

Homocysteine: a promising biomarker of folate antagonist chemotherapy

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SUMMARY

For decades it has been well known that elevated levels of homocysteine (Hcy) 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 compartment. Recently, it has been postulated that homocysteine may be a marker of „pharmacodynamic effect“ of methotrexate but studies attesting to this role are only emerging. Here, we explore genetic disease of folate and homocysteine metabolism and discuss links between these model disorders with pharmacology and pharmacogenetics of folate antagonists used in the clinic and outline possible ways how homocysteine may be a biomarker of antifolate therapy.

Key words: homocysteine, biomarker, pharmacogenetics, methotrexate.

Introduction

For decades it has been well known that elevated levels of homocysteine (Hcy) 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 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 [3].

Metabolism of sulfur-containing amino acids is biochemically integrated with a group of reactions termed „one-carbon transfer reactions“ channeling various oxidative states of a methyl moiety towards further cellular utilization, such as synthesis of precursors of nucleic acids, and has been comprehensively detailed elsewhere [1, 101]. Homocysteine is a normal metabolic intermediate arising from demethylation of methioni-

ne whose methyl moiety is then utilized for a variety of essential reactions [4]. Three vitamins serve as cofactors influencing homocysteine levels: pyridoxal 5'-phosphate (vitamin B₆) for the cystathionine-forming reaction and folate (vitamin B₁₀) and cobalamin (vitamin B₁₂) for the methionine-resynthesizing reaction, where 5-methyltetrahydrofolate 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 metabolism of sulfur-containing amino acids. In other words – its pivotal metabolic significance stems 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, which actively extracts plasma homocysteine, remethylation process takes place in every eukaryotic cell. It is catalyzed by either betaine-dependent methyltransferases, E.C. 2.1.1.3 and 2.1.1.5 confined to the liver [5] or, alternatively, by a ubiquitous 5-methyltetrahydrofolate-utilizing transferase, E.C., 2.1.1.13 [6] also called methionine synthase.

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 observed in human plasma. Traditionally recognized forms are i) homo-

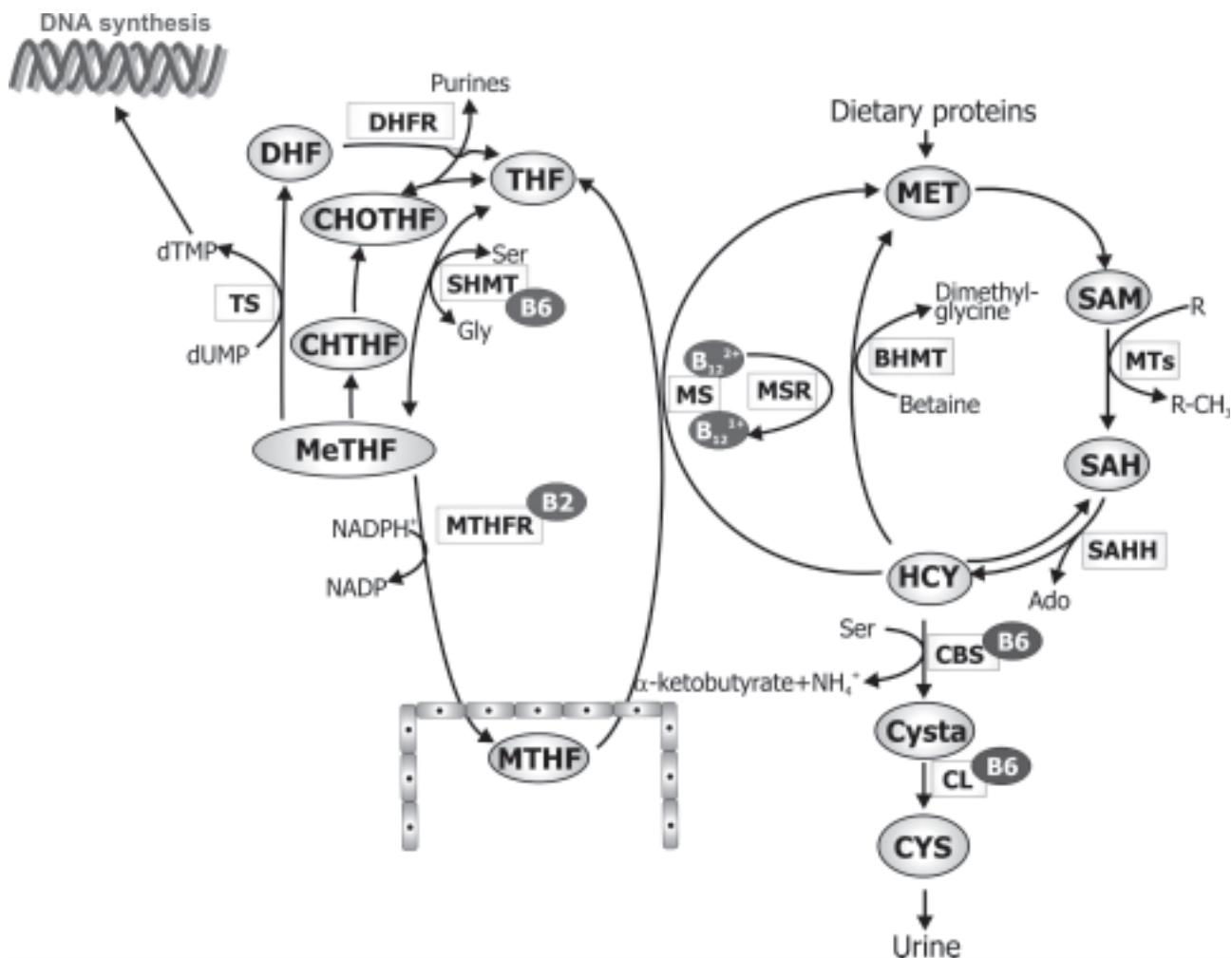


Fig. 1. Physiological chemistry of one-carbon transfer and transsulfuration reactions; extracellular compartment contains only MTHF, which is the main plasmatic form of folate – 5-methyltetrahydrofolate. Intracellular compartment contains all other species; DHFR – dihydrofolate reductase, DHF – dihydrofolate, THF – tetrahydrofolate, CHOTHF – formyltetrahydrofolate, SHMT – serine/glycine hydroxymethyltransferase, B₆ – pyridoxal 5'phosphate, Ser – serine, Gly – glycine, CHTHF – methenyltetrahydrofolate, MeTHF – methylenetetrahydrofolate, dTMP – thymidine monophosphate (also thymidylate), dUMP – deoxyuridine monophosphate (also deoxyuridylate), TS – thymidylate synthase, MTHFR – methyltetrahydrofolate reductase, B₂ – riboflavin, MET – methionine, MS – methionine synthase, B₁₂ – cobalamin, MSR methionine synthase reductase, BHMT – betaine hydroxymethyltransferase, SAM – S-adenosyl methionine, MTs – methyltransferase, i.e. any SAM-utilizing methyltransferase methylating a substrate denoted R to a methylated product denoted R-CH₃, SAH – S-adenosylhomocysteine, SAHH – S-adenosylhomocysteine hydrolase, Ado – adenosine, HCY – homocysteine, CBS – cystathionine beta synthase, Cysta – cystathionine, CL – cystathionine lyase, CYS – cysteine eliminated to urine as cystine, i.e. cysteine-cysteine disulfide, NADP(H) – nicotinamide adenine dinucleotide phosphate (H – hydrogenated, i. e. reduced).

cysteine, ii) homocysteine disulfide (alias homocystine), iii) cysteine-homocysteine mixed disulfide, and iv) homocysteine linked to plasma proteins by means of –SH groups, also abbreviated as 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 [7] reflecting the fact that total homocysteine is indeed what is generally measured by techniques employing 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 „to-

tal homocysteine“ (tHcy) easily measurable by various techniques [8].

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 epsilon-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 [9]. Several other homocysteine-related precursor and/or daughter species occur *in vivo* as well, such as homocystic acid and S-adenosylhomocysteine but their role in pathogenesis of homocysteine-related disorders has not yet been elucidated.

Homocysteine is related to a number of disease conditions

Homocysteine became an attractive molecule since 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 beta-synthase (CBS) deficiency after methionine load [10]. Since then, a large number of studies investigated the possible role of moderately elevated plasma homocysteine levels in premature vascular disease – for review see [11] and references therein. However, despite a large body of evidence on the possible and perhaps causative role of homocysteine in 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 etc [12].

Pathogenesis of homocysteine-related vascular damage remained poorly understood until recent work of Jakubowski [13] 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 formation of homocysteine thiolactone modify proteins posttranslationally in all organisms 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 „total homocysteine“ levels and that homocysteine-induced protein damage can trigger autoimmune response thus putatively contributing to pathology of human disease [9, 13].

Homocysteine, folates and one-carbon transfer reactions

Since homocysteine intercepts transsulfuration metabolism and metabolism of one-carbon transfer folate-dependent reactions, brief mention on folate physiology will help understand this important point further (see also Figure 1). Folate physiology has been extensively reviewed [14, 15]. The term „folate“ denotes a group of pteridine ring-containing compounds covalently linked to paraaminobenzoic 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 the positions 5- and 10- of the pteridine heterocycle.

After ingestion, folates from food are reduced and methylated in the gut at the N5 position of a pteridine ring giving rise to 5-methyltetrahydrofolate (5-methyl-THF, monoglutamate form) that is the principal form of folate occurring in plasma within the low nanomolar range 5–25 nmol/l [16]. The 5-methyltetrahydrofolate is then taken up by cells from plasma through two classes of cell membrane transporters and concentrated up to a micromolar range within the intracellular compartment; nevertheless, in many tissues 5-methylTHF remains

the main intracellular form of folate. Two distinct systems exist for transport of folates and antifolates across mammalian cell membranes: i) a Reduced Folate Carrier (RFC) is a high-capacity, low-affinity system also responsible for import of methotrexate at micromolar concentrations [17] and ii) a Folate-Binding Proteins – a family of membrane-associated receptors (FBP) – a low-capacity, high-affinity transport system that operates at nanomolar concentrations of extracellular folate [18]. 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 [19]. This modification is crucial in keeping the intracellular pool of folates available for essential reactions such as 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_m s and also serve as intracellular storage forms of folate [20].

However, before this intracellular mechanism can take effect, 5-methyltetrahydrofolate derived either from plasma or generated from other reactive folate species such as 5,10- methyleneTHF through the MTHFR-catalyzed reaction must be demethylated to liberate reduced tetrahydrofolate, thus restoring the metabolically active tetrahydrofolate pool. This reaction mechanism operates in every cell (a methionine synthase reaction) and transfers the methyl moiety of 5-methylTHF to a ubiquitous metabolic intermediate – homocysteine – utilizing cobalamin as a cofactor. Furthermore, this reaction is essentially unidirectional and has no alternative in eukaryotic cells so every molecule of 5-methylTHF once formed in every eukaryotic cell must pass through the methionine synthase 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 (see Figure 1). Indeed, upon cobalamin deficiency a condition known as „methyl trap“ occurs [16]. In this situation, all active folates are „trapped“ as 5-methylTHF – thus being metabolically dead – despite the fact that high concentrations of folate (but in the form of 5-methylTHF) are found in plasma and intracellularly, for example in erythrocytes. Clinically, this condition presents as megaloblastic anemia (coined „pernicious“ anemia) with concurrent laboratory finding of hyperhomocysteinemia.

Homocysteine and folate antagonists

Folate antagonists were brought to clinical practice by Farber in 1948 [21] 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, [22]) although a number of new folate antagonists targeting more specifically various metabolic steps within folate metabolism have been brought to the clinic. Recent knowledge on their mechanism of action and modes of resistance has been

excellently reviewed [23]. 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 used predominantly for treatment of children with acute lymphoblastic leukemia, non-Hodgkin's lymphoma and osteosarcoma [22, 24]. Low-dose methotrexate therapy is also used in the clinic but rather for treatment of non-oncological, chronic disorders, where immune derangement is believed to be responsible for pathogenesis, such as rheumatoid arthritis, psoriasis and ulcerative colitis [22, 25, 26].

Methotrexate is a tight-binding inhibitor of dihydrofolate reductase (DHFR), the enzyme responsible for reduction of folates to their metabolically active tetrahydrofolate form. Traditionally, decreased rate 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 tetrahydrofolate cofactors to their inactive dihydrofolate form [27]. Several other enzymes in purine biosynthetic pathway are also inhibited by methotrexate. A major mechanism of anticancer action of methotrexate has been therefore attributed to inhibition of DNA synthesis through depletion of nucleotides and misincorporation of dUMP into DNA and also RNA to some extent [22, 23, 27, 28]. Another determinant of the methotrexate effect appears to be an excess of binding sites on dihydrofolate reductase, because intracellular levels of this target enzyme are 20–30 times higher than required to maintain necessary tetrahydrofolate pool [29].

A novel mechanism of methotrexate action has been recently described by Casey et al. [30]. They showed that 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 intracellular concentration of S-adenosylhomocysteine, which is a potent inhibitor of cellular methyltransferases [31]. Methyltransferase inhibition leads to disruption of Ras-signaling pathway and cessation of cell growth. The authors, however, do not refute the traditionally described mode of action of methotrexate [30]. 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“. 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 [31].

Besides sporadic reports [32, 33, 34], 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 bone marrow of patients with acute lymphoblastic

leukemia cultivated *ex vivo* with methotrexate resulted in changes in expression of the p53 protein and in upregulation of several p53-regulating genes [see also 35, 36, 37]. 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 [38, 39, 40]. 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 a 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 as well [41 and web-linked comments]. Consequently, drug dosage based on BSA is precise but lacks biological accuracy necessary to tailor therapy more specifically to variables such as: i) individual needs of an ii) individual patient at a iii) given time during the course of disease. Therefore, a search for biomarkers has been continuing and speeded up in recent years with the recent advent in pharmacogenomics [42] and predictive oncology [102].

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 potentially fulfilling criteria for a biomarker. Indeed, antifolates have been shown to exert profound effect on homocysteine concentrations at cellular levels [40] and in methotrexate-treated patients [43, 44, 45] but evidence gathered from these studies was rather descriptive and unable to build up a foundation for defining a predictive model. It was not until recently, when clinical introduction of a new antifolate pemetrexed – belonging to a group coined „multi-target 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 et al. [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 B₁₂ 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 reemerged in the literature and recently, it has been directly postulated that homocysteine is a marker of „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, the pre-

-therapy plasma levels of folate were discriminators of toxic and nontoxic chemotherapy courses in the same patient [42, and manuscript submitted].

Homocysteine, methotrexate and neurotoxicity

The primary toxic effects of methotrexate are myelosuppression 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 [22, 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 forms [52].

Three principal hypotheses have been brought up 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 recycling of bipterin 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 bipterin metabolism such as „nonclassical“ variants of phenylketonuria. Smith et al. published an interesting observation linking bipterin 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 methionine synthase reaction exclusively regenerating tetrahydrofolate (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 ce-

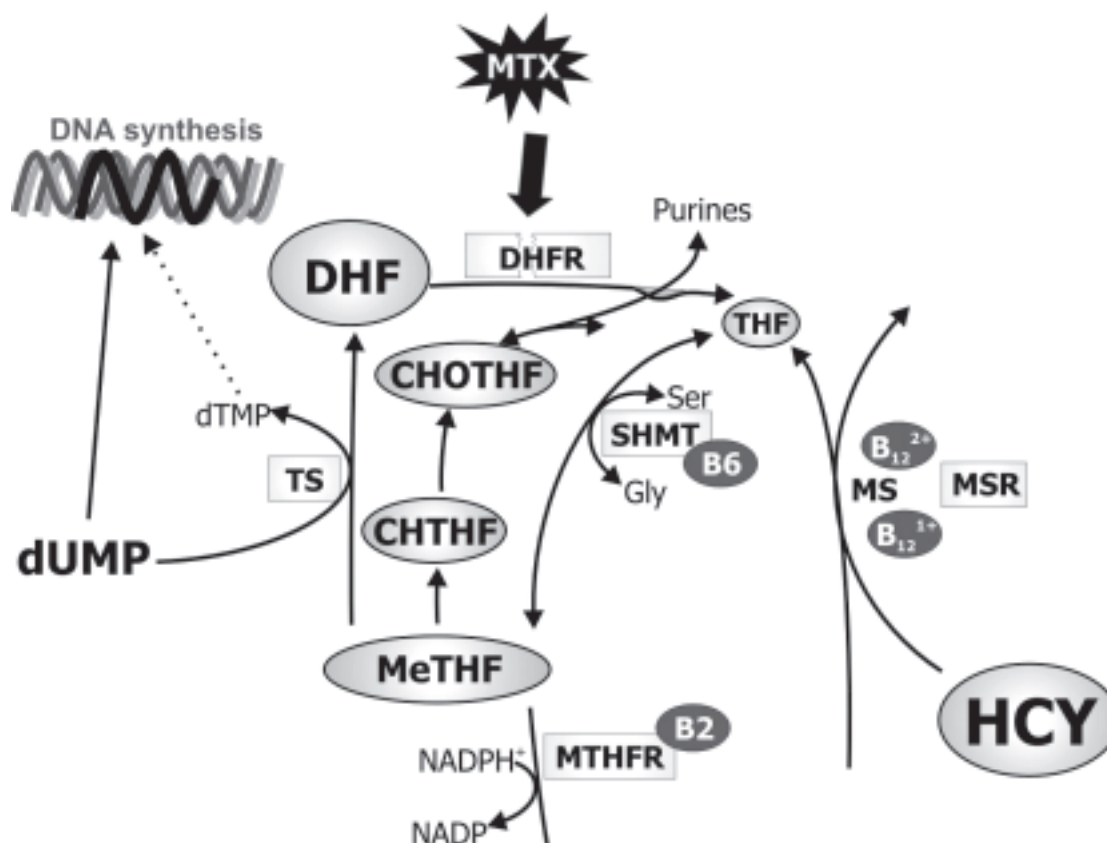


Fig 2. A section of the Figure 1 showing methotrexate (MTX, all other abbreviations identical with Figure 1) hitting its primary target – DHFR – and downstream event related to homocysteine

Rapid depletion of THF leads to a cessation of a methionine synthase (MS) reaction with subsequent buildup 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. Buildup of dUMP results in its erroneous incorporation into nucleic acids.

rebrospinal fluid has been reported recently by Quinn [59]. They studied serial cerebrospinal fluid 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 that 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

Considering scientifically-based concepts of pharmacotherapy, then 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 area-under-curve [60]. Inter- and intraindividual variabilities in a patient's response to a given drug are recognized determinants of therapy efficacy, because these factors critically influence drug exposure [61]. Genetic background of a drug recipient recently emerged as a critical determinant of individual clinical outcome and a need of dose individualization recently led to convergence of pharmacogenomics and biomarker research [42]. 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-oriented clinical decision. Assessing 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 following example shows that it is, in fact, already in the clinical practice. Before administration of warfarin, genotype information can identify carriers of CYP2C9 variant(s) and information whether a desired effect has been achieved can be timely assessed using thromboplastin test (expressed as INR = international normalized ratio) values during the course of 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 antineoplastics.

Pharmacology, pharmacokinetics and indications of methotrexate have been authoritatively described elsewhere [22]. Of pharmacogenetic interest may be its biotransformation resulting in a proportion of methotrexate being processed presumably through a liver cytochrome-P450 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-P450 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 potential pharmacogenetic importance is dihydrofolate reductase itself. Unfortunately, reports from the literature deal mostly with increase of expression of dihydrofolate reductase as a mechanism of antifolate resistance that develops in the tumor cells upon exposure to folate antagonists [23]. In pediatric acute lymphoblastic leukemias higher occurrence of dihydrofolate reductase 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 dihydrofolate reductase – and possibly other enzymes participating in pteridine metabolism – in 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 biotin 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 natural course of these disorders, for example by occurrence of intracranial calcifications [65, 67] but these reports are rather anecdotal.

In summary, although genetic disorders affecting dihydrofolate reductase 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 of them enzyme deficiency was not demonstrable in the cell culture [103]. One possible explanation of the complexity of this phenomenon is that biallelic germline mutation severely hampering function of such a critical enzyme as dihydrofolate reductase 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. [104]. They showed that the length of repeats in the promoter sequence of thymidylate synthase (2rpt/2rpt versus 3rpt/3rpt variant) may be a determinant of toxicity/efficacy of thymidylate synthase inhibitors, such as fluoropyrimidines and 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 (see Figure 2). The occurrence of a 3rpt/3rpt variant was also associated with disturbances in plasma levels of homocysteine [70].

Unresolved issues: methotrexate, folate pretreatment and interactions

Despite 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 lympho-

ma. 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 et al. [71] were able to demonstrate a better outcome of children with individualized dosing of methotrexate compared to conventional dosing based on body surface area. Supplementation with folate and vitamin B₁₂ prior to methotrexate therapy remains an open and unresolved issue. Hills and Ivey [72] showed that concurrent daily administration of folinic acid with weekly oral methotrexate prevented improvement of psoriasis, whereas folinic acid given every day except the day of methotrexate resulted in marked improvement. This finding is of substantial importance because 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 rather unwarranted suggested supplementation with folate and B₁₂ prior to antifolate therapy. Until experimentally proven, administration of cosubstrates and cofactors (as folate and vitamin B₁₂) prior to therapy with antifolates [48] should be probably 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 [20, 22, 73]. Unfortunately, studies addressing this issue are rarely found in the literature.

Our interim data investigating 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 pretreatment value (unpublished). This rise is sharply interrupted at the moment of administration of folinic acid, which occurs in the 42nd hour from the beginning of high dose methotrexate (5 g/m²) infusion according to the ALL-BFM 95 protocol [24]. In summary, current – though incomplete – knowledge offers up to three possible tools for functional assessment of antifolate therapy. They may indicate i) the rate of transformation to a less efficient and toxic compound: 7-hydroxymethotrexate, ii) the rate of body tetrahydrofolate depletion: elevation of homocysteine with/without methylmalonic acid during and after drug administration and iii) pre-therapy levels of plasma folate, homocysteine and methylmalonic acid whose disturbances may be associated with impaired drug tolerance and possibly drug antitumor effect.

Open question: biomarkers in the context of „functional“ pharmacogenomics

Considering biological reality at all levels – genomic, metabolic, cellular, organ and patient – probably no single-gene and/or single-pathway approach will be sufficient enough to bring accurate and comprehensive answer to a complex issue even though seemingly well defined – such as administration of a drug with „known“ metabolism. In a recent review summarizing current knowledge on pharmacogenomics encompassing also

pharmacogenomic aspects of antifolate treatment, Ulrich et al. [104] discussed benefits and drawbacks of different pharmacogenomic approaches including array technologies. They conclude that rather combinations of methods and approaches may help elucidate underlying mechanisms of drug-related toxicities and help bring such knowledge to clinical practice. Indeed, the current belief that genetic methods will help elucidate mechanisms responsible for adverse reactions may not be entirely accurate in an aspect that genetic methods including microarray technologies are – by their very nature – morphological. As such they may not be adequate if a need is to obtain functional and time-dependent information – such as estimates of organ-localized enzyme activities along multiple metabolic pathways [74]. 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 occurring within one family. Such a complexity, paradoxically, increased a 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 – tandem mass spectrometry, MALDI/SELDI-TOF, etc. Such an armamentarium of methods concurrently 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 as well. 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 manifestation of a complex clinicopathological response. Homocysteine as a biomarker of antifolate effect is subject of current investigation. As we outlined here, its potential is based on metabolic uniqueness and stoichiometry of the methionine synthase reaction where per each mol of 5-methylTHF consumed, one mol of homocysteine is remethylated to methionine. It follows that all conditions that affect supply and/or generation of 5-methylTHF – such as indirect action of antifolates through inhibition of DHFR and subsequent depletion of 5-MeTHF – lead to 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 fo-

late levels and tissue stores will play important role. Deciphering these events will be an important goal in defining the role of homocysteine as a biomarker of antifolate chemotherapy.

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Přehled odborných akcí garantovaných Českou společností klinické biochemie ve druhém pololetí roku 2006

11.–13. 9. 2006, Pardubice

Vitaminy 2006

6th International Conference VITAMINS - Health Ingredients Metabolism Analysis

13.–15. 9. 2006, Praha

Atherosklerosa-Hyperhomocysteinemie 2006

17.–19. 9. 2006, Pardubice

FONS 2006 - Symposium klinické biochemie

10. 10. 2006, Hradec Králové

Pracovní konference ČSKB - Méně časté monoklonální gamapatie

17.–19. 11. 2006, Praha

Education & Training in Clinical Chemistry & Laboratory Medicine (II. mezinárodní symposium)

2. 11. 2006, Praha

Metabolismus alkoholu a jeho důsledky na zdraví člověka

Pracovní den Sekce biochemických laborantů ČSKB

15. 11. 2006, Brno

Pracovní den ČSKB - Informační technologie v klinických laboratořích

6.–7. 12. 2006, Karlova Studánka,

Státní léčebné lázně

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