Pharmacogenetic and metabolic biomarkers in predicting drug response:

Inborn errors of metabolism as functional models of pharmacogenetic diseases with specific emphasis on disorders of purine and pyrimidine metabolism

...a PhD thesis

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Introduction

This thesis summarizes the author's (DV) experimental work done over the last 15 years. Its text is divided into three chapters. The Chapter A brings up the theoretical foundations of inborn errors of metabolism, their pharmacogenetic implications, the evolution of pharmacogenetics, the basic aspects of human drug response and concepts of applying and utilization of endogenous metabolic biomarkers in assessing the rate of response to various pharmaceuticals, the specifics of disorders of purine and pyrimidine metabolism and their implications for anticancer chemotherapy. The Chapter B contains comprehensive discussion of author's published results where each of the papers is commented in depth along with their implications for laboratory diagnosis in clinical laboratory practice. Each of the discussed papers is also corroborated with notes on achieved scientific feedback in the form of their respective quotations – journal citations are presented as listed in the ISI Web of Science as of October 2005 together with citations of respective papers in monographs and/or reference textbooks. Various laboratory methods used and/or developed are not discussed in details as their description is comprehensively given in the respective publications; only the significant outcomes are mentioned in more detailed discussion. Besides the published work this chapter also brings several interim yet unpublished results that are subjects of the author's current research and/or close international collaboration. The Chapter C summarizes the author's interpretation of current knowledge on pharmacogenetics and its applicability in contemporary clinical pharmacological practice. Next, the author brings his view on the further development in the field of pharmacogenetics and biomarker assessment and discussed their pros and cons and sets forth several hypotheses to attempt an implementation of pharmacogenetic principles to improve current pharmacological practice with specific emphasis of cancer treatment.

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CHAPTER A

Inborn errors of metabolism and pharmacogenetics:
historical aspects, basic concepts, terms and definitions

The foundation of scientific discipline that we today call "pharmacogenetics" can be tracked back to the beginning of the 20th century. Theoretical foundations of pharmacogenetics are derived from the concept of "chemical individuality" that was first raised by a British physician Sir Archibald Garrod in 1902. He noticed that people with alkaptonuria, a disease with impaired degradation of a tyrosine metabolite, homogentisic acid, tend to produce urine that turns dark on standing and/or alkalinization, have black ochronotic pigmentation of cartilage and collagenous tissues, and arthritis, especially characteristic in the spine. Carefully observing his patients he noticed that this feature is transmitted vertically throughout the family pedigree as a genetic trait. Although knowing nothing about genes at that time he was the first who gave "Mendelian interpretation" to this phenomenon. In his 1902 publication he concluded that *"...we are dealing with individualities in metabolism and not with the results of morbid processes, the thought naturally present itself that these are merely extreme examples of variations of chemical behaviour which are probably present everywhere in minor degree and just as no two individuals of a species are absolutely identical in bodily structure neither are their chemical processes carried out on the same line"* (1). Later, in 1909, he derived the concept of "inborn error of metabolism", which he correctly perceived as a subset of his much broader concept – the concept of chemical individuality (2).

Inborn errors of metabolism

In striking similarity to Johann Gregor Mendel, whose landmark discoveries today called Mendel´s laws were forgotten to be rediscovered at the beginning of the 20th century, Garrod´s concept of an inborn error almost came in vain before its reinvention during late fifties – in 1949, a symposium of The Biochemical Society of London was devoted exclusively to a discipline called "biochemical genetics". Garrod´s contribution to genetics was widely acknowledged because of providing two important principles i) *that "...whenever possible, a genetic difference should be measured by a chemical test"* and ii) *that "...if we knew enough every gene would be assigned a specific chemical effect in the body"* (3). In view of the recent level of scientific knowledge, these principles proved fundamental for modern basic and clinical genetics – in fact, contemporary multidisciplinary subspecialties such as pharmacogenetics have been built mainly on these principles. Considering examples of inborn errors and pharmacogenetics one can see that it is an endogenous chemical substance overloading a given biochemical pathway

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that is a poisonous and pathogenic element in an inborn error whereas an exogenous compound overloading the same biochemical pathway with an artifactual substrate (such as a drug) may be the cause of a manifest clinical phenotype in an otherwise healthy individual.

Mendelian inheritance, monogenic diseases and ecogenetics

Without being really noticed by contemporary scientists, the manuscript of the lecture entitled "Versuche über Pflanzen-Hybride" (Treatises on Plant Hybrids) written by the Moravian-Silesian Augustinian monk Johann Gregor Mendel appeared in 1865. Mendel described his observations on the inheritance of various characteristics ("factors") in cross-bred pea plants as manifested in the phenotypes of subsequent generations. In crossing pea plants over and over again, he noticed some patterns to the inheritance of traits from one set of pea plants to the next. By carefully analyzing his pea plant numbers and their shape he discovered two visionary principles of inheritance later acknowledged Mendel's Laws: The Law of Dominance and Segregation and, The Law of Independent Assortment. Much later, essentially with the discoveries of Watson and Crick on DNA and its structure, biochemistry and molecular biology explained the molecular basis of Mendel's Laws in the form of a gene expression in cells and a gene transmission in the germline. Importantly, Mendel's work made possible for the first time to exploit the genetic resources of organisms systematically. Mendel's name marks not only the beginning of genetics as a scientific discipline in its own right but also the beginning of the systematic use of mathematics, the importance of quantification in measurements and applied statistics in biology (4).

During Garrod's period, a gene remained a statistical entity until the publication by Beadle and Tatum (5) who assigned to the gene a biochemical "descendant" – an enzyme. Thus, it was essentially the idea of a "biochemical phenotype" that made the concepts of "chemical individuality" and an "inborn error" consistent with clinical thinking by providing i) a functional definition of a gene and ii) a direct link among the gene, its product with a specific biochemical function and a clinically apparent/measurable phenotype of a given trait. This is a concept that holds true for what we know today as "monogenic disorders".

Understanding peculiarities of human drug response started about 150 years ago. At that time, intriguing effects of foreign substances to the human body were observed for the first time but putting more progress to experimental work was not possible until methodological discoveries by the end of 19th and beginning of the 20th centuries (6). This progress also paralleled development of knowledge in the field of organic chemistry. As was pointed out in more particular above, a rediscovery of the Mendel's laws led significant thinkers of the early 20th century, Garrod, Cuenot and Bateson to suggest that genetic material played a significant

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role in directing chemical transformations in the body. Garrod´s work with a hypnotic compound "sulfonal" led him to suggest that inborn factors were implicated not only in elimination of an endogenous substance accumulating in the body due to an "inborn error of metabolism (such as alkaptonuria)" but also exogenous substances that are foreign to the body. Another significant observation was that foreign substances ingested were often excreted in a form that differed from that ingested. Extending his observation he made a point that an individual can transform many foreign substances to nontoxic conjugates such as hippurates, and glucuronates, a mechanism enabling them to be excreted harmlessly (7). Demonstration by Snyder that transmission of taste blindness in children was inherited from their parents as an autosomal recessive Mendelian trait belongs to the first pharmacogenetic phenomena discovered revealing a high order of specificity that might be expected in human response to chemicals (8). In terms of Garrod´s concept of chemical individuality, these phenomena later proved their heritable nature.

The major categories of drug metabolizing enzymes were discovered about 50 years later, i.e. at the time when a substantial number of "classical" inborn metabolic disorders was thought already described (9). The studies of Williams (10) yielded that humans and animals were capable of biotransforming a vast number of foreign chemicals by surprisingly few metabolic pathways. In these studies, Williams raised a new and far-reaching principle of drug metabolism, specifically that the metabolic disposition in humans and animals occurs in two phases: a Phase I consisting of the chemical modification of a parent drug such as oxidation, reduction, or hydrolysis, which is followed by a Phase II, when a parent drug modified during the Phase I was subsequently conjugated to yield a more water soluble and polar conjugate, more prone to be excreted from the body.

Pharmacogenetics and its context in the environment-mediated response

Variations of patient response to suxamethonium, primaquine and isoniazid were the first phenomena investigated from the genetic standpoint during the late fifties and sixties (11). Hereditary variations in response to antimalarics emerged as especially instructive cases of "chemical individuality" concept where an underlying inborn error of metabolism – deficiency of glyceraldehyde 3-phosphate dehydrogenase translated to an unusual sensitivity to primaquine and similar drugs. The difference was shown to be sex-specific (12). One of the first clinically effective antituberculotic agents, isoniazid, was shown to produce peripheral neuropathy in a subset of patients. Later, it was proven that isoniazid is metabolized by acetylation and that the human population is essentially divided into "rapid" and "slow" acetylators according to the rate of the transfer of the acetyl moiety to the exogenous compounds (13). The true beginning of pharmacogenetics emerged by publication of a paper by

Motulsky (14) who was the first to fuse the concepts of biochemistry, pharmacology and genetics into a confluential scientific discipline devoted to person-to-person variations in the drug response caused by unique genetic variations. Subsequently, the term "pharmacogenetics" itself was proposed by Vogel in 1962 (15). As follows from what has been discussed previously, pharmacogenetics encompasses a wide range of pharmacologic and toxicological phenomena that is – at least in theory – essentially unlimited. The fact that a majority of "pharmacogenetic" examples are derived from administration of drugs is historically of technical nature, since drugs are usually administered under rigorous conditions in contrast to the environmental pollutants and endogenous compounds present in foodstuff as well. Here, terms such as "environmental genetics" and/or "ecogenetics" appear more accurate and, understandably, pharmacogenetics lies essentially within their scope (16).

The rationale for a pharmacogenetic investigation is often based on isolated observations such as case reports on "side effects" and/or unexpected "idiosyncratic" and/or epidemiological studies. Typically, it takes significant experimental effort to classify and elucidate these phenomena at the level of genotype, pharmacobiochemical phenotype and clinical phenotype. It follows that significant obstacles may occur in achieving these goals and that application of a wide array of techniques including testing for putative biomarkers of drug response and close collaboration of scientists with different expertise is necessary (17). To underline these difficulties, one may emphasise that whereas epidemiological studies usually raise a suspicion that a particular "ecotoxic" (drug, carcinogen, physical entity such as radiation, etc) compound may be responsible for a downstream morbid phenomenon they do not offer causative explanation of pathology observed because of technical and ethical limitations.

In contrast with many other genetic disciplines where establishing experimental animal models led to significant discoveries in elucidating specific metabolic and regulatory pathways, creating a laboratory animal model for a human pharmacogenetic condition is difficult due to the very nature of pharmacogenetic phenomena – recalling the Garrodian thesis of chemical individuality one may easily derive that this functional characteristics may widely differ among various mammalian species. Indeed, mammals can suffer from a variety of morbid conditions that are in their nature "inborn errors" but they may not necessarily parallel pathophysiology observable in humans. To demonstrate this, two interesting examples pertinent to inborn errors of purine and pyrimidine metabolism can be mentioned – the mouse model of Lesch-Nyhan syndrome and orotic aciduria in Holstein cattle strain. Establishing a laboratory mouse strain deficient for an enzyme hypoxanthine-guanine phosphoribosyltrasferase (HGPRT) did not lead to the clinical phenotype expected since – in contrast to humans – this enzyme probably does not play such an essential role in recycling purine metabolic intermediates as it does in humans (18). The first example proved technical feasibility of creating a full biochemical phenotype of the Lesch-Nyhan syndrome including universally decreased activity of HGPRT and

Genetic basis of pharmacology and human drug response

Xenochemicals administered to individuals exert characteristic and more or less reproducible effects when administered to mammal individuals. Responses that are induced by administrations of drugs are mostly due to physiological and biochemical properties of i) compounds administered and ii) features of the cells and tissues that are subject to treatment. Any drug administered to an individual is subject of essentially two critical pharmacobiochemical processes determining additively the response to the drug: the process of pharmacokinetics and pharmacodynamics. A central "dogma" of pharmacology holds that an active drug must be present at the receptor site to exert its response. However, exerting its response in a complex system such as mammals, the therapeutic compound must be administered in a way that can produce efficient concentrations of an active substance at the receptor sites. As a majority of drugs currently used in clinical practice act reversibly, the magnitude and characteristics of their responses are, in principle, related to the concentration of the transient complexes formed between the drug and the receptor sites. In quantitative terms, it is quite difficult to determine the exact concentration of the receptor-ligand (i.e. a drug) complexes, but fortunately, since these processes obey the "law of mass action" one can express the concentration of the drug/receptor complexes in quantitative terms and consequently, the drug/receptor complexes would parallel the concentration of a free drug delivered to the receptor site, which is essentially governed by drug plasma concentration. Taking together, processes leading to elimination of an administered substance are categorized as "pharmacokinetics" and processes responsible for modulating the pharmacological action are coined "pharmacodynamics". Because both biological effects are governed through action of genetically determined protein molecules one can derive that substantial variations can be expected both in pharmacokinetic and pharmacodynamic arms of the drug response.

Inborn errors of purine and pyrimidine metabolism: History and physiological chemistry

Purines and pyrimidines fulfill a wide range of diverse biologic function ranging from energy transport and handling to storage and management of genetic information. In fact, purines were discovered by Arthur Kossel in 1879 (22) but their physiological significance has not been appreciated before the elucidation of their biochemical role of the structural components of nucleic acid. Possessing a peculiar physicochemical property – the capability of creating hydrogen bridges – they play a fundamental role in keeping the dynamic structure of nucleic acids, DNA and RNAs. All major purine (adenine and guanine) and pyrimidine (cytosine and thymine/uracil) bases are synthesises through their respective *de novo* pathways.

Unlike a number of other biochemical cellular processes, purine and pyrimidine de novo synthesis pathways require significant substrate and energy expenditure, a point that phylogenetically led to the evolution of metabolic shunts effectively recycling purines and pyrimidines in the form of their corresponding nucleosides.

As one can surmise from what was explained above, an "inborn error" can appear literally in every protein-driven reaction (enzymes, regulatory proteins etc). Although current estimates of collective incidence of inborn errors of metabolism reach up to 10 % of all pediatric hospitalisations (23, 24) the actual incidence of purine and pyrimidine diseases is surprisingly low regarding both the number of cases diagnosed but also a number of disorders discovered to date. The known disorders of pyrimidine metabolism consist of two defects affecting pyrimidine biosynthesis, uridine monophosphate synthase and uridine monophosphate hydrolase deficiencies, and three defects in the degradation pathway of the pyrimidine ring, dihydropyrimidine dehydrogenase, dihydropyrimidinase and ureidopropionase (25). To make a "pharmacogenetic" point here it is important to say that all pyrimidine drug analogs used in the treatment of human disease represented by an antitumor agent 5-fluorouracil and 5-fluorocytosine used for treatment o fungal infections are degraded by the three enzyme pyrimidine degradation pathway already mentioned. Although a mechanism for recycling of pyrimidine species exists – a group of enzymes called pyrimidine nucleoside kinases and represented typically by thymidine kinase (E.C. 2.7.1.21) and deoxycytidine kinase E.C 2.7.1.74 - no apparent inborn error in the salvage pathway has been described to date despite the fact that this mechanism plays a significant role under certain drug-induced conditions. Upon inhibition of the thymidylate synthase by antagonists of folic acid and resulting depletion of thymidylic acid essential for continuation in cell division (26), thymidine kinase may overcome a critical depletion of thymidylic acid by rephosphorylating the respective nucleoside thymidine (27). The disorders originating in malfunction of purine ring metabolism and its subsequent metabolic handling are known from mediaeval ages and are represented mainly by gout (28). However, the molecular and pathophysiological basis has been described only during the last 30 years (29). Familial gout and gouty nephropathy belong, together with xanthinuria, to the disorders of purine degradation and elimination. Two inborn errors are known in purine recycling: deficiency of hypoxanthine, guanine phosphoribosyltransferase, HGPRT, E.C 2.4.2.8, and deficiency of adenine phosphoribosyltransferase E.C 2.4.2.7. Interestingly, despite intensive effort devoted to their research within last 30 years, the only known disorders in purine *de novo* synthesis described was the deficiency of adenylosucinate lyase (29, 30). Several hypotheses have been brought up at that time to explain this peculiar observation; the most plausible explanation seemed that due to general metabolic indispensability of purine ring-containing compounds inborn errors affecting their biosynthesis are probably lethal. However, in 2004 a novel, clinically devastating de novo purine synthesis disorders was found and termed AICARibosiduria (31).

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To summarize, the research in disorders affecting purine biosynthesis remains very challenging due to the biological character of putative enzyme defects but also due to the technical reasons. Despite the advent of biological tandem mass spectrometry and other techniques with in vivo applicability such as nuclear magnetic resonance, technical limitations (the marker molecule stability in mass spectrometric application, extractability from complex matrix mixtures, etc.) pose major obstacles to the progress needed to understand more on their biology, biochemistry and pathophysiology.

CHAPTER B

Chronology of author´s contribution to diagnosis of purine and pyrimidine inborn errors and transition to pharmacogenetics: Discussion of published results.

Screening and diagnosis of purine and pyrimidine disorders:

During the 90th no systematic screening of purine and pyrimidine disorders was performed. Testing of suspect clinical cases was done in specialized centres only mostly by two dimensional TLC and HPLC techniques. The screening of a newly discovered disorder, adenylosuccinate lyase deficiency, was especially difficult and the development of two new techniques appeared in the literature (32, 33). In 1994, DV developed a combined screening method employing high-performance TLC, the principle of which was to put together a method of screening disorders of saccharide metabolism and succinylpurines, markers of ADSL. To achieve this, the author combined chromatography of urinary saccharides and succinylpurines on a plate with a fluorescent thin layer support; succinylpurines were detected by their UV absorption plus their reaction with naphtoresorcinol due to a ribose moiety in their molecule along with other urinary sugars. Validation and clinical implementation for this novel methodological approach was done with the aid of the grant IGA1831-2 (34, Grant report ADSL, evaluated as category "A", 35). The author's work on the diagnosis of purine and pyrimidine diseases continued during his clinical fellowship at Mayo Clinic (1994 – 1996) in the Laboratory of Metabolic Diseases. After initial method validation the combined TLC screening method was introduced as a diagnostic screening procedure at Mayo. The clinical efficacy of this technique was subsequently proven by diagnosing the first case of adenylosuccinate lyase deficiency outside Europe (36). Of note is that despite the advent of tandem mass spectrometry-based techniques in recent years this technique remained on the panel of clinical tests until now (37). The newly diagnosed case of ADSL deficiency was interesting from a number of views. Clinically, the dominant feature was profound hypotonia but psychomotor retardation was completely absent. Laboratory studies revealed excretion of both succinylnucleosides within an expected range. However, the enzyme activity was not completely absent but decreased to about 40 % of activity of normal control fibroblasts. Substantial effort was devoted to refine an available method for the measurement of enzyme activity of ADSL because of significant lability of this tetrameric enzyme. The development of a technique of enzyme extraction to a glycerol-containing medium improved enzyme activity recovery from cell lysates. To include an internal control, measurement of the true ADSL activity was accomplished both as substrate elimination plus product formation (see the fulltext for details). The comprehensive approach to the diagnosis of purine and pyrimidine diseases based on HPLC was published (38). In this

paper the authors highlighted several methodological improvements over techniques used: solubility and recovery issues for several purine ring-containing diagnostic compounds, improved detection of 2,8 dihydroxyadenine, efficient separation of succinylpurines, the use of structurally and chemically similar internal standard 3-methyluridine, updated reference ranges for the most frequent diagnostic purine and pyrimidine compounds occurring in urine and clarification of nature of major interferences from common drugs used in the clinic.

Transition to pharmacogenetics

As discussed earlier in the Chapter A, metabolic diseases can be clearly considered model diseases for a number of drug- and patient-related adverse reactions. Rapid development of new pyrimidine ring-containing drugs during 90th aimed at the use as antivirotics against hepatitis C revealed severe mitochondrial-related liver toxicity (39). These events occurred before the advent of knowledge on "apoptosis", a phenomenon that was subsequently shown to be, *at least in part,* mitochondria-related through the release of the "death effector" molecules such as cytochrome c. Clearly, this was an unexpected finding that linked together processes thought to be functionally independent – the metabolic processes of electron transport and oxidative phosphorylation with the cellular events of cell senescence and death. These findings also interrelated several fundamental cellular and biochemical processes and concepts: chemical individuality, cell death and drug/receptor interactions (for discussion and author's input see also reference 40). An author's contribution to the discussion on 5-fluorouracil toxicity published in 1998 (41) attempted to clarify several misconcepts and increase awareness of clinical oncologists to the inborn error of metabolism-based adverse drug reaction to 5-fluorouracil.

Taken together, it was this theoretical basis together with his clinical and laboratory experience that led the author to the definition of the three specific pharmacogenetic and biomarker projects on the predictive aspects of patient response to the medication used in current anticancer therapy setting:

1) *Biomarkers in monitoring methotrexate pharmacodynamics*
2) *Thiopurine methyltransferase and response to 6-thiopurine drugs*
3) *Pharmacogenetic implications of fluoropyrimidine chemotherapy.*

In the following paragraphs, these projects are presented in the chronological order reflecting their completeness as of October 2005 and each of them will be discussed further in more details.

Project 1: Biomarkers in monitoring methotrexate pharmacodynamics

Methotrexate has been in clinical use since its introduction by Farber in 1948 (42) and remains the key component of the antileukemic therapy of childhood ALL. Plasma homocysteine was found to be elevated in children with ALL treated with methotrexate (43). Even though several attempts have been undertaken to clarify the role of homocysteine during methotrexate chemotherapy no systematic clinical and/or pilot study has been performed yet. A hypothesis *that "methotrexate is a pharmacodynamic biomarker of high-dose methotrexate chemotherapy"* has been studied by a group of investigators led by DV and most of the results already published (44, Grant report NC7104-3 (evaluated as "category A") and refs. 17, 21). To summarize briefly, the results obtained imply that i) plasma homocysteine behaves as a dose-dependent biomarker of methotrexate administered and ii) that the magnitude of elevation of plasma homocysteine in response to high-dose methotrexate treatment is directly related to the plasma pretreatment level of folate. These studies thus contributed to establishing a link between a preexisting condition – plasma folate concentration, and the pharmacological drug effect – antifolate effect of methotrexate, through an endogenous metabolic molecule (a biomarker) – homocysteine. The potential clinical implication of these observations lies in the fact that overtly high levels of plasma folate may, at least to some extent, neutralize antimetabolic effect of high-dose methotrexate.

Project 2: Thiopurine methyltransferase and response to 6-thiopurine drugs

Methylation of an exogenous compound, pyridine, was first described by His in 1884 (45). Three distinct methylation pathways specific for S-, O-, and N- methylation were found in human tissues. Thiopurine-containing drugs have been in clinical use as anticancer agents for decades since their discovery by subsequent Nobelists Elion and Hitchings in 1951 (46) and, similarly to methotrexate, remain the mainstay of antileukemic chemotherapy. Discovery by Weinshilboum led to understanding that a specific enzyme (thiopurine methyltransferase, E.C.2.1.1.67) is responsible for detoxication of 6-thiopurine containing anticancer drugs. Individuals with decreased activity of this enzyme were shown to be susceptible for increased hematologic toxicity, whereas those with increased activity may fail to respond optimally and may have higher relapse rate (47). The gene locus encoding TPMT was found at 6.p22.3 (48) and first molecular genetic studies revealed a single nucleotide transversion G>C at the position 238 (49). Recent most frequent mutation that are subject of clinical testing are given in OMIM (50). A first indication of genetic heterogeneity in TPMT was discovered in 1980 (51). The enzyme assay used originally for measurement of enzyme activity was that of Weinshilboum utilizing radiolabelled S-adenosylmethionine as a cofactor of the TPMT-catalyzed reaction and 6-

mercaptopurine as its substrate. In principle it was a single endpoint assay measuring the amount of the reaction product over the specified period of time (1 hour) when the reaction was stopped. The surrogate cell lysate (typically red blood cell lysate) was prepared from washed erythrocytes and the reaction proceeded in a phosphate buffer for 60 min at 37 °C. The reaction product, C^{14}-labelled 6-methylmercaptopurine, was then extracted to the organic phase consisting of toluene and isoamylalcohol and radioactivity of the extract was considered a measure of the rate of its formation through a TPMT-catalyzed reaction. A newer but still a single endpoint assay has been published and introduced in the clinical laboratory practice (52) based on a more sensitive and specific detection of the reaction product 6-MMP using tandem mass spectrometry. However, none of the TPMT assays used to date addressed two important issues of enzyme activity measurement i) the rate of the product formation and ii) an adequate reaction blanking.

Employing original reaction conditions the author devised and set up "a rate measurement" that enabled to monitor the reaction course (see the TPMT protocol in "Supplemental Data") when measuring patient specimens. Further experiments came up with several important conclusions. The assay as published has not been blanked properly, since the reaction product is apparently formed in the "reaction blank" mixture (full reaction mixture with no cell lysate added) but not in the "sample blank mixture" (full reaction mixture with no substrate, 6-MP, added). Next, it has not been possible to set up the reaction mixture to reach the zero-order kinetics since the reaction velocity always increased with increased amount of the methylating cofactor, S-adenosylmethionine. Third, the reaction is linear only within a relatively narrow range of hematocrit meaning that controlling this preanalytical factor contributes significantly to the activity obtained. Through collaboration, these results – specifically the problem of the reagent blank – were subsequently confirmed (Dr. John O'Brien, Biochemical Genetics Laboratory, Mayo Clinic, Rochester MN, USA); currently, a protocol is being devised to eliminate the influence of such factors. However, it follows that the results of TPMT methods published to date in the literature did not take into consideration the reagent blank problem and therefore their numerical values include the reagent blank bias accounting for up to 25 % of TPMT activity. The nature of this phenomenon is unclear but most probably lies in the strong alkylating propensity of S-adenosylmethionine. To summarize, these phenomena may be of substantial significance and will be the subject of further collaboration and joint and consensual publications once unequivocally proven by independent laboratories.

An integrated clinical approach to the diagnosis of TPMT-dependent susceptibility to adverse reaction to 6-mercaptopurine drugs was designed by the author and introduced into the clinical practice at the Department of Pediatric Oncology, Children's Hospital, Brno. It delineates a genotype/phenotype correlation in an each individual patient scheduled for 6-MP containing (see Supplemental Data II). As soon as the diagnosis of ALL is established a pre-therapeutic specimen is tested for i) the red blood cell activity of TPMT using the technique

described above and ii) for the occurrence of TPMT mutation supposed to be responsible for decreased TPMT activity. An individualized report on the genotype/pharmacophenotype correlation is established and an interpretive result is released to the attending physician.

Project 3) Practical pharmacogenetic implications of fluoropyrimidine chemotherapy

A fluorinated pyrimidine analogue 5-fluorouracil has been extensively used in chemotherapy of solid tumors since its synthesis by Duschinsky in 1957 (53). Its pharmacological effect in neoplastic tissues results from its activation to 5-FdUMP, which is a suicide inhibitor of thymidylate synthase, and from interference with RNA following incorporation of fluorinated nucleotide into its structure. As mentioned above, naturally occurring pyrimidine bases are metabolized via a three enzyme degradation pathway consisting of dihydropyrimidine dehydrogenase (DHPD, E.C.1.3.1.2), dihydropyrimidinase (DHP, E.C. EC 3.5.2.2) and ureidopropionase (E.C.3.5.1.6) resulting in final formation of β-alanine and β-aminoisobutyric acid from uracil and thymine, respectively. Fluorinated pyrimidines are degraded by the same set of enzymes. The first familial case of severe 5-fluorouracil toxicity was described in 1985 (54) and more comprehensive pharmacological description subsequently published by Diasio (55) who suggested that polymorphism in *DPYD* gene encoding DHPD may lead to impairment in drug degradation. Interestingly, an autosomal recessive phenotype of the respective metabolic disease – complete deficiency of DHPD – was described later by Berger and coworkers (56) and called "hereditary thymine-uraciluria"; however, a large variation of clinical phenotypes has been associated with this condition. Since DHPD deficiency is inherited as an autosomal recessive trait, severe 5-fluorouracil toxicity attributable to DHPD deficiency may reach 1-3 % of unselected group of cancer patients according to several reports (57). Furthermore, a significant finding concerning the genetic basis of fluoropyrimidine toxicity was published recently, where 5-fluorouracil toxicity was also associated with the second enzyme in pyrimidine degradation pathway, dihydropyriminase (58). Collectively, alterations in physiological chemistry of both dihydropyrimidine dehydrogenase (DHPD) and dihydropyrimidinase (DHP) may account for substantial level of genetically determined fluoropyrimidine toxicity.

Despite the advent of new anticancer drugs during the last decade, 5-fluorouracil and its newer pharmacological formulas such as capecitabine remain – in striking similarity to methotrexate and 6-mercaptopurine – the fundamental components of chemotherapy of solid tumors. Therefore, evaluation of genetic susceptibility to fluoropyrimidine toxicity in the clinical oncology practice gains practical significance. Similarly to TPMT, an integrated approach to the clinical testing of fluoropyrimidine susceptibility was proposed and is being a subject of clinical evaluation at the Masaryk Memorial Cancer Institute, Brno. In view of the

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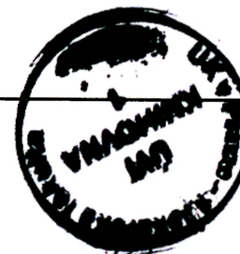
recent knowledge that an alteration in function of two Phase I enzymes, DHPD and DHP – i.e. not just DHPD as was previously thought – can be the cause of fluoropyrimidine toxicity, a three-level protocol was proposed for the patients scheduled for therapy. It has been suggested that heterozygotes for DHPD and DHP may excrete larger amount of uracil, thymine and their corresponding dihydroderivatives, dihydrouracil, dihydrothymine and 5-hydroxymethyluracil than controls. Phenotypic assessment encompasses two levels of testing. First, prior to therapy, a random urine specimen is taken and analyzed for the excretion of natural pyrimidines (59) using modified stable isotope dilution GC/MS (see Appendix IV for the brief protocol). Second, at the start of fluoropyrimidine therapy blood is taken for evaluation of plasma pharmacokinetic profile of 5-fluorouracil elimination and 5,6 -fluorodihydrouracil appearance using HPLC (see Appendix IV for the brief protocol). The third level of testing consists of sequencing the *DPYD* gene encoding DHPD. At present, the data gathered through application of this approach are released to clinicians involved in this comprehensive project for pre-clinical interpretation.



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CHAPTER C

Author's interpretation of current knowledge on pharmacogenetics, its applicability in contemporary clinical pharmacological practice and setting of future prospects

Classification of pharmacogenetic conditions

To date, "pharmacogenetic" disorders have been categorised according to a number of criterions; probably the most comprehensive classification was proposed by Weber (60). However, a majority of similar classifications is usually based on the principal pharmacological mechanism – typically whether the affected point lies in the pharmacokinetic and/or pharmacodynamic pathway – making these classifications difficult to understand for clinicians. Therefore, to make an alternative view of "pharmacogenetic" disorders in the context of Mendelian inheritance and chemical individuality we may consider them in several subgroups according to their clinical phenotype (Figure).

To accomplish this, three phenotypic differences may be defined as proposed in the Table 1. The first phenotypic group is a Garrodian phenotype of a metabolic disease with the ocurrence of both clinical and biochemical phenotype in an affected individual. The second phenotypic groups is a compound phenotypic group and is shown here for the purpose of completion, since individuals with the classical phenotype already expressed may not live long enough to be treated for an acquired condition, such as cancer, that would be subsequently treated with drugs of pharmacogenetic importance. The third group encompasses truly pharmacogenetic phenotype in which a morbid phenotype has not manifested, does not exist and/or it is not known. This group can be further separated into disease entities based on various forms of heterozygosity, such as SNPs, compound heterozygotes and into those with truly unknown and/or nonexisting morbid phenotype even in homozygous state such as TPMT. Of interest is the recent discovery of the TATA box mutation showing that disturbance in the regulatory element leads to a common condition, *Gilbert syndrome,* with important pharmacogenetic implications. Applying these categories back to the pharmacologic classification it is reasonable to state that a vast majority of "pharmacogenetic disorders" important in current medical practice has their genetic basis and biochemical background in the alteration of the activity of enzymes important in pharmacokinetic Phases I and/or II. Less frequently, this situation applies to the pharmacodynamic arm of the human drug response although several practical examples can be delivered. The Table 2 describes an integrated pharmacogenetic and/or biomarker approach for pharmacologic management of important anticancer drugs. Here, the author suggests a multi-level testing to assess an individual patient

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response including a proposal for the test hierarchy ("reference" tests) depending on the quality of information delivered by the respective testing. Of note is that in some examples, a pharmacophenotype testing is considered being of higher order just because of the relevance of information provided. On the other hand, some examples, such as mutations in gene regulatory elements (such as TATA box in UGT1A1) provide an accurate application for the nucleic acid based technologies.

Conclusions and Future prospects

From the practical standpoint, pharmacogenetics achieves its clinical appreciation only if it can provide a clinician with two fundamental informations that current laboratory medicine is not able to deliver. First, an individualized predictive information on a potential individual drug toxicity and second, on the potential dose adjustment. Although vast amount of knowledge has been already gathered in pharmacogenetics a long way remains before fragmented research in particular and fragmented disciplines will be put together into a confluential interpretive information presentable and understandable to clinicians who would then be able to use – and, of course, not to use – such a piece of information for making specific, individualized therapeutic decisions. To generate such an information, reliable methodological inputs are necessary at the levels of genotype AND pharmacophenotype given described complexity of pharmacogenetic phenomenons. It becomes increasingly clear that probably no single methodological approach – including the "array-based" technologies – will be robust enough to deliver a clinically accurate predictive information (for discussion see the author's paper 17, and reference therein). Until these frontiers are reached much work remains in understanding pharmacogenetics in its broader and functional concepts that appear inevitable in the light of the recent discoveries in genomics, proteomics and metabolomics.

In summary, perceiving pharmacogenetics a multifaceted and multilevel biological issue led the author to raise and clinically evaluate the concept of a multilevel testing summarised in this thesis based on assessment of individual susceptibility using a correlative genotype/pharmacophenotype evaluation. Results already published, presented and discussed in this thesis aspire to bring contribution to this exciting yet mostly underexplored interdisciplinary field between clinical medicine, basic and applied biochemistry, genetics and pharmacology.

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Pharmacogenetic perspective

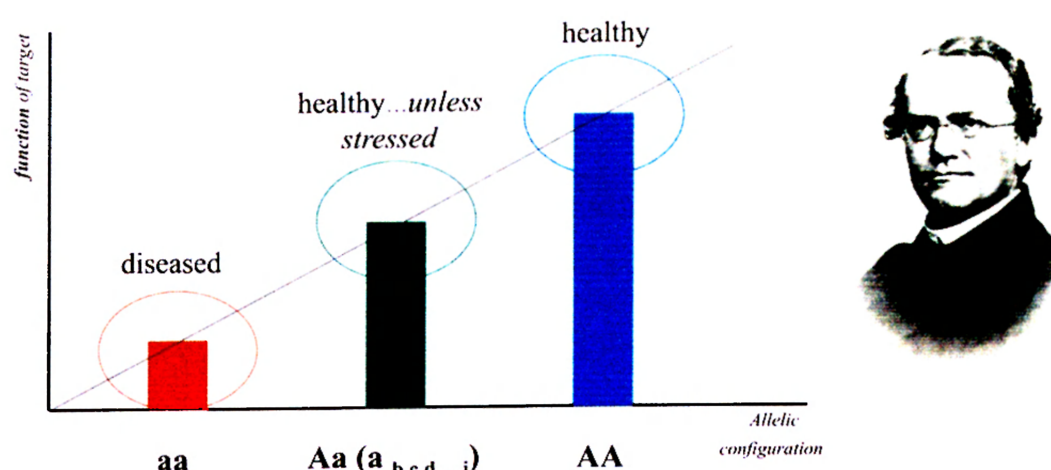

Figure. Problems in contemporary pharmacogenetics: relations between allelic configuration of an individual and function of a target.

Assessing pharmacogenetics background (x-axis in the figure) may identify those patients who are a priori at lifelong risk of side effects from administration of a particular drug; mutations/polymorphisms of this kind occur in somatic, non-tumor cells and are inherited as germline mutations, or may emerge as new mutations. Typically, those individuals are healthy heterozygotes for a monogenic disease, where a "pharmacogenetic" disease appears after overloading the otherwise metabolically sufficient pathway by a drug metabolized though that pathway. These side effects are caused by decreased function of a "target" (see y-axis in the figure, which is mostly enzymatic activity), which, – in contemporary pharmacogenetics – is/are most frequently an enzyme(s) participating in phase I and II elimination reactions. The occurrence of a mutation and/or polymorphisms may not translate linearly into biochemical activity of the target structure (enzyme, substrate carrier etc.), since an enzyme may be expressed preferentially in a specific organ (liver, etc) or its function is dominant within a specific organ (for example: liver dihydropyrimidine dehydrogenase is responsible for elimination of a major portion of 5-fluoropyrimidines). This complexity makes the use of solely genetic methods insufficient and necessitates their complementation with "pharmacophenotyping". The phenomenon is quantitative, since it is not only quality (i.e. a metabolic congener) but also quantity of that congener(s) and rate(s) of its appearance/disappearance that determine the degree of toxicity observed clinically. Therefore, these quantitative aspect necessitates complementation of genetic tools such as analysis of endogenous biomarkers and direct determination of metabolites for accurate interpretation.

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Table 1. Examples of known disorders whose pharmacogenetics is important in anticancer chemotherapy (irrelevant fields are left blank)

Disorder (target affected)	**Classical phenotype of metabolic disease (morbid + biochemical)**			**Pharmacogenetic phenotype in already diseased individual**			**Pharmacogenetic phenotype only, i.e. in hitherto healthy persons**
	Age at presentation	**Morbid phenotype**	**Biochemical phenotype**	**Age at presentation**	**Morbid phenotype**	**Pharmacogenetic phenotype**	
Lesch-Nyhan syndrome (HGPRT deficiency)	Boys, early childhood	CNS derangement, automutilation	hyperuricaemia	As in already affected individuals	As in already affected indivduals	Impaired activation of purine containing drugs delivered to Lesch Nyhan patients (6-mercaptopurine, allopurinol)	Not known (but possible with delayed activation of purine containing drugs)
Uridine glucuronosyl transferase deficiency (mutations in ORF, i.e. Crigler Najjar syndromes I and II)	Early childhood	Jaundice	hyperbilirubinemia	As in already affected individuals	As in already affected individuals	If treated: impaired glucuronidation of a number of xenochemicals	Not known (and probably not existing)
Uridine glucuronosyltransferase deficiency (mutation in regulatory element, i.e. Gilbert syndrome)	Childhood, young adulthood	Mild jaundice, variable and sometimes inapparent	Fluctuating, mild unconjugated hyperbilirubinemia	As in already affected individuals	As in already affected individuals	impaired glucuronidation of drugs metabolized by glucuronidation, SN-38 glucuronide (active metabolite on irinotecan)	Not yet described, those individuals with delayed glucuronidation without previous hyperbilirubinemia may be reclassified as having Gilbert syndrome
Thymidylate synthase		Not known (Mendelian homozygosity for TS deficiency would lead probably to lethal phenotype)			Not known		Impaired response to fluoropyrimidine therapy due to decreased affinity of TS to 5-FdUMP
Thiopurine methyltransferase deficiency		Not known			Not known	Impaired detoxication of 6-thiopurine drugs	Significantly impaired detoxication of 6-thiopurine drugs
Thiopurine methyltransferase superactivity		Not known			Not known	Hypothetical	Increased elimination of 6-thiopurine drugs leading to decreased efficacy
Dihydropyrimidine dehydrogenase deficiency	During childhood	Variable: from nearly lack of phenotype through various degree of CNS damage to acute life-threatening episodes	Increased excretion of uracil, thymine and 5-hydroxymethyluracil	As in already affected individuals	As in already affected individuals	If treated: grossly impaired elimination of fluoropyrimidines in phenotypically affected individuals	Augmented toxicity of fluoropyrimidines due to decreased activity of liver DHPD in heterozygotes
Dihydropyrimidinase deficiency	During childhood	Mostly CNS involvement	Increased excretion of dihydrouracil and dihydrothymine	As in already affected individuals	As in already affected individuals	If treated: grossly impaired elimination of fluoropyrimidines in phenotypically affected individuals	Augmented toxicity of fluoropyrimidines due to decreased activity of liver DHPD in heterozygotes

Table 2. Using pharmacogenetic and/or biomarker approach for pharmacologic management of important anticancer drugs

DRUG	CONDITION	diagnostic approach		genotype/phenotype correlation and suggested pharmacological action			note:
		genotyping	pharmacophenotyping /biomarkers	*yes:* *mutation(s) found and pathological pharmacophenotype*	*no:* *mutation(s) found and normal pharmacophenotype*	*no:* *mutation(s) not found (unless sequenced) and abnormal pharmacophenotype*	
Thiopurines	TPMT deficiency	screening for selected mutations	TPMT activity (measured with reagent blank)	*dose reduction and/or drug elimination*	*drug can be administered but „wait-and-watch" approach taken*	*drug elimination*	suggested „reference" test: TPMT activity
Fluopyrimidines	DHPD and DHP deficiencies	screening for selected mutations and/or sequencing	i) urinary excretion of natural pyrimidines, ii) plasma 5-DHFU appearance after first 5-FU administration	*dose reduction and/or drug elimination*	*drug can be administered but „wait-and-watch" approach taken*	*immediate drug elimination*	suggested „reference" test: plasma 5-DHFU
Topoisomerase inhibitors (irinotecan)	prodrug activation to SN-38 through liver carboxyesterase	none known	plasma SN-38 appearance after first irinotecan administration	*not known*			suggested „reference" test: plasma SN-38
	SN-38 elimination through UGTA1	i) ORF mutations: Crigler-Najjar syndrome	not necessary	*drug not be used*			suggested „reference" test: mutation analysis
		ii) TATAA box mutations: Gilbert syndrome	not necessary	*drug dose reduced („wait and watch") or drug not be used*			suggested „reference" test: mutation analysis
Taxanes	CYP3A4 and 2A9	screening for selected mutations	plasma 2-hydroxypaclitaxel (active) and 6-alfa hydroxypaclitaxel (inactive but toxic) after first administration	*dose reduction and/or drug elimination*	*drug can be administered but „wait-and-watch" approach taken*	*drug not be used, no active metabolite formed*	suggested „reference" test: plasma hydroxytaxanes
Methotrexate	counteraction of endogenous folates,	none known	plasma pretreatment folate and homocysteine	*not applicable due to multiple mechanisms*			decreased amount of antidote leucovorine if high plasma folate and low homocysteine after MTX administration
	CYP dependent drug elimination	CYP not known	plasma 7-hydroxymethotrexate	*not applicable due to unknown CYP genotype*			watch and wait approach depending of 7-hydroxymethotrexate levels

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Supplemental Data I

a) Fulltexts of Published Papers and their Citations (Web of Science, as of October 2005)

- Valik D, Jones JD. Adenylosuccinase deficiency and disorders of carbohydrate metabolism - The integrated screening test. Pediat Res 37 (4): A154-A154 Part 2 APR 1995

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b) Invited Lectures at International Meetings and Grant Report

- TDM Renaissance and Pharmacogenomic Forum III, AACC, Baltimore 2004

- IGA 1831-2/1995, listed as reference 34

- IGA NC7104-3/2004, listed as reference 44

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Clinica Chimica Acta 249 (1996) 197–200

Letter to the Editor

Adenylosuccinate lyase deficiency and disorders of saccharide metabolism; experience with combined screening test[1]

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Dear Editor,

Adenylosuccinate lyase (ASL) deficiency (E.C. 4.3.2.2) was described in 1984 in children suffering from mental retardation and seizures [1]. The ASL gene has been located to the chromosome 22q13.1 → 13.2 [2]; DNA analysis revealed the missense mutation resulting in Ser^{413} → Pro substitution leading to a structural instability of the gene product [3]. Several methods have been described for "selective" screening of ASL deficiency; however, thin-layer chromatography of urinary monosaccharides appears to be the most suitable for screening of de novo purine synthesis defects [4].

Our experience with the new three-step TLC method that combines screening of ASL deficiency with disorders of saccharide metabolism [5] is discussed. In brief the high-resolution TLC plate was spotted with 7 μl of native urine for creatinine concentration between 10 and 70 mg/dl;

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12 μl were applied if creatinine was less than 10 mg/dl and 4 μl if higher than 70 mg/dl. The plate was developed in butanol/ethylacetate/isopropanol/glacial acetic acid/water (7:20:12:7:5) to 4 cm distance from the start, dried and developed again exactly to the edge. The plate was observed under UV light, 254 nm, and the dark bands were marked with a soft pencil. Finally, the plate was sprayed with a naphthoresorcinol reagent, heated and bands of mono- and disaccharides were located and the presence of two dark bands subsequently reacting blue with R_f 0.35 (succinyladenosine) and 0.29 (succinylaminoimidazole-carboxamide, riboside, SAICAr) was assessed. The succinyladenosine standard was prepared as described in [1]. The method was evaluated using four specimens from patients with ASL deficiency (3 urine specimens from ASL-deficient patients were provided by Dr. Jakub Krijt, Center from Hereditary Metab. Disorders, Prague, The Czech Republic, 1 by Dr. Georges van den Berghe, International Inst. of Cellular and Molecular Pathology, Belgium; 3 shown in Fig. 1), which consistently yielded two dark bands with R_f 0.35 and 0.29 (step I) that turned blue after treatment with naphthoresorcinol (step II) — the band with R_f 0.35 comigrated with succinyladenosine (Fig. 1) and the band with R_f 0.29 diazotisable (SAICAr) when rechromatographed and sprayed with Pauly reagent (step III) [6]. All four specimens contained succinyladenosine and SAICAr by HPLC with diode-array detection. The spectral characteristics of the R_f 0.29 band was consistent with SAICAr and that of R_f 0.35 with succinyladenosine.

Our technique extends the original concept [1] by integrating screening for succinylnucleosides with TLC of urinary mono- and disaccharides. The method also indicates the presence of oligosaccharides in a specimen as naphthoresorcinol-positive bands between the zone of lactose and the origin. We believe that this approach is beneficial since TLC of urinary saccharides is frequently performed and evaluations of succinylnucleosides and even oligosaccharides are not commonly available or not requested. The lack of specificity of clinical presentation of ASL deficiency and disorders of oligosaccharide metabolism further underscores the need for integrated screening.

Succinylnucleosides are not normally detected in urine. In positive specimens they typically occur isolated on the TLC plate. Common drugs (cephalosporins, aminoglycosides, paracetamol, ibuprofen) or their metabolites appear on TLC as dense UV absorbing zones; however, they can usually be discriminated by their distinctive reactivity with naphthoresorcinol. The distortion of saccharide bands may occur due to matrix effects, salts, etc. requiring dilution and rechromatography to get exact band match; this is notable for the fructose-glucose area where other compounds reactive with naphthoresorcinol can occur. Results of very diluted

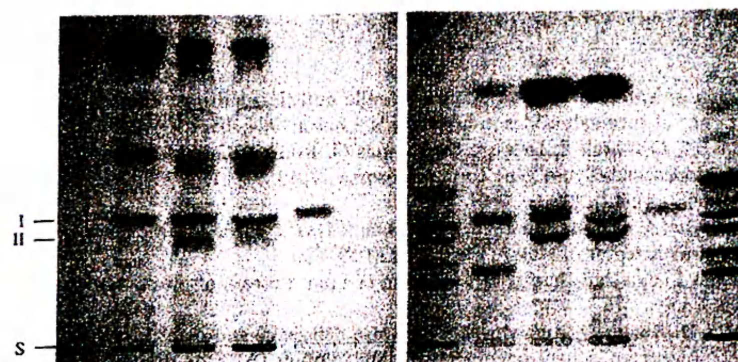

Fig. 1. (a) left panel: The HPTLC plate (Kieselgel 60, cat. No. 5628/5 with fluorescence indicator, sample applied quantitatively as a narrow streak using a Hamilton syringe) illuminated by the UV light 254 nm. Dark bands correspond to compounds absorbing at 254 nm, therefore quenching background fluorescence. Lanes 1–6 from left: standard of mono- and disaccharides giving no signal under UV; lanes 2, 3, 4: 7 μl of urine from ASL-deficient patients; lane 5; succinyladenosine standard (1 nmol). Vertical axis: I, succinyladenosine; II, SAICAr (diazotisable in step III); S, start. (b) right panel: The identical plate after spraying with naphthoresorcinol/sulfuric acid reagent and heating. Lanes 1–6 from left: standard of mono and disaccharides, 45 mg/dl each, 2 μl applied per lane; order from start — inulin, raffinose, lactose, maltose, sucrose, galactose, fructose, glucose, xylose, rhamnose. lanes 2,3,4: patients as in the left panel; lane 5: succinyladenosine standard. Vertical axis: I, succinyladenosine; II, SAICAr; S, start.

specimens with creatinine < 10 mg/dl and those with severe drug-related interferences should be interpreted with caution and retested. In routine operation the method proved to be reliable and robust and unequivocally identified four specimens from patients with ASL deficiency available to us. So far, we have tested approx. 3500 routine specimens at J.G. Mendel Children's Hospital, Brno, The Czech Republic (600) and Mayo Clinic, Rochester, MN, USA (2900). In this skewed group, for which we had no clinical information available, we identified 5 specimens with high galactose (3 within 100–200 mg/dl, 2 > 1000 mg/dl — classical galactosemia; normal < 30 mg/dl), 5 cases of pathological oligosacchariduria (urinary oligosaccharides not requested) and 1 hereditary fructose intolerance. We have not yet identified a new case of adenylosuccinate lyase deficiency.

Acknowledgement

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First U.S. Case of Adenylosuccinate Lyase Deficiency With Severe Hypotonia

Dalibor Valik MD*†, Philip T. Miner, MD‡§, and James D. Jones, PhD†

Adenylosuccinate lyase (ASL) deficiency is a defect in purine de novo synthesis pathway. The disease has variable clinical presentation involving psychomotor retardation, seizures, hypotonia, and autism. The presence of succinyladenosine and succinylamino-imidazole carboxamide riboside (SAICA riboside) in body fluids characterizes the biochemical phenotype. All cases of ASL deficiency described to date have been diagnosed in Europe. Using a high-resolution thin-layer chromatography (TLC) technique combining screening for ASL deficiency and disorders of saccharide metabolism, we found the first case of this disease in the US. The patient presented with delayed motor development and profound hypotonia. The family history and routine laboratory tests were negative. Screening for metabolic disorders detected the presence of succinyladenosine and SAICA riboside in urine. The activity of ASL in the patient's skin fibroblasts was 43% of controls (patient, mean = 1.20 nmol/min/mg of protein, s = 0.21, n = 3; controls, mean = 2.78 nmol/min/mg of protein, s = 0.61, n = 7). In a 15-month-old girl with profound hypotonia, we established the diagnosis of ASL deficiency by demonstrating succinyladenosine and SAICA riboside in urine and decreased residual activity of ASL in skin fibroblasts. **© 1997 by Elsevier Science Inc. All rights reserved.**

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Introduction

Adenylosuccinate lyase (ASL) deficiency (E.C. 4.3.2.2) was described in 1984 in children with mental retardation and seizures [1,2]. The deficiency of this purine de novo synthesis enzyme results in intracellular accumulation of SAICAr (succinylamino-imidazole carboxamide ribotide) and S-AMP (succinyladenosine monophosphate). Their dephosphorylated counterparts, SAICA riboside and succinyladenosine, also termed succinylnucleosides or succinylpurines, occur in urine and cerebrospinal fluid (CSF) of patients and form the biochemical phenotype of the disorder. The inheritance of the disease is autosomal recessive, and the ASL gene has been located to the chromosome 22q13.1 → q13.2 [3,4]. The incidence of the disorder and the relation of genotype, which appears to be heterogeneous, to biochemical and clinical phenotypes are not known [5].

Several methods have been suggested to screen for the disease [6]. However, screening is not routinely performed even in high-risk populations of children with psychomotor retardation, hypotonia, and seizures. We recently introduced a three-step high-resolution thin-layer chromatography (TLC) method that combines screening for ASL deficiency with disorders of saccharide metabolism [7]. With this method, we have tested more than 4,600 specimens in urine in a period of 1 year. Clinical information is generally not provided with these specimens. A first U.S. case of ASL presenting with uncommonly mild clinical characteristics was recently identified and is the subject of our study.

Case Report

A 15-month-old girl was referred for evaluation of developmental delay. The parents reported that she could not achieve a sitting position on her own but, if so placed, was able to maintain support. She rolled over at 5 months of age but did not begin rolling over on her own volition until 7 to 8 months of age. She began crawling between the ages of 10 and 11 months. At times, the parents have noticed that one eye or the other deviates, especially when she is tired. They believe that she can see

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and hear. She eats with no difficulty and sleeps well. The parents have not observed any staring episodes with unresponsiveness, tonic-clonic convulsions, or any unusual movements of her extremities. She does not cry a lot and is not irritable.

She is the 4,149-g product born to a gravida 2 woman after a normal pregnancy and uncomplicated labor. Her Apgar scores were 8 and 9 at 1 and 5 min, respectively. Clinical evaluation is negative for serious illnesses, injuries or known allergies. The family history is noncontributory and specifically is negative for neurologic diseases. Her parents are white and are nonconsanguinous.

At examination, her weight was 10 kg (10th percentile); head circumference was 47 cm (67th percentile). The anterior fontanel measured 2 × 2 cm and was flat. Except for a light pink capillary hemangioma on the right side of her torso, there were no skin lesions, heart murmurs, bruits, or organomegaly. No unusual movements were observed during the examination, in particular, there were fasciculations of the tongue or distal extremities.

She is alert and cognitively responsive. She moves all four extremities equally well, but resistance to passive and active movements is markedly decreased. Reflexes are very brisk bilaterally with downgoing plantar responses. Cerebellar functions are normal. Eye contact is excellent, and she follows the examiner with her eyes in all visual fields. Fundi are normal. Cranial nerve functions are intact. Her gag reflex is slightly increased. She can place support on her legs in a standing position, but hyperextends at the knees. Her language and social interactions are appropriate for age.

The EEG and electromyogram (EMG) are normal. Magnetic resonance imaging (MRI) of the brain shows a small area of increased T_2 signal in the centrum semiovale on the right. It also demonstrates some patchy areas of increased T_2 signal along the posterior aspect of the trigones in the periventricular white matter that extend, especially on the right, to the ependymal surface. The hemogram, liver function tests, plasma lactate and pyruvate, ceruloplasmin, thyroid-stimulating hormone (TSH), chromosome analysis, and fragile X analysis are normal. A quadriceps muscle biopsy examined by light and electron microscopy is normal. The screening for metabolic disorders demonstrates the presence of SAICA riboside and succinyladenosine in urine.

Methods

Inborn errors of metabolism screening included chemical testing (reducing substances, ketones, blood, pH), high-resolution TLC [7] for mono-, di-, and oligosaccharides and succinylnucleosides, glycosaminoglycans, and high-performance liquid chromatography (HPLC) for amino acids in plasma and urine.

Quantitation of succinylnucleosides in urine was performed by reverse-phase HPLC. Chemicals were purchased from Sigma, unless otherwise stated. Samples were prepared by pipetting 50 µl urine and 50 µl water to the Ultrafree *10,000* NMWL (Millipore, No. UFC3LGCNB) membrane filter; 25 µl internal standard (400 µmol/L 3-methyluridine) was added, the mixture was ultrafiltered, and 20 µl was analyzed. SAICA riboside and succinyl-AMP (No. A 4778) were used for succinylnucleoside quantitation [1,8].

Skin fibroblasts were cultured in minimum essential medium (MEM) with 5 mmol/L glutamine, 10% fetal calf serum, and antibiotics. For measurement of ASL activity, cells were trypsinized and washed with phosphate-buffered saline (PBS) and 200 µl 40 mmol/L Tris HCl, pH = 7.4, added to the cell pellet. The cells were frozen at −80°C for 5 min, thawed by addition of 200 µl buffer A [9] and centrifuged. Incubation medium was prepared from 500 µl 40 mmol/L Tris HCl, pH = 7.4, 150 ml 1 mol/L KCl, 150 µl 1 mmol/L dithiothreitol, and 200 µl 2 mmol/L EDTA. Incubation mixture was prepared from 100 µl of the medium, 100 µl 1 mmol/L S-AMP, 50 µl 1 mmol/L α,β-methyleneadenosine diphosphate (MADP), and 200 µl cell extract, with incubation for 60 min at 25°C. Aliquots (50 µl) were taken at time 0, 15, 30, 45, and 60 min and mixed with 100 µl 10% perchloric acid (PCA) (vol/vol) and centrifuged; 80 µl was analyzed by identical HPLC protocol to quantitate AMP (product) and S-AMP (substrate) of the ASL-catalyzed reaction.

Results

Identification of Succinylnucleosides in a Patient Specimen. The urine from the index case arrived at the laboratory with no clinical information for the screening of metabolic disorders. Reducing substances, ketones, amino acids, and other chemical tests were negative; creatinine level was 44 mg/dl. On high-resolution TLC, two ultraviolet-absorbing bands with R_f = 0.35 and 0.29, which normally are not seen, were observed. The bands formed blue color complexes after subsequent spraying with napthoresorcinol. Rechromatography followed by spraying with Pauly reagent [7,8] identified the band with R_f = 0.29 as SAICA riboside. The native urine specimens were chromatographed next, along with known cases of ASL deficiency (Fig 1). The presence of succinyladenosine and SAICA riboside was demonstrated in the specimen by comparison with succinyladenosine standard and four known cases. A second specimen of urine collected 2 weeks later which had a creatinine level of 7 mg/dl yielded identical findings.

Succinylnucleoside Conformation and Quantitation by HPLC. HPLC analysis of the filtered urine specimens from a known case of ASL and from the patient are shown in Figure 2; additional confirmation was furnished by preliminary fractionation of urine by TLC (Fig. 2). The TLC plate area from SAICA riboside to succinyladenosine was marked with a soft pencil, silica gel-excised, eluted with water, and analyzed by HPLC. Chromatographic and spectral data were similar to known cases of ASL deficiency and concentrations of SAICA riboside and succinyladeno-

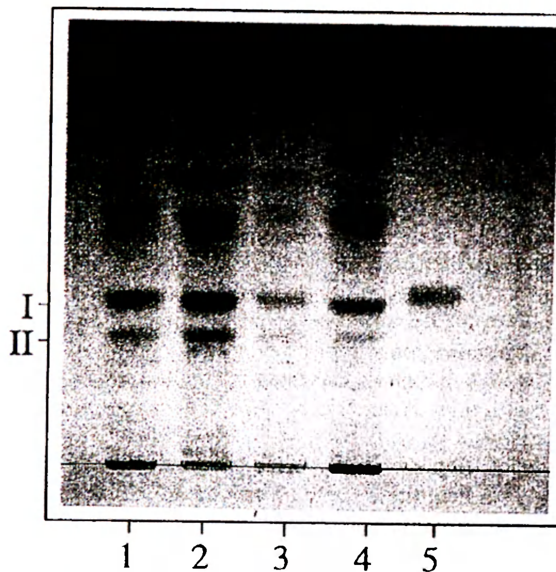

Figure 1. High-resolution thin-layer chromatography of urine of known cases and the index case. Horizontal axis: Lane 1: KC4a, known case 4a (7 µl of urine applied); lane 2, IC1, index case specimen 1 (7 µl applied); lane 3, IC2, index case specimen 2 (12 µl applied); lane 4, KC1, known case 1 (7 µl applied); lane 5, succinyladenosine standard. Vertical axis: I, succinyladenosine; II, SAICA riboside (diazotizable).

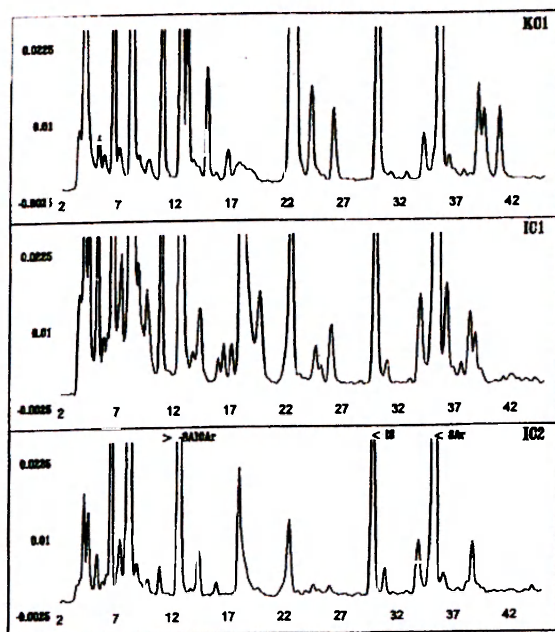

Figure 2. High-performance liquid chromatography of urine of the known case and the index case. Vertical axis: absorbance units full scale at 270 nm. Horizontal axis: Time (min). Instrumentation and conditions: Waters 600E gradient module was connected to WISP 712 and 990 photodiode-array detector. A Supelco LC-18-S "nucleoside" column with Supelguard LC-18-S precolumn thermostatted to 27°C was eluted at 0.8 ml/min with the mixture of 25 mmol/l ammonium acetate (instrument channel A) and acetonitrile.methanol:water, 100:250:450 (B) and water (C) using conditions as follows: Isocratically A50:B3:C47 (%) from 0 to 8 min, linear gradient to A50.B20:C30 at 35 min, linear gradient to A50:B45:C5 at 38 min and isocratically to 45 min, and then equilibrated with the initial mixture for 25 min before next injection. Quantitation was performed from heights for each analyte at 254, 270, and 285 nm against an internal standard (IS) 3-methyluridine using 990 software (Waters). KC1, known case 1; IC1, index case urine specimen 1; IC2, index case urine specimen 2. SAICAriboside, IS, and succinyladenosine (SAr) are indicated on the IC2 chromatogram.

sine was determined (Table 1); the ratio of succinyladenosine to SAICA riboside was 2.5 and 2.6 in the first and the second specimens, respectively [5].

Measurement of ASL activity in skin fibroblasts. A linear increase in AMP and decrease in S-AMP (r_{AMP} = 0.99, r_{SAMP} = 0.98, n = 10) was observed. The slope of both responses was also a function of protein concentration. ASL activity was expressed as average of activity calculated from substrate consumption plus that from product formation.

Control fibroblasts harvested in similar growth phases had activities from 2.35 to 3.74 nmol/min/mg protein (mean = 2.78, s = 0.61, n = 7). The ASL activities in the patient's fibroblasts were 0.89, 1.33 and 1.36 nmol/min/mg of protein (mean = 1.20 nmol/min/mg protein, s = 0.21, n = 3). The cell extracts contained insignificant amounts of hypoxanthine, inosine, and succinyladenosine throughout the incubation. When incubated at 37°C, activity of control was 3.9 to 5.1 nmol/min/mg protein and that of the patient was 1.9-2.1 nmol/min/mg protein (n = 2). A substantial amount of inosine was formed at 30 min when increased consumption of AMP by 5′-nucleotidase

Table 1. Concentrations of SAICA riboside and SAr in urine of known cases and the index patient*

Patient	SAICA riboside (μmol/L)	SAICA riboside (μmol/ mmol cr)	SAr (μmol/L)	SAr (μmol/ mmol cr)
Known case 1	286.0	851.4	1,138.2	3,388.5
Known case 2	230.4	21.73	1,613.4	152.2
Known case 3	5.5	4.8	384.2	334.1
Known case 4a	968.7	377.8	2,136.4	833.4
Known case 4b	120.7	455.1	284.4	1,072.4
Index case 1	1,071.4	275.35	2,729.4	701.6
Index case 2	163.3	263.9	423.8	684.9

*Urine specimens from known cases were sent to the laboratory in a filter paper and extracted with water.

Abbreviations:
SAICA = Succinylamino-imidazole carboxamide
SAr = Succinyladenosine

was indicated, despite the presence of its inhibitor MADP. The reactions AMP → adenosine → inosine influence the linearity of product formation at this temperature.

Discussion

Twenty cases of ASL deficiency had been described by December 1994 (nationality of patients: Belgium, The Netherlands, The Czech Republic, Spain, Italy, Turkey, Morocco; Dr. H. Anne Simmonds, Purine Research Laboratory, London, UK, personal communication; most were diagnosed in Belgium and The Netherlands). In 1986, Roesel et al. [10] reported an infant with lissencephaly, seizures, immobile gut activity, and respiratory distress syndrome requiring intensive life support who died at 36 days of age. The infant had "increased excretion of SAICA riboside," as evidenced by treating amino acid TLC by Pauly reagent [10], but the presence of succinyladenosine was not reported. Succinyladenosine is, however, more stable, less prone to interferences, and occurs in even higher concentrations in urine of ASL-deficient patients. No further reports on this case have appeared in the literature.

The TLC signal in both specimens of urine from the index case was easily distinguished from common interfering substances. The concentration of SAICA riboside was quite high in both specimens tested, presumably because they were relatively fresh. Instability of SAICA riboside can lead to false negativity in methods aimed solely at detecting this compound as is evidenced by the low concentration of SAICA riboside we detected in urine of the "known case 3," which was the result of specimen handling (freezing/thawing, alkaline urine, bacteriuria). Direct diazodetection of SAICA riboside in urine is prone to multiple interferences. HPLC of succinylnucleosides in native urine requires high chromatographic resolution to detect and quantify these compounds unequivocally.

To our knowledge, we have identified the first case of

ASL deficiency in the United States, using a screening technique that detects succinylnucleosides concurrently with urinary saccharides. The diagnosis was made by identifying the pathognomonic biochemical phenotype of the disorder and demonstrating about 40% residual activity of ASL in the patient's fibroblasts. The clinical presentation of our patient has been mild and is dominated by muscle hypotonia, which probably reflects the early age of diagnosis; the patient is currently treated with adenine and allopurinol according to the method of Van den Berghe [5]. The clinical picture may change as the natural history of the disorder proceeds. The incidence of this disease is unknown, because specimens from ASL-deficient patients go through testing systems of most metabolic laboratories unrecognized. The features of our patient, as yet unique and resembling clinically but not biochemically the "type II [5]," further broadens the spectrum of the clinical presentation of the disorder [1,2,4]. Autism, often considered a dominant symptom of the disease, may be a secondary phenomenon superimposed on the damaged central nervous system [11]. The finding of the first case after evaluation of 3,800 specimens in the 12-month period supports the need to focus screening of metabolic disorders on the vast group of children with psychomotor retardation, autism, muscular wasting, seizures, hypotonia, and/or motor delay. ASL deficiency should be considered in the differential diagnosis of such patients.

This work was supported in part by Mayo Foundation and by Project No. IGA MZCR 1831-2. Until December 1995, 3800 specimens were analyzed by high-resolution TLC at Mayo Clinic, Rochester, MN, and 800 were analyzed at J. Gregor Mendel Children's Hospital, Brno, The Czech Republic. Three urine specimens from ASL-deficient patients were provided by Dr. Jakub Krijt, Center for Hereditary Metabolic Disorders, Prague, The Czech Republic (known cases 2-4, 4a, 4b are two different specimens from the same patient); one was provided by Dr. Georges van den Berghe, International Institute of Cellular and Molecular Pathology, Brussels, Belgium (known case 1).

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Hereditary Disorders of Purine and Pyrimidine Metabolism: Identification of Their Biochemical Phenotypes in the Clinical Laboratory

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• *Objective*: To describe a laboratory approach to the diagnosis of hereditary diseases of purine and pyrimidine metabolism and emphasize clinical situations in which these disorders should be considered in the differential diagnosis.

• *Design*: Disease-specific patterns were identified in random specimens of ultrafiltered urine by using gradient high-performance liquid chromatography with diode-array detection, and reference ranges were established for uric acid, hypoxanthine, xanthine, and uracil expressed per creatinine in random specimens of urine.

• *Material and Methods*: Diagnostically significant purines and pyrimidines were separated with use of a Supelco LC-18-S nucleoside column eluted with 25 mmol/L ammonium acetate buffer and acetonitrile-methanol-water. Biologic fluids were prepared by ultrafiltration after addition of 3-methyluridine as internal standard. We used specimens negative for screening of metabolic disorders to establish reference ranges.

• *Results*: Disease-specific patterns were identified in specimens with purine and pyrimidine disorders and several urea cycle disorders characterized by increased production of pyrimidine.

• *Conclusion*: The approach described identified disease-specific patterns of purine and pyrimidine disorders and several urea cycle disorders. We suggest that testing for purine and pyrimidine disorders be done in specimens evaluated in metabolic laboratories for "screening for inborn errors of metabolism."

(*Mayo Clin Proc 1997;72:719-725*)

CV = coefficient of variation; HPLC = high-performance liquid chromatography; TLC = thin-layer chromatography; UV = ultraviolet

Purines and pyrimidines fulfill a wide range of diverse biologic functions, ranging from energy transport to storage and management of genetic information.[1] Important physiologic roles such as signaling, transmitting, and modulating,[2-4] reproduction,[5] and reactive oxygen species scavenging[6] have been described or postulated for many purines and pyrimidines. Some purines have been introduced into clinical practice as active pharmacologic agents.[7] Several features unique to purine and pyrimidine metabolism have been recognized. Their synthesis is tightly controlled in a cell-type-specific and cell-cycle-specific manner, and substantial recycling occurs in vivo in a tissue-dependent manner. Purines are salvaged primarily as free bases, and pyrimidines are usually salvaged as nucleosides.[8,9] Because extensive turnover of purines and pyrimidines occurs in rapidly dividing cells, inhibition of their synthesis de novo or recycling is a target of cytotoxic therapy.[8]

Numerous genetic disorders of purine and pyrimidine metabolism (Table 1) have been described in the past 40 years,[10-18] with the exception of primary idiopathic gout (which was described earlier). Additional enzymatic defects remain to be characterized because some variants of purine and pyrimidine de novo synthesis defects probably lead to a lethal phenotype in utero.[19] The laboratory diagnosis remains difficult because of generally nonspecific clinical manifestations crossing several medical specialties, existence of disease variants, lack of physician awareness, and, to some extent, complexity and unavailability of necessary expertise and rapidly evolving laboratory techniques.[18] The determinations of serum uric acid and urinary uric acid/creatinine ratio are simple and valuable tests that imply the diagnostic possibility of purine metabolism-related disorders. Primary diagnostic procedures are based on identification of metabolites in urine and plasma by high-performance liquid chromatography (HPLC)[18] and two-dimensional thin-

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Table I.—**Purine and Pyrimidine Disorders With Recognized Clinical Phenotypes***

Disorder	Compounds measured	Principal clinical manifestation	Treatment
HGPRT deficiency (and less severe variants)	Uric acid, hypoxanthine (u/p)[10-16]	Neurologic, renal	Allopurinol—renal symptoms
FJHN	Uric acid (u/p)[10-16]	Renal	Allopurinol—renal symptoms
PRPPS superactivity	Uric acid (u/p)[10-16]	Neurologic, renal	Allopurinol—renal symptoms
APRT deficiency	2,8-Dihydroxyadenine (u)[10-16]	Renal	Allopurinol—renal symptoms
AMPDA deficiency	None	Muscular weakness	Not available—ribose orally (?)[10-16]
XO deficiency	Xanthine, hypoxanthine (u)[17]	Renal	Diet
MoCF deficiency	Xanthine, *S*-sulfocysteine (u)[17]	Renal, neurologic	Diet (?)
ADA deficiency	Deoxyadenosine, adenosine (u/p)[10-16]	SCID, T-cell dysfunction	PEG-ADA, BMT
PNP deficiency	Inosine, guanosine, and their deoxyanalogues (u/p)[10-16]	Neurologic, immunodeficiency	Not available
ASL deficiency	SAICAr, succinyladenosine (u/csf)[17]	Neurologic	Not available
ADA superactivity	Low ATP in erythrocytes[18]	Anemia (Diamond-Blacklan syndrome)	Not available
UMPS deficiency	Orotate, orotidine (u)[17,18]	Neurologic, hematologic	Uridine
UMPH deficiency	UMP, CMP increased in erythrocytes[17]	Anemia, basophilic spotting	Not available
DPD deficiency	Thymine, uracil (u)[17]	Neurologic	Not available
DPH deficiency	Dihydrothymine, dihydrouracil (u)[17]	Neurologic	Not available
DPD heterozygotes	None[18]	Adverse reaction to 5-fluorouracil	Dose adjustment
6-TPMT deficiency or superactivity	None[18]	N-6 substituted purine intolerance, nonresponsiveness	Dose adjustment

*ADA = adenosine deaminase; AMPDA = adenosine monophosphate deaminase; APRT = adenine phosphoribosyltransferase; ASL = adenylosuccinate lyase; ATP = adenosine triphosphate; BMT = bone marrow transplantation; CMP = cytidine monophosphate; csf = cerebrospinal fluid; DPD = dihydropyrimidine dehydrogenase; DPH = dihydropyrimidinase; FJHN = familial juvenile hyperuricemic nephropathy; HGPRT = hypoxanthine, guanine phosphoribosyltransferase; MoCF = molybdenum cofactor; p = plasma; PEG-ADA = polyethylene glycol-modified ADA; PNP = purine nucleoside phosphorylase; PRPPS = phosphoribosylpyrophosphate synthase; SAICAr = succinoaminoimidazole-carboxamide riboside; SCID = severe combined immunodeficiency syndrome; TPMT = 6-thiopurine methyltransferase; u = urine; UMP = uridine monophosphate; UMPH = uridine monophosphate hydrolase; UMPS = uridine monophosphate synthase; XO = xanthine oxidase.

layer chromatography (TLC).[20] Second-level testing to confirm a disease-specific pattern has used identical methods but involving prior fractionation (mostly anion-exchange techniques) to eliminate the analytic interferences invariably present in clinical specimens.[20-22] An automated method requiring four sets of ultraviolet (UV) absorbance detectors and column switching has been applied to evaluation of ultrafiltered urine for purine and pyrimidine disorders.[23] A sophisticated HPLC procedure has been developed for identification of hypermodified nucleosides in biologic fluids by using phenylboronate gel for covalent extraction of compounds with vicinal *cis*-diol functionality, such as ribonucleosides.[24]

Herein we describe our approach to the diagnosis of purine and pyrimidine disorders and emphasize clinical situations in which physicians should consider these diseases as diagnostic possibilities. Our goal was to work with random urine specimens and avoid often-imposed restrictions. Requirements for a "low-purine" diet, 24-hour urine collection, and "no drugs" before sample collection are impractical and often unachievable for patients, physicians, and parents.

We established a single two-mode reverse-phase HPLC procedure: a gradient *mode I* for profiling of purine and pyrimidine compounds in unextracted biologic fluids and an isocratic, short *mode II* for achieving efficient uracil/pseudouridine and 2,8-dihydroxyadenine/uric acid and orotic acid separation. The procedure analyzes native ultrafiltered materials and uses diode-array detection and in-line three-wavelength quantification. For extended testing, we used phenylboronic acid[24] and TLC fractionation procedures. Reference values have been published for normally occurring metabolites (uracil, hypoxanthine, xanthine, and uric acid) in random specimens.[25] We demonstrate the diagnostic power of the method by using examples of purine and pyrimidine deficiency syndromes and urea cycle disorders characterized by intermittent pyrimidine overproduction.

MATERIAL AND METHODS

Instrumentation.—A Waters 600E gradient module was connected to a WISP 712 through a narrow-bore tubing. Samples were injected on a Supelco LC-18-S "nucleoside" column (250 by 4.6 mm) equipped with a Supelguard LC-18-S precolumn[24] maintained at 24°C. The detection was done with a Waters 990 diode-array detector and the 990 software (Waters).

Chromatography.—Solvent A was 25 mmol/L ammonium acetate, pH adjusted to 4.02 ± 0.02 with glacial acetic acid (stable for 1 week), prepared from 500 mmol/L of stock solution (stored refrigerated, stable for 3 months) and diluted with helium-degassed HPLC grade water. Solvent B was acetonitrile:methanol:water (100:250:450) made fresh for each run (stable for about 30 hours). Solvent C was degassed HPLC grade water (fresh daily), and solvent D was methanol (a washing solvent only—not used for separation).

For purine and pyrimidine profiling (*mode I*), the gradient setting was as follows (in percent, programmed as "table 1" in the instrument): 0 to 8 minutes isocratic A50:B3:C47, 8 to 35 minutes linear to A50:B20:C30, 35 to 38 minutes linear to A50:B47:C3, 38 to 44 minutes isocratic A50:B47:C3, 44 to 45 minutes linear to A50:B3:C47, and 45 to 70 minutes isocratic column equilibration A50:B3:C47. Analysis was recorded at 235 to 400 nm from 2 to 45 minutes.

For *mode II*, the setting was as follows (instrument "table 2"): 0 to 15 minutes isocratic A30:C70, 15 to 16 minutes linear A30:B67:C3, 16 to 24 minutes isocratic A30:B67:C3, 24 to 25 minutes linear A30:C70, and 25 to 40 minutes isocratic column equilibration A30:C70.

Instrument Calibration.—Stock solutions (1 mmol/L) in water (alkalinized with saturated Li_2CO_3, if necessary) of orotic acid, uracil, uric acid (dissolved in saturated Li_2CO_3), 2,8-dihydroxyadenine (dissolved in saturated Li_2CO_3), hypoxanthine, xanthine, uridine, thymine, inosine, deoxyinosine, guanosine, deoxyguanosine, thymidine, adenosine, and deoxyadenosine were diluted to 50 mmol/L of working standard of each compound and 500 mmol/L of uric acid. Succinyladenosine was prepared as described previously.[26] Working standard (100 μL) was pipetted into the Ultrafree 10,000 NMWL (Millipore, catalog no. UFC3 LGCNB), and 25 μL of internal standard (400 mmol/L of 3-methyluridine[24]) was added, vortexed, and centrifuged at 8,000 rpm (*g*) for 15 minutes. Ultrafiltrate (50 μL) was injected, and calibration tables at 254, 270, and 285 nm were generated from peak heights. *Mode II* was calibrated by using external standards (50 mmol/L) run with each series of specimens.

Sample Preparation. **Plasma.**—Heparinized or ethylenediaminetetraacetic acid-treated plasma (100 μL) was pipetted into the Ultrafree unit, and 25 μL of internal standard was added, vortexed, and centrifuged. Alternatively (cerebrospinal fluid, amniotic fluid), higher volumes at a 4:1 ratio were processed, and 50 to 80 μL of plasma (and up to 180 μL of amniotic fluid, cerebrospinal fluid, or other low concentration material) was injected onto a column.

Urine With Creatinine.—For samples with less than 10 mg/dL, before measurement the urine was warmed for about 10 minutes to 55°C to dissolve precipitated material. Preparation was thereafter identical to that for plasma; the injection volume was 20 μL. For samples of 10 to 100 mg/dL, (50 μL of urine + 50 μL of water) + 25 μL of internal standard were pipetted into the Ultrafree unit, and 20 μL was injected. For 100 to 150 mg/dL, (25 μL of urine + 75 μL of water) + 25 μL of internal standard were pipetted into the unit, and 20 μL was injected. For samples greater than 150 mg/dL, the procedures were the same as for 100 to 150 mg/dL, but 10 μL was injected. For *mode II*, sample preparation and injection volumes were identical, but no internal standard was added.

Urine Fractionation for Extended Testing.—For fractionation, 100 μL of urine was mixed with 25 μL of internal standard, and 20 μL was applied per 15-mm lane as a narrow streak on the HPLC plate (Merck catalog no. 5628/5, with a fluorescence indicator to enable visual identification of zones). Three lanes were applied per specimen, and a fourth lane was a standard of 3-methyluridine, thymine, and guanosine. The plate (up to the end) was developed once in butanol:glacial acetic acid:water (8:1:1). After migration, the plate was inspected under UV light (254 nm). Three fractions were isolated (one fraction from each lane); thus, an internal standard could be included in each fraction: (1) from the internal standard band (included) to thymine (included); (2) from the internal standard band (included) to the front edge of guanosine; and (3) the internal standard band only plus the area from guanosine (included) to the start (included). Each silica gel fraction scraped from the plate was quantitatively transferred to the Ultrafree unit, 300 μL of water was added, vortexed, and centrifuged, and 150 μL of ultrafiltrate (corresponding to 10 μL of original urine) was injected onto the HPLC column.

Reference Range Study Specimens.—Random morning urine specimens submitted to the laboratory for screening of inborn errors of metabolism were tested for amino acids, monosaccharides, disaccharides, and oligosaccharides, and succinylnucleosides.[27] Organic acids and glycosaminoglycans were used when no abnormality was found.

Quality Control.—Chemtrak normal or pathologic general chemistry control serum (Medical Analysis Systems, Inc.) was used to assess quantification accuracy of uric acid determinations. The material was processed as plasma (50 μL) and analyzed once per new batch of the buffer stock substrate. Periodically, a Supelco nucleoside test mixture (catalog no. 4-7310) was analyzed to verify quantification

accuracy of several nucleoside determinations (inosine, uridine, and guanosine).

RESULTS

Chromatography.—The column efficiency expressed in theoretical plates was n = 29,000 and 68,000 for guanosine and deoxyadenosine, respectively. The internal standard retention time coefficient of variation (CV) was 0.51%. The within-run precision (CV) ranged from 3.6% for inosine to 5.1% for adenine; between-run precision ranged from 5.7% for guanosine to 6.25% for uracil. The recovery of ultrafiltrate ranged from 96.8% for hypoxanthine to 100.5% for guanosine; the recovery after TLC fractionation was 42% for deoxyadenosine and 83% for deoxyguanosine or better for bases and other analytes. Separations of reference mixture (50 μL of Chemtrak + 50 μL of calibration solution) and disease-specific metabolites are shown in Figure 1.

Identification of Disease-Specific Patterns.—*Dihydropyrimidine dehydrogenase deficiency* is biochemically characterized by thymine and uracil in the urine. The concentration of thymine in our specimen reached only 129 mmol/L. Uracil was present at a concentration of about 250 mmol/L. Microbial degradation of pseudouridine (a ubiquitous nucleoside originating from degradation of transfer RNA[24]) during shipment also contributed to this increase. This specimen did not contain 5-hydroxymethyluracil, which would appear between uracil and pseudouridine peaks.

Both metabolites indicative of *adenylosuccinate lyase deficiency* were chromatographically resolved and identified; succinyladenosine yielded a clear and quantifiable signal at 285 nm, where absorption of hippuric acid appearing before succinyladenosine is minimal. The pattern of *Lesch-Nyhan syndrome* treated with allopurinol showed high peaks for uric acid, hypoxanthine, allopurinol, and oxypurinol. A characteristic excretion profile of inosine, guanosine, deoxyinosine, and deoxyguanosine in conjunction with minimal excretion of uric acid is indicative of *purine nucleoside phosphorylase deficiency*. *Xanthine oxidase deficiency* and *molybdenum cofactor deficiency* showed increased levels of xanthine and minimal excretion of uric acid. *Adenine phosphoribosyltransferase deficiency* biochemically manifests with excretion of normally undetectable 2,8-dihydroxyadenine, characteristically with an absorption maximum at 306 nm; both analytic modes identified the condition, and mode II was better for metabolite quantification.

Urea cycle disorders are characterized by pyrimidine overproduction. The pattern of acute relapse of *ornithine carbamoyltransferase deficiency* showed increased excretion of orotic acid and highly increased excretion of uracil and uridine; uridine excretion disappears in remission, and uracil, orotic acid, or both remain increased in asymptomatic patients. We observed almost an identical pattern of orotic acid, uracil, and uridine excretion in a newborn with *argininosuccinate lyase deficiency* during an acute attack; this patient subsequently died. A male patient with mental retardation, behavioral problems, and psychiatric symptoms, in whom argininosuccinate lyase deficiency was diagnosed at age 14 years, excreted normal amounts of orotic acid and uracil but no uridine; his nitrogen balance was maintained by massive excretion of argininosuccinate.

The patterns shown were identified in nonfractionated urine (reconstituted from filter papers used for mailing except for dihydropyrimidine dehydrogenase sent frozen and urea cycle disorders diagnosed in the laboratory) on the basis of retention times and 254/270/285 nm ratios and in-line UV spectroscopic evaluation. The reference values for uric acid, hypoxanthine, xanthine, and uracil are shown in Figure 2.

DISCUSSION

Chromatography.—Our HPLC of purines and pyrimidines proved to be a stable method with minimal sample preparation and fully suitable for screening up to 17 specimens a day. Column temperature control is recommended because retention times of several compounds (for example, succinylnucleosides) vary up to 1 minute/1°C. This chromatographic system remains stable for about 2,000 analyses if precolumns are changed after about 200 injections, as evidenced by reproducible retention times (CV for internal standard retention time, less than 0.5%) and separation of xanthine and uridine. Although tetrahydrofuran improved symmetry of the adenine peak, it introduced variability with retention times because of its volatility and was found unnecessary for separation.[18,21] Quantification at two to three wavelengths is needed to obtain valid results. Purity of the peak is ensured if calculated concentrations at different wavelengths match. If interferences occur, the sample is fractionated with use of normal-phase TLC; the same approach is taken as the confirmatory technique when the presence of a metabolite of interest is indicated. Once calibrated, the system is stable for several months. Use of strong hydroxides (for example, NaOH and KOH) to "facilitate" dissolving of uric acid and 2,8-dihydroxyadenine results in their decomposition; therefore, saturated Li_2CO_3 is the solvent of choice. Specific identification of dihydropyrimidines was done by *mode I* for dihydrothymine and *mode II* for dihydrouracil, but ammonium acetate buffer was replaced by otherwise identical ammonium phosphate buffer to eliminate absorbance of acetate at 200 to 240 nm. Mixtures of urine spiked with dihydropyrimidines were evaluated because urine specimens from a patient with dihydropyrimidinase deficiency[28] were unavailable.

Sample Preparation.—Several hundred compounds absorbing in the UV range 230 to 320 nm can be present in urine. Normal-phase TLC offers different fractionation se-

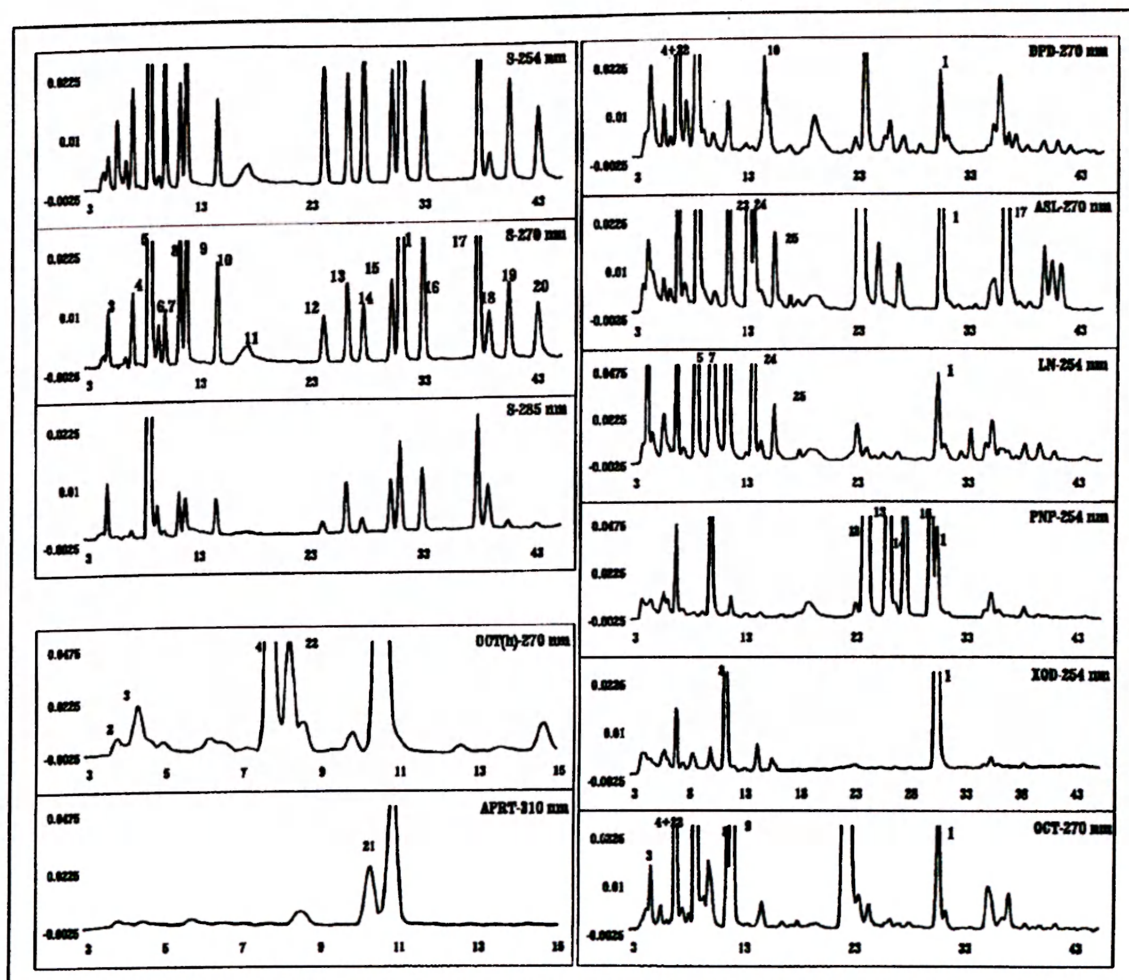

Fig. 1. Analyses scanned from 235 to 320 nm for two-mode high-performance liquid chromatography (y-axes in absorbance units, full scale; x-axes in minutes). *S-254, S-270,* and *S-285 nm*, Standard three-wavelength, mode I chromatogram of a mixture of standard + Chemtrak abnormal serum (50 mL + 50 mL) + 25 mL of internal standard, shown at selected wavelengths. Numbering of peaks is identical throughout all chromatograms: *1* = internal standard; *2* = orotidine; *3* = orotic acid; *4* = uracil; *5* = uric acid; *6* = tyrosine; *7* = hypoxanthine; *8* = xanthine; *9* = uridine; *10* = thymine; *11* = adenine; *12* = inosine; *13* = guanosine; *14* = deoxyinosine; *15* = deoxyguanosine; *16* = thymidine; *17* = succinyladenosine; *18* = tryptophan; *19* = adenosine; *20* = deoxyadenosine; *21* = 2,8-dihydroxyadenine; *22* = pseudouridine; *23* = succinoaminoimidazole-carboxamide riboside; *24* = oxypurinol; *25* = allopurinol. *OCT(h)-270 nm*, Mode II, female heterozygous for ornithine carbamoyltransferase deficiency, 6 hours after allopurinol load, characterized by increased excretion of orotidine, orotic acid, and highly increased uracil; uridine not detectable. *APRT-310 nm*, Mode II, adenine phosphoribosyltransferase deficiency. *DPD-270 nm*, Mode I, dihydropyrimidine dehydrogenase deficiency. *ASL-270 nm*, Mode I, adenylosuccinate lyase deficiency, treated with aminoimidazole-carboxamide riboside, adenine, and allopurinol. *LN-254 nm*, Mode I, Lesch-Nyhan syndrome, treated with allopurinol. *PNP-254 nm*, Mode I, purine nucleoside phosphorylase deficiency. *XOD-254 nm*, Mode I, xanthine oxidase deficiency. *OCT-270 nm*, Mode I, male hemizygous for ornithine carbamoyltransferase deficiency during an acute attack, characterized by increased excretion of orotic acid, uracil, and uridine.

lectivity in comparison with anion-exchange chromatography and yields no fraction overlap; thus, compounds difficult to separate (uracil + 5-hydroxymethyluracil + pseudouridine; succinyladenosine + hippuric acid) segregate fully in different fractions. The use of internal standard throughout the procedure allows quantitative data handling. The recovery is also satisfactory for deoxynucleosides except for deoxyadenosine, for which degradation occurs even if alka-

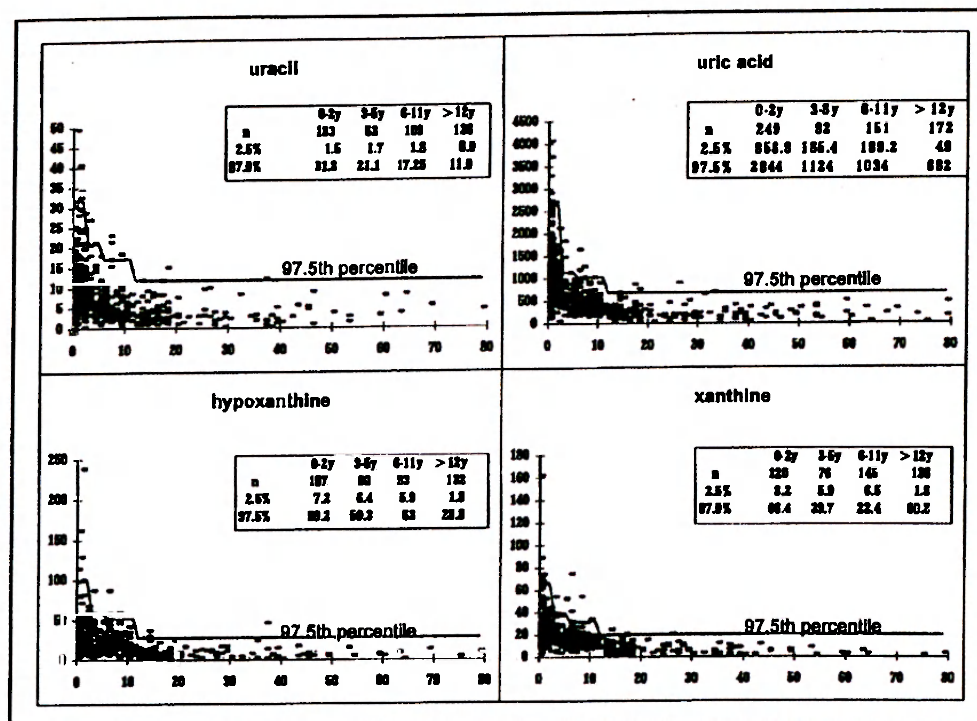

Fig. 2. Distributions of concentrations versus age of subjects (y-axes in mmol/mmol creatinine; x-axes in years) and reference ranges for uracil, uric acid, hypoxanthine, and xanthine. Age-groups: younger than 2 years, 3 to 5 years, 6 to 11 years, and 12 years or older; n = number of measurements within a group.

line solvents are used (probably because of the acidic character of silica gel). We prefer ultrafiltration[23] over trichloroacetic acid[21] with back-extraction or organic solvent deproteination. Hypoxanthine in plasma can be evaluated only if formed elements are separated in a refrigerated centrifuge within 15 minutes after venipuncture.

Interferences.—The technique reveals about 40 major peaks that correspond to endogenous and exogenous compounds in urine. Interferences due to new drugs and their metabolites remain an analytic challenge because the specimens analyzed are frequently from patients receiving therapy. One metabolite of ibuprofen, commonly used by pediatricians, appears between hypoxanthine and xanthine, and a second metabolite coelutes with inosine, both having an "inosine-like" UV spectrum (the identity of both metabolites is confirmed by gas chromatography-mass spectrometry). Acetaminophen (confirmed by gas chromatography-mass spectrometry) appears as a major peak after the 3-methyluridine peak and before hippuric acid and *N*-acetyltyrosine. Acycloguanosine elutes at about 22 minutes, separated from measured compounds; however, one of its metabolites coelutes with hypoxanthine. Spectral comparisons by the diode-array detector of peaks and known compounds have proved valuable in evaluation of this chromatographic procedure and in detecting interferences.

Reference Values.—Clinically insignificant gender and year-to-year differences in serum uric acid concentration were established in a group ranging from 0 to 11 years of age; the steady downward trend we observed for all four compounds is caused by the increase in creatinine excretion with advancing age. Other factors such as diet may influence excretion of uric acid. We do not think that this is a critical confounding factor, however, because finding of true hyperuricosuria, proved also by a 24-hour urine collection, should elicit differential diagnostic testing (for example, for purine disorders, glycogenoses, β-oxidation diseases, and lactic acidosis).

The pathobiochemistry and relationships of genotypes to biochemical and clinical phenotypes of purine and pyrimidine disorders remain poorly understood despite the progress in identifying new entities during past decades. Nothing is known about the mechanism of induction of severe central nervous system symptoms. We suggest that all patients who have psychomotor retardation, seizures, or muscle wasting

and hypotonia should be screened by a panel of tests, including studies of purines and pyrimidines. Reasonable approaches are either (1) to screen for purine and pyrimidine disorders after eliminating other metabolic disorders as diagnostic possibilities (van Gennip AH. Personal communication) (bearing in mind existence of variants that can shift the age at manifestation to later decades of life) or (2) to screen specimens sent to the metabolic laboratories for "screening for metabolic disorders." Indeed, both groups represent a highly skewed sample of patients with substantial probability of occurrence of metabolic pathologic conditions. In fact, we recently identified the first US patient with adenylosuccinate lyase deficiency by analysis of a urine specimen submitted for screening for inborn errors of metabolism.[29]

ACKNOWLEDGMENT

Urine specimens from patients with purine and pyrimidine disorders were kindly made available to us by Dr. A. H. van Gennip (dihydropyrimidine dehydrogenase), Dr. G. Van den Berghe (adenylosuccinate lyase), and Dr. J. Krijt (hypoxanthine or guanine phosphoribosyltransferase, adenine phosphoribosyltransferase, purine nucleoside phosphorylase, orotate phosphoribosyltransferase, and xanthine oxidase deficiency).

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Letters to the Editor

Encephalopathy, lactic acidosis, hyperammonaemia and 5-fluorouracil toxicity

Sir,

I read with interest the recent article by Yeh and Cheng (1997) concerning toxicity of the 5-fluorouracil/leucovorin (HDFL) treatment protocol. The clinical presentation of side-effects was encephalopathy associated with laboratory findings of hyperammonaemia, lactic acidosis and hypotriglyceridaemia. This phenomenon was present in 5.7% of patients treated with the HDFL protocol. The authors conclude that ammonia as the end-product of 5FU metabolism overloads capacity of the Krebs cycle and that under HDFL treatment a large amount of fluoroacetic acid directly inhibits the ATP-producing Krebs cycle (Yeh and Cheng, 1997). I have the following comments to make: one should distinguish between the Krebs–Henseleit cycle, which is ureasynthetic, and the Krebs tricarboxylic acid cycle, which generates redox equivalents for the electron transport chain and consequent synthesis of ATP. Both these cellular processes require intact mitochondria to operate. In hepatic encephalopathy it is of interest to assess the clinical stage of encephalopathy using available scoring scales (i.e. Glasgow Coma Scale). The profile of plasma amino acids informs about the elevation of glutamine as this amino acid is an essential part of the ammonia disposal system and is also related to acid–base balance (Guder et al, 1987). Moreover, other constituents of the ureagenic cycle are also evaluated. Alanine and pyruvate measurements are of value for assessment of the redox status and optimally, AKBR is suggested as the best prognostic and functional test of cytoplasmic (lactate/pyruvate) and mitochondrial (beta-hydroxybutyrate/acetacetate) redox state (Asonuma, 1991; Saibara, 1994; Takahashi, 1997). Hypotriglyceridaemia is usually accompanied by elevated serum free fatty acids and hypoglycaemia usually develops because of liver gluconeogenesis failure. Altogether, these findings indicate severe disturbance of the liver function in terms of (a) impaired ureagenesis (elevated ammonia, elevated glutamine, decreased urea, alkalosis); (b) impaired oxidative phosphorylation (lactate/pyruvate); and (c) impaired synthesis of complex lipoproteins. Interpretation of these findings is, in my opinion, consistent with hepatic dysfunction; the authors assumed that none of their patients had hepatic or renal dysfunction.

The frequency of this complication, which was 5.7% in the group under study, is high and could imply a certain genetic basis. I wish to offer the following differential diagnostic considerations to complement those discussed by Yeh and Cheng. Dihydropyrimidine dehydrogenase deficiency in its *incomplete form* remains a possibility (Wei et al, 1996) as the complexity of the compound-heterozygous status in which both alleles harbour different functionally more or less relevant mutations (also called polymorphisms) may trigger a complex clinical response under stress conditions (i.e. substrate loading superimposed on disease status). Mitochondrial disorders are another possibility (OMIM 1996). Their genetics are based on the populational rather than on a Mendelian basis and their spectrum of clinical presentation is extremely broad. Loss of structural integrity of mitochondria leads to disruption of the proton gradient and consequently to failure of energy (ATP) production. In the liver, these events result in failure of the Krebs–Henseleit ureasynthetic cycle and consequent hyperammonaemia. Lactic acidosis develops because of derangement (regardless of the nature) of the cellular redox status and microvesicular fatty infiltration (in the liver) appears because of inability to create and export complex lipoproteins. Again, one can hypothesize that a stress situation superimposed on a genetically altered background (i.e. certain proportion of *primarily malfunctioning mitochondria*) may trigger a response such as this. Liver toxicity of several pyrimidine derivatives has been previously demonstrated for AZT (Mondica-Napolitano, 1993) and fialuridine (Lewis et al, 1996) and the mechanism was related to *damaged mitochondria*. The inability of mitochondria to synthesize thymidine de novo may be the single most important reason for mitochondrial toxicity of pyrimidine analogues (Shaw and Locarnini 1995). The point raised by Yeh and Cheng is important for two reasons: (a) assessment of patients with this pathological reaction to HDFL treatment should be comprehensive and in addition should include determinations of plasma amino acids and pyruvate; (b) when these side-effects occur the clinician in charge should consider consultation with a medical geneticist to review possibilities mentioned above in the context of the patient's family history. Gaining experience and knowledge could perhaps result in delineation of a test protocol assessing the *pharmacogenetic background* of patients scheduled for HDFL treatment. Notably, the importance of pharmacogenetics in cancer treatment has already been recognized for thiopurine methyltransferases (Szumlanski et al 1996) and dihydropyrimidine dehydrogenases (Meinsma et al, 1995; Beuzeboc, et al, 1996).

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5-Fluorouracil-related encephalopathy: at least two distinct pathogenetic mechanisms exist – reply

Sir,

We greatly appreciate Dr Valik's comments, which have provided new information for studying the possible pathogenetic mechanisms of HDFL-related encephalopathy. However, we would like to take this opportunity to clarify some of the points that we raised in the original article (Yeh et al, 1997).

The two Krebs cycles mentioned by Dr Valik, i.e. the Krebs tricarboxylic acid cycle (Krebs citric acid cycle) and the Krebs–Henseleit urea cycle, correspond to the Krebs cycle and the urea cycle in our article respectively. We considered that these two simplified terms are well accepted by readers and are widely used in the literature (Lehninger et al, 1993*a,b*; Mayes et al, 1993; Rodwell et al, 1993). We believed that the metabolic encephalopathy observed by us was a new pathogenetic entity, which differed significantly from the more common hepatic encephalopathy. Therefore, we chose not to use the traditional clinical grading system for hepatic encephalopathy (Sherlock et al, 1954, 1989). We have indeed documented that none of our patients had hepatic or renal dysfunction in terms of the conventional biochemical criteria (Yeh et al, 1997). Also, none of them had hypoglycaemia.

The relatively high overall incidence (5.7%) of HDFL-related encephalopathy in our patients did not necessarily imply a genetic basis for this condition. Instead, all our evidence has indicated that dihydropyrimidine dehydrogenase (DPD) deficiency, even in its incomplete form, is probably not the pathogenetic mechanism of HDFL-related encephalopathy. 5-FU treatment in patients with DPD deficiency should result in severe mucosal and haematological toxicities (Tuchman et al, 1985; Diasio et al, 1988), which were definitely not observed in all our 16 patients. Also, the encephalopathy due to DPD deficiency should characteristically present at the *first* exposure to drug, and should have low or no catabolic products (such as ammonia) of 5-FU (Tuchman et al, 1985; Diasio et al, 1988; Takimoto et al, 1996). Among our 16 patients, the encephalopathy developed at the first exposure to HDFL in eight patients, but developed at or after the second exposure in another eight patients. On rechallenge with HDFL, only 8 out of 12 patients developed recurrent encephalopathy. These observations strongly argued that DPD deficiency is the cause of HDFL-related encephalopathy.

We suggest that there are at least two distinct pathogenetic entities of 5-FU-related encephalopathy. The first is the 'DPD deficiency type'. DPD deficiency results in failure of the first step of 5-FU catabolism and leads to 5-FU accumulation (Tuchman et al, 1985; Diasio et al, 1988; Takimoto et al, 1996). High concentration of 5-FU penetrates into cerebrospinal fluid (CSF) and causes acute demyelination of the neurons. After discontinuation of 5-FU, it usually takes weeks to months for remyelination to occur (Kerr et al, 1984; Takimoto et al, 1996). High plasma level of 5-FU should also cause severe gastrointestinal (GI) and marrow toxicities (Tuchman et al, 1985; Diasio et al, 1988; Takimoto et al, 1996). And few or no catabolites (FUPA, 5-fluoroureidopropionic acid; FBAL, 2-fluoro-β-alanine; ammonia, etc.) should be detected because of the failure of 5-FU catabolism. The second is the '5-FU catabolite type' (Yeh et al, 1997). The major catabolic pathway of 5-FU is intact. However, the relatively large dose of 5-FU results in transient accumulation of 5-FU catabolites (including ammonia). If the disposal of the latter is not adequate, such as under conditions of malnutrition and/or impaired Krebs cycle, transient encephalopathy ensues. No demyelinating changes and no severe GI or marrow toxicities should be observed, and recovery from encephalopathy usually occurs within a few days (Yeh et al, 1997). Theoretically, a specific DPD inhibitor (such as 5-ethynyluracil) may protect the patients from the encephalopathy of the 5-FU catabolite type (Davis et al, 1994).

We, however, cannot completely exclude other possible mechanisms of the hyperammonaemia observed in our patients. The one raised by Dr Valik, which hypothesized that a stress situation superimposed on a genetically altered background in urea cycle (i.e. incomplete form of urea cycle enzyme deficiencies, such as ornithine transcarbamoylase deficiency) is certainly a possibility that deserves further exploration (Sinatra et al, 1975; Snodgrass et al, 1976). We also agree with Dr Valik that patients with HDFL-related encephalopathy should have an examination of plasma amino acids (glutamine, arginine, etc.) and the intermediates of urea cycle (ornithine, citrulline, argininosuccinate, etc.) (Rodwell

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et al, 1993; Lehninger et al, 1993*b*). This may help further clarify the pathogenetic mechanisms. And, along the same line, a detailed pharmacogenetic study is also indicated.

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ARTICLES

Release of Cytokeratin-18 and -19 Fragments (TPS and CYFRA 21-1) Into the Extracellular Space During Apoptosis

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Abstract Serum fragments of cytokeratins-18 and -19 (measured as TPS and CYFRA 21-1, respectively) have traditionally been considered as markers of tumor proliferation, although the evidence is scarce for a causative relationship between proliferation and levels of TPS and CYFRA 21-1. We examined whether apoptosis might produce TPS and CYFRA 21-1 fragments. MCF-7 breast cancer cells were treated with mitomycin C or agonistic anti-CD95 antibody, and levels of TPS and CYFRA 21-1 in tissue culture supernatants were compared with the frequency of cells exhibiting the following markers of cell death: intracellular cytokeratin-18 cleavage, surface staining with annexin-V, propidium iodide uptake, DNA fragmentation. Twenty-four hours after inducing apoptosis, levels of TPS and CYFRA 21-1 were elevated $\geq$ 4-fold in culture supernatants. Elevations in TPS and CYFRA 21-1 coincided with apoptosis measured by the first three cell death markers but preceded DNA fragmentation. These mitomycin C- and CD95-mediated elevations were completely inhibited by co-incubation with the caspase inhibitors Z-VAD.fmk and Z-IETD.fmk, respectively. We conclude that TPS and CYFRA 21-1 can be abundantly released into the extracellular space during the intermediate stage of epithelial cell apoptosis. J. Cell. Biochem. 85: 670–677, 2002. © 2002 Wiley-Liss, Inc.

Key words: tumor marker; proliferation; caspase; breast cancer; CD95/Fas

Apoptosis is a process in which damaged or unneeded cells commit suicide. Apoptosis requires activation of a biochemical pathway of proteases known as caspases (cysteine proteases with specificity for aspartic acid residues). Apoptosis can be intitiated by release of cytochrome c from mitochondria or by signaling through cell surface death receptors such as CD95 (Fas) [reviewed in Green, 2000]. The substrates of caspases are diverse. Through cleavage, caspases can activate other caspases and inactivate both DNA repair enzymes and structural proteins such as actin and certain cytokeratins. Cleavage of these substrates prepares for, and participates in, the dismantling of the cell in an orderly fashion that may substantially reduce inflammation [Bellamy et al., 1995], although perhaps not completely [Restifo, 2000].

Serum fragments of cytokeratins-18 and -19 can be detected and referenced as TPS (Tissue Polypeptide Specific antigen) and CYFRA 21-1 (Cytokeratin-19 Fragment), respectively. Serum concentrations of TPS and CYFRA 21-1 correlate with disease progression in various malignancies and thus have been used as tumor markers [Nisman et al., 1998; Rebhandl et al., 1998; Tempfer et al., 1998; Nagler et al., 1999; Doweck et al., 2000]. TPS and CYFRA 21-1 have traditionally been considered as proliferation markers, although the evidence for this association is limited, and the biology behind the release of soluble cytokeratin fragments

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actually remains poorly understood. Interestingly, intracellular cleavage of cytokeratin-18 was recently shown to be mediated by caspases 6, 7, and 3 during apoptosis [Caulin et al., 1997]. Cleavage of cytokeratin-19 has been postulated to occur through spontaneous caspase 3 activity, resulting in release of CYFRA 21-1 into tissue culture supernatants [Dohmoto et al., 2001]. We therefore investigated whether the apoptotic program in epithelial cells can induce release of TPS and CYFRA 21-1 into the extracellular space in vitro.

MATERIALS AND METHODS

Cell Culture and Reagents

MCF-7 breast adenocarcinoma cells were plated at subconfluence in 75 cm^2 tissue culture flasks (Nalge Nunc International, Denmark) and maintained in 5% CO_2 at 37°C in Dulbecco's modified eagle's medium (DMEM) containing 10% fetal bovine serum, glutamine, and antibiotics. Agonistic anti-CD95 IgM antibody (clone CH11) and antagonistic anti-CD95 antibody (ZB4) were purchased from Immunotech (Westbrook, ME). Cycloheximide (CHX) (Sigma, St. Louis, MO) was solubilized in culture medium immediately prior to use. Mitomycin C (Sigma) was solubilized in distilled water and stored at −20°C until use. Z-VAD.fmk was obtained from RBI/Sigma (MA) and Z-IETD.fmk from R&D Systems (Weisbaden-Nordenstadt, Germany). M30 antibody was obtained from Boehringer Mannheim (Mannheim, Germany). Monoclonal antibodies CO8 [Bartek et al., 1991] and DC-10 [Lauerova et al., 1988] were developed in our laboratory and are specific for human cytokeratin-18; monoclonal antibody BA-17 is specific for cytokeratin-19 [Bartek et al., 1986]; the epitopes recognized by these antibodies have not been characterized.

Immunoblotting

Total cellular protein lysates were prepared by harvesting cells in hot Laemmli electrophoresis sample buffer. Proteins were separated by SDS–polyacrylamide gel electrophoresis (SDS–PAGE) on 10 or 12.5% gels, and transferred onto a nitrocellulose membrane in a Bio-Rad Mini Trans-Blott Electrophoretic Transfer Cell for 2 h at 4°C, applying 150 mA in transfer buffer (242 mmol Tris, 190 mmol glycine, 20% methanol). Prestained molecular weight markers (Bio-Rad, Hercules, CA) were run in parallel. The blotted membranes were blocked with 5% milk and 0.1% Tween 20 in phosphate buffered saline (PBS) for 2 h and probed overnight with specific monoclonal antibodies (final concentration 1 μg of antibody in 1 ml of PBS containing 5% milk). After washing 3 × in PBS plus 0.1% Tween 20, peroxidase-conjugated rabbit anti-mouse immunoglobulin antiserum (Dako, Denmark) diluted 1:1,000 in PBS containing 5% milk and 0.1% Tween 20 was used as the secondary antibody. To visualize peroxidase activity, ECL reagents (Amersham Pharmacia Biotech, Buckinghamshire, UK) were used according to the manufacturer's instructions.

Quantitation of Soluble Cytokeratin Fragments

The amounts of soluble cytokeratin-18 fragments in tissue culture supernatants were determined using the TPS enzyme immunoassay (Beki Diagnostics, Bromma, Sweden), which employs the M3 antibody specific for cytokeratin-18 residues 322–340 [Rydlander et al., 1996]. The TPS assay was used as recommended by the manufacturers, followed by quantitation using a Cobas CORE analyzer (Roche, Basel, Switzerland). Soluble cytokeratin-19 fragments were measured using the highly specific [Bodenmuller et al., 1994] CYFRA 21-1 electrochemiluminescence immunoassay (Roche Diagnostics, Mannheim, Germany) on an Elecsys 2010 (Roche Diagnostics). Briefly, after establishing that centrifugation over a wide range of speeds had no effect on the levels of TPS and CYFRA 21-1 in the supernatant of normal and apoptotic cultures, 400 μl of tissue culture supernatants were centrifuged at 800*g* for 20 min to pellet floating cells, and the supernatants were stored at −20°C until analysis. After thawing, 100- and 5-fold dilutions were typically used for TPS and CYFRA 21-1 assays, respectively, as determined experimentally to be within the linear measuring ranges of the assays. Pelletted cells were pooled with the main fraction of floating and adherent cells during simultaneous staining for apoptosis, described below.

Detection of Apoptosis

All apoptotic parameters were quantitatively assessed on an EPICS® XL flow cytometer (Coulter, Hialeah, FL) using the manufacturer's analysis software. DNA content (sub-G1) analyses were performed as previously described [Sheard et al., 1999]. Cells containing

intracellular cytokeratin-18 fragments were stained using the M30 antibody that is specific for a neo-epitope of fragmented cytokeratin-18 expressed during early apoptosis [Leers et al., 1999]. Briefly, adherent and floating cells were pooled, fixed for 2 h in methanol at −20°C, washed 1 × in washing solution (PBS, 0.1% Triton X-100), washed 1 × in staining solution (PBS, 0.1% albumin, 0.1% Triton X-100), incubated for 2 h at room temperature in the dark with the M30 antibody diluted 1:250 in staining solution, washed 2 × in washing solution, and resuspended in 0.5 ml PBS. Subcellular debris were eliminated from analysis by appropriate gating on forward scatter and side scatter parameters.

Expression of phosphatidyl serine on the outer leaflet of the surface membrane, which occurs on both apoptotic and non-apoptotic dead cells, was quantitated using the Annexin-V assay (Boehringer Mannheim). Cells were trypsinized, washed 2 × in cold incubation buffer (10 mmol HEPES/NaOH, pH 7.4, 140 mmol NaCl, 5 mmol $CaCl_2$), and resuspended in 100 µl of staining solution containing 1 µg/ml propidium iodide and annexin-V-fluorescein diluted 1:50 in incubation buffer. Cells were incubated for 10 min and analyzed by flow cytometry using a 530 nm bandpass filter to detect annexin-V-fluorescein and a 620 nm bandpass filter to detect propidium iodide.

RESULTS

Intracellular Fragmentation of Cytokeratin-18 During Apoptosis

To examine cleavage of cytokeratin-18 during the apoptotic program, we induced apoptosis in MCF-7 breast carcinoma cells with mitomycin C or anti-CD95 antibody and stained intracellularly with the M30 antibody. Induction of cell death by mitomycin C elicited easily detectable levels of cleaved, intracellular cytokeratin-18 in a time-dependent manner (Fig. 1A). Similarly, signaling through the CD95 death receptor also induced a substantial level of intracellular cytokeratin-18 fragments (Fig. 1B), which was preceded by activation of caspase-8 detected 14 h after treatment (data not shown). CD95-mediated generation of intracellular cytokeratin-18 fragments was almost completely blocked by pretreatment with an antagonistic antibody (Fig. 1B). To confirm these findings, immunoblotting was performed using two distinct anti-cytokeratin-18 antibodies. A strong band corresponding to unfragmented cytokeratin-18 was observed at 45 kDa in all treatment groups, and a second band exhibiting increased migration (indicative of formation of a smaller fragment) was observed at ~29 kDa, 24 and 48 h after treatment with either mitomycin C or anti-CD95 agonistic antibody (Fig. 2A). Furthermore, immunoblotting with the M30 antibody

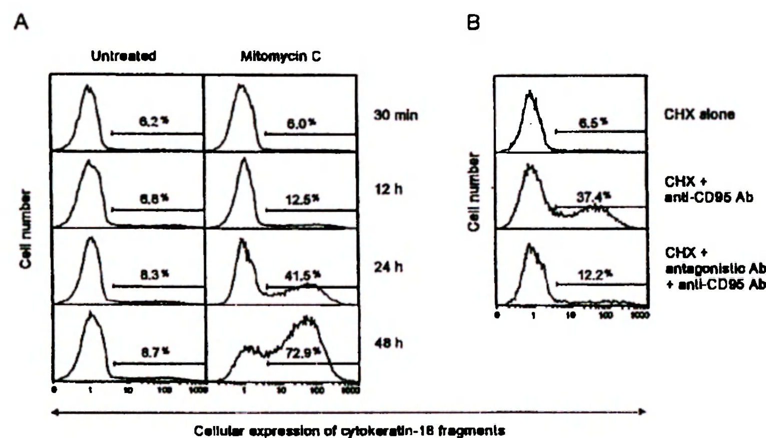

Fig. 1. Detection of intracellular cytokeratin-18 cleavage in MCF-7 cells after apoptosis-inducing treatments. A: Cellular expression of cytokeratin-18 fragments after treatment with mitomycin C. Cells were treated at various times with mitomycin C (8 µg/ml) and harvested together for staining. Plasma membranes were permeabilized, and cells were stained with the M30 antibody for flow cytometric analysis. B: Expression of cytokeratin-18 fragments after treatment with anti-CD95 agonistic antibody. Cell cultures were pretreated with the CD95-sensitizing agent CHX (3 µg/ml), and one culture received an antagonistic anti-CD95 antibody (0.5 µg/ml), as shown. Six hours later, specified cultures were treated with agonistic antibody (0.1 µg/ml), incubated for an additional 46 h, and stained as above. Results are representative of three independent experiments.

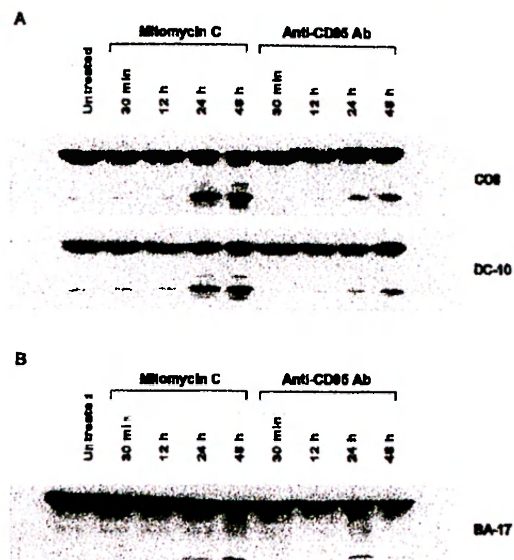

Fig. 2. Detection of intracellular cytokeratin-18 and -19 cleavage by immunoblotting. Cultures of MCF-7 cells were treated together and harvested at the time-points shown. Floating cells were pelletted by centrifugation, adherent cells were rinsed 3x with PBS and lysed, and floating cells were pooled with corresponding lysates before immunoblotting. A: Cytokeratin-18 cleavage. Upper and lower gels were probed with distinct anti-cytokeratin-18 antibodies, clones C08 and DC-10. Mitomycin C was used at a concentration of 8 μg/ml. Anti-CD95 antibody-treated cultures were pretreated with CHX (3 μg/ml) for 6 h prior to addition of 0.1 μg/ml of the anti-CD95 agonistic antibody. B: Cytokeratin-19 cleavage was detected using anti-cytokeratin-19 antibody BA-17.

identified similar bands of cytokeratin-18 fragments within cells (data not shown). Paralleling cytokeratin-18 fragmentation, cleavage of cytokeratin-19 (~40 kDa) was also observed, with fragments detected at ~27 kDa in treated cells (Fig. 2B). Collectively, these results indicate that cytokeratin-18 and -19 are cleaved intracellularly during apoptosis in MCF-7 cells.

Cellular Release of TPS and CYFRA 21-1 During Apoptosis

To examine whether cytokeratin fragments might be released from cells during apoptosis, aliquots were collected from tissue culture supernantants of mitomycin C-treated cells and assayed for their content of soluble cytokeratin-18 and cytokeratin-19 fragments by TPS and CYFRA 21-1 assays. Although no increase in soluble cytokeratin fragments was detectable within the first 12 h of treatment with 8 μg/ml mitomycin C, a greater than 4-fold increase was observed 24 and 48 h after treatment (Fig. 3A). A lower dose of 2 μg/ml, chosen to approximate the serum concentration of mitomycin C after bolus administration of 20–25 mg/m^2 [McEvoy, 1999], consistently induced a small increase in soluble cytokeratin levels. A low spontaneous level of apoptosis in untreated cultures correlated with a detectable level of soluble cytokeratin fragments in corresponding culture supernatants. No fragments were detectable in cell-free culture medium containing 10% fetal bovine serum (data not shown).

The increases in soluble fragments of cytokeratins induced by mitomycin C correlated temporally with the initiation of apoptosis. Large increases in the frequencies of cells exhibiting intracellular fragments of cytokeratin-18, as well as phosphatidyl serine exposure on the outer leaf of the plasma membrane and uptake of the vital dye propidium iodide, were detected 24 and 48 h after mitomycin C treatment (Fig. 3A). In contrast, an increase in the frequency of cells exhibiting DNA fragmentation (a late marker of apoptosis in most epithelial cells) was not detectable until 48 h after treatment with mitomycin C (Fig. 3A) or agonistic anti-CD95 antibody (Fig. 3B and data not shown).

A 5–7-fold increase in levels of soluble cytokeratin fragments was observed during CD95-mediated apoptosis (Fig. 3B). Pretreatment with an antagonistic anti-CD95 antibody substantially blocked both apoptosis and the release of soluble cytokeratin fragments induced by agonistic antibody. Taken together, these results show that the same monoclonal antibody-based assays used for detection of cytokeratin-18 and -19 fragments in patient sera also detect cytokeratin-18 and -19 fragments in cell culture supernatants after apoptosis induction. Thus, drug-induced and death receptor-mediated apoptosis can involve the release of TPS and CYFRA 21-1 from epithelial cells.

An Essential Role of Caspases in Producing Extracellular TPS and CYFRA 21-1

To examine the role of caspases in the production of soluble cytokeratin fragments during apoptosis, cells were simultaneously treated with mitomycin C and the broad-spectrum caspase-inhibitor Z-VAD.fmk. Z-VAD (25 μmol) abrogated both the intracellular cleavage of cytokeratin-18 and the release of TPS and CYFRA 21-1 occurring 24 h after mitomycin C

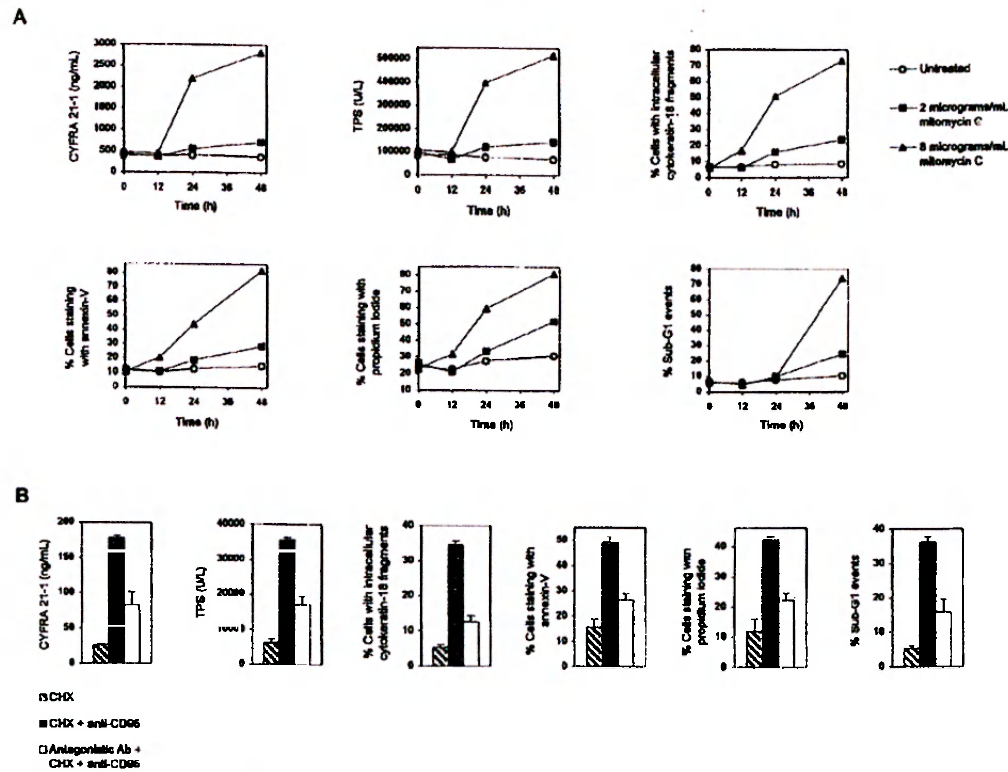

Fig. 3. Generation of TPS and CYFRA 21-1 during apoptosis. MCF-7 cells were induced to undergo apoptosis and cultures were assayed for TPS, CYFRA 21-1 and for both intracellular and surface markers of cell death. Results are representative of three independent experiments. A: Time-course for detection of TPS and CYFRA 21-1 during mitomycin C-induced apoptosis. Cell cultures were plated together, treated at different time-points with mitomycin C (8 μg/ml, triangles; 2 μg/ml, squares; untreated, circles), and aliquots of tissue culture supernatants were collected at the same time for analyses. Simultaneous assays were performed to determine the percentages of cells exhibiting. (i) intracellular fragments of cytokeratin-18, (ii) surface staining with annexin-V, (iii) uptake of the vital dye propidium iodide, and (iv) a sub-G1 amount of DNA. B: Generation of TPS and CYFRA 21-1 during CD95-mediated apoptosis. MCF-7 cells were pretreated with CHX (3 μg/ml) and antagonistic anti-CD95 antibody for 6 h, and subsequently incubated for 48 h with agonistic anti-CD95 antibody. Means of triplicates are shown ± standard deviations.

treatment (Fig. 4A). Z-VAD did not completely eliminate mitomycin C-induced cell death as measured by annexin V and propidium iodide staining, indicating that a fraction of cells had undergone caspase-independent death in the presence of the caspase inhibitor, without releasing TPS or CYFRA 21-1.

To confirm the essential role of caspases in generating soluble cytokeratin-18 and -19 fragments in culture supernatants, quantitation of TPS, CYFRA 21-1, and apoptosis was performed 24 h after treatment of cells with anti-CD95 agonistic antibody in the presence or absence of 10 μmol Z-IETD.fmk (a specific inhibitor of caspase-8, the apical caspase in the CD95 signaling pathway). Z-IETD.fmk strongly inhibited both the generation of soluble cytokeratins and cell death after signaling through CD95 (Fig. 4B). Altogether, these results obtained with two distinct caspase inhibitors indicate that caspase activity is critically required for drug-induced and death receptor-induced increases in the levels of extracellular TPS and CYFRA 21-1.

DISCUSSION

The data presented here demonstrate that apoptosis-inducing treatment of MCF-7 epithelial cells causes multi-fold elevations in extracellular TPS and CYFRA 21-1 concommitantly with significant increases in intracellular cytokeratin fragments, exposure of phosphatidyl serine on the outer leaflet of the plasma

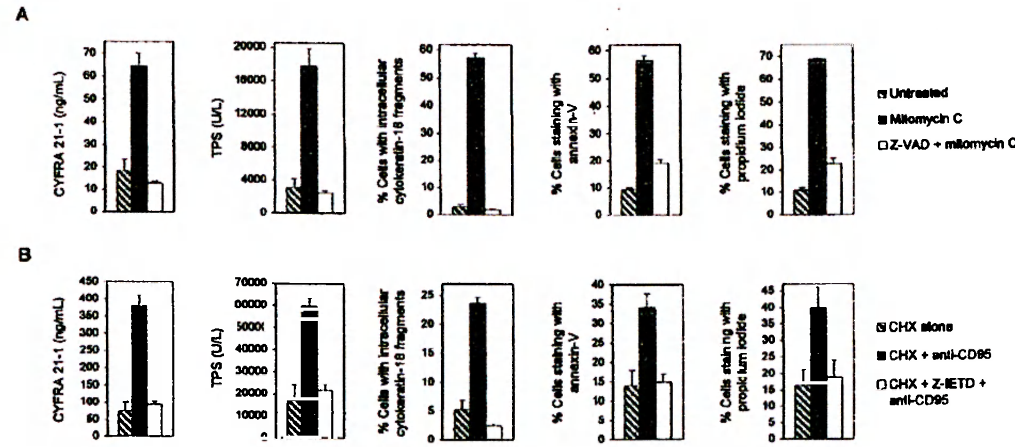

Fig. 4. Caspase inhibitors suppress the generation of intracellular cytokeratin-18 fragments, TPS, and CYFRA 21-1 after treatment of cells with apoptosis-inducing stimuli. **A**: Effect of the pan-caspase inhibitor Z-VAD.fmk on the levels of TPS, CYFRA 21-1, and cell death after mitomycin C treatment. MCF-7 cells were incubated with mitomycin C (8 μg/ml) and Z-VAD.fmk (25 μmol) for 24 h. Means of triplicates are shown ± respective standard deviations. Results are representative of three independent experiments. **B**: Effect of the caspase-8 inhibitor Z-IETD.fmk on the levels of TPS, CYFRA 21-1, and cell death induced by anti-CD95 antibody treatment. MCF-7 cells were treated with CHX overnight (12 h), certain flasks were treated with Z-IETD.fmk (10 μmol) for 1 h, and then specified cultures were incubated with agonistic anti-CD95 antibody for 24 h prior to analyses.

membrane, and loss of propidium iodide exlusion, but preceding the late apoptotic event of DNA fragmentation. Abrogation of these elevations by caspase inhibitors indicates that release of TPS and CYFRA 21-1 into the extracellular space occurs specifically during apoptosis, since caspases are not active in necrosis. Furthermore, mitomycin C-treated cells dying by caspase-independent death in the presence of the Z-VAD caspase inhibitor did not release measurable TPS and CYFRA 21-1, at least within the time period examined (Fig. 4A). Based on these findings, we conclude that cellular release of TPS and CYFRA 21-1 occurs during the intermediate stage of apoptosis, as a consequence of caspase activation.

Intracellular cleavage of cytokeratin-18 has previously been reported to occur during etoposide-induced apoptosis of SNG-M human endometrial adenocarcinoma cells and HR-9 mouse parietal endodermal cells [Caulin et al., 1997] as well as in TRAIL-induced apoptosis of MCF-7 cells [MacFarlane et al., 2000]. The data presented here extend these findings by suggesting that increased levels of cytokeratin-18 and -19 fragments can serve not only as intracellular markers, but also as an extracellular markers of apoptosis.

In contrast with necrosis, apoptosis is believed to be a form of cell death that results in an intact plasma membrane and/or membrane-bound apoptotic bodies, without inducing inflammation [Bellamy et al., 1995]. However, the presence of TPS and CYFRA 21-1 in the extracellular space indicates that apoptotic cells can release intracellular components. Consistent with this finding, it has been recently argued that apoptosis can be accompanied by a low or even substantial level of inflammation [Restifo, 2000]. Interestingly, a third form of cell death, termed autophagic cell death, has recently been proposed in which degradation of organelles occurs without substantial cleavage of cytokeratins [Bursch et al., 2000]. Thus, treatment of MCF-7 cells with inducers of autophagic cell death such as tamoxifen did not result in significant cleavage of cytokeratins [Bursch et al., 2000].

In numerous clinical laboratories, levels of TPS and CYFRA 21-1 in the sera of cancer patients have been determined as an indirect measure of tumor progression [Nisman et al., 1998; Rebhandl et al., 1998; Tempfer et al., 1998; Nagler et al., 1999; Doweck et al., 2000], although the utility of this approach has been challenged for some tumor types [Plebani et al., 1993; Wollenberg et al., 1996; Theyer et al., 1999; Valik and Nekulova, 2000]. Since the release of TPS and CYFRA 21-1 can correlate with apoptosis in vitro, further studies

are warranted to determine whether apoptotic epithelial cells can be a significant source of extracellular, soluble cytokeratins in vivo. Notably, levels of TPS have been reported to be elevated in sera of patients with hepatitis B or alcoholism [Kao et al., 1994; Gonzalez-Quintela et al., 2000], and elevated levels of CYFRA 21-1 were reported in sera of patients with interstitial pneumonia [Dobashi et al., 1999; Fujita et al., 1999]. Whether the source of elevated TPS and CYFRA 21-1 is apoptotic cells or regenerating cells is unclear. Given the current data, it has become an open question whether the constitutive low level of apoptosis occurring in most tumors is sufficient to generate a cumulative and detectable increase in the serum concentration of TPS and CYFRA 21-1.

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PERSPECTIVE

Homocysteine: exploring its potential as a pharmacodynamic biomarker of antifolate chemotherapy

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For decades it has been well known that elevated levels of homocysteine are harmful to humans on the basis of clinical observations derived from classical model diseases, such as inherited metabolic disorders. This group of diseases includes classical homocystinuria and several other inherited diseases affecting the so-called 'transsulfuration pathways'. Homocysteine lies in a metabolic checkpoint that interconnects one-carbon-transferring reactions with metabolism of sulfur-containing amino acids, since every molecule of 5-methyltetrahydrofolate derived either from plasma or generated from other folate species must be demethylated to liberate the reduced tetrahydrofolate. This unidirectional mechanism operates in every cell and has no alternative in eukaryotic cells. Antifolates are a group of anticancer agents targeting various metabolic steps within folate metabolism. They exert an indirect influence on the rate of appearance/disappearance of homocysteine from cellular and plasma compartments. Recently, it has been postulated that homocysteine may be a marker of the 'pharmacodynamic effect' of methotrexate, but studies attesting to this role are only now emerging. Here, we explore the genetic disease of folate and homocysteine metabolism and discuss the links between these model disorders with pharmacology and pharmacogenetics of folate antagonists used in the clinic. We outline possible ways of how homocysteine may be used as a biomarker of antifolate therapy.

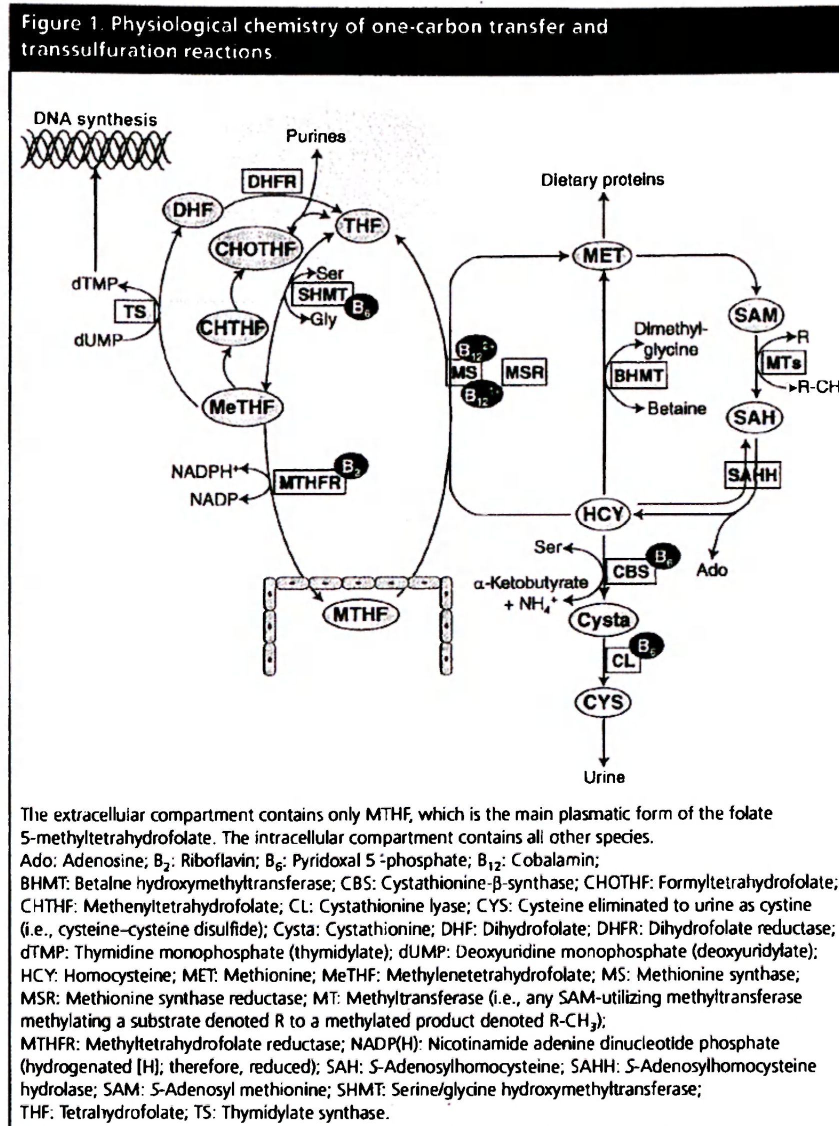
For decades it has been well known that elevated levels of homocysteine are harmful to humans on the basis of clinical observations derived from classical model diseases, such as inherited metabolic disorders. This group of diseases includes classical homocystinuria and several other inherited diseases affecting the so-called 'transsulfuration pathways' [1,101], the purpose of which is to preserve sulfur within an eukaryotic organism. Metabolic disturbances in these pathways, whether induced by an inherited disease or environmental condition, such as pyridoxine, folate and/or cobalamin deficiencies, commonly lead to an increase in concentrations of several plasma sulfur-containing amino acids; namely, homocysteine, homocystine, and the mixed disulfide cysteine–homocysteine [2]. In fact, all three measurable species are various oxidative forms of two amino acids: cysteine and homocysteine. Dominant clinical features of these disorders include severe, progressive neurological damage and, importantly, increased diathesis (propensity) for hypercoagulation and vascular disease [1].

The metabolism of sulfur-containing amino acids is biochemically integrated with a group of reactions termed 'one-carbon transfer reactions'. These reactions channel various oxidative states of a methyl moiety toward further cellular utilization, such as the synthesis of precursors of nucleic acids, and has been comprehensively detailed elsewhere [1,101]. Homocysteine is a normal metabolic intermediate arising from the demethylation of methionine, whose methyl moiety is then utilized for a variety of essential reactions [3]. Three vitamins serve as cofactors influencing homocysteine levels: pyridoxal 5′-phosphate (vitamin B_6) for the cystathionine-forming reaction; and folate (vitamin B_{10}) and cobalamin (vitamin B_{12}) for the methionine-resynthesizing reaction, where 5-methyltetrahydrofolate (5-methyl-THF) serves as a cosubstrate and methylcobalamin as a cofactor (Figure 1). Homocysteine, therefore, lies in an important metabolic branch point that interconnects one-carbon-transferring reactions with the metabolism of sulfur-containing amino acids. In other words, its pivotal metabolic significance lies in its ability to 'shuttle' between completing methionine degradation or replenishing the methionine pool. Whereas methionine degradation through the pyridoxine-dependent cystathionine pathway occurs mainly in the liver, which actively extracts plasma homocysteine, the remethylation process takes place in every eukaryotic cell. It is catalyzed by either betaine-dependent

Keywords: biomarker, homocysteine, methotrexate, pharmacogenetics

future medicine

Figure 1. Physiological chemistry of one-carbon transfer and transsulfuration reactions



The extracellular compartment contains only MTHF, which is the main plasmatic form of the folate 5-methyltetrahydrofolate. The intracellular compartment contains all other species.
Ado: Adenosine; B_2: Riboflavin; B_6: Pyridoxal 5'-phosphate; B_{12}: Cobalamin;
BHMT: Betaine hydroxymethyltransferase; CBS: Cystathionine-β-synthase; CHOTHF: Formyltetrahydrofolate; CHTHF: Methenyltetrahydrofolate; CL: Cystathionine lyase; CYS: Cysteine eliminated to urine as cystine (i.e., cysteine–cysteine disulfide); Cysta: Cystathionine; DHF: Dihydrofolate; DHFR: Dihydrofolate reductase; dTMP: Thymidine monophosphate (thymidylate); dUMP: Deoxyuridine monophosphate (deoxyuridylate); HCY: Homocysteine; MET: Methionine; MeTHF: Methylenetetrahydrofolate; MS: Methionine synthase; MSR: Methionine synthase reductase; MT: Methyltransferase (i.e., any SAM-utilizing methyltransferase methylating a substrate denoted R to a methylated product denoted R-CH_3);
MTHFR: Methyltetrahydrofolate reductase; NADP(H): Nicotinamide adenine dinucleotide phosphate (hydrogenated [H]; therefore, reduced); SAH: *S*-Adenosylhomocysteine; SAHH: *S*-Adenosylhomocysteine hydrolase; SAM: *S*-Adenosyl methionine; SHMT: Serine/glycine hydroxymethyltransferase;
THF: Tetrahydrofolate; TS: Thymidylate synthase.

methyltransferases, E.C. 2.1.1.3 and 2.1.1.5, which are confined to the liver [4], or, alternatively, by a ubiquitous 5-methyl-THF-utilizing transferase, E.C. 2.1.1.13 (methionine synthase [MS]) [5].

Different forms of homocysteine are found in plasma

Several forms of homocysteine coexist in human plasma. The high reactivity of the thiol group in its molecule is responsible for its 'sticky' properties resulting in a number of daughter species, which are observed in human plasma. Traditionally recognized forms are:

- homocysteine
- homocysteine disulfide (homocystine)
- cysteine–homocysteine mixed disulfide
- homocysteine linked to plasma proteins by means of -SH groups (Hcy-*S*-protein), which is represented by a major portion of homocysteine bound to albumin [2]

Because of this complexity, plasma homocysteine is often referred to as total homocysteine and/or homocyst(e)ine [6], reflecting the fact that total homocysteine is indeed what is generally measured by techniques employing the initial reduction step before analysis. Such a common step reduces soluble disulfides and liberates the Hcy-*S*-protein portion of homocysteine – which is quantitatively dominant – to give rise to a uniform (but, in fact, artifactual) species 'total homocysteine' (tHcy), which is easily measurable by various techniques [7].

More understanding of the plasma forms of homocysteine has been recently derived from experiments by Jakubowski, who showed that homocysteine links to proteins through an ε-amino group of lysine (Hcy-*N*-protein), and that a number of other and potentially toxic forms occur *in vivo*; specifically, homocysteine thiolactone and *S*-nitroso-homocysteine [8]. Several other homocysteine-related precursor and/or daughter species occur *in vivo* as well, such as homocysteic acid and *S*-adenosylhomocysteine, but their role in the pathogenesis of homocysteine-related disorders has not yet been elucidated.

Homocysteine is related to a number of disease conditions

Homocysteine became an attractive molecule in 1976 when Wilcken and Wilcken published their observation that, in male subjects with angiographic evidence for ischemic heart disease, plasma cysteine–homocysteine mixed disulfide concentrations were elevated to the same extent as in obligate heterozygotes for cystathionine-β-synthase (CBS) deficiency after methionine load [9]. Since then, a large number of studies investigated the possible role of moderately elevated plasma homocysteine levels in premature vascular disease (for a review, see [10] and references therein). However, despite a large body of evidence on the possible and, perhaps, causative role of homocysteine in the pathogenesis of vascular disease, this concept has not yet gained universal acceptance. Rather, mildly elevated plasma levels of homocysteine in patients with coronary artery disease may be a 'bystander' effect caused by environmental factors, such as lifestyle, smoking and/or low vitamin intake [11].

Pathogenesis of homocysteine-related vascular damage remained poorly understood until recent work by Jakubowski [12], who presented convincing evidence that homocysteine may be incorporated into proteins by an epigenetic mechanism. He showed that homocysteine can enter the protein biosynthetic apparatus and, through the formation of homocysteine thiolactone, modify proteins post-translationally in all the organisms that were investigated, including humans. Furthermore, he demonstrated that levels of homocysteine bound by amide or peptide linkages (Hcys-*N*-protein) in human plasma proteins are directly related to plasma tHcy levels and that homocysteine-induced protein damage can trigger autoimmune response, thus putatively contributing to the pathology of human disease [8,12].

Homocysteine, folates and one-carbon transfer reactions

Since homocysteine intercepts transsulfuration metabolism and the metabolism of one-carbon transfer folate-dependent reactions, a brief background on folate physiology will further help to understand this important point (see also Figure 1). Folate physiology has been extensively reviewed [13,14]. The term 'folate' denotes a group of pteridine-ring-containing compounds covalently linked to para-aminobenzoic acid and a glutamate moiety. Owing to redox properties of their pteridine ring, these compounds can exist *in vivo* in different oxidative states and carry a methyl moiety either at and/or between positions 5 and 10 of the pteridine heterocycle.

After ingestion, folates from food are reduced and methylated in the gut at the N-5-position of a pteridine ring, giving rise to 5-methyl-THF (monoglutamate form), which is the principal form of folate occurring in plasma within the low nanomolar range of 5–25 nmol/l [15]. The 5-methyl-THF is then taken up by cells from the plasma through two classes of cell membrane transporters and concentrated up to a micromolar range within the intracellular compartment; nevertheless, in many tissues, 5-methyl-THF remains the main intracellular form of folate. Two distinct systems exist for the transport of folates and antifolates across mamalian cell membranes:

- a reduced folate carrier (RFC) is a high-capacity, low-affinity system also responsible for the import of methotrexate at micromolar concentrations [16]
- a folate-binding protein (FBP), a family of membrane-associated receptors, which provide a low-capacity, high-affinity transport system that operates at nanomolar concentrations of extracellular folate [17]

To conserve a majority of cellular folates, eukaryotic cells developed a mechanism to add up to

seven moieties of glutamic acid through the action of folylpolyglutamate synthetase [18]. This modification is crucial in keeping the intracellular pool of folates available for essential reactions, such as the synthesis of purine bases and thymidylate. In fact, polyglutamylated forms are now believed to be the active cosubstrates (this denomination should be preferred over the more traditional term cofactors) of folate-dependent reactions due to their generally lower K_ms, and they also serve as intracellular storage forms of folate [19].

However, before this intracellular mechanism can take effect, 5-methyl-THF, derived either from plasma or generated from other reactive folate species, such as 5,10-methylene-THF through the methyltetrahydrofolate reductase (MTHFR)-catalyzed reaction, must be demethylated to liberate reduced THF, thus restoring the metabolically active THF pool. This reaction mechanism operates in every cell (a MS reaction) and transfers the methyl moiety of 5-methyl-THF to an ubiquitous metabolic intermediate, homocysteine, by utilizing cobalamin as a cofactor. Furthermore, this reaction is essentially unidirectional and has no alternative in eukaryotic cells so every molecule of 5-methyl-THF, once formed, in every eukaryotic cell, must pass through the MS reaction to regenerate active THF. It is, therefore, this mechanism that essentially links one-carbon transfer processes to the metabolism of sulfur-containing amino acids, employing homocysteine as a metabolic shuttle (Figure 1). Indeed, upon cobalamin deficiency, a condition known as 'methyl trap' occurs [15]. In this situation, all active folates are 'trapped' as 5-methyl-THF despite the fact that high concentrations of folate (in the form of 5-methyl-THF) are found in plasma and intracellularly (e.g., in erythrocytes). Clinically, this condition presents as megaloblastic anemia (coined 'pernicious' anemia) with concurrent laboratory findings of hyperhomocysteinemia.

Homocysteine and folate antagonists

Folate antagonists were brought to clinical practice by Farber in 1948 [20] and have remained the mainstay of anticancer therapy in a number of pediatric and adult malignancies. The active compound most often used clinically is methotrexate (amethopterin, 4-amino,10-methylpteroylglutamic acid [21]), although a number of new folate antagonists targeting more specifically various metabolic steps within the folate metabolism have been brought to the clinic. Recent knowledge on their mechanisms of action and modes of resistance has been excellently reviewed [22]. At present, a 'high-dose' administration format ramping up to tens of grams per square meter is the preferred dosing of methotrexate in pediatric oncology, where it is used predominantly for the treatment of children with acute lymphoblastic leukemia (ALL), non-Hodgkin's lymphoma, and osteosarcoma [21,23]. Low-dose methotrexate therapy is also used in the clinic, but commonly for treatment of non-oncologic, chronic disorders, where immune derangement is believed to be responsible for pathogenesis, such as rheumatoid arthritis, psoriasis, and ulcerative colitis [21,24,25].

Methotrexate is a tight-binding inhibitor of dihydrofolate reductase (DHFR), the enzyme responsible for the reduction of folates to their metabolically active THF form. Traditionally, decreased rates of thymidylate synthesis from deoxyuridylic acid is considered a critical determinant of methotrexate cytotoxicity since this is the only one-carbon transfer reaction that oxidizes the THF cofactors to their inactive dihydrofolate form [26]. Several other enzymes in the purine biosynthetic pathway are also inhibited by methotrexate. A major mechanism of anticancer action of methotrexate has been, therefore, attributed to the inhibition of DNA synthesis through the depletion of nucleotides and misincorporation of deoxyuridine monophosphate (dUMP) into DNA, as well as RNA to some extent [21,22,26,27]. Another determinant of the methotrexate effect appears to be an excess of binding sites on DHFR, because intracellular levels of this target enzyme are 20–30 times higher than the required amount to maintain the necessary THF pool [28].

A novel mechanism of methotrexate action has been recently described by Casey and co-workers [29]. They showed that the inhibition of isoprenylcysteine carboxyl methyltransferase, which is responsible for carboxylmethylation of a G protein Ras and its accurate localization into the cell membrane, is a critical component of antiproliferative action of methotrexate. The inhibition of homocysteine remethylation due to folate-depleting antimetabolite methotrexate led to an increase in the intracellular concentration of *S*-adenosylhomocysteine, which is a potent inhibitor of cellular methyltransferases [30]. Methyltransferase inhibition leads to a disruption of the Ras-signaling pathway and the cessation of cell growth. The authors, however, do not refute the traditionally described mode of action of

methotrexate [29]. Rather, they raise an important point, stating that 'the relative contribution of any single mechanism may vary from tumor to tumor and cell to cell' [29]. Furthermore, it may be reasonable to speculate that interference with cellular methylation at the metabolic level may translate itself into disturbances of methylation occurring at the levels of DNA, proteins and also small molecules, such as polyamines, which, in general, possess regulatory properties [30].

Besides sporadic reports [31-33], cellular events occurring downstream from the inhibition of DNA synthesis by methotrexate have remained largely unexplored. Recent data from our laboratory (unpublished) are indicating that chemotherapy-naive lymphoblasts isolated from the bone marrow of patients with ALL cultivated *ex vivo* with methotrexate resulted in changes in expression of the p53 protein and in upregulation of several p53-regulating genes [34-36]. The p53-dependent cellular events (apoptosis and/or G1 arrest) may, thus, be the downstream events occurring in response to DNA damage caused by dUMP misincorporation and subsequent DNA strand breaks [37-39]. These preliminary results may broaden our understanding of the mechanism of methotrexate action at the cellular level going beyond the traditional DHFR inhibition effect.

Homocysteine as a marker of antifolate effects

Despite the major progress achieved in cancer therapy at genomic, proteomic and 'metabolomic' levels in recent years, standard drug dosing is still mostly based on body-surface-area (BSA) calculations. Due to its very nature, such an approach completely ignores individuality at the level of a tumor and a host ([40] and web-linked comments). Consequently, drug dosage based on BSA is precise but lacks the biological accuracy necessary to tailor therapy more specifically to variables such as the individual needs of an individual patient at a given time during the course of the disease. Therefore, a search for biomarkers has been ongoing and has speeded up in recent years with the present advents in pharmacogenomics [41] and predictive oncology [42].

Under optimal conditions, a marker of biological response to a given drug should be an *a priori* predictor of toxicity and/or efficacy, or both. Homocysteine may be an endogenous species that potentially fulfills this criteria for a biomarker. Indeed, antifolates have been shown to exert a profound effect on homocysteine concentrations at cellular levels [39] and in methotrexate-treated patients [43-45], but evidence gathered from these studies has been rather descriptive and unable to build a foundation for defining a predictive model. It was not until recently, when the clinical introduction of a new antifolate, pemetrexed, which belongs to a group coined 'multitarget antifolates', promoted publications of initial but yet sparse data relating its antifolate effect to plasma levels of homocysteine and methylmalonic acid [46]. In another study coordinated by researchers from Eli Lilly laboratories, Niyikiza and co-workers [47] showed that 'homocysteine and methylmalonic acid may be markers to predict and avoid toxicity from pemetrexed therapy'. They pointed out that supplementation of folic acid and vitamin B_{12} prior to pemetrexed administration may be protective against pemetrexed-induced toxicity 'while maintaining or possibly improving efficacy' [48]. These results appear attractive, nevertheless, given recent knowledge, this statement seems to be inadequately supported by literary data. However, studies investigating relations of homocysteine and folate antagonists re-emerged in the literature and, recently, it has been directly postulated that homocysteine is a marker of the 'pharmacodynamic effect' of methotrexate [49]. In line with this concept is our recent observation that in a patient treated with high-dose methotrexate, homocysteine plasma levels paralleled development and the course of nearly fatal neurotoxicity. Furthermore, it was noted that the pre-therapy plasma level of folate were discriminators of toxic and non-toxic chemotherapy courses in the same patient [41].

Homocysteine, methotrexate, and neurotoxicity

The primary toxic effects of methotrexate are myelosupression and orointestinal mucositis occurring 5–14 days after dosing. The development of toxic reactions is related to the concentration of the drug and duration of exposure [21,50]. Neurotoxicity of various severity occurs in some children treated with high-dose methotrexate and remains one of the most serious adverse effects of this therapy in pediatric oncology [51]. Neurotoxicity occurs in two clinically distinct forms differing by the onset of symptoms: an early-onset and a late-onset form [52].

Three principal hypotheses have been suggested to elucidate methotrexate-induced neurotoxicity; nevertheless, experimental and clinical data remain sparse and somewhat conflicting. An attractive hypothesis has been raised by Cronstein [53]. He showed increased levels of

locally liberated adenosine in the site of methotrexate-treated inflammation. Since adenosine is a molecule with significant neuromodulatory properties, antagonists of adenosine receptors have been thought to alleviate symptoms of neurotoxicity and, indeed, were brought to the clinic in 1995 [54]. Interference of methotrexate with the recycling of biopterin species led to a hypothesis that disturbances of metabolism in dopamine and serotonin [55] may result in clinically manifested neurotoxicity. In support of this concept are inherited disorders of biopterin metabolism, such as 'non-classical' variants of phenylketonuria. Smith *et al.* published an interesting observation linking biopterin metabolism with folate antagonists and concluded that in DHFR deficiency, cerebral calcifications may develop in a similar distribution to that seen in congenital folate malabsorption and methotrexate toxicity. Furthermore, they were able to demonstrate that symptoms are ameliorated by 5-formyltetrahydrofolic acid but exacerbated by folic acid [56]; the reason is that folinic acid (leucovorin [5-formyltetrahydrofolic acid]) bypasses the otherwise indispensable MS reaction exclusively regenerating THF (Figure 2). Microvascular injury mediated by homocysteine represents the third category of hypotheses to explain methotrexate-induced neurotoxicity: this concept is derived from knowledge that sequelae of methotrexate therapy include focal neurological deficits, white matter lesions, and mineralizing microangiopathy [57,58].

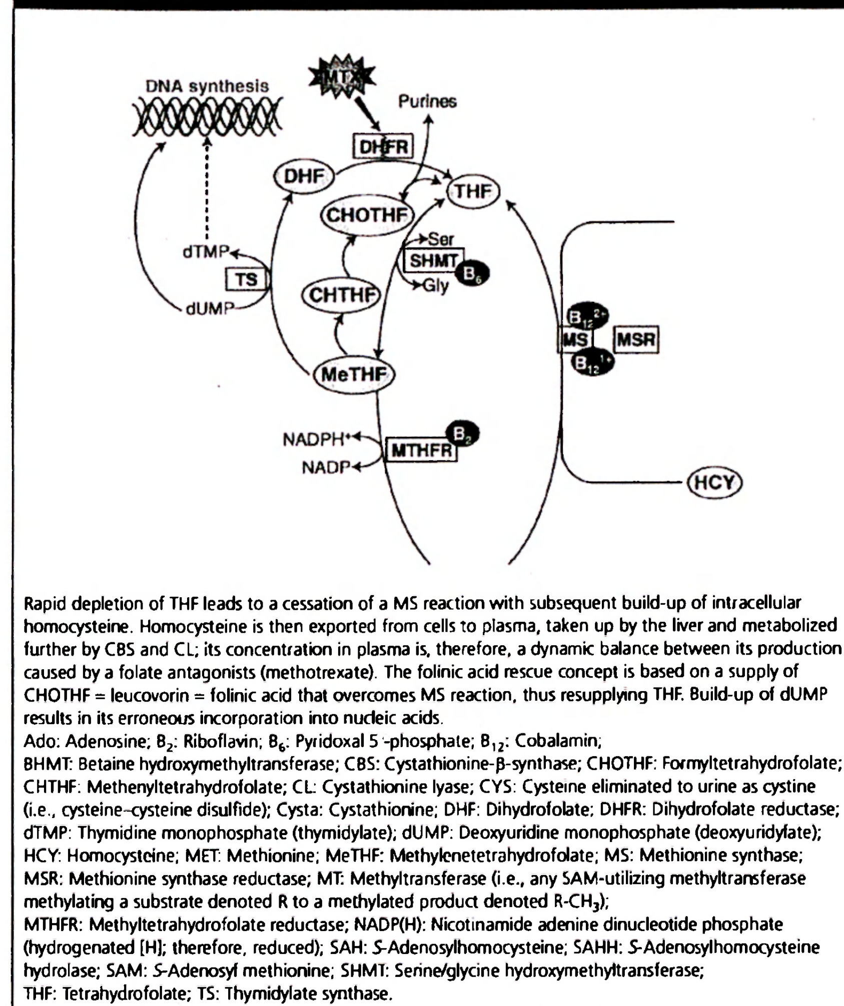
An interesting observation regarding methotrexate-related neurotoxicity and homocysteine levels in cerebrospinal fluid (CSF) has recently been reported by Quinn [59]. They studied serial CSF samples from a child during two courses of intraventricular methotrexate and found a rapid and reproducible depletion in CSF of reduced folates and *S*-adenosylmethionine, which was accompanied by marked increases in homocysteine and adenosine; they were not able to detect any sulfur-containing excitatory amino acids.

Future prospective: homocysteine, pharmacogenomics and assessment of response

When considering scientifically based concepts of pharmacotherapy, a central 'dogma' of pharmacology is that the effect of a given drug is directly related to an overall drug exposure expressed as achieved concentrations over a period of time. Upon integration, this parameter can be quantitatively expressed as the area under curve [60]. Inter- and intra-individual variabilities in a patient's response to a given drug are recognized determinants of therapy efficacy, because these factors have a critical influence on drug exposure [61]. The genetic background of a drug recipient recently emerged as a critical determinant of individual clinical outcome and a need of dose individualization has led to a convergence of pharmacogenomics and biomarker research [41]. The reasons are obvious. Owing to the complexity of genotype–phenotype interactions and generally unknown and/or poor agreement between transcriptome and proteome [62], a multi-level assessment may be more informative for an individual and patient-orientated clinical decision. Assessing a patient's pharmacogenetic background may identify those individuals who are *a priori* at lifelong risk of side effects from a given therapy. Then, follow up of a target biomarker(s) over a period of time may provide information on treatment efficacy and (under optimal scenario) dose prediction and/or dose tuning. Even though this concept may sound idealistic at present, the following example shows that it is, in fact, already in clinical practice. Before the administration of warfarin, genotype information can identify carriers of *CYP2C9* variant(s), and information regarding whether a desired effect has been achieved can be timely assessed using a thromboplastin test (expressed as an international normalized ratio [INR]) that provides values during the course of the treatment [63]. 'Functional' and pharmacogenetic assessment of biological action of methotrexate is much less straightforward. However, several tools have recently emerged to facilitate dynamic monitoring of clinical efficacy of this useful but quite toxic antineoplastic.

Pharmacology, pharmacokinetics and indications of methotrexate have been authoritatively described elsewhere [21]. Of pharmacogenetic interest may be its biotransformation resulting in a proportion of methotrexate being processed presumably through a liver cytochrome-containing hydroxylating system to a 7-hydroxymethotrexate. This compound retains some of the antifolate effect and toxicity of methotrexate but mostly competes with a parent drug for the cell membrane folate transporters. It is not yet clear which of the cytochrome superfamily members are responsible for this hydroxylation, but this metabolic event may be one of the pharmacogenetic determinants influencing exposure to methotrexate by governing the rate of its disappearance. The second determinant of

Figure 2. A section of Figure 1 showing methotrexate hitting its primary target, DHFR, and the downstream events related to homocysteine.



Rapid depletion of THF leads to a cessation of a MS reaction with subsequent build-up of intracellular homocysteine. Homocysteine is then exported from cells to plasma, taken up by the liver and metabolized further by CBS and CL; its concentration in plasma is, therefore, a dynamic balance between its production caused by a folate antagonists (methotrexate). The folinic acid rescue concept is based on a supply of CHOTHF = leucovorin = folinic acid that overcomes MS reaction, thus resupplying THF. Build-up of dUMP results in its erroneous incorporation into nucleic acids.
Ado: Adenosine; B_2: Riboflavin; B_6: Pyridoxal 5´-phosphate; B_{12}: Cobalamin;
BHMT: Betaine hydroxymethyltransferase; CBS: Cystathionine-β-synthase; CHOTHF: Formyltetrahydrofolate; CHTHF: Methenyltetrahydrofolate; CL: Cystathionine lyase; CYS: Cysteine eliminated to urine as cystine (i.e., cysteine–cysteine disulfide); Cysta: Cystathionine; DHF: Dihydrofolate; DHFR: Dihydrofolate reductase; dTMP: Thymidine monophosphate (thymidylate); dUMP: Deoxyuridine monophosphate (deoxyuridylate); HCY: Homocysteine; MET: Methionine; MeTHF: Methylenetetrahydrofolate; MS: Methionine synthase; MSR: Methionine synthase reductase; MT: Methyltransferase (i.e., any SAM-utilizing methyltransferase methylating a substrate denoted R to a methylated product denoted R-CH_3);
MTHFR: Methyltetrahydrofolate reductase; NADP(H): Nicotinamide adenine dinucleotide phosphate (hydrogenated [H]; therefore, reduced); SAH: *S*-Adenosylhomocysteine; SAHH: *S*-Adenosylhomocysteine hydrolase; SAM: *S*-Adenosyl methionine; SHMT: Serine/glycine hydroxymethyltransferase;
THF: Tetrahydrofolate; TS: Thymidylate synthase.

potential pharmacogenetic importance is DHFR itself. Unfortunately, reports from the literature deal mostly with an increase in expression of DHFR as a mechanism of antifolate resistance that develops in the tumor cells upon exposure to folate antagonists [22]. In pediatric ALLs, higher occurrence of DHFR overexpression is reported in T-lineage lymphoblasts compared with B-precursor lymphoblasts [64]. Nevertheless, essentially no data have been published regarding genetically determined decreased expression of DHFR, and possibly other enzymes participating in pteridine metabolism, somatic cells and concurrent treatment with antifolates. A search throughout model diseases, such as inherited diseases of folate/pteridine metabolism, reveals that links have been postulated between defects in bioterin and phenylalanine metabolism, and folate-related disorders on one side [55,56,65,66] and observed side effects of antifolates on the other. Folate antagonists may, in some way, mimic the natural course of these disorders (e.g., by occurrence of intracranial calcifications [65,67]), but these reports are rather anecdotal.

In summary, although genetic disorders affecting DHFR have been described, their clinical

presentation is heterogeneous and polymorphic. Moreover, some of the described diseases were later reclassified as different diagnostic entities and, in some disorders, enzyme deficiency was not demonstrable in the cell culture [101]. One possible explanation of the complexity of this phenomenon is that the bi-allelic germline mutation severely hampers the function of such a critical enzyme as DHFR and may be lethal. Indirect support for this concept is provided by the fact that folate deficiency causes fetal morphological malformations (specifically, neural tube defects) and increases the rate of spontaneous abortions [68,69]. The third possible pharmacogenetic determinant of antifolate treatment was described recently by Ulrich *et al.* [70]. They showed that the length of repeats in the promoter sequence of the thymidylate synthase gene (*TYMS*) (2rpt/2rpt versus 3rpt/3rpt variant) may be a determinant of toxicity/efficacy of thymidylate synthase inhibitors, such as fluoropyrimidines and the newer generation of antifolates. To recall, this is the first downstream reaction indirectly inhibited by methotrexate and, more specifically, by several newer antifolates, such as raltitrexed and pemetrexed (Figure 2). The occurrence of a 3rpt/3rpt variant was also associated with disturbances in plasma levels of homocysteine [71].

Unresolved issues: methotrexate, folate pretreatment, and interactions

Despite the rapid development of new cancer treatment modalities, high-dose methotrexate with folinic acid rescue will probably remain the key component in chemotherapy of pediatric malignancies, particularly acute lymphoblastic leukemia and non-Hodgkin's lymphoma. Hepatotoxicity and granulocytopenia are, in fact, regularly occurring unwanted, albeit reversible, side effects, but neurotoxicity remains the most feared complication among pediatric oncologists. Therefore, tools to avoid unnecessary toxicity and optimize treatment efficacy are needed. Evans and co-workers [72] were able to demonstrate a better outcome of children with individualized dosing of methotrexate compared to conventional dosing based on BSA. Supplementation of methotrexate with folate and vitamin B_{12} prior to methotrexate therapy remains an open and unresolved issue. Hills and Ive [73] showed that the concurrent daily administration of folinic acid with weekly oral methotrexate prevented an improvement of psoriasis, whereas folinic acid given every day except the day of methotrexate resulted in a marked improvement. This finding is of substantial importance because the authors assessed the clinical outcome – a similar design is not possible in contemporary oncology. Their conclusions are in line with the 'folinic acid rescue' concept, but they do not support the suggested folate and vitamin B_{12} supplementation prior to antifolate therapy. Until experimentally proven, the administration of cosubstrates and cofactors (e.g., folate and vitamin B_{12}) prior to therapy with antifolates [48] should be judged with caution. Such coadministration may, in fact, result in numerous interactions at the levels of membrane transport and intracellular enzyme targets, in addition to effects on polyglutamylation [19,21,74]. Unfortunately, studies addressing this issue are rarely found in the literature.

Our interim data investigating the time course of plasma homocysteine levels before, during and after administration of high-dose methotrexate clearly demonstrated that homocysteine levels rise in average to 200% of the pretreatment value (unpublished). This rise is sharply interrupted at the moment of administration of folinic acid, which occurs in hour 42 from the beginning of high-dose methotrexate (5 g/m^2) infusion according to the ALL-Berlin-Franfurt-Münster (BFM) 95 protocol [23]. In summary, current, although incomplete, knowledge offers up to three possible tools for the functional assessment of antifolate therapy. They may indicate:

- the rate of transformation to a less efficient and toxic compound: 7-hydroxymethotrexate
- the rate of body THF depletion: elevation of homocysteine with/without methylmalonic acid during and after drug administration
- pretherapy levels of plasma folate, homocysteine and methylmalonic acid, whose disturbances may be associated with impaired drug tolerance and, possibly, drug antitumor effects

Open question: biomarkers in the context of 'functional' pharmacogenomics

When considering biological reality at all levels (i.e., genomic, metabolic, cellular, organ, and patient), probably no single-gene and/or single-pathway approach will be sufficient enough to provide an accurate and comprehensive answer to a complex issue, even if it is seemingly well-defined, such as the administration of a drug with 'known' metabolism. In a recent review summarizing current knowledge on pharmacogenomics that also encompassed pharmacogenomic aspects of antifolate treatment, Ulrich *et al.* [70] discussed the benefits and

Highlights

- Homocysteine interconnects one-carbon-transferring metabolic reactions with metabolism of sulfur-containing amino acids and its pivotal metabolic significance is based in its ability to 'shuttle' between completing methionine degradation or replenishing the methionine pool.
- Whereas methionine degradation through pyridoxine-dependent cystathionine pathway occurs mainly in the liver, the remethylation process takes place in every eukaryotic cell and is catalyzed by an ubiquitous 5-methyl-THF-utilizing transferase called MS.
- Various forms of homocysteine occur in human plasma; a number of them are putatively involved in the pathogenesis of homocysteine-related disorders.
- Folate antagonists interfere with the metabolism of homocysteine by competing with natural reduced folate forms. This metabolization interference occurs at various levels: DHFR; folate transport/export through cell membrane; and intracellular folate polyglutamation.
- Because of the competitive nature of the interaction between homocysteine and antifolates, homocysteine was implicated as a biomarker of action of folate antagonists. Sporadic reports published, to date, described disturbances in homocysteine levels after treatment with folate antagonists.

drawbacks of different pharmacogenomic approaches, including array technologies. They concluded that a combination of methods and approaches may be better to help elucidate underlying mechanisms of drug-related toxicities and help bring such knowledge to the clinic.

Indeed, the current belief that genetic methods will help elucidate the mechanisms responsible for adverse reactions may not be entirely accurate, as genetic methods, including microarray technologies, are by their very nature morphological. As such, they may not be adequate in obtaining functional and time-dependent information, such as estimates of organ-localized enzyme activities along multiple metabolic pathways [75]. Inherited diseases of metabolism may again serve as instructive biological models. It has been shown that a variety of underlying mutations may be responsible for a given inherited disease. Many of these mutations were subsequently shown to be 'private' mutations occuring within one family. Such a complexity, paradoxically, increased the need for comprehensive genotype/metabolic phenotype and biomarker testing to gather interpretable and diagnostically relevant information. Translating this experience to contemporary 'postgenomic' pharmacogenetics, one may conclude that genomic methods are already in place, and proteomic and metabolomic methods are under rapid development in parallel with the advent of a variety of mass spectrometric techniques, such as tandem mass spectrometry and MALDI/SELDI-TOF. Such an armamentarium of methods, when combined with accurate clinical interpretation of data, may help accomplish the goal of individualized treatment.

Outlook and conclusions

Significant discovery of a new mechanism of homocysteine binding to proteins may promote research on biological interactions of such modified proteins with cellular and tissue environment, including carcinogenesis and the role of the immune system. Protein homocysteinylation may play a role in the development of toxicity of folic acid antagonists, specifically in its subacute forms, such as neurotoxicity, although no single mechanism will probably be responsible for the manifestation of a complex clinicopathological response. Homocysteine as a biomarker of the antifolate effect is subject to current investigation. As we outlined here, its potential is based on metabolic uniqueness and stoichiometry of the MS reaction where for every mole of 5-methyl-THF consumed, 1 mol of homocysteine is remethylated to methionine. It follows that all conditions that affect the supply and/or generation of 5-methyl-THF, such as the indirect action of antifolates through the inhibition of DHFR and subsequent depletion of 5-methyl-THF, lead to an increase of homocysteine. However, this phenomenon, although with sound theoretical foundation, is likely to be more complex *in vivo*, where a number of collateral and/or interacting events, such as rates of polyglutamylation, folate/antifolate transport and antifolate transformation/metabolism, in relation to endogenous folate levels and tissue stores will play important roles. Deciphering these events will be an important goal in defining the role of homocysteine as a biomarker of antifolate chemotherapy.

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Severe Encephalopathy Induced by the First but Not the Second Course of High-Dose Methotrexate Mirrored by Plasma Homocysteine Elevations and Preceded by Extreme Differences in Pretreatment Plasma Folate

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Key Words

Encephalopathy · Methotrexate · Folate · Homocysteine

Abstract

Plasma homocysteine has recently been associated with the occurrence of methotrexate-related neurotoxicity. We observed extreme elevations of homocysteine in a 9-year-old boy presenting with leukemia treated with the ALL-BFM 95 protocol. Coma occurred at about the 71st hour from the first methotrexate administration, and lasted for 30 h but MRI and CT studies showed no intracranial pathology. The second course of high-dose methotrexate was administered with no complications. Homocysteine areas under the curve (AUC) were calculated as the sum of areas of rectangles during the 6-hour intervals from T_0 to T_{72} hours (AUC_{0-72}) and methotrexate AUCs were evaluated using MW/PHARM3.3 software. The AUC of homocysteine during the first, toxic course was 5.2 times higher than AUC during the second administration, whereas AUC of methotrexate also differed by a factor of 5. Plasma concentrations of folate prior to the first and the second courses, respectively, were 4.4 versus 45 μmol/l making this difference the most striking discriminator between the two courses. Mutation analysis showed that the patient was heterozygous for the C677T mutation in the MTHFR gene. We suggest that plasma homocysteine, pretreatment plasma folate and possibly the presence of MTHFR mutations may be biomarkers of methotrexate toxicity and possibly its antifolate effect targeted towards the tumor as well.

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Introduction

Methotrexate has been in clinical use for nearly five decades and remains an important part of therapy of childhood lymphoblastic malignancies. However, mechanisms of its action, resistance and individual susceptibility are complex and incompletely understood. Furthermore, significant and sometimes fatal toxicity associated with high-dose methotrexate (HDMTX)-containing protocols still remains an unresolved issue [1–3]. Recently, plasma homocysteine has been associated with the occur-

D.V. presented an invited lecture containing part of this data at the TDM Renaissance and Pharmacogenomic Forum III organized by American Association of Clinical Chemistry (www.aacc.org), Baltimore, Md., February 2004.

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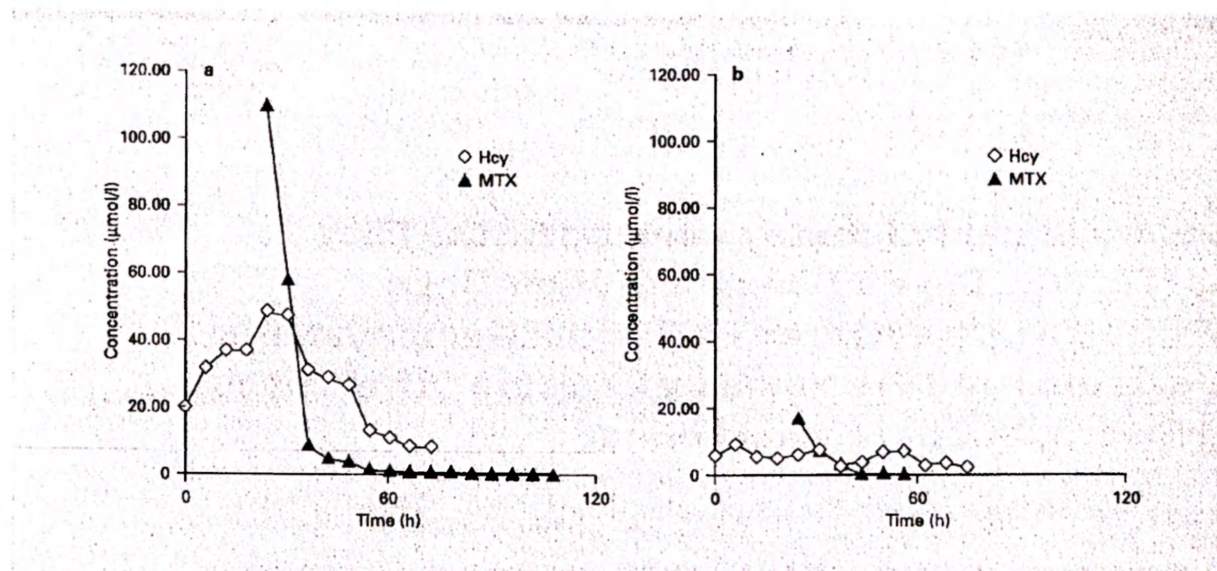

Fig. 1. Courses 1 (**a**) and 2 (**b**) are shown, each delivering the same dose of methotrexate. x axis denotes time from initiation of methotrexate infusion till the end of homocysteine sampling. **a** First course with neurotoxicity and coma: initial (T_0) plasma folate = 4.4 nmol/l, homocysteine AUC = 28.4 $\mu mol \cdot l^{-1} \cdot h^{-1}$, and methotrexate AUC = 603.2 $\mu mol \cdot l^{-1} \cdot h^{-1}$. **b** Second, nontoxic course, which followed 2 weeks after course 1: initial plasma folate = 45 nmol/l, homocysteine AUC = 5.4 $\mu mol \cdot l^{-1} \cdot h^{-1}$, and methotrexate AUC = 120.4 $\mu mol \cdot l^{-1} \cdot h^{-1}$. Hcy = Homocysteine; MTX = methotrexate.

rence of neurotoxicity in children treated with HDMTX [4] and it has been suggested but not proven that the presence of the TT genotype in the methylenetetrahydrofolate reductase gene (MTHFR) is a possible risk factor for augmented toxicity of HDMTX treatment [5]. In our ongoing study we hypothesized that pretreatment levels of plasma folate and/or homocysteine, and/or the magnitude of their elevations may be predictors of in vivo pharmacobiochemical action of methotrexate.[1] Recently, we observed severe encephalopathy provoked by the first but not the second course of HDMTX mirrored by substantial plasma homocysteine elevations and preceded by one order of magnitude differences in the pretreatment plasma folate levels.

[1] Our IRB-approved protocol allows for a maximum of 0.5 ml blood sampling in a 6-hour interval during and after the 24-hour HDMTX infusion for determination of homocysteine in all samples and folate and B_{12} in the pretreatment (i.e. T_0) specimens. Criteria for enrollment are age at diagnosis more than 1 year, diagnoses of ALL and/or NHL, and the treatment protocol specifying a dose of 5 g/m². Endpoints are changes in laboratory markers of folate metabolism and occurrence of significant toxicity. Goals are (1) to gather data enabling a putative dose and/or timing individualization and (2) assessing p53 induction in blasts after HDMTX to evaluate drug effect in the cellular context.

Case Report

A 9-year-old boy with an unremarkable family history presenting with petechiae, thrombocytopenia and leukocytosis was subsequently diagnosed with acute, hybrid, bcr-abl-positive leukemia. Therapy was commenced with the ALL-BFM 95 [6, 7] protocol for high-risk patients (course HR1) and was free from complications until day 31, when he developed febrile neutropenia. The condition was controlled with antibiotic and antifungal systemic therapy. By day 33 of treatment, no bone marrow remission was achieved and the therapy thus continued with methotrexate administration during a 24-hour infusion (T_0–T_{24}). The plasma level of methotrexate reached 110 µmol/l by the T_{24} hour of administration and decreased slowly; therefore, hydration was increased and an augmented dose of leucovorin was administered at the T_{42} hour according to the protocol recommendations. At about the T_{70} hour, the patient began to feel uncomfortable but clinical and laboratory findings were normal. Approximately 1 h later, alteration of consciousness occurred scoring down to 5 points on the Glasgow coma scale. Inhalation of oxygen led to a transient improvement; neurological findings and fundoscopy done at that time were normal. Due to acute CNS toxicity attributable to methotrexate a dose of 3 mg/kg of syntophylline was administered but with no apparent benefit [8]. His condition deteriorated further and convulsions not responding to diazepam subsequently occurred necessitating the introduction of artificial ventilation with antiedematous therapy. After 30 h of coma the patient was weaned off ventilation, regained conscious-

ness and was transferred back to the oncology ward. MRI and CT studies did not show any diffuse or focal intracranial pathology. Methotrexate plasma levels dropped below the threshold level of 0.2 μmol/l at T_{108} after the start of infusion with significant and prolonged hepatotoxicity [presenting as gross elevation of ALT 40.0 μkat/l, AST 14.8 μkat/l and GMT 11.6 μkat/l, by International Federation of Clinical Chemists (IFCC) methods]. Because of this complication, the HR1 course was terminated. Following 2 weeks of clinical consolidation, a second and identical course of HDMTX was administered under increased clinical alert but without pretreatment. Surprisingly, no concomitant clinical complications and/or laboratory abnormalities were observed.

Blood for homocysteine and folate determinations was drawn into closed K_3EDTA-containing tubes, plasma was spun and immediately recovered to prevent prolonged cell contact and frozen until assay. Homocysteine was determined using Abbott AxSym and folate using Roche Elecsys technologies according to the manufacturers' instructions. The trend of plasma homocysteine concentrations in relation to time of methotrexate administration was parametrized using the area-under-curve (AUC) method. Homocysteine AUCs were calculated as the sum of areas of rectangles during the consecutive 6-hour time interval from T_0 to T_{72} hours (i.e. AUC_{0-72}) whereas methotrexate AUCs were calculated by applying a two-compartment model using MW/PHARM3.3 software. Here, we considered the T_{24} MTX measurement the first datapoint ($T_{24\text{-MTX}}$); then we used all subsequent available measurements and extrapolated the curve to infinity ($AUC_{24\text{ to infinity}}$) thus obtaining the 'elimination phase AUC'. To ascertain differences between the first and second course of administration we compared the respective AUCs for homocysteine and methotrexate (fig. 1a, b). The AUC of homocysteine during the first and clinically toxic course was 5.2 times higher than AUC during the second administration. AUCs of methotrexate during the first versus the second course also differed by a factor of 5. Initial serum concentrations of folate measured in the T_0 specimens were 4.4 versus 45 μmol/l making this difference the most striking discriminator between the first, i.e. toxic, and the second, clinically unremarkable course. We did not observe any other substantially different clinical and/or laboratory parameter between these two respective courses of drug administration. Mutation analysis showed that the patient was heterozygous for the C677T mutation in the MTHFR gene.

Conclusion

Individualization of therapy is one of the leading issues in contemporary oncology, which – approached from the pharmacogenomic, functional and/or biomarker perspective – is believed to add predictive information on the dosage adjustment and/or efficacy versus toxicity ratio for a specific drug and/or protocol. In this patient, differences in methotrexate pharmacokinetics in relation to pretreatment levels of plasma folate were nearly quantitatively mirrored by substantial homocysteine elevations impressively paralleling his severe clinical presentation. We think that a heterozygous form of the MTHFR polymorphism seen here was rather a bystander than a causative phenomenon because, despite its presence, an antifolate toxicity was putatively overridden by an epigenetic phenomenon – approximately 10 times higher levels of plasma folate. We hope that this result may stimulate further efforts in elucidating the role of plasma folate and homocysteine as potential predictors of a methotrexate pharmacobiochemical effect [9]. Importantly, AUCs rather than the 'square meter-normalized drug dose' [10] are the target parameters determining the exposure of a tumor to a drug level achievable within the context of a patient's actual clinical condition. Furthermore, striking differences in pretreatment plasma folate levels may extend the debate on the 'folate overrescue concept' [11] with possible critical reappraisal of leucovorin/methotrexate dosage and/or timing of administration. Evidence on the relation of timing and dosage of leucovorin and methotrexate is rather scarce and inconsistent – a randomized study evaluating high-dose protocols with leucovorin rescue and Capizzi scheme with no leucovorin rescue is currently ongoing [12]. A clinically relevant study evaluating the dosage of leucovorin with and 24 h after low-dose methotrexate in psoriatic patients where the ultimate endpoint was clinical improvement of cutaneous lesions was published by Hills and Ive [13]. They concluded that the administration of folinic acid with methotrexate had no effect whereas the administration of folinic acid 24 h following methotrexate led to a marked improvement. Because of the impossibility to set up this design in oncology looking for suitable biomarkers is a logical step. Taken together, to interpret our data in the context of the study of Hills and Ive [13] we think that further effort is needed to find out whether doses and/or timing of the administration of leucovorin used in high-dose protocols to 'antagonize' folate antagonists may in fact lead to folate accumulation over time with a subsequent decrease of their antifolate effect during consecutive courses of high-dose chemotherapy.

Acknowledgment

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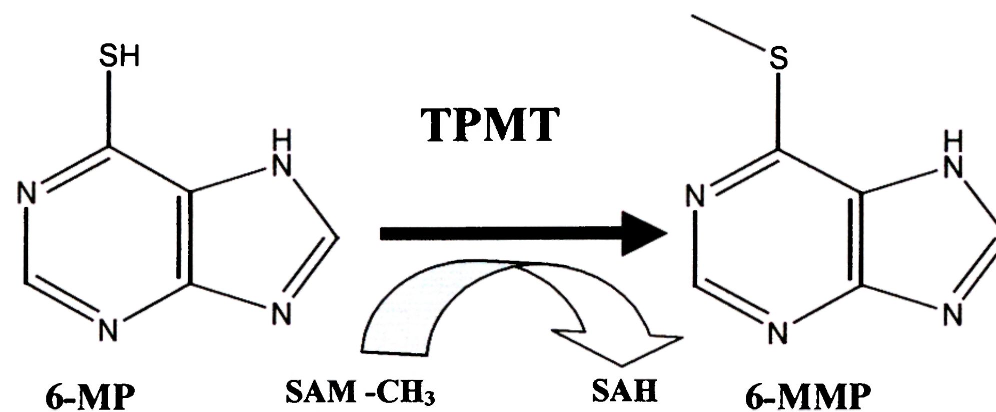
Supplemental Data II: Interim results and study protocols

1) TPMT ASSAY

Principle



Thiopurine Methyltransferase (TPMT) facilitates the metabolism of 6-mercaptopurine (6-MP) to 6-methylmercaptopurine (6-MMP) with S-adenosyl-L-methionine (SAM) providing the methyl group. The amount of 6-MMP formed is measured by HPLC and the rate of this formation is an indication of the amount of TPMT present in a patient's red blood cell lysate. Reactions which would interfere with the specific methylation shown above such as formation of methylthiopurines other than 6-MMP, oxidation of 6-MP by xanthine oxidase, and oxidation of sulfhydryl groups are eliminated in the following ways:

- phosphate buffers are used because other buffers allow for the formation of methylthiopurines other than 6-MMP;
- allopurinol is included to inhibit xanthine oxidase which would otherwise result in substrate depletion.

Sample preparation

One mililiter of whole blood (K_3EDTA or heparin) where the complete blood count was performed before taken prior to therapy with mercaptopurines is pipetted onto a microcrystallic cellulose column equilibrated with cold isotonic saline and is allowed to flow freely. The column effluent containing red blood is collected, its hematocrit measured adjusted with isotonic saline to the value between 0,1 – 0,3 and recorded. One hundred microliters of this

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suspension is pipetted to 0,4 ml of HPLC grade water and cells are allowed to lyse for 10 min at room temperature. The cell lysate can be processed either immediately or stored at – 70 °C.

Reaction mixture

TPMT activity reaction for one patient consists of three reaction vials i) patient sample vial ii) reagent blank vial and iii) sample blank vial. Their actual composition is in the Table.

	lysate	6-MP 120 mmol/l	Phosphate buffer 1 mol/l pH 7,5	DTT 9,0 mmol/l	Allopurinol 3,2 mmol/l	SAM in buffer 25 mmol/l of buffer
Sample	500	50	---	100	20	50
Sample blank	500	---	50	100	20	50
Reagent blank	---	50	500	100	20	50

Chromatographic conditions

The WATERS ALLIANCE chromatographic system equipped with Supelcosil LC-18-S nucleoside column with the corresponding precolumn is used form measurement of TPMT activity. Prior to analysis the system is set to the following conditions: flowrate 1.0 ml/min, column temperature 40 °C and autosampler temperature 37 °C. The vials are incubated in the autosampler compartment of the chromatograph. The gradient is programmed to allow separation of the reaction product, 6-methylmercaptopurine within 15 min cycle including column equilibration. A sampling cycle is 15 min and aliquots of vials are sampled in a way that the reaction blank vial is sampled in 0, 30, 60 and 90 min and the sample vial in 15, 45, 75 and 105 min; the sample blank vial is injected once at 120 min. With each reaction course a pure standard of 6-methylmercaptopurine dissolved in DMSO is analyzed and its nominal area is used for quantitation of the reaction product formed.

Signal processing and enzyme activity calculation

The signal of the "sample blank" should be zero and is not taken into calculations. The regression line of the "reagent blank" is subtracted form the regression line of the "patient sample" thus correcting for the nonspecific methylation of 6-MP (Figure). An amount of the 6-MMP formed reflects the TPMT activity per minute. The total TPMT activity is then expressed by multiplying this value by the respective dilutions and 1/hematocrit to obtain activity corrected to 1 ml of the packed red blood cells.

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2) URINARY EXCRETION OF NATURAL PYRIMIDINES BY GAS CHROMATOGRAPHY/MASS SPECTROMETRY

Pyrimine bases uracil and thymine are natural compounds of human urine. In this method the amount of excreted thymine is determined by stable-isotope dilution gas chromatography mass spectrometry. Uracil is also measured by this method but its excretion depends also on other fators that not related to dihydropyrimidine dehydrogenase activity. Increased amount of uracil is observed in urines with bacterial contamination, where it originates from degradation of pseudouridine by bacteria. Also, during procedure of sample derivatization for GCMS analysis a significant amount of pseudouridine can be hydrolyzed to spuriously increase concentration of uracil.

Specimen preparation: An amount of urine corresponding to 0,25 mg of creatinine is acidified and then extracted with ethylacetate. After evaporation under a stream of nitrogen, the dry residue is silylated with BSTFA/1 % TMCS and analyzed.

Equipment and instrument conditions:
Agilent GCMS 6890 gas chromatograph equipped with 5973 quadrupole mass spectrometer Injector: 250 °C, split mode, 1 μl injection volume, split ratio 1:5. Capillary column HP5 (19091J-102) 5% Phenylmethylsiloxan 25,0 m x 20 um x 0,33 μm, constant flow 0,8 ml/min. Temperature ramps are set as follows: 80 °C for 4 min, then 8 °C/min up to 120 °C, then 5 °C/min up to 290 °C, then 290 °C during 9 min (total 52 min). The retention time of uracil is 13,8 min with the time window 13,0 - 14,5 min), two mass ions with m/z 241 and 255 with the dwell time 30 ms are detected. Thymine is detected within the time window 14,5 - 19,0 min and has the retention time 15,3 min. The mass detector scans for mass ions with m/z 255+257 and 270+272 with the dwell time 30 ms. The mass fragments m/z = 257 and 272 correspond to trimethylsilylderivatives of the N^{15}-labelled thymine used as the stable isotope-labelled internal standard.
Results: Peaks of uracil and thymine are inspected for consistency and the area of the thymine peak is compared with the respective reference values being at present in preparation. Heterozygotes for DHPD deficiency are expected to have higher excretion rate of thymine than normal homozygous controls.

3) PLASMATIC LEVELS OF 5-FLUOROURACIL AND 5,6-DIHYDROFLUOROURACIL

Treatment of gastrointestinal malignancies includes 5-fluorouracil administered as a part of two different protocols. The first protocol is a bolus administration with folinic acid coined

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"FUFA/Mayo", where no concomitant anticancer drugs are administered. The second protocol is the "de Gramont" protocol administering 5-fluorouracil in the form of 48 intravenous infusion. The purpose of this method is to monitor the time course of conversion of the parental molecule 5-fluorouracil to its daughter metabolite 5,6-dihydrofluorouracil, which is formed upon action of liver dihydropyrimidine dehydrogenase. Individuals at risk of toxicity due to either dihydropyrimidine dehydrogenase and/or dihydropyrimidinase will exert alterations of plasmatic appearance/disappearance of these two compounds.

Specimen preparation: 300 µl of lithium heparin plasma is mixed with 50 µl of 5-chlorouracil, 50 µmol/l used as the internal standard and 100 µl of HPLC grade water. Then, 20 µl of 70 % perchloric acid is added to the mixture, vortexed and centrifuged. The supernatant is transferred to Waters autosampler vials and analyzed by HPLC.

Equipment and instrument conditions:

A chromatographic system Waters Alliance equipped with 3 µm Atlantis dC18 reverse phase column 33.9 x 150 mm is connected to Waters DAD and fluorescence detectors. The respective channels of the system are filled with: A = 25 mmol/l ammonium phosphate, pH = 6.0; B = acetonitrile 40 % + water 60 %; C = water and D = methanol. Compounds are eluted with the gradient program as follows:

	Time	Flow	%A	%B	%C	%D	Curve
1		0,4	9.0	1.0	90.0	0.0	
2	7.00	0,4	9.0	1.0	90.0	0.0	6
2	8.00	0,4	9.0	14.0	77.0	0.0	6
4	15.00	0,4	9.0	26.0	65.0	0.0	6
5	15.10	0,4	9.0	0.0	11.0	80.0	6
6	19.90	0,4	9.0	0.0	11.0	80.0	6
7	20.00	0,4	9.0	1.0	90.0	0.0	6

The system is calibrated with negative plasma-based matrix calibrators with added 5,6-dihydrofluorouracil and 5-fluorouracil and 5-chlorouracil is used as an internal standard. A typical chromatogram of the calibration curve from 0 µmol/l to 50 µmol/l of each analyte is shown below; peaks are extracted from diode-array data from 200 –300 nm using the WATERS MAXPLOT detector function between 200 – 225 nm to visualize 5,6-dihydrofluorouracil

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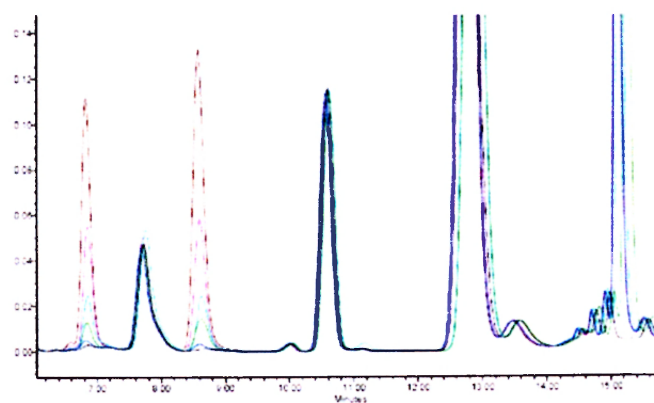
Peaks: Rt – 6.85 min = 5,6-dihydrofluorouracil
Rt – 8.82 min = 5-fluorouracil
Rt – 15.2 min = 5-chlorouracil (internal standard)



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