FdUMP and dUMP, respectively. This is accomplished by the pyrophosphatase enzyme dUTP hydrolase (Caradonna & Cheng, 1980; Ingraham et al., 1980). The second mechanism involves the DNA repair enzyme uracil glycosylase. This repair enzyme removes incorporated uracil or 5-FU from DNA through the hydrolysis of the uracil and 5-FU deoxyribose glycosyl bond of dUMP and FdUMP residues in the DNA (Ingraham et al., 1980; Mauro, De Riel, Tallarida, & Sirover, 1993). This is followed

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

by subsequent repair of the apyrimidinic site by apyrimidine endonuclease (Caradonna & Cheng, 1980).

Later studies by Schuetz et al., (1988) confirmed that DNA repair does occur following incorporation of 5-FU; however, this repair was also shown to produce damage to the DNA structure leading, to inhibition of DNA elongation, in addition to DNA fragmentation.

Catabolism

Until recently, most of the research in 5-FU metabolism was focused on the anabolic activation of the drug. This was the obvious pathway of interest since early cellular studies of 5-FU demonstrated that the antitumor effects (as well as the host cytotoxicity) could be attributed to the conversion of 5-FU to nucleotides (Heidelberger, 1965; Kessel, Hall, & Wodinsky, 1966). However, as discussed earlier, the catabolic pathway actually regulates the amount of 5-FU available for the anabolic pathway (Diasio & Harris, 1989; Heggie et al., 1987). The importance of the catabolic pathway was clearly demonstrated in clinical studies conducted in the early 1980s (Woodcock, Martin, Damin, Kemeny, & Young, 1980). In these studies, 5-FU was coadministered with thymidine (which is metabolized to thymine). Since the catabolic (degradative) pathway for 5-FU is identical to that of the naturally occurring pyrimidines (uracil and thymine), the coadministration of thymidine with 5-FU resulted in competition for the catabolic pathway and ultimately caused decreased clearance of 5-

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

FU and a corresponding increase in the availability of 5-FU for anabolism (Au, Rustum, Ledesma, Mittelman, & Creaven, 1982). These early studies, along with others demonstrating the extent to which an administered dose of 5-FU is eliminated through the catabolic pathway (Heggie et al., 1987), generated intense interest in fully characterizing the catabolic pathway.

The first studies in the catabolism of fluoropyrimidines (Chadhuri, Mukherjee, & Heidelberger, 1959; Mukherjee & Heidelberger, 1960) were complicated by technical limitations in which open column low pressure chromatography was used in an attempt to identify metabolites. Progress in delineation of the catabolic pathway did not proceed until the early 1980s, when reverse phase high pressure liquid chromatography (HPLC) methodology (Diasio & Wilburn 1979; Sommadossi, Gewirtz, Diasio, Aubert, & Cano, 1982) was successfully used to resolve virtually all of the metabolites of 5-FU (catabolites and anabolites). These and subsequent studies conducted in isolated rat hepatocytes (Sommadossi, Cross, Gerwirtz, Goldman, & Diasio, 1985) and isolated perfused rat liver (Sweeny, Barnes & Diasio, 1988), as well as clinical pharmacokinetic studies in cancer patients receiving 5-FU (Heggie et al., 1987), clarified and defined the catabolic pathway shown in Figure 5.

The degradative catabolic pathway for uracil, thymine, and 5-FU begins with the reduction of the double bond between C-5 and C-6 of the pyrimidine ring (Figure 5A). This reaction

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

uses NADPH as the hydrogen donor and is catalyzed by dihydropyrimidine dehydrogenase (DPD, EC 1.3.1.2). The pyrimidine ring is then hydrolyzed between N-3 and C-4 (Figure 5B) in a reaction catalyzed by dihydropyrimidinase (DHPase, EC 3.5.2.2). The opened pyrimidine ring is next cleaved between N-1 and C-2 by ß-ureido-propionase (ß-alanine synthase, EC 3.5.1.6) to yield carbon dioxide, ammonia, and the corresponding ß amino acid (Figure 5C).

Examination of metabolites in plasma, urine, and bile (Heggie et al., 1987), using radiolabeled 5-FU, demonstrated that over 24 hr, α-fluoro-ß-alanine (FBAL) represented the major catabolite in urine (>95%). Approximately 2-3% of the total administered radiolabeled dose of 5-FU was identified in bile as previously unknown metabolites. These novel metabolites were later identified (Sweeny, Barnes, Heggie, & Diasio, 1987; Sweeny, Martin, & Diasio, 1988), using HPLC and mass spectrometry analysis, as conjugates of FBAL and either cholic acid or N-chenodeoxy-cholic acid (Figure 5D). These previously unknown bile acid conjugates were shown to be conjugated by the cytosolic liver enzyme cholyl-CoA-amino acid N-acyltransferase (EC 2.3.1.13, BAT) (Johnson, Barnes, & Diasio, 1990; Johnson, Barnes, Kwakye, & Diasio, 1991). The observation that the FBAL-chenodeoxycholate bile acid conjugate could produce cholestasis in the isolated perfused rat liver (Sweeny, Barnes, & Diasio, 1988) motivated further studies which ultimately resulted in the purification, characterization, and cDNA cloning of BAT (Falany, Johnson,

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Barnes, & Diasio, 1994; Johnson et al., 1991). Following a brief description of each of the enzymes involved in the catabolic pathway, a separate section will describe in detail the enzymology and pharmacology of DPD (the focus of this study).

DPD

DPD is the initial and rate limiting enzyme involved in pyrimidine catabolism (Lu, Zhang, & Diasio, 1992). This enzyme catalyzes the reversible reduction of uracil, thymine, and 5-FU to their corresponding dihydropyrimidines (Figure 5A). In the past several years, a significant number of studies have emphasized the importance of the DPD enzyme as a major determinant which influences both host cell toxicity and the antitumor effectiveness of 5-FU (Diasio & Harris, 1989; Fleming et al., 1992). In addition, there have been several reports describing cancer patients with an inherited deficiency of DPD who develop severe toxicity (including death) to fluoropyrimidine based chemotherapy (Diasio, Beavers & Carpenter, 1988; Fleming et al., 1992; Lu, Zhang & Diasio, 1993a; Morrison, Bastian, Dela Rosa, Diasio, & Takimoto, 1997; Tuchman et al., 1985). These studies have motivated research in the biochemical and molecular characterization of this important catabolic enzyme (Lu et al., 1992; Yokota et al., 1994). The purification, characterization and pharmacology of DPD will be discussed in detail below.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

DHPase

The second enzyme in pyrimidine catabolism is DHPase. This enzyme catalyzes the reversible hydrolysis of dihydrouracil, dihydrothymine, and dihydrofluorouracil into ß-ureidopropionic acid, ß-ureidoisobutyrate, and a-fluoro-ß-ureidopropionic, respectively (Figure 5B). In early studies, DHPase was partially purified and characterized from calf and rat liver (Maguire & Dudley, 1978; Wallach & Santiago, 1957). More recently, DHPase has been purified to homogeneity from cow liver (Brooks, Jones, Kim, & Sander, 1983; Lee, Cowling, Sander, & Pettigrew, 1986) and rat liver (Kikugawa et al., 1994). Both the bovine and rat liver enzymes were shown to be tetramers with a molecular weight of 226 and 215 kDa, respectively. In addition, each DHPase enzyme was shown to contain 4 mol Zn^{2+} per mol of active enzyme, presumably 1 atom Zn^{2+} per subunit (Brooks et al., 1983; Kikugawa et al., 1994; Lee et al., 1986).

In the early and mid-1990s, several case reports described pediatric patients who were deficient in DHPase (Duran, Robers, & de Bree, 1991; Henderson, Ward, Simmonds, Duley, & Davies, 1993; Ohba et al., 1995; Putman et al., 1997). These pediatric patients exhibited neurological abnormalities including febrile illness, seizures, and gross microcephaly, as well as developmental retardation. In an attempt to delineate the molecular basis for this disease, the cDNA coding for the rat (Matsuda et al., 1996) and human (Hamajima et al., 1996) DHPase has recently been cloned.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

These studies have revealed significant amino acid identity between DHPase and proteins thought to be related to nervous system development (CRMP-62 and TOAD-64). However, the frequency and ultimate molecular basis for DHPase deficiency remains to be determined and continues to be a growing and active area of research (Hamajima et al., 1996).

ß-Alanine Synthase (ß-Ureidoproprionase)

ß-Alanine synthase is the third enzyme involved in pyrimidine catabolism (Figure 5C). This enzyme irreversibly cleaves ß-ureidopropionate, ß-ureidoisobutyrate, and a-fluoro-ß-ureidopropionate to ß-alanine, ß-aminoisobutyrate, and a-fluoro-ß-alanine, respectively. Carbon dioxide is generated from the second carbon of the pyrimidine ring, while ammonia is generated from N-3 (Figure 5C). ß-Alanine synthase has recently been purified to homogeneity and characterized from rat liver (Matthews, Liao, Kvalnes-Krick, & Traut, 1992). The purified rat liver enzyme was shown to be a hexamer with a subunit molecular weight of 42 kDa, an isoelectric point of 6.7, and a K_m of 8 µM. The enzyme was shown to be sensitive to denaturation if heated and required the addition of reducing agents to minimize denaturation during the purification process. In addition, ß-alanine synthase was shown to be a dissociating enzyme that is converted to an inactive trimer by the product ß-alanine or is converted to the larger enzymatically active hexamer form in response to the substrate N-

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

carbamoyl-ß-alanine (Matthews & Traut, 1987; Matthews et al., 1992).

Importance of DPD in 5-FU Chemotherapy

As mentioned earlier, most (more than 80%) of an administered dose of 5-FU is inactivated and eliminated through the catabolic pathway (Heggie et al., 1987). However, the cytotoxicity of 5-FU in host and tumor cells occurs following anabolism of 5-FU to nucleotides. Preclinical studies have shown that inhibition of pyrimidine catabolism results in decreased clearance of 5-FU with concurrent increases in plasma 5-FU concentrations. This ultimately results in increased anabolism of 5-FU with corresponding increases in cytotoxicity (Diasio, Lu, Zhang, & Shihanian, 1995). Since DPD is the initial and rate-limiting step in pyrimidine catabolism (Lu et al., 1992), several studies have focused on the relative importance of the DPD step as a major determinant of both the toxicity and the antitumor effectiveness of 5-FU. These studies will be described below.

Effect of DPD on 5-FU Pharmacokinetics

DPD has been shown to influence 5-FU pharmacokinetics. Studies in mice by Iigo et al. (1989) demonstrated that co-administration of 5-FU and (E)-5-(2-bromovinyl)-2'-deoxyuridine (a prodrug which is metabolized to (E)-5-(2-bromovinyl) uracil, a potent inhibitor of DPD) results in a significant decrease in 5-FU clearance. Later studies by Fleming et al.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

(1992) examined whether DPD enzyme activity levels could be correlated to 5-FU clearance. These studies examined both plasma 5-FU levels and DPD levels in peripheral blood mononuclear (PBM) cells in 57 patients receiving 5-FU by 5-day continuous infusion (1,000 mg/M^2). The results demonstrated a significant linear correlation between DPD activity and 5-FU clearance ($r=0.716$, $p<0.0001$). The authors of this study, as well as others (Etienne et al., 1992), have concluded that monitoring DPD activity in PBM cells may be useful in 5-FU based chemotherapy for identifying patients at risk for altered 5-FU metabolism.

Additional studies have shown that the level of DPD in the tumor can have a major effect on the likelihood that the tumor will respond to 5-FU (Etienne et al., 1995; Milano, Etienne, & Fischel, 1995). Those tumors with high levels of DPD enzyme activity have been shown to catabolize 5-FU efficiently, resulting in reduced 5-FU available for the anabolic pathway. Thus, increased DPD expression in certain tumors may be the basis for some cases of drug resistance (nonresponders) following 5-FU chemotherapy. These studies have motivated several researchers to search for drugs (such as ethynluracil and iodouracil) that inhibit DPD (Porter, Chestnut, Taylor, Merrill, & Spector, 1991; Spector et al., 1994). These researchers (and others) have shown that administration of 5-FU with a DPD inhibitor results in improved antitumor effectiveness (Baker et al., 1996; Fischel et al., 1997; Masaaki, Araki, Nakajima, & De Clercq, 1988).

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Last, pharmacokinetic studies of plasma 5-FU levels in patients receiving 5-FU during a 5-day continuous venous infusion at a constant rate demonstrated that the plasma 5-FU levels varied throughout the day with a circadian pattern (Petit, et al., 1988). Subsequent studies in patients receiving 5-FU by continuous infusion showed that the plasma 5-FU levels varied inversely with a circadian variation in DPD enzyme activity from PBM cells (Harris, Song, Soong, & Diasio, 1990). These authors have suggested that time-modified administration of 5-FU could improve chemotherapeutic response.

DPD Deficient Patients

The importance of DPD activity in 5-FU chemotherapy and toxicity is best illustrated by examining what happens to patients who are DPD deficient. In 1985 Tuchman et al. described a breast cancer patient who developed profound toxicity and eventually died following adjuvant treatment with cyclophosphamide, methotrexate, and 5-FU (CMF regimen). Prior to her death, elevated levels of uracil and thymine were detected in both the serum and the urine of the patient and her brother. Although direct measurement of DPD enzyme activity was not performed, the authors of this study suggested familial pyrimidinemia, possibly secondary to DPD deficiency, as a cause for altered 5-FU metabolism and increased toxicity.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Subsequent studies in this laboratory described a 40-year-old female breast cancer patient who developed grade IV myelosuppression following each cycle of CMF chemotherapy (Diasio et al., 1988). After her third cycle of CMF chemotherapy, she developed cerebellar ataxia and cerebral encephalopathy progressing to obtundation 1 week later. Treatment was discontinued and her neurological status slowly returned to normal over 4 months. This patient was assayed for DPD activity and demonstrated an undetectable level of enzyme activity in her PBM cells. Pharmacokinetic studies were conducted on this patient by administration of a radiolabeled "test" dose of 5-FU (25 mg/M^2, 600 μCi (^{3}H-6)-5-FU) equivalent to 22 times less than the standard dose of 550 mg/M^2. Plasma, urine, and cerebrospinal fluid were sampled at various times to determine the elimination half-life and clearance of 5-FU. This patient was shown to present altered 5-FU pharmacokinetics with prolonged elimination half-life of 159 min and significantly reduced 5-FU clearance of 70 ml/min/m^2 (as compared to patient controls who had an apparent elimination half-life of 13±7 minutes and total plasma clearance of 594±198 ml/min/m^2). Approximately 90% of the drug was recovered in the urine of the patient within 24 hr of administration (as compared to 9.8±1.6% in patient controls). As with the original patient described by Tuchman et al.(1985), this patients baseline plasma uracil and thymine concentrations were also elevated.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

There have now been more than 17 patients reported with DPD deficiency who experienced severe toxicity after 5-FU administration (Harris, Carpenter, & Diasio, 1991; Lyss, Lilenbaum, Harris, & Diasio, 1993; Lu et al., 1993a; Houyauet al., 1993; Beuzeboc, Pierga, Lyonnet, Couturier, & Pouillart, 1996; Beuzeboc et al., 1996; Takimoto et al., 1996). Although the frequency of DPD deficiency in the population remains to be determined, the number of case reports continues to increase, suggesting that this pharmacogenetic syndrome may be more frequent than originally thought.

Characterization of Human DPD

The importance of DPD to 5-FU based chemotherapy, along with the increasing number of confirmed DPD deficient cancer patients, has resulted in a number of studies focused on the biochemical and kinetic characterization of the DPD enzyme. Early studies reported the purification of DPD to varying degrees from the liver of several species, including bovine (Grisolia & Cardoso, 1957; Porter et al., 1991), rat (Fritzen, 1960; Fujimoto, Matsuda, Kikugama, Kaneko, & Tamak, 1990; Shiotani & Weber, 1981), mouse (Sanno, Holzer, & Schimke, 1970), and pig (Goedde, Agarwal, & Eickhoff, 1970; Podschun, Cooks, & Schnackerz, 1990); however, in most of these preparations, the DPD enzyme was not purified to homogeneity.

In 1992 Lu et al. reported the first purification to homogeneity of human liver DPD. The purified enzyme was

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

reported to be a dimer with a subunit mass of 105 kDa. Kinetic studies were conducted with uracil, thymine, and 5-FU with reported Km values of 4.9, 4.8, and 3.3 μM, respectively (Lu et al., 1992). These data suggest that 5-FU is the preferred substrate over both uracil and thymine and agree with previously published data obtained from crude liver homogenates (Naguib, el Kouni, & Cha, 1985). In addition, for the first time, a specific polyclonal antibody was generated against human liver DPD.

The purification methodology developed to purify human liver DPD was slightly modified in later studies to allow purification to homogeneity of rat, pig, and bovine liver DPD (Lu, Zhang & Diasio, 1993b). These studies generated specific polyclonal antibodies against pig (Podschun, Wahler, & Schnackerz, 1989) and rat (Lu et al. 1993b) liver DPD in addition to amino-terminal and internal amino acid sequence data for bovine (Porter, Chestnut, Merrill, & Spector, 1992) and human liver DPD (Lu et al., 1992).

Utilizing the pig polyclonal antibody, the full length DPD cDNA has recently been cloned from pig liver (Yokota et al., 1994). In the same study, the pig liver DPD cDNA was used as a probe to clone a partial DPD cDNA from a human liver cDNA library. This report was rapidly followed by the independent cDNA cloning of bovine liver DPD cDNA in our laboratory (Albin, Johnson, & Diasio, 1996).

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Characterization of DPD Deficient Patients

Initial studies characterizing DPD deficient patients were conducted using the specific polyclonal antibody raised against human liver DPD. Peripheral blood mononuclear (PBM) cytosol from 15 DPD deficient patients was examined by Western blot analysis (Lu et al., 1993a; Diasio et al., 1995) to determine DPD protein levels. In all of these cases of DPD deficiency, DPD protein was either undetectable or significantly reduced compared to controls with normal DPD activity. Whether this decrease in DPD levels reflects a decrease in transcription, translation, mRNA stability, or increased protein degradation remains to be determined.

With the availability of the partial cDNA sequence for human liver DPD, it has also become possible to examine, at the molecular level, patients who were previously identified as being DPD deficient. Initial reports by our laboratory and others have reported possible DPD mutations associated with DPD deficiency, including a missense base pair (bp) substitution (Albin, Johnson, Shahinian, Wang, & Diasio, 1995) and exon skipping (Meinsma, Fernandez-Salguero, Van Kuilenburg, Van Gennip, & Gonzalez, 1995; Wei, Mcleod, McMurrough, Gonzalez, & Fernandez-Salguero, 1996). Currently, little is known about the functional effects or the population frequency of these sequence differences in cDNA of the DPD enzyme.

In summary, for over 40 years 5-FU has been one of the most effective and widely used anticancer chemotherapeutic drugs. Over the past decade, a number of studies have demon-

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

strated the critical role of DPD in 5-FU pharmacokinetics, toxicity, and efficacy. The identification of cancer patients with an inherited deficiency of DPD has also heightened interest in the characterization of human DPD. Further characterization of the DPD enzyme should be useful in the development of specific diagnostic tests that may predict both host toxicity and tumor response prior to administration of 5-FU.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

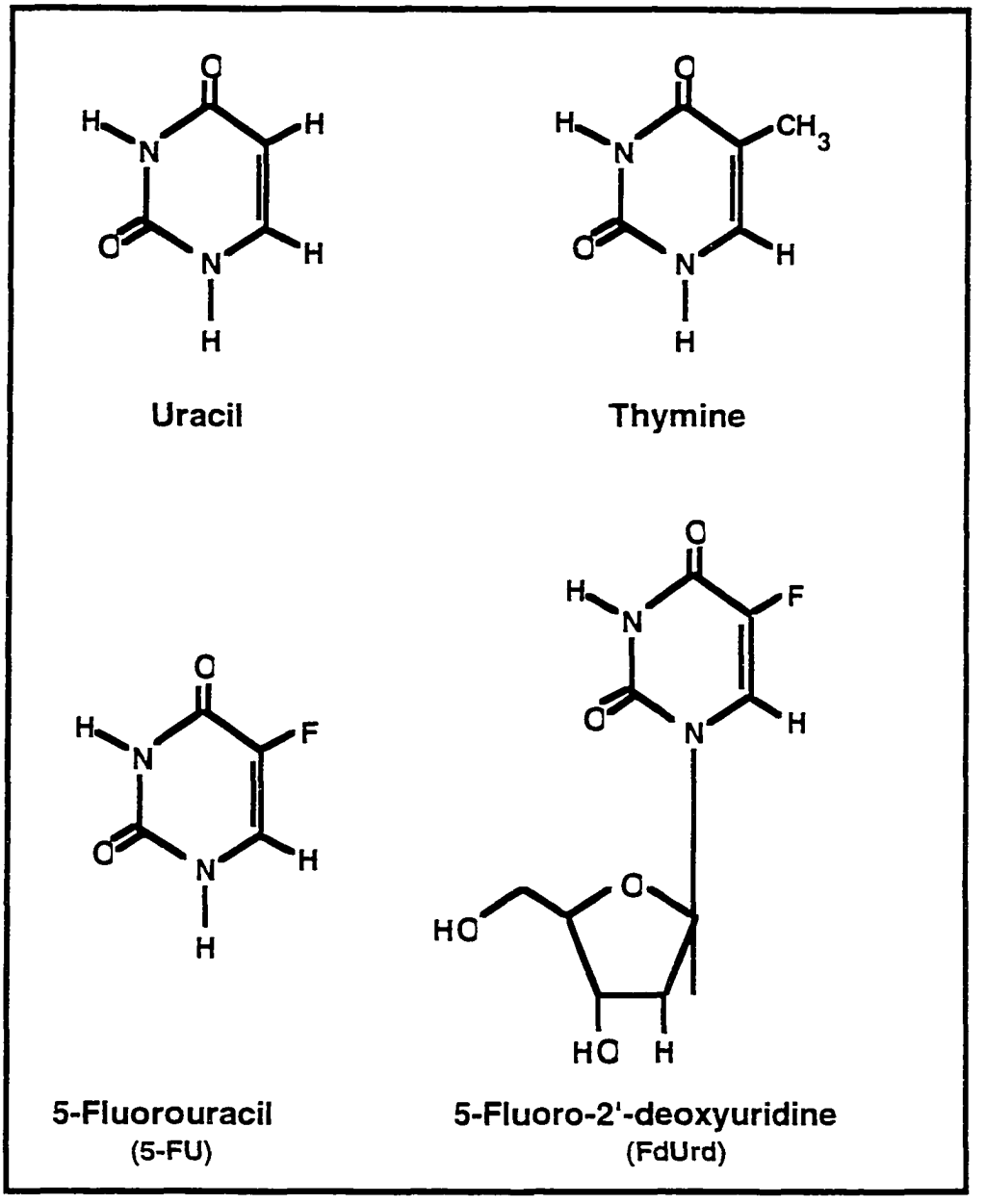


Figure 1. Chemical structures of uracil, thymine, 5-fluoro-uracil, and 5-fluoro-2'-deoxyuridine.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

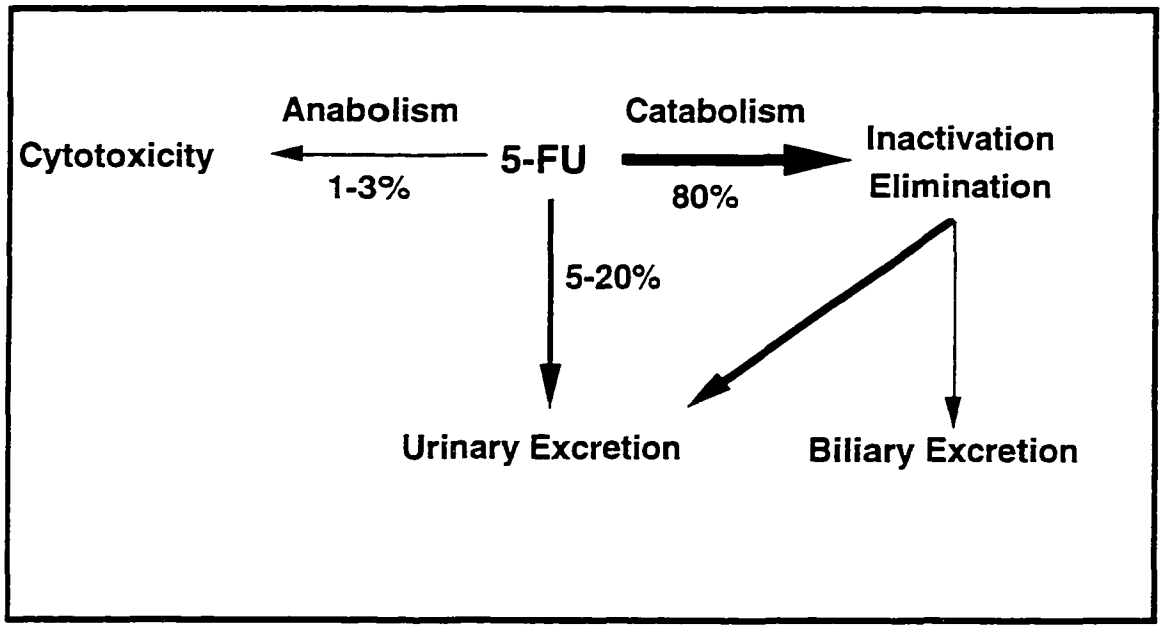


Figure 2. Metabolism of 5-fluorouracil in humans.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

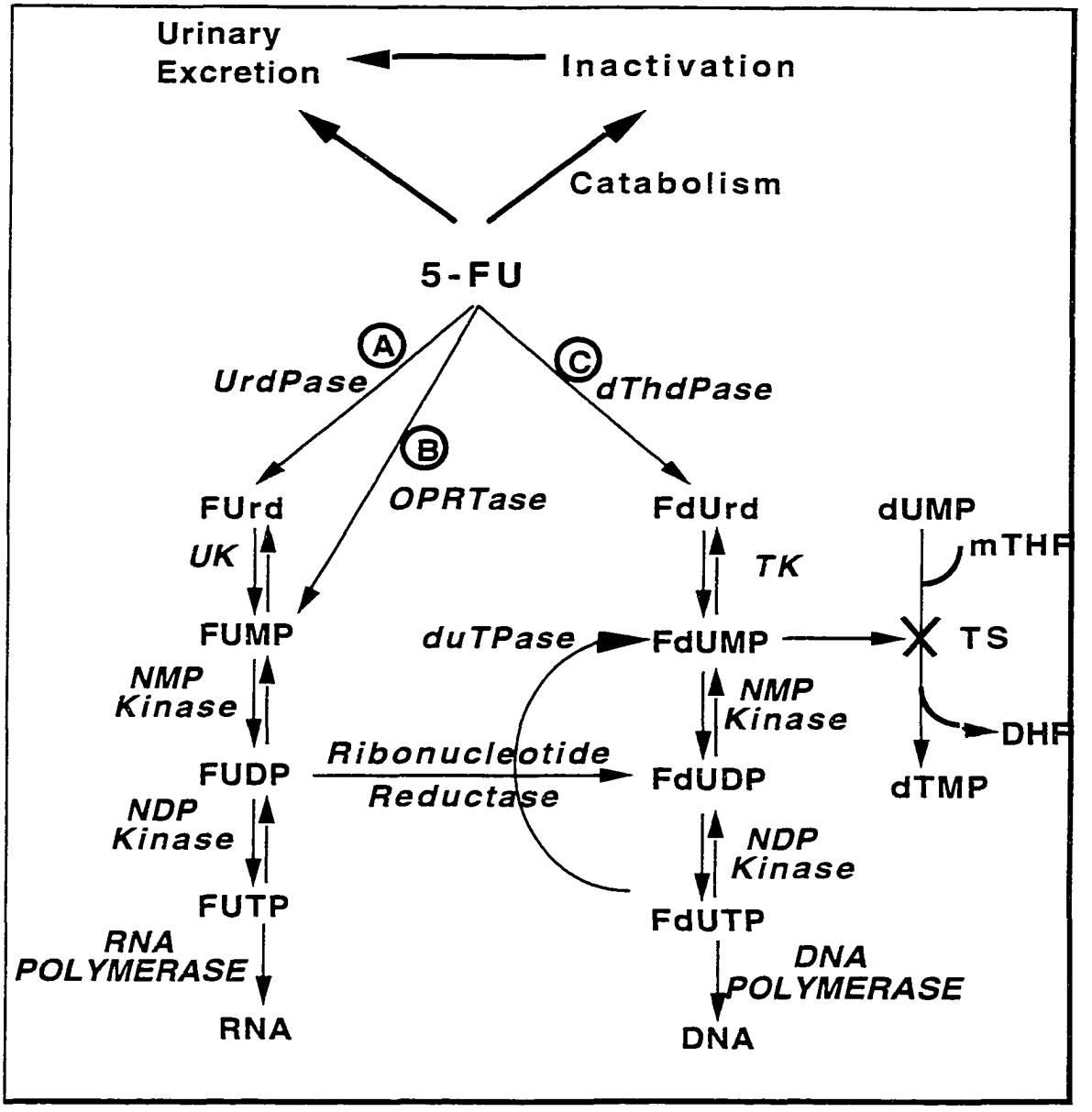


Figure 3. Anabolism of 5-fluorouracil and 5-fluoro-2-deoxyuridine in humans. UrdPase=uridine phosphorylase; UK=uridine kinase; NMP Kinase=nucleoside monophosphate kinase; NDP Kinase=nucleoside diphosphate kinase; ORTase=orotate phosphoribosyltransferase; dThdPase=thymidine phosphorylase; TK=thymidine kinase; TS=thymidylate synthetase.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

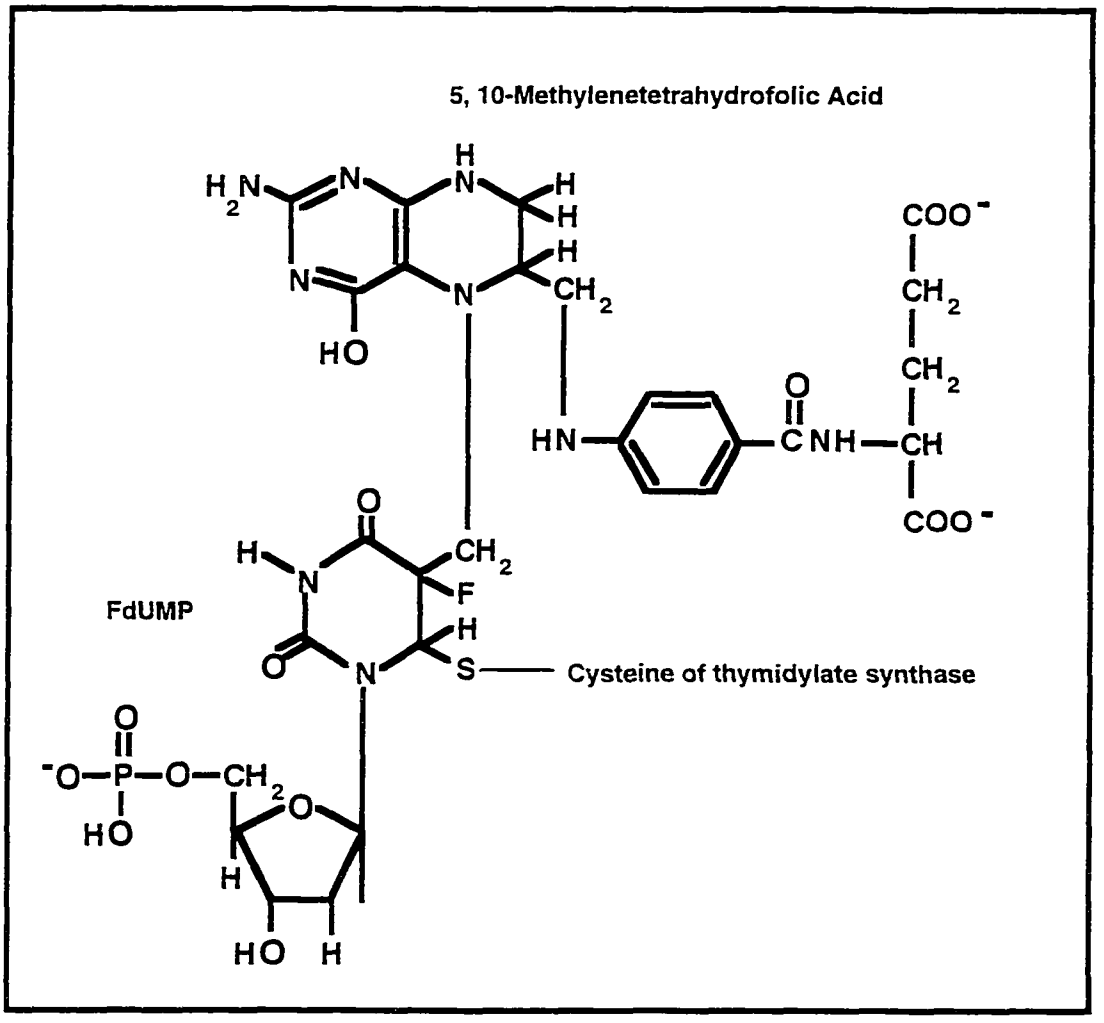


Figure 4. Inactivation of thymidylate synthase (TS) through formation of a ternary complex containing 5, 10 methylenetetrahydrofolic acid, 5-fluoro-2-deoxy-uridine monophosphate (FdUMP), and TS.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

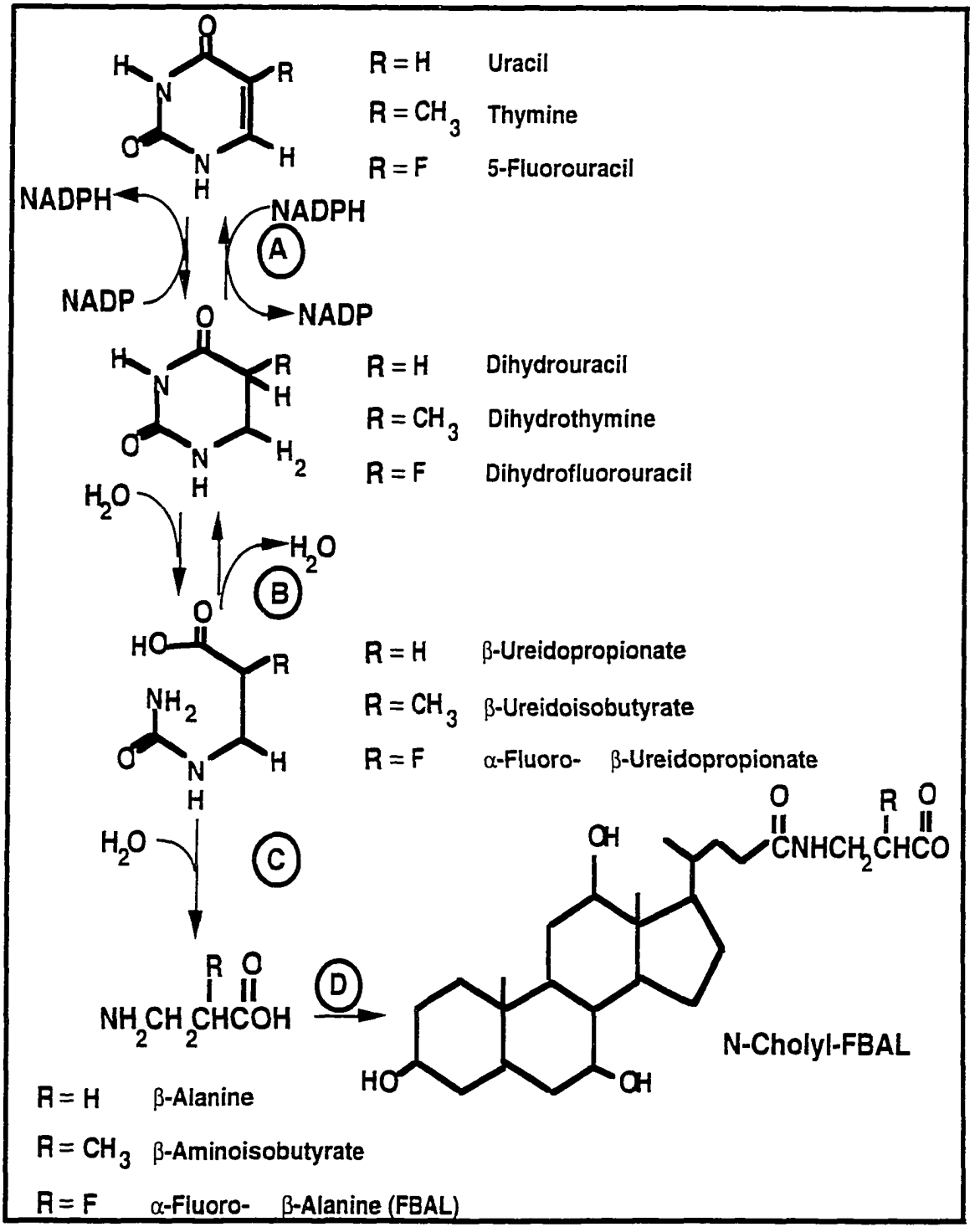


Figure 5. Inactivation and elimination of 5-FU through the catabolic pathway.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

SPECIFIC AIMS

The primary objective of this dissertation research was to improve clinical 5-FU chemotherapy through a molecular characterization of the human DPD enzyme. Following review from my committee, the following specific aims were proposed:

1. Develop a method for the direct measurement of DPD enzyme activity. (a) Design a specific semiautomated DPD radioassay. (b) Perform assay validation experiments, including recovery and stability of reaction products, optimal reaction conditions, linearity with time, and protein concentration, and kinetic properties. (c) Utilize the radioassay to screen cancer patients DPD activity prior to administration of 5-FU with the possibility of identifying new DPD deficient cancer patients.

2. Determine the structural organization of the DPD gene (intron/exon boundaries). (a) Delineate the structural organization of the DPD gene, including determination of the intron/exon splice sites and estimation of intron lengths.

3. Develop a strategy for cloning the complete cDNA coding for human DPD. (a) To clone and sequence the complete human lymphocyte DPD cDNA from a healthy donor with normal enzyme activity. (b) To clone and sequence the complete cDNA coding for human liver DPD. (c) To compare lymphocyte and liver DPD cDNA.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

4. Perform a preliminary molecular characterization of three DPD deficient patients. (a) To screen the genomic DNA of three DPD deficient patients for an A (control) to T (deficient) missense mutation previously identified in a DPD deficient patient. (b) To screen the genomic DNA of the same three DPD deficient patients for a G (control) to A (deficient) mutation previously identified in the invariant GT splice site of a Dutch DPD deficient patient.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

SEMI-AUTOMATED RADIOASSAY FOR DETERMINATION OF DIHYDRO-PYRIMIDINE DEHYDROGENASE (DPD) ACTIVITY; SCREENING CANCER PATIENTS FOR DPD DEFICIENCY, A CONDITION ASSOCIATED WITH 5-FLUOROURACIL TOXICITY

by

MARTIN R. JOHNSON, JIEMING YAN, LINGNING SHAO, NICOLAS ALBIN AND ROBERT B. DIASIO

Journal of Chromatography B, 696, 183-191, 1997

Copyright
1997
by
Elsevier Science

Used by permission

Format adapted for dissertation

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

ABSTRACT

Dihydropyrimidine dehydrogenase (DPD) catalyzes the reduction of the naturally occurring pyrimidines, uracil and thymine, and the fluoropyrimidine anticancer drug, 5-fluorouracil (5-FU) to 5,6-dihydropyrimidines. Previous studies have demonstrated that cancer patients who are DPD deficient exhibit severe toxicity (including death) following treatment with 5-FU. To date, the direct measurement of DPD enzyme activity has been the only reliable method to identify DPD deficient cancer patients. We now report a semi-automated radioassay for measuring DPD activity in human peripheral lymphocytes. Following incubation of lymphocyte cytosol (at a fixed protein concentration of 200 μg) with [6-14C]5-FU at timepoints ranging from 0 to 30 min, samples are ethanol precipitated, filtered and analyzed by HPLC. Determination of radioactivity is accomplished using an in-line flow scintillation analyzer with automatic quantitation of peaks. This method provides the first specific assay for DPD enzyme acvity which is rapid, reproducible and sensitive enough to be used in the routine screening of cancer patients for DPD deficiency prior to treatment with 5-FU.

INTRODUCTION

Dihydropyrimidine dehydrogenase (EC 1.3.1.2, dihydrouracil dehydrogenase, dihydrothymine dehydrogenase, DPD), is the initial and rate-limiting enzyme involved in the reduction of both uracil and thymine to dihydrouracil and dihydro-

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

thymine, respectively (Traut & Loechel, 1984; Westernak, 1980). DPD catalyzes the first reaction in the three-step catabolic pathway leading to the formation of ß-alanine (Mathews, Liao, Kvalnes-Krick, & Traut, 1992). Clinical interest in DPD activity originated with studies which demonstrated that this enzyme catabolizes more than 85% of an administered dose of the antineoplastic agent 5-fluorouracil (5-FU) to dihydrofluoro-uracil (Daher, Harris, & Diasio, 1990). Recent studies have shown that DPD regulates the amount of 5-FU available for metabolism, thereby affecting its pharmacokinetics, toxicity and efficacy (Baccanari, Davis, Knick, & Spector, 1993; Harris, Song, Song, & Diasio, 1990).

Deficiency in DPD activity was shown to be responsible for severe 5-FU toxicity during cancer chemotherapy. Administration of standard doses of 5-FU to DPD deficient patients resulted in mucositis, granulocytopenia, neuropathy and even death (Diasio, Beavers, & Carpenter, 1988; Harris, Carpenter, & Diasio, 1991). Since the initial reports several years ago, there have been an increasing number of cases reported suggesting that this disorder may be more frequent (3% based on population studies of DPD activity from peripheral blood mononuclear (PBM) cells) than initially thought (Milano & Etienne, 1994). The frequent use of 5-FU in cancer chemotherapy, the critical role of DPD in 5-FU pharmacokinetics and the clinical significance of DPD deficiency suggests the potential value of determining DPD activity in cancer pa

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

potential value of determining DPD activity in cancer patients prior to 5-FU treatment.

In the present study we describe a new HPLC based, semi-automated radioassay for measuring DPD activity in human peripheral lymphocytes. This assay utilizes [6-14C]5-FU; peak detection using an in-line flow scintillation analyzer[1]; and computer software allowing automatic quantitation of peaks. The system allows for continuous multiple sampling with automatic integration and quantitation of dihydrofluorouracil. All chromatograms are stored in data archives following GLP procedures.

To determine whether this assay was useful in the clinical diagnosis of DPD deficiency, samples from recently diagnosed cancer patients were obtained and assayed for DPD activity. These data resulted in the identification of two profoundly DPD deficient cancer patients. These patients are unique in that they were diagnosed as DPD deficient prior to treatment with 5-FU thus avoiding the life threatening toxicity associated with this syndrome. We conclude that this assay is rapid and simple enough for use in the detection and or screening of DPD deficient patients prior to treatment with 5-FU.

EXPERIMENTAL

Chemicals

Histopaque, bovine serum albumin, and NADPH were purchased from Sigma (St. Louis, MO, USA). [6-14C]5-FU (56

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

mCi/mmol) was obtained from New England Nuclear (Boston, MA, USA). The purity of the unlabelled and labelled 5-FU was confirmed by HPLC to be greater than 99%. All other solvents and reagents were purchased in the highest grade available. The primary buffer (buffer A) contained 35 mM potassium phosphate (pH 7.4), 2.5 mM magnesium chloride and 10 mM 2-mercaptoethanol. Since NADPH, the cofactor in the enzyme reaction, is light sensitive and unstable with long term storage, it was freshly prepared.

Patients and Controls

One hundred healthy volunteer (age range 19-58 years) samples were collected from individuals who had never been treated with 5-FU. This normal population consisted of faculty, staff and students from this University. Samples from eighty recently diagnosed cancer patients were obtained during initial oncology office visits, prior to any chemotherapy treatment. Informed consent was obtained from both healthy volunteers and cancer patients using an institutionally approved protocol.

Isolation of Mononuclear cells

Since DPD activity is known to follow a circadian pattern of enzyme activity, all blood samples were obtained between 8 a.m. and 9 a.m. Blood samples (30-60 ml) were drawn from a peripheral vein into a 60 cc syringe containing 5 ml of heparin (1000 units/ml). The syringe was inverted to

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

mix heparin and blood after collection. The blood sample was then layered (in 30 ml aliquots) onto 15 ml of warm (37°C) Histopaque. The blood was fractioned by centrifugation at 2500 g for 30 min at 25°C, and the peripheral blood mononuclear (PBM) cells (located between the plasma fraction and Histopaque) were carefully transferred to a clean 50 cc conical centrifuge tube. Residual Histopaque was removed by washing the PBM cells with 25 ml cold (4°C) phosphate-buffered saline (PBS) followed by centrifugation at 1200 g for 10 min at 4°C for a total of three times.

Preparation of Cytosol

The fresh PBM cells were resuspended in 300 µl of cold (4°C) buffer A and disrupted (four times for 10 s with a 30 s interval between sonication) on a VirSonic 50 sonicator at 50% power. Following centrifugation at 14,000 g for 30 min at 4°C, the supernatant (PBM cytosol) was removed for use in the subsequent enzyme assays. The protein concentration of the cytosol was determined using the method outlined by Bradford (Bradford et al., 1976) using bovine serum albumin as the standard.

DPD Assay

The standard assay mixture contained 200 µM NADPH, 8.23 µM [6-14C]5-FU (56 mCi/mmol), buffer A and 200 µg cytosolic protein in a final volume of 1 ml. Incubations were performed at 37°C. At 5 min time points (from 0 to 30 min), 130 µl of

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

the reaction sample was transferred to a clean eppendorf tube and the reaction was stopped by adding an equal volume of ice cold 100% ethanol. This mixture was then vortexed and placed in a -70°C freezer for at least 10 min to facilitate protein precipitation. The samples were centrifuged for 10 min at 14,000 g and filtered through a 0.2 µm Acrodisc filter (Gelman Sciences, Ann Arbor, MI, USA) prior to HPLC analysis.

HPLC Analysis

A high performance liquid chromatograph (Hewlett-Packard 1050) was equipped with an automatic injector and an on-line radioisotope flow detector (Radiomatic FLO-ONE Beta, Packard Instrument, Meriden, CT, USA). This system utilizes a Dell XPS P90 computer running Flo-one software which allows continuous multiple sampling with integration and quantitation of peaks of radioactivity. All analyses were performed on 50 µl of sample using two reversed phase columns (25 x 0.46 cm) connected in series and packed with Hypersil ODS (Jones Chromatography, Lakewood, CO, USA)[2]. Elution was carried out isocratically at a flow rate of 1 ml/min with a mobile phase consisting of 1.5 mM potassium phosphate-5.0 mM tetrabutylammonium hydrogen sulfate (pH 8.5). Eluent from the columns was directed via a low dead volume connection into the on-line radioisotope flow detector for quantitation of catabolites. A non-gelling scintillation fluid (Ultima-Flo AP, Packard., Downers Grove, IL, USA) was used for all analysis at an effluent to scintillation fluid ratio of 1:3 (v/v).

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Calculation of DPD Activity

Integration and quantitation of peaks of radioactivity was calculated using the Flo-one software provided with the on-line radioisotope flow detector. A quench correction was made using a special quench curve correction program incorporated into the software of this instrument. The quench of a standard radiolabelled sample (while in the mobile phase used during the HPLC analysis) is determined by the channels ratios method (Kessler et al., 1982). The channels ratios vs. the counting efficiency is stored by the system software. When an actual sample is analyzed with the same mobile phase, the dpm is automatically corrected for quenching. Since there is no dilution in vitro by endogenous metabolites, the nmoles of the dihydrofluorouracil can be calculated directly from the peak area.

To determine the specific activity of patient samples, nmoles of dihydrofluorouracil formed (y) were plotted against time (x). The slope of the graph (products formed/min) was then calculated by linear regression analysis. The slope was then divided by the amount of protein added to obtain the final result (DPD activity expressed as nmol/min/mg protein).

Assay Validation

The intra-assay (within-run) precision was assessed by analyzing five replicates of three concentrations of PBM cytosol (100, 150 and 250 μg) on the same day. Inter-assay (between-day) precision was determined by measuring the same

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

controls used for the intra-assay precision study (which was run in duplicate) over 5 days. The precision is expressed as the coefficient of variation (Kessler et al., 1982) where:

$$\%C.V. = \frac{\text{(standard deviation)}}{\text{mean}} \times 100$$

Recovery

The mean efficiency of extraction was determined for both pre-filtered and posted filtered samples using known amounts of [14C]5-FU. Recovery of [14C]5-FU and [14C] dihydrofluorouracil were evaluated as follows: 20 replicate assays were incubated for 15 min at 37°C under the standard conditions described above (200 μM NADPH, 8.23 μM [6-14C]5-FU (56 mCi/mmol), buffer A and 200 μg cytosolic protein (from a healthy donor) in a final volume of 1 ml). All samples were terminated by adding an equal volume (1 ml) of ice cold 100% ethanol. Five samples were immediately mixed with 6 ml scintillation cocktail and quantitated in a Beckman (Fullerton, CA, USA) LS 6000 liquid scintillation counter. The remaining 15 samples were vortexed and placed in a -70°C freezer for 10 min and then centrifuged for 10 min at 14,000 g. The supernatant from five samples was mixed with 6 ml scintillation cocktail and the total radioactivity in each sample quantitated as described above. The supernatant from the remaining 10 samples was filtered through a 0.2 μm Acrodisc filter. Five of these samples were then mixed with scintillation cocktail and quantitated. The remaining five samples were injected on the HPLC and quantitated by collecting 1 min

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

fractions and counting the samples in the same liquid scintillation counter described above. Total radioactivity eluted from the columns was determined by measuring the sum of the two peaks.

Stability of the Reaction Products

The stability of [14C]5-FU and [14C]dihydrofluorouracil in ten terminated reaction mixtures (equal volumes of ethanol and reaction mix) were evaluated over a 3 month period with samples being stored at -20°C. In addition the stability of ten terminated reaction mixtures was evaluated during storage at room temperature over 48 hr.

Kinetic Studies

Kinetic studies were performed on PBM cell cytosol obtained from previously characterized control individuals with normal DPD activity (0.18-0.67 nmol/min/mg). Initial reaction rates were determined for various concentrations of 5-FU (0.5, 1.0, 2.0, 3.0, 4.0, 5.0, 6.0 and 8.2 μM) in the presence of 200 μM NADPH. Reactions were performed in buffer A (pH 7.4) at 37°C. The incubation time and protein concentration were adjusted so that no more than 20% of the limiting substrate was consumed. Estimation of apparent Km values was performed by fitting these data to the Michaelis-Menton equation by non-linear regression analysis (Cleland et al., 1979).

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

RESULTS

Recovery of 5-FU and Dihydrofluorouracil From HPLC

This HPLC chromatographic technique unambiguously resolves dihydrofluorouracil from 5-FU with retention times of 7(±1) and 19(±1) min, respectively, as shown in Figure 1. In preliminary experiments, multiple injections of known amounts of [6-14C]5-FU demonstrated that the total radioactivity applied to the columns was recovered in 25 min (97.5% ± 2.0%) with a coefficient of variation of only 5.5% using the autosampler (n=33). To determine the amount of sample lost during the ethanol extraction and filtration step of the assay, the recovery of [14C]5-FU and [14C]dihydrofluorouracil were evaluated for pre-filtered and post filtered assays as described in the experimental section above (results summarized in Table 1). Briefly, these data demonstrate that less than 5% of the sample is lost during the extraction process.

Assay Validation

Intra-assay precision (%C.V.) for each PBM cytosol protein concentration (100, 150 and 250 µg) tested (n=5 for each concentration) did not exceed 6.5%. The inter-assay precision was evaluated over 5 days using the same protein concentrations described above (100, 150 and 250 µg). Inter-assay variation did not exceed 8%.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Stability of the Reaction Products

The mean (±S.D.) ratio of calculated [14C]5-FU and [14C]dihydrofluorouracil concentrations (new/old) suggest that the enzyme reaction products are stable in a 50% ethanol mixture for at least 3 months at -20°C: 1.13 (±0.21) for [14C]5-FU (n=10) and 0.92 (±0.43) for [14C] dihydrofluorouracil (n=10). These data were not affected by at least three freeze/thaw cycles. Evaluation of the 50% ethanol extracts at room temperature demonstrated that both [14C]5-FU and [14C]dihydrofluorouracil were stable (no detectable change) over 48 hr.

Optimal Conditions and Detection Limits of the PBM-DPD Assay

Previous studies by our laboratory optimized the assay conditions for quantitation of PBM-DPD activity in both fresh and frozen PBM samples (Lu, Zhang, & Diasio, 1993). Briefly, these conditions require that the enzyme reaction be maintained at pH 7.4 and 37°C with an NADPH concentration of 200 μM. By controlling the reaction time and the amount of PBM protein cytosol added to each assay, formation of product was maintained below 20%. Both substrate and product inhibition have been observed with PBM-DPD activity. The minimal detection limit for this assay system was determined to be 0.005 nmol of dihydrofluorouracil.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Linearity of PBM-DPD Assay
With Time and Protein

The rate of dihydrofluorouracil formation was initially examined with respect to incubation time. Human PBM cell cytosol (200 µg) was incubated at 0, 5, 10, 15, 20, 25 and 30 min in the presence of 8.23 nmol [6-14C]5-FU in a total volume of 1.0 ml. A linear rate of dihydrofluorouracil formation was observed during the first 30 min of incubation at 37°C (Figure 2).

To establish whether there was a direct relationship between the amount of protein added and the observed DPD activity, various amounts of human PBM cell cytosol (25-250 µg) were incubated for 15 min. Under these conditions, a linear increase in the rate of dihydrofluorouracil formation was observed (Figure 3).

Kinetic Properties

Previous studies using [3H]pyrimidines showed that uracil, thymine, and 5-FU were substrates for purified DPD with Km values of 4.9, 4.8 and 3.3 µM, respectively (Lu, Zhang, & Diasio, 1992). Kinetic studies performed using this semi-automated assay system demonstrated that DPD activity varied with increasing pyrimidine concentrations conforming with linear Michaelis-Menten kinetics. These studies produced a km value of 3.0 µM for 5-FU (Figure 4).

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Population Distribution of PBM-DPD Activity

Samples from 100 healthy volunteers (age range 19-58 years) samples were collected from faculty, staff and students from this University who had never been treated with 5-FU. These data agree with previous studies (Milano et al., 1994; Lu et al., 1993) which report that the range of PBM DPD activity has a normal Gaussian distribution with no differences observed with increasing age. The mean DPD activity in this population was calculated to be 0.431 nmol/min/mg (compared to 0.425 in a previous study (Lu et al., 1993) by our laboratory of 124 healthy volunteers). The SD was calculated to be 0.153 with a SE of 0.091.

New DPD Deficient Patients

Collaboration with medical oncologists within our university allowed us to use this new assay system to screen cancer patients for DPD activity prior to treatment with 5-FU. Samples from 80 recently diagnosed cancer patients were obtained during an initial office visit and assayed for DPD activity using the method described above. These data identified two profoundly DPD deficient patients with less than 10% of the mean DPD activity in the general population and below the lower limit of 99% distribution range (Lu et al., 1993). A complete characterization of these patients will be presented elsewhere.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

DISCUSSION

Deficiency of DPD has been shown to be responsible for a pharmacogenetic syndrome in which administration of standard doses of 5-FU is associated with severe and potentially life-threatening toxicity, including mucositis, granulocytopenia, neuropathy and even death. Since 5-FU remains one of the most commonly used antineoplastic agents, determining the level of DPD activity of cancer patients prior to receiving 5-FU could preclude a DPD deficient patient from potentially fatal toxicity. However, determination of DPD activity of cancer patients prior to beginning chemotherapy has been difficult due to the time consuming, labor intensive methods currently in use (Harris et al., 1990; Diasio et al., 1988; Lu et al., 1993).

In the present study, we describe a rapid, sensitive, semi-automated radioassay for measuring DPD activity in human peripheral lymphocytes. This assay was developed to screen for DPD deficiency in high-risk patients, such as those with family members previously demonstrating toxicity to 5-FU. The principle features of this system are: a HPLC system equipped with an automatic injector allowing multiple sample loading, an in-line flow analyzer for automatic peak detection and a computer software package that automatically converts peak area to nmol of product formed. This system was evaluated for linearity with time and protein concentration and further verified by determining the Km value for 5-FU which agreed

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

with previously published results (Lu et al., 1992; Shiotani & Weber, 1981).

Previously described spectroscopic DPD assay methods measuring NADPH oxidation (Queener, Morris, & Weber, 1971; Shiotani et al., 1981) lack specificity; this suggests the possibility, particularly in crude preparations, that the activity of NADPH oxidizing enzymes rather than DPD activity would be measured. Other researchers (Fernandez-Salguero, Gonzalez, Etienne, Milano, & Kimura, 1995; Sommadossi et al., 1982) have described specific assays which utilize radio-labelled 5-FU. These assays separate the reduced product of the reaction (dihydrofluorouracil) by either TLC or HPLC. Samples are then quantitated by autoradiography (for TLC) or by collecting fractions and counting them in a scintillation counter (for HPLC). Unfortunately, these methods are not easily automated, time consuming, expensive and labor intensive.

The clinical diagnosis of DPD deficiency is difficult since the appearance of life threatening toxicity secondary to exposure to 5-FU is typically the first symptom of this pharmacogenetic syndrome. The aim of this study was to develop a simple, sensitive and accurate method for determining DPD enzyme activity in cancer patients prior to and/or during 5-FU treatment. When one considers (a) the frequent use of 5-FU in cancer patients, (b) the severe toxicity to this drug in DPD deficient patients and (c) the potential value of using an individual's DPD activity to attenuate 5-FU chemo-

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

therapy, the benefits of determining a cancer patients DPD activity becomes apparent. Unfortunately, previously described assays for DPD activity are too expensive and labor intensive to be offered in most cancer treatment centers.

Prior to any large scale screening of DPD activity in patients, clear criteria for the identification of deficient patients must be determined. The precise level of DPD deficiency (or lack of enzyme activity) required to observe toxicity secondary to treatment with 5-FU remains to be determined. Earlier population studies of PBM-DPD activity in 124 healthy volunteers (Lue et al., 1993) and 185 cancer patients (Milano et al., 1994) demonstrated that DPD activity followed a unimodal Gaussian distribution. These studies suggested that toxicity following treatment with 5-FU occurs when DPD activity levels are below 0.182 and 0.1 nmol/min/mg, respectively. While both these studies agree that complete DPD deficiency is an extremely rare event, neither study attempts to define the limits of DPD deficiency at which toxicity to 5-FU occurs. One of the clear applications of this new assay method will be to screen enough patients to correlate DPD enzyme activity levels with levels of 5-FU toxicity and 5-FU antitumor effectiveness. The DPD assay method described above is a significant improvement on existing methods and is suitable for screening cancer patients for DPD deficiency prior to administration of 5-FU.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

FOOTNOTES

[1]Additional information (limits of detection, cost, etc.) may be obtained on the internet at http://www.packardinst.com /fsa.htm

[2]Attempts in our laboratory to reduce cost and analysis time by using a single HPLC column proved unsuccessful. While it is possible to separate 5-FU and FUH_2 under these conditions, the peaks become broader with a single column and the assay becomes significantly less sensitive.

ACKNOWLEDGMENTS

This work was supported by USPHS Grant CA 62164.

REFERENCES

Baccanari, D., Davis, S.T., Knick, V.C., & Spector, T. (1993). 5-Ethynyluracil (776C85): a potent modulator of the pharmacokinetics and antitumor efficacy of 5-fluorouracil. Proceedings of the National Academy of Sciences of the United States of America, 90, 11064-11068.

Bradford, M.A.(1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analyti cal Biochemistry, 72, 254-258.

Cleland, W.W. (1979). Statistical analysis of enzyme kinetic data. Methods in Enzymology, 63, 103-138.

Daher, G.C., Harris, B.E., & Diasio, R.B. (1990). Metabolism of pyrimidine analogues and their nucleosides. Pharmacology & Therapeutics, 48, 189-222.

Diasio, R.B., Beavers, T.L., & Carpenter, J.T. (1988). Familial deficiency of dihydropyrimidine dehydrogenase. Biochemical basis for familial pyrimidinemia and severe

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

5-fluorouracil-induced toxicity. Journal of Clinical Investigation, 81, 47-51.

Harris, B.E., Carpenter, J.T., & Diasio, R.B. (1991). Severe 5-fluorouracil toxicity secondary to dihydropyrimidine dehydrogenase deficiency: a potentially more common pharmacogenetic syndrome. Cancer, (Philadelphia) 68, 499-501.

Harris, B.E., Song, R., Soong, S.J., & Diasio, R.B.(1990). Relationship between dihydropyrimidine dehydrogenase activity and plasma 5-fluorouracil levels with evidence for circadian variation of enzyme activity and plasma drug levels in cancer patients receiving 5-fluorouracil by protracted continuous infusion. Cancer Research, 50, 197-201.

Kessler, M.J.(1982). A sensitive radioactivity detector for HPLC. American Laboratory, 8, 52-63.

Lu, Z.H., Zhang, R., & Diasio, R.B.(1992). Purification and characterization of dihydropyrimidine dehydrogenase from human liver. Journal of Biological Chemistry, 267, 17102-17109.

Lu, Z., Zhang, R., & Diasio, R.B. (1993). Dihydropyrimidine dehydrogenase activity in human peripheral blood mononuclear cells and liver: population characteristics, newly identified deficient patients, and clinical implication in 5-fluorouracil chemotherapy. Cancer Research, 53, 5433-5438.

Lyss, A.P., Lilenbaum, R.C., Harris, B.E., & Diasio, R.B. (1993). Severe 5-fluorouracil toxicity in a patient with decreased dihydropyrimidine dehydrogenase activity. Cancer Investigation, 2, 239-240.

Matthews, M.M., Liao, W., Kvalnes-Krick, K.L., & Traut, T.W. (1992). beta-Alanine synthase: purification and allosteric properties. Archives of Biochemistry & Biophysics, 293, 254-263.

Milano, G., & Etienne, M.C. (1994). Potential importance of dihydropyrimidine dehydrogenase (DPD) in cancer chemotherapy. Pharmacogenetics, 4, 301-306.

Queener, S.F., Morris, H.P., & Weber, G. (1971). Dihydrouracil dehydrogenase activity in normal, differentiating and regnerating liver and in hepatomas. Cancer Research, 31, 1004-1009.

Salguero, P.F., Gonzalez, F.J., Etienne, M.C., Milano, G., & Kimura, S. (1995). Correlation between catalytic activity and protein content for the polymorphically ex-

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

pressed dihydropyrimidine dehydrogenase in human lymphocytes. Biochemical Pharmacology, 50, 1015-1020.

Shiotani, T., & Weber, G. (1981). Purification and properties of dihydrothymine dehydrogenase from rat liver. Journal of Biological Chemistry, 256, 219-224.

Sommadossi, J.-P., Gewirtz, D.A., Diasio, R.B., Aubert, C., Cano, J.P., & Goldman, I.D. (1982). Rapid catabolism of 5-fluorouracil in freshly isolated rat hepatocytes as an-alyzed by high performance liquid chromatography. Journal of Biological Chemistry, 257, 8171-8176.

Traut, T.W., & Loechel, S.(1984). Pyrimidine catabolism: Individual characterization of the three sequential enzymes with a new assay. Biochemistry, 23, 2533-2539.

Wasternak, C. (1980). Degradation of pyrimidines and pyrimidine analogs--pathways and mutual influences. Pharmacology & Therapeutics, 8, 629-651.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

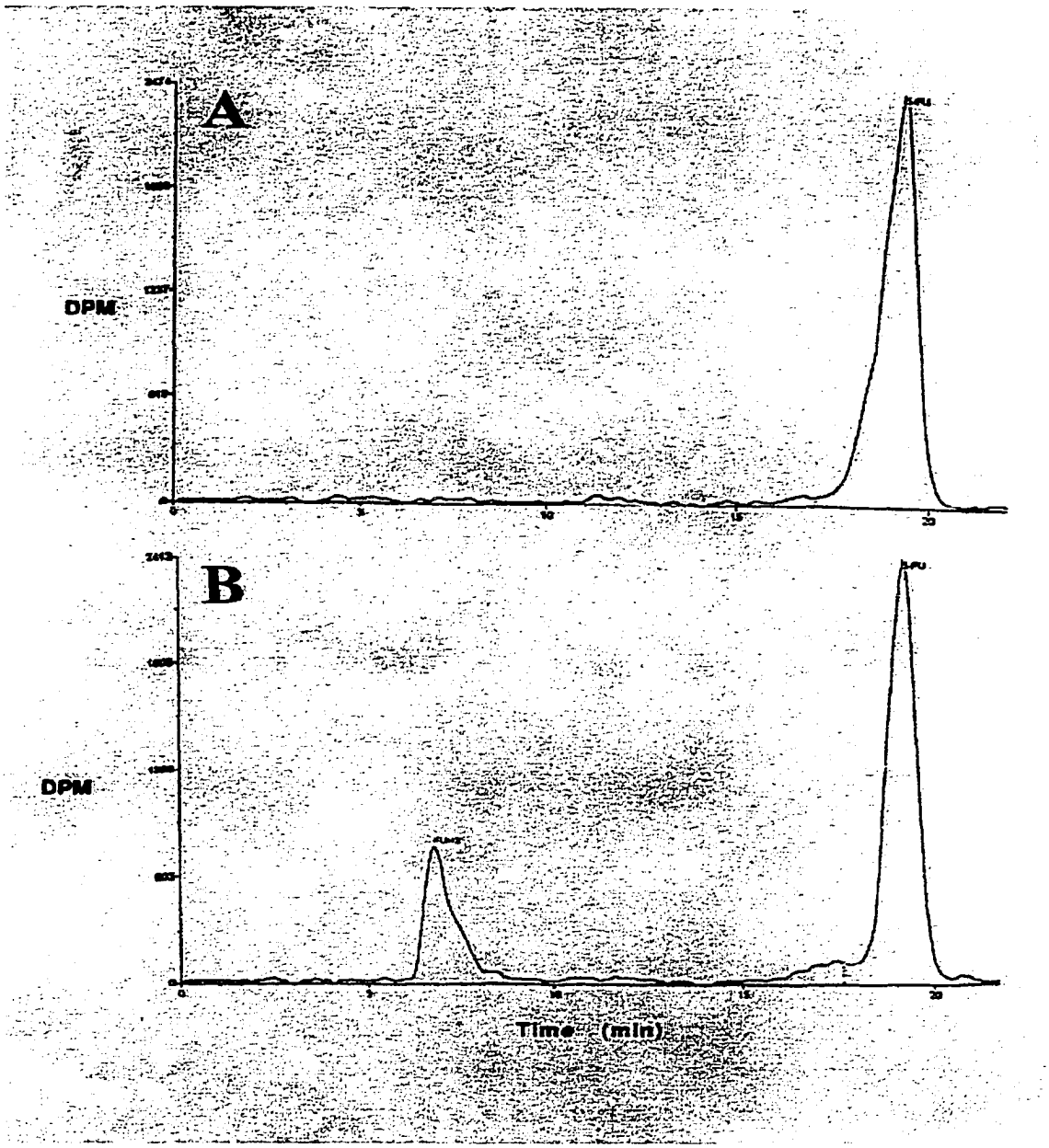


Figure 1. HPLC elution pattern showing the radioactive profile of (A) [6-^{14}C]5-FU and (B) [6-^{14}C]5-FU incubated with 250 µg human lymphocyte cytosol for 30 min at 37°C in the presence of 200 µM NADPH in buffer A. Reactions were terminated and injected on HPLC as described under Experimental.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Table 1

Recovery of [^{14}C]FUra and [^{14}C]Dihydrofluorouracil for Pre-filtered and Post Filtered Assays

Sample (n=5)	Mean Radioactivity (DPM)	Extraction Recovery (%)	Coefficient of Variation (C.V.)
Crude assay mixture	1023252 ± 2753	100%	0.27%
Ethanol precipitated crude mixture	1020114 ± 2567	99.7%	0.25%
Ethanol precipitated filtered	1004833 ± 31150	98.2%	3.1%
Total radioactivity eluted from HPLC (sum of two peaks)	979252 ± 46024	95.7%	4.7%

Replicate assays (n=20) were incubated for 15 min at 37°C in the presence of 200 µM NADPH, 8.23 µM [6-^{14}C] FUra (56 mCi/mmol), buffer A and 200 µg cytosolic protein (from a healthy donor) in a final volume of 1 ml. Samples were subsequently extracted as described in the Experimental section. Total mean radioactivity was determined in the crude assay mixture (n=5) and assigned a value of 100%. Recovery from each step in the extraction process is reported as a percent compared to the total radioactivity determined in the crude assay mixture.

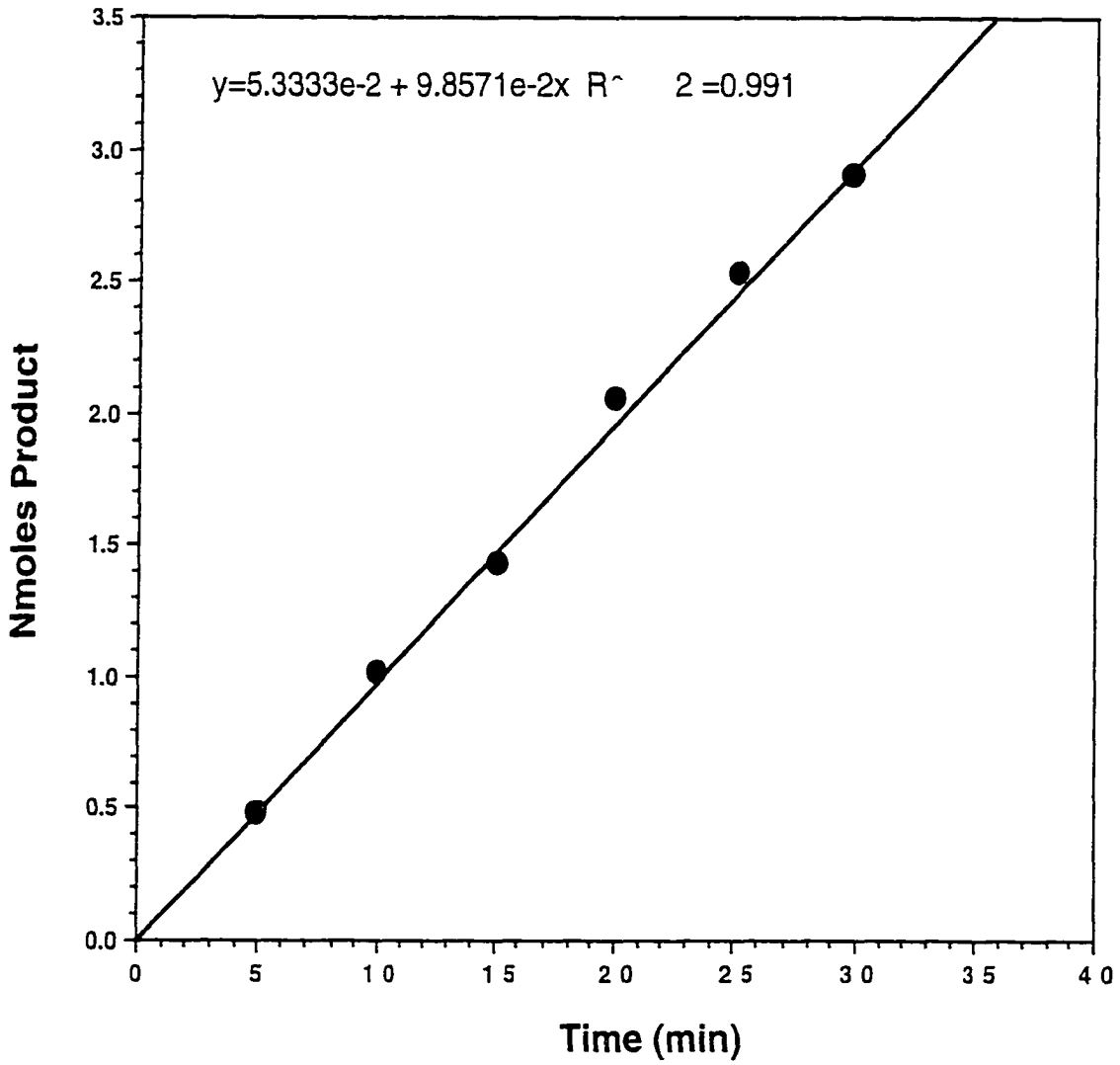


Figure 2. Production of dihydrofluorouracil was evaluated over a 30 min incubation. All samples contained 200 µg human lymphocyte cytosol, 8.23 nmol [6-^{14}C]5-FU and 200 µM NADPH in a total volume of 1.0 ml. Reactions were incubated at 37°C, terminated and injected on HPLC as noted under Experimental.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

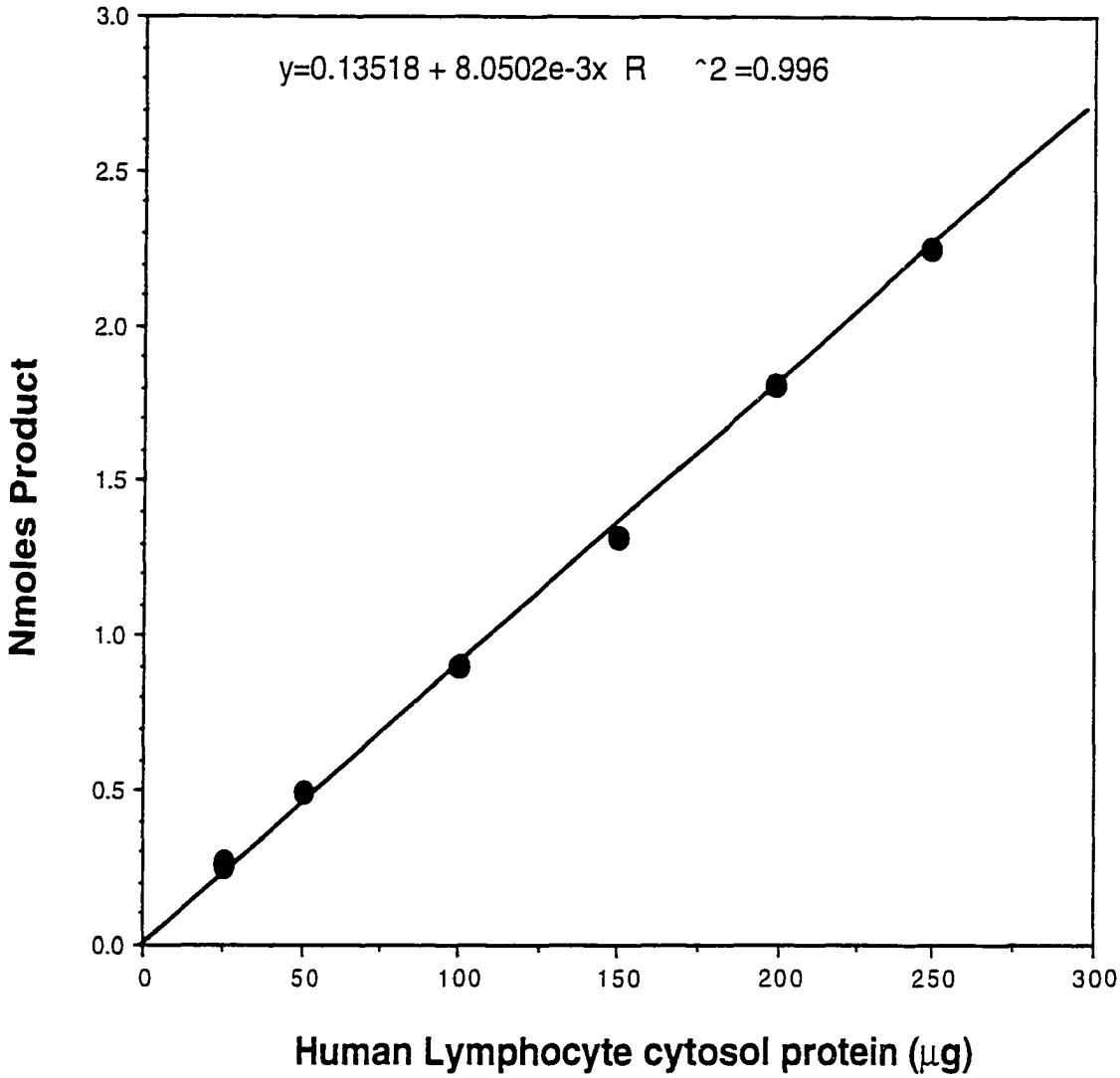


Figure 3. Production of dihydrofluorouracil in a 15 min incubation with varying amounts of human lymphocytic cytosolic protein (25-250 µg).

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

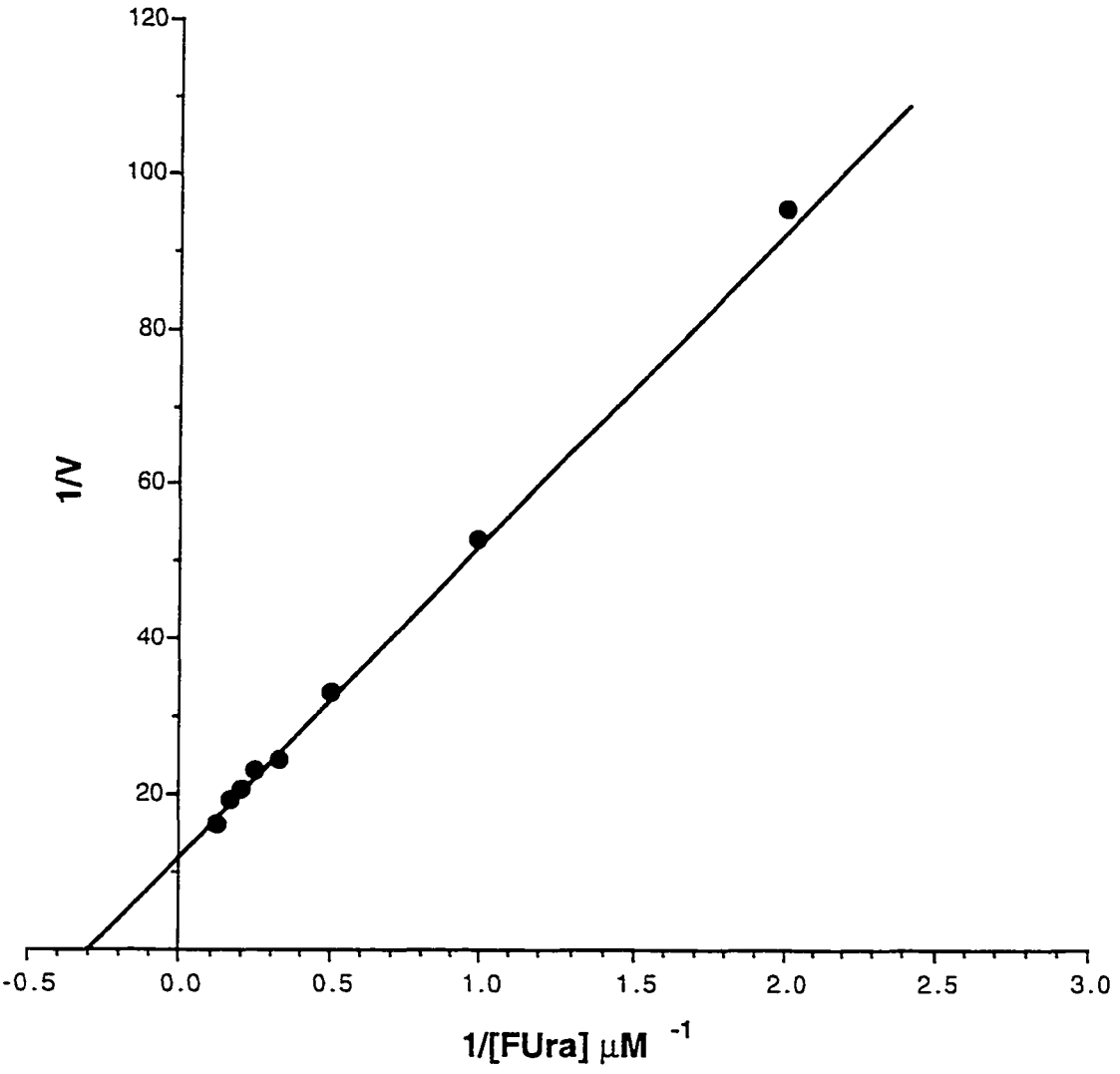


Figure 4. Double-reciprocal plot of the rate of reaction as a function of the concentration of [6-^{14}C]5-FU. The rate of dihydrofluorouracil formation was determined in the presence of 200 μM NADPH using the following concentrations of [6-^{14}C]5-FU (0.5, 1.0, 2.0, 3.0, 4.0, 5.0, 6.0 and 8.2 μM).

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

STRUCTURAL ORGANIZATION OF THE HUMAN *DIHYDROPYRIMIDINE DEHYDROGENASE* GENE

by

MARTIN R. JOHNSON, KANGSHANG WANG, SILKE TILLMANNS, NICOLAS ALBIN, AND ROBERT B. DIASIO

Cancer Research,57, 1660-1663, 1997

Copyright 1997
by
American Association for Cancer Research

Used by permission

Format adapted for dissertation

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

ABSTRACT

Deficiency of the pyrimidine catabolic enzyme, dihydropyrimidine dehydrogenase (DPD), has been shown to be responsible for a pharmacogenetic syndrome in which administration of 5-fluorouracil (5-FU) is associated with severe and potentially life-threatening toxicity. Following the recent availability of the cDNA for DPD, there were initial reports of several molecular defects (point mutations, deletions due to exon skipping) that were suggested as a potential molecular basis for DPD deficiency, even before the complete physical structure of the DPD gene was known. To understand the mechanism responsible for DPD deficiency, we have determined the genomic structure and organization of the human DPD gene. The gene is approximately 150 kb in length, and it consists of 23 exons, ranging in size from 69 to 1404 bp. The sequences of intronic regions flanking the exon boundaries have been determined. The physical map of the DPD gene should permit development of rapid assays to detect point mutations or small deletions in the DPD gene associated with 5-FU toxicity.

INTRODUCTION

5-Fluorouracil (5-FU), synthesized in 1957 and studied extensively by Heidelberger et al., (1975), today continues to be widely used in the management of several common malignancies, including cancers of the colon, breast, and skin. The anticancer effects and toxicity of 5-FU are directly

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

related to anabolism of 5-FU into its nucleotides, which can then produce cytotoxicity through inhibition of thymidylate synthase activity or incorporation into RNA and/or DNA (Grem, et al., 1996).

In humans, more than 85% of administered 5-FU is degraded through the catabolic pathway (Heggie, Sommadossi, Cross, Huster, & Diasio, 1987). DPD (EC 1.3.1.2; also known as dihydrouracil dehydrogenase, dihydrothymine dehydrogenase, and uracil reductase) catalyzes the initial and rate-limiting step in pyrimidine catabolism, the reduction of uracil or thymine to 5,6-dihydrouracil or 5,6-dihydrothymine (Sommadossi et al., 1982). Thus, DPD regulates the availability of 5-FU for anabolism, thereby affecting its pharmacokinetics, toxicity, and efficacy (Diasio & Lu, 1994).

Several recent studies have described a pharmacogenetic disorder in which individuals with absent or significantly decreased DPD activity develop life-threatening toxicity following exposure to 5-FU (Diasio, Beavers, & Carpenter, 1988; Etienne et al., 1994; Harris, Carpenter, & Diasio, 1991; Lu, Zhang, & Diasio, 1993; Takimoto et al., 1996). Administration of standard doses of 5-FU to DPD-deficient patients has resulted in severe life-threatening toxicity, including mucositis, granulo-cytopenia, neuropathy, and even death. Since the initial reports several years ago, there have been an increasing number of cases reported suggesting that this disorder may be more frequent than initially thought (Lu, et al., 1993; Milano & Etienne, 1994). Popula-

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

tion studies in 124 healthy volunteers (Lu, et al., 1993) and 185 cancer patients (Milano et al., 1994) have demonstrated that approximately 3% of the individuals tested were partially DPD deficient with enzyme activity below the 95% distribution range of DPD enzyme activity (below 0.064 nmol/min/mg). The recent availability of the DPD cDNA (Johnson, Albin, Wang, & Diasio, 1995; Yokota et al., 1994), has allowed investigators to examine, at the molecular level, patients who were previously phenotyped (by enzyme assay) as DPD deficient. These studies have demonstrated polymorphisms (Albin, Johnson, Shahinian, Wang, & Diasio, 1995) and exon skipping (Meinsma, Salguero, Van kuilenburg, Van Gennip, & Gonzalez, 1995; Wei, McLeod, McMurrough, Gonzalez, & Salguero, 1996) as possible bases for this pharmacogenetic syndrome.

These observations have raised the possibility that there are multiple causes for DPD deficiency. An understanding of the structure and organization of the DPD gene should enhance our ability to identify mutations and alternatively spliced regions of this gene. Here, we report the genomic structure and organization for the human DPD gene.

MATERIALS AND METHODS

Library Screening

Clones representing the human genomic locus of the DPD gene were isolated from a titered P1 human genomic library (Genome System Inc., St. Louis, Missouri). The library was

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

screened using PCR and the following primers: sense, TAGGA-AAAGCACTGCAGTACCTTGG, and antisense, CTGGTAGCCAGAATCATTACA-GGTCATG. Specific cycling conditions were evaluated prior to screening the P1 library using purified genomic DNA. The conditions used to screen the library were 10 µM each primer, 60 mM Tris-HCl (pH 8.5), 15 mM $(NH_4)_2SO_4$, 2.5 mM $MgCl_2$, 2.5 mM each dNTP, and 1 unit of AmpliTaq DNA polymerase in a total volume of 50 µl. The samples were amplified in an MJ model PTC-100 thermal cycler (MJ Research, Inc. Watertown, MA) programmed for a temperature-step cycle of 1 min at 95°C, 1 min at 58°C, and 1 min at 72° C. This cycle was repeated for a total of 30 cycles.

Identification of Exon-Intron Boundaries

Approximately 10% of the exon-intron boundaries were identified by direct cycle sequencing (New England Biolabs) of the P1 genomic clone (Pierce, Sternber, & Sauer, 1992) using primers designed from DPD cDNA. The remaining boundaries were determined using suppression PCR, as follows (Clontech Laboratories Inc., Palo Alto, CA). Genomic DNA was digested with EcoRV, ScaI, DraI, PvuII, and SspI separately. An adaptor was ligated to the ends of the DNA fragments, and a small aliquot of each digested-ligated DNA was used as a template for PCR using adaptor primers and gene-specific primers designed from DPD cDNA (Siebert, Chenchik, Kellogg, Lukyanov, & Lukyanov, 1995). Amplified products were subcloned into the pCRII vector (Invitrogen) and sequenced

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

using the universal T7 and M13 primers bordering the multiple cloning site. DNA sequencing was accomplished using the dideoxy chain termination procedure (Sequenase Version 2, United States Biochemical Corp.). Exon-intron boundaries were identified by the presence of consensus splice junctions at sites where the sequence of the genomic product differed from the published DPD cDNA sequence. The computer program Mac-Vector and AssemblyLIGN were used for comparison of DNA sequences.

Sizes of introns were determined by exon-exon PCR amplification products using LA PCR (Takara Shuzo Co., Kyoto, Japan) with a buffer supplied by the manufacturer. The final reaction volume was 50 µl and contained 10 µM each primer and 50 ng of genomic DNA as a template. Cycling conditions were as follows: denaturation at 98°C for 20 s and annealing and extension at 68°C for 15 min. Following the first 14 cycles, an autosegment extension of 15 s per cycle (at the annealing and extension step temperature of 68°C) was added for the remaining 16 cycles (Barnes et al., 1994; Cheng, Fockler, Barnes, & Higuchi, 1994). Most runs included an initial 15-min hold at 94°C and a final 10-min hold at 72°C. All samples were run using a manual "hot start" technique in which Mg^{2+} was withheld until samples had been incubated at 94°C. A positive control that amplified a region of the human β-globin cluster (21.5 kb) was run with each reaction and used the following primers: sense, ACATGATTAGCAAAAGGGCCTAGCTTGG-ACTCAGA and antisense, TGCACCTGCTCTGTGATTATGACTATCCCACAGTC.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Primer Extension

Oligonucleotide (CCAGTGACAAACCCTCCTTGCG) was phosphorylated with [γ-^{32}P]ATP and hybridized to 30 µg of total RNA prepared from freshly isolated peripheral blood mononuclear cells (Lu et al., 1993). Annealing proceeded in 10 mM Tris-HCl (pH 8.3), 1 mM EDTA, and 250 mM KCl at 60°C for 1 hr, followed by incubation at room temperature for 1.5 hr. Reverse transcription was performed in 16.7 mM KCl, 13.3 mM $MgCl_2$, 23.3 mM Tris-HCl (pH 8.3), 13.3 mM DTT, 0.33 mM dNTPs, and 0.133 mg/ml actinomycin D with 200 units of Moloney murine leukemia virus RNaseH$^-$ reverse transcriptase (Life Technologies, Inc.). The extension products were fractionated on an 8.0 M urea-6% polyacrylamide gel and analyzed by autoradiography (MacKnight & Kingsbury, 1982).

5'-Rapid Amplification of cDNA Ends (5'-RACE).

Total RNA was prepared from freshly isolated peripheral blood mononuclear cells as described above. 5'-RACE was performed according to the manufacturer's instructions (Clontech, Palo Alto, CA). Briefly, cDNA was generated using a modified lock-docking oligo(dT) primer which contained two degenerate nucleotides at the 3' end (Borson, Sato, & Drews, 1992). Second-strand synthesis was performed using a combination of Escherichia coli DNA polymerase I, Rnase H, and Escherichia coli DNA ligase provided by the manufacturer. T4 DNA polymerase was then used to create blunt ends on the double stranded cDNA. An "adaptor primer" was then ligated to

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

both ends of the double-stranded cDNA using T4 DNA ligase. PCR was then performed using an "anchor primer" complementary to the "adaptor primer" and a specific DPD cDNA primer located in exon 2. The amplified products were purified, subcloned, and sequenced as described above.

RESULTS AND DISCUSSION

In the initial phase of this study, we screened a titered P1 human genomic library with specific primers (designed within exon 22 of the DPD DNA sequence). Clones representing the human genomic locus of the DPD gene were identified by amplification of a 135-bp product. Three independent P1 clones were isolated, and the boundaries for exons 21-23 were identified by direct thermal cycle sequencing using exon-specific primers. Unfortunately, PCR and hybridization analysis of the three P1 clones demonstrated that they did not contain exons 1-20. The relatively large size of the introns isolated for the DPD gene from the P1 screening (Ogata, Fujibuchi, & Kanehisa, 1996), along with previous reports describing difficulties in screening phage libraries (Meinsma et al., 1995), suggested that the average insert size of most commercial λ phage libraries (14-20 kb) would be too small to be useful for this particular gene. We therefore elected to use a recently described technique known as suppression PCR (Siebert et al., 1995, Maiti et al., 1996, Johansson & Karlsson, 1996). This method had a particular advantage over traditional library screening with regard to the DPD gene in

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

that only the boundary of each splice site, junction is amplified from genomic DNA and subcloned. Following amplification of each splice site the PCR products were purified on low melting point agarose and subcloned into the pCRII vector for sequence analysis.

Previous studies have mapped the DPD gene to chromosome 1p22 (Salguero, Kimura, Gonzalez, & Yamada, 1994). We now report the organization and structure of the DPD gene, which consists of 23 exons spanning a region approximately 150 kb in length. The size and sequence of intronic regions flanking the exon boundaries has also been determined and is summarized in Table 1. All intron-exon boundaries were found to conform to the canonical GT-AG rule. A physical map encompassing the entire coding region of the DPD gene is shown in Figure 1. Exons are numbered 1-23, with exon 15 (69 bp) being the smallest and exon 23 (1404 bp) being the largest. The translation start site (ATG) was located in exon 1. Exon 23 contained 168 nucleotides of coding sequence, followed by the translation stop codon (TAA) and a 1236-nucleotide 3' untranslated region. The human liver DPD cDNA sequence published by Yokota et al. (Yokota et al., 1994) did not contain the complete 3' untranslated region. Subsequent studies in our laboratory identified the complete human lymphocytic DPD cDNA sequence (GenBank accession no. U20938), which contained this region and was used in our comparison of genomic and cDNA sequence searching for consensus splice junctions where

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

the sequence of the genomic product differed from the lymphocytic DPD cDNA sequence.

Primer extension experiments were undertaken to determine the precise size of exon 1. Freshly isolated RNA from PBM cells was used for this analysis. The band indicated by the arrow (Figure 2) is the reverse-transcribed product whereas the other bands represent known DNA sequences that were included for determination of molecular size. Comparison of the nucleotides in the known sequence with the mobility of the DPD mRNA lane demonstrates that the distance from the primer to the 5' end of the message coding for DPD is 104 nucleotides. This result suggested that there were approximately 20-25 additional nucleotides in the mRNA sequence that had not been identified previously by cDNA cloning (Yokota et al., 1994; Johnson et al., 1995). We used 5'-RACE to clone and sequence these additional nucleotides located upstream from the published DPD cDNA sequence. PCR with an anchor primer and an internally nested primer located 193 bp from the 5' end of the published human DPD cDNA sequence resulted in the amplification of a cDNA approximately 214 bp in length. Following subcloning, 10 clones were selected and sequenced. All of the inserts examined contained the human DPD cDNA sequence previously reported; however, 8 of the 10 clones extended further upstream for an additional 21 nucleotides. These data suggest that exon 1 is 140 nucleotides long and contains 101 nucleotides of 5'-untranslated region, fol-

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

lowed by the initiating methionine and 36 nucleotides of coding sequence.

Intron sizes were determined by PCR amplification using genomic DNA as a template with gene-specific primer pairs from adjacent exons. The use of nested PCR allowed verification of the sizes of the introns. Only PCRs gave a single well-defined product (Figure 3) were used for estimation of intron sizes. Introns 5 and 10 were the largest, with sizes greater than 20 kb, whereas introns 9 and 17 were the smallest.

The recent examination of two unrelated DPD-deficient patients has suggested exon skipping (Meinsma et al., 1995; Wei et al., 1996) as a possible mechanism for DPD deficiency. These studies demonstrated a 165-bp deletion in the mRNA of both patients. Genomic DNA analysis of these patients revealed a G to A mutation in the GT 5′ splicing recognition sequence of the intron preceding the mutation. It is now clear from the current study that exon 14 represents the skipped exon and that the G to A point mutation in the invariant GT splice donor site is located at the 5' end of intron 14. However, our examination of this region of the DPD gene in two additional well-characterized DPD-deficient patients has demonstrated that neither patient exhibits this particular G to A mutation (full details of this study will be published elsewhere).

The aim of this study was to determine the structure and organization of the DPD gene. These data could then be used

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

as a foundation for rapidly scanning the DPD gene for mutations in genomic DNAs in an attempt to define the molecular basis of DPD enzyme deficiency. The data presented in this study should allow the development of genetic assays that could clarify the role of the DPD gene in deficient patients. The clinical diagnosis of DPD deficiency is difficult because the appearance of life-threatening toxicity secondary to exposure to 5-FU is the first symptom of this pharmacogenetic syndrome. Until now, only phenotypic DPD enzyme assays have been available to diagnose patients as DPD deficient. These assays are expensive and labor intensive and are not available in most cancer treatment centers. Thus, there is a great need for a more direct diagnostic method, preferably available to the patient prior to receiving 5-FU. The characterization of the genomic structure of the DPD gene provides the basis for the development of simple genetic tests. To date, all DPD-deficient patients have been analyzed without a complete understanding of the DPD gene structure and organization. The stability of DNA in comparison to RNA along with the ease with which DNA samples may be extracted and purified represents a clear diagnostic advantage. In addition, the development of genomic DNA based assays would allow analysis of archive paraffin-embedded samples from deceased DPD-deficient patients. These samples would allow researchers to build pedigrees on families (including determination of heterozygotes), who, until now, were impossible to study. Although the size and complexity of the DPD gene makes scan-

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

ning the gene for mutations a challenge, we believe that recent advances in the field of molecular biology (such as single-stranded conformational polymorphism) may be used for identifying additional mutations in the DPD gene.

FOOTNOTES

1Supported by USPHS Grant CA 62164. N.A. is a recipient of a grant from the N.C.I./E.O.R.T.C. Exchange Program and the ARC. S.T. was supported by the German-American Fulbright Commission (Commission for Educational Exchange between the United States and Germany).

REFERENCES

Albin, N., Johnson, M.R., Shahinian, H., Wang, K., & Diasio, R.B.(1995). Initial characterization of the molecular defect in human dihydropyrimidine dehydrogenase deficiency. Proceedings for the American Association for Cancer Research 36, 211.

Barnes, W.M. (1994). PCR amplification of up to 35-kb DNA with high fidelity and high yield from λ bacteriophage templates. Proceedings of the National Academy of Sciences, United States of America, 91, 2216-2220.

Borson, N.D., Sato, W.L., & Drewes, L.R. (1992). A lock-docking oligo(dT) primer for 5' and 3' RACE PCR. PCR Methods & Applications, 2, 144-148.

Cheng, S., Fockler, C., Barnes, W.M., & Higuchi, R. (1994). Effective amplification of long targets from cloned inserts and human genomic DNA. Proceedings of the National Academy of Sciences, United States of America, 91, 5695-5699.

Diasio, R.B., Beavers, T.L., & Carpenter, J.T.(1988). Familial deficiency of dihydropyrimidine dehydrogenase. Journal of Clinical Investigation, 81, 47-51.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Diasio, R.B., & Lu, Z-H. (1994). Dihydropyrimidine dehydrogenase activity and fluorouracil chemotherapy. Journal of Clinical Oncology, 12, 2239-2242.

Etienne, M.C., Lagrange, J.L., Dassonville, O., Fleming, R., Thyss, A., Renee, N., Schneider, M., Demard, F., & Milano, G. (1994). Population study of dihydropyrimidine dehydrogenase in cancer patients. Journal of Clinical Oncology, 12, 2248-2253.

Grem, J.L.(1996). Fluoropyrimidines. Chabner, B.A. and Longo, D.L. (Eds.), Cancer Chemotherapy and Biotherapy Second (pp 149-197, Philadelphia, New York: Lippincott-Raven.

Harris, B.E., Carpenter, J.T., & Diasio, R.B. (1991). Severe 5-fluorouracil toxicity secondary to dihydropyrimidine dehydrogenase deficiency: A potentially more common pharmacogennetic syndrome. Cancer, (Philadelphia) 68, 499-501.

Heggie, G.C., Sommadossi, J.P., Cross, D.S., Huster, W.J., & Diasio, R.B.(1987). Clinical pharmacokinetics of 5-fluorouracil and its metabolites in plasma, urine, and bile. Cancer Research, 47, 2203-2206.

Heidelberger, C.(1975). Flurornated pyrimidines and their nucleosides. Sartorelli A.C. & Johns, D.G. (Eds.), Antineoplastic and immunosuppressive Agents (pp. 193-231). Berlin, New York: Springer-Verlag.

Johansson, M.& Karlsson, A. (1996). Cloning and expression of human deoxyguanosine kinase cDNA. Proceedings of the National Academy of Sciences, United States of America, 93, 7258-7262.

Johnson, M.R., Albin, N., Wang, K., & Diasio, R.B. (1995). cDNA cloning of dihydropyrimidine dehydrogenase from human lymphocytes. Proceedings for the American Association for Cancer Research, 36, 503.

Lu, Z., Zhang, R., & Diasio, R.B. (1993). Dihydropyrimidine dehydrogenase activity in human peripheral blood mononuclear cells and liver: Population characteristics, newly identified deficient patients and clinical implication in 5-fluorouracil chemotherapy. Cancer Research, 53, 5433-5438.

MacKnight, S.L., & Kingsbury, R.(1982). Transcriptional control signals of a eukaryotic protein-coding gene. Science Washington DC, 217, 316-324.

Maiti, S., Doskow, J., Li, S., Nhim, R.P., Lindsey, J.S., & Wilkinson, M.F.(1996). The Pem homeobox gene. Androgen-dependent and -independent promoters and tissue-specific

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

alternative RNA splicing. Journal of Biological Chemistry, 271, 17536-17546.

Meinsma, R., Salguero, P.F., Van Kuilenburg, A.B., Van Gennip, A.H.P., & Gonzalez, F.J.(1995). Human polymorphism in drug metabolism: Mutation in the dihydropyrimidine dehydrogenase gene results in exon skipping and thymine uraciluria.DNA and Cell Biology, 14, 1-6.

Milano, G. & Etienne, M.C.(1994). Potential importance of dihydropyrimidine dehydrogenase (DPD) in cancer chemotherapy. Pharmacogenetics 4, 301-306.

Ogata, H., Fujibuchi, W., & Kanehisa, M. (1996). The size differences among mammalian introns are due to the accumulation of small deletions. FEBS Letters, 390, 99-103.

Pierce, J.C., Sternber, N., & Sauer, B. (1992). A mouse genomic library in the bacteriophage P1 cloning system: Organization and characterization. Mammalian Genome, 3, 550-558.

Salguero, P.F., Kimura, S., Gonzalez, F.J., & Yamada, K. (1994). Assignment of the human dihydropyrimidine dehydrogenase gene (DPYD) to chromosome region 1p22 by fluorescence in situ hybridization. Genomics, 24, 613-614.

Siebert, P.D., Chenchik, A., Kellogg, D.E., Lukyanov, K.A., & Lukyanov, S.A. (1995). An improved PCR method for walking in uncloned genomic DNA. Nucleic Acids Research, 23, 1087-1088.

Sommadossi, J.P., Gewirtz, D.A., Diasio, R.B., Aubert, C., Cano, J.P., & Goldman, I.D.(1982). Rapid catabolism of 5-fluorouracil in freshly isolated rat hepatocytes as analyzed by high performance liquid chromatography. Journal of Biological Chemistry, 257, 8171-8176.

Takimoto, C.H., Lu, Z.H., Zhang, R., Liang, M.D., Larson, L.V., Cantilena, L.R., Grem, J.L., Allegra, C.J., Diasio, R.B., & Chu, E. (1996). Severe neurotoxicity following 5-fluorouracil-based chemotherapy in a patient with dihydropyrimidine dehydrogenase deficiency. Clinical Cancer Research, 2, 477-481.

Wei, X., McLeod, H.D., McMurrough, J., Gonzalez, F.J., & Salguero, P.F. (1996). Molecular basis of the human dihydropyrimidine dehydrogenase deficiency and 5-fluorouracil toxicity. Journal of Clinical Investigation, 98, 610-615.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Yokota, H., Fernandez-Salguero, P., Furuya, H., Lin, K., McBride, O.W., Podschun, B., Schnackerz, K.D., & Gonzalez, F.J.(1994). cDNA cloning and chromosome mapping of human dihydropyrimidine dehydrogenase, an enzyme associated with 5-fluorouracil toxicity and congenital thymine uraciluria. Journal of Biological Chemistry, 269, 23192-23196.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Table 1

Intron-Exon Boundaries of the Human DPD Gene

Exon	Exon Size (bp)	3' Intron Splice Site	Exon Sequence (5' to 3')	5' Intron Splice Site	Intron Length (bp)
1	140+[a]		.....CATCGAG	gtacggactt	3554
2	111	ctgtctttag	AGTATCC.....CTGCTTT	gtaagtacca	10610
3	83	tatgttgcag	AATTGTG.....CAATGAG	gtaagtctgg	5055
4	88	tatttcctag	ATGCCTG.....AAACAAG	gtaaattcag	12613
5	166	taatttgcag	AACTATT.....TACTGAG	gtatgtatga	>20000
6	193	ttccccgtag	GTATTCA.....GTTTAAG	gtaatgccta	7762
7	82	ttcttttcag	TACTTCT.....TGTAAAG	gtaaatgaaa	5643
8	88	tttcttatag	ATAATTT.....GGAATAG	gtaagtagtt	8414
9	108	tgttacttag	GTTTGCC.....AAAGCAG	gtataacata	2236
10	170	catcattcag	GAATGTG.....TGAGGAG	gtaaaatgga	>20000
11	211	tttgttttag	ATGGAAC.....CCTAAAG	gtacagtgct	4610
12	185	gtttttcag	TAAAAGA.....CGTACAG	gtaggcattt	5296
13	216	tattttgcag	TCACAAT.....TGATAAG	gtaagaaaat	4323
14	165	ctttcatcag	GACATTG.....AGACAAC	gtaagtgtga	4972
15	69	ctttttaaag	ATTGTGA.....GTCTGAG	gtaatggtta	9080
16	91	ttgtttaaag	GATTCTG.....GATCCAG	gtaaggacct	6902
17	114	acaggcccag	AGCTGGT.....AAGGAAG	gtaagaactt	843
18	121	gtcttgcaag	GTGGTGC.....TGTCTGG	gtaggtgttg	2948
19	142	ttttgtgtag	GACAGCA.....CCTCCAG	gtcattgtgt	4011
20	180	tcttttctag	GTATGCA.....GGACAAG	gtatgagctt	4894
21	144	ttcctttag	AAACTGC.....CATCAAG	gtaaaaatta	5107
22	141	ctatttttag	GATGTAA.....CTACCAG	gtaagaatcc	2753
23	1404	tctgttgcag	GCTATAC		

[a] Transcription start site not determined.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

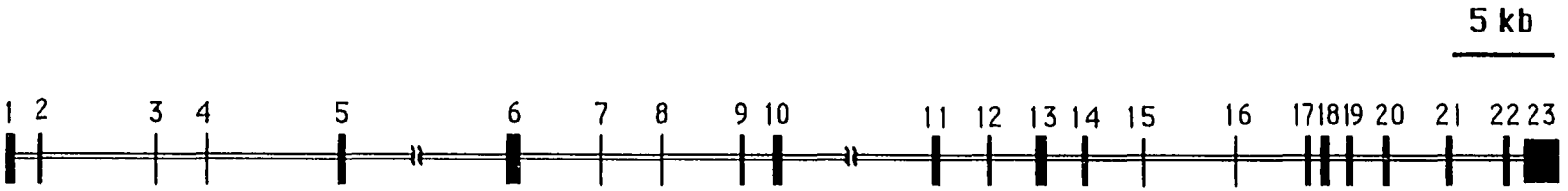


Figure 1. Organization of the DPD gene. Schematic map of the human DPD gene; ■, exons 1-23. The initiating ATG is located in exon 1, and the stop codon (TAA) is located in exon 23.

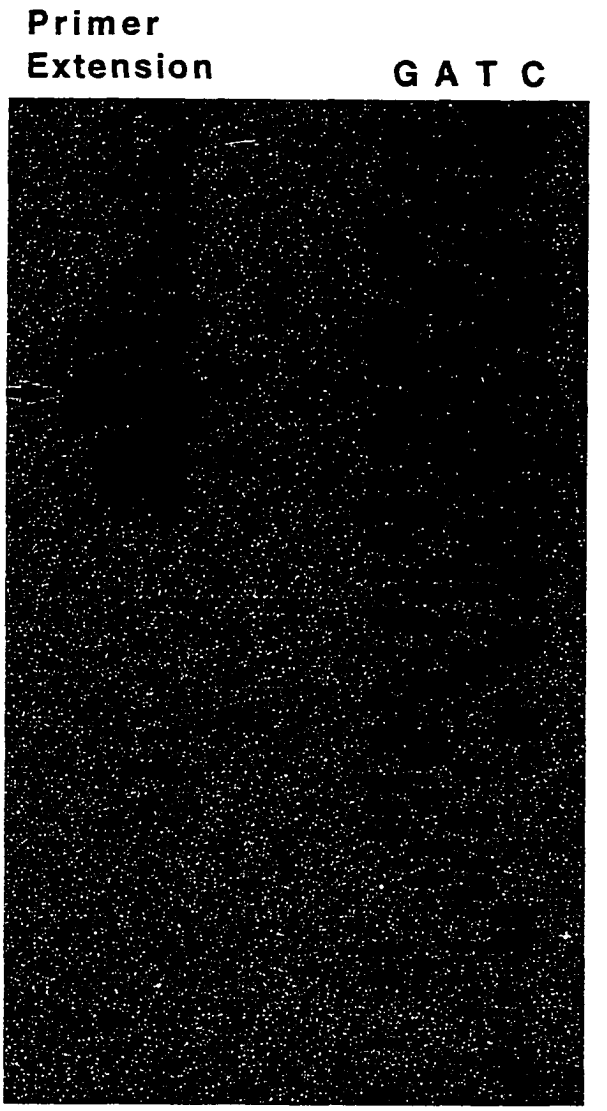


Figure 2. Determination of the size of exon 1 by primer extension. Primer extension was performed as described in "Materials and Methods." One major (arrow) product was detected.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

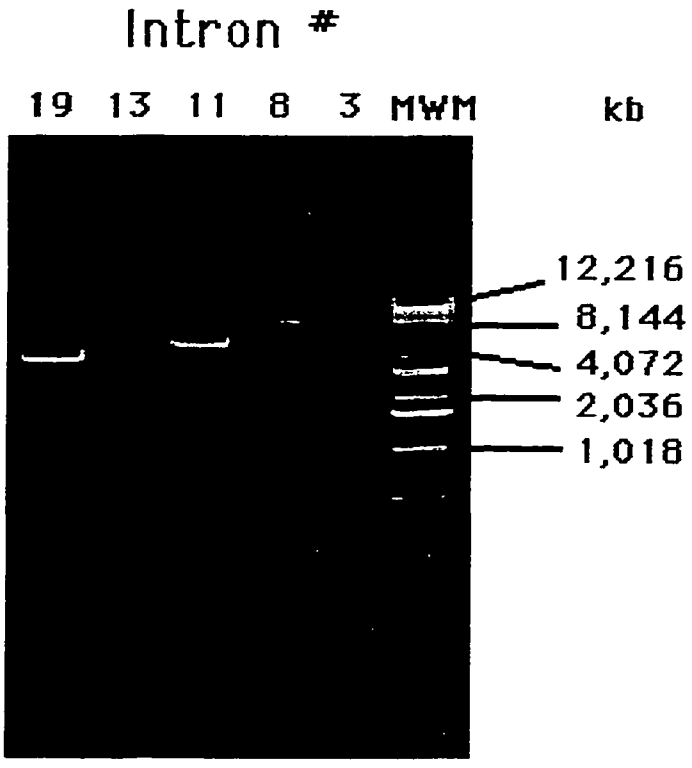


Figure 3. Sizing of introns for the human DPD gene. Sizes of introns were determined by exon-exon PCR amplification products, using either the P1 clone or genomic DNA as the template. PCR products for introns 19, 13, 11, 8, and 3 are shown (from left to right) resolved on a 1 % agarose gel.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

cDNA CLONING AND CHARACTERIZATION OF HUMAN LYMPHOCYTE AND LIVER DPD

by

MARTIN R. JOHNSON

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

INTRODUCTION

As discussed earlier, the recent availability of a partial human liver DPD cDNA sequence (Yokota et al., 1994) has allowed the preliminary characterization of patients previously phenotyped (by enzyme assay) as DPD deficient. These studies suggest that exon skipping is the molecular basis for DPD deficiency (Meinsma et al., 1995). This conclusion was based on the identification of a G to A mutation in a Dutch pediatric patient. This mutation is located in the GT 5′ splicing recognition sequence of the intron preceding the mutation (Wei et al., 1996). As described above, we have recently characterized the DPD gene and have determined that exon 14 represents the skipped exon and that the G to A point mutation in the invariant GT splice donor site is located at the 5' end of intron 14. However, our examination of this region of the DPD gene in three additional well characterized DPD deficient patients has demonstrated that none of these patients exhibits this particular G to A mutation. These observations have raised the possibility that there are multiple causes for DPD deficiency. In addition, alignment of control and DPD deficient patient lymphocyte DPD cDNA with the previously described partial human liver DPD cDNA revealed significant sequence differences between liver and lymphocyte DPD cDNA.

In order to provide a basis for the systematic identification of the genetic polymorphisms/mutations responsible for DPD deficiency, we have undertaken the following additional studies: (a) cloning and sequencing the complete cDNA coding

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

for human lymphocyte DPD from healthy donors with normal DPD activity, (b) examination of putative sequence differences between human liver and human lymphocyte DPD cDNA, and (c) cloning and sequencing portions of the DPD gene from three DPD deficient patients to determine whether these patients demonstrated sequence differences previously associated with DPD deficiency. The serial studies described below show the complete nucleotide sequence of lymphocyte and liver DPD cDNA for the first time. These data should help investigators to identify genetic differences between normal and DPD deficient individuals. In addition, we provide evidence that there are multiple causes for DPD deficiency.

MATERIALS AND METHODS

Materials

The materials were purchased from following sources: the RNAzol B from Biotecx (Houston, TX), the First-Strand cDNA Kit from Pharmacia (Piscataway, NJ), the PCR reagents from Perkin Elmer (Norwalk, CT), the TA cloning kit from Invitrogen (San Diego, CA), the restriction enzymes from New England Biolabs (Beverly, MA), and the coupled in vitro transcription and translation system from Promega (Madison, WI). Specific oligonucleotides were synthesized by Midland (Midland, TX). [^{35}S]Methionine (1000 Ci/mmol) was purchased from Amersham (Arlington Heights, IL). All other reagents were of molecular biology grade.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Human Subjects

Healthy volunteers. Whole blood was obtained from 20 individuals having normal DPD levels (as determined by enzyme activity and Western blot analysis). Peripheral blood mononuclear (PBM) cells were then separated using Histopaque (Sigma, St. Louis, MO) as previously described (Johnson, Yan, Shao, Albin, & Diasio, 1997).

DPD deficient patients. Whole blood was obtained from three DPD deficient patients. The first patient (R.F.) is a 41-year-old female breast cancer patient who developed severe gastrointestinal (nausea, prolonged vomiting, diarrhea, and stomatitis) and hematologic toxicity following CMF adjuvant chemotherapy (Harris et al., 1991). This patient was hospitalized 8 days after treatment with neutropenia (leukocyte count of 1,100 with 5% neutrophils) and fever. In addition, the patient reported some mild neurologic toxicity (ataxia) that persisted for approximately 2 weeks.

The second patient (L.R.) is a Norwegian born 50-year-old Caucasian man diagnosed with recurrent metastatic hepatocellular carcinoma, fibrolamellar variant. This patient developed grade II diarrhea, nausea, and vomiting (NCI toxicity scale) in addition to dehydration and confusion (Takimoto et al., 1996) following treatment with 5-FU. After his second cycle of chemotherapy, he developed cerebellar ataxia and cerebral encephalopathy progressing to obtundation one week later (Takimoto et al., 1996).

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

The third patient (H.Y.) is a 77-year-old Caucasian man who received topical 5-FU for the treatment of skin cancer. This patient also developed grade II diarrhea, nausea, and vomiting along with neurologic toxicity (ataxia). This patient represents the first DPD deficient patient exhibiting severe 5-FU toxicity following treatment with a topical preparation of the drug. A full characterization of this patient with a complete case report is currently being prepared by this laboratory.

Preliminary analysis of all three patients has demonstrated undetectable levels of PBM DPD enzyme activity and elevated plasma and urine uracil levels. In addition, Western blot analysis of PBM cytosol from both R.F. and L.R. demonstrated decreased DPD protein levels.

Human Liver

Human autopsy liver tissue was obtained from the National Disease Research Interchange, Philadelphia, PA. The human liver biopsy samples were obtained from the Tissue Procurement Service located at this university. The liver tissue was stored in an ultra-low temperature freezer (-80°C) until needed. Prior to RNA isolation, the liver was ground into a fine powder under liquid nitrogen using a mortar and pestle.

RNA Isolation and First-Strand cDNA Synthesis

Total RNA was isolated from liver tissue and PBM cells using RNAzol B following the manufacturer's instructions.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Random primed cDNAs were prepared from 0.25 µg total RNA using a Pharmacia first-strand cDNA synthesis kit. cDNA synthesis reactions were performed in a 33 µl total reaction volume following the manufacturer's instructions. The reaction mix was treated with 1 µl RNase H and incubated for 30 min at 37°C prior to PCR amplification.

PCR Amplification and Subcloning of the PCR Products

The amplification of DPD cDNA was performed in a 50 µl reaction volume containing 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 2.5 mM $MgCl_2$, plus dNTPs (10 µM each), 10 µmol of each primer, 5 µl template cDNA (see cDNA synthesis above), and 2.5 units of Thermus aquaticus (Taq) polymerase. The samples were amplified in an MJ model PTC-100 thermal cycler (MJ Research, Inc., Watertown, MA) programmed for a temperature-step cycle of 95°C (1 min), 65°C (2 min), and 72°C (3 min). This cycle was repeated for a total of 5 steps, after which the 65°C annealing temperature was decreased to 63°C and the cycling continued for an additional 25 steps, with a 10 min extension at 72°C after the final cycle. Six different primer pairs were used to generate three overlapping human lymphocyte DPD cDNA fragments, as shown in Figure 1. These three partial DPD cDNA clones together span the entire coding region and include the 5′ and 3′ untranslated regions of human lymphocyte DPD. PCR primer 1 was based on 5′ RACE data recently obtained during the characterization of the DPD

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

gene. Primers 2, 3, 4, and 5 were designed based on the consensus sequence data obtained from a partial human liver DPD cDNA clone and a bovine liver DPD cDNA clone. Primer 6 is a bifunctional primer composed of oligo d(T) attached to an anchor domain which contains a Not I restriction site. The PCR products were purified by electrophoresis in low melting point agarose and subcloned directly into the pCR II vector (Invitrogen, San Diego, CA). Three independent RT/PCR reactions were sequenced for each fragment of the DPD cDNA clone to eliminate the possibility of RT or Taq polymerase errors.

Genomic DNA Preparation and Analysis

Genomic DNA was prepared from human PBM cells from both normal donors and DPD deficient patients as previously described (Marmur, 1961). Approximately 200 ng of genomic DNA was used as a template in the PCR reaction. Following denaturation at 95°C for 2 min, 35 cycles of denaturation at 95° C (1 min), annealing at 55°C (1 min), and extension at 72°C (1 min) were performed. Analysis of genomic DNA was carried out with PCR amplification using two sets of primers. The first set of primers, Primer 7 (sense 5'-TAGGAAAAGCACTGCAGTAC CTTGG-3') and Primer 8 (antisense 5'-CCTGGTAGCCAGAATCATTACAGG TCATG-3'), corresponded to positions 2875-2899 and 2981-3009, respectively, of the DPD cDNA sequence. These primers were designed to amplify a 135 bp fragment from exon 22 in genomic DNA that contained the single base pair mutation described by Albin et al. (1995). The second set of primers, Primer 9

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

(sense 5'-ATCAGTGAGAAAACGGCTGCATATTGG-3') and Primer 10 (antisense 5'-CCAACTTATGCCAATTCTCTTGTTTTAGATG-3') border the G to A point mutation in the invariant GT splice donor site reported by Meinsma et al. (1995). These primers were designed to amplify an 87 base pair fragment from the 5' end of intron 14. Following amplification, the PCR products were purified on 2.4% low melting point agarose gel and subcloned into the pCR II vector (Invitrogen).

DNA Sequencing

Amplicons subcloned into the pCR II vector were subjected to double-stranded sequencing by the dideoxynucleotide chain termination method using Sequenase 2.0 and [α-^{35}S]dATP to label the newly synthesized strands (Sequenase Version 2, United States Biochemical Corporation). The ^{35}S-labeled products were resolved on 6% polyacrylamide-urea gels. The complete cDNA sequence was obtained by using commercially available or custom-made primers derived from cloned sequences. Sequence gels were read manually and analyzed using MacVector 4.1 Sequence Analysis software (IBI, New Haven, CT). Sequence analysis was repeated three times in each direction.

In vitro Transcription and Translation of Human DPD cDNA

The DPD open-reading frame was PCR amplified from normal control and two DPD deficient patients (R.F. and L.R.) as

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

described above using specific primers which bordered the open-reading frame and included the initiating ATG in the sense primer and the stop codon TAA in the antisense primer: Primer 11 (sense 5' -TGTAGGCACTGCCATGGCCCCTGTG-3') and Primer 12 (antisense 5'-TTCACAAATCACCTTAACACACC-3'). The 3104 base pair PCR product, containing the 3075 base pair open-reading frame, was purified by electrophoresis in low melt agarose and subcloned directly into the pCR II vector downstream from a T7 promoter site. In vitro transcription and translation were conducted with the TNT™ T7 coupled reticulocyte lysate system (Promega) using either [^{35}S]methionine for labelling of the synthesized proteins or unlabelled amino acid for Western blot analysis. Incubations were performed at 30°C. At 0 and 60 min time points, 5 µl of the reaction samples were transferred to a separate eppendorf tube and the reaction was stopped by placing it in boiling water for 1 min. The translated products were resolved on a 7.5% SDS-polyacrylamide gel (Lu et al., 1991). The gels using [^{35}S]methionine for labeling of the synthesized proteins were vacuum dried at 65°C and exposed to autoradio-graph film for 1 min. Gels containing unlabeled proteins were transferred to nitrocellulose and immunoblotted as described (Lu et al., 1992).

RESULTS AND DISCUSSION

Human Lymphocyte DPD cDNA

The nucleotide sequence and deduced amino acid sequence of full length human lymphocyte DPD cDNA is shown in Figure 2

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

and is also available through the National Center for Biological Information (http://www.ncbi.nlm.nih.gov/ Genebank accession number U20938). The ATG start codon has the canonical flanking sequence for a translational start site with the customary GCC at position -3 to -1 and the standard G at position 4 (Kozak, 1991). The complete human lymphocyte DPD cDNA sequence is 4409 base pairs long and contains a 101 nucleotide 5'-nontranslated region and an open-reading frame of 3075 bases encoding a 1025 amino acid protein. The termination codon (TAA) is followed by 1230 nucleotides of 3' nontranslated region containing at least two polyadenylation signal sequences (AAUAAA).

Comparison of the translated human lymphocyte sequence to that of bovine liver reveals 92% identity; if conserved substitutions are considered, the overall homology increases to 94% over the 1025 overlapping amino acids. Figure 3 compares the deduced amino acid sequences of human, bovine, and pig DPD. The translated amino acid sequence of human lymphocyte DPD shows 93% identity to pig liver DPD. These analyses suggest that mammalian DPD was relatively conserved through evolution.

Other sequences identified as similar to DPD include dihydroorotate dehydrogenase (Quinn, Stephenson, & Switzer, 1991), thioredoxin reductase, and glutamate synthase with amino acid sequence partial identities of 40, 37, and 38%, respectively. These values are too low to support a common ancestry with DPD (Doolittle, 1981). However, it is interest-

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

ing to note that dihydroorotate dehydrogenase (a flavoprotein using FAD as a cofactor) catalyzes the fourth step in pyrimidine biosynthesis.

Homology of Human Lymphocyte and Liver DPD cDNA

A previous study suggested that human lymphocytes contained a different isozyme of DPD from that of the liver (Naguib et al., 1985). This was based on kinetic data which suggested the absence of allosterism in lymphocyte DPD and its presence in the liver enzyme. In addition, differences in Km values for the liver enzyme versus that of the lymphocyte were observed. Comparison studies utilizing different types of tissues have suggested that lymphocytes contain about five times more DPD activity when compared to the activity found in liver (Naguib et al., 1985). The fact that human lymphocytes are typically used for assessing DPD activity and will ultimately be the most accessible source of RNA for the molecular characterization in surviving DPD deficient patients, combined with the possibility that there were liver and lymphocyte isozymes of DPD, persuaded us to clone and sequence human lymphocyte DPD cDNA and compare it to the partial human liver DPD cDNA sequence reported by Yokota et al. (1994).

Alignment of the complete human lymphocyte DPD cDNA sequence with the partial human liver DPD cDNA (Yokota et al., 1994) revealed 18 nucleotide differences (Table 1). Because the previous study did not clone the complete 3'

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

untranslated region for the human liver DPD cDNA, we were unable to include this region in our comparison. To verify these apparent sequence differences, we cloned the DPD cDNA from three human liver samples using the methods described above. The human liver DPD cDNA clones were then sequenced in the regions corresponding to the putative nucleotide differences. In all three liver samples, the sequence obtained was identical to that originally sequenced from lymphocyte DPD cDNA (Figure 2). Initial attempts to clone the complete 3' untranslated region from human liver produced incomplete clones similar to the one previously reported by Yokota et al. (1994). The complete 3' untranslated region for human liver DPD cDNA was finally cloned by using human liver biopsy samples as the RNA source instead of the autopsy liver samples that were previously used. This suggests that the DPD mRNA may be extremely sensitive to degradation at the 3' end. These data demonstrate that there is no difference between human lymphocyte and human liver DPD at the level of mRNA.

In vitro Transcription and Translation of Human Lymphocyte DPD cDNA

An in vitro transcription/translation method was initially used to verify that the cloned cDNA translated a protein equivalent in size and immunoreactivity to DPD. Reactions were performed using either [^{35}S]methionine for labeling of the synthesized proteins or unlabeled amino acids for Western blot analysis. Resolution of the [^{35}S]-labeled

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

product by SDS-PAGE showed a single specific protein band with an estimated molecular mass of 108,000 daltons (Figure 4A). Western blot analysis of reactions using unlabeled methionine demonstrated a similar protein band which immunoreacted with the specific rabbit anti-human DPD polyclonal antibody and was identical in size to a purified human DPD control (Figure 4B). These data suggest that the cloned cDNA codes for a protein of the predicted size and immunoreactivity of normal DPD.

Analysis of DPD Deficient Patient Genomic DNA

The availability of the complete cDNA sequences for human lymphocyte and liver DPD, in addition to the characterization of the DPD gene, has permitted preliminary sequence analysis in patients previously characterized as DPD deficient. The first sequence data for a DPD deficient patient (R.F.) was reported by Albin et al. (1995). The cDNA from this patient revealed a single base change from the control sequence; A (control) to T (deficient) at position 2947. Translation of this cDNA resulted in a nonconservative amino acid substitution (Asp To Val) at codon 949. Subsequent subcloning and sequence analysis of multiple PCR reactions flanking this region from 20 individuals having normal DPD activity demonstrated that this nucleotide substitution was present only in the DPD deficient patient's cDNA.

These preliminary data were extended in the current studies to determine whether this mutation was homozygous or

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

heterozygous in the proband and to establish the parental genotype. Genomic DNA (prepared from PBM cells) from both the DPD deficient proband and both parents was purified as described above. A PCR reaction was performed using Primers 7 and 8 (described above) to amplify the 135 bp product. Sequence analysis of several clones from multiple PCR reactions indicated that the DPD deficient patient was homozygous and that the parents were both heterozygous for the single A (control) to T (deficient) base pair difference. In addition, this region of the genomic DNA of two separate unrelated DPD deficient patients (L.R. and H.Y.) was PCR amplified and sequenced. These data demonstrated that neither of these two patients exhibited the base pair difference observed in patient R.F.

As described earlier, a second G to A point mutation in the invariant GT splice donor site located at the 5' end of intron 14 has been identified in a Dutch pediatric DPD deficient patient (Wei et al., 1996). These studies prompted us to examination of this region of the DPD gene (using Primers 9 and 10 as described above) in the three DPD deficient patients R.F., L.R., and H.Y. Sequence analysis of the resulting 87 base pair PCR products demonstrated that none of these patients exhibited this particular G to A mutation (Table 2). These observations raise the possibility that there may be multiple mutations leading to DPD deficiency.

While the importance of the identified sequence differences remains to be determined, genetic polymorphisms have

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

been identified in a number of other enzymes which participate in the metabolism of chemotherapeutic drugs. The wide degree of genetically determined individual variation has a profound effect on the metabolism of several anticancer drugs like 6-mercaptopurine, 6-thioguanine (Krynetski et al., 1995), and amonafide (Ratain et al., 1995), affecting their pharmacokinetics, toxicity, and efficacy. In the case of thiopurine, a single point mutation in the *S*-methyltransferase gene was associated with loss of enzyme activity that significantly affected metabolism of 6-thioguanine and 6-thiopurine. Due to the narrow therapeutic index for most anticancer drugs, these pharmacogenetic factors may prove critical for optimizing the dose/response relationship in specific individuals.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Table 1

Putative Variance Between Human Liver and Lymphocyte DPD cDNA

Position	Human Lymphocyte	Human Liver	Codon (lymph to liver)
81	C	Gap	5' UT Region
506	T	A	Silent (Leu)
1136	T	C	Silent (Phe)
1175	T	A	Silent (Arg)
1235	A	G	Silent (Gly)
1997	T	C	Silent (Phe)
2830	A	G	Asn to Ser
3235	T	C	3' UT Region
3370	T	Gap	3' UT Region
3614	T	A	3' UT Region
3690	A	Gap	3' UT Region
3739	A	Gap	3' UT Region
3741	A	G	3' UT Region
3907	A	C	3' UT Region
3948	G	A	3' UT Region
3959	C	T	3' UT Region
3964	T	C	3' UT Region
3966	T	C	3' UT Region

Comparison of human lymphocyte DPD cDNA sequence with a previously reported partial human liver DPD cDNA (Wei et al., 1996) revealed 18 putative nucleotide differences.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

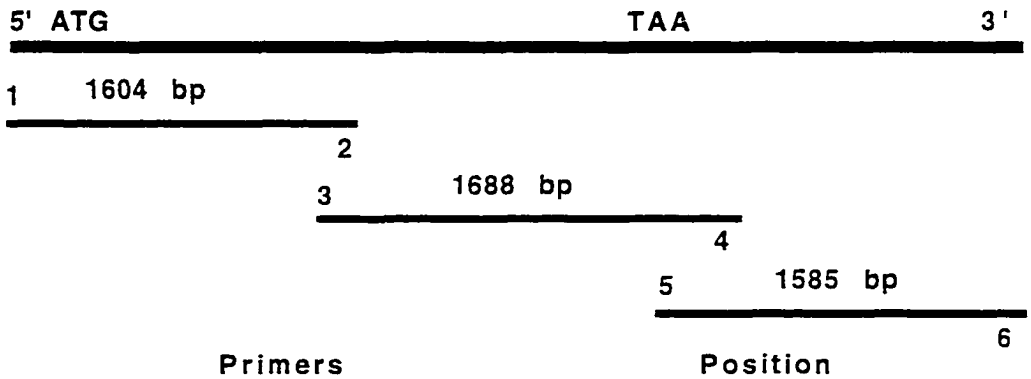
Table 2

Analysis of Three DPD Deficient Patients (R.F., L.R. & H.Y.)

Patient	Activity	Western	Mutations	
			A to T[1]	G to A[2]
R.F.	Undetectable	Low	Yes (homozygote)	No
L.R.	Undetectable	Undetectable	Neither mutation found	
H.Y.	Undetectable	ND[3]	Neither mutation found	

[1] Albin et al., 1995
[2] Wei et al., 1996
[3] Only genomic DNA is available for this patient.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.



	Primers	Position
1	TTTCGACTCGCGCTCCGGCTGCTGTCACTTG	1 - 31
2	CCAAGAAGCTTGCTTTCCATCATTCACCGA	1575 - 1604
3	GTTTGTTCGGACAGAGCAAGATGAAACTGG	1322 - 1351
4	CCTGGTAGCCAGAATCATTACAGGTCATG	2981 - 3009
5	TAGGAAAAGCACTGCAGTACCTTGG	2875 - 2899
6	AACTGGAAGAATTCGCGGCCGCAGGAAT	Poly A+ Tail

Figure 1. Six different primer pairs were used to generate three overlapping human lymphocyte DPD cDNA fragments.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

```
      TTCGACTCGCGCTCCGGCTGCTGTCACTTGGCTCTCTGGCTGGAGCTTGAGGACGCAAGGAGGGTTTGTCACTGGCAGACTCGAGACTGT    91

 92   AGGCACTGCC ATG GCC CCT GTG CTC AGT AAG GAC TCG GCG GAC ATC GAG AGT ATC CTG GCT TTA AAT CCT   161
                 M   A   P   V   L   S   K   D   S   A   D   I   E   S   I   L   A   L   N   P    20

162   CGA ACA CAA ACT CAT GCA ACT CTG TGT TCC ACT TCG GCC AAG AAA TTA GAC AAG AAA CAT TGG AAA AGA   230
 21    R   T   Q   T   H   A   T   L   C   S   T   S   A   K   K   L   D   K   K   H   W   K   R    43

231   AAT CCT GAT AAG AAC TGC TTT AAT TGT GAG AAG CTG GAG AAT AAT TTT GAT GAC ATC AAG CAC ACG ACT   299
 44    N   P   D   K   N   C   F   N   C   E   K   L   E   N   N   F   D   D   I   K   H   T   T    66

300   CTT GGT GAG CGA GGA GCT CTC CGA GAA GCA ATG AGA TGC CTG AAA TGT GCA GAT GCC CCG TGT CAG AAG   368
 67    L   G   E   R   G   A   L   R   E   A   M   R   C   L   K   C   A   D   A   P   C   Q   K    89

369   AGC TGT CCA ACT AAT CTT GAT ATT AAA TCA TTC ATC ACA AGT ATT GCA AAC AAG AAC TAT TAT GGA GCT   437
 90    S   C   P   T   N   L   D   I   K   S   F   I   T   S   I   A   N   K   N   Y   Y   G   A   112

438   GCT AAG ATG ATA TTT TCT GAC AAC CCA CTT GGT CTG ACT TGT GGA ATG GTA TGT CCA ACC TCT GAT CTT   506
113    A   K   M   I   F   S   D   N   P   L   G   L   T   C   G   M   V   C   P   T   S   D   L   135

507   TGT GTA GGT GGA TGC AAT TTA TAT GCC ACT GAA GAG GGA CCC ATT AAT ATT GGT GGA TTG CAG CAA TTT   575
136    C   V   G   G   C   N   L   Y   A   T   E   E   G   P   I   N   I   G   G   L   Q   Q   F   158

576   GCT ACT GAG GTA TTC AAA GCA ATG AGT ATC CCA CAG ATC AGA AAT CCT TCG CTG CCT CCC CCA GAA AAA   644
159    A   T   E   V   F   K   A   M   S   I   P   Q   I   R   N   P   S   L   P   P   P   E   K   181

645   ATG TCT GAA GCC TAT TCT GCA AAG ATT GCT CTT TTT GGT GCT GGG CCT GCA AGT ATA AGT TGT GCT TCC   713
182    M   S   E   A   Y   S   A   K   I   A   L   F   G   A   G   P   A   S   I   S   C   A   S   204
```

Figure 2. Nucleotide and predicted amino acid sequence of human lymphocyte DPD.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

```
714   TTT TTG GCT CGA TTG GGG TAC TCT GAC ATC ACT ATA TTT GAA AAA CAA GAA TAT GTT GGT GGT TTA AGT  782
205    F   L   A   R   L   G   Y   S   D   I   T   I   F   E   K   Q   E   Y   V   G   G   L   S  227

783   ACT TCT GAA ATT CCT CAG TTC CGG CTG CCG TAT GAT GTA GTG AAT TTT GAG ATT GAG CTA ATG AAG GAC  851
228    T   S   E   I   P   Q   F   R   L   P   Y   D   V   V   N   F   E   I   E   L   M   K   D  250

852   CTT GGT GTA AAG ATA ATT TGC GGT AAA AGC CTT TCA GTG AAT GAA ATG ACT CTT AGC ACT TTG AAA GAA  920
251    L   G   V   K   I   I   C   G   K   S   L   S   V   N   E   M   T   L   S   T   L   K   E  273

921   AAA GGC TAC AAA GCT GCT TTC ATT GGA ATA GGT TTG CCA GAA CCC AAT AAA GAT GCC ATC TTC CAA GGC  989
274    K   G   Y   K   A   A   F   I   G   I   G   L   P   E   P   N   K   D   A   I   F   Q   G  296

990   CTG ACG CAG GAC CAG GGG TTT TAT ACA TCC AAA GAC TTT TTG CCA CTT GTA GCC AAA GGC AGT AAA GCA 1058
297    L   T   Q   D   Q   G   F   Y   T   S   K   D   F   L   P   L   V   A   K   G   S   K   A  319

1059  GGA ATG TGC GCC TGT CAC TCT CCA TTG CCA TCG ATA CGG GGA GTC GTG ATT GTA CTT GGA GCT GGA GAC 1127
320    G   M   C   A   C   H   S   P   L   P   S   I   R   G   V   V   I   V   L   G   A   G   D  342

1128  ACT GCC TTT GAC TGT GCA ACA TCT GCT CTA CGT TGT GGA GCT CGC CGT GTG TTC ATC GTC TTC AGA AAA 1196
343    T   A   F   D   C   A   T   S   A   L   R   C   G   A   R   R   V   F   I   V   F   R   K  365

1197  GGC TTT GTT AAT ATA AGA GCT GTC CCT GAG GAG ATG GAA CTT GCT AAG GAA GAA AAG TGT GAA TTT CTG 1265
366    G   F   V   N   I   R   A   V   P   E   E   M   E   L   A   K   E   E   K   C   E   F   L  388

1266  CCA TTC CTG TCC CCA CGG AAG GTT ATA GTA AAA GGT GGG AGA ATT GTT GCT ATG CAG TTT GTT CGG ACA 1334
389    P   F   L   S   P   R   K   V   I   V   K   G   G   R   I   V   A   M   Q   F   V   R   T  411
```

Figure 2. (Continued)

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

```
1335  GAG CAA GAT GAA ACT GGA AAA TGG AAT GAA GAT GAA GAT CAG ATG GTC CAT CTG AAA GCC GAT GTG GTC 1403
 412   E   Q   D   E   T   G   K   W   N   E   D   E   D   Q   M   V   H   L   K   A   D   V   V   434

1404  ATC AGT GCC TTT GGT TCA GTT CTG AGT GAT CCT AAA GTA AAA GAA GCC TTG AGC CCT ATA AAA TTT AAC 1472
 435   I   S   A   F   G   S   V   L   S   D   P   K   V   K   E   A   L   S   P   I   K   F   N   457

1473  AGA TGG GGT CTC CCA GAA GTA GAT CCA GAA ACT ATG CAA ACT AGT GAA GCA TGG GTA TTT GCA GGT GGT 1541
 458   R   W   G   L   P   E   V   D   P   E   T   M   Q   T   S   E   A   W   V   F   A   G   G   480

1542  GAT GTC GTT GGT TTG GCT AAC ACT ACA GTG GAA TCG GTG AAT GAT GGA AAG CAA GCT TCT TGG TAC ATT 1610
 481   D   V   V   G   L   A   N   T   T   V   E   S   V   N   D   G   K   Q   A   S   W   Y   I   503

1611  CAC AAA TAC GTA CAG TCA CAA TAT GGA GCT TCC GTT TCT GCC AAG CCT GAA CTA CCC CTC TTT TAC ACT 1679
 504   H   K   Y   V   Q   S   Q   Y   G   A   S   V   S   A   K   P   E   L   P   L   F   Y   T   526

1680  CCT ATT GAT CTG GTG GAC ATT AGT GTA GAA ATG GCC GGA TTG AAG TTT ATA AAT CCT TTT GGT CTT GCT 1748
 527   P   I   D   L   V   D   I   S   V   E   M   A   G   L   K   F   I   N   P   F   G   L   A   549

1749  AGC GCA ACT CCA GCC ACC AGC ACA TCA ATG ATT CGA AGA GCT TTT GAA GCT GGA TGG GGT TTT GCC CTC 1817
 550   S   A   T   P   A   T   S   T   S   M   I   R   R   A   F   E   A   G   W   G   F   A   L   572

1818  ACC AAA ACT TTC TCT CTT GAT AAG GAC ATT GTG ACA AAT GTT TCC CCC AGA ATC ATC CGG GGA ACC ACC 1886
 573   T   K   T   F   S   L   D   K   D   I   V   T   N   V   S   P   R   I   I   R   G   T   T   595

1887  TCT GGC CCC ATG TAT GGC CCT GGA CAA AGC TCC TTT CTG AAT ATT GAG CTC ATC AGT GAG AAA ACG GCT 1955
 596   S   G   P   M   Y   G   P   G   Q   S   S   F   L   N   I   E   L   I   S   E   K   T   A   618
```

Figure 2. (Continued)

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

```
1956  GCA TAT TGG TGT CAA AGT GTC ACT GAA CTA AAG GCT GAC TTT CCA GAC AAC ATT GTG ATT GCT AGC ATT 2024
 619   A   Y   W   C   Q   S   V   T   E   L   K   A   D   F   P   D   N   I   V   I   A   S   I  641

2025  ATG TGC AGT TAC AAT AAA AAT GAC TGG ACG GAA CTT GCC AAG AAG TCT GAG GAT TCT GGA GCA GAT GCC 2093
 642   M   C   S   Y   N   K   N   D   W   T   E   L   A   K   K   S   E   D   S   G   A   D   A  664

2094  CTG GAG TTA AAT TTA TCA TGT CCA CAT GGC ATG GGA GAA AGA GGA ATG GGC CTG GCC TGT GGG CAG GAT 2162
 665   L   E   L   N   L   S   C   P   H   G   M   G   E   R   G   M   G   L   A   C   G   Q   D  687

2163  CCA GAG CTG GTG CGG AAC ATC TGC CGC TGG GTT AGG CAA GCT GTT CAG ATT CCT TTT TTT GCC AAG CTG 2231
 688   P   E   L   V   R   N   I   C   R   W   V   R   Q   A   V   Q   I   P   F   F   A   K   L  710

2232  ACC CCA AAT GTC ACT GAT ATT GTG AGC ATC GCA AGA GCT GCA AAG GAA GGT GGT GCC AAT GGC GTT ACA 2300
 711   T   P   N   V   T   D   I   V   S   I   A   R   A   A   K   E   G   G   A   N   G   V   T  733

2301  GCC ACC AAC ACT GTC TCA GGT CTG ATG GGA TTA AAA TCT GAT GGC ACA CCT TGG CCA GCA GTG GGG ATT 2369
 734   A   T   N   T   V   S   G   L   M   G   L   K   S   D   G   T   P   W   P   A   V   G   I  756

2370  GCA AAG CGA ACT ACA TAT GGA GGA GTG TCT GGG ACA GCA ATC AGA CCT ATT GCT TTG AGA GCT GTG ACC 2438
 757   A   K   R   T   T   Y   G   G   V   S   G   T   A   I   R   P   I   A   L   R   A   V   T  779

2439  TCC ATT GCT CGT GCT CTG CCT GGA TTT CCC ATT TTG GCT ACT GGT GGA ATT GAC TCT GCT GAA AGT GGT 2507
 780   S   I   A   R   A   L   P   G   F   P   I   L   A   T   G   G   I   D   S   A   E   S   G  802

2508  CTT CAG TTT CTC CAT AGT GGT GCT TCC GTC CTC CAG GTA TGC AGT GCC ATT CAG AAT CAG GAT TTC ACT 2576
 803   L   Q   F   L   H   S   G   A   S   V   L   Q   V   C   S   A   I   Q   N   Q   D   F   T  825
```

Figure 2. (Continued)

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

```
2577  GTG ATC GAA GAC TAC TGC ACT GGC CTC AAA GCC CTG CTT TAT CTG AAA AGC ATT GAA GAA CTA CAA GAC 2645
 826   V   I   E   D   Y   C   T   G   L   K   A   L   L   Y   L   K   S   I   E   E   L   Q   D   848

2646  TGG GAT GGA CAG AGT CCA GCT ACT GTG AGT CAC CAG AAA GGG AAA CCA GTT CCA CGT ATA GCT GAA CTC 2714
 849   W   D   G   Q   S   P   A   T   V   S   H   Q   K   G   K   P   V   P   R   I   A   E   L   871

2715  ATG GAC AAG AAA CTG CCA AGT TTT GGA CCT TAT CTG GAA CAG CGC AAG AAA ATC ATA GCA GAA AAC AAG 2783
 872   M   D   K   K   L   P   S   F   G   P   Y   L   E   Q   R   K   K   I   I   A   E   N   K   894

2784  ATT AGA CTG AAA GAA CAA AAT GTA GCT TTT TCA CCA CTT AAG AGA AAC TGT TTT ATC CCC AAA AGG CCT 2842
 895   I   R   L   K   E   Q   N   V   A   F   S   P   L   K   R   N   C   F   I   P   K   R   P   917

2843  ATT CCT ACC ATC AAG GAT GTA ATA GGA AAA GCA CTG CAG TAC CTT GGA ACA TTT GGT GAA TTG AGC AAC 2921
 918   I   P   T   I   K   D   V   I   G   K   A   L   Q   Y   L   G   T   F   G   E   L   S   N   940

2922  GTA GAG CAA GTT GTG GCT ATG ATT GAT GAA GAA ATG TGT ATC AAC TGT GGT AAA TGC TAC ATG ACC TGT 2990
 941   V   E   Q   V   V   A   M   I   D   E   E   M   C   I   N   C   G   K   C   Y   M   T   C   963

2991  AAT GAT TCT GGC TAC CAG GCT ATA CAG TTT GAT CCA GAA ACC CAC CTG CCC ACC ATA ACC GAC ACT TGT 3059
 964   N   D   S   G   Y   Q   A   I   Q   F   D   P   E   T   H   L   P   T   I   T   D   T   C   986

3060  ACA GGC TGT ACT CTG TGT CTC AGT GTT TGC CCT ATT GTC GAC TGC ATC AAA ATG GTT TCC AGG ACA ACA 3128
 897   T   G   C   T   L   C   L   S   V   C   P   I   V   D   C   I   K   M   V   S   R   T   T  1009

3129  CCT TAT GAA CCA AAG AGA GGC GTA CCC TTA TCT GTG AAT CCG GTG TGT TAA GGTGATTTGTGAAACAGTTGCTG 3202
1010   P   Y   E   P   K   R   G   V   P   L   S   V   N   P   V   C  stop                        1025
```

Figure 2. (Continued)

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

```
3203  TGAACTTTCATGTCACCTACATATGCTGATCTTTTAAAATCATGATCCTTGTGTTCAGCTCTTTCCAAATTAAAACAAATATACATTTTCT 3293
3294  AAATAAAAATATGTAATTTCAAAATACATTTGTAAGTGTAAAAAATGTCTCATGTCAATGACCATTCAATTAGTGGTCATAAAATAGAATA 3384
3385  ATTCTTTTCTGAGGATAGTAGTTAAATAACTGTGTGGCAGTTAATTGGATGTTCACTGCCAGTTGTCTTATGTGAAAAATTAACTTTTTTG 3475
3476  TGGCAATTAGTGTGACAGTTTCCAAATTGCCCTATGCTGTGCTCCATATTTGATTTCTAATTGTAAGTGAAATTAAGCATTTTGAAACAAA 3566
3567  GTACTCTTTAACATACAAGAAAATGTATCCAAGGAAACATTTTATCATTAAAAATTACCTTTAATTTTAATGCTGTTTCTAAGAAAATGTA 3657
3658  GTTAGCTCCATAAAGTACAAATGAAGAAAGTCAAAAAATTATTTGCTATGGCAGGATAAGAAAGCCTAAAATTGAGTTTGTAGAACTTTAT 3748
3749  TAAGTAAAATCCCCTTCGCTGAAATTGCTTATTTTTGGTGTTGGATAGAGGATAGGGAGAATATTTACTAACTAAATACCATTCACTACTC 3839
3840  ATGCGTGAGATGGGTGTACAAACTCATCCTCTTTTAATGGCATTTCTCTTTAAACTATGTTCCTAACAAAATGAGATGATAGGATAGATCC 3930
3931  TGGTTACCACTCTTTTGCTGTGCACATACGGGCTCTGACTGGTTTTAATAGTCACCTTCATGATTATAGCAACTAATGTTTGAACAAAGCT 4021
4022  CAAAGTATGCAATGCTTCATTATTTAAGAATGAAAAATATAATGTCGATAATATATATTAAGTGTGCCAAATCAGTTTGACTACTCTCTGT 4112
4113  TTTAGTGTTTATGTTTAAAAGAAATATATTTTTTGTTATTATTAGATAATATTTTTGTATTTCTCTATTTTCATAATCAGTAAATAGTGTC 4203
4204  ATATAAACTCATTTATCTCCTCTTCATGGCATCTTCAATATGAATCTATAAGTAGTAAATCAGAAAGTAACAATCTATGGCTTATTTCTAT 4294
4295  GACAAATTCAAGAGCTAGAAAAATAAAATGTTTCATTATGCACTTTTAGAAATGCATATTTGCCACAAAACCTGTATTACTGAATAATATC 4385
4386  AAATAAAATATCATAAAGCATTTTAAAAAAAAAAAAAAAAAATTCCTCGGCCGCGAATTCTTCC                          4449
```

Figure 2. (Continued)

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Figure 3. Comparison of the amino acid sequences of human lymphocyte with those of human, bovine, and pig liver DPD.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Figure 3. (Continued)

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Figure 3. (Continued)

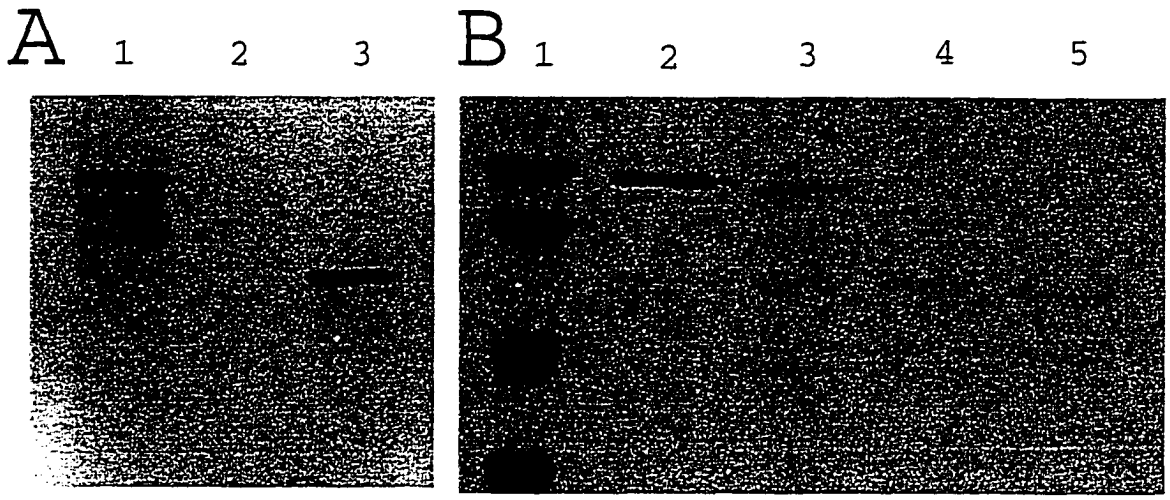


Figure 4. In vitro transcription/translation analysis of cDNA coding for DPD. In A, [^{35}S]-labeled products from an in vitro transcription/translation analysis of DPD cDNA were resolved by SDS-PAGE and visualized by autoradiography. Lane 1: 5 µl of the reaction products; Lane 2: 5 µl of a control reaction which contained empty vector with no DPD cDNA insert; Lane 3: 5 µl of the reaction products produced by a luciferase positive control. In B, Western blot analysis of an in vitro transcription/translation reaction using unlabeled methionine. Reaction products were analyzed using a specific rabbit anti-human DPD polyclonal antibody. Lane 1: molecular weight markers; Lane 2: purified human liver DPD; Lane 3: 30 µl of the reaction products; Lane 4: an empty vector control with no DPD cDNA insert; Lane 5: a control reaction run with heat inactivated T7 polymerase.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

SUMMARY AND CONCLUSION

The critical role of DPD in 5-FU pharmacokinetics, the frequent use of 5-FU as a cancer chemotherapeutic agent, and the clinical significance of DPD deficiency have prompted several recent studies characterizing this important catabolic enzyme (Khor et al., 1997; Etienne et al., 1995; Lu et al., 1992, 1993a, 1993b; Porter et al., 1991; Podschun et al., 1989). The focus of this dissertation research has been the cDNA cloning and characterization of the DPD gene.

Initial studies in this project involved the development of a specific semiautomated radioassay for the determination of DPD enzyme activity (Johnson, Yan, Shao, Albin, & Diasio, 1997). The need for a simple, sensitive, and accurate method for determining DPD enzyme activity became apparent when attempts to utilize currently available assays for DPD activity proved too expensive, labor intensive, and/or non specific. The assay developed during this dissertation research project represents a significant improvement on existing methods and can be used to screen for DPD deficiency in high-risk patients, such as those with family members previously demonstrating toxicity to 5-FU.

During the development of the radioassay for DPD, concurrent studies in our laboratory and others focused on cloning DPD cDNA from bovine (Albin et al., 1996), pig, and

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

human (Yokota et al., 1994) liver. With the availability of a partial cDNA structure for human liver DPD, we initially undertook molecular studies with two previously characterized DPD deficient patients (R.F. and L.R.) who had markedly decreased enzyme activity and almost absent DPD protein on Western blot (Harris et al., 1991; Takimoto et al., 1996). Subsequent sequencing of the cDNA of both DPD deficient patients identified one missense mutation (A to T) at position 2947 in the open reading frame of patient R.F.(Albin et al., 1995). Translation of the cDNA revealed that this sequence difference results in a nonconservative amino acid change (Asp to Val) at codon 949. Analysis of the second DPD deficient patient's DPD cDNA (L.R.) demonstrated no sequence differences.

Another group of investigators reported a 165 base pair deletion in the messenger RNA of two unrelated DPD deficient patients (Meinsma et al., 1995). Later studies by the same group revealed a G to A mutation in the GT 5' splicing recognition sequence of the intron preceding the mutation resulting in exon skipping (Wei et al., 1996). While these authors concluded that this G to A mutation was the basis for all DPD deficiency, we determined that neither DPD deficient patient R.F. nor L.R. contained this particular mutation.

An understanding of the structural organization of the human DPD gene would enhance our ability to identify mutations and alternatively spliced regions of this gene. In addition, the characterization of the DPD gene would allow

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

analysis of archive paraffin-embedded samples from deceased DPD deficient patients. Data resulting from these studies could then be used to build pedigrees on families who until now have been impossible to study. We therefore decided that it was critical to characterize the DPD gene with delineation of the exons, estimation of intron lengths, and, most important, characterization of exon-intron splice sites.

The results of this study demonstrated that the DPD gene, which is located on chromosome 1p (Takai, Fernandez-Salguero, Kimura, Gonzalez, & Yamada, 1994), is approximately 150 kb in length, consisting of 23 exons and ranging in size from 69 bp to 1404 bp (Johnson et al., 1997). Furthermore, the characterization of the DPD gene has revealed that exon 14 represents the skipped exon reported by Meinsma et al. (1995) and that the G to A mutation reported by Wei et al. (1996) occurs in the invariant GT splice donor site at the 5' end of intron 14. Prior to this study, all DPD deficient patients had been analyzed without a complete understanding of the DPD gene structure and organization. Delineation of the exons and, most important, characterization of exon-intron splice sites allow the PCR amplification of individual exons using genomic DNA as a template. This eliminates the complete dependence on RNA samples which are not available from a significant number of patients exhibiting toxicity to 5-FU.

During the course of these studies with DPD deficient patients, we noted that alignment of control lymphocyte DPD

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

cDNA with the previously described human liver DPD cDNA (Yokota et al., 1994) revealed significant sequence differences. In order to provide the basis for a systematic identification of genetic polymorphisms/mutations associated with DPD deficiency, the following additional studies were conducted: (a) The complete lymphocyte DPD cDNA was cloned and sequenced from healthy donors with normal DPD activity, (b) the complete liver DPD cDNA was cloned and putative sequence differences between human liver and human lymphocyte DPD cDNA were examined, (c) regions of the DPD gene containing the A to T mutation (Albin et al., 1995) or the G to A mutation (Wei et al., 1996) were cloned and sequenced from two DPD deficient patients (L.R. and H.Y.) to determine whether these patients contained these sequence differences previously associated with DPD deficiency and (d) the A to T mutation (Albin et al., 1995) identified in DPD deficient patient R.F. cDNA was amplified from the patient's genomic DNA to determine whether the proband was homozygous or heterozygous for this particular mutation. In addition, the parental genotype of patient R.F. was determined.

These studies showed that human lymphocyte and liver DPD cDNA cloned in our laboratory demonstrated 100% sequence identity. Thus, there is no difference between human lymphocyte and liver DPD at the level of mRNA. Presently there is no evidence for the existence of isozymes of DPD. These data represent the first complete (including 5' & 3' untranslated regions) sequence analysis of human DPD cDNA and provide

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

information that will be useful in the characterization of DPD deficient patients.

A summary of our findings on the analysis of the DPD deficient patients follows: (a) The complete sequencing of the DPD cDNA of two previously characterized DPD deficient patients (R.F. and L.R.) by our laboratory has revealed one missense mutation (A to T) at position 2947 in the open reading frame (exon 22) of patient R.F., resulting in a non conservative amino acid change (Asp to Val) at codon 949; (b) sequence analysis of R.F.'s parent's genomic DNA has shown both parents were heterozygote for the A to T missense mutation, (c) evaluation of DPD deficient patient L.R.'s DPD cDNA revealed no sequence differences and (d) examination of the genomic DNA from three DPD deficient patients (R.F., L.R., and H.Y.) failed to demonstrate a G to A mutation (Wei et al., 1996) in the invariant GT splice donor site at the 5' end of intron 14 (Johnson et al., 1997).

In the course of this dissertation research, a novel and specific semiautomated radioassay for the determination of DPD enzyme activity has been developed. In addition, the structural organization of the DPD gene (including delineation of the intron/exon boundaries and estimation of intron lengths) has been determined. The complete cDNA coding for human lymphocyte and liver DPD has been cloned and sequenced for the first time, and these data have been used to screen three DPD deficient patients for previously identified mutations associated with DPD deficiency. These studies suggest

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

the possibility that there are multiple causes for DPD deficiency. The results presented in this dissertation provide the basis for future molecular and pharmacological studies of this enzyme. In the following section, the direction for future studies will be discussed.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

FUTURE STUDIES

The present studies demonstrate the significant progress made in the cloning and sequence analysis of the DPD cDNA from normal and DPD deficient individuals. The results presented in this dissertation provide the basis for future molecular and pharmacological studies of this enzyme, particularly mechanisms responsible for DPD deficiency and the characterization of transcriptional regulatory elements of human DPD gene.

We believe that the following studies will improve our understanding of the molecular basis for DPD deficiency and be useful in the development of specific diagnostic tests that may predict both host toxicity and tumor response prior to administration of 5-FU: (a) the development of an expression system in which in which a functional comparison between normal and mutated DPD cDNA may be evaluated, (b) the determination of the frequency of the two previously described mutations identified in DPD deficient patients, (c) the performance of a comprehensive molecular characterization on those DPD deficient patients exhibiting neither of the previously identified mutations (L.R. and H.Y.) and (d) the identification and characterization of transcriptional regulatory elements of the human DPD gene.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Expression of Normal and Mutated DPD

Full elucidation of the effects of the A to T (Albin et al., 1995) or the G to A (Wei et al., 1996) mutations currently associated with DPD deficiency will require the development of an expression system in which a functional comparison between normal and mutated DPD cDNA may be evaluated. Early studies in our laboratory failed to produce enzymatically active DPD in several prokaryotic enzyme expression systems (data not shown). In addition, preliminary studies using transiently transfected baby hampster kidney (BHK) cells confirmed the production of recombinant DPD enzyme (as determined by Western Blot analysis). However, analysis of cytosol prepared from these cells demonstrated no detectable DPD enzyme activity (data not shown). Further studies are needed to develop a stable eukaryotic cell line in which active wild-type and mutated DPD enzyme may be expressed and compared. These studies should include pulse chase experiments which will ultimately define the half-life of normal and mutated DPD protein and mRNA.

The lack of enzyme activity from recombinant DPD produced in the BHK cell line suggests the absence of some currently unknown subunit or cofactor necessary for enzymatically active DPD in this particular cell line. Analysis of cytosol obtained from wild type BHK cells demonstrated that they contain no endogenous DPD enzyme activity. Thus, the choice of cell lines used to produce recombinant DPD may be critical in obtaining active recombinant DPD enzyme. To test

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

this possibility, cell lines which contain measurable levels of DPD enzyme activity such as GK-5 human lymphoblastoma or Molt-4 human T lymphocyte cells could be tranfected and assayed for increases in baseline DPD enzyme levels.

Once established, these stable cell lines could be used in kinetic studies examining whether there are differences between mutated and nonmutated DPD enzyme activity and substrate specificity. In addition, since immunoblot analysis from individuals containing either of these mutations suggests decreased DPD protein levels, the relationship between these mutations and decreased transcription, decreased translation, unstable mRNA, or increased DPD protein degradation could also be evaluated.

Determine the Frequency of the Mutations Identified in DPD Deficient Patients

Several autosomal recessive disorders such as methylenetetrahydrofolate reductase deficiency (Rozen, 1996), pyruvate carboxylase deficiency (Robinson, MacKay, Chun, & Ling, 1996), pyruvate dehydrogenase deficiency (Robinson et al., 1996), thiopurine S-methyltransferase deficiency (Krynetski et al., 1996), and argininosuccinate synthetase deficiency (Kobayashi, Jackson, Tick, O'Brien, & Beaudet, 1990) have demonstrated multiple genotypes resulting in enzyme deficiency. Although patients diagnosed with these diseases can be identified by a single phenotype (enzyme deficiency), molecular characterization of affected individuals reveals a heterogeneity of mutations occurring at differ-

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

ent frequencies in the population. Thus far, preliminary data from DPD deficient patients have demonstrated at least two mutations associated with DPD deficiency. Since all DPD deficient patients examined in this study do not exhibit both mutations (patient R.F. contains the A to T mutation, while patients L.R. and H.Y. contain neither mutation), we have concluded that both these mutations occur at different frequencies in the population.

In order to identify, characterize, and determine the frequency of both mutations, DNA or RNA must be obtained from patients who are DPD deficient. Thus, an initial requirement of this study would be the identification of patients with DPD deficiency. In addition, a significant number of normal controls (approximately 100) who would be phenotypically assayed for DPD enzyme activity would also have to be examined to determine the frequency of these mutations in a normal population. By determining what proportion of the DPD deficient patients have a specific molecular defect, it should be possible to determine the most common molecular defects associated with DPD deficiency.

Comprehensive Molecular Characterization on DPD Deficient Patients L.R. and H.Y.

While the studies described above will determine the frequency of known mutations in the population of DPD deficient patients, we have already identified at least two DPD deficient patients (L.R. and H.Y.) who contain neither the A to T nor the G to A mutation. These data support the hypothe-

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

ses that there are multiple causes for DPD deficiency and that there are additional mutations yet to be determined in DPD deficient patients. For those patients exhibing neither of the previously identified mutations, a comprehensive characterization should be carried out including (a) quantitation of immunoreactive protein by Western blot analysis, (b) quantitation of mRNA coding for DPD by S1 nuclease protection assay or quantitative PCR, (c) Southern blot analysis, and (d) screening for the presence of any genetic differences between normal and DPD deficient patients by using DPD gene specific primers and PCR methodology.

Identify and Characterize Transcriptional Regulatory Elements of the Human DPD Gene

There are several reasons for undertaking an examination of the transcriptional regulatory elements of the human DPD gene. These include (a) the identification of two DPD deficient individuals who contained neither of the mutations currently associated with DPD deficiency, and with no clear abnormality in one of these patient's DPD cDNA (patient L.R.), we believe that the transcriptional regulatory elements which control DPD expression must be considered a theoretical site for genetic defects causing DPD deficiency, (b) DPD expression follows a circadian pattern (Harris et al., 1990), suggesting regulatory elements which control expression, (c) it is clear from previous studies from our laboratory that DPD expression varies in different tissues throughout the body (Lu & Diasio, 1996), suggesting regula-

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

throughout the body (Lu & Diasio, 1996), suggesting regulation in different tissues, and (d) since increased DPD expression in certain tumors may be the basis for 5-FU resistance (Jiang, Lu, He, & Diasio, 1997), knowledge of the transcriptional regulatory elements which control DPD expression in tumors may be useful in the future to improve 5-FU therapy.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

GENERAL LIST OF REFERENCES

Albin, N., Johnson, M.R., & Diasio, R.B. (1996). cDNA cloning of bovine liver dihydropyrimidine dehydrogenase. DNA Sequence, 6, 243-250.

Albin, N., Johnson, M.R., Shahinian, H., Wang, K., & Diasio, R.B.(1995). Initial characterization of the molecular defect in human dihydropyrimidine dehydrogenase deficiency. Proceedings for the American Association for Cancer Research 36, 211.

Armstrong, R.D., Lewis, M., Stern, S.G., & Cadman, E.C. (1986). Acute effect of 5-fluorouracil on cytoplasmic and nuclear dihydrofolate reductase messenger RNA metabolism. Journal of Biological Chemistry, 261, 7366-7371.

Au, J.L., Rustum, Y.M., Ledesma, E.J., Mittelman, A., & Creaven, P.J. (1982). Clinical pharmacological studies of concurrent infusion of 5-fluorouracil and thymidine in treatment of colorectal carcinomas. Cancer Research, 42, 2930-2937.

Baker, S.D., Khor, S.P., Adjei, A.A., Doucette, M., Spector, T., Donehower, R.C., Grochow, L.B., Sartorius, S.E., Noe, D.A., Hohneker, J.A., & Rowinsky, E.K.(1996). Pharmacokinetic, oral bioavailability, and safety study of fluorouracil in patients treated with 776C85, an inactivator of dihydropyrimidine dehydrogenase. Journal of Clinical Oncology, 14, 3085-3096.

Benz, C., & Cadman, E. (1981). Modulation of 5-fluorouracil metabolism & cytotoxicity by antimetabolite pretreatment in human colorectal adenocarcinoma HCT-8. Cancer Research, 41, 994-999.

Bertino, J.R., Levitt, M., McCullough, J.L., & Chabner, B. (1971). New approaches to chemotherapy with folate antagonists: Use of leucovorin "rescue" and enzymic folate depletion. Annals of the New York Academy of Sciences, 186, 486-495.

Beuzeboc, P., Pierga, J.Y., Lyonnet, D.S., Couturier, J., & Pouillart, P. (1996). Toxicite severe au 5-fluorouracile chez une femme traitee pour un cancer du sein, presentant une osteogenese imparfaite et un deficit en dihydropyrimidine dehydrogenase [Severe 5-fluorouracil toxicity in a woman treated for breast cancer with

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

concurrent osteogenesis imperfecta and dihydropyrimidine dehydrogenase deficiency]. Bulletin du Cancer, 83, 324-327.

Beuzeboc, P., Pierga, J.Y., Stoppa-Lyonnet, D., Etienne, M.C. Milano, G., & Pouillart P.(1996). Severe 5-fluorouracil toxicity possibly secondary to dihydropryrimidine dehydrogenase deficiency in a breast cancer patient with osteogenesis imperfecta. European Journal of Cancer, 32A, 369-370.

Bleyer, W.A. (1989). New vistas for leucovorin in cancer chemotherapy. Cancer, 63, 995-1007.

Bowen,D., Diasio, R.B., & Goldman, I.D. (1979). Distinguishing between membrane transport and intracellular metabolism of fluorodeoxyuridine in Ehrlich ascites tumor cells by application of kinetic and high-performance liquid chromatographic techniques. Journal of Biological Chemistry, 254, 5333-5339.

Bowen, D., White, J.D., & Goldman, I.D. (1978). A basis for fluoropyrimidine-induced antagonism to methotrexate in Ehrlich ascites tumor cells in vitro Cancer Research, 38, 219-222.

Brooks, K.P., Jones, E.A., Kim, B.D., & Sander, E.G. (1983). Bovine liver dihydropyrimidine amidohydrolase: Purification, properties, and characterization as a zinc metalloenzyme. Archives of Biochemistry & Biophysics, 226, 469-483.

Caradonna, S.J., & Cheng, Y.C. (1980). The role of deoxyuridine triphosphate nucleotidehydrolase, uracil-DNA glycosylase, and DNA polymerase in the metabolism of FUdR in human tumor cells. Molecular Pharmacology, 18, 513-520.

Carrico, C.K., & Glazer, R.I. (1979). Effect of 5-fluoroura cil on the synthesis and translation of polyadenylic acid-containing RNA from regenerating rat liver. Cancer Research, 39, 3694-3701.

Chabner B.A., & Collins, J.M. (Eds.). (1990). Cancer chemo therapy: Principles and practice. Philadelphia: Lippincott.

Chadhuri, N.K., Mukherjee, K.L., & Heidelberger, C. (1959). Studies on fluorinated pyrimidines: Vol 7. The degradative pathway. Biochemical Pharmacology, 1, 328-341.

Cheng, Y.C., & Nakaymaa, K. (1983). Effects of 5-fluoro-2'-deoxyuridine on DNA metabolism in HeLa cells. Molecular Pharmacology, 23, 171-174.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Chu, E., Lai, G.M., Zinn, S., & Allegra, C.J. (1990). Resis tance of a human ovarian cancer line to 5-fluorouracil associated with decreased levels of 5-fluorouracil in DNA. Molecular Pharmacology, 38, 410-417.

Cisneros, R.J., Silks, L.A., & Dunlap, R.B. (1988). Mechanis tic aspects of thymidylate synthase: Molecular basis for drug design. Drugs Future, 13, 859-881.

Cohen, M.B., & Glazer, R.I. (1985). Cytotoxicity and the in- hibition of ribosomal RNA processing in human colon carcinoma cells. Molecular Pharmacology, 27, 308-313.

Cory, J., Breland, J.B., & Carter, G.L. (1979). Effect of 5- fluorouracil on RNA metabolism in Novikoff hepatoma cells. Cancer Research, 39, 4905-4913.

Coustere, C., Mentre, F., & Sommadossi, J.P. (1991). A math- e matical model of the kinetics of 5-fluorouracil and its metabolites in cancer patients. Cancer Chemotherapy and Pharmacology, 28, 123-129.

Daher, G.C., Harris, B.E., & Diasio, R.B. (1990). Metabolism of pyrimidine analogues and their nucleosides. Pharma- cology & Therapeutics, 48, 189-222.

Danenberg, P.V.(1984). The role of reduced folates in the enhanced binding of FdUMP to dTMP synthetase. In the current status of 5-fluorouracil-leucovorin calcium combination, pp 5-11 H.W. Bruckner and Y.M. Rustum (Eds.), New York: John Wiley & Sons.

Danenberg, P.V., & Danenberg, K.D. (1978). Effect of 5,10- methylene tetrahydrofolate on the dissociation of 5- fluoro-2'-deoxyuridylate from thymidylate synthetase: Evidence for an ordered mechanism. Biochemistry, 17, 4018-4024.

Danenberg, P.V., & Lockshin, A. (1981). Fluorinated pyrimi- dines as tight-binding inhibitors of thymidylate synth- ase. Pharmacology & Therapeutics, 13, 69-90.

Danenberg, P.V., Shea, L.C., & Danenberg, K.D. (1990). Effect of 5-fluorouracil substitution on the self-splicing ac- tivity of tetrahymena ribosomal RNA. Cancer Research, 50, 1757-1763.

DeVita, V.T. Jr., Hellman, S., & Rosenberg, S.A. (1989). Cancer: Principles and practice of oncology (3rd ed.). Philadelphia: Lippincott.

Diasio, R.B., Beavers, T.L., & Carpenter, J.T. (1988). Familial deficiency of dihydropyrimidine dehydrogenase. Biochemical basis for familial pyrimidinemia and severe

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

5-fluorouracil-induced toxicity. Journal of Clinical Investigation, 81, 47-51.

Diasio, R.B., & Harris, B.E. (1989). Clinical pharmacology of 5-fluorouracil. Clinical Pharmacokinetics, 16, 215-237.

Diasio, R.B., Kuilenburg Van, A.B.P., Lu, Z., Zhang, R., Lenthe Van, H., Bakker, H.D., & Gennip Van, A.H. (1995). Determination of diydropyrimidine dehydrogenase (DPD) in fibroblasts of a DPD deficient pediatric patient and family members using a polyclonal antibody to human DPD. Purine and Pyrimidine Metabolism in Man, 8, 7-10.

Diasio, R.B., Lu. Z., Zhang, R., & Shahinian, H.S. (1995). Fluoropyrimidine catabolism. Cancer Treatment & Research, 78, 71-93.

Diasio, R.B., & Wilburn, M.E. (1979). Effect of column temperature on resolution of fluoropyrimidines by reverse phase high performance liquid chromatography. Journal of Chromatography Science, 17, 565-567.

Dolnick, B.J., & Pink, J.J. (1983). 5-Fluorouracil modulation of dihydrofolate reductase RNA levels in methotrexate-resistant KB cells. Journal of Biological Chemistry, 258, 13299-13306.

Domin, B.A., Mahony, W.B., & Zimmerman, T.P. (1993). Transport of 5-fluorouracil and uracil into human erythrocytes. Biochemical Pharmacology, 46, 503-510.

Doolittle, R.F. (1981). Similar amino acid sequences: Chance or common ancestry?. Science, 214, 149-159.

Duran, M., Robers, P., & de Bree, P.K. (1991). Dihydropyrimidinuria: a new inborn error of pyrimidine metabolism. Journal of Inherited Metabolic Diseases, 14, 367-370.

Ensminger, W.D., Rosowsky, A., Raso, V., Levin, D.C., Glode, M., Come, S., Steele, G., & Frei, E. (1978). A clinical-pharmacological evaluation of hepatic arterial infusions of 5-fluoro-2'-deoxyuridine and 5-fluorouracil. Cancer Research, 11, 3784-3792.

Etienne, M.C., Cheradame, S., Fischel, J.L., Formento, P., Dassonville, O., Renee, N., Schneider, M., Thyss, A., Demard, F., & Milano, G. (1995). Response to fluorouracil therapy in cancer patients: The role of tumoral dihydropyrimidine dehydrogenase activity. Journal of Clinical Oncology, 13, 1663-1670.

Etienne, M.C., Milano, G., Fleming, R.A., Thyss, A., Renee, N., Schneider, M., & Demard, F.(1992). Dihydropyrimidine

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

dehydrogenase activity in lymphocytes: Predictive factor for 5-fluorouracil clearance. Bulletin du Cancer, 79, 1159-1163.

Falany, C.N., Johnson, M.R., Barnes, S., & Diasio, R.B. (1994). Glycine and taurine conjugation of bile acids by a single enzyme: Molecular cloning and expression of human liver bile acid CoA: Amino acid N-acyltransferase. Journal of Biological Chemistry, 269, 19375-19379.

Fischel, J.L., Formento, P., Etienne, M.C., Spector, T., Renee, N., & Milano, G.(1997). Dual modulation of 5-fluorouracil cytotoxicity using folinic acid with a dihydropyrimidine dehydrogenase inhibitor. Biochemical Pharmacology, 53, 1703-1709.

Fleming, R.A., Milano, G., Gaspard, M.H., Bargnoux, P.J., Thyss, A., Plagne, R., Renee, N., Schneider, M., & Demard, F.(1993). Dihydropyrimidine dehydrogenase activity in cancer patients. European Journal of Cancer, 29A, 740-744.

Fleming, R.A., Milano, G., Thyss, A., Etienne, M.-C., Renee, N., Schneider, M., & Demard, F.(1992). Correlation between dihydropyrimidine dehydrogenase activity in peripheral mononuclear cells and systemic clearance of fluorouracil in cancer patients. Cancer Research, 52, 2899-2902.

Fritzen, P. (1960). Properties and assay of dihydrouracil dehydrogenase of rat liver. Journal of Biological Chemistry, 235, 719-725.

Fujimoto, S., Matsuda, K., Kikugama, M., Kaneko, M., & Tamak, N. (1990). Effect of vitamin B2 deficiency on rat liver dihydropyrimidine dehydrogenase activity. Journal of Nutritional Science & Vitaminology, 37, 89-98.

Garmont, A.D., Krulik, M., Cady, J., Lagadec, B., Maisani, J.E., Loisear, J.P., Grange, J.D., Gustavo, G.C., Benedicte, D., Louvet, C., Seroka, J., Dray, C., & Debray, J. (1988). High-dose folinic acid and 5-fluorouracil bolus and continuous infusion in advanced colorectal cancer. European Journal of Clinical Oncology, 9, 1499-1503.

Goedde, H.W., Agarwal, D.P., & Eickhoff, K. (1970). Purification and properties of dihydrouracil dehydrogenase from pig liver. Hoppe-Seylers Zeitschrift fur Physiologische Chemie, 351, 945-951.

Grisolia, S., & Cardoso, S.S. (1957). The purification and properties of hydropyrimidine dehydrogenase. Biochimica et Biophysica Acta, 25, 430-431.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Hakala, M.T. (1984). Basis for the effect of leucovorin on the activity and site of action of 5-fluorouracil in human cells. In H.W. Bruckner and Y.M. Rustum (Eds.), The current status of 5-fluorouracil-leucovorin calcium combination (pp. 33-39). New York: John Wiley & Sons.

Hamajima, N., Matsuda, K., Sakata, S., Tamaki, N., Sasaki, M., & Nonaka, M. (1996). A novel gene family defined by human dihydropyrimidinase and three related proteins with differential tissue distribution. Gene, 180, 157-163.

Hardy, L.W., Finer-Moore, J.S., Montfort, W.R., Jones, M.O., Santi, D.V., & Stroud, R.M. (1987). Atomic structure of thymidylate synthase: Target for rational drug design. Science, 235, 448-455.

Harris, B.E., Carpenter, J.T., & Diasio, R.B. (1991). Severe 5-fluorouracil toxicity secondary to dihydropyrimidine dehydrogenase deficiency: A potentially more common pharmacogenetic syndrome. Cancer, 68, 499-501.

Harris, B.E., Song, R., Soong, S.J., & Diasio, R.B. (1990). Relationship of dihydropyrimidine dehydrogenase activity and plasma 5-fluorouracil levels: Evidence for circadian variation of 5-fluorouracil levels in cancer patients receiving protracted continuous infusion. Cancer Research, 50, 197-201.

Heggie, G.D., Sommadossi, J.P., Cross, D.S., Huster, W.J., & Diasio, R.B. (1987). Metabolism of 5-fluorouracil in cancer patients with quantitation of fluorouracil catabolites in plasma, urine, and bile over time. Cancer Research, 47, 2203-2206.

Heidelberger, C.(1965). Fluorinated pyrimidines. Progress in Nucleic Acid Research & Molecular Biology, 4, 1-50.

Heidelberger, C., Chaudhuri, N.K., Danenberg P., Mooren, D., Griesbach, L., Duschinsky, R., Schnitzer, R.J., Pleven, E., & Scheiner J.(1957). Fluorinated pyrimidines, a new class of tumor-inhibitory compounds. Nature, 179, 663-666.

Heimer, R., & Sartorelli, A.C. (1989). RNA polymerase II transcripts as targets for 5-fluorouridine cytotoxicity: Antagonism of 5-fluorouridine actions by a-amanitin. Cancer Chemotherapy & Pharmacology, 24, 80-86.

Henderson, M.J., Ward, K., Simmonds, H.A., Duley, J.A., & Davies, P.M. (1993). Dihydropyrimidinase deficiency presenting in infancy with severe developmental delay. Journal of Inherited Metabolic Diseases, 16, 547-576.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Herrick, D.J., & Kufe, D.W. (1984). Lethality associated with incorporation of 5-fluorouracil into preribosomal RNA. Molecular Pharmacology, 26, 135-140.

Herrick, D.J., Major, P.P., & Kufe, D.W. (1982). Effect of methotrexate on incorporation and excision of 5-fluorouracil residues in human breast carcinoma DNA. Cancer Research, 42, 5015-5017.

Houghton, J.A., & Houghton, P.J.(1980). 5-fluorouracil in combination with hypoxanthine and allopurinol: Toxicity and metabolism in xenografts of human colonic carcinomas in mice. Biochemical Pharmacology, 29, 2077-2080.

Houghton, J.A., & Houghton, P.J.(1983). Elucidation of path ways of 5-fluorouracil metabolism in xenografts of human colorectal adenocarcinoma. European Journal of Cancer Clinical Oncology, 19, 807-815.

Houyau, P., Gay, C., Chatelut, E., Canal, P., Roche, H., & Milano, G. (1993). Severe fluorouracil toxicity in a patient with dihydropyrimidine dehydrogenase deficiency. Journal of National Cancer Institute, 85, 1602-1603.

Iigo, M., Nishikata, K., Nakajima, Y., Hoshi, A., Okudaira, N., Odagiri, H., & De Clercq E. (1989). Enhancing effect of bromovinyldeoxyuridine on antitumor activity of 5'-deoxy-5-fluorouridine against adenocarcinoma 755 in mice. Correlation with pharmacokinetics of plasma 5-fluorouracil levels. Biochemical Pharmacology, 38, 1885-1889.

Ingraham, H.A., Tseng, B.Y., & Goulian, M.(1980). Mechanism for exclusion of 5-fluorouracil from DNA. Cancer Research, 40, 998-1001.

Ingraham, H.A. Tseng, B.Y., & Goulian, M. (1982). Nucleotide levels and incoporation of 5-fluorouracil and uracil into DNA of cells treated with 5-fluorodeoxyuridine. Molecular Pharmacology, 21, 211-216.

Jacquez, J.A., (1962). Permeability of Ehrlich cells to uracil, thymine and fluorouracil. Proceedings of the Society for Experimental Biology & Medicine, 109, 132-135.

Jiang, W., Lu, Z., He, Y., & Diasio, R.B. (1997). Dihydropyrimidine dehydrogenase activity in hepatocellular carcinoma; implication for 5-fluorouracil-based chemotherapy. Clinical Cancer Research, 3, 395-399.

Johnson, M.R., Barnes, S., & Diasio, R.B. (1990). 2-Fluoro-β-alanine, a previously unrecognized substrate for bile

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

acid CoA: amino acid: N-acyltransferase from human liver. Biochemical Pharmacolocology, 40, 1241-1246.

Johnson, M.R., Barnes, S., Kwakye, J., & Diasio, R.B. (1991). Purification of human liver cholyl CoA: amino acid: N-acyltransferase. Journal of Biological Chemistry, 262, 10227-10233.

Johnson, M.R., Wang, K., Tillmanns, S., Albin, N., & Diasio, R.B. (1997). Structural organization of the human dihydropyrimidine dehydrogenase gene. Cancer Research, 57, 1660-1663.

Johnson, M.R., Yan, J., Shao, L., Albin, N., & Diasio, R.B. (1997). Semi-automated radioassay for determination of dihydropyrimidine dehydrogenase (DPD). activity; Screening cancer patients for DPD deficiency, a condition associated with 5-fluorouracil toxicity. Journal of Chromatography B. 696, 183-191.

Kanamaru, R., Kakuta, H., Sato, T. Ishioka, C., & Wakui, A. (1986). The inhibitory effects of 5-flluorouracil on the metabolism of preribosomal and ribosomal RNA in L-1210 cells in vitro. Cancer Chemotherapy Pharmacology, 17, 43-46.

Kanamaru, R., & Wakui, A. (1988). Mechanism of action of anticancer drugs from the viewpoint of RNA metabolism. Japanese Journal of Cancer Chemotherapy, 15, 1011-1018.

Kemeny, N. (1996). Colorectal cancer- an undertreated disease. Anti-Cancer Drugs, 7, 623-629.

Kessel, D., Deacon, J., Coffey, B., & Bakamajian, A. (1972). Some properties of a pyrimidine phosphoribosyltransferase from murine leukemia cells. Molecular Pharmacology, 8, 731-739.

Kessel, D., & Hall, T.C. (1967). Studies on drug transport by normal human leukocytes. Biochemical Pharmacology, 16, 2395-2403.

Kessel, D., Hall, T.C., & Wodinsky, I. (1966). Nucleotide formation as a determinant of 5-fluorouracil response in mouse leukemias. Science, 154, 911-913.

Keyomarsi, K., & Moran, R.G. (1988). Mechanism of cytotoxicity synergism of fluoropyrimidines and folinic acid in mouse leukemia cells. Journal of Biological Chemistry, 263, 14402-14409.

Khor, S.P., Amyx, H., Davis, S.T., Nelson, D. Baccanari, D.P., & Spector T. (1997). Dihydropyrimidine dehydroge-

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

nase inactivation and 5-fluorouracil pharmacokinetics: Allometric scaling of animal data, pharmacokinetics and toxicodynamics of 5-fluorouracil in humans. Cancer Chemotherapy & Pharmacology, 39, 233-238.

Kikugawa, M., Kaneko, M., Fujimoto-Sakata, S., Maeda, M., Kawasaki K., Takagi, T., & Tamaki, N. (1994). Purification, characterization and inhibition of dihydropyrimidinase from rat liver. European Journal of Biochemistry, 219, 393-399.

Kobayashi, K., Jackson, M.J., Tick, D.B., O'Brien, W.E., & Beaudet A.L.(1990). Heterogeneity of mutations in argininosuccinate synthetase causing human citrullinemia. Journal of Biological Chemistry, 265, 11361-11367.

Kobayashi, K., & Ratain, M.J. (1993). Individualizing dosing of cancer chemotherapy. Seminars in Oncology, 20, 30-42.

Kozak, M. (1991). Structural features in eukaryotic mRNAs that modulate the initiation of translation. Journal of Biological Chemistry, 266, 19867-19870.

Krynetski, E.Y., Schuetz, J.D., Galpin, A.J., Pui, C.H., Relling, M.V., & Evans, W.E. (1995). A single point mutation leading to loss of catalytic activity in human thiopurine S-methyltransferase. Proceedings of the National Academy of Sciences of the United States of America, 92, 949-953.

Krynetski, E.Y., Tai, H.L., Yates, C.R., Fessing, M.Y., Loennechen, T., Schuetz, J.D., Relling, M.V., & Evans, W.E. (1996). Genetic polymorphism of thiopurine S-methyltransferase: Clinical importance and molecular mechanisms. Pharmacogenetics, 6, 279-290.

Kufe, D.W., Major, P.P., Egan, E.M., & Loh, E. (1981). 5-Fluoro-2'deoxyuridine. Incorporation in L1012 DNA. Journal of Biological Chemistry, 256, 8885-8888.

Kufe, D.W. Scott, P., Fram, W., & Major, P. (1983). Biologic effect of 5-fluoro-2'-deoxyuridine incorporation in L1210 deoxyribonucleic acid. Biochemical Pharmacology, 32, 1337-1340.

Laufman, L.R., Krzeczowski, R.R., & Segal, M. (1987). Leucovorin plus 5-fluorouracil: An effective treatment for metastatic colon cancer. Journal of Clinical Oncology, 5, 1394-1400.

Lee, M.H., Cowling, R.A., Sander, E.G., & Pettgrew, D.W. (1986). Bovine liver dihydropyrimidine amidohydrolase: pH dependencies of inactivation by chelators and steady

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

state kinetic properties. Archives of Biochemistry & Biophysics, 248, 368-378.

Lockshin, A., & Danenberg, P.V. (1981). Biochemical factors affecting the tightness of 5-fluorodeoxyurdylate binding to human thymidylate synthetase. Biochemical Pharmacology, 30, 247-257.

Lu, Z., & Diasio, R.B. Polymorphic drug-metabolizing enzymes. In Principles of Antineoplastic Drug Development and Pharmacology R.L. Schilsky, G.A. Milano, and M.J. Ratain (Eds.)., Marcel Dekker, New York, NY, 1996, pp. 28305.

Lu, Z.H., Zhang, R., & Diasio, R.B.(1992). Purification and characterization of dihydropyrimidine dehydrogenase from human liver. Journal of Biological Chemistry, 267, 17102-17109.

Lu, Z., Zhang, R., & Diasio, R.B. (1993a). Dihydropyrimidine dehydrogenase activity in human peripheral blood mononuclear cells and liver: Population characteristics, newly identified deficient patients, and clinical implication in 5-fluorouracil chemotherapy. Cancer Research, 53, 5433-5438.

Lu, Z.H., Zhang, R., & Diasio, R.B. (1993b). Comparison of dihydropyrimidine dehydrogenase from human, rat, pig and cow liver. Biochemical and immunological properties. Biochemical Pharmacology, 46, 945-952.

Lyss, A.P., Lilenbaum, R.C., Harris, B.E., & Diasio, R.B. (1993). Severe 5-fluorouracil toxicity in a patient with decreased dihydropyrimidine dehydrogenase activity. Cancer Investigation, 2, 239-240.

Maguire, J.H., & Dudley, K.H. (1978). Partial purification and characterization of dihydropyrimidinases from calf and rat liver. Drug Metabolism and Disposition, 6, 601-605.

Mandel, H.G. (1969). The incorporation of 5-fluorouracil into RNA and its molecular consequences. Progress in Molecular and Subcellular Biology, 1, 82-135.

Marmur, J. (1961). A procedure for the isolation of DNA from microorganisms. Journal of Molecular Biology, 3, 208-218.

Masaaki, I., Araki, E., Nakajima, Y., & De Clercq, E. (1988). Enhancing effects of bromovinyldeoxyuridine on antitumor activity of 5-fluorouracil against adenocarcinoma 755 in mice. Increased therapeutic index and correlation with increased plasma 5-fluorouracil levels. Biochemical Pharmacology, 37, 1609-1613.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Matsuda, K., Sakata, S., Kaneko, M., Hamajima, N., Nonaka, M., Sasaki, M., & Tamaki, N.(1996). Molecular cloning and sequencing of a cDNA encoding dihydropyrimidinase from the rat liver. Biochimica et Biophysica Acta, 1307, 140-144.

Matthews, M.M., Liao, W., Kvalnes-Krick, K.L., & Traut, T.W.(1992). beta-Alanine synthase: purification and allosteric properties. Archives of Biochemistry & Biophysics, 293, 254-263.

Matthews, M.M., & Traut, T.W.(1987). Regulation of N-carbamoyl-beta-alanine amidohydrolase, the terminal enzyme in pyrimidine catabolism, by ligand-induced change in polymerization. Journal of Biological Chemistry, 262, 7232-7237.

Mauro, D.J., De Riel, J.K., Tallarida, R.J., & Sirover, M.A. (1993). Mechanisms of excision of 5-fluorouracil by uracil DNA glycosylase in normal human cells. Molecular Pharmacology, 43, 854-857.

Meinsma, R., Fernandez-Salguero, P., Van Kuilenburg, A.B., Van Gennip, A.H., & Gonzalez, F.J.(1995). Human polymorphism in drug metabolism: Mutation in the dihydropyrimidine dehydrogenase gene results in exon skipping and thymine uraciluria. DNA and Cell Biology, 14, 1-6.

Milano, G., Etienne, M.C., & Fischel, J.L. (1995). La dihydropyrimidine deshydrogenase intratumorale et son inhibition: Une nouvelle approche dans la pharmacomodulation du 5-fluoro-uracile [Intratumoral dihydropyrimidine dehydrogenase and its inhibition: A new approach in the pharmacokinetics of 5-fluorouracil]. Annales de Gastro enterologie et de Hepatologie, 31, 103-105.

Morrison, G.B., Bastian, A., Dela Rosa, T., Diasio, R.B., & Takimoto, C.H.(1997). Dihydropyrimidine dehydrogenase deficiency: A pharmacogenetic defect causing severe adverse reactions to 5-fluorouracil-based chemotherapy. Oncology Nursing Forum, 24, 83-88.

Mukherjee, K.L., & Heidelberger, C., (1960). Studies on fluorinated pyrimidines. Vol. 9. The degradation of 5-fluoro-2'-deoxyuridine-2-^{14}C in cancer patients. Cancer Research, 23, 49-66.

Myers, C.E., Young, R.C., & Chabner, B.A. (1975). Biochemical determinants of 5-fluorouracil response in vivo. The role of deoxyuridylate pool expansion. Journal of Clinical Investigation, 56, 1231-1238.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Naguib, F.N., el Kouni, M.H., & Cha, S. (1985). Enzymes of uracil catabolism in normal and neoplastic human tissues. Cancer Research, 45, 5405-5412.

Ohba, S., Kidouchi, K., Sumi, S., Imaeda, M., Takeda, N., Yoshizumi, H., Tatematsu, A., Kodama, K., Yamanaka, M., Kobayashi, M., & Wada, Y. (1995). Dihydropyrimidinuria: The first case in Japan. Advances in Expermental Medicine and Biology, 370, 383-386.

Parker, W.B., Kennedy, K.A., & Klubes, P. (1987). Dissociation of 5-fluorouracil into DNA and its cytotoxicity in murine T-lymphoma (S-49). cells. Cancer Research, 47, 979-982.

Petit, E., Milano, G., Levi, F., Thyss, A., Bailleul, F., & Schneider M. (1988). Circadian rhythm-varying plasma concentration of 5-fluorouracil during a five-day continuous venous infusion at a constant rate in cancer patients. Cancer Research, 48, 1676-1679.

Petrelli, N., Herrera, L., Rustum, Y., Burke, P., Creaven, P., Stulc, J., Emrich, L.J., & Mittelman, A. (1987). A prospective randomized trial of 5-fluorouracil versus 5-fluorouracil and high-dose leucovorin versus 5-fluorouracil and methotrexate in previously untreated patients with advanced colorectal carcinoma. Journal of Clinical Oncology, 5, 1559-1565.

Podschun, B., Cooks, P.F., & Schnackerz, K.D. (1990). Kinetic mechanism of dihydropyrimidine dehydrogenase from pig liver. Journal of Biological Chemistry, 265, 12966-12972.

Podschun, B., Wahler, G., & Schnackerz, K.D. (1989). Purification and characterization of dihydropyrimidine dehydrogenase from pig liver. European Journal of Biochemistry, 185, 219-224.

Porter, D.J., Chestnut, W.G., Merrill, B.M., & Spector T. (1992). Mechanism-based inactivation of dihydropyrimidine dehydrogenase by 5-ethynyluracil. Journal of Biological Chemistry, 267, 5236-5242.

Porter, D.J., Chestnut, W.G., Taylor, L.C., Merrill, B.M., & Spector, T. (1991). Inactivation of dihydropyrimidine dehydrogenase by 5-iodouracil. Journal of Biological Chemistry, 266, 19988-19994.

Putman, C.W., Rotteveel, J.J., Wevers, R.A., van Gennip A.H., Bakkeren, J.A., & De Abreu R.A. (1997). Dihydropyrimidinase deficiency, a progressive neurological disorder? Neuropediatrics, 28, 106-610.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Quinn, C.L., Stephenson, B.T., & Switzer, R.L. (1991). Functional organization and nucleotide sequence of the Bacillus subtilis pyrimidine biosynthetic operon. Journal of Biological Chemistry, 266, 9113-9127.

Ramberg, E.S., Ishaq, M., Rulf, S., Moeller, B., & Horowitz, J. (1978). Inhibition of transfer RNA function by replacement of uridine and uridine-derived nucleosides with 5-fluorouridine. Biochemistry, 17, 3978-3985.

Ratain, M.J., Rosner, G., Allen, S.L., Costanza, M., Van Echo, D.A., Henderson, C., & Schilsky, R.L. (1995). Population pharmacodynamic study of amonafide: A cancer and leukemia group B study. Journal of Clinical Oncology, 13, 741-747.

Reyes, P., & Hall, T.C. (1969). Synthesis of 5-fluorouridine 5'-phosphate by a pyrimidine phosphoribosyltransferase of mammalian origin. Vol. II. Correlation between the tumor levels of the enzyme and the 5-fluorouracil-promoted increase in survival of tumor-bearing mice. Biochemical Pharmacology, 18, 2587-2590.

Robinson, B.H., MacKay, N., Chun, K., & Ling, M.(1996). Disorders of pyruvate carboxylase and the pyruvate dehydrogenase complex. Journal of Inherited Metabolic Diseases, 19, 452-462.

Rozen, R. (1996). Molecular genetics of methylenetetrahydrofolate reductase deficiency. Journal of Inherited Metabolic Diseases, 19, 589-594.

Sanno, Y., Holzer, M., & Schimke, R.T.(1970). Studies of a mutation affecting pyrimidine degradation in inbred mice. Journal of Biological Chemistry, 245, 5668-5676.

Santi, D.V., & McHenry, C.S. (1972). 5-Fluoro-2'-deoxyuridylate: Covalent complex with thymidylate synthetase. Proceedings of the National Academy of Sciences, USA, 69, 1855-1857.

Santi, D.V., McHenry, C.S., & Sommer, H. (1974). Mechanism of interaction of thymidylate synthetase with 5-fluorodeoxyuridylate. Biochemistry, 13, 471-481.

Schuetz, J.D., Collins, J.M., Wallace, H.J., & Diasio, R.B. (1986). Alteration of the secondary structure of newly synthesized DNA from murine bone marrow cells by 5-fluorouracil. Cancer Research, 46, 119-123.

Schuetz, J.D., & Diasio, R.B. (1985). The effect of 5-fluorouracil on DNA chain elongation in intact bone marrow cells. Biochemical & Biophysical Research Communications, 133, 361-367.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Schuetz, J.D., Wallace, H.J., & Diasio, R.B. (1984). 5-Fluorouracil incorporation into DNA of CF-1 mouse bone marrow cells as a possible mechanism of toxicity. Cancer Research, 44, 1358-1363.

Schuetz, J.D., Wallace, H.J., & Diasio, R.B.(1988). DNA repair following incorporation of 5-fluorouracil into DNA of mouse bone marrow cells. Cancer Chemotherapy & Pharmacology, 21, 208-210.

Schwartz, P.M., & Handschumacher, R.E. (1979). Selective antagonism of 5-fluorouracil cytotoxicity by 4-hydroxy-pyrazolopyrimidine (allopurinol). in vitro. Cancer Research, 39, 3095-3101.

Scrip's cancer chemotherapy report. (1996).Scrip World Pharmaceutical News. London: PJB Publications Ltd.

Shiotani, T., & Weber, G. (1981). Purification and properties of dihydrothymine dehydrogenase from rat liver. Journal of Biological Chemistry, 256, 219-224.

Sköld, O. (1958). Enzymic ribosidation and ribotidation of 5-fluorouracil by extracts of the Ehrlich-ascites tumor. Biochimica et Biophysica Acta, 29, 651.

Sommadossi, J.P., Cross, D.S., Gewirtz, D.A., Goldman, I. D., & Diasio, R.B. (1985). Evidence from rat hepatocytes of an unrecognized pathway of 5-fluorouracil metabolism with the formation of a glucuronide derivative. Cancer Research, 45, 2450-2455.

Sommadossi, J.P., Gewirtz, D.A., Diasio, R.B., Aubert, C., & Cano, J.P. (1982). Rapid catabolism of 5-fluorouracil in freshly isolated rat hepatocytes as analyzed by high performance liquid chromatography. Journal of Biological Chemistry, 357, 8171-8176.

Spector, T., Porter, D.J.T., Nelson, D.J., Baccanari, D.P., Davis, S.T., Almond, M.R., Khor, S.P., Amyx, H., Cap, S., & Rustum, Y.M. (1994). 5-Ethynluracil (776c85), a modulator of the therapeutic activity of 5-fluorouracil. Drugs of the Future, 19, 565-571.

Sweeny, D.J., Barnes, S., & Diasio, R.B. (1988a). Production of cholestasis by 2-fluoro-β-alanine-chenodeoxycholic acid: Possible role of this metabolite in the cholestasis associated with hepatic arterial infusion of fluoropyrimidines. Proceedings of the American Association for Cancer Research, 29, 486.

Sweeny, D.J., Barnes, S., Heggie, G.D., & Diasio, R.B. (1987). Metabolism of 5-fluorouracil to a N-cholyl-2-

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

fluoro-β-alanine conjugate: A novel role for bile acids in drug conjugation. Proceedings of the National Academy of Sciences, United States of America, 84, 5439-5443.

Sweeny, D.J., Martin, M., & Diasio, R.B. (1988). N-chenodeoxycholyl-2-fluorob-b-alanine: A biliary metabolite of 5-fluorouracil in man. Drug Metababolism and Disposition, 16, 892-894.

Takai, S., Salguero, P.F., Kimura, S., Gonzalez, F.J., & Yamada, K. (1994). Assignment of the human dihydropyrimidine dehydrogenase gene (DPYD). to chromosome region 1p22 by fluorescence in situ hybridization. Genomics, 24, 613-614.

Takimoto, C.H., Lu, Z.H., Zhang, R., Liang, M.D., Larson, L.V., Cantilena, L.R., Grem, J.L., Allegra, C.J., Diasio, R.B., & Chu, E. (1996). Severe neurotoxicity following 5-fluorouracil-based chemotherapy in a patient with dihydropyrimidine dehydrogenase deficiency. Clinical Cancer Research, 2, 477-481.

Tanaka, M., Kimura, K., & Yoshida, S.(1984). Increased incorporation of 5-fluorodeoxyuridine into DNA of human T-lymphoblastic cell lines. Ganno Rinsho, 75, 986-992.

Tennvall, J., Albertsson, M., Biorklund, A., Wennerberg, J., Anderson, H, Andersson, T., Elner, A., & Mercke, C. (1991). Induction chemotherapy (cisplatin + 5-fluorouracil). and radiotherapy inadvanced squamous cell carcinoma of the head and neck.Acta Oncologica, 30, 27-32.

Tuchman, M., Stoeckeler, J.S., Kiang, D.T., O'Dea, R.F., Rammaraine, M.L., & Mirkin, B.L. (1985). Familial pyrimidinemia and pyrimidinuria associated with severe fluorouracil toxicity. New England Journal of Medicine, 313, 245-249.

Wallach, D.P., & Santiago, G. (1957). The purification and properties of hydropyrimidine hydrase. Journal of Biological Chemistry, 251, 6909-6914.

Wei, X., Mcleod, H.L., McMurrough, J., Gonzalez, F.J., & Salguero, P.F. (1996). Molecular basis of the human dihydropyrimidine dehydrogenase deficiency and 5-fluorouracil toxicity. Journal of Clinical Investigation, 98, 610-615.

Wilkinson, D.S., Tisty, T.D., & Hanas, R.J. (1975). The inhibition of ribosomal RNA synthesis and maturation in Novikoff hepatoma cells by 5-fluorouridine. Cancer Research, 35, 3014-3020.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Wohlhueter, R.M., Mclvar, R.S., & Plageman, P.G.W. (1980). Facilitated transport of uracil and 5-fluorouracil, and permeation of orotic acid into cultured mamalian cells. Journal of Cell Physiology, 194, 309-319.

Woodcock, T.M., Martin, D.S., Damin, L.A., Kemeny, N.E., & Young, C.W. (1980). Combination clinical trials with thymidine and fluorouracil: A phase I and clinical pharmacologic evaluation. Cancer, 45, 1135-1143.

Yin, M.B., & Rustum, Y.M. (1991). Comparative DNA strand breakage induced by 5-FU and FdUrd in human ileocecal adenocarcinoma (HCT-8). cells: Relevance to cell growth inhibition. Cancer Communications, 3, 45-51, 1991.

Yokota, H., Salguero, P.F., Furuya, H., Lin, K., McBride, O.W., Podschun, B., Schnackerz, K.D., & Gonzalez, F.J. (1994). cDNA cloning and chromosome mapping of human dihydropyrimidine dehydrogenase, an enzyme associated with 5-fluorouracil toxicity and congenital thymine uraciluria. Journal of Biological Chemistry, 269, 23192-23196.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

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

Name of Candidate Martin Ray Johnson

Major Subject Pharmacology

Title of Dissertation cDNA Cloning and Characterization of the Dihydropyrimidine Dehydrogenase Gene: an Enzyme Associated with 5-Fluorouracil Metabolism

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 he may be recommended for the degree of Doctor of Philosophy.

Dissertation Committee:

Name		Signature
Dr. Robert Diasio	**, Chair**	
Dr. Denise R. Shaw		
Dr. Bob LeBoeuf		
Dr. Jeffrey B. Smith		
Dr. William B. Parker		

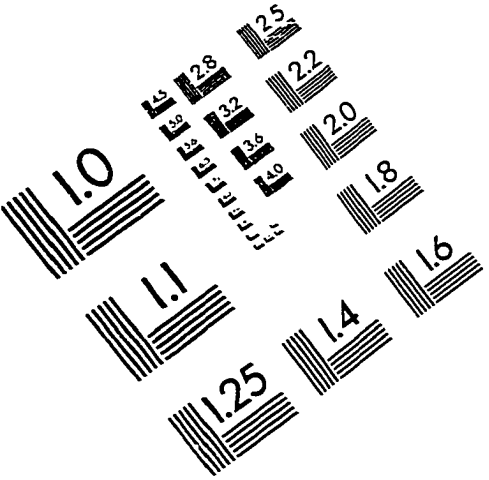
Director of Graduate Program

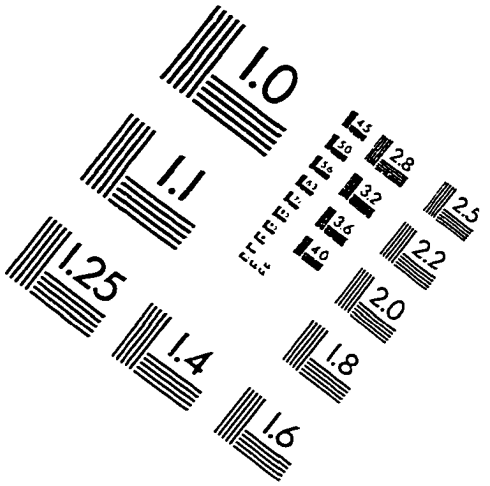
Dean, UAB Graduate School

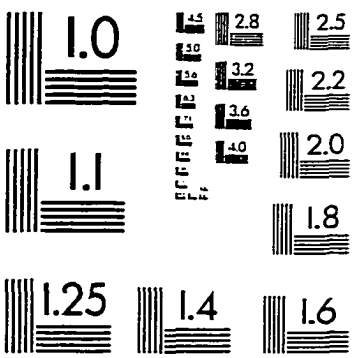
Date 4/8/98

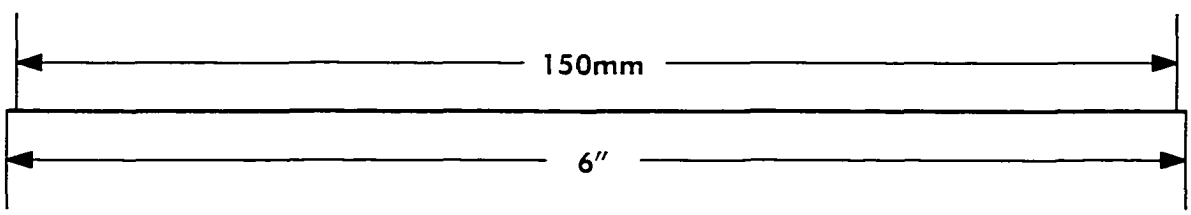
Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

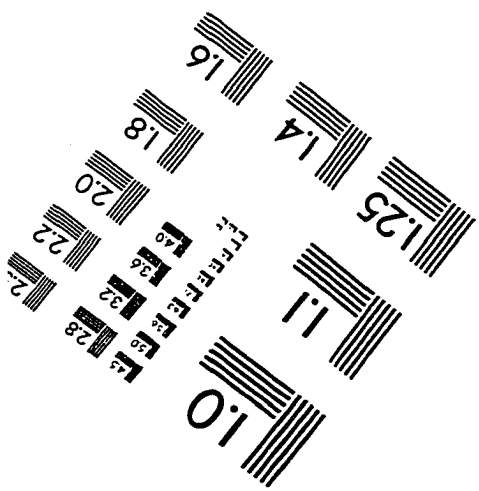
IMAGE EVALUATION TEST TARGET (QA-3)

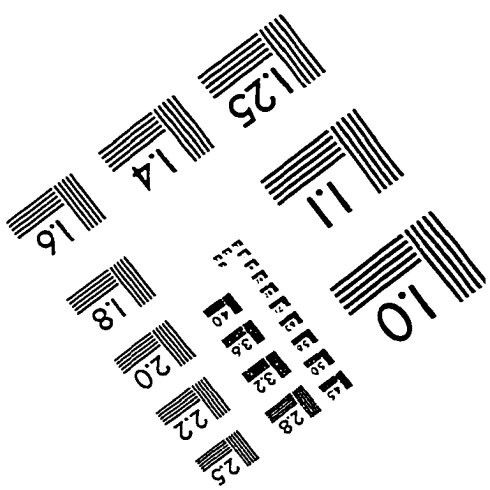












APPLIED IMAGE, Inc
1653 East Main Street
Rochester, NY 14609 USA
Phone: 716/482-0300
Fax: 716/288-5989

© 1993, Applied Image, Inc., All Rights Reserved

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.