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**Abstracts**

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## Unexpected Reactions in Pteridine Chemistry

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The pteridine ringsystem can be regarded as a prototype of a broad variety of unexpected, unique reactions.

Nature is using various pteridine derivatives as cofactors to catalyse important biosynthetic transformations such as aromatic hydroxylations, reduction of formyl to methyl groups, methyl transfer reactions or built-up of new heterocyclic ringsystems. The biosynthesis of the pteridine nucleus is also based on a unique intramolecular ring-enlargement reaction converting GTP into 7,8-dihydroneopterin-3'-triphosphate. Riboflavin biosynthesis affords also a pteridine precursor to form the dimethylbenzene moiety.

Chemical reductions of lumazines lead to dimeric structures which can also been achieved by electrochemical reductions of lumazine-aldehydes. Photochemical reactions showed cleavages of sidechains and formation of unusual 5,8-dihydro-pteridine derivatives and in special cases demethylation reactions have been observed. Thermolyses of 7-azidolumazines give rise to an interesting ring-contraction forming a new type of purine-ylides. Reinvestigation of an old purine synthesis turned out to form pteridines instead. A new chemical approach for the synthesis of pteridines was developed on the basis of these findings. Simple substitution reactions often end up in tele-substitutions at different sites of the nucleus.

Characteristic examples of those unexpected reactions will be discussed in detail and the mechanistic aspectcs explained for better understanding.

## Selective Formation of 6-hydroxyalkylpterins

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6-Hydroxyalkylpterin, such as biopterin and neopterin, belongs to one of the most biologically important groups of pterin derivatives. Generally synthesis of these compounds has been achieved by Gabriel-Coleman synthesis that is the condensation of triaminopyrimidine with a sugar derivative, but it is well known that this type ring-formation has not usually proceeded in regioselective manner. Consequently, isomeric 7-hydroxyalkylpterin, separation of which from desired 6-hydroxyalkylpterin is rather difficult, is sometimes obtained in significant yield. For example, due to the problem, naturally occurring L-erythro-biopterin which is a practical precursor for pharmaceutical supply of (6R)-tetrahydrobiopterin has been synthesized using regioselective reaction from phenylhydrazone derivative of L-rahmnose. In 1992, we reported Gabriel-Coleman type regioselective pterin ring-formation using

a,b-epoxyaldehyde, but due to unsatisfactory yields it has not been considered for long time. In this paper, we would like to describe improvement of the reaction and application to synthesis of L-erythro-biopterin and sepiapterin.

**Synthetic Study of Methanopterin 1 Introduction of Amino Alkyl Group into C6 Substituents**

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There exists a folate analogue known as methanopterin that is isolated from an archaeabacteria, Methanosarcina thermophila, which produces methane from $CO_2$ under anaerobic conditions. In the methane-producing metabolic process, tetrahydromethanopterin is known to work as a cofactor for the reduction of the C1 unit like a tetrahydrofolate. We would like to announce synthesis of the key intermediate, 2-amino-4-cyclohexyloxy-6-[2-(4-hydroxyethylphenyl)amino]ethyl-7-methylpterin derivative, for total synthesis of methanopterin. The compound was synthesized from protected 6-acetyl-2-amino-4-cyclohexyloxy-7-methylpterin, by imine formation with aniline derivatives followed by selective reduction. The starting material, 6-acetyl-7-methylpterin, was prepared using the improved method independent of strongly toxic selenium dioxide in high yield. Condensation of the acetylpterin derivative with para substituted aniline derivatives was carried out using BF3 catalyst together with molecular sieves, a dehydrating agent, to give corresponding imine derivatives. These imines were reduced to corresponding amine derivatives using ZrCl4 and NaBH4. It was also succeeded to carry out reductive amination of 6-acetyl-7-methylpterin, instead of the two-step conversion, using 2-picoline-borane. Oxidation of the amine derivative with hydroxyethyl group by tetra-n-propylammonium perruthenate afforded the benzaldehyde derivative due to C-C bond cleavage.

**Novel Routes to Blocked Dihydropteridines**

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Within the Suckling group there have been recent advances with regards to a diversity orientated synthesis at the C2 position on pteridines and analogous structures. Whilst this approach often utilises the Isay synthesis and is not usually a useful means to obtain blocked dihydropteridines, it is proof of concept with regards to diversity at C2. This poster will present results from within the group regarding a C2 diversity orientated synthesis utilising the Boon approach, a method which is far more useful in synthesising blocked dihydropteridines. This would allow access to many interesting compounds with potential in the fields of antifungal (1), antibacterial (2) and anticancer activity (3).

Nitric oxide (NO) is an inorganic free radical which plays an important role in mammalian biology, and is an important cell messenger. NO is derived from nitric oxide synthase (NOS) a multidomain enzyme which has three iso-forms; iNOS, nNOS and eNOS. All iso-forms require specific co-factors to function properly (4). An important co-factor of these enzymes is tetrahydrobiopterin (BH4). BH4 is a pteridine which plays an important role as a single electron donor within the eFectron transfer cascade, which leads to the production of NO

A blocked dihydropteridine, analogous to BH4, called WSG-1002 has shown significant biological activity as a NOS activator (5). It is thought that WSG-1002 acts much in the same way as BH4, in that it donates an electron, to the NOS enzyme. With this and other biological activities in mind, an analogue of WSG-1002 was synthesised with the aim of increasing the electron density in the pteridine ring system, and therefore enhancing electron donor ability. In order to obtain such compounds, several synthetic approaches have been used including a novel "activating - protection group" allowing access to previously unobtainable analogues. Biological

results from enzyme assays will also be presented.

**First Synthesis of a Representative, Natural Pterin glycoside: 2'-O-(alpha-D-glucopyranosyl)biopterin**

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Some pterins having a hydroxyalkyl side-chain at C-6 have been found as glycosidic forms in certain prokaryotes, representative examples being 2'-O-(alpha-D-glucopyranosyl)biopterin {1} isolated from various kinds of cyanobacteria [1] and limipterin [2'-O-(2-acetamido-2-deoxy-beta-D-glucopyranosyl)biopterin] isolated from a green sulfur photosynthetic bacterium [2]. Glycosides of other pterins such as ciliapterin (L-threo-biopterin), neopterin and 6-hydroxymethylpterin have also been isolated from cyanobacteria, anaerobic photosynthetic bacteria and chemoautotrophic archaebacteria. We have undertaken a synthetic exploration of various types of glycosides of biopterin and related pterins owing to a marked interest in their physiological functions and biological activities as well as the structural proof of those natural products; limipterin and two kinds of ciliapterin glycosides have recently been prepared [3]. We present here the first synthesis of 2'-O-(alpha-D-glucopyranosyl) biopterin {1}, which remained unprepared since its first discovery in 1958.

In our synthetic scheme, a key, versatile precursor N2-(N,N-dimethylaminomethylene)-1'-O-(4-methoxybenzyl)-3-[2-(4-nitrophenyl)ethyl]-biopterin {2} was prepared from D-xylose in 14 steps involving condensation with 2,4,5-triamino-6-hydroxypyrimidine, while a novel glycosyl donor 4,6-di-O-acetyl-2,3-di-O-(4-methoxybenzyl)-alpha-D-glucopyranosy bromide {3} was efficiently prepared from D-glucose in 8 steps. Glycosylation of the precursor {2} with the donor {3} in the presence of silver triflate and tetramethylurea predominantly afforded the corresponding alpha-D-glucopyranoside, from which 2'-O-(alpha-D-glucopyranosyl)biopterin {1} was obtained by the successive removal of the protecting groups.

**Quantitative Determination of Both Reduced and Oxidized Forms of Biopterin by a Single Fluorometric HPLC**

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Tetrahydrobiopterin (BH4) has been measured extensively for human disorders related to aromatic amino acid hydroxylation and nitric oxide synthesis. While BH4 is functional in the fully reduced form, it can easily be oxidized to dihydrobiopterin (BH2) and fully oxidized form (biopterin) in vivo. We developed a biochemical method to distinguish BH4 from its oxidized forms by employing BH4:UDP-glucose α-glucosyltransferase (BGluT), which catalyzes glucosyl transfer from UDP-glucose to BH4. The recombinant enzyme isolated from Escherichia coli selectively catalyzed BH4 in the presence of BH2 to yield an equimolar amount of BH4-glucoside. Therefore, an acidic iodine oxidation of the reaction mixture followed by a single fluorometric HPLC procedure allowed simultaneous determination of both reduced and oxidized forms of biopterin. The quantitative method was validated using authentic biopterins and animal tissues.

**Molecular Basis of Antifolate Efficacy in the Treatment of Chronic Inflammatory Diseases**

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For many decades, the folate antagonist methotrexate (MTX) is considered the anchor drug in the treatment of chronic inflammatory diseases such as rheumatoid arthritis (RA). In clinical practise, MTX is combined with other disease modifying antirheumatic drugs or with biological agents including antibodies or soluble receptors to proinflammatory cytokines. Despite the proven efficacy of MTX in RA treatment there is room for further improvement of antifolates-based therapies due to the onset of drug resistance phenomena and toxicity after long term chronic exposure (1, 2). From this perspective new generation of rationally designed folate antagonists deserve further exploration of as potential candidates to elicit anti-inflammatory responses. Here we show that the type of membrane transport system for antifolates expressed in immune effector cells implicated in the pathophysiology of RA (e.g. activated T-cells and macrophages) requires different targeting strategies for therapeutic interventions. Notably, activated T cells from RA patients dominantly express the classical reduced folate carrier (RFC) system that facilitated more efficient uptake and more potent inhibition of those novel generation folate antagonists for which RFC harbors a high affinity and/or those that are efficiently polyglutamylated. In fact, in 5-6 out 7 RA patients clinically unresponsive to MTX, novel folate antagonists like PT523, ralitrexed, pemetrexed, GW1843 and plevitrexed were capable of inhibiting the release of pro-inflammatory cytokines from activated T cells. Activated macrophages in inflamed synovial tissues may require an alternative targeting strategy as they express Folate Receptor isoform beta (FRb) as their main transport route for antifolates. Screening of candidate drugs revealed the thymidylate synthase inhibitor BGC945/ ONX0801 was a prototypical antifolate with a high binding affinity for FRb and a low affinity for RFC thereby allowing selective FR targeting and reducing potential toxic side effects of FR-targeted therapies (3).

Collectively, increasing knowledge of mechanisms of action of antifolates and rational targeting of specific immune effector cells may assist in further improved antifolate-based treatment option for chronic inflammatory diseases.

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**Hopkins Lecture**
**The Ups and Downs of GTP Cyclohydrolase I**

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Whether within vascular endothelial cells or monoaminergic neurons the control of GTP Cyclohydrolase I (GCH1) gene expression underlies the physiology and functional genetics of BH4-requiring systems. Our studies have focused on the regulation of GCH1 transcription and have demonstrated that the proximal promoter located within 140 bp of the primary transcription start site is critical for basal and regulated transcription but not for cell type-specific gene expression. Moreover, we have shown that three DNA binding elements located with the proximal promoter, the CCAAT-box, the CRE and the GC-box and the proteins that bind to these elements, namely

NF-Y, CREB and C/EBP and Sp1 and 3, have essential and unique functions in basal and regulated transcription. Of particular interest to us is the C/EBP family of transcription factors because of the common involvement of these proteins and GCH1 in differentiation and the inflammatory response. We have also begun to address the role of chromatin remodeling in GCH1 expression. To date these studies suggest that there is little or no relationship between GCH1 gene transcription and; 1) acetylation and methylation histone marks located on nucleosomes associated with the proximal promoter; 2) methylation of CpG dinucleotides within the proximal promoter CpG island and; 3) the amount of transcription factor proteins and RNA Polymerase II bound to the proximal promoter. The GCH1 proximal promoter thus seems to be functioning similarly in cell types that express high or low levels of GCH1. Our studies also show, however, that inhibition of histone deacetylase activity can result in an increase in GCH1 transcription but only in cells with low basal GCH1 expression. In contrast, no effect of inhibiting DNA methylation on GCH1 expression was observed in these same cells. These results indicate that chromatin remodeling complexes containing histone deacetylase but not DNA methyltransferase activity are actively involved in control of the GCH1 gene and possibly underlie cell type-specific expression of GCH1. Moreover, these results suggest a mechanism through which GCH1 gene transcription may be "poised" for activation in cell types that do not constitutively express GCH1. (Funded by NINDS grant NS26081)

**The Biosynthesis of Aurodrosopterin, a Minor Red Eye Pigment of Drosophila**

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Dihydropterin deaminase, which catalyzes the conversion of 7,8-dihydropterin to 7,8-dihydrolumazine was purified 5850-fold to apparent homogeneity from Drosophila melanogaster. Its molecular mass was estimated to be 48 kDa by gel filtration and SDS-PAGE, indicating that it is a monomer under native conditions. The pI value, temperature and optimal pH of the enzyme were 5.5, 40°C, and 7.5, respectively. Interestingly, the enzyme had much higher activity for guanine than for 7,8-dihydropterin. The specificity constant (Kcat/Km) for guanine (8.6 x 106 M-1os-1) was 860-fold higher than that for 7,8-dihydropterin (1.0 x 104 M-1os-1). The structural gene of the enzyme was identified by MALDI-TOF mass spectrometry analysis as CG18143, located at region 82A1 on chromosome 3R. The cloned and expressed CG18143 exhibited both 7,8-dihydropterin and guanine deaminase activities. Flies with mutations in CG18143, SUPor-P/Df (3R) A321R1 transheterozygotes, had severely decreased activities in both deaminases compared with the wild type. Among several red eye pigments, the level of aurodrosopterin was specifically decreased in the mutant, and the amount of xanthine and uric acid also decreased considerably, to 76% and 59% of wild type amounts, respectively. In conclusion, dihydropterin deaminase encoded by CG18143 is involved in the biosynthesis of aurodrosopterin by providing one of its precursors, 7,8-dihydrolumazine, from 7,8-dihydropterin. Dihydropterin deaminase also functions as guanine deaminase, an important enzyme for purine metabolism.

**Pharmacogenomics of Colon and Non-small Cell Lung Cancer (NSCLC): Potential for Tailored Therapy?**

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Therapy of both colon cancer and NSCLC include thymidylate synthase (TS) inhibitors. For colon cancer either 5-fluorouracil (5FU) or an

oral prodrug (e.g. capecitabine, UFT or S-1) is always part of a combination regimen, while pemetrexed (PMX) is used for NSCLC. 5FU inhibits thymidylate synthase (TS) by formation of a ternary complex together with 5,10-methylene-tetrahydrofolate (CH2-THF). PMX (as a polyglutamate) is a very potent inhibitor of TS. Resistance to both 5FU and PMX has been associated with overexpression of TS, while a disturbed folate homeostasis due a polymorphism (C677T) in methylenetetrahydrofolate reductase (MTHFR) may increase CH2-THF levels and enhance TS inhibition. For 5FU, degradation by dihydropyrimidine dehydrogenase (DPD) and for PMX, decreased transport (either the reduced folate carrier or the proton-coupled folate transporter) or polyglutamylation may confer resistance. In retrospective studies a high TS level was associated with poor response in both diseases. In a prospective study in colon cancer we also demonstrated that pre-treatment analysis of TS and DPD almost doubled the response rate (RR) to 5FU-leucovorin (LV) treatment to 35% and overall survival (OS) to 23.4 months. In order to simplify pre-treatment selection, polymorphisms in the promoter region have been analyzed. The 5'untranslated region (5ÚTR) of human TS mRNA contains tandemly repeated elements in the TS enhancer region (TSER). In vitro translation experiments showed a relation between the number of TSER and TS expression, and it was hypothesized that these repeats would predict for TS expression in the tumor allowing to select patients on TS polymorphism in e.g. white blood cells. However, these polymorphisms were indeed associated with increased TS levels in normal tissues, but not in tumors, possibly because TSER germline genotypes may not be in agreement with tumors, one of the reasons being a frequent deletion of chromosome 18p11.32 in colon tumors. Hence there is more evidence that this polymorphism may predict for toxicity rather then for the antitumor effect. For MTHFR contradictory results have published with higher and lower RR in C677T patients, although the C677T genotype was also associated with a higher incidence of 5FU associated side effects. Loss of Heterozygosity (LOH) such as loss of chromosome 18p11.32 is characteristic for colon tumors and was more frequently observed (p=0.015) in patients responding to capecitabine-irinotecan (CAPIRI) treatment. Therefore genome-wide DNA copy number profiles (with oligonucleotide array CGH) were used for hierarchical clustering enabling to separate responders from non-responders, not only CAPIRI (p=0.03), but also 5FU-LV (p=0.04). In conclusion, both analysis of single genes and gene clusters offer potential possibilities to tailor therapy, although the whole genome approach may be of more for combination therapy.

*Conflicts of Interest*

*This abstract is intended as the invited lecture; there are no other conflicts of interest*

**Cell Cycle Modulation Enhances the Cytotoxicity of Thymidylate Synthase Inhibitors**

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Thymidylate synthase (TS) is a cell cycle regulated enzyme. It increases during proliferation and usually has a higher activity during the S-phase in order to provide the cell with sufficient dTTP to facilitate DNA synthesis. Hence inhibition of cyclin dependent kinases (CdK) may lead to a decrease of TS and enhance the inhibition of TS. A number of CdKs control progress of the cell cycle together with checkpoint kinases (ChK1 and ChK2) which are usually activated by phosphorylation mediated by protein kinases such as protein kinase C (PKC). Both staurosporine (STS) and UCN-01 are inhibitors of PKC, but STS also inhibits CdK2, while UCN-01 inhibits CdK2, 4 and 6 as well as ChK1, cyclin D and pRb. We investigated the interaction between 5-fluorouracil (5FU) and STS or UCN-01 in syngeneic colon cancer cells, either wild type for p53 (LovoB2) or mutated (Lovo175x2). Cell growth inhibition was evaluated using the the sulphorodamine B (SRB) test, synergism was evaluated using the multiple drug effect analysis yielding combination indices (CI: synergism < 0.9; antagonism: > 1.1), cell cycle distribution and cell death by FACS analysis, cell cycle proteins by

western blots, while TS expression was measured by radioactive assays. 5FU was combined with STS or UCN-01 using simultaneous and sequential schedules. Cytotoxicity of 5FU was enhanced by UCN-01 (LovoB2, CI=0.4; Lovo175x2, CI=0.6) but less for STS (CI=0.8-0.9); interaction was more favourable when 5FU preceded STS. At IC80 values, 5FU (5 □M) induced an S-phase arrest (2-3 fold) in both cell lines, 0.5 □M UCN-01 a slight decrease in G2-M arrest but 0.05 □M STS not. 5FU and UCN-01 combinations all decreased G2-M phase. STS and 5FU combinations led to a similar S-phase accumulation as with 5FU. Induction of cell kill after 48 hr by UCN-01 was independent of p53, as it was 2-3 fold higher (25%) in Lovo175x2 cells compared to LovoB2, for STS this was similar for both cell lines (5-10%), as well as for 5FU (2-5%). Simultaneous 5FU and STS or UCN-01 resulted in additive cell kill in both cell lines, but 5FU followed by STS cell kill increased to 30% in both cell lines, but not for 5FU followed by UCN-01. At a molecular level 5FU caused an increase in TS levels (predominantly as the ternary complex), STS down-regulated TS partially, but UCN-01 completely, which was associated with a similar decrease in E2F. STS, UCN-01 and 5FU treatment also decreased TS catalytic activity in both cell lines. 5FU caused a transient appearance of pChK expression at 24 hr which was enhanced by UCN-01 and STS.

In conclusion, cell cycle abrogation caused differential effects on 5FU, depending on scheduling, possibly by the interaction with the E2F-pRB complex and the ChK1 regulation.

**Histidine Phosphorylation, or Tyrosine Nitration, Affects Thymidylate Synthase Properties**

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Thymidylate synthase (TS; EC 2.1.1.45), a target in chemotherapy, catalyzes the N5,10-methylenetetrahydrofolate (meTHF)-assisted C (5)-methylation of dUMP.

Possible phosphorylation of TS, previously reported [Samsonoff et al. J. Biol. Chem. 272, 13281-13285 (1997)], prompted us to examine this in more detail. TS preparations highly purified in the presence of phosphatase inhibitors, including endogenous TS forms from L1210 parental and FdUrd-resistant cells, as well as mouse, rat, human and Trichinella spiralis recombinant TSs expressed in bacterial cells, contained a low proportion of phosphorylated forms, as analyzed with the Pro-Q® Diamond Phosphoprotein Gel Stain following SDS-PAGE, but MS analysis of the bands revealed no phosphorylated amino acid residues. By contrast, MS analysis of IEF fractions of TS preparations from parental and FdUrd-resistant mouse leukemia L1210 cells, whose differing sensitivity to inactivation by FdUMP and its analogues was previously found not due to mutations [Cie□la et al. Acta Biochim. Pol. 53, 189-198 (2006)], demonstrated phosphorylation of Ser10 and Ser16 in the resistant, but not the parental, enzyme.

Enrichment of phosphorylated fractions of each of the four recombinant TS preparations using metal oxide/hydroxide affinity chromatography on Al (OH)3 beads, yielding always ? 1% of the total protein, allowed to demonstrate that TS phosphorylation is responsible for a 3-4-fold lower Vmaxapp, with unaltered Kmapp for either substrate or cofactor, and ability to repress in vitro translation of TS cognate, as well as luciferase, mRNA. Surprisingly, while MS analyses did not reveal the presence of phosphorylated residues in any of the fractions investigated, 31P NMR spectroscopy demonstrated clearly the presence of phosphorylated residues only in the phosphorylated enzyme fractions. Further analyses of the 31P NMR spectra (including their time-dependent changes following acidification), and comparison with those of synthetic phosphoramide derivatives of basic amino acids (Lys, Arg and His), and

commercially available phospho-amino acids, revealed the presence of phosphorus in a phosphoramide (acid-labile) bond, pointing to modification of histidine residue (s).

Biological nitration of protein tyrosine residues, that may affect protein function, is associated with over 50 diseases, including cancer, associated with intensified NO biosynthesis. Each human, mouse and Ceanorhabditis elegans recombinant TS preparation, incubated in vitro in the presence of $NaHCO_3$, $NaNO_2$ and $H_2O_2$, underwent tyrosine nitration, leading to a Vappmax 2-fold lower following nitration of 1 (with human or C. elegans TS) or 2 (with mouse TS) tyrosine residues per monomer. Enzyme interactions with dUMP, meTHF or 5-fluoro-dUMP were not distinctly influenced.

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**Methotrexate-induced Senescence in Human Adenocarcinoma Cells is Associated with Induced Expression of p53 Effector Genes**

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OBJECTIVE: A classic antifolate methotrexate (MTX) has been reported to induce in cancer cells a wide array of chemotherapeutic responses, including apoptosis, differentiation and growth arrest. Chemotherapeutic efficacy of MTX was also found to be impaired due to development of various resistance mechanisms. In the present study senescence-like growth arrest is demonstrated to constitute the main type of human colorectal adenocarcinoma C85 cells response to 1 μM MTX, a dose corresponding to clinically relevant drug concentration attained with high-dose MTX therapy.

METHODS AND RESULTS: The following hallmarks of accelerated senescence in C85 cells were identified: (i) positive staining for senescence-associated β-galactosidase activity, (ii) growth arrest occurring at the G1 and S phases of the cell cycle, (iii) decrease in DNA synthesis level, (iv) increased expression of cyclin-dependent kinase (CDK) inhibitor, p21waf1/cip1, and decreased expression of p16INK4a CDK inhibitor. The latter finding pointed to p53, rather than p16-pRB-dependent signaling pathway as underlying induction of apparent senescence phenotype. Analysis of comparative expression microarray data performed in Ingenuity Pathways Analysis software (Ingenuity Systems) allowed to identify a set of p53 pathway effector genes whose expression is upregulated during C85 cells treatment with MTX, followed by subsequent recovery in drug-free media. Those genes are classified as exerting various biological functions, including inhibition of angiogenesis, promotion of cell survival, cell cycle arrest and DNA repair. Certain p53 effector genes, including those underlying the process of senescence, were identified as upregulated only at the initial exposure to MTX, but not during the recovery period. Importance of p53 signaling in MTX-induced senescence in C85 cells is further stressed by overexpression of genes regulating acetylation of p53 protein.

CONCLUSION: Induction of accelerated senescence in C85 cells exposed to MTX seems to be dependent on a neat regulation of p53 activity and p53 effector functions.

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**Determination of Folate Monoglutamates in Biofortified Rice with UPLC-MS/MS**

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Folates are essential nutrients for human beings. To reduce folate deficiencies and consequences thereof, such as spina bifida, in developing areas like Northern China, biofortification of rice by means of genetic modification is one of

the approaches. We successfully overexpressed two plant genes involved in the folate pathway in rice (1) and evaluated these interventions by a validated UPLC-MS/MS method.

Rice was homogenized with an Ultra-Turrax® or a Retsch mill®. After heating, a tri-enzyme treatment (α-amylase, protease and deconjugase) was performed for the complete extraction of folates trapped in the complex matrix and to hydrolyse the polyglutamates to their corresponding monoglutamate form. Subsequently, the extract was centrifuged, ultrafiltrated, and injected in the UPLC system, on an Acquity UPLC® HSS T3 column (150 x 2.1 mm, 1.8-µm particle size), coupled to tandem mass spectrometry and eluted under gradient conditions.

The LOD and LOQ of the individual folates ranged between 0.06 and 0.45 µg/100g, and 0.14 and 1.07 µg/100g, respectively. Intra- and inter-day reproducibility were below 15 % and accuracy was within the acceptable limits (85-115%). Matrix effects were compensated for by use of isotopically labelled internal standards. Most folates in rice extracts were stable when kept at -20 and -80°C, but degraded after three freeze-thaw cycles or at 4°C, even when stored protected from light.

This method was applied to quantify folates in wild type and in genetically modified rice. Wild type rice had folate concentrations around 20 µg/100g, while some biofortified lines yielded up to 1000 µg/100g. 5-Methyl-THF was the dominant natural folate form (± 90% of total folate content for biofortified rice). These data correlate very well to previously reported results (1), which were analysed with an HPLC-MS/MS method (2).

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**Application of Microbiological Assay and Liquid Chromatography-ionisation Mass Spectrometry for the Profiling of Rice Grain Folates**

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This work is an effort to measure naturally occurring folates in rice grains by high performance liquid chromatography-tandem mass spectrometry (LC-MS/MS) and to validate against/compare with microbiological assay (MA) using Lactobacillus rhamnosus (NCIMB 10463) as a test organism. Fifty one rice cultivars of different ecosystems and types were assayed for their individual total folate content using MA. Profile of some of the rice varieties with relatively high, medium and low folate concentration based on the MA results were analysed using a LC-MS/MS procedure. The analytes were extracted from the grains using a mono-enzyme (?-amylase) treatment, commercially-available folate standards and internal standards (MTX, tri-MTX and hexa-MTX) were spiked into each corresponding sample replicates, and analysed by LC-MS/MS in the negative ion mode using electrospray ionisation. The application of the system was verified by analyzing a certified reference material (CRM 121-wholemeal flour) and a plant quality control (spinach). The results showed that LC-MS/MS gave good separation of the major monoglutamate folate forms - 5-methyl tetrahydrofolate, 5-formyl tetrahydrofolate. In agreement to the results evidenced in other studies comparing folate values determined chromatographically and microbiologically, this study revealed lower values for folate concentration in rice grains. However, recovery tests showed that extraction method employed gave low % recovery for most forms of folate especially for the mostly unstable polyglutamates as solid-phase extraction was not used due to its incompatibility with the current system being used. An alternative method but mass spectrometry-friendly extraction should be explored in the future using this novel chromatography

technique.

**Evolution of Structure, Function and Regulation in Phenylalanine Hydroxylase**

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The aromatic amino acid hydroxylases (AAAH) constitute a family of tetrameric tetrahydrobiopterin (BH4)-dependent enzymes which in mammalian includes phenylalanine hydroxylase (PAH), tyrosine hydroxylase (TH) and tryptophan hydroxylase 1 and 2 (TPH1 and 2). Neuronal TH and the TPHs are rate limiting enzymes in the synthesis of catecholamines and serotonin, respectively, while liver PAH is fundamentally catabolic and responds with positive cooperativity to increased concentration of the substrate. Dysfunction of the PAH system, as found in the disease phenylketonuria (PKU), leads to accumulation of L-Phe in plasma and neurological damage in untreated patients. The cooperative activation of mammalian PAH by L-Phe represents a protection mechanism that safeguards L-Phe homeostasis in blood in order to (i) avoid PKU and (ii) provide a continuous supply of tyrosine for protein synthesis. In addition to the cooperative response to phenylalanine binding, PAH is regulated by phosphorylation at Ser16, which acts synergistically with L-Phe activation, and by BH4, which has an inhibitory effect on PAH that protects the enzyme from degradation in the resting state.

We have studied the structure, function and regulation of PAH isolated from bacteria (bPAH), the protozoan Dictyostelium discoideum (DictyoPAH) and the nematode Caenorhabditis elegans (cePAH), and perform comparative analysis with the human form of the enzyme (hPAH) (1-3). Our results support that PAH from prokaryotes and early eukaryotes has a main anabolic function and uses L-Phe as a precursor of tyrosine-derivatives such as melanins. While bPAH is monomeric, DyctioPAH and cePAH are tetrameric but both lack the sophisticated regulatory mechanisms found in the mammalian enzyme, including the positive cooperativity for L-Phe. This indicates that the importance of proper regulation and prompt response of the mammalian enzyme to elevated substrate level have evolved with the complexity of the nervous system and the need for protection against neurotoxic L-Phe levels.

In addition to extensive site-directed mutagenesis we have constructed structural models of all PAH forms based on crystal structures of truncated forms and homology modelling. Molecular dynamics simulations have been applied to these models to get insights into the conformational changes related to function and regulation of hPAH by both the substrate and biopterin cofactor at an atomic level. Our results have also contributed to better understand the molecular pathogenic mechanisms behind PKU and to define the evolutionary history of the AAAH family (2).

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**Phosphorylated Tyrosine Hydroxylase Formed Insoluble Aggregates by Inhibition of Ubiquitin Proteasome System in NGF-differentiated PC12D Cells**

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OBJECTIVE: Tyrosine Hydroxylase (TH) is a rate-limiting enzyme for the biosynthesis of catecholamines including dopamine (DA), and requires tetrahydrobiopterin (BH4) as a cofactor. Parkinson's disease (PD) is caused by degenera-

tion of nigro-striatal dopaminergic neurons, and formation of protein aggregates, called Lewy body, is a pathological feature. The cause of dopaminergic neuron-specificity is yet unclear. In order to see possible involvement of TH on the etiology of PD, we examined the effect of proteasomal inhibition on the TH protein in NGF-differentiated PC12D cells.

METHODS: To approach the biochemical character and metabolic pathway of TH, we used DA biosynthesizing cells, PC12D cell line. Cells were cultured in the DMEM medium and treated with MG-132 or PSI, which is 26S or 20S proteasomal inhibitor, respectively. For double-labeling immunofluorescent analyses, cells were fixed and stained with each antibody or Thioflavine-S. For the Western blot analyses, cells were lysed and ultracentrifuged to separate soluble and insoluble components.

RESULTS: We found that p40-TH formed cytoplasmic aggregates which are Thioflavine-S positive, following the proteasomal inhibition by MG-132 or PSI. Choline acetyltransferase (ChAT), an enzyme for biosynthesis of acetylcholine, did not form such aggregates. p40-TH positive spots were colocalized with ubiquitin. In the Western blot analysis, phosphorylated TH was accumulated in the insoluble fractions.

CONCLUSIONS: We showed for the first time that p40-TH readily made insoluble aggregates and that p40-TH was degradated through ubiquitin-proteasomal system in PC12D cells. It might be possible that insolubilization of p40-TH may be involved in formation of Lewy bodies in dopaminergic neurons in the brain of PD patients.

## Alkylglycerol Monooxygenase Candidate Genes

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Alkylglycerol monooxygenase (glyceryl ether monooxygenase, E.C. 1.14.16.5) is a tetrahydrobiopterin - dependent, membrane associated enzyme with unknown sequence. We have developed a novel, highly sensitive assay for this enzyme, which allows the detection of alkylglycerol monooxygenase activities in nanogram amounts of protein, making it suited for testing activity in mammalian cells transfected with expression plasmids encoding alkylglycerol monooxygenase candidate genes. From the handling of the tetrahydrobiopterin cofactor, alkylglycerol monooxygenase resembles more the aromatic amino acid hydroxylases than the nitric oxide synthases. Regarding the chemistry of the substrate hydroxylation reaction, alkylglycerol monooxygenase differs from both classes of known tetrahydrobiopterin dependent enzymes, i.e. from the aromatic amino acid hydroxylases and the nitric oxide synthases, in that an aliphatic carbon centre is hydroxylated.

Based on the similarity in handling of the tetrahydrobiopterin cofactor, we were looking with in silico methods in protein databases for candidates with similarities to aromatic amino acid hydroxylases in the primary sequence where tetrahydrobiopterin is bound, or, in motifs for tetrahydrobiopterin binding with respect to the position of amino acids in the primary sequence. In addition, we used two different methods to look for homologues of aromatic amino acid hydroxylases with respect to the tertiary structure, or with respect to properties predicted to contribute to a similarity in tertiary structure. Finally, we analysed partially purified fractions of rat liver alkylglycerol monooxygenase for proteins by mass spectrometry.

Our investigations using the primary sequence and primary sequence motifs yielded the conclusion that no protein with significant similarity with aromatic amino acid hydroxylases can be found in mammalian protein databases. Using

homology searches with two predicted structure based approaches, and of mass spectral data of partially purified fractions, produced a list of candidates, which we filtered according to comparison of data on subcellular localization and expression in tissues in comparison with experimental data we have collected on alkylglycerol monooxygenase activity. The most promising candidates will now be selected and expression plasmids will be transfected into mammalian cells to test for alkylglycerol monooxygenase activity.

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**Alkylglycerol Monooxygenase: Properties of an Orphan Enzyme**

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Among the 1299 orphan enzymes which have been characterised regarding their function but have not yet been linked to a gene, there is also the tetrahydrobiopterin-dependent enzyme alkylglycerol monooxygenase (glyceryl ether monooxygenase, [EC 1.14.16.5]). This enzyme cleaves alkyl glycerols into the corresponding aldehydes and glycerol. The physiological role of alkylglycerol monooxygenase has also not yet been clarified in detail but there is increasing evidence that alkylglycerols have important roles in both cellular structure and signalling and are required for proper brain function.

Since we could not purify the protein so far due to activity losses, we sought for a deeper insight into the properties of alkylglycerol monooxygenase in tissue homogenates to improve the chances of getting a pure protein together with its sequence information.

We were able to show that alkylglycerol monooxygenase accomplishes its catalytic function by the use of a metal ion which is not heme-bound like the iron in the tetrahydrobiopterin-dependent nitric oxide synthase but is more loosely attached in analogy to the histidines-bound iron in the aromatic amino acid hydroxylases.

Another property that shows the similarity of alkylglycerol monooxygenase to the aromatic amino acid hydroxylases is the co-factor handling which was tested with two pterin analogs.

We also investigated the tissue expression profile of this enzyme in both rat and mouse. Besides an overall similar expression pattern with high activity in the liver, the gastrointestinal tract and fat tissue, an interesting difference between the two species could be detected: While rats have a much higher activity in their livers ($1253.5 \pm 46.0$ pmol/ (mg*min)), mice livers display peak values which are about 50 times lower.

Our data show that alkylglycerol monooxygenase is biochemically related to the family of aromatic amino acid hydroxylases and, based on its broad tissue distribution, imply that it might contribute to the important role of alkylglycerols in physiology.

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**Direct Monitoring of Fatty Aldehyde Dehydrogenase Activity by Pyrenedecanal**

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Degradation of alkylglycerols in cells into glycerol and long chain fatty aldehydes is catalysed by the tetrahydrobiopterin-dependent enzyme alkylglycerol monooxygenase (glyceryl ether monooxygenase, EC 1.14.16.5). In a consecutive enzymatic reaction the toxic aldehydes are then further converted into the corresponding acids. This second reaction is catalysed by the NAD+-dependent enzyme fatty aldehyde dehydrogenase (EC 1.2.1.48, gene symbol: ALDH3A2). Fatty aldehyde dehydrogenase is a membrane-bound protein that accepts a wide variety of aliphatic aldehydes (typically 6-24 carbon atoms) as substrate. Missense mutation in the ALDH3A2 gene can cause the autosomal recessive disorder Sjögren-Larsson Syndrom (SLS). Symptoms such as ichthyosis, mental retardation and spasticity are caused by accumulation of fatty aldehydes in cells, which leads to the formation of Schiff base adducts with amine containing lipids or proteins.

We developed a direct and sensitive HPLC assay for the measurement of fatty aldehyde dehydrogenase activity using the novel fluorescent labelled substrate pyrenedecanal. Activity distribution of fatty aldehyde dehydrogenase in mouse tissues showed highest activity levels in liver, stomach, ovaries and testes and only marginal activities were measureable in heart and skeletal muscle.

We measured a residual activity of 5.5 ± 2.7% in skin fibroblasts of an SLS patient, as compared to a healthy control. For the measurement we needed 20-50 times less protein than is required for currently used assays.

The novel assay was also adapted for the measurement of enzyme activity in slices of SDS gels run under mild conditions. Activity migrates at about 100 kDa indicating the presence of a homodimer. Presence of the protein in the peak fraction was verified by MALDI-TOF mass spectrometry.

Thus, direct monitoring of fatty aldehyde dehydrogenase by the novel substrate pyrenedecanal and HPLC determination of the product pyrenedecanoic acid allows the reliable and sensitive detection of enzyme activity in nanogram amounts of microsomal or microgram amounts of fibroblast protein.

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**Hopkins Lecture**
**Enzymology of Mammalian Mitochondrial One-carbon Metabolism**

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Previous studies in our laboratory showed that isolated, intact adult rat liver mitochondria are able to oxidize the three-carbon of serine and the N-methyl carbon of sarcosine to formate without the addition of any other cofactors or substrates [Garcia-Martinez and Appling (1993) Biochemistry 32, 4671-4676]. It was proposed that these one-carbon units are transferred to tetrahydrofolate (THF) to form 5,10-methylene-THF (CH2-THF) via the activities of serine hydroxymethyltransferase or sarcosine dehydrogenase, and then oxidized to formate by mitochondrial CH2-THF dehydrogenase, 5,10-methenyl-THF (CH+-THF) cyclohydrolase, and 10-formyl-THF synthetase activities. Two mammalian mitochondrial folate-interconverting enzymes have been identified to date: a bifunctional CH2-THF dehydrogenase/CH+-THF cyclohydrolase, encoded by the MTHFD2 gene [Belanger and MacKenzie (1989) J. Biol. Chem. 264, 4837-4843]; and a monofunctional 10-formyl-THF synthetase encoded by the MTHFD1L gene [Walkup and Appling (2005) Arch. Biochem. Biophys. 442, 196-205]. Because the MTHFD2 gene is not expressed in most adult tissues, and the MTHFD1L enzyme is a monofunctional 10-formyl-THF synthetase, the enzyme (s) responsible for conversion of CH2-

THF to 10-formyl-THF in adult mammalian mitochondria remain unknown.

A new mitochondrial CH2-THF dehydrogenase isozyme, encoded by the MTHFD2L gene, has now been identified. This gene is present in all sequenced vertebrate genomes, including mouse and rat. The mammalian MTHFD2L gene is composed of 8 exons, encoding a protein of about 338 amino acids homologous to the mitochondrial bifunctional dehydrogenase/cyclohydrolase and to the dehydrogenase/cyclohydrolase domain of cytoplasmic C1-THF synthase. The MTHFD2L proteins have a predicted N-terminal mitochondrial targeting sequence. Importantly, the MTHFD2L proteins possess the four critical dehydrogenase/cyclohydrolase residues that are substituted in the MTHFD1L-encoded monofunctional mitochondrial C1-THF synthase. The recombinant protein exhibits robust CH2-THF dehydrogenase activity, and is localized to mitochondria when expressed in CHO cells. The MTHFD2L gene is expressed in adult tissues, thus completing the pathway from CH2-THF to formate in adult mammalian mitochondria.

## Genotype-predicted Tetrahydrobiopterin ($BH_4$)-Responsiveness and Molecular Genetics in Croatian Patients with Phenylalanine Hydroxylase (PAH) Deficiency

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Specific mutations in the gene encoding phenylalanine hydroxylase (PAH), located on chromosome 12q22-24.1, are linked to tetrahydrobiopterin (BH4; sapropterin)-responsive phenylketonuria (PKU). Diagnosis is usually done through the newborn screening for PKU, followed by a BH4 loading test. So far, more than 60 mutant alleles, presenting with a substantial residual PAH activity (average ~47%), were identified in more than 500 patients worldwide. We investigated the predictive value of BH4-responsive PAH mutations in Croatian population. From a group of 127 PKU patients, 62 were selected (based on the genotype) as potentially BH4-responsive and 39 loaded with BH4 (20 mg/kg). The overall frequency of BH4-responsiveness (>30% blood phenylalanine reduction within 24 hours) was 36% (14 out of 39 patients with 23 different genotypes), significantly less than expected. The best responders were patients with mild hyperphenylalaninemia (4/4; 100%), followed by mild PKU (8/9; 89%), and classical PKU (2/26; 8%). The most common BH4-responsive genotypes were p.E390G/p.R408W and p.P281L/p.E390G. These genotypes correspond for approximately >30% residual PAH activity. The p.E390G mutation was 100% associated with BH4-responsiveness, regardless of the second allele (p.R408W, p.P281L, p.F55Lfs, p.L249P). With regard to the predicted relative PAH-activity of recombinantly expressed mutant alleles, there was a significant ($p<0.002$) difference between BH4-responders and non-responders.

In a general Croatian PKU population, disease-causing mutations were identified on 226 alleles (99%). There were 35 different mutations: 21 missense, 8 splice site, 3 nonsense, 2 single nucleotide deletions, and one in-frame deletion. Four mutations are reported for the first time: p.E76D, p.L333P, p.G346E, and IVS8-2A>G. Five mutations accounted for over two thirds of investigated alleles: p.L48S, p.R261Q, p.P281L, p.E390G, and p.R408W. Thus, the Croatian PKU population seems to be more homogenous than some other Mediterranean or Central European populations.

This study reveals the importance of a full genotype for the prediction of BH4-responsiveness. In contrast to previous assumption and with exception of the p.E390G mutation, single allele mutations are not reliable for the selection of potential PKU candidates for pharmacological therapy with BH4.

**Molecular and Biochemical Basis of Tetrahydrobiopterin Responsiveness in Phenylketonuria**

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Tetrahydrobiopterin (BH4)-responsive phenylalanine hydroxylase (PAH) deficiency is characterized by the reduction of blood phenylalanine level after a BH4 administration. Phenylalanine breath test (PBT) quantitatively measures the conversion of L-[1-13C] phenylalanine to 13CO2 and is useful for in vivo PAH activity. The present study is designed to determine whether in vivo PAH activity is consistent with the clinical phenotype and genotype and whether the determination of this parameter is useful for controlling BH4-responsive PAH deficiency.

Methods: The patients were diagnosed as having mild phenotype of PAH deficiency. Single- and/or four-dose of BH4 loading tests revealed a decline of more than 20% and/or 30%, respectively, of blood phenylalanine. The patients were diagnosed as BH4-responsive PAH deficiency and followed by a BH4 treatment for more than one months. For the phenylalanine breath test, $^{13}$C-phenylalanine was administered orally at a dose of 10 mg/kg (maximum of 200 mg) after an overnight fast. In vivo PAH activity was expressed as the ratio of the total amount of $^{13}CO_2$ in expiration during 120 min for administered dose of $^{13}$C-phenylalanine. The effect of BH4 was examined by PBT with BH4 administration (10 mg/kg/day) for three days and/or a long term-BH4 treatment.

Results: The patients with more than a 5 years long-term BH4 treatment (6-20mg/kg/day) showed 2-8 mg/dl of blood phenylalanine with relaxed diet therapy or free. All patients had at least a mild mutation in one or two alleles, which were R241C, A373T, and P407S/severe mutation and R241C/R241C. After a BH4 treatment, the patients showed stable blood phenylalanine level under the infectious disease and several stress. In addition, the compliance and the QOL of the patient have been improved remarkably. After BH4 loading, in vivo PAH activity in mild PKU patients (R241C/ severe mutation) increased from 1.09 to 3.26 (PAH activity level in the mild HPA). PAH activity in a mild HPA patient (R241C/ R241C) increased from 2.74 to 7.22, which can maintain 2mg/dl in the blood phenylalanine. The effect of the BH4 on PBT supports the result of the long-term treatment mentioned above.

Conclusion: The in vivo PAH activity and the genotype were related to BH4-responsiveness. Until now, finding BH4-responsiveness in PKU patients was referred to be important. However, setting the optimal dose of BH4 and the blood phenylalanine level will be also important, and PBT and genetic diagnosis will become indicators.

**BH4 Responsive Phenylketonuria and BH4 Deficiency Phenylketonuria in Korea**

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*BH4 Responsive Phenylketonuria in Korea*

Of the 152 patients who were diagnosed and treated for hyperphenylalaninemia in Department of Pediatrics, Soonchunhyang University Hospital from 1999 to 2009, 10 patients were BH4 responsive PKU. Among these 10 patients, 6 patients were male and 4 patients were female. On BH4 loading test, opposed to the fact that the phenylalanine level decreased to nearly normal range on PTPS (6-pyruvoyl-tetrahydropterin synthase) deficiency patients, that of BH4 responsive

classic PKU patients were decreased to 40.6% after 8 hours. Four patients were treated only with BH4 whereas the other 6 patients were treated with BH4 and phenylalanine-free milk. The gene mutations of these patients were as following: R241C/A259T (3 patients), R241C/T278I (1 patient), Y356X/R408Q (1 patient), R64H/ R243Q (1 patient) and R241C/R243Q (2 patients).

As we report, many of the classic phenylketonuria patients respond to BH4 loading test. Therefore, all patients who were detected on neonatal screening test must be challenged by BH4. Instead of the phenylalanine-restrict diet, BH4 treatment would be expected to improve their quality of life substantially in BH4 responsive PKU patients.

*BH4 deficiency Phenylketonuria in Korea*

Of the 152 patients who were diagnosed and treated for hyperphenylalaninemia in Department of Pediatrics, Soonchunhyang University Hospital from 1999 to 2009, 15 patients were BH4 deficiency PKU. Among these 15 patients, 13 patients were 6-pyruvoyltetrahydropterin synthase (PTPS) deficiency, 1 patient was dihydropteridin reductase (DHPR) deficiency. 14 BH4 deficiency PKU patients except for 1 patient with DHPR deficiency were treated with L-dopa, 5-hydroxytryptophan and BH4. 1 patient with DHPR deficiency were treated with L-dopa, 5-hydroxytryptophan and leukovorin. The gene mutation of these patients were as following: 259C>T/12S 1-291A>G (1 patient), 272A>G347GA>G (1 patient), 155A>G/259C>T (1 patient), 259C>T/317C>T (1 patient), P87S/T106M (1 patient), N52S/exon3skipping (1 patient) and C.259C>T/C.259C>T (2 patients). The gene mutation of DHPR deficiency patient was c.253T>C/c.515C>T.

## Optimizing the Use of Sapropterin (BH4) in the Management of Phenylketonuria

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In PKU a Phe-restricted diet is essential to reduce blood Phe levels and prevent long-term neurological impairment and other adverse sequelae. This diet is commenced within the first few weeks of life and current recommendations favor lifelong diet therapy. The observation of clinically significant reductions in blood Phe levels in a subset of patients with PKU following oral administration of 6R-tetrahydrobiopterin (BH4), a cofactor of PAH, raises the prospect of oral pharmacotherapy for PKU. An orally active formulation of BH4 (sapropterin dihydrochloride) is now commercially available.

Firstly, patients who may respond to this treatment need to be identified. We propose an initial 3-day test: first day blood Phe monitoring at -8, -16, and -24h, second and third day a challenge with 20 mg/kg and subsequent Phe monitoring at 8, 16, and 24h, followed by a 1-4-week trial of sapropterin and subsequent adjustment of the sapropterin dosage and dietary Phe intake to optimize blood Phe control in responders.

Clinical studies suggest that treatment with sapropterin provides better Phe control and increases dietary Phe tolerance, allowing significant relaxation, or even discontinuation, of dietary Phe restriction. Overall, sapropterin represents a major advance in the management of PKU.

*Conflicts of Interest*
*supported by Merck Serono*

**Lower Plasma Neopterin Concentration in Dopa-responsive Dystonia Patients**

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OBJECTIVE: Partial defects of guanosine 5'-triphosphate cyclohydrolase I (GTPCH) cause dopa-responsive dystonia (DRD). To identify DRD chemically, determination of decrease of GTPCH activity in cultured fibroblasts, measurement of neopterin and biopterin concentration in CSF, or prolonged hyperphenylalaninemia after phenylalanine loading test has been proposed. We diagnosed several DRD patients by using the method of direct sequencing of GCH1 gene, which was coding GTPCH. Then, we measured their plasma neopterin concentration and compared to controls.

PATIENTS AND METHODS: We examined 47 Japanese in this study. We identified mutations in GCH1 gene of 7 DRD patients. We measured neopterin and biopterin concentration in plasma and cerebrospinal fluids (CSF) of these patients. We also examined 7 non-dystonic controls and 33 dystonic but dopa non-responsive controls.

RESULTS: In DRD patients, 3 patients had missense mutations and 3 patients had nonsense mutations. The rest of them had deletion of exon 2 and 3, which has been identified in another institute. In missense mutations, 2 nonrelated patients had an A190V mutation and another patient had a T106I mutation. In nonsense mutations, two mutations, which were K107fs and M211fs, were identified. The M211fs mutation was observed in a sibling case. A190V and K107fs were novel mutations. Plasma neopterin concentration of DRD patients (6.80 ± 2.72 nM) was significantly lower than those of non-dystonic controls (22.36 ± 6.59 nM; $p<0.001$) or dopa non-responsive dystonic controls (17.83 ± 6.08 nM; $p<0.001$). No significant difference was observed between non-dystonic controls and dopa non-responsive dystonic controls. Neopterin and biopterin concentration in CSF of DRD group were significantly lower than those of non-dystonic controls, or dopa non-responsive dystonic controls as plasma neopterin concentration. Plasma biopterin concentration did not show any difference among those groups.

CONCLUSIONS: DRD patients showed lower plasma neopterin concentration in addition to lower CSF neopterin and biopterin concentration. The relationshipbetween the decrease of plasma neopterin concentration and neurological symptoms in DRD was still unknown . However, lower plasma neopterin concentration in DRD patients would indicate the loss of their GTPCH activity. Measurement of plasma neopterin concentration could improve the diagnostic classification with DRD vs dopa non-responsive dystonia patients.

**Phenylketonuria: Correlations Between Lumbar Bone Mineral Density and Vitamin and Mineral Blood Concentrations**

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The pathophysiology of reduced bone mineral density (BMD) in subjects with phenylketonuria (PKU) remains obscure.

OBJECTIVE: To investigate relationships between Z-scores of lumbar BMD and vitamins, minerals, blood phenylalanine concentrations, variation in phenylalanine concentrations, and severity of PKU.

DESIGN: Retrospective cohort study.

METHODS: BMD was examined in 53 Dutch PKU patients (mean age 16.7, range 2 - 35 years) using dualenergy X-ray absorptiometry scans (DXA-scans) of the lumbal spine. Subjects were divided in three age groups: < 10.0 yrs (n 12), 10.0-19.9 yrs (n 23), and ≥ 20.0 yrs (n 18). Correlations between Z-scores of BMD and blood concentrations of vitamins, minerals and phenylalanine, variation in phenylalanine concentrations, and severity of PKU were investigated. Subjects with reduced BMD and normal BMD for chronological age were compared to analyze pos-

sible differences in the parameters mentioned.

RESULTS: BMD was statistically significant reduced in all age groups, without statistically significant differences between age groups. Z-score of BMD showed statistically significant negative correlations to blood calcium concentrations (R2 -0.485, p 0.004). Other vitamins and minerals were not statistically significant correlated to Z-scores of BMD. No statistically significant correlations were found between Z-score of BMD and blood phenylalanine concentrations, variation in phenylalanine concentrations, and severity of PKU.

CONCLUSIONS: Reduced BMD in PKU seems to start at early age, not to progress with age, and does not seem to be associated with toxicity or deficiency of phenylalanine, nutritional deficiencies of vitamins and minerals, or severity of PKU.

**Gene Therapy for Phenylketonuria Exploiting Liver or Muscle for Phenylalanine Clearance**

Beat Thöny

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PKU is a frequent inherited disorder of amino acid metabolism caused deficiency of hepatic phenylalanine hydroxylase (PAH) resulting in accumulation of Phe and its metabolites in blood and other tissues. Treatment consists of Phe intake restriction, which prevents severe neurological damage in PKU patients, although mild neuropsychological findings, such as poor school performance, a slight reduction in intelligence quotient, and the presence of tremor may arise, especially when careful dietary compliance is not achieved. We have previously shown life-long therapeutic correction of PKU in a PKU mouse model by using a recombinant AAV2 pseudotype-8-mediated transfer of the murine PAH gene to liver after portal vein or tail vein administration.1 Recently, we demonstrated effective long-term correction for PKU also following intramuscular delivery of a recombinant triple-cistronic AAV2 serotype 1 expressing ectopically in muscle tissue PAH along with two essential genes for tetrahydrobiopterin biosynthesis.2 This non-invasive approaches are the basis to develop strategies for an efficient and safe gene therapy procedure for PKU.

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**Strategies to Restore Endothelial BH4 Levels and NO Synthesis as Potential Pharmacotherapy for Diabetetic Vasculopathy**

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Oxidation of BH4, in the setting of diabetes and other chronic vasoinflammatory conditions, results in cofactor insufficiency and uncoupling of endothelial NO synthase (eNOS), manifest by a switch from NO to superoxide production. We previously demonstrated that eNOS uncoupling is not simply a consequence of BH4 insufficiency, rather it results from a diminished ratio of BH4 vs. its catalytically-incompetent oxidation product, 7,8,-dihydrobiopterin (BH2). In support, [3H]-BH4 binding studies established that BH4 and BH2 bind eNOS with equal affinity (Kd " 80 nM) and BH2 can rapidly and efficiently replace BH4 in preformed eNOS-BH4 complexes. Whereas the total biopterin pool of murine endothelial cells (EC) is unaffected by 48 h exposure to diabetic glucose levels (30 mM), BH2 levels increased from undetectable to 40% of total biopterin. This BH2 accumulation was associated with a diminished capacity for NO synthesis by

eNOS and accelerated superoxide production. Since superoxide production was suppressed by NOS-inhibitor treatment, eNOS was implicated as a principal superoxide source. Importantly, BH4 supplementation of EC (in low and high glucose-containing media) revealed that A23187-evoked NO bioactivity correlates with intracellular BH4:BH2, and not absolute intracellular levels of BH4. Reciprocally, superoxide production was found to negatively correlate with intracellular BH4:BH2. Using an untargeted metabolite profiling approach, 42 molecular ions species were identified as uniquely accumulating in high vs. low glucose-treated EC, including the 6-dihydroxypropyl cleavage products of oxidized BH4, biopterin and pterin. Whereas chronic BH4 supplementation was associated with a worsening of high glucose-induced oxidation of BH4, therapy was afforded by treatment with a superoxide-dependent NO releasing agent. Together, these findings implicate diminished intracellular BH4:BH2 (rather than BH4 depletion per se) as the molecular trigger for NO insufficiency in high glucose treated EC and diabetes and suggest a novel chemotherapeutic approach for diabetic vasculopathy.

**Roles of Heme Oxygenase-1 in the Antiproliferative and Antiapoptotic Effects of Nitric Oxide on Jurkat T Cells**

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Nitric oxide (NO) has been shown to exert antiproliferative and antiapoptotic effects on human T cells. Heme oxygenase-1 (HO-1), which degrades heme into biliverdin, free iron (Fe2), and carbon monoxide (CO), has also been known to have antiproliferative and antiapoptotic effects. Recent evidence suggests that HO-1 is an important cellular target of NO; whether HO-1 expression contributes to the antiproliferative and/or antiapoptotic effects mediated by NO remains to be investigated. In the present study, we examined the effects of NO on HO-1 expression and possible roles of HO-1 in T cell proliferation and apoptosis. Using human Jurkat T cells, we found that the NO donor sodium nitroprusside (SNP) induced HO-1 expression and that preincubation with SNP suppressed

T cell proliferation induced by concanavalin A and apoptosis triggered by anti-Fas antibody. Suppressions of T cell proliferation and apoptosis comparable with SNP were also observed when the T cells were preincubated with the HO-1 inducer cobalt protoporphyrin. A phosphorothioate-linked HO-1 antisense oligonucleotide blocked HO-1 expression, and subsequently abrogated the antiproliferative and antiapoptotic effects of SNP. Overexpression of the HO-1 gene after transfection into Jurkat T cells resulted in significant decreases in T cell proliferation and apoptosis. The CO donor tricarbonyldichlororuthenium (II) dimer mimicked the antiproliferative effect of SNP, and the Fe2donor FeSO4 blocked anti-Fasinduced apoptosis. Taken together, our results suggest that NO induces HO-1 expression in T cells and that suppressions of T cell proliferation and apoptosis afforded by NO are associated with an increased expression of HO-1 by NO.

**Tetrahydrobiopterin, Nitric Oxide Synthase and FDG microPET for Monitoring the Hypoxic Ischemic Effect in Neonatal Pigs**

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OBJECTIVE: Brain injury induced by perinatal hypoxia-ischemia (HI) is a significant neurological problem in neonates. It has been reported that nitric oxide (NO) and tetrahydrobiopterin (BH4) play an important role in ischemic neuronal death. In this study, we investigated BH4 levels and the activity of NO synthase (NOS) using a newborn piglet model of HI as well as the level of brain injury by FDG-microPET imaging. We also examined the distribution of intravenously administered BH4 into the brain.

METHODS: Newborn piglets were ventilated with 6% oxygen, and both common carotid arteries were clamped for 45 minutes to induce HI. Control piglets were sham-treated. Blood samples were collected at 0, 4, 8, 12 hours after HI. Biopterin and NO concentration in brain tissue were measured at 12 hours after HI. To examine the distribution of BH4 into the brain, BH4 was administered intravenously (1mg/kg/hour) for 4 hours to both control and HI piglets, and measured biopterin levels in plasma and brain tissue. Biopterin concentrations in the plasma and brain were determined by HPLC and $NO_2$- and $NO_3$- concentrations by a commercial kit. FDG-microPET images were obtained using a microPET P4 system before and at 24 hours after HI.

RESULTS: In HI models, the level of biopterin, the degradation product of BH4, increased in the plasma after the HI insult, but not in the brain until 12 hours after HI. The NOS activity, as measured by $NO_2$- and $NO_3$- concentrations, was found in the brain in the HI group, but not in the controls, and increased 12 hours after insults. MicroPET imaging showed that (18)F-FDG uptake in the brain was significantly reduced after 24 hours of HI induction. After BH4 administration, the brain biopterin level showed no significant changes in the control animals but was increased in the HI group.

DISCUSSION: We identified an imbalance in biopterin distribution between the plasma and brain tissue in the acute phase of HI. This finding suggests that the production of biopterin in the CNS was reduced during the acute phase of HI, compared to other organs. The initial shortage of biopterin in the hypoxic-ischemic brain may have affected the severity of brain damage. MicroPET imaging showed low activities of brain tissues after HI. Neuronal damage seemed to be enhanced by the increase in superoxide production due to insufficiency of biopterin production in the brain. This study suggests the therapeutic possibility of supplying BH4 directly to the brain to prevent neuronal damage by hypoxic ischemic events.

**Critical Role for BH4 Recycling by DHFR in Regulation of eNOS Coupling**

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Tetrahyrobiopterin (BH4) is a required cofactor for the synthesis of nitric oxide by endothelial nitric oxide synthase (eNOS). BH4 bioavailability within the endothelium is a critical factor in regulating the balance between NO and superoxide production by eNOS (eNOS coupling). BH4 levels are determined by the activity of GTP cyclohydrolase I (GTPCH), the rate-limiting enzyme in de novo BH4 biosynthesis, and by oxidative loss of BH4 to 7,8-dihydrobiopterin (BH2) and other oxidized pterin species. BH4 can be regenerated from BH2 by dihydrofolate reductase (DHFR), but the importance of DHFR in maintaining eNOS function in endothelial cells remains unclear. We investigated the role of DHFR in endothelial cells and in a novel cell line stably expressing doxycyline-regulated human GCH cDNA and eNOS-GFP fusion protein. Pharmacological inhibition of DHFR activity by methotrexate, or genetic knockdown of DHFR protein by RNA interference, reduced intracellular BH4 and increased BH2 levels, resulting in enzymatic uncoupling of eNOS, as indicated by increased eNOS-dependent superoxide but reduced NO production. While a decreased BH4:BH2 ratio was induced by exposure of cells to DHFR-specific siRNA, total biopterin levels were attenuated approximately 90% by GTPCH-specific siRNA, with no change in BH4:BH2 ratio. In our cell culture model of eNOS uncoupling, DHFR inhibition exacerbated the already diminished BH4:BH2 ratio with concomitant changes in NO and superoxide production. Taken together, these data define a key role for DHFR protein in the maintenance of eNOS coupling. We report that while GTPCH protein is fundamental in regulating the amount of intracellular BH4, DHFR protein activity is critical to eNOS by determining BH4:BH2 ratio and thus in maintaining eNOS coupling.

**Tyrosine Modification of Beta2-tubulin and its Potential Nitric Oxide Signaling in Cardiomyogenesis**

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The importance of nitric oxide (NO) in cardiac development and cardiomyocyte differentiation from embryonic stem (ES) cell has been suggested; however, it remains undiscovered that what occurs at molecular level as the consequence of endogenous NO production. Employing cardiomyocyte differentiation from mouse P19CL6 ES cell line, we found out that beta2-tubulin, a component of microtubule, underwent a new type of posttranslational modification, tyrosine nitrosylation. Furthermore, we revealed that the modification mediates the interaction of beta2-tubulin with Op18/stathmin, a microtubule destabilizer. Western blot with nitrotyrosine antibody exihibited a prominant immunoreactivity on 50-kDa protein, in parallel with activation of endothelial NO synthase (eNOS) and increased NO production; meanwhile, treatment with NOS inhibitor L-NAME suppressed both endogenous NO production and the immunoreactivity. Through electrospray ionization tandem mass spectrometry (ESI-MS/MS) we identified the immunoreactive 50-kDa as beta2-tubulin and further revealed that beta2-tubulin was tyrosine nitrosylated, including two susceptible residues, Tyr-106 and Tyr-340. More interestingly, the tyrosine nitrosylation enhanced the interaction of beta2-tubulin with Op18/stathmin, a microtubule destabilizer and predisposed the modified beta2-tubulins into depolymerized tubulin pool. Additionally, the immunoreactive beta2-tubulins were observed enriched not only in spontaneously beating cell clusters but also in mouse embryonic heart (E11.5) containing left ventricle and aorta. In this study we for the first time discovered that as a new target molecule of NO beta2-tubulin undergoes the tyrosine nitrosylation in physiological process, which was suggested in our recent report, as well as this modification could be involved in protein-protein interaction as a new NO signaling.
Keywords: Nitric oxide, eNOS, tyrosine nitrosylation, beta2-tubulin, cardiomyocyte differentiation, Op18/stathmin, embryonic stem cell.

**Cofactor Rather than Antioxidative Aactivity of Tetrahydrobiopterin Prevents Ischemia Reperfusion Injury Associated Severe Graft Pancreatitis Following Murine Pancreas Transplantation**

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OBJECTIVE: Tetrahydrobiopterin (H4B) is an essential co-factor of nitric oxide synthases (NOS) and a strong antioxidant. Following oxidative stress associated H4B depletion, NOS is known to produce oxygen radicals rather than nitric oxide by uncoupling, a phenomenon

thought to contribute to endothelial dysfunction. In a murine pancreas transplantation model we observed that H4B substitution lead to reduced ischemia reperfusion injury (IRI), a major cause for the occurrence of severe graft pancreatitis. To differentiate cofactor activity from antioxidant effects we compared in this study H4B supplementation with tetrahydroneopterin (H4N), a pterin with similar redox behaviour but no NOS cofactor activity, and with the antioxidant vitamine C (VitC).

METHODS: Male syngenic C57BL6 (H-2b) mice were used as size-matched donor and recipient pairs. Murine cervical pancreas transplantation was performed. To induce graft pancreatitis grafts were subjected to 16h cold ischemia time (CIT) as well as to 45min warm ischemia time. Different treatment regiments were applied: untreated animals, H4B 50mg/kg i.m., H4N 50mg/kg i.m. and VitC 350mg/kg i.m., each administered to the donor prior to organ retrieval. Non transplanted animals served as controls. After 2h of reperfusion intravital fluorescence microscopy was used for analysis of graft microcirculation by means of functional capillary density (FCD) and capillary diameter (CD). Parenchymal damage was quantified by histology (H&E) and by performing nitrotyrosine-immunostaining to determine peroxynitrite formation.

RESULTS: Following prolonged CIT only pancreatic grafts treated with H4B prior to organ explantation displayed markedly higher values of FCD and CD compared to non treated animals ($p<0.01$ and $p<0.05$, respectively). In contrast, pre-treatment of donor animals with H4N as well as with VitC did not improve microcirculation. Compared to non treated animals application of both pterins and VitC significantly attenuated peroxynitrite formation ($p<0.05$). However, early parenchymal damage after murine pancreas transplantation was significantly reduced only by H4B pre-treatment ($p<0.05$), which lead also to markedly longer recipient survival rates compared to non-treated animals ($p<0.001$).

CONCLUSION: H4B attenuates IRI related severe graft pancreatitis primarily due to its NOS cofactor activity rather than due to its antioxidative capacity and might be a promising agent preventing IRI associated severe parenchymal damage.

## Metabolic and Genetic Regulation of the Folate-dependent Methionine Cycle

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The folate-dependent methionine cycle is required for homocysteine remethylation to methionine and for the demethylation of 5-methyl-THF to THF to allow folate accumulation by tissues. The cycle consists of four enzymes, cytosolic serine hydroxymethyltransferase (cSHMT), methylenetetrahydrofolate reductase (MTHFR), B12-dependent methionine synthase (MSR), and the accessory protein methionine synthase reductase (MSRR). cSHMT catalyzes the formation of methylene-THF, the entry substrate for the cycle, but is not absolutely required as methylene-THF can be produced by reduction of 10-formyl-THF generated from histidine catabolism or mitochondrial one carbon metabolism. We have investigated the regulation of the methionine cycle using mice with interruptions in the MTHFR and MSR genes. The metabolic and pathological effects of vitamin B12 deficiency in the rodent have also been studied.

Although MTHFR is considered the rate-limiting enzyme in the methionine cycle, the metabolic flux through the cycle was also responsive to MSR levels. The influence of changes in folate status and enzyme levels, and long range regulatory interactions, on the metabolic fluxes through the metabolic cycles of one carbon metabolism were modeled using a program developed by Fred Nijhout and Mike Reed (Duke University).

Although the MSR null mouse is embryonically lethal, the MTHFR/MSR double null survives, demonstrating the lethality of the 'methyl trap'. Common phenotypes shared by the MTHFR null and MTHFR/MSR double null mice included decreased body weight, hyperhomocysteinemia and a lower SAM/SAH ratio. No methyl-cobalamin or 5-methyl-THF was detectable in the dou-

ble knockout mice. Brain lipid composition and myelin formation in neonatal pups was normal, even when fed a vitamin B12 deficient diet. Myelin synthesis rates also appeared normal in the B12 deficient mouse although remyelination rates were impaired after a temporary induced demyelination.

cDNA microarray studies were performed to better understand the underlying mechanisms behind the phenotypic changes. Postnatal day (P1) mouse brain and liver from MTHFR null, MTHFR/MSR double null MTHFRand wild type mice were used. Differentially expressed genes were confirmed by real time PCR. Several categories of genes of interest were found including some iron-related genes, growth factors, lipid metabolism genes, and ubiquitin related genes. Many of the changes observed were consistent with induction of stress response genes. H-ferritin and hepcidin expression were significantly increased in knockout animals. Vitamin B12 deficiency influenced an overlapping set of genes and the effect was magnified in mice heterozygous for the MTHFR or MSR deletions.

**Functional Lessons Derived from Mutant proton-coupled Folate Transporters from Patients with Hereditary Folate Malabsorption**

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A novel carrier for folate uptake displaying optimal transport activity at acidic pH (5.5) has been recently identified and termed proton-coupled folate transporter (PCFT/SLC46A1) (Qiu et al., Cell, 2006). FPCFT recognizes folic acid (FA), reduced folates and methotrexate (MTX) with comparable high affinities (Km= 1-5 μM) and hence plays a key role in intestinal folate absorption within the acidic microenvironment of the small intestine as well as in folate uptake across the choroid plexus-cerebrospinal fluid (CSF) barrier, although the latter tissue resides in a physiological pH environment. Recently, various loss-of-function mutations in the PCFT gene were shown to constitute the molecular basis of hereditary folate malabsorption (HFM; OMIM 229050) (Zhao et al., Blood, 2007; Lasry et al., Blood, 2008). HFM is a rare autosomal recessive disorder caused by impaired intestinal folate absorption with folate deficiency characterized by anemia, hypoimmunoglobulinemia and recurrent infections. In various HFM patients, neurological abnormalities including seizures and mental retardation emerge during early childhood; the latter are attributable to impaired folate transport into the central nervous system and the poor folate levels in the CSF. Hence, similar to folate receptor ?, PCFT appears to play a functional role in folate transport into the CSF and brain.

We have recently shown that some inactivating PCFT mutations from HFM patients with loss of FA uptake, clustered in the PCFT residue R113, thereby suggesting a functional role for this specific amino acid in FA uptake (Lasry et al., Blood, 2008). Herein, using site-directed mutagenesis we show that unlike non-conservative substitutions, a conservative R113H mutant was targeted to the plasma membrane and retained 9% of wild type (wt) PCFT FA influx at pH 5.5, thereby allowing for a transport kinetic study. R113H displayed a dramatic increase (80-fold) in the FA transport Km while retaining wt Vmax, hence indicating a major fall in folate substrate affinity while preserving substrate translocation capability. Furthermore, R113H transfectants displayed a substantial decrease in the FA growth requirement relative to mock transfectants. Homology modeling based on the crystal structures of the close E. coli transporter homologues, the multidrug resistance transporter EmrD and glycerol-3-phosphate revealed that the R113H rotamer properly protrudes into the cytoplasmic face of the minor cleft normally occupied by the wt R113. Thus, these novel findings constitute the first demonstration that a basic amino acid at position 113 of PCFT is

absolutely required for the binding and hence transport of the negatively charged folate substrate, and that certain R113 substitutions markedly decreasing substrate affinity may not interfere with carrier mobility.

## Role of Autoantibodies in Cerebral Folate Deficiency Syndrome and other Pediatric Disorders

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OBJECTIVE: There are over 80 autoimmune diseases annually affecting nearly 24 million people in the U.S. alone. Although the causes of these diseases are not well understood; genetic, environmental and immunological factors are believed to play important roles. The core features of autoimmune disease are systemic inflammation and the production of autoantibodies. Recently, there has been increasing interest in determining the possibility that abnormal maternal autoantibodies might be associated with adverse pregnancy outcomes. This has been reported by Rothenberg and colleagues with respect to neural tube defects (NTDs) in a small cohort. In our studies, we have evaluated immunological responses that might contribute to the risk of Cerebral Folate Deficiency (CFD) Syndrome and NTDs such as spina bifida as well as considered the possible modifying factors, both genetic and environmental.

METHODS: For our NTD studies, serum samples from a cohort of mid-gestational serum specimens from 29 mothers with a pregnancy complicated by spina bifida and 76 mothers with unaffected children were obtained from the California Expanded α-Feto Protein Screening Program, and examined for the presence of IgG- and IgM antibodies to human folate receptor (FR) and bovine folate binding protein (FBP), and inhibition of folic acid binding to FR and FBP. The paraoxonase activity of each specimen was also determined, as were antibody titers to IgG and IgM antibodies to C reactive protein (CRP). For the CFD studies, the same immune parameters were examined in five sibships, including parents and unaffected siblings in addition to an interrogation of their hFRalpha gene.

RESULTS: Associations were observed between both autoantibodies against FR, and IgM autoantibodies against FBP with pregnancies complicated by an NTD. There was also an association between NTD pregnancies and serum that blocked FA binding to FR. Paraoxonase activity was associated with NTD risk when modeled with IgG antibodies against FBP. In contrast to the recent publications by Ramaekers and colleagues, we did not see an elevation in antibody titers to the folate receptor or in blocking of folate binding in CFD cases compared with unaffected controls. We did observe a highly significant elevation in anti-CRP titers versus controls.

CONCLUSIONS: These data support the hypothesis that the presence of high titers of autoantibodies and blocking of folate binding to FR should be regarded as a significant risk factor for NTDs; paraoxonase activity may modify this risk. The immunological triggers contributing to elevated NTD risk appear to differ from those factors that may contribute to the etiology of CFD.

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## Regulation of Folate Receptor Internalization by Protein Kinase C Signaling

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The glycosyl-phosphatidylinositol anchored folate receptor (FR) mediates selective delivery of a broad range of experimental drugs to the receptor rich tumors but molecular mechanisms controlling FR internalization have not been adequately studied. FR quantitatively recycles between the cell surface and endocytic compartments via a Cdc42-dependent pinocytic pathway. Protein kinase C (PKC) activators including diacylglycerol and phorbol ester have previously been reported to increase the proportion of FR on the cell surface. We have identied the α-subtype of PKC as the mediator of phorbol ester action on FR recycling and obtained evidence that activated PKCα is recruited to FR-rich membrane microdomains where, in association with its receptor RACK1, it inhibits FR internalization; the activation state of Cdc42 remains unaltered. We have also found that the PKC substrate, annexin II, is required for FR internalization. The studies clarify a molecular mechanism for the regulation of FR recycling through PKC and offer a potential means of controlling FR internalization for more effective delivery of cancer therapeutics.

**Differential Responses to Folic Acid Versus Natural 5-methyltetrahydrofolate in Humans**

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Since 1945 when folic acid (FA, pteroylglutamic acid) was first synthesized at American Cyanamid, this oxidized form of folate has been used to treat anemia, decrease the risk of neural tube defects, and in clinical trials to test prevention of a number of diseases. By the early 1950's it was discovered that all of the biochemical functions of this vitamin are carried out by a family of one-carbon derivatives of tetrahydrofolic acid. Folic acid administered to most laboratory animals is rapidly converted to tetrahydrofolic acid by dihydrofolate reductase. Increasing evidence has shown that this is not necessarily the case in humans. We have demonstrated that human liver has about 55 times less DHFR activity per gram than rats. This simple fact has the consequence of not only incomplete bioavailability as active folate cofactors, but also considerable exposure of humans to unmetabolized FA. Cmax for unmetabolized FA in plasma escalates rapidly as the dose is increased higher than the Daily Value (0.4 mg). The level of active tetrahydrofolates in the plasma following oral FA does not increase above a dose of 1.0 mg, indicating saturation of DHFR. Fasting plasma levels of unmetabolized FA in samples taken from subjects in the United States who are not consumers of multivitamins or breakfast cereals averages about 0.7 nM. This steady state level is increased after chronic consumption of FA, but appears to nearly plateau with a dose of 5 mg/d. This suggests saturation of a tight binding site within humans that has a very slow off-rate for FA. A placebo controlled 10 week study of FA (2.5 and 5.0 mg/d) vs 5-methyltetrahydrofolate revealed several biochemical and physiological parameters, such as blood pressure and changes in plasma thiols, that were specific to each form of folate. Moreover, several changes elicited by 2.5 mg/d FA were reversed at 5.0 mg/d FA. These studies indicate that synthetic folic acid and the natural 5-methyltetrahydrofolate do not always produce the same effects in humans, especially at high dose. Understanding these differences may be important in optimizing the benefits of folate.

*Conflicts of Interest*

*Supported by NHLBI, Merck Eprova and Pamlab*

**Erythrocyte Folate Status in Patients with Thyroid and Breast Cancers**

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OBJECTIVE: There are accumulating data on the effects of attenuating levels of folate on modulation of cancer risk. There are well established

data on cervix and colorectal cancer. Moreover there are less clear cut data regarding breast cancer, lung cancer and etc. The mechanism of anticancer effect of folate is unclear but may be due to DNA methylation and synthesis. The effect of folate seems to be modulated by alcohol, methionin, MTHFR polymorphism. Animal studies revealed that folate may decrease or increase cancer risk depending dosage and timing. Also it may be postulated that the dietary intake or blood levels do not reflect the cellular level in the cancer tissue. The aim of the present study was to determine the presence of alterations in the primary folate reserves in patients with thyroid and breast cancers.

PATIENTS AND METHODS: A modified microbiological method was used determine the erythrocyte folate levels in patients with thyroid and breast cancers.

RESULTS: The breast cancer patients (n=24) were found to have higher folate levels when compared to control group (n=25). In addition; the patients with malignant thyroid cancer (n=18) were found to have higher folate levels when compared to control group but did not reach statistical significance. The results of folate levels of patients with benign breast (n=27) and thyroid diseases (n=35) were also evaluated and a tendency to have higher levels with comparison to control group were determined; nevertheless it did not reach statistical significance.

CONCLUSIONS: Although our results show that there are enhanced erythrocyte levels in patients with malignant and benign disease we believe that further studies with folate measurements in cancer tissue are needed to confirm our results.

**MTHFR, MTR, and MTRR Polymorphisms in Relation to p16 (INK4A) Gene Promoter Hypermethylation in Colorectal Mucosa and Clinical Outcome of Patients with Colorectal Cancer**

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OBJECTIVE: Recently, we analyzed the hypermethylation status of the p16 (INK4a) gene promoter in normal mucosa obtained from 180 patients with CRC. The results showed that hypermethylation of the tumor suppressor gene p16 (INK4a) was associated with inferior survival of CRC patients. In the present study, germ line polymorphisms in the folate- and methyl-associated genes methylenetetrahydrofolate reductase (MTHFR), methionine synthase (MTR), and methionine synthase reductase (MTRR) were analyzed in the same patient cohort in order to find a possible link between the genotype and the p16 (INK4a) hypermethylation status.

PATIENTS AND METHODS: Genomic DNA was extracted from blood of patients (n=180) and controls (n=300). Genotype analyses of the genes MTHFR, MTR, and MTRR were run on an ABI PRISM® 7900HT sequence detection system (Applied Biosystems) using real-time PCR and TaqMan chemistry. Allele and genotype frequencies were correlated to the presence or absence of p16 (INK4a) hypermethylation.

RESULTS: Neither genotype distributions nor allele frequencies of the patient group were significantly different from that of the control group. Furthermore, none of the clinical or pathological parameters (gender, age, tumor location, tumor differentiation grade, and tumor stage) were found to be associated with genotypes. When patients with tumor stages II-III were subgrouped according to the MTHFR, MTR, or MTRR genotypes and dichotomized by the p16 (INK4a) hypermethylation status in mucosa the results showed that patients with MTRR66 genotypes AA/AG had a significantly worse prognosis if p16 (INK4a) was hypermethylated in the mucosa (p=0.0076). When stage II patients were analyzed separately the p16 (INK4a) hypermethylation status was found to be a prognostic marker for patients with genotypes MTHFR 677CC (p=0.05) and MTRR 66AA/AG (p=0.0012).

CONCLUSION: The results indicate a relationship between genetic germ-line variants of the genes MTHFR and MTRR, and the p16 (INK4a)

hypermethylation status in mucosa which affects the clinical outcome of CRC patients.

## Murine Models of Inherited Pterin and Monoaminergic Neurotransmitter Disorders

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Various transgenic mouse models, mostly knock-out mice, for almost all the described human defects in primary biogenic amine metabolism are available today: PAH, TH, TPH1, TPH2, DbH, PNMT, MAO-A and for COMT. The only exception is the gene encoding AADC. Regarding secondary disorders of neurotransmitter metabolism, mouse models were generated for defects in the tetrahydrobiopterin metabolism, i.e. DRD, PTPS, SR, PCD and DHPR, plus for the Lesch-Nyhan syndrome and Menkes disease. No animal models are thus far available for the autosomal recessive form of GTPCH deficiency. A long list of knock-out animals has been generated with functions in release, clearance, transport, or receptor signalling in the monoamine neurotransmitter system, including tryptophan hydroxylase deficiency, but no corresponding human disorders could be assigned thus far. From a technical point of view, it is a question of time to collect mouse mutants for all genes coding for neurometabolic enzymes or proteins. However, not every mouse model bears the same potential of usefulness, as most commonly complete knockout mice are generated, whereas genetic variations in humans often are not null mutations but rather single-nucleotide alterations or only polymorphisms. Moreover, regarding phenotypical variations of diseases, effects of protein modifiers and the genetic background have to be considered. Yet mice are inbred strains with a constant or specific background in contrast to patients. Another level of complexity or challenge for modelling of human disorders lies in behavioral characterization of mice, for example ptosis, depression, ADHD, and schizophrenia. Furthermore, reference values and specimens or samples for diagnosis are not always comparable between mice and humans, e. g. CSF collection from lumbar punction is not possible in mice.

## BH4 Metabolism in the Brains of KO Mice

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OBJECTIVE: Metabolism of tetrahydrobiopterin (BH4) had been extensively studied in the liver, because relatively high levels of BH4 and BH4-synthesizing activities were present in the liver in order to metabolize phenylalanine by the action of phenylalanine hydroxylase (PAH). BH4 also acts as a cofactor for tyrosine hydroxylase (TH) and tryptophan hydroxylase (TPH) in the brain. In order to examine the BH4 metabolism in the brain, we analyzed knock-out mice of BH4-relating enzymes.

METHODS: We produced pyruvoyltetrahydropterin synthase (Pts) knock-out mice. Sepiapterin reductase (Spr) knock-out mice and dihydropteridine reductase (Dpr) knock-out mice were produced by Lexicon Pharmaceuticals Inc.

RESULTS: Both Pts and Spr are the enzymes for de novo biosynthesis of BH4 from GTP. Disruption of the gene for Pts or Spr resulted in great reduction of BH4 both in the liver and in the brain. Although Pts homozygous knock-out mice died within 2 days after birth, most of Spr-null mice could be alive beyond 2 weeks. Because deficiency of BH4 was milder in Spr-null mice than that in Pts-null mice, deficiencies of monoamine levels in the brain were also milder in Spr-null mice. The difference would suggest presence of alternative pathway to make BH4 in vivo or presence of enzyme (s) that catalyzes similar reaction as Spr.

CONCLUSIONS: Our data suggest that there may be some differences in BH4 metabolism between the brain and the liver.

**New Mouse Models for BH4 Deficiency by Targeting the 6-pyruvolytetrahydropterin Synthase Gene Pts**

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Tetrahydrobiopterin (BH4) deficiencies are a highly heterogeneous group of rare disorders, which primarily are due to recessive mutations in the 6-pyruvoyltetrahydropterin synthase (PTPS). Mutations in its gene PTS lead to a broad range of phenotypes ranging from hyperphenylalaninemia to abnormal monoamine neurotransmitter biosynthesis. 81% of the PTPS patients are associated with a severe or central phenotype, i.e. catecholamine and serotonin neurotransmitter deficiency, whereas 19% of the PTPS patients have a mild or peripheral phenotype, i.e. only hyperphenylalaninemia. The molecular mechanisms for these phenotypic distinctions are unknown. Previous studies have shown that a complete Pts-knock-out (Pts-ko) for the mouse Pts gene exhibited perinatal lethality which made it difficult to analyze the pathophysiology of the diseases and to perform therapeutic studies (1). Due to the perinatal lethality of the Pts-ko mouse and in order to investigate a milder form of BH4 deficiency, a second Pts mouse was generated by 'knocking-in' a single codon exchange in the Pts gene (Pts-ki). The mouse arginine 15 to cysteine (Pts-R15C) mutation was chosen because it is equivalent to the human R16C-PTS mutation which has shown to be associated with a peripheral or mild phenotype that do not synthesize BH4 in peripheral organs but apparently lead to normal BH4 and neurotransmitter levels in the central nervous system. The Pts-ki allele was generated by homologous recombination in embryonic stem cells and morulae aggregation. The homozygous Pts-ki targeted mice showed neither signs of hyperphenylalaninemia nor significant differences in monomaine neurotransmitter metabolites in the brain, but reduced PTPS enzyme activity and elevated neopterin levels in brain and liver. Currently we are breeding and characterizing compound heterozygous Pts-ki/Pts-ko mice in order to obtain a mouse model with a potentially more severe phenotype then the homozygous Pts-ki mouse and at the same time with no perinatal lethality like homozygous Pts-ko mice.

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**Sepiaptein Reductase Knockout Mice: Biochemical and Histochemical Analyses**

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OBJECTIVE: Human sepiapterin reductase (SPR) deficiency has unique characteristics that it does not cause hyperphenylalainemia and considerable amount of BH4 is generated in the patients, and that they develop neurological symptoms caused by monoamine neurotransmitter deficiency. To clarify the biochemical background of SPR deficiency, we analyzed Spr-knockout (KO) mice made by Lexicon Pharmaceuticals.

METHODS and RESULTS: First we examined whether Spr-KO mice showed the similar phenotypes observed in the human patients. Most of the Spr-KO mice were alive for 2 to 4 weeks after birth, and the period was much longer than pyruvoyltetrahydropterin synthase (Pts)-KO mice, which die within 2 postnatal days. Spr-KO mice showed severe growth retardation, hypo-activity, and limb-clasping in the tail suspension test. Some mice lived beyond 6 weeks, and they showed tremor-like symptoms. We analyzed the tissues from 17 days old Spr-KO mice, and found the elevated liver phenylalanine levels and the

low BH4 and monoamine levels in their brains. We also found that the TH protein level was significantly decreased in the brains of Spr-KO mice. Immunohistochemincal study showed that the decrease of the TH protein was severe in nerve terminals.

To explore the reason of the difference in the life span between Spr-KO and Pts-KO mice, we performed the biochemical analysis of neonatal Spr-KO mice. BH4 levels in the brain or peripheral tissues of Spr-KO neonates were lower than those of wild type, but less severe than those of Pts-KO neonates. We found that the levels of monoamines and their metabolites in neonatal brains of Spr-KO mice were higher than those of Pts-KO neonates. In addition, in contrast to Pts-KO mice, the TH protein levels were not severely decreased in Spr-KO neonates examined by Western blot and immunohistochemical analyses.

CONCLUSION: These results represent that there are some difference (s) in BH4 metabolism between human and mouse and that Spr-defect elicits a milder effect in neonatal mice than Pts-defect does.

**Low-dose Tetrahydrobiopterin Supplementation Reduces Atherosclerosis Progression in ApoE-KO Mice via Effects on vascular T Cell Infiltration**

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Tetrahydrobiopterin supplementation improves endothelial function in models of vascular disease by maintaining endothelial nitric oxide synthase coupling and nitric oxide bioavailability. However, the cellular mechanisms through which this leads to reduced atherosclerosis remain unclear. We have used a pharmaceutical formulation of tetrahydrobiopterin (BH4) to investigate the effects of BH4 supplementation on atherosclerosis progression in ApoE-KO mice. Single oral dose pharmacokinetic studies revealed rapid BH4 uptake from the gastrointestinal tract into ApoE-KO plasma and organs. While in most organs BH4 levels returned to baseline by 8 hours after oral dosing, BH4 levels in aorta remained markedly increased at 24 hours, suggesting an active mechanism for BH4 retention. Daily oral BH4 supplementation in 8 week old chow-fed ApoE-KO mice for 8 or 12 weeks had no effect on plasma lipids or hemodynamic parameters, but resulted in significantly reduced aortic root atherosclerosis only at 10 mg/kg BH4, compared with placebo treated animals. Higher BH4 doses (50, 500 mg/kg) caused significantly reduced weight gain. Notably, atherosclerosis progression was reduced even after BH4 supplementation was delayed until 16 weeks of age. BH4 supplementation led to a striking reduction in vascular infiltration of T cells, both within plaques and in the surrounding adventitia, but importantly had no effect on circulating leukocytes. Thus, BH4 supplementation reduces atherosclerosis through a reduction in vascular T cell infiltration mediated by salutary effects on endothelial nitric oxide bioavailability.

*Conflicts of Interest*

*The presenting author and the study are funded by BioMarin Pharmaceutical Inc, 105 Digital Drive, Novato CA, USA. Charles A. O'Neill is employee of BioMarin Pharmaceutical Inc.*

*Disclosures*

*The presenting author and the study are funded by BioMarin Pharmaceutical Inc, 105 Digital Drive, Novato CA, USA. Charles A. O'Neill is employee of BioMarin Pharmaceutical Inc.*

**Autophagy in Response to Tetrahydrobiopterin Deficiency**

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We demonstrate that impaired metabolism of BH4 induces autophagy in mouse model. Autophagic vesicles were accumulated in various tissues of BH4 deficient mice in were the Spr gene encoding sepiapterin-reductase (Spr) catalyzing final steps of BH4 biosynthesis. Autophagy induction in tissues of BH4 deficienct mice was confirmed by the conversion of LC3-I into lipidated LC3-II form, an autophagic marker. Autophagy induction in BH4 deficient Spr-/- mice is attributable to a paucity of tyrosine as was ascertained by ultrastructure analysis showing the clearance of autophagic vesicles in the liver of Spr-/- mice after the replacement therapy by the supplementation of tyrosine for 4 weeks. Supplementation of tyrosine inactivates autophagy in the liver of Spr-/- mice. It is anticipated that the knockout of Spr gene that affects the availabilities of BH4 and tyrosine induces autophagy in mice. Autophagy in response to tyrosine deficiency was also proved in vitro. Autophagy was induced in NIH3T3 cells grown in tyrosine-limited media. Autophagic pathway in this tyrosine metabolism impaired mouse model seems to be activated by inhibiting mTOR signaling since the phosphorylation of S6K, a substrate for mTOR), was reduced in the liver of Spr-/- mice.

**Pteridine Glycosyltransferases: Structure and Function**

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Pteridine glycosides have various kinds of sugars attached to the side chain at C-6 of pterin rings. Most of their chemically identified structures were biopterin-glycosides except a few 6-hydroxymethylpterin or neopterin glycosides. Hitherto, they have been found in a few classes of prokaryotes such as cyanobacteria, anaerobic photosynthetic bacteria, and archaebacteria, although the function is unknown. The final step of pteridine glycoside synthesis is glycosyltransfer which is catalyzed by pteridine glycosyltransferases (PGTs). Recently bacterial genome sequencing is so fast that we could find more than a hundred PGT homologs from them. As we were interested in their substrate specificity depending on protein sequences, we started to investigate their structure and function by preparing recombinant proteins of the homologs one by one. Here we report a finding of new PGTs which catalyze xylosyl group transfer from UDP-xylose to tetrahydrobiopterin.

**Hyperglycemia Effect on Tetrahydropteridine Synthesis in Dictyostelium Discoideum Ax2**

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Dictyostelium discoideum Ax2 produces not only BH4 but also its stereoisomer D-threo-H4-biopterin (DH4) in a high amount. Dictyostelium is a multicellular organism growing as separate, independent cells but interact to form multicellular structures by starvation. Since many of the underlying molecular and cellular processes involved in the multicellular stage have remained fundamentally unchanged throughout evolution, Dictyostelium provides a powerful system for basic biomedical research in cell and developmental biology. In order to see if pteridines are involved in oxidative stress imposed by glucose, glucose effect on Dictyostelium was investigated at the molecular and cellular levels. Higher glucose concentrations in the medium increased oxidative stress as determined by increased reactive oxygen species but decreased mitochondrial activity, thereby inhibiting cell growth. Under hyperglycemia conditions, pteridine synthesis was decreased together with lower activities of the biosynthetic enzyme activities, whereas gene expressions of GTP cyclohydrolase I (GTPCH)

and dihydropteridine reductase (DHPR) were increased much. Particularly, DHPR mRNA was increased up to 30-fold, suggesting a role of regeneration pathway in oxidative stress conditions.

**Dihydropteridine Reductase (DHPR) Activity in Thyroid and Breast Cancer Patients**

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OBJECTIVE: Tetrahydrobiopterin (BH4) is the cofactor in the synthesis, and regulation of neurotransmitters, oxidative cleavage of ether lipids and nitric oxide production. It is known that many diseases may cause changes in BH4 concentration and/or DHPR enzyme activity. There are limited numbers of studies correlating DHPR activity in malignancies. The main goal of the present study was to evaluate alterations in the DHPR activity in patients with various human cancers.

PATIENTS AND METHODS: The patient group consisted of ninety-seven patients with thyroid/breast disorders which were grouped on the basis of their clinical status, while thirty healthy subjects as the control group were included in the study. The exclusion criteria of the study were; obscure histopathologic diagnosis prior to definitive treatment, history of chemotherapy or radiotherapy for tumors other than the thyroid/breast cancer, the patients with familial pattern of the diseases, the patients who had a history of thyroid/breast or other organ malignancies and presence of active infection at the time of sample collection. The 6th edition of the "AJCC Cancer Staging System" was used for staging. The changes in the enzyme activity were measured with a method based on reduction of cytochrome C in presence of NADH. The alteration in the DHPR activity was compared with the mean enzyme activity of the control group.

RESULTS: The breast cancer patients (n=24) were found to have lower DHPR activity when compared to both of the benign breast patients (n=15) and the controls (n=30). In addition; the patients with thyroid cancer (n=16) were found to have higher DHPR activity when compared to both the benign thyroid patients (n=42) and the control group. There was a significant difference in terms of DHPR activities between the control group and the patients with malign thyroid disease ($p=0.037$). There were not any significant differences in DHPR activities between the controls and the benign thyroid patients ($p>0.05$) or patients with benign and malign thyroid ($p>0.05$). On the other hand we observed significant differences between the benign breast patients and benign thyroid patients ($p=0.020$) and also malign breast patients and malign thyroid patients ($p=0.000$).

CONCLUSIONS: Our results suggest that DHPR activity in malignant thyroid disorders seems to be highest among all groups. This may be due to the fact that thyroid is an oxidatively active organ and majority of thyroid tumors are well differentiated functional tumors.

**Screening of Urinary Neopterin Levels in Turkish Adolescents**

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OBJECTIVE: During cellular immune activation, with the stimulation of interferon gamma (IFN-γ), monocyte/macrophages release neopterin, which is then excreted by urine. Increase in neopterin levels in body fluids has been reported to be related to many pathological states including transplant rejection or infectious complications, autoimmune diseases and neurodegenerative di-

seases. In healthy subjects with different ages, from newborns to adults, neopterin levels were reported to be decreasing with age. The assessment of the urinary neopterin levels in different age groups may provide information on the healthy profile of each group. Comparison of neopterin levels with this profile may be important in diagnosis of the diseases at early period, which may be of useful in early treatment. The aim of this study was to evaluate the urinary levels of neopterin in adolescents as there is no detailed study especially in healthy subjects on pteridine levels of young people in Turkey.

PATIENTS AND METHODS: To assess the urinary neopterin levels, 75 adolescents (aged between 10 and 18 years) who were admitted to Hacettepe University Ihsan Dogramaci Children's Hospital Adolescent Unit and who were not taking any medication were included in this study. Urinary neopterin levels of the volunteer adolescents were analyzed by HPLC.

RESULTS: According to our preliminary results, neopterin levels ranges between 52.80 - 481.94 μmol/mol creatinine with an average of 152.16 ± 9.23 μmol/mol creatinine (mean ± S.E.M.)

CONCLUSIONS: No correlation was observed between the age and urinary neopterin levels of the volunteers at this age interval. Urinary neopterin levels in adolescents between ages 15 and 18 were reported to be 144 - 65 μmol/mol creatinine. 6.7% and 12% of our volunteers were above and below this level, respectively. The possibility of pathologies and relationship between other parameters linked with pteridine metabolism of volunteers having too low and too high neopterin levels should be investigated. The study will be continued with increased number of patients to provide healthy results in adolescent profile.

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**Severe PTPS Deficiency in an Adult Lawyer with Normal IQ**

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The patient was screened for PKU at birth and a diagnosis of classic PKU (plasma phenylalanine (phe) 1200 μmol/L) was made. No BH4 loading test, DHPR assay or urinary pterins were analysed at that time. A low-phe diet was introduced in the first month of life with good metabolic control and progressive tolerance increase so that free diet was introduced at age 3 years without any evidence of plasma phe increase in the following months and years.

The patient refers a normal life (sport at agonistic levels), with good metabolic control, until the age of 14 years, when he experienced the first panic attack (followed by 8 attacks in the following 2 years) that occurred before a sport competition. He started treatment with alprazolam and consulted many psychologists until the age of 27 when he asked for a consult in our Metabolic Department. In the meanwhile he attended Milan University with good results. He got a degree despite many episodes of dystonic movements, related to stress induced panic attacks in particular before university examinations.

At admission in our Metabolic Department the patient (age 26 years) presented with normal IQ (103 Wechsler Adult Intelligence Scale, WAIS), referring frequent episodes characterized by flexor spasm (hands), involuntary movements of arms, tongue protrusion, hypersalivation, all occurring during the clinical evaluation. An MR of the brain was made and was normal. Plasma phe concentration was 381 μmol/L. Prolactin plasma level was 75 ng/ml (nv 3-20) . Cerebrospinal - fluid neurotransmitters and pterins analysis showed a pattern suggestive for severe PTPS deficiency (HIAA 8 nmol/L, HVA 25 nmol/L, 3OMD 10 nmol/L, Neo 54 nmol/L, Bio 3.6 nmol/L). Enzyme assay on red blood cells was performed and therapy with neurotransmitters, BH4 and folinic acid was immediately started. Since therapy was introduced the patient has shown progressive improvement of dystonic movements and no panic attacks had occurred.

This is the first case of severe PTPS deficiency diagnosed in adult life in a patient with normal IQ and dystonic movements, with onset of clinical symptoms in late adolescence.

## 6-pyruvoyl Tetrahydropterin Synthase Deficiency - Clinical and Molecular Profiles of Six Malaysian Patients

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6-pyruvoyl-tetrahydropterin synthase (PTPS) deficiency is an autosomal recessive disorder resulting in not only hyperphenylalaninemia, but also neurological symptoms because of lack of dopamine, catecholamine and serotonin. We review 6 patients (5 males, 1 female) from 5 unrelated families. All of them were Chinese. In all patients, the diagnosis were confirmed by BH4 loading test, analysis of urinary/CSF pterins and mutational analysis of the PTS gene. Five patients presented clinically with psychomotor retardation, dystonia and oculogyric crises. In three patients the oculogyric crises were initially misdiagnosed as epilepsy and treated with anticonvulsant without clinical improvement. Three of them have long delayed diagnosis which ranges from 7.5 to 18 years. One patient was diagnosed early by newborn screening in view of positive family history. One patient has homozygous 259C>T mutation. The other five patients have 259C>T mutation in one allele and one of the following mutation in another allele: 155A>G, 286G>A and 116_119del. All patients were treated with BH4 and neurotransmitter precursors. The most important predictor of outcome was age at diagnosis. Three patients with early diagnosis before 2 years have good neurocognitive outcome with treatment, whereas three patients with long delayed diagnosis have less favourable outcome.

## Tetrahydrobiopterin/Total Biopterin Ratio in Human Urine

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Tetrahydrobiopterin (BH4) in biological samples has been measured extensively for clinical diagnosis and biomedical research. While BH4 is functional in the fully reduced form, it can be oxidized to dihydrobiopterin (BH2) and fully oxidized form (biopterin) in vivo especially under oxidative stress conditions. Therefore, ratio of BH4 to total biopterin (BH2+biopterin) in plasma was shown to be important in the associated pathological conditions. Although it was known that human urine contains a huge amount of reduced and oxidized forms of BH4, we could not find a population study. We determined them in a hundred people in the age range of 20-72. A high level of variation was found between individuals in BH4 and oxidized forms, while the ratio of BH4 to total biopterin was relatively constant irrespective of age and sex. The ratio was also maintained fairly constant in a day, while the amounts of both BH4 and oxidized forms varied much. It remains several questions how the ratio is maintained and if it has any physiological significance.

## Role of Tetrahydrobiopterin in Neuronal Apoptosis in Drosophila Melanogaster

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Tetrahydrobiopterin (BH4) is an essential cofactor for nitric oxide synthase and neurotransmitter-producing-enzymes such as tyrosine hydroxylase and tryptophan hydroxylase. Although its biochemical function is well known,

the role of BH4 in neuronal apoptosis is not clearly understood. BH4 protects neuronal cells from ROS (Reactive Oxygen Species) as well as RNS (Reactive Nitrogen Species) in vitro, while BH4 per se causes dopaminergic cell death. In this study, we elucidate how BH4 is involved in neuronal apoptosis in Drosophila. Sepiapterin reductase (SR) is known to catalyze the last step of de novo BH4 biosynthesis. In order to induce specific alteration of BH4 content in Drosophila brain, we first generated a SR mutant by transposon-mediated dysgenesis, and several transgenic flies expressing different levels of SR using neuronal cell specific UAS-gal4 system. The loss-of-function mutant SRDEL exhibits only 3% of sepiapterin reductase activity compared to wild type. In addition, the mutant shows increased resistance to paraquat, an ROS producer.

**Crystal Structure of Dictyostelium Discoideum Phenylalanine Hydroxylase in Complex with Dihydrobipterin and L-norlucine (Fe (III)-BH2-NLE)**

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Phenylalanine hydroxylase (PAH, PheOH, phenylalanine 4-monooxygenase, EC 1.14.16.1) is very important for neural systems in higher animals. It hydrolyses the essential aromatic amino acid L-phenylalanine (L-Phe) to L-tyrosine (L-Tyr) in the presence of the specific cofactor tetrahydrobiopterin (BH4) and dioxygen (O2). Dictyostelilum discoideum PAH shows significant similarities to other mammalian PAH, but it exhibits higher activity with tetrahydrodictyopterin (DH4), a tetrahydrobiopterin (BH4) stereoisomer abundant in Dictyostelilum, than with the natural cofactor BH4 and shows different allosteric regulation.

To understand dual cofactor specificities and enzyme regulation mechanism, dicPAH 1-415 (residue 1-415) was expressed, purified and crystallized for structural analysis. Diffraction-quality crystals of ternary complex of dicPAH 1-415 -Fe (III)-BH2-norleucine have been obtained using the hanging-drop vapor-diffusion method. The crystal diffracted to a resolution limit of 2.1 AA and belonged to space group P21 with unit cell parameters a = 71.52, b = 85.67, c = 73.48 A, β = 110.08°. The structure has been solved by molecular replacement using a human PAH catalytic domain model. The missing N-terminal regulatory domain could be modeled into the electron density obtained from the molecular replacement. Model building and structural analysis is now in progress. Analysis of the structures between dicPAH and mammalian PAH, may reveal structural determinants for different allosteric regulation and dual cofactor specificities.

**Uncovering Enzymatic Transformations Involved in Biosynthesis of 7-deazapurine Containing Compounds**

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Deazapurine-containing secondary metabolites comprise a broad range of structurally diverse nucleoside analogs found throughout biology including various antibiotics produced by species of Streptomyces bacteria and the hypermodified tRNA bases queuosine and archaeosine. Despite early interest in deazapurines as antibiotic, antiviral, and antineoplastic agents, the biosynthetic route toward deazapurine production had remained largely elusive for more than 40 years.

We have utilized genomic and biochemical studies to identify a paradigm for the biosynthesis of deazapurine-containing compounds. Interestingly, it is becoming increasingly clear that homologs of enzymes that are usually associated with pathways for biosynthesis of folate or biopterin have been co-opted for biosynthesis of deazapurine-containing compounds. For instance, recent studies in our laboratory have shown that a bacterial GTP cyclohydrolase I (GCH I) catalyzes the first step in the pathway (1). The 7,8-dihydroneopterin triphosphate is in turn utilized by

a 6-pyruvoyltetrahydropterin synthase homolog, which unlike the mammalian enzyme, catalyzes its conversion to 6-carboxy-5,6,7,8-tetrahydropterin (2). These studies highlight the interconnections between primary and secondary metabolism and provide glimpses into evolution of novel secondary metabolism pathways.

The presentation will discuss our most recent successful efforts at in vitro reconstitution of various steps of the biosynthetic pathways leading to various deazapurine-containing compounds

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**Expression of the Drosophila melanogaster Punch Gene Encoding GTP Cyclohydrolase I**

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The Drosophila melanogaster Punch gene encodes the GTP cyclohydrolase I (GTPCHI) that has been reported to be involved in the rate-limiting step of the de novo synthesis of tetrahydrobiopterin. The expression of the Punch gene has been known to be regulated by two different promoters (P1 and P2). In order to identify the important DNA elements in the Punch gene expression, we characterized the 5'-flanking DNA regions required for P1 and P2 promoter activities using transient expression assay. Our results showed that the DNA regions between -98 and +31, and between -73 and +35 are required for efficient P1 and P2 promoter activities, respectively, and that the regions between -98 and -56 and between -73 and -41 may contain critical elements required for the gene expression. By aligning the nucleotide sequences in the P1 and P2 promoter regions of the Drosophila melanogaster and Drosophila virilis GTPCHI genes, several conserved elements including palindromic sequences in the regions critical for P1 and P2 promoter activities were identified. Western blot analysis of transgenic flies transformed using P1 or P2 promoter-lacZ fusion plasmids further revealed that P1 promoter expression is restricted to the late pupal and adult stages but that the P2 promoter driven expression is constitutive throughout fly development. In addition, X-gal staining of the embryos and imaginal discs of transgenic flies suggests that the P2 promoter is active from stage 13 of embryo and is generally active in most regions of the imaginal discs at the larval stage.

**Synthesis and Cytotoxicity Activity of Aminopyrimidine Derivatives as Potential Dihydrofolate Reductase Inhibitors**

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Dihydrofolate reductase (DHFR) is an essential enzyme in both eukaryotic and prokaryotic cells and it has been the subject of a large number of studies in the field of medicinal chemistry for a long time. Thymidylate synthase (TS) is another key enzyme in the process of cell proliferation which along with methylated form of DHFR catalyses the reductive methylation of deoxyuridylate to deoxythymidylate.

Inhibitors of DHFR and TS enzymes in cancer, microbial and protozoan cells provide compounds that have found clinical indications as antitumor,

antimicrobial and antiprotozoal agents. The elegant works by Gangjee et al. during the last decade indicate that aminopyrimidines have the potential to be both TS and DHFR inhibitors. In the present study a group of aminopyrimidine derivatives have been synthesized as potential DHFR inhibitors using the reaction between DMF-DMA and a proper ketone with at least one alpha hydrogen to obtain 3-dimethylamino-1-(aryl)-2-propen-1-one intermediate which in reaction with guanidine affords the desired aminopyrimidine derivative.

Cytotoxic activities of the compounds against CHO and OV2008 cell lines were evaluated using both MTT and Clonogenic assays. DHFR inhibitory activities of the compounds were also determined in order to study the mechanism of action for these derivatives.

A few number of compounds showed high activities against methotrexate-resistant cells.

**Synthesis and Antimicrobial Activity of Aminopyrimidine Derivatives as Potential Dihydrofolate Reductase Inhibitors**

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A few 2-aminopyrimidine derivatives were synthesized as potential inhibitors of Thymidylate synthase (TS) and Dihydrofolate reductase (DHFR) enzymes.

The key intermediate in the synthesis of these analogs was 3-dimethylamino-1-(aryl)-2-propen-1-one which was prepared by the reaction of an appropriate ketone and N,N-dimethylformamide dimethyl acetal in DMF as the solvent. The reaction of this intermediate and guanidine HCl in the presence of a strong base such as sodium methoxide gave the aminopyrimidine derivatives.

The structures of all target compounds and intermediates were verified using $^{1}H$- and $^{13}C$-NMR spectroscopy as well as Mass spectrometry.

Minimum inhibitory concentration (MIC) for the compounds were determined as a measure of antimicrobial activities against a group of microorganisms such as E. coli, S. aureus, P. aeruginosa, and some fungi such as C. albicans.

Dihydrofolate reductase inhibitory activities were assayed spectrophotometrically in a solution containing dihydrofolate, NADPH, Tris HCl, 2-mercaptoethanol and EDTA at pH 7.4 and 30°C. The reaction was inhibited with an amount of enzyme yielding a change in O.D. at 340 nm of 0.015/min.

Trimethoprime was used as a standard DHFR inhibitor and some of the tested compounds showed comparable activities.

**Crystal Structure of Mouse Thymidylate Synthase**

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Thymidylate synthase (TS) catalyzes the reductive methylation of dUMP, the reaction being the only de novo source of thymidylate required for DNA synthesis. TS is an important target in chemotherapy, as its inhibition leads to apoptosis of dividing cells. Between many TS crystal structures reported, only two, rat and human, are of metazoan origin. Human TS loop 181-197 was observed to populate two conformers, one of them, apparently inactive, stabilized by a pair of hydrogen bonds from Arg163 to the carbonyls of Ala191 and Leu192. As mouse TS, containing lysine residue in the corresponding position, has been hypothesized unable to populate the inactive conformer (Lovelace et al., Biochemistry, 46, 2823-2830, 2007), learning the

crystal structure of the mouse enzyme was of interest.

Recombinant mouse TS (mTS) was overexpressed in E.coli, purified and released from the phosphorylated fraction as previously described (Fr□czyk et al., Acta Biochim. Pol. 54-S3, 37-38, 2007). Crystallization experiments (hanging drop diffusion) resulted in the diffraction quality crystals of rec-mTS protein alone and complexed with dUMP. Data were collected from three single flash-frozen crystals at the Max-Lab Lund University using X-rays with wavelengths of 1,038 and 0,908 A. Data were processed with Denzo and Scalepack. Both structures were determined by molecular replacement carried out with the CCP4 package, using the rat TS ternary complex without ligands as the search model. The crystal structures of mTS and mTS-dUMP were determined at resolutions of 1,94 and 1,7 A, respectively, and their correctness was evaluated using Sfcheck&Procheck from the CCP4 suite.

The structures, consisting of one and three dimers per asymmetric part of the unit cell for mTS and mTS-dUMP, respectively, showed an overall similarity to the corresponding structures of human and/or rat TSs, as expected for proteins with highly conserved primary structures. In the mTS-dUMP, as well as mTS structure, the active site loop 175-191, equivalent to the human enzyme 181-197 loop, populated the active conformation, with the catalytic Cys 189 located at a close, but non-covalent distance from dUMP. The molecule of dUMP was bound in a manner similar to that observed in the corresponding complexes of other mammalian and bacterial TSs, with the ligand anchored in the active site by several H-bonds to its phosphate moiety from four arginine and single serine residues. The orientation of dUMP was secured by H-bonds between the conserved Asn 220 and the O4 and N3-H moieties of the pyrimidine ring. The active site in the mTS structure held a single sulfate anion, bound at nearly the same location as dUMP phosphate moiety in the mTS-dUMP structure and stabilized by H-bonds with the pair of arginine residues from the quartet coordinating the phosphate in the other structure.

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**Membrane Transport Properties and Biological Activity of Stereo-isomers of Glycinamide Ribonucleotide Formyltransferase (GARFT) Inhibitors AG2032 and AG2034**

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OBJECTIVE: To characterize membrane transport efficacy via the classical reduced folate carrier (RFC) and / or Folate Receptor (FR) of two series of folate-based glycinamide ribonucleotide formyltransferase (GARFT): (6S)-AG2034 and its R-stereoisomer AG2033, and for (6R,S) AG2032 and 6S-(AG2037) and 6R-(AG2038) stereoisomers. Beyond this, the biological activity of these compounds was evaluated as a function extracellular/intracellular folate status.

METHODS: The following cell line models were used: human CEM/WT leukemia cells (harboring wild type RFC), CEM/7A cells (overexpressing RFC) and CEM/MTX-RFCmut cells (harboring a mutated RFC (E45K) allowing more efficient folic acid uptake leading to 7-fold higher intracellular folate levels as compared to CEM/WT). Murine L1210 leukemia cells lacking RFC activity but with high expression of the alpha isoform of FR served as a model to assess receptor-mediated uptake of the GARFT inhibitors. Competition experiments for [3H]methotrexate (MTX) uptake and [3H]folic acid binding were used to measure transport affinities for RFC and binding affinities for FR, respectively.

RESULTS: While AG2033 displayed an affinity for RFC transport similar as to MTX, all other GARFT inhibitors elicited a 4-5 fold better RFC transport efficiency than MTX. Consistently, these compounds displayed 6-10 fold greater growth inhibitory potency against CEM-7A cells (IC50: 3.9-11 nM) than CEM/WT cells (IC50: 29-66 nM). Interestingly, AG2037 retained potent growth inhibitory potential against CEM/MTX-RFCmut cells with high intracellular folate levels.

The series of 5 GARFT inhibitors displayed proficient binding affinities for FR, ranging from 5-fold lower than that of folic acid (AG2038), 2.5-fold lower than folic acid (AG2032 and AG2037) and 1.2-1.7 fold higher than folic acid (AG2034 and AG2033, respectively). Consistently, these were potent inhibitors of growth of L1210-FR cells (IC50: 5.6 - 24 nM) when no competing folates were available, while excess folic acid abrogated growth inhibitory effects due to competitive binding to FR.

CONCLUSIONS: A novel series of folate-based GARFT showed dual transport capacities for both RFC and FR. Furthermore, notably AG2032 and AG2034 retained proficient biological activity regardless of extra/intracellular folate status.

**Determination of Folates in Tumor and Adjacent mucosa of CRC Patients Using LC-MS/MS**

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A liquid chromatography (LC)-electrospray ionization tandem mass spectrometry (LC-MS/MS) method has been developed for the determination of 5,10-methylenetetrahydrofolate (methyleneTHF), tetrahydrofolate (THF) and 5-methyltetrahydrofolate (methylTHF) in mucosa and tumor tissues. The folate extraction method involved homogenization, heat treatment and folate conjugase treatment to hydrolyze polyglutamyl folate to monoglutamyl folate. Before analyze on LC-MS/MS, a simple and fast sample purification with ultrafiltration (molecular weight cut-off membrane, 5kDa) was performed. Folates and internal standard were analyzed simultaneously by LC-MS/MS using selected reaction monitoring, which allowed a higher specificity. Folates were detected and quantified using positive electrospray (ESI). The mobile phase was a binary gradient mixture of 0.1% acetic acid and acetonitrile. The detection limits in the tissue for methyleneTHF, THF and methylTHF were found to be 30, 5.5 and 1.3 fmol, respectively. Tomudex was used as an internal standard. Measurement repeatability (RSD) ranged from 4-9 % for all analytes over 3.5 hour of analysis and the variability over four days ranged from 3-22 % for all analytes. Tissue from tumor and adjacent mucosa from 75 colorectal cancer patients were analyzed and correlated to clinicopathological factors. Mean concentration of methyleneTHF and THF was found to be significantly higher in the tumor compared with mucosa. The results show that the LC-MS/MS method has a great advantages over other previously used methods because of its high sensitivity and selectivity. The aim of this study was to develop a LC-MS/MS method to analyze folates in mucosa and tumor in patients with colorectalcancer (CRC).

**Profiling of Rice Grain Folates Using Microbiological Assay and Liquid Chromatography-tandem Mass Spectrometry**

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OBJECTIVE: Folate is an essential B vitamin for normal growth and development preventing neural tube defects (NTDs), cardiovascular disease (CVD), cancer and anemia among other diseases. However, intake is often suboptimal in population where rice is the staple food, hence enhancing the folate content of the rice grain is a biofortification target. Two folate profiling methods have been used to compare folate levels in a range of rice grains.

MATERIALS AND METHODS: Naturally occurring folates in rice grains were measured by high performance liquid chromatography-tandem mass spectrometry (LC-MS/MS) and microbiological assay (MA) using Lactobacillus rhamnosus (NCIMB 10463) as a test organism. LC-MS/MS procedure which allowed a full profile of all individual mono- and polyglutamated folate

species was applied. The analytes were extracted from the grains following a mono-enzyme (α-amylase) treatment, commercially-available folate standards and internal standards (MTX, tri-MTX and hexa-MTX) were spiked into each corresponding sample replicates, and analysed by LC-MS/MS in the negative ion mode using electrospray ionisation. The application of the system was verified by analyzing a certified reference material (CRM 121-wholemeal flour) and a plant quality control (spinach).

RESULTS: The results showed that LC-MS/MS gave good separation of the major monoglutamate folate forms - 5-methyl tetrahydrofolate (3.5 - 59.2 μg/100g) and 5-formyl tetrahydrofolate (0.6 - 8.7 μg/100g). In agreement to the results evidenced in other studies comparing folate values determined chromatographically and microbiologically, this study revealed lower values for folate concentration in rice grains compared with MA results. The major polyglutamated forms of folate detected in unpolished rice grains were identified as 5-CH3-H4PteGlu4, 5-CH3-H4PteGlu5 and 5-CHO-PteGlu5.

CONCLUSIONS: While MA provides the total folate concentration, only LC-MS/MS can quantify the individual folate forms including the polyglutamates.

**Approaches to the Development of Inhibitors of Parasitic Pteridine Metabolism**

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Dihydrofolate reductase (DHFR) is an enzyme that reduces dihydrofolate (DHF) to tetrahydrofolate (THF) and is in great demand by cells which proliferate rapidly, e.g. cancer, bacterial and parasitic cells. This feature makes it a logical therapeutic target. Antifolates have been designed to target DHFR and are used as effective cancer chemotherapies and antibacterials (1, 2).

Pteridine reductase 1 (PTR1) is capable of reducing biopterin and folate from the oxidised and dihdro-state to their tetrahydro-state (3). It has been implicated in the pathogenesis of Trypanosomatid type parasitic diseases and is understood to be responsible, in part, for resistance of these parasites to antifolates, which in theory should be effective against these parasites (4). By upregulating PTR1 production when antifolates are introduced, parasites are able to continue to reduce DHF to THF and, therefore, PTR1 acts as a metabolic by-pass. Thus, it is an important therapeutic target.

PTR1 was studied using a computational tool called SID (Simple Intrasequence Difference), which was developed at the University of Strathclyde (5). SID was used to highlight any vulnerable interfaces created by protein chain folds and to define the surface accessible regions through which these interfaces could be accessed by a ligand.

This analysis of PTR1 has yielded a potential allosteric site, which became the focus for further computational analysis, ultimately leading to docking studies, yielding a large list of "hits". After using some stringent criteria this list was cut down to a more reasonable 57 compounds, five of which were picked as a pteridine based scaffold could be incorporated into their structure. The pteridine analogues of the original five, after being designed on paper, were then docked in silico and their interactions with the allosteric site were enhanced by further modifications.

The molecules identified formed templates for synthesis using the diversity-oriented methods developed at the University of Strathclyde.

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