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

Expanded abstract of 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 therapy. The Chapter B contains comprehensive discussion of author's published results where each of the papers is commented in depth along with 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 shown 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 Besides the published work this chapter also brings several interim yet unpublished results that are subjects of the author's current research and/or close international collaboration.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.

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 90-- 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. 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 the ribose moiety 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 (an author's paper, ref. 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 (MML Test Catalog, ref 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 (an author's paper, ref. 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

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. 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 (an author's paper, ref. 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 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. 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, DV – principal investigator, evaluated as "category A" and author's papers, 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

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 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. The gene locus encoding TPMT was found at 6.p22.3 and first molecular genetic studies revealed a single nucleotide transversion G>C at the position 238. Recent most frequent mutation that are subject of clinical testing are given in OMIM. A first indication of genetic heterogeneity in TPMT was discovered in 1980. 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 produced 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 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 F. 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. 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. 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 and more comprehensive pharmacological description subsequently published by Diasio 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 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. 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. Collectively, alterations in physiological chemistry of both dihydropyrimidine dehydrogenase (DHPD) and

dihydropyrimidinase (DHP) may account tor 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 recent knowledge that an alteration in function of two Phase I enzymes, DPHD and DHP – and not just DPHD 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 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.

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. 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 .

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 intergrated 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 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 a vast amount of knowledge has been gathered in pharmacogenetics a long way remains before fragmented research in particular and ultimately fragmented disciplines will be put together into a confluential interpretive information presentable and understandable to a clinician who would then be able to use – and, of course, not to use – such a piece of information for making a 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.

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
Times Cited: 0

- Valik D, Miner PT, Jones JD. First US case of adenylosuccinate lyase deficiency with severe hypotonia. Pediat Neurol 16 (3): 252-255 APR 1997
Times Cited: 11 9 (in ISI web of science)
2 (monographs: MMBID 8th Edition, C. Scriver, Ed McGraw-Hill 2000 and Fernandes J, Saudubray J-M, Van den Berghe G. Eds Inborn Metabolic Diseases, Diagnosis and Treatment, 3rd Edition. Springer-Verlag, Berlin 2000)

- Valik D, Jones JD. Hereditary disorders of purine and pyrimidine metabolism: Identification of their biochemical phenotypes in the clinical laboratory. Mayo Clin Proc 72 (8): 719-725 AUG 1997
Times Cited: 6

- Valik D. Encephalopathy, lactic acidosis, hyperammonaemia and 5-fluorouracil toxicity
British J Cancer 77 (10): 1710-1711 MAY 1998
Times Cited: 0

- Sheard MA, Vojtesek B, Simickova M, and Valik D.. Release of cytokeratin-18 and -19 fragments (TPS and CYFRA 21-1) into the extracellular space during apoptosis. J Cell Biochem 85 (4): 670-677 2002
Times Cited: 11

- Valik D, Radina M, Sterba J, Vojtesek B. Homocysteine: exploring its potential as a pharmacodynamic biomarker of antifolate chemotherapy. Pharmacogenomics 5 (8): 1151-62, 2004
Times Cited: 1

- Valik D, Sterba J, Baiciova V, Demlova R. 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. Oncology 69, 269-72, 2005

Times cited: 0

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