Amino Acids (2006) 30: 311–316
DOI 10.1007/s00726-006-0301-6

Amino Acids

Addendum: Abstracts from the 9th International Congress on Amino Acids and Peptides*

Vienna, Austria, August 8–12, 2005

Oxidative Stress and Protein Modification by Reactive Oxygen and Nitrogen Species

An overview on the biological relevance of oxidative damage to proteins

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The relevance of protein oxidative damage to disease and aging has gained increasing recognition since the pioneering studies of Stadtman et al. The classic protein carbonyl assay, introduced by this researcher, robust and easy to perform, has played a key role in hundreds of studies documenting increased levels of steady-state oxidative protein damage in association with such diverse degenerative ailments as Alzheimer's and Parkinson's diseases, atherosclerosis, rheumatoid arthritis, cataract, or amyotrophic lateral sclerosis. Increased levels of carbonyls have also been documented in aging in disparate models including C. elegans, rats, or human progeric cell lines. Recent analytical advances, based on mass spectrometry techniques, have allowed to identify and quantitate specific protein carbonyl derivatives, namely, glutamic and aminoadipic semialdehyde residues, present in model oxidized proteins and in biological samples. These compounds add to the growing list of specific oxidation products derived from direct oxidation of the protein backbone that includes, among others, o- and dityrosine and methionine sulfoxide, or from adduction to proteins of reactive species generated through oxidation of lipids and carbohydrates, such as carboxymethyl-lysine, MDA-lysine or 4-hydroxynonenal-lysine. Quantitation of these specific products in specific proteins isolated from relevant biological samples, combined with assessment of resulting changes in function and/or structure of such proteins, is one of the immediate challenges in the field. This will allow establishing relationships of cause and effect. As an example, recent work on oxidative modifications in the prion protein, PrP, will be discussed.

Medicinal Chemistry

Proteasome activities during ageing in various tissues of transgenic mice for the human SOD-1 gene: a model for trisomy 21

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Premature aging is one of the Down syndrome characteristics; it has been proposed that enhanced oxidative stress present in DS might be the cause of some aspects of this condition. Inhibition of proteasome activities have been shown during normal aging and might play a causal role in mediating neuron death in certain diseases and such a role can also be played by a chronic low-level proteasome inhibition. Increasing evidence suggests that the activity of the proteasome can be altered by conditions characterized by enhanced oxidative stress. So far nothing is known on proteasome activities in Down syndrome. We have used as a first model to evaluate proteasome activities in animal models for DS, transgenic mice overexpressing the human SOD-1 gene (Tg-hSOD mice). Three activities of the proteasome (tryspine-like, chymotrypsine-like and peptidylglutamyl-peptide-hydrolase) were assayed in Tg-hSOD-1 and control mice from 2 to 24 months. In cerebral hemisphere and cerebellum from control mice almost no modification was observed during aging. By contrast, proteasome activities from 7 and 24 months Tg-hSOD were slightly decreased in cerebral hemisphere when compared to control tissues and severely decreased in cerebellum.

Interestingly, proteasome activities in thymus reflect the fact that this tissue is a natural clock. Indeed, proteasome activities in thymus decrease slightly from control mice aged from 80 to 240 days when compared to 15 days old controls. SOD1 overexpression induces the same decrease as in controls but much more pronounced confirming that SOD1 overexpressinf is deleterious.

These results show for the first time that a dysregulation of the antioxidant enzymes balance can alter differently proteasome activities according to tissues in this simple model of DS. Further experiments are performed to understand how this inhibition could be overcome.

* Editor's Note: The abstracts to follow form an addendum to those already published in Amino Acids 29 (1) (2005) (G. L.)

Plant Amino Acids

An *L,L*-diaminopimelate aminotransferase defines a novel variant of the lysine biosynthesis pathway in plants

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The complete set of reactions needed for lysine biosynthesis in plants has been determined with the identification of a transaminase that specifically catalyzes the inter-conversion of tetrahydrodipicolinate and *L,L*-diaminopimelate using glutamate/2-oxoglutarate as amino donor/acceptor. The same reaction requires 3 genes/enzymes in the acyl-diaminopimelate pathway of bacteria. The gene encoding *L,L*-DAP:2-oxoglutarate aminotransferase was identified from *Arabidopsis thaliana*. The cDNA derived from the gene was able to complement a *dapD* and *dapE* double mutant of *Escherichia coli* indicating that *L,L*-DAP aminotransferase can function in the lysine biosynthetic direction under in vivo conditions. The recombinant enzyme produced as a His-tagged form in *E. coli* showed a specific activity of 2.5 micromol min^{-1} mg^{-1} protein in the biosynthetic direction and 22.3 micromol min^{-1} mg^{-1} protein in the reverse direction. The apparent K_m values were 71 microM for *L,L*-DAP, 50 microM for tetrahydrodipicolinate, 8.3 mM for 2-oxoglutarate and 2.0 mM for glutamate. The enzyme was found to be able to use *L,L*-DAP but not its isomer *m*-DAP, or several other related diamine compounds. These results demonstrate that lysine biosynthesis in plants is distinct from the pathways that have been defined in microorganisms.

Regulation of amino acid transporter gene expression by nitrogen and carbon metabolites

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In higher plants, amino acids are the currency of nitrogen exchange between the sites of primary assimilation and the import-dependent tissues. The partitioning of amino acids in this resource allocation process requires the activity of several classes of amino acid transporters in the plasma membrane. The transcript of AAP1, a proton-amino acid symporter, in mature leaf tissue is regulated by nutrient status and environmental cues. After 7 days nitrogen starvation, AAP1 message is highly induced after feeding 30 min in 25 mM NO_3^- or 10 mM NH_4^+ or 5 mM amino acids such as glutamine and glutamate. AAP1 is also induced in dark-adapted plants after 3 hours of illumination. Light dependent changes in expression may be mediated by a specific photoreceptor or by photosynthesis-dependent increases in leaf sugar content. Both 1% sucrose or glucose feeding induces AAP1 message in dark-adapted plants, suggesting light induction might be an indirect effect of sugar-signaling. However, we can not rule out a role for a photoreceptor in regulation of AAP1 message, because far red light illumination decreases the sucrose-dependent induction. Parallel experiments using expression profiling have identified transcription factors associated with nitrate-dependent changes in gene expression, and recent results describing these transcription factors will be presented.

Functional genomics of amino acid and related metabolism by integrated analysis of transcriptomics and metabolomics in *Arabidopsis thaliana*

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Integrated analysis of metabolome and transcriptome can provide the clues for identification of unknown gene function and understanding of multi-layer systems. To identify the function of particular genes and to find new molecular networks in *Arabidopsis thaliana*, we investigated global gene expression profile by DNA microarray and metabolite profile by combination of different mass spectrometric technologies including LC-MS, GC-MS and FT-MS. Nutritional stress of nitrogen and sulfur resulted in global change of metabolome that could be correlated with the modulation of gene expression, indicating the presence of several gene-to-metabolite networks. In particular, glucosinolate production was notably modified by these stresses, and thus the genes showing similar pattern of expression were identified as the candidates involved in glucosinolate metabolism. The metabolite profiles of *pap1-D* mutant and *pap1* cDNA transgenic lines over-expressing a *Myb* gene in *A. thaliana* indicated that these plants produce an elevated level of particular anthocyanin molecules. The comprehensive gene expression and metabolite profiles of these lines suggested the function of novel genes that are responsible for modification and storage of anthocyanins. Subsequently the functions of those genes were unequivocally identified by analysis of the T-DNA insertion lines and the enzymatic activities of recombinant proteins.

Proteomics

Proteome analysis involving off-line 2D-LC of intact proteins, proteolytic digestion and capillary RP-LC-MS/MS analysis

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The limitations of 2D GE have stimulated the development of alternative techniques for large scale proteome analysis. More recently shotgun proteomics, i.e. multidimensional LC-MS/MS of digested complex protein samples, has been established as a robust technique for protein identification. This method is capable of identifying large number of proteins, often on the basis of a few peptides only. The sample complexity and wide dynamic range impairs though the detection and sequencing of low abundant peptides.

In this study we describe an off-line multidimensional LC method (IEX-RP) for separation of intact proteins coupled with fraction collection. The fractionated proteins are digested by trypsin in

solution in 384 well plates and identified by capillary LC-MS/MS. Both the micro-preparative RP separation of proteins as well as the separation of peptides from tryptic digests are performed on PS-DVB monolithic columns with i.d.'s of respectively 500 and 200 µm. Chromatographic separations of proteins and peptides are optimized. All steps are reproducible and require a minimum of sample handling.

The separation of intact proteins followed by digestion in well plates reduces the sample complexity and enhances the detection of low abundance proteins. Methods were developed for the analysis of standard protein mixtures as well as more complex proteins samples, such as bacterial lysates. This off-line 2D-LC approach allows detection of low abundance proteins in complex protein samples and can also indicate changes in protein expression under physiologic stress.

The role of the MORF/MRG family of proteins during development and aging

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We have been involved in studies of cellular senescence, or the limited ability of normal cells to divide in culture, which is in striking contrast to the unlimited growth potential (immortality) of tumor cells. Cell fusion studies of normal with immortal cells in culture, done in our laboratory, have shown that senescence is dominant over the immortality phenotype exhibited by tumor cell lines and that this occurs irrespective of tumor type, activated oncogene expression or p53 status. This has also led to the identification of MORF4 (mortality factor on human chromosome 4), as a cell senescence inducing gene. MORF4 is a member of a family of novel transcription factors of which only three members are expressed: MORF4 and the MORF related genes on chromosomes 15 and X (MRG15 and MRGX). The three proteins share many similar protein motifs: nuclear localization signals, leucine zipper and non-basic helix-loop-helix domains, and various potential phosphorylation sites. MORF4 is a truncated version of the other two proteins as MRG15 has a chromodomain motif at its amino terminus; and MRGX a novel amino terminal region, both of which are absent in MORF4. We have found that MRG15 and MRGX are present in multiple nucleoprotein complexes and activate or repress various gene promoters in a cell type dependent fashion. MRG15 has received the highest interest in the scientific literature because it is highly conserved, with orthologs present from yeast to human. It has been found to be a component of the Tip60/NuA4 histone acetyltransferase complex in yeast, drosophila and human cells. Inactivation of either dMRG15 or Tip60 in Drosophila results in an embryonic lethal phenotype and inability to repair double strand breaks. We have generated MRG15 null mice and these are embryonic lethal exhibiting many developmental defects in heart, lung, skin and brain. These appear to be, in part, due to defects in proliferation as BrDU labeling is decreased in vivo and null MEFs proliferate poorly when compared with wild type cells. MRGX is expressed only in vertebrates and null mice show no obvious phenotype. The double knockout of MRG15 and X is lethal at an even earlier embryonic stage the MRG15 null alone. Both MRG 15 and X protein levels decline about 2–3 fold at senescence and nucleoprotein complexes change dramatically in young versus old liver. Thus there is an emerging role for these proteins in development, replicative senescence and aging. Supported by grants from NIA AG2752 and the Ellison Medical Foundation (OMP-S) and DOD DAMD17-03-1-0324 (JGJ).

Modifications of proteasome activities in thymus of mice overexpressing human Cu/Zn SuperOxide Dismutase (hSOD1) and Amyloid Precursor Protein (hAPP), a model of Down Syndrome

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Premature aging is one of the Down syndrome (DS) characteristics and it has been proposed that enhanced oxidative stress present in DS might be the cause of some aspects of this condition. The proteasome is a complex enzymatic system, known to remove alterated proteins during aging and which is very sensitive to stress conditions. Two genes of chromosome 21, SOD1 (Cu/Zn SuperOxide Dismutase) and APP (Amyloid Precursor Protein) might be important to study aging and indeed, we have previously shown that SOD1 overexpression induces an early thymic involution, which is an aging characteristic present in trisomy 21 patients.

We thus evaluated the proteasome enzymatic activities in the thymus of transgenic mice for SOD1, APP and both transgenes and compared them to control thymus.

We showed that these proteasome activities are decreased in SOD1 transgenic mice in comparaison to control at different ages (15, 30 and 80 days) but are similar in hAPP transgenic mice. In double transgenic mice APP/SOD1, the proteasome activities are also decreased in comparison to controls but much less than in the transgenic mice for human SOD1 transgene only. These results show for the first time at the biochemical level an interplay between the two genes APP and SOD1. Further experiments will be made to understand the respective roles of APP and SOD1 on proteasome activities.

Induction of Mac-2 binding protein by telomerase catalytic subunit (hTERT) in gastric cancer

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Gastric cancer is the one of the most common cancers in Japan, China, and Korea. The etiology of stomach cancer remains unclear, but it has been shown to involve a number of risk factors, such as dietary factors and infections with *Helicobacter pylori*. However, the underlying molecular events critical to the development of gastric cancers are largely undetermined.

Mac-2 binding protein (Mac2BP) is a ligand of galectin-3, secreted highly glycoprotein present in the extracellular matrix of several tissues and in extracellular fluids such as serum and milk. High expression level of Mac-2BP is associated with a shorter survival, the occurrence of metastasis or a reduced response to chemotherapy in patients with different types of malignancy. Therefore, it is suggested to influence tumor proliferation and metastasis formation.

In this study, we performed the microarray using a hTERT over-expressing telomerase negative cell line SW13, and revealed that Mac2BP mRNA was highly induced by hTERT over-expression. And microarray data was also reproved by RT-PCR and Northern blot analysis. In order to characterize the function of Mac-2BP in gastric tumor, we analyzed the Mac2BP mRNA expression in SNU gastric tumor cell line. High level of Mac-2BP transcripts were appeared in almost of SNU cell lines except for SNU 484. In addition, Mac-2BP mRNA expression was in accordance with hTERT expression pattern. Furthermore, mRNA expression level of Legumain, which is known as a human solid tumor marker, was similar to that of Mac-2BP. An immunohistochemistry

followed by confocal staining analysis revealed that highly expression of Mac-2BP protein was significantly appeared in stomach cancer tissues. These results suggest that Mac-2BP expression induced by hTERT may be associated with a gastric tumor progression.

Bioinformatics solutions in proteomics

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Proteomics analysis – by definition – requires dealing with large amounts of data. Data that may be generated during the analysis of multiple 2D gels or multiple LC-MS/MS runs. In addition each of these analysis philosophies may require pre-fractionation on the protein level and in the end data analysis and validation across such multiple experiment proteomics projects.

The following aspects will be covered in the contribution:

Detailed analysis of MS/MS spectra for PTM discovery and de novo sequencing.
Data validation on the spectrum level.
Data validation for LC-MS/MS experiments.
ProteinScape 1.3: A database system for 2D gel and LC based protein identification and quantitation that is the Bioinformatics backbone for the HUPO Brain Proteome Program.
Automated algorithms to reduce manual validation efforts.
Requirements for quantitative proteomics approaches.

Synthesis

Aromatic and heteroaromatic α-trifluoromethyl α-amino acids

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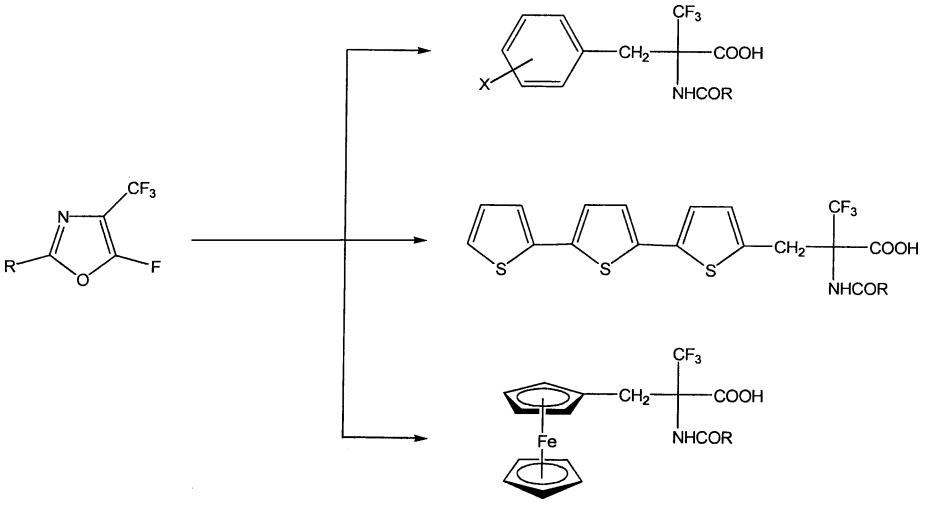
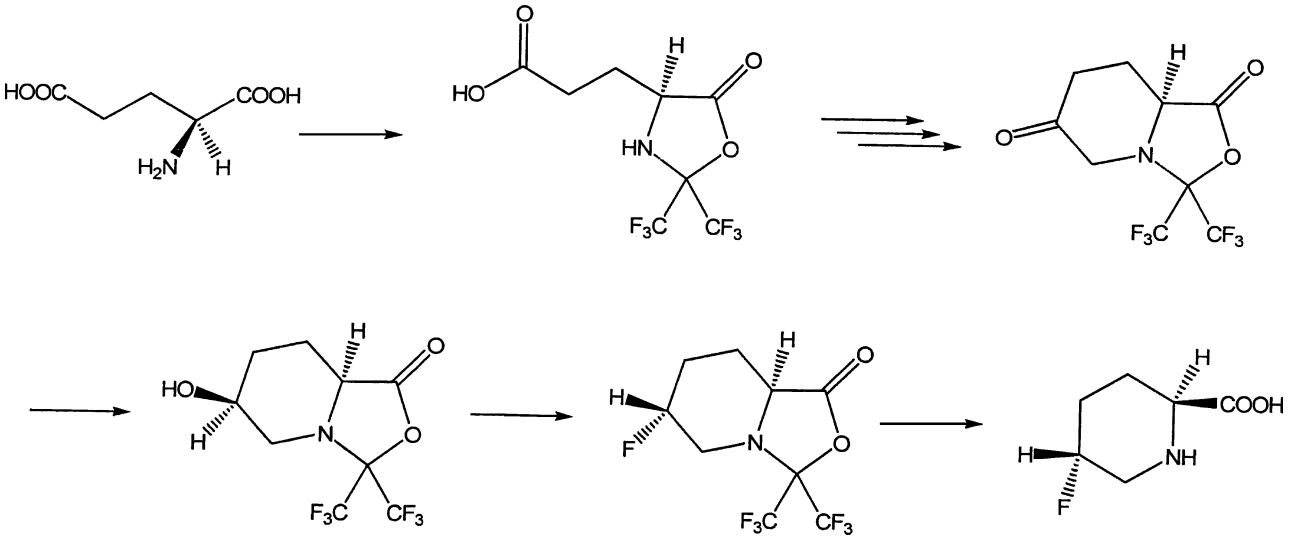
It is well documented that noncovalent aromatic-aromatic interactions are important in determining molecular conformation, stability and activity of biological relevant molecules. Phe, Tyr and Trp often are incorporated into strategical positions of hydrophobic pockets to bind aromatic substrates.

Modulation of pharmacokinetic properties by fluorine substitution has become a well-established strategy for lead optimization and resulted in a large number of fluoro-containing drugs in clinical use. Introduction of a trifluoromethyl group into α-position of an amino acid improves proteolical stability, lipophilicity, transport rates and permeability through certain body barriers. Because of the high electron density, the trifluoromethyl group is capable to participate in hydrogen bonding and may act as coordinative site in metal complexes. Furthermore, the trifluoromethyl group can serve as powerful NMR lable for spectroscopic studies of metabolism and conformation.

Herein, we disclose a concise route to trifluoromethyl substituted amino acids with aromatic, and heteroaromatic groups in the side chain starting from 5-fluoro-4-trifluoromethyl-1,3-oxazoles. The design and synthesis of aromatic bis-amino acid derivatives has attracted considerable interest mainly due to their presence as subunits in many peptide antibiotics. It is desirable to have a repertoire of building blocks that reliably induce a desired conformation in a peptide. Consequently, efforts have been undertaken to develop new methodology for the construction of scaffolds that induce well-defined secondary structure motifs or allow to switch between two different secondary structure motifs.





Synthesis of 4- and 5-substituted pipecolic acids

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The development of synthetic methodology for pipecolic acid derivatives is of current interest, because they are constituents of antibiotics and represent valuable building blocks *i. a.* for targeted drug and protein design as well as for cis/trans peptide bond isomerization studies. A major drawback of peptide drugs is their rapid degradation by proteases, their low lipophilicity and the lack of transport systems to direct peptides into cells. With increasing success the design of peptides and proteins of enhanced activity and stability is developed by incorporation of non-proteinogenic and non-natural amino acids. The advantage of applying fluoro-modification to stabilize peptides and proteins is that this strategy is complementary to other existing stabilization methods.

There are two fundamentally different strategies by which fluorine and perfluoroalkyl groups can be introduced into target molecules. Direct substitution of hydrogen or other suitable functional groups by fluorine and perfluoroalkyl groups in a late step of the reaction sequence. Alternatively, fluorine and perfluoroalkyl groups can be incorporated *via* fluoro-containing building blocks. Despite the potential hazard of explosions, if not properly handled, DAST has gained widespread popularity as a fluorinating reagent for the displacement of hydroxy groups and carbonyl oxygen atoms.

Herein we describe a new efficient approach to hydroxy- and oxopipecolic acid derivatives using hexafluoracetone as protecting and activating agent and their subsequent transformation into the corresponding fluoro-substituted derivatives on treatment with DAST.

Synthesis and biological activity of novel peptide ester prodrugs of acyclovir

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Four peptides ester prodrugs (Gly, Val) of 9-[(2-hydroxyethoxy) methyl]guanine (acyclovir, ACV) containing thiazole, oxazole ring were synthesized. LC/MS was used to characterize the new prodrugs. Both ^{1}H NMR and ^{13}C NMR spectra of the four prodrugs of ACV were measured and assigned based on spectral comparison with compounds of similar structures.

Transport

Oligomerization of neurotransmitter transporters – a prerequisite for ER-export and for reverse transport?

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Neurotransmitter transporters retrieve neurotransmitters from the synaptic cleft. A large subfamily thereof (12 in man) are the Na^{+}/Cl^{-}-dependent neurotransmitter transporters. They share conserved structural features (e.g. a hydrophobic membrane-embedded core of twelve transmembrane spanning segments) and have several functional properties in common (e.g. they utilise the Na^{+}-gradient for cotransport of the substrate; substrate flux is associated with an ionic current). In addition, they are the site of action of therapeutic (antidepressants, antiepileptics) and illicit drugs (cocaine, amphetamine, ecstasy etc.). Amphetamine and its congeners induce reverse transport: i.e. the transporters for noradrenaline, dopamine and serotonin extrude the neurotransmitter while taking up the amphetamines. The underlying mechanism has remained elusive. Neurotransmitter transporters form constitutive homo-oligomers. The interaction is driven by several transmembrane segments (i.e. helices 2, 5 and 11/12); this precludes a simple dimeric arrangement. The biological role of this oligomerization is not understood. However, several observations with the GABA-transporter-1 (GAT1) are consistent with the interpretation that oligomerization is a prerequisite for export of these transporters from the endoplasmic reticulum (ER). In addition,

experiments with a concatemeric transporter (serotonin transporter = SERT fused to GAT1) show that amphetamine-induced transport reversal is contingent on oligomerization; in other words: amphetamine takes two to tango.

Significance of cloning and identification of polymorphisms of the serotonin transporter: application to human studies

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Collaborations are now evolving between bench and bedside research to examine the amino acid/monoamine family of transporters applicable to human disease processes. To illustrate one example, our group began with the known rat sequence of the serotonin (5-HT) monoamine transporter. We cloned the same in human, and then identified allelic variants in the population. The best studied is a functional variation in the length of the promoter region, 5-HTTLPR (5-HTT long promoter region). We developed techniques to readily characterize the serotonin transport kinetics in human platelets and synaptosomes (the latter from surgical pathology specimens). Using this approach, we could study both the genotype and phenotype of the transporter in humans. This enabled us to find that a difference in human platelet 5-HTT Vmax correlates with differences in human brain 5-HTT Vmax. We also observed that the 5-HTTLPR deletion is associated with lower platelet 5-HTT Vmax. We found that differences in the 5-HTTLPR, and differences in platelet 5-HT transport kinetics, independently correlated with differences in human temperament. We have also found that platelet 5-HTT Vmax is lower in psychiatric depression, although the 5-HTTLPR deletion does not appear to explain why. Furthermore, both platelet 5-HTT Vmax and 5-HTTLPR are associated with human behavioral differences, including different patterns of alcohol use, and different patterns of antidepressant response. We will discuss this translational research in the context of our current efforts to optimize antidepressant pharmacotherapy utilizing these biomarkers.

Verleger: Springer-Verlag GmbH, Sachsenplatz 4–6, 1201 Wien, Austria – Herausgeber: Prof. Dr. Gert Lubec, Universitäts-Kinderklinik, Währinger Gürtel 18–20, 1090 Wien, Austria – Redaktion: Sachsenplatz 4–6, 1201 Wien, Austria – Hersteller: Satz und Umbruch: Thomson Press (India) Ltd., Chennai; Offsetdruck: Novographic Druck G.m.b.H., Sagedergasse 29, 1120 Wien, Austria – Verlagsort: Wien – Herstellungsort Wien – Printed in Austria.