

Leiden/Amsterdam
Center for Drug Research

Universiteit Leiden/Vrije Universiteit Amsterdam



LACDR Spring Symposium

May 12th, 2011
09.00 h – 17.00 h

Auditorium
Vrije Universiteit Amsterdam
De Boelelaan 1105
1081 HV Amsterdam

SYMPOSIUM PROGRAM

09.00 h Preparation posters by the PhD students and reception with coffee and tea

09.30 h Opening and introduction by **Prof.dr. Nico Vermeulen, Director of Research**

09.45 h **Prof.dr. Kostas Kostarelos (School of Pharmacy / London)**
Professor of Nanomedicine and Head of the Centre for Drug Delivery Research,
School of Pharmacy, London, UK
"How to Transform Novel Nanomaterials to Clinically-relevant Nanomedicine Tools: The Carbon Nanotube Experience"

10.30 h coffee / tea break

11.00 h **Bart Lammers, Division of Biopharmaceutics**
"Augmented atherogenesis in mice lacking both macrophage ABCA1 and apoE"

11.15 h **Ferry Heus, Division of BioMolecular Analysis**
"Development of a miniaturized fluorescence detection system for the hyphenation of nano-LC to on-line biochemical assays"

11.30 h **Daan van Schalkwijk, Division of Analytical BioSciences**
"Computational model-based diagnostic markers improve cardiovascular risk prediction"

11.45 h **Lisa Frederiksson, Division of Toxicology**
"Drug-induced liver injury – NFkappaB in control of life or death"

12.00 h **Poster presentation by all LACDR PhD students and lunch**

14.00 h **Clara Blad, Division of Medicinal Chemistry Leiden**
"Allosteric enhancement of the nicotinic acid receptor: a new approach towards cardiovascular health"

14.15 h **Jeroen van Smeden, Division of Drug Delivery Technology**
"Human stratum corneum lipid analysis by LC-MS and its applicability on skin diseases?"

14.30 h **Joost Westerhout, Division of Pharmacology**
"Prediction of human brain target site concentrations: the preclinical approach using microdialysis and in silico modeling"

14.45 h **Stephanie de Beer, Division of Molecular Toxicology**
"Structural rationalization of selective cytochrome P450 BM3 metabolism"

15.00 h **Liane Klok, Division of Medical Pharmacology**
"A receptor for the stress hormone cortisol modulates resilience to major depressive disorder in females"

15.15 h **Danny Scholten, Division of Medicinal Chemistry Amsterdam**
"Molecular Determinants of Chemokine Receptor CXCR7 Regulation"

15.30 h coffee / tea break

15.45 h **Dr. Veronique de Vroey and Dr. Jerry Snoeks (Janssen Research & Development)**
Director Discovery Screening and Liaison for Academic and Industry Relations,
Janssen Research & Development, A Division of Janssen Pharmaceutica NV, Beerse, Belgium
"The Current Challenges of the Pharmaceutical Industry"

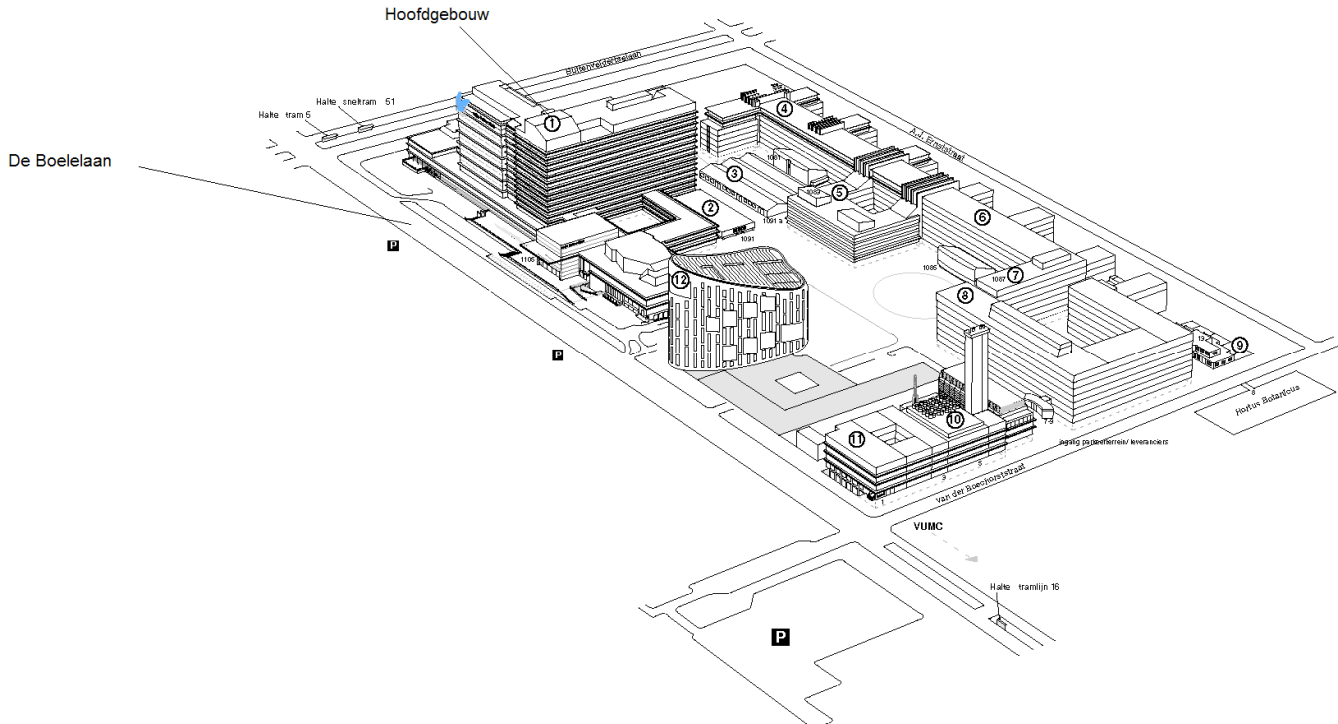
16.30 h Presentation of the winners of the PhD competition

16.45 h Closing by **Prof.dr. Meindert Danhof, Director of Research**

17.00 h Drinks!

Voting procedure PhD competition:

- On re-entering the lecture hall at 11.00 hrs, you will receive the ballot paper and a pen.
- This paper has to be completed immediately after the final lecture, and will be collected at 15.30 hrs.
- Note: points have to be given to all candidates. Incomplete forms are invalid.**
- You can only vote when you attend the symposium the whole day.**



Address:

Hoofdgebouw VU
 De Boelelaan 1105
 1081 HV Amsterdam

Public Transport (recommended)

From Central Station Amsterdam

Express tram 51 (16 minutes)
 Tram 5 (25 minutes)

From Station Zuid / WTC

Express tram 51 (1 minute), direction Poortwachter
 Tram 5 (1 minute), direction Poortwachter
 It is a 10 minute walk to the VU from Station Zuid/WTC

By Car

The A-10 Amsterdam ring can be reached from all directions. Follow the A-10 to the Zuid/Amstelveen exit S 108. Turn left at the end of the slip road onto Amstelveenseweg: after about three hundred yards (at the VU Hospital building) turn left again onto De Boelelaan. The VU can be reached via city routes S 108 and S 109.

Parking

There is a limited amount of parking space available around the VU itself in De Boelelaan and also in the A.J. Ernststraat, at the back of the VU. These spaces have parking meters. There is even more parking space on the east side of Buitenveldertselaan at the junction with Willem van Weldammelaan, within 5 minutes walking distance of the VU. A number of parking places for the handicapped are reserved in front of the VU Main Building and within its grounds.



Prof.dr. Kostas Kostarelos

Nanomedicine Laboratory, Centre for Drug Delivery Research,
School of Pharmacy, London University, UK

How to Transform Novel Nanomaterials to Clinically-relevant Nanomedicine Tools: The Carbon Nanotube Experience

A lot of effort is currently invested in the development of various types of nanomaterials such as quantum dots, metallic and semi-conducting nanoparticles, carbon nanotubes (CNT), all designed for a variety of biomedical applications. Carbon nanotubes in particular differ dramatically in terms of structural characteristics (diameter, length, size distribution), surface (chemical composition of coated or grafted groups, aspect ratio, hydrophobicity) and colloidal properties (degree of aggregation, dispersibility). These differences result in diverse biological profiles *in vitro* and *in vivo*.

Even within the same type of CNT dramatic structural, surface and chemical differences exist based on manufacturing or chemical treatment specifications that will determine their biological profiles *in vivo*. This leads to the need for very careful determination of the material characteristics and their correlation with pharmacological performance and any adverse effects that may occur.

The nanometer-scale dimensions of CNT make quantities of milligrams possess a large number of cylindrical, fibre-like particles, with a concurrent high total surface area. The large aspect ratio will also depend on their degree of bundling and aggregation of nanotubes in solution. Concerning the toxicity of CNT, *in vitro* studies have indicated that chemically functionalised CNT produce less cytotoxic effects than aqueous dispersions of pristine CNT (non-covalently functionalised).

However, the toxicity of CNT does not only depend on the degree of surface functionalisation and the different toxicity of functional groups. Batches of pristine CNT (non-purified and/or non-functionalised) readily after synthesis contain impurities such as amorphous carbon and metallic nanoparticles (catalysts: Co, Fe, Ni and Mo), which can also be the source of toxic effects have been reported in studies using pristine CNT.

In this talk, specific examples of engineering carbon nanotube material to achieve control over their pharmacology (localisation and retention in specific tissues) on administration and their toxicological impact will be shown. Such engineering exercises are considered essential for the development of CNT in medicine.



Bart Lammers

Division of Biopharmaceutics
LACDR, Leiden

Augmented atherogenesis in mice lacking both macrophage ABCA1 and apoE

B. Lammers, M. Hoekstra, R.B. Hildebrand, D. Ye, I. Meurs, Y. Zhao, Th.J.C. Van Berkel, M. Van Eck

Division of Biopharmaceutics, LACDR, Leiden University, The Netherlands.

Objective:

ABCA1 protects against atherosclerosis by facilitating cholesterol efflux from macrophage foam cells in the arterial wall to extracellular apolipoprotein (apo) A-I. In contrast to apoA-I, apoE is secreted by macrophages and can, like apoA-I, induce ABCA1-mediated cholesterol efflux. Yet, the combined effect of macrophage ABCA1 and apoE on lesion development is unexplored.

Methods:

LDL receptor knockout (KO) mice were transplanted with bone marrow from ABCA1/apoE double KO (dKO) mice, their respective single knockouts, and wild-type (WT) controls and were challenged with a high-fat/high-cholesterol diet for 9 weeks.

Results:

In vitro cholesterol efflux experiments showed no differences between ABCA1 KO and dKO macrophages. The serum non-HDL/HDL ratio in dKO transplanted mice was 1.7-fold and 2.4-fold ($p < 0.01$) increased compared to WT and ABCA1 KO transplanted mice, respectively. The atherosclerotic lesion area in dKO transplanted animals ($650 \pm 94 \times 10^3 \mu\text{m}^2$), however, was 1.9-fold ($p < 0.01$) and 1.6-fold ($p < 0.01$) increased compared to single knockouts (ABCA1 KO: $341 \pm 20 \times 10^3 \mu\text{m}^2$; apoE KO: $402 \pm 78 \times 10^3 \mu\text{m}^2$, respectively) and 3.1-fold increased ($p < 0.001$) compared to WT ($211 \pm 20 \times 10^3 \mu\text{m}^2$). Moreover, when normalized for serum cholesterol exposure, macrophage ABCA1 and apoE independently protected against atherosclerotic lesion development ($p < 0.001$).

Conclusions:

Combined deletion of macrophage ABCA1 and apoE results in a defect in cholesterol efflux and, compared to ABCA1 KO transplanted mice, elevated serum total cholesterol levels, thereby inducing a more dramatic and significant increase in atherosclerosis.



Ferry Heus

Division of Biomolecular Analysis
LACDR, Amsterdam

Development of a miniaturized fluorescence detection system for the hyphenation of nano-LC to on-line biochemical assays

F. Heus, H. Lingeman, M. Giera, J. Kool, and W.M.A. Niessen

Division of BioMolecular Analysis, LACDR, VU University, Amsterdam, the Netherlands

In order to screen complex mixtures for bioactive compounds in natural samples, numerous systems have been developed where liquid chromatography (LC) is directly coupled to a continuous-flow bioassay and mass spectrometry. This allows for simultaneous bioaffinity profiling and compound elucidation. Usually, the bioassay consists of the target protein and a fluorescently labelled tracer ligand which are continuously mixed post-column with (possible) ligands eluting from the LC-column effluent. A fluorescence detector (FD) is then used for on-line bioassay readout.

A limitation of this on-line screening approach can be the relatively high consumption of expensive reagents and/or precious (natural) samples. Here, a methodology is presented where sample – and assay consumption is reduced by decreasing the overall flow rates of the screening setup.

In the chromatographic part of the system this is achieved by implementing nano-LC separations. The bioaffinity part of the system was miniaturized by utilizing a microfluidic chip with a 6 μL incubation chamber, where the continuous flow bioassay is mixed with the nano-LC effluent. Additionally, a miniaturized LED-induced fluorescence detection system was developed, capable of sensitively monitoring typical continuous “low-flow” bioassays.

The microfluidic screening setup was applied to a fluorescent enhancement assay based on the Acetylcholine Binding Protein (AChBP; analogue of the binding domain of the nicotinic acetylcholine receptor) with a fluorescent probe (DAHBA). The binding protein and the probe were infused together into the chip by a syringe pump at a flow rate of 5 $\mu\text{L}/\text{min}$, where they mixed continuously with the nano-LC effluent set at 500 nL/min . The system is currently applied for the screening of snake – and cone snail venom, which contain neurotoxic peptides with high affinity to the AChBP. These valuable compounds, when identified, can then be used for further pharmacological research to e.g. brain and muscle related nicotinic acetylcholine receptors and maybe as lead compounds for drug discovery as biopharmaceuticals.



Daan van Schalkwijk

Division of Analytical Biosciences / TNO Quality of Life
LACDR, Leiden

Computational model-based diagnostic markers improve cardiovascular risk prediction

D.B. van Schalkwijk,^{1,2,3} **A.A. de Graaf**,¹ **B. v.d. Werff**,¹ **L.D. Parnell**,⁴ **B. v Ommen**,¹ **J.M. Ordovás**,⁴ **J. vd Greef**^{1,2}

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²Division of Analytical BioSciences, LACDR, Leiden University, the Netherlands;

³The Netherlands Bioinformatics Centre (NBIC);

⁴The Nutrition and Genomics Laboratory, JM-USDA Human Nutrition Research Center on Aging at Tufts University, Boston, MA, USA.

Background

Cardiovascular disease continues to be a major killer worldwide. Improved diagnostics contribute to early detection and timely treatment of this disease.

Methods

Current measurements of lipoproteins, the cholesterol-carrying particles in human blood, can distinguish up to 40 subfractions in a 'lipoprotein profile'. In medical practice, this information is much harder to interpret than the standard 'bad' LDL and 'good' HDL cholesterol. Therefore, we developed a computational model called Particle Profiler to interpret detailed lipoprotein profile data, and extract medically relevant information. The markers we derive are called 'lipoprotein metabolic ratios', which are different ratios of the metabolic production, lipolysis, and uptake processes that lipoproteins undergo.

We applied Particle Profiler to NMR lipoprotein profiles measured in 1981 subjects from the Framingham offspring cohort and calculated lipoprotein metabolic ratios. We applied stepwise cross-validated logistic regression to select classical risk factor variables and lipoprotein metabolic ratios that contributed to predicting 10-year risk for general cardiovascular disease.

Results

Six lipoprotein metabolic ratios were consistently found to contribute to cardiovascular risk prediction after cross-validation. Adding the six selected lipoprotein metabolic ratios to a multivariate logistic regression model with the best performing classical risk markers and NMR lipoprotein derived markers, resulted in a significantly improved predictive power as measured by the 'area under the receiver operating characteristic (ROC) curve'.

Conclusion

Six calculated lipoprotein metabolic ratios significantly improved cardiovascular risk prediction above the best predicting 'classical' variables, similar to those included in the Framingham risk score. The improvement based on our diagnostic markers more than doubled the improvement obtained by adding total and HDL cholesterol to non-cholesterol risk factors.



Lisa Frederiksson

Division of Toxicology
LACDR, Leiden

Drug-induced liver injury – NFkappaB in control of life or death

L. Fredriksson, G. Benedetti, B. Herpers, Z. Di, J. Meerman, M. de Graauw and B. van de Water

Division of Toxicology, LACDR, Leiden University, the Netherlands

Drug-induced liver injuries (DILIs) are the major cause of drug failures and are often idiosyncratic in nature. We hypothesize that idiosyncratic DILI occurs due to crosstalk between drug reactive metabolite and cytokine stress signaling. To study this hypothesis, human hepatoma HepG2 cells were exposed to diclofenac, which causes idiosyncratic DILI in humans, in the presence of the pro-inflammatory cytokine TNF α . Diclofenac itself induced a mild concentration-dependent apoptosis of HepG2 cells. While TNF α itself was not cytotoxic, it strongly enhanced the diclofenac-induced apoptosis.

Using a siRNA screening approach in combination with live cell imaging of apoptosis, diclofenac/TNF α -induced apoptosis was identified as death-receptor dependent involving the intrinsic, mitochondrial death pathway. Under normal conditions the pro-apoptotic signaling pathways that are activated down-stream of the TNF- receptor are controlled by the activation of the transcription factor NF- κ B and the resulting gene transcription. Live cell imaging of HepG2 cells expressing GFP-p65 demonstrated that diclofenac causes a delay in the NF- κ B oscillatory nuclear-to-cytosol translocation pattern in association with reduced NF- κ B transcriptional activity. Imaging-based siRNA screening of this NF- κ B oscillatory response identified critical signaling components responsible for the diclofenac-mediated delay in TNF α -induced NF- κ B translocation. Some of these candidate genes are essential in the life/death signaling balance under diclofenac/TNF α exposure conditions.

Together our data suggest a model whereby diclofenac-mediated stress signalling suppresses TNF α -induced survival signalling routes and sensitizes cells to apoptosis and consequently the onset of DILI. We anticipate that our work will enable us to identify mechanism-based biomarkers that can predict idiosyncratic-like DILI in a pre-clinical drug development setting.



Clara Blad

Division of Medicinal Chemistry
LACDR, Leiden

Allosteric enhancement of the nicotinic acid receptor: a new approach towards cardiovascular health

C.C. Blad, J.P. van Veldhoven, C. Klopman, D. Wolfram, G.J.P. van Westen, J. Brussee, A.P. IJzerman

Division of Medicinal Chemistry, LACDR, Leiden University, The Netherlands

Introduced 50 years ago, nicotinic acid is still the best drug for increasing HDL cholesterol in parallel to reductions in (V)LDL cholesterol and plasma triglycerides. However, a high dose of 1-2 g/day is needed, and the side effects of nicotinic acid severely limit patient compliance. Nicotinic acid-mediated lipid modification and the most common side effect, skin flushing, are both caused by the direct activation of the recently identified G protein-coupled receptor HCA₂.

We have now explored a new class of HCA₂ ligands, the pyrazolopyrimidines, which we synthesized in our laboratory (figure 1). Interestingly, these compounds bind to the receptor at a site that is different from the nicotinic acid binding site, and thus are allosteric modulators for HCA₂. The pyrazolopyrimidines (partially) activate the receptor on their own, but they can also increase the potency of nicotinic acid on HCA₂ by binding simultaneously. Therefore, the nicotinic acid dose could be lowered up to 10 times if it were given as a combination therapy with an allosteric modulator.

Excitingly, the allosteric modulators also increase the potency of endogenous HCA₂ ligand 3-hydroxybutyrate (3-OHB). Administration of a pyrazolopyrimidine alone may already increase receptor activation by the natural ligand sufficiently to induce healthy changes in circulating lipids. In this case, the receptor reacts to the natural fluctuations in 3-OHB concentration, so the side-effects are expected to be minimal.

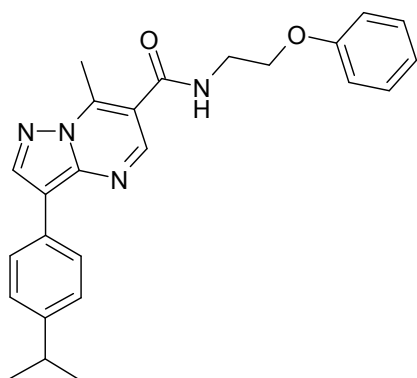
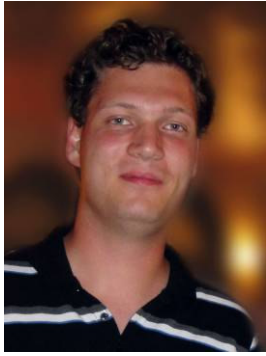


Figure 1. Example of an allosteric modulator for HCA₂



Jeroen van Smeden

Division of Drug Delivery Technology
LACDR, Leiden

Human stratum corneum lipid analysis by LC-MS and its applicability on skin diseases?

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³ Netherlands Metabolomics Centre, LACDR, Leiden University, Leiden, The Netherlands

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An increasing number of people suffer from skin diseases like atopic eczema, with a current occurrence of over 15% in Western Europe. In recent years, several studies reported strong evidence of an impaired barrier function of the skin in patients with atopic eczema¹⁻². It is known that the lipids in the outermost layer of the skin, the stratum corneum (SC), are crucial for a competent skin barrier function, as the lipid matrix is the only continuous pathway in the SC³⁻⁴.

The SC lipids consist mainly of three lipid classes: Cholesterol (CHOL), Free Fatty Acids (FFAs) and Ceramides (CERs). Analyzing the stratum corneum lipid composition of patients suffering from atopic eczema is expected to lead to a better understanding of the impaired skin barrier function in these patients. For decades, the determination of the lipid composition was primarily performed using thin layer chromatography and/or gas chromatography, which resulted in tremendous knowledge of the lipid composition in stratum corneum⁵⁻⁷. Recently, more comprehensive methods are developed to analyze the stratum corneum lipids, like liquid chromatography coupled to mass spectrometry (LC-MS). This revealed the presence of additional lipid subclasses, in particular with respect to CERs⁸. However, robust and reliable analytical MS methods for analyzing skin lipids are few and far between, and no method is reported that analyzes all three main lipid classes in a single run.

Here, we describe a novel method for the combined analysis of the 3 main SC lipids (viz. CHOL, FFAs, CERs) by means of LC-MS, all analyzed in one dedicated run. We observed CHOL, as well as all known CER classes, as well as one new ceramide subclass which was fully identified. Concerning the FFA lipid class, to our knowledge for the first time, we could quantitatively determine the amounts of more than 15 different FFAs present in human SC using this LC-MS method. In total, the number of all lipid components exceeds 250. In subsequent studies the method was used to compare the lipid composition in SC from healthy volunteers and of patients suffering from atopic eczema. We observed significant differences - with possible biological relevance - compared to normal human SC. Patients suffering from eczema showed significant lower levels of certain CER subclasses (CER subclasses with long acyl chains), a result that is supported by *in vitro* data.

Future research needs to focus on more profound studies for elucidating the biological background of all observed differences in SC lipids, but the method described here proves to be both robust and reliable for analyzing all these lipids in a quick, straightforward and very detailed manner.

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1. Di Nardo, A., P. Wertz, A. Giannetti, and S. Seidenari. 1998. Ceramide and cholesterol composition of the skin of patients with atopic dermatitis. *Acta Derm Venereol* **78**: 27-30
 2. Jungersted, J. M., L.I. Helligren, G. B. E. Jemec, and T. Agner. 2008. Lipids and skin barrier function – a clinical perspective. *Contact Dermatitis* **58**: 255-262
 3. Madison, K. C., 2003. Barrier function of the skin: "la raison d'etre" of the epidermis. *J Invest Dermatol* **121**: 231-241
 4. Wertz, P.W., and B. van den Bergh. 1998. The physical, chemical and functional properties of lipids in the skin and other biological barriers. *Chem Phys Lipids* **91**: 85-96
 5. Ponc, M., A. Weerheim, P. Lankhorst, and P. Wertz. 2003. New acylceramide in native and reconstructed epidermis. *J Invest Dermatol* **120**: 581-588
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 7. Stewart, M. E., and D. T. Downing. 1995. Free sphingosines of human skin include 6-hydroxysphingosine and unusually long-chain dihydrosphingosines. *J Invest Dermatol* **105**: 613-618
 8. Masukawa, Y., H. Narita, E. Shimizu, N. Kono, Y. Sugai, T. Oba, R. Homma, J. Ishikawa, Y. Takagi, T. Kitahara, Y. Takema, and K. Kita. 2008. Characterization of overall ceramide species in human stratum corneum. *J Lipid Res* **49**: 1466-1476



Joost Westerhout

Division of Pharmacology
LACDR, Leiden

Prediction of human brain target site concentrations: the preclinical approach using microdialysis and in silico modeling

J. Westerhout¹, M. Danhof¹, and E.C.M. de Lange¹

¹ Division of Pharmacology, LACDR, Leiden University, the Netherlands

Purpose

To be able to predict CNS drug effects in human a more mechanistic understanding is needed on the individual contribution of mechanisms involved in brain target site distribution and ultimate drug effects. Moreover, insight is needed on the variability of such contributions, as a result of species, gender, and age differences as well as by disease state, diet, drug treatment, etc. So far, CSF concentrations are often used as a surrogate marker for brain target site concentrations during drug development. However, the use of CSF concentrations for the prediction of CNS drug effects is not that simple and straightforward, as a generally applicable relationship between CSF concentrations and brain target site concentrations does not exist.¹

The intracerebral microdialysis technique allows quantitative investigation of the processes that determine drug distribution into and within the CNS. This technique can be applied in multiple sites in the brain. With integrative cross-compare designed studies important mechanisms can be influenced in a well-controlled and systematic manner, for example specific inhibition of an efflux transporter. With the use of advanced mathematical modeling procedures the data obtained may be analyzed to dissect contributions of individual mechanisms in animals as links to the human situation.

Methods

Acetaminophen (i.v. infusion of 15 mg/kg in 10 min) was used as a model drug for passive blood-brain transport. Acetaminophen concentrations in brain ECF, CSF (from the lateral ventricle (CSF_{LV}) and cisterna magna (CSF_{CM})), and plasma were determined by HPLC and electrochemical detection. Quinidine (i.v. infusion of 10 or 20 mg/kg in 10 min) was used as a model drug for active blood-brain transport by P-glycoprotein (P-gp). The specific impact of P-gp mediated transport was studied by pre-administration of the P-gp blocker tariquidar (i.v. infusion of 15 mg/kg in 10 min). Quinidine concentrations in brain ECF, CSF, and plasma were determined by HPLC and fluorescence detection.

Results

The acetaminophen concentration-time profiles for the brain ECF are similar to the plasma concentration-time profiles, whereas the CSF_{LV} and CSF_{CM} concentration time profiles are ~4-fold lower. For quinidine, important differences between CSF and brain ECF concentrations were found, being differentially affected by P-gp transport.

Discussion

Differences between the brain ECF and CSF concentration-time profiles could be caused by: (1) the difference in surface area between the blood-brain barrier (BBB) and blood-CSF barrier (BCSFB); (2) The high turnover rate of CSF, causing a diluting effect of CSF; (3) the difference in expression and activity of active transport systems at the BBB and BCSFB. Population-based PK analysis is currently being performed, using NONMEM[®], to reveal the impact of the passive and active transport processes on the brain ECF – CSF relationship.

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Stephanie de Beer

Division of Molecular Toxicology
LACDR, Amsterdam

Structural rationalization of selective cytochrome P450 BM3 metabolism

S.B.A. de Beer¹, L. A. H. van Bergen¹, K. Keijzer¹, H. Venkataraman¹, V. Rea¹, J.N.M. Commandeur¹, N.P.E. Vermeulen¹, and D.P. Geerke¹

¹ Division of Molecular and Computational Toxicology, LACDR, VU University, Amsterdam, the Netherlands

Computational modeling and simulation methods have proven to be a useful tool to rationalize typical shifts in metabolism patterns between mutant enzymes (e.g. *Proteins* 2008,71:336-52). Cytochrome P450 BM3 is a well-studied, soluble, fatty acid hydroxylase from *Bacillus megaterium*, and is a very promising biocatalyst due to its high catalytic activity and stability.

In the present study, the structural basis for shifting patterns in Cytochrome P450 BM3 regio-selectivity in testosterone (TES) and nordione (NOR) metabolism was investigated, by combining molecular docking studies and molecular dynamics (MD) simulations. A plasticity model is obtained, from which regioselectivity can be understood by taking the flexibility of the protein into account.

The computational findings, supported by the experimental results, made it possible to rationalize the altered mutant selectivity towards steroids. In accordance with experiment, several independent docking studies involving BM3 mutants showed new insights into the orientation of TES and NOR within the active site. It was found that in the binding cavity of the wild type enzyme testosterone can more easily adopt orientations corresponding with formation of multiple hydroxylated metabolites. In contrast, BM3 with additional mutations in the cavity can hydroxylate TES with a higher regio-selectivity. The structurally similar compound NOR does not show such a pattern, but shows only one hydroxyl metabolite for all mutants.

These new insights into the effect of the active site's topology on substrate selectivity will open up new possibilities for future engineering and design of selective drug metabolizing P450 BM3 biocatalysts.



Liane Klok

Division of Medical Pharmacology
LACDR, Leiden

A receptor for the stress hormone cortisol modulates resilience to major depressive disorder in females

M.D. Klok¹, E.J. Giltay², A.J.W. Van der Does^{2,3}, S.A. Vreeburg⁴, B.W.J.H. Penninx⁴, D.I. Boomsma⁵, J.M. Geleijnse⁶, N. Antypa³, N. van Leeuwen⁷, F.G. Zitman², E.R. de Kloet¹, and R.H. de Rijk¹

¹Division of Medical Pharmacology, LACDR, Leiden University, The Netherlands

²Department of Psychiatry, Leiden University Medical Center, The Netherlands

³Institute of Psychology, Leiden University, The Netherlands

⁴Department of Psychiatry, VU University Medical Center, Amsterdam, The Netherlands

⁵Department of Biological Psychology, VU University Amsterdam, The Netherlands

⁶Division of Human Nutrition, Wageningen University, The Netherlands

⁷Department of Molecular Cell Biology, Leiden University Medical Center, The Netherlands

A fundamental question in mental health research is which factors can tip the balance from vulnerability to resilience. Clearly, genes and stressful environmental factors play a role. Here we focus on the mineralocorticoid receptor (MR), which mediates effects of the hormone cortisol in the brain, facilitating appraisal of stressful information, emotional arousal and behavioural response selection.

We tested whether common MR gene variants are functional *in vitro* and whether they associate with the risk for depression. Three common MR gene variants could be identified that affect MR expression and functioning *in vitro* and are linked to variability in activity of the neuroendocrine stress system (the hypothalamic-pituitary-adrenal axis) *in vivo*. Genetic association analysis showed that one of these MR gene variants, that results in the highest MR expression, associates with heightened dispositional optimism in one study group, with less thoughts of hopelessness, rumination and fewer diagnosis of depression in a second study group and with a lower risk for depression in a third and large depression cohort study. All effects were restricted to women, which is interesting considering the two times higher prevalence of depression in women. Moreover, results from an association study among a large group of depressed patients indicated interaction between antidepressants and MR genotype.

To conclude, common and functional MR gene variants resulting in altered receptor expression may confer inter-individual variability in resilience to psychopathology in women. We propose the MR to be an important target for antidepressant treatment with the MR genotype providing guidance for selecting a specific antidepressant and dose.

This research was supported by the Netherlands Brain Foundation (Hersenstichting Nederland), the Dutch Ministry of Health, Welfare and Sport, NWO, the Geestkracht program of the Netherlands Organisation for Health Research and Development (Zon-Mw, grant number 10-000-1002), International Research Training Group IRTG funded by the DFG and NWO grant, psychiatric hospital Rivierduinen and the Royal Netherlands Academy of Arts and Sciences.



Danny Scholten

Division of Medicinal Chemistry
LACDR, Amsterdam

Molecular Determinants of Chemokine Receptor CXCR7 Regulation

D.J. Scholten, M. Canals, S. de Munnik, M. Han, M.J. Smit, and R. Leurs

Division of Medicinal Chemistry, LACDR, Faculty of Science, VU University Amsterdam, The Netherlands.

The chemokine receptor CXCR7 belongs to the superfamily of G protein-coupled receptors and recognizes two endogenous peptide ligands, CXCL11 and CXCL12. CXCL11 also binds to CXCR3, and CXCL12 to CXCR4. CXCR7 has been implicated in tumor development and progression, but the mechanism behind it remains unclear.

Here we report on the signaling and regulation of CXCR7, using several pharmacological assays, including radioligand binding, [³⁵S]-GTPγS accumulation, β-arrestin recruitment, immunocytochemistry and receptor internalization experiments. In summary, we show that ligand binding to CXCR7 does not result in classical G protein mediated signaling, but leads to recruitment of β-arrestin as well as clathrin- dependent internalization and recycling of the receptor. Moreover, this receptor regulation critically depends on residues in the C-terminus of CXCR7.

Our results suggest that CXCR7 is not a classical chemokine receptor, as it seems to be biased towards β-arrestin mediated pathways. Whether CXCR7 is directly involved in tumorigenesis, or indirectly by scavenging of CXCL11 or CXCL12, thereby modulating their availability to CXCR3 or CXCR4, still remains to be elucidated.



Dr. Veronique de Vroey¹ and Dr. Jerry Snoeks²

Director Discovery Screening¹
Liaison for Academic and Industry Relations²



Janssen R&D, Janssen Pharmaceutica NV, Beerse, Belgium

The Current Challenges of the Pharmaceutical Industry

The pharmaceutical industry is clearly facing tough challenges: an increasing cost of R&D, patent expirations, a decline in R&D productivity, increased expectations for innovative healthcare solutions from the market and the buyers, all leading to a growing innovation crisis. Additionally the traditional fully integrated business model of big Pharma underlies the crisis and the venture capital model is faltering.

Revitalizing the industry requires new business models to enable Open Innovation. What does this mean and how can it be done? During this talk we will give you an in depth insight in the challenges and the responses to this changing landscape in the industry.

Jerry Snoeks

Jerry has been with J&J for almost 25 years, currently as Director Operations & Process Quality within Drug Safety Sciences (DSSc). Main responsibilities focus on general operational management and GLP compliance. Next to that he leads the Beerse DSSc external innovation project “Collabres”, which now became a global cross-divisional initiative. Before that he was 10+ years responsible for strategic alliances with external service providers within Clinical Development. Jerry holds a BSc Clinical Chemistry, Ing. Biochemistry and MSc Information Management.

Veronique De Vroey

Veronique joined the company 14 years ago and is Director Screening Lab Operations within JnJ’s Community of Research excellence and Advanced Technologies (C.R.E.A.Te) . Main responsibilities focus on Operational Management for the discovery research projects and she is also involved in External Relationships. Veronique is Bio-Engineer in Cell& Gene Technology and graduated at the Katholic University of Leuven (KUL) in Belgium.

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1. IDENTIFICATION OF GENES IN THE BRAIN INVOLVED IN FEMALE SEXUAL FUNCTION USING EUMAMA

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The central serotonin (5-HT) system plays a key role in regulating female sexual behavior. Circuitries involving specific hypothalamic nuclei and cortical brain areas are hypothesized to form the neural substrate of female sexual function. Sexual receptivity in female rodents is suppressed by activation of 5-HT_{1A} and inhibition of 5-HT₃ receptors, but facilitated by 5-HT_{2A/C} receptor activation. Administration of the 5-HT_{1A} agonist *R*-(+)-8-hydroxy-2-(di-*n*-propylamino)tetralin (8-OH-DPAT) attenuates sexual receptivity. In a non-human primate model of female sexual dysfunction, we found that 8-OH-DPAT administered daily for 5-6 weeks decreases female sexual receptivity and increases aggressive interactions between male-female common marmoset (*Callithrix jacchus*) pairmates.

Here, we aim to reveal transcriptomic mechanisms that induce the observed behavioral alterations using a marmoset-specific DNA microarray (EUMAMA). Eight female marmosets were ovariectomized and implanted with estradiol-filled capsules. Before the animals were sacrificed and the brains prepared for expression analysis, the females were daily injected over 17 weeks with 0.1 mg/kg 8-OH-DPAT, or vehicle. Coronal brain sections were obtained by cryosectioning, and the medial prefrontal cortex (mPFC), medial preoptic area (mPOA), CA1 area of the hippocampus (CA1) and dorsal raphé nucleus (DRN) were excised by laser microdissection. RNA was isolated, amplified and hybridized to the EUMAMA array.

Chronic 8-OH-DPAT affected gene expression the strongest in the DRN, where 9% ($p < 0.05$) of the genes present on the EUMAMA were differentially regulated, followed by the mPOA (5%), mPFC (4%) and CA1 (3%). While the majority of differentially expressed genes was downregulated in the postsynaptic projection areas (mPFC 71%, mPOA 64%, CA1 59%), this was not the case in the presynaptic DRN (48%). Gene expression changes were subtle, with only 2% (CA1) – 12% (DRN) of differentially expressed genes showing fold changes larger than 2. The majority of expression changes was below 1.5-fold, ranging from 50% in DRN to 73% in CA1. Analysis of gene clusters will elucidate the key genomic processes through which 8-OH-DPAT affects female sexual function.

This work was supported by a collaborative grant with Boehringer-Ingelheim and grants from the Netherlands Organization for Scientific Research (NWO) (836.06.010; MEERVOUD to N.A. Datson) and TI Pharma T5-209. ERdK was supported by the Royal Netherlands Academy of Science.

2. INTEGRIN RECEPTORS IN CELLULAR MECHANOTRANSDUCTION

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Chemical and physical cues from the extracellular matrix (ECM) are used as input for an integrated mechanochemical sensory system that controls cell behavior. Integrin transmembrane receptors connect the ECM to intracellular cytoskeletal network. Integrins are expected to play an important role in cellular responses to forces derived from the ECM that have been implicated in stem cell differentiation as well as cancer progression. Vice versa, these receptors mediate ECM remodeling in response to cytoskeletal dynamics, which is critical for cell motility. In my project, such bidirectional force transmission will be studied for various integrins using designed 2D and 3D ECM substrates and the previously developed genetic models. The aim will be to elucidate how switching between different integrins alter mechanocoupling between ECM and cytoskeleton.

3. ADJUVANT EFFECT OF CATIONIC LIPOSOMES: MORE THAN CHARGE ALONE?

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Cationic liposomes are known as potent adjuvants for antigen delivery. The purpose of this study is to understand if their adjuvanticity is based on the positive charge alone, or if the chemistry of the positive headgroup is also important.

Several cationic liposomes formulations, containing one cationic compound (DDA, DPTAP, DC-Chol or DPePC) and a neutral lipid (DPPC), were prepared at two molar ratios (10 and 50 mol% cationic compound). The liposomes were loaded with influenza hemagglutinin (HA) and characterized with dynamic light scattering, laser doppler velocimetry and differential scanning calorimetry (DSC).

The adjuvanticity of the cationic liposomes (loaded with 0.5 and 2µg HA/dose) was tested *in vivo* in a mice model (immunized twice). The mouse sera were analyzed for HA-specific antibodies by ELISA and hemagglutination inhibition (HI) assay.

The obtained liposomes were similar in size (about 200 nm), charge (+50 mV) and membrane fluidity (melting temperature about 40°C, except for the 50% DC-Chol liposomes which did not show any phase transition). The immunization study showed that the 50% DC-Chol liposomes elicited significantly higher total IgG and IgG1 titers compared to the other liposomal and non-adjuvanted HA group. A similar trend, although not statistically significant, was observed for the IgG2a titers and the HI titers.

These results show that the adjuvanticity of cationic liposomes not only depends on the charge but also on the physicochemical properties of the charged compound. Nevertheless the significant difference observed with 50% DC-Chol liposomes raises another question: is it an effect of the DC-Chol head group or because of the membrane fluidity? The effect of bilayer fluidity on the immunogenicity of cationic liposomes is currently under investigation.

4. STRUCTURAL RATIONALIZATION OF REGIO-SELECTIVE STEROID METABOLISM BY CYTOCHROME P450 BM3 MUTANTS

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Computational modeling and simulation methods have proven to be a useful tool to rationalize typical shifts in metabolism patterns between mutant enzymes (e.g. *Proteins* 2008,71:336-52). Cytochrome P450 BM3 is a well-studied, soluble, fatty acid hydroxylase from *Bacillus megaterium*, and is a very promising biocatalyst due to its high catalytic activity and stability.

In the present study, the structural basis for shifting patterns in Cytochrome P450 BM3 regio-selectivity in testosterone (TES) and nordione (NOR) metabolism was investigated, by combining molecular docking studies and molecular dynamics (MD) simulations. A plasticity model is obtained, from which regioselectivity can be understood by taking the flexibility of the protein into account.

The computational findings, supported by the experimental results, made it possible to rationalize the altered mutant selectivity towards steroids. In accordance with experiment, several independent docking studies involving BM3 mutants showed new insights into the orientation of TES and NOR within the active site. It was found that in the binding cavity of the wild type enzyme testosterone can more easily adopt orientations corresponding with formation of multiple hydroxylated metabolites. In contrast, BM3 with additional mutations in the cavity can hydroxylate TES with a higher regio-selectivity. The structurally similar compound NOR does not show such a pattern, but shows only one hydroxyl metabolite for all mutants. These new insights into the effect of the active site's topology on substrate selectivity will open up new possibilities for future engineering and design of selective drug metabolizing P450 BM3 biocatalysts.

5. RELEVANCE OF QT-RR CORRELATIONS IN THE ASSESSMENT OF QTc-INTERVAL PROLONGATION IN CLINICAL TRIAL SIMULATIONS

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Correction for changes in heart rate is a fundamental step to the evaluation of QTc-interval prolongation. Yet, clinical trial simulations for thorough QT (TQT) studies often rely on approximations to evaluate design factors such as group size. The aim of the investigation was to develop a model-based approach to describe the correlation between QT and RR intervals in healthy volunteers.

A large pool (339 males and 437 females) of healthy volunteer ECG data has been used for the analysis. Data was split into two subsets to allow for the external validation of the final model. The analysis was performed using a non-linear mixed effects approach using NONMEM VI. Model building selection was based on changes in the objective function (OFV) and goodness of fit plots (GOF). Model validation has been carried out internally (simulations and NPDE) and externally (GOF and NPDE).

Among the different functions used in the evaluation of the QT-RR correlation, a parabolic function allowed the best model performance. Age and gender were the only available covariates; gender has been found to be significant both on slope [181(13.5) females and 166(12.8) males] and exponent [0.85(0.13) females; 0.74(0.13) males]. Inter-occasion variability on slope and exponent was also identified as a significant random effect. Distributions of simulated and real QT values were comparable. Goodness of fit plots clearly showed the ability of the model to predict data from a different subset of studies. Parameter estimates were subsequently used as part of a thorough QT study simulation.

The final model allows a reliable and realistic simulation of QT-interval profiles starting from a physiological set of RR values. In the context of clinical trial simulations, the availability of such a model represents a concrete improvement in the evaluation of drug-induced QTc-interval prolongation.

6. SYSTEMS TOXICOLOGY APPROACH TO UNRAVEL IMMUNE RESPONSE-MEDIATED NEPHROTOXICITY

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The kidneys and liver are the two major target organs of drug-induced toxicity. In both organs, inflammation plays an important role in the enhancement of the primary injury caused by the drugs. The proinflammatory cytokine Tumor necrosis factor alpha (TNF- α) is one of the main cytokines involved in the inflammatory processes in these organs and activates mainly the NF- κ B pathway.

Here we have shown that exposure to TNF- α enhances the cell death induced by cisplatin in the mouse kidney cells IM-PTECs (immortalized proximal tubular epithelial cells) and induced by diclofenac in the human liver HepG2 cells (Hepatoma G2 cells).

We are using a systems toxicology approach to unravel mechanisms and signalling pathways via which TNF- α enhances drug toxicity. This approach consists in three complementary studies: a screen for nephrotoxic compounds that show synergism with TNF- α , comparison of gene expression profiles of these compounds and a siRNA functional genomic screen for NF- κ B translocation.

In conclusion, TNF- α enhanced cisplatin-induced nephrotoxicity and diclofenac-induced hepatotoxicity via interfering with NF- κ B activation. The synergistic effect of TNF- α was associated with altering both NF- κ B translocation and gene transcription. We also observed with other nephrotoxic compounds a synergistic effect with TNF- α but the mechanisms responsible have to be elucidated. siRNA screening can be used to identify downstream pathways of TNF- α enhanced drug toxicity.

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Introduced 50 years ago, nicotinic acid is still the best drug for increasing HDL cholesterol in parallel to reductions in (V)LDL cholesterol and plasma triglycerides. However, a high dose of 1-2 g/day is needed, and the side effects of nicotinic acid severely limit patient compliance. Nicotinic acid-mediated lipid modification and the most common side effect, skin flushing, are both caused by the direct activation of the recently identified G protein-coupled receptor HCA₂.

We have now explored a new class of HCA₂ ligands, the pyrazolopyrimidines, which we synthesized in our laboratory (figure 1). Interestingly, these compounds bind to the receptor at a site that is different from the nicotinic acid binding site, and thus are allosteric modulators for HCA₂. The pyrazolopyrimidines (partially) activate the receptor on their own, but they can also increase the potency of nicotinic acid on HCA₂ by binding simultaneously. Therefore, the nicotinic acid dose could be lowered up to 10 times if it were given as a combination therapy with an allosteric modulator.

Excitingly, the allosteric modulators also increase the potency of endogenous HCA₂ ligand 3-hydroxybutyrate (3-OHB). Administration of a pyrazolopyrimidine alone may already increase receptor activation by the natural ligand sufficiently to induce healthy changes in circulating lipids. In this case, the receptor reacts to the natural fluctuations in 3-OHB concentration, so the side-effects are expected to be minimal.

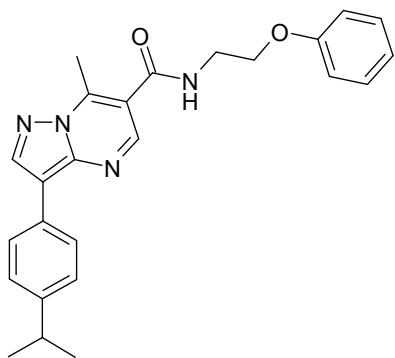


Figure 1. Example of an allosteric modulator for HCA₂

8. DEVELOPMENT OF A GENERIC METHOD FOR THE PREPARATION AND IDENTIFICATION OF DRUG-PROTEIN ADDUCTS USING P450 BM3 MUTANTS

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Life-threatening idiosyncratic drug reactions still constitute a major problem for the pharmaceutical industry. Covalent modification of proteins by chemically reactive metabolites is presumably involved in the etiology of most of these reactions. In this regard, it is important to study the structure and properties of protein adducts. In addition, there is a need for reference adducts to confirm observations from *in vivo* experiments.

The aim of this research is the development of a potentially generic method for the production and identification of protein adducts. Highly active P450 BM3 mutants were used for bioactivation of known hepatotoxicants. The model protein Human Glutathione-S-Transferase P1 (hGSTP1-1) was used to trap the formed reactive metabolites. Using affinity chromatography, the (modified) target protein could be separated from the biocatalyst. Covalent modification was established by mass spectrometry. Protein adducts were identified first by searching for peptides modified by known reactive metabolites of the drugs studied. Based on the observed adducts, a number of key MS/MS ions were identified for each of the targeted peptides. These diagnostic ions were then used to screen for protein adducts with unanticipated mass shifts. Using this methodology, novel drug adducts of troglitazone to hGSTP1-1 were identified.

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The purpose of this project is to determine the carcinogenic potential of several insulin analogues that are currently used as therapeutics by diabetic patients. Several epidemiological studies in Germany and Sweden have suggested a link between the use of some insulin analogues and cancer incidence in diabetic patients. The current hypothesis is that this carcinogenic potential of these insulin analogues is caused by an increased affinity for the insulin like growth factor 1 receptor (IGF1R).

In vitro exposure experiments with several insulin analogues will be performed in human breast cancer cell lines (MCF-7) that either over express the insulin receptor (with a knockdown of the IGF1R) or the insulin like growth factor 1 receptor (with a knockdown of the insulin receptor). By measuring the activation of downstream signalling cascades we hope to get a better understanding of the mechanisms behind insulin (analogue) signalling.

In vivo exposure experiments will be performed in a **p53^{R270H/+}WAPCre** mouse model that will develop (spontaneously) within 80 weeks human-like (Li Fraumeni) breast tumours. The hypothesis is that exposure to insulin analogues will decrease the time for tumour development.

Affymetrix chips will be used for a high throughput gene expression analysis on the *in vitro* and *in vivo* tissue. Hopefully genesets can be determined to distinguish between insulin receptor signalling and IGF1R signalling. By combining the *in vitro* with the *in vivo* data we hope to be able to make predictions about carcinogenic potential of insulin analogues based on *in vitro* data, this might lead to a decrease in the number of laboratory animals used in carcinogenicity studies in the future.

10. USE OF STABLE ISOTOPE METABOLIC TRACER TO MEASURE DYNAMIC BILE ACID RECONJUGATION *IN VITRO* AND *IN VIVO* BY UPLC/TOF-MS

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The purpose of this study was to evaluate the use of D₄-cholic acid (D₄-CA) as a metabolic tracer to measure the metabolism and reconjugation fate of bile acids *in vitro* and *in vivo*.

Stable isotope tracers are becoming an increasingly important research tool for many disease areas as it enables the measurement of small but significant biological changes which may not be apparent when monitoring static endogenous concentrations of particular metabolites. One such tracer, D₄-cholic acid (D₄-CA), has been used for measuring bile acid (BA) metabolism in humans and in cell-based systems. Slc27a5, also known as fatty acid transport protein 5 (FATP5), is the hepatic BA-CoA ligase that re-conjugates BAs during enterohepatic BA recycling and is exclusively expressed in the liver.

Here, we show the utility of using D₄-CA as a tracer for the re-conjugation of BAs *in vitro* and *in vivo*, and describe a method for high chromatographic and mass spectral resolution in the detection and quantification of D₄-BA metabolites and endogenous BAs. Using Slc27a5-cKD mice, we showed a significant reduction in the re-conjugation of D₄-CA to D₄-Taurocholic acid (D₄-TCA), as well as other conjugated BA metabolites, in plasma (p= 0.0031). Further conjugation derived from D₄-CA, such as taurotetrahydroxy cholanoil, were also reduced in the Slc27a5-cKD mouse plasma. The UPLC/TOF-MS method described allowed a rapid measure of many D₄ and endogenous BA alterations. Analysis of the bile resulted in the detection of 39 different BA metabolites from a 13 min analytical run, which is at least two-fold faster than current published protocols.

Finally, these data demonstrate the utility of D₄-CA as an *in vitro* and *in vivo* tracer to gauge BA reconjugation and describe a novel high resolution UPLC/TOF-MS method of quantifying BA levels in various biological fluids.

11. CHARACTERISATION OF ADULT RATS WITH A HISTORY OF NEONATAL GLUCOCORTICOID EXPOSURE: IMPACT OF EARLY HANDLING

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Synthetic glucocorticoids such as dexamethasone (dex) are the common treatment for prematurity-associated morbidity such as bronchopulmonary dysplasia. Despite the short term benefit on lung development, both human and animal studies have reported adverse effects of dex treatment on neurodevelopment, suggesting that the benefits might not outweigh the adverse effects.

Previous studies in our laboratory, investigating the long-lasting effects of neonatal dex treatment in rats, have revealed relatively mild effects. However, our within-litter design required daily marking of the neonates from postnatal day (pnd) 1-21, for discrimination between littermates. This resulted in a substantial amount of neonatal handling, which is known to induce pronounced and long-lasting phenotypic alterations that could potentially overrule the dex-induced alterations. To investigate the use of handling as an intervention strategy to rescue dex-induced alterations, we compared the effects of dex on adult phenotype in a handling vs non-handling context. Rat pups were injected with tapering doses of dex or saline (sal) on pnd 1, 2 and 3. Half of the animals were daily handled for 15 min on pnd 1-21, whereas the other half was not handled.

We report that for startle reactivity the effects of handling and dex treatment work in the same direction meaning that they reduce reactivity. However, in other paradigms we observed interaction effects. Spatial learning is affected by dex treatment, which can be fully restored by handling, whereas for sensory motor gating and endocrine stress responsiveness we observed that dex treatment decreases the sensitivity for handling. Additionally, the freezing response to a foot shock is solely affected by handling without effects of drug treatment. We conclude that neonatal exposure to glucocorticoids and handling interacts in shaping some aspects of the behavioural and endocrine phenotype of the animal in adulthood. The support by EU LifeSpan is gratefully acknowledged.

12. MATURATION OF GFR IN PRETERM AND TERM NEONATES REFLECTED BY CLEARANCE OF DIFFERENT ANTIBIOTICS

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Objective:

Throughout infancy, renal function matures resulting in differences in glomerular filtration rate (GFR) at different stages of development. These developmental changes in GFR were previously quantified in (pre)term neonates aged up to 1 month on the basis of the clearance of amikacin. In this developmental renal excretion model^[1], the maturation of GFR was predicted by birth weight (BWb) and postnatal age (PNA).

The aim of this study is to assess model performance when this developmental renal excretion model^[1] is used to describe maturation in clearance of other renally excreted antibiotics in (pre)term neonates. Using this approach a distinction is being made between system specific and drug specific information in paediatric pharmacokinetic models.

Methods:

For the netilmicin dataset, 386 netilmicin concentrations were available from 97 (pre)term neonates (BWb 470-3000 g, PNA 1-30 days)^[2]. The vancomycin dataset contained 752 vancomycin concentrations from 273 preterm neonates (BWb 385-2550 g, PNA 1-30 days)^[3].

A pharmacokinetic model was developed for both netilmicin or vancomycin using the developmental renal excretion model for amikacin clearance in neonates (1):

$$CL_i = CL_p * \left\{ \left(\left(\frac{BWb}{BWb_{median}} \right)^{1.34} \right) * \left(1 + 0.213 * \left(\frac{PNA}{PNA_{median}} \right) \right) \right\}$$

Using this approach, CL_p is considered a drug specific property and was therefore estimated for each of the drugs separately. The remaining information in this equation is considered system specific information which can be applied for all renally excreted drugs.

The descriptive and predictive performance of models developed using the developmental renal excretion model^[1] were compared with comprehensive covariate models^[4] for netilmicin or vancomycin respectively, by evaluation of the objective function (OFV), basic goodness-of-fit plots, NPDE and the individual and population parameter estimates versus most predictive covariate^[4].

Results:

The descriptive and predictive properties of the models developed using the developmental renal excretion model, were similar compared to the comprehensive covariate models for basic goodness-of-fit plots and NPDE. In agreement the models that were developed using the developmental renal excretion model, in the comprehensive covariate models BWb and PNA were identified as most predictive covariates for clearance. The comprehensive covariate models had only a slightly lower objective function (netilmicin $p < 0.05$, vancomycin $p < 0.001$) compared to the models using the developmental renal excretion model.

Conclusion:

Use of the developmental renal excretion model quantifying maturation in GFR mediated amikacin clearance for the analysis of netilmicin and vancomycin clearance in neonates, results in adequate descriptive and predictive performance. We conclude that the application of system specific information may lead to optimization of sparse data analysis in children.

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13. THE THREE-HIT HYPOTHESIS OF PSYCHOPATHOLOGY: SCHIZOPHRENIA ENDOPHENOTYPES AND ENHANCED STRESS REACTIVITY CO-PRECIPIRATE FOLLOWING ADVERSE LIFE EVENTS IN GENETICALLY SUSCEPTIBLE RATS

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Purpose:

Schizophrenia is a complex mental disorder driven by genetic and environmental risk factors. This study sought to test, in rodents, the "three-hit hypothesis" of schizophrenia by examining the interaction between predisposing genes (G), adverse early-life experience (ELE), and exposure to a stressful environment in adolescence (AE).

Methods:

G: we used the pharmaco-genetically selected apomorphine susceptible (APOSUS) line, which is characterized by schizophrenia-like phenotypes.

ELE: poor mother-pup interaction (i.e. low licking & grooming) was used as a marker of early-life adversity causing epigenetically programmed stress hyper-responsiveness.

AE: post-weaning isolation rearing was used as unfavourable environment for late brain maturation, a condition known to produce sensorimotor gating deficits.

Phenotypes: Animals were tested on apomorphine-induced gnawing for dopamine sensitivity, pre-pulse inhibition (PPI) of acoustic startle for sensorimotor-gating, spontaneous alternation for working memory and conditioned emotional response for ACTH, prolactin and corticosterone (CORT) stress-reactivity.

Results:

1. APOSUS individuals are, in contrast to wildtype controls, resistant to exogenous CORT. APOSUS, apart from their high gnawing behaviour, display enhanced working memory and, after contextual fear-conditioning, increased freezing behaviour and release of ACTH and prolactin, but not of CORT.
2. Adult APOSUS rats having experienced as pups poor maternal care develop a baseline PPI-deficit and show enhanced stress-induced CORT secretion together with a dramatic release of ACTH.
3. Additional isolation rearing abolished baseline PPI in the low maternal care APOSUS offspring. Also, their working memory is impaired and CORT reactivity is still enhanced.

Mechanism:

The enhanced stress reactivity found in the adult APOSUS animals exposed to early-life adversity is already present from the first week of life and corresponds to priming of the amygdala circuitry. The analysis of aberrant glucocorticoid responsive genes in this pathway will reveal a novel susceptibility drug targets for psychosis.

Conclusion:

Our data support the three-hit hypothesis of psychopathology: early-life adversity enhances vulnerability of the genetically predisposed individuals to a later psycho-social stressor resulting in a severe schizophrenia-like phenotype.

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14. DISCOVERY OF NOVEL CXCR4 AND CXCR7 SIGNALING PARTNERS BY REVERSE PHASE PROTEIN ARRAY (RPPA)

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Chemokine receptors belong to the family of G protein coupled receptors. They play a key role in the regulation of the immune system, but appear also to be implicated in inflammatory diseases and tumor growth and metastasis. The role of CXCR4-CXCL12 axis in cancer has been extensively studied and is well established. In 2005, CXCR7 was identified as a new chemokine receptor. Initial studies showed that increased expression of CXCR7 was associated with enhanced tumor formation. Moreover, a CXCR7 inhibitor effectively inhibits *in vivo* cancer development in mice. Besides, CXCR7 is present in tumor samples from breast, prostate and lung cancer patients.

Despite the clear promise of CXCR7 as new anti-cancer target, little information is available on the biochemical signalling pathway(s) activated by this receptor that explain the oncogenic properties of CXCR7. Unlike other chemokine receptors, this receptor shows no coupling to Gai protein. We have found that CXCR7 induces β -arrestin recruitment, a non G protein signalling pathway. Moreover, CXCR7 binds chemokines CXCL12 and CXCL11, known to bind respectively to CXCR4 and CXCR3.

In collaboration with Ram and colleagues (MD Anderson Cancer Center, Texas USA) we have performed a reverse phase protein array (RPPA) on cancer cell lines expressing both CXCR4 and CXCR7 or CXCR7 alone. We determined the expression of 177 cancer related proteins upon a time course stimulation with CXCL12. Several (phosphorylated) proteins were either up- or downregulated by CXCL12 stimulation. Moreover, small molecule inhibitors targeting CXCR4 or CXCR7 were used to determine which receptor is responsible for the observed changes. This data is used to create a model that shows which signalling networks are activated by either chemokine receptor.

15. PHENOTYPE IDENTIFICATION OF CELLULAR TUBULOGENESIS BY HIGH THROUGHPUT SCREENING AND 3D MULTIPARAMETRIC IMAGING

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European legislation will inevitably restrict the use of animal testing and so effective alternatives will be required by the pharmaceutical industry and research laboratories. Compared to conventional 2D assays, 3D in vitro cell culture models offer a more physiologically relevant context in which to investigate the effects of xenobiotics on biological systems and will most likely play a significant role in future in vitro testing. We are developing high throughput high content imaging-based 3D assays for nephrotoxicity and hepatotoxicity testing. These utilize well-differentiated multicellular renal and hepatic organoids (cultured tissues) which express fluorescent stress-reporter genes and develop ductal epithelial characteristics under the regulation of reproductive hormones. Key goals will be the identification of toxins that influence these phenotypic changes.

In order to effectively capture the 3D information in high throughput format - involving many thousands of images per 96-well or 384-well plate, complex image stacks are collected by epifluorescence microscopy. This allows faster imaging than confocal microscopy but the disadvantage is that out-of-focus information is also captured, which appears as "blur", presenting a challenge for 3D reconstructions. A new fully automatic image process method - Depth of Field Clustering (EDFS) - was developed to define the region of interest (ROI) while eliminating the out-of-focus parts in each image slice. Subsequently, 2D ROI contour refinement by active contour and 3D labelling were implemented for 3D reconstruction. The obtained 3D reconstructions were compared with a benchmark - 3D ROI's obtained from confocal microscopy by manual segmentation and labelling. No significant difference is obtained from the comparison which indicates the high accuracy of our 3D reconstruction method for images derived from epifluorescence microscopy.

To quantify the phenotype of the cultured tissues, a list of 3D morphologic parameters was defined and measured from the 3D structure. We applied 3 multi-parametric tests to identify drug induced toxicity and compared these tests with each other as well as with single parametric tests. We found that the Wilks Lambda test gave the best performance among 3 tests, significantly increasing the sensitivity compared to single parameter analyses.

16. ROLE OF HUMAN GLUTATHIONE S-TRANSFERASES ON THE INACTIVATION OF DICLOFENAC REACTIVE METABOLITES

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Conjugation of reactive intermediates (RIs) to glutathione (GSH) is considered an important detoxification mechanism that can be spontaneous and/or mediated by glutathione S-transferases (GSTs). Given this role and the high abundance of GSTs in the liver and other tissues, genetically determined deficiencies (polymorphisms) in GSTs may be risk factor for adverse drug reactions related to the formation of reactive drug metabolites.

While the exact mechanism remains unknown, idiosyncratic adverse drug reactions due to the anti-inflammatory drug diclofenac have been proposed to be caused by the generation of reactive metabolites. Oxidative metabolism of diclofenac in the liver by cytochromes P450 forms hydroxy diclofenac metabolites, the further oxidation of which generates electrophilic quinone imines. The reactive quinone imines can be detoxified via conjugation to GSH either spontaneously or enzymatically via GSTs. While previous studies have identified GSH conjugates of diclofenac, no research has focused on the roles of GSTs in the conjugation of GSH with diclofenac RIs.

A single bacterial P450 mutant was selected for bioactivation reactions and the effects of four recombinant human GSTs, hGST A1-1, hGST M1-1, hGST P1-1, and hGST T1-1, on the formation of GSH conjugates of diclofenac were studied. Addition of hGST A1-1, hGST M1-1, and hGST P1-1 increased total GSH conjugation, with hGST M1-1 showing the highest activity and hGST A1-1 the lowest. hGST P1-1 showed the different conjugate profile with highest activity towards the conjugates formed from hydroxy metabolite 5-OH diclofenac. While hGST T1-1 generated fewer GSH conjugates than observed in chemical conjugation alone, a novel primary conjugate of a hydroxy diclofenac metabolite was identified.

hGSTs may thus play an important role in the inactivation of diclofenac quinone imines, and further investigation is warranted to confirm if polymorphisms of these enzymes contribute to idiosyncratic diclofenac liver injury.

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Traditionally, hepatotoxic properties of compounds are tested using animal studies. Disadvantages of *in vivo* studies are that large numbers of animals are required, that the exposures may affect animal welfare, that such studies are not fully predictive for effects in humans, and that they are associated with high cost. Alternative systems for hepatotoxicity are currently developed to accommodate these ethical, scientific and economical drawbacks. One of the main purposes of this project is to validate the zebrafish embryo (ZFE), which is considered as a non-licensed animal model until 5 days post fertilization (dpf) as an alternative testing model for hepatotoxicity. For this purpose, ZFEs (and adult zebrafish for comparison) were exposed to 12 model hepatotoxicants/controls, including those inducing three defined phenotypic endpoints, necrosis, steatosis, and cholestasis. Histopathology revealed compound-specific effects in a dose-dependent manner in ZFE liver. For analysis of genomic markers of hepatotoxicity in this model, the first step was to optimize toxicogenomic analysis comparing adult zebrafish liver (aZFL) and the whole ZFE using deep sequence technology. Genes related to hepatotoxic phenotypes were equally expressed in both adult zebrafish liver and whole ZFE, whereas genes related to development showed higher expression in the ZFE. These results suggest that the ZFE model has potential for assessment of hepatotoxicity. For further validation, toxicogenomic profiles will be generated using micro arrays in ZFE which will be compared to profiles obtained in other alternative models, and to adult zebrafish, mice and rats *in vivo*.

18. DEVELOPMENT OF HUMAN RECONSTRUCTED ATOPIC ECZEMA SKIN MODELS AS A TOOL FOR SCREENING PURPOSES

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Atopic Eczema (AE) is a common skin disease, especially in industrialized countries, that affects mostly children. Currently, the occurrence of AE in children is approximately 20% and its prevalence is rapidly increasing. AE is clinically characterized by erythema and severe itching, which has enormous impact on the quality of life of the patients as well as their relatives. In 50% of the patients, presence of AE leads to development of asthma or allergic rhinitis. AE is characterized by an impaired barrier function and an inflammatory immune response. A defective barrier allows antigens to penetrate the skin more easily, thereby inducing the activation of the immune system. This emphasizes the important role of the barrier function of the skin of AE patients, especially in the onset of the disease. An important protein involved in the barrier formation and maintenance is filaggrin. Recent research has shown that 50% of the AE patients have loss of function mutations in the filaggrin gene.

So far, no representative AE models are present, which hampers the development of novel therapies for AE. The purpose of this project is to generate and fully characterise a novel human skin equivalent (HSE) that closely mimics the properties of AE skin. This model will be used to examine in detail AE features and for screening of newly developed drugs for AE treatment.

To mimic AE and to investigate the barrier function in AE, two approaches will be applied to generate an AE-HSE. First, an AE-HSE will be established by culturing AE-derived biopsies onto fibroblast-populated collagen matrices. Second, an AE-HSE will be established by genetic modification of the filaggrin gene in keratinocytes. Subsequently, these models will be used to assess various barrier properties of AE skin, including epidermal morphogenesis and filaggrin expression. In addition, the effect of supplementation of various cytokines (cytokine data obtained from *in vivo* AE studies), will be evaluated to determine the involvement of the immune system in the disease. Finally, the optimal AE-HSE that mimics the AE phenotype will be used to screen new formulations and drugs developed for AE treatment.

Generation of an AE-skin model that gives us better insight into biological processes of AE. Furthermore, this model will be used as tool to screen new formulations.

19. SUBSTRATE-DEPENDENT BIOSYNTHESIS OF TASTE AND FLAVOUR COMPOUNDS BY *SACCHAROMYCES CEREVISIAE* DURING INDUSTRIAL FERMENTATION

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Baker's yeast *Saccharomyces cerevisiae* is widely used in industrial biotechnology applications, ranging from production of biofuels and chemicals, to base products for the pharma industry, to ingredients for the food and feed industry. In the latter group of appliance, the production of yeast extracts from baker's yeast presents an important example. Those yeast extract products are generally being used for the manufacturing of sauces, soups and take away meals in order to generate a 'meaty' taste and to strengthen savoury flavour in general. This practice plays a significant role in reducing salt content in various food products, *i.e.* to prevent the development of high blood pressure.

The biosynthesis of taste and flavour compounds by yeast *Saccharomyces cerevisiae* presents an interesting and highly innovative platform for the comprehensive analysis of control and regulation of secondary metabolism, from both a fundamental and an applied science point of view. Substrate and growth conditions of the production process of yeast products determine the regulation of specific metabolite formation and hence, taste and flavour 'performance' of the yeast extract product. However, post-fermentation treatment of the yeast broth plays a crucial if not essential role in determining the final taste and flavour quality of the industrial yeast extract. Therefore, a thorough statistical analysis is required to understand the cause-effect relationship on the biosynthesis of taste and flavour key components in yeast.

This research project aims at understanding which and how yeast metabolites determine taste and flavour 'performance' of industrial yeast extracts, how this relates to the yeast physiology and/or the downstream autolysis treatment and, last but not least, unravelling the yeast intracellular molecular control mechanisms that govern the biosynthesis of these metabolites.

This is a joint research project between the Netherlands Metabolomics Centre (Leiden University & DSM) and the TU Delft/Kluyver Centre for Genomics of Industrial Fermentation.

20. INTERSPECIES DIFFERENCES IN MOXIFLOXACIN-INDUCED QTc-INTERVAL PROLONGATION

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Objectives:

Assessment of the propensity of non-antiarrhythmic drugs in prolonging QT/QTc-interval is critical for the progression of compounds into clinical development. Different animal models are used in pre clinical assays to assess QTc-interval prolongation liability. However, it's unclear how the QTc-interval changes in dogs and primates can be translated into accurate risk of QTc-interval prolongation in humans, as proposed by the ICH E-14 guidelines, *i.e.*, >10 msec. The aim of this investigation is to characterise interspecies differences in QTc-interval prolongation following administration of moxifloxacin to dogs, cynomolgus monkeys and healthy subjects.

Methods:

ECG and pharmacokinetic data from experiments in conscious beagle dogs, cynomolgus monkeys and clinical trials in healthy volunteers were evaluated. First, pharmacokinetic models were developed to obtain drug concentrations at the time of each QT-interval measurement. Data analysis was performed using a model-based approach which takes into account the concentration-effect relationship, translating drug effects in terms of the

probability of QTc-interval prolongation. NONMEM VII and WinNONLIN 4.1 were used for pharmacokinetic data analysis, whilst PKPD modelling was performed in WinBUGS v1.4.3.

Results:

Thanks to model parameterisation, drug-specific and systemic specific parameters could be estimated separately and the overall probability associated with QTc-interval prolongation >10msec compared across species. Measurement noise and feeding procedures are important sources of variability and as such affect parameter estimates in dogs and monkeys.

Conclusions:

The magnitude of the QTc-prolonging effect of moxifloxacin at peak concentrations seems to reflect species differences in sensitivity to hERG inhibition. The differences in the slope of the concentration-effect relationship across species suggest that monkeys are slightly more sensitive than dogs to drug effects.

21. FRAGMENT GROWING INDUCES CONFORMATIONAL CHANGES IN ACETYLCHOLINE-BINDING PROTEIN: A STRUCTURAL AND THERMODYNAMIC ANALYSIS

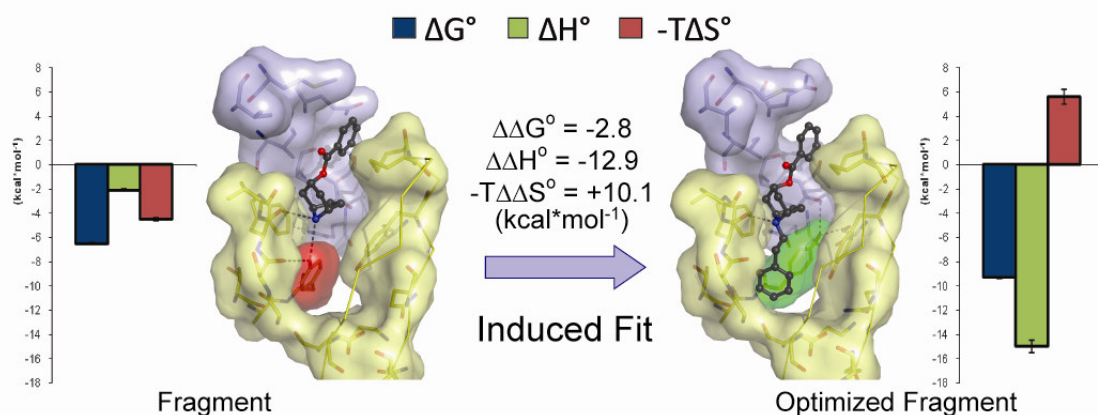
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Optimization of fragment hits towards high affinity lead compounds is a crucial aspect of fragment-based drug discovery (FBDD). In the current study, we have successfully optimized a fragment by growing into a ligand-inducible subpocket of the binding site of acetylcholine-binding protein (AChBP). This protein is a soluble homolog of the ligand binding domain (LBD) of Cys-loop receptors.¹ The fragment optimization was monitored with X-ray structures of ligand complexes and systematic thermodynamic analyses using surface plasmon resonance (SPR) biosensor analysis and isothermal titration calorimetry (ITC). Using site-directed mutagenesis and AChBP from different species, we find that specific changes in thermodynamic binding profiles, are indicative of interactions with the ligand-inducible subpocket of AChBP. This study illustrates that thermodynamic analysis provides valuable information on ligand binding modes and is complementary to affinity data when guiding rational structure- and fragment-based discovery approaches.²

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22. DEVELOPMENT OF HUMAN RECONSTRUCTED ATOPIC ECZEMA SKIN MODELS: MIMICKING ATOPIC ECZEMA INFLAMMATORY MICROENVIRONMENT *IN VITRO*

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Atopic eczema (AE) is the most common pruritic chronic inflammatory skin disease affecting 17% of European children¹. AE is characterised by a defective skin barrier and clinically manifested as dry skin, swellings, erythema and pruritic rash.

The barrier function of the skin is located in the uppermost layer of the skin i.e. stratum corneum (SC). The SC consists of protein-rich corneocytes embedded in an intercellular lipid matrix. Increased penetration of harmful/allergic substances due to a defective skin barrier in AD triggers a Th2 immune response. As a consequence, over-expression of Th2 cytokines IL-4, IL-13, IL-31 and secretion of TSLP by keratinocytes occurs in AE skin. The over-expression of Th2 cytokines results in reduced expression of an important epidermal differentiation protein, filaggrin². Filaggrin is involved in terminal differentiation of keratinocytes and formation of the skin barrier. Loss-of-function mutations in filaggrin have shown to be also present in 50% of AE patients. Its post-translational products: natural moisturizing factors (NMF) and *trans*-urocanic acid, maintain hydration levels and the pH of the SC respectively.

Progress in development of novel therapies have been hampered due to the lack of feasible and validated *in vitro* models that can be used for drug screening before animal testing. Therefore, the purpose of this project is to generate and fully characterise a novel human skin equivalent (HSE) that closely mimics properties of AE skin. The derived AE HSEs serve as a tool to examine in detail the features of AE skin and the screening of drugs for AE treatment.

We aim to mimic AE skin inflammatory microenvironment and in so doing, alter filaggrin expression and barrier properties to AE conditions. To this effect, healthy human epidermal skin equivalents were generated *in vitro* and the culture medium was supplemented with human recombinant IL-4, IL-13, IL-31 and TNF- α at concentrations of 15ng/ml and 30ng/ml.

Interleukin supplementation resulted in intercellular oedema/spongiosis and delayed K10 expression, an epidermal differentiation protein, in epidermal skin models as shown by immunohistochemical analysis. Spongiosis and delayed K10 expression are histological hallmarks of lesional AE skin. Therefore in conclusion, supplementation of epidermal skin models with interleukins alters epidermal morphology and protein expression to mimic some histological hallmarks of AE *in-vitro*.

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23. ON-LINE MODIFICATION, IDENTIFICATION AND BIOLOGICAL CHARACTERIZATION OF P38 KINASE INHIBITORS BY ELECTROCHEMICAL OXIDATION COUPLED TO A HYPHENATED SCREENING ASSAY.

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Protein kinases represent an important class of drug targets in modern drug discovery. p38 mitogen-activated protein kinase α (p38 α) is heavily involved in the cellular response to inflammatory stimuli. The development of technologies that facilitate the design and identification of kinase inhibitors can significantly contribute to the success-rate of lead discovery. Nowadays electrochemical oxidation is used to form oxidation products of drugs to support drug metabolite identification studies. In the present work we describe the integration of electrochemical oxidation with a hyphenated screening assay. In this way, lead compounds are modified by electrochemical oxidation while binding of the products to p38 α is assessed and chemical information is obtained by high resolution mass spectrometry. With the integration of formation, characterization and identification the sample handling steps are greatly reduced. The risk of sample loss, contamination or degradation is thereby significantly reduced while a comprehensive set of data on the products is obtained.

24. MONITORING THE AGGREGATION STATE OF THERAPEUTIC IgG AGGREGATES IN HUMAN SERUM AND PLASMA

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The purpose of this work was to monitor whether the aggregation state of stressed therapeutic monoclonal IgG changes once brought into contact with human serum or plasma.

A therapeutic human IgG was covalently labeled with Alexa Fluor 488 dye. Aggregates of unlabeled and labeled IgG were obtained by pH shift and heat stress. The effect of the label on the aggregation state of the IgG was tested by size exclusion chromatography (SEC). Unstressed and stressed labeled IgG formulations were diluted in formulation buffer, in human serum and in human plasma. The aggregates were analyzed with fluorescent activated cell sorting (FACS), confocal microscopy and single particle tracking (SPT) analysis.

SEC indicated that the Alexa 488 dye does not affect the aggregation state of the IgG formulations. FACS and confocal microscopy showed that the unstressed IgG does not aggregate in serum and plasma, and that the aggregates of both stressed formulations are still present after dilution in serum or plasma. SPT showed that the nanometer sized heat-induced aggregates do not change their average size once in contact with serum or plasma, but the pH shift-induced aggregates become smaller once in contact with either of these biological fluids.

Depending on the type of IgG aggregates, IgG aggregates can drastically change their characteristics once in contact with human serum and plasma.

25. INDUCTION OF REGRESSION OF ATHEROSCLEROSIS BY INTERRUPTION OF THE OX40-OX40L PATHWAY

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T cells are important players in atherosclerosis and are activated through antigen stimulation and engagement of costimulatory molecules such as OX40 (TNFRSF4) and OX40L (TNFSF4). The OX40-OX40L pathway is important for the proliferation and survival of T cells, provides B cell stimulation, and OX40L is associated with cardiovascular disease. Since most patients suffering from cardiovascular disease have established atherosclerotic lesions, we aimed to induce regression of atherosclerosis by a combination of anti-inflammatory treatment and cholesterol lowering.

LDLr^{-/-} mice were fed Western-type diet for 10 weeks, whereafter the mice received Chow diet to lower plasma cholesterol levels and were treated with anti-OX40L or PBS for 10 weeks. A significant 29- and 38% reduction in lesion size was observed in the aortic root and the aortic arch of the anti-OX40L treated mice, respectively. The interruption of the OX40-OX40L pathway reduced the Th2 response as shown by decreased GATA-3 and IL-4 levels. As a consequence IgG1 and IgE levels were decreased, which was reflected in a reduced mast cell presence and activation within the lesions. Interestingly, IL-5 production was increased by T cells, NK cells and B1 cells, and the atheroprotective oxLDL-specific IgM production by B1 cells was consequently increased. The increase in production of IL-5 and thus IgM was mediated by the production of IL-33 by antigen presenting cells upon OX40L blockade.

Interruption of the OX40-OX40L signaling pathway combined with cholesterol lowering induces regression of atherosclerosis through a reduction in mast cell activation and the induction of the atheroprotective anti-oxLDL IgM mediated by IL-5.

26. DICLOFENAC INHIBITS TNFA-INDUCED NF-κB NUCLEAR SHUTTLING CAUSING SYNERGISTIC HEPATOCYTE APOPTOSIS VIA CASPASE-8

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Drug-induced liver injuries (DILIs) are the major cause of drug failures and are often idiosyncratic in nature. We hypothesize that idiosyncratic DILI occurs due to crosstalk between drug reactive metabolite and cytokine stress signaling. To study this hypothesis, human hepatoma HepG2 cells were exposed to diclofenac, which causes idiosyncratic DILI in humans, in the presence of the pro-inflammatory cytokine TNFα. Diclofenac itself induced a mild concentration-dependent apoptosis of HepG2 cells. While TNFα itself was not cytotoxic, it strongly enhanced the diclofenac-induced apoptosis.

Using a siRNA screening approach and a live cell imaging of apoptosis technique, diclofenac/TNFα-induced apoptosis was identified as death-receptor dependent involving the intrinsic, mitochondrial death pathway. In addition, diclofenac itself caused sustained activation of the stress kinase JNK, and knock-down of this gene also resulted in an inhibition of the induced apoptosis. Under normal conditions these two pro-apoptotic signaling pathways that are activated down-stream of the TNF- receptor are controlled by the activation of the transcription factor NF-κB and the resulting gene transcription.

By using immunofluorescence staining of wild type HepG2 cells and live cell imaging of HepG2 cells expressing GFP-p65 we show that diclofenac causes a delay in the NF-κB oscillatory nuclear-to-cytosol translocation pattern in association with reduced NF-κB transcriptional activity. The anti-apoptotic role of p65 was evident since both inhibition of IKK as well as stable lentiviral shRNA-based knock down of p65 sensitized hepatocytes towards diclofenac/TNFα-induced cytotoxicity.

Together our data suggest a model whereby diclofenac-mediated stress signalling suppresses TNFα-induced survival signalling routes and sensitizes cells to apoptosis and consequently the onset of DILI. We anticipate that our work will enable us to identify mechanism-based biomarkers that can predict idiosyncratic-like DILI in a pre-clinical drug development setting.

27. STAPHYLOCOCCAL CELL WALL COMPONENTS AFFECT ATHEROSCLEROSIS DEVELOPMENT VIA TLR2

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Pattern-recognition receptors enable antigen-presenting cells (APCs) to recognize the *Staphylococcus aureus* (*S. aureus*) cell wall and mount an inflammatory response to this microbe. We (Frodermann et al., J Infect Dis 2011; in press) have recently shown that TLR2 signaling induced by the *S. aureus* cell wall can also result in a modulatory IL-10 response by human APCs.

We show here that this IL-10 response to *S. aureus* cell wall is conserved in APCs of C57BL/6 and LDLr^{-/-} mice *in vitro*. Moreover, we observed that pre-exposure to the *S. aureus* cell wall results in an increased IL-10 and a decreased IL-12 response by macrophage-derived foam cells. The *S. aureus* cell wall thus shifts APCs *in vitro* towards an anti-inflammatory phenotype.

To determine whether this effect is able to prevent atherosclerotic lesion formation *in vivo*, we administered i.p. injections of *S. aureus* cell wall to LDLr^{-/-} mice three days prior to Western-type diet (WTD, 0.25% cholesterol) start. We continued to administer *S. aureus* twice a week during eight weeks of WTD. In the first week, we saw a significant increase of IL-10 in the serum. After four weeks, we saw a significant increase of CD8⁺ T cells and a significant decrease of monocytes circulating in the blood. These results may indicate a beneficial effect of *S. aureus* on atherosclerotic lesion formation.

28. AUTOMATED IMAGING AND MULTIPARAMETRIC ANALYSIS OF TUMOR FOCI BURDEN IN ZF XENOTRANSPLANTATION MODEL

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An automated imaging and image analysis platform is developed for quantification of tumor foci burden in zebrafish embryos, xenotransplanted with mammalian cancer cell lines. Our results show that the image analysis algorithm has a high accuracy, predictability, reproducibility, and fast computational speed.

Development of such an automated platform represents a step toward high-throughput screening of zebrafish xenotransplanted embryos for identifying novel anti-cancer drug targets.

29. THE MINERALOCORTICOID RECEPTOR AT THE PLASMA MEMBRANE, EXAMINING SINGLE MOLECULES WITH TIRF MICROSCOPY

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In addition to their well-known function as transcription factor, many steroid receptors are now found to act also as initiators of rapid signaling in response to their steroid ligands. Importantly, for this latter function the receptors seem to localize at the outside of the plasma membrane, as rapid effects can also be initiated by membrane-impermeable conjugates of the ligands. For the mineralocorticoid receptor (MR), a rapid function was found in neurons of the hippocampus and amygdala. Here, the fast actions of corticosterone on glutamate release require the MR and these effects can be reproduced with BSA-conjugated corticosterone.

However, the absolute proof of membrane localization of the MR remains difficult as the bulk of the receptor population is located in the cytoplasm, directly bordering the membrane. One method to specifically image membrane proteins is TIRFM (total internal reflection fluorescence microscopy). With TIRFM an evanescent wave is created that penetrates only 100 nm into the sample. We combined this technique with real-time, single molecule imaging to study the kinetics of single molecules of MR bound to yellow fluorescent protein (YFP-MR) at the membrane. We expect that membrane-associated MR will have different kinetics than cytoplasmic located MR.

YFP-MR molecules are clearly detectable in TIRFM mode in CHO cells. We compared the kinetics of YFP-MR in TIRFM (mostly membrane) and in wide-field microscopy (cytoplasm). We found that the kinetics of YFP-MR were significantly slower near the membrane than in the cytoplasm, while this was not the case for a membrane-associated control (YFP-caax) and a cytoplasmic control (YFP-YFP). We suggest this shift in kinetics for YFP-MR is a clear indication for the existence of a population of membrane-associated MR.

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30. THE RELATIONSHIP BETWEEN BINDING KINETICS AND FUNCTIONAL EFFICACY OF ADENOSINE A_{2A} RECEPTOR AGONISTS

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The adenosine A_{2A} receptor (A_{2A}R) belongs to the superfamily of G protein-coupled receptors (GPCRs) and is a promising therapeutic target. Traditionally, the discovery of novel A_{2A}R agents started from assessments performed in equilibrium, largely ignoring the kinetic aspects of the ligand-receptor interaction. The aim of the study is to test the binding kinetics of A_{2A}R agonists and explore a possible relationship with their functional efficacy.

We used a radioligand competition association assay to determine the binding kinetics of unlabelled A_{2A}R ligands. Functional efficacies of these agonists were determined in a cAMP assay.

A correlation was observed between the dissociation rate of agonists (i.e. residence time) and their functional efficacy. In other words, the longer the agonist stayed on the receptor the higher the efficacy. Notably, the affinity of A_{2A}R agonists was not correlated to their functional efficacy.

This study shows that residence time of A_{2A}R agonists is linked to their efficacy unlike their affinity. Hence, more attention should be paid to drug-target residence time to improve the efficacy of new generations of A_{2A}R ligands.

31. DEVELOPMENT OF A MINIATURIZED FLUORESCENCE DETECTION SYSTEM FOR THE HYPHENATION OF NANO-LC TO ON-LINE BIOCHEMICAL ASSAYS

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In order to screen complex mixtures for bioactive compounds in natural samples, numerous systems have been developed where liquid chromatography (LC) is directly coupled to a continuous-flow bioassay and mass spectrometry. This allows for simultaneous bioaffinity profiling and compound elucidation. Usually, the bioassay consists of the target protein and a fluorescently labelled tracer ligand which are continuously mixed post-column with (possible) ligands eluting from the LC-column effluent. A fluorescence detector (FD) is then used for on-line bioassay readout.

A limitation of this on-line screening approach can be the relatively high consumption of expensive reagents and/or precious (natural) samples. Here, a methodology is presented where sample – and assay consumption is reduced by decreasing the overall flow rates of the screening setup.

In the chromatographic part of the system this is achieved by implementing nano-LC separations. The bioaffinity part of the system was miniaturized by utilizing a microfluidic chip with a 6 µL incubation chamber, where the continuous flow bioassay is mixed with the nano-LC effluent. Additionally, a miniaturized LED-induced fluorescence detection system was developed, capable of sensitively monitoring typical continuous “low-flow” bioassays.

The microfluidic screening setup was applied to a fluorescent enhancement assay based on the AcetylCholine Binding Protein (AChBP; analogue of the binding domain of the nicotinic acetylcholine receptor) with a fluorescent probe (DAHBA). The binding protein and the probe were infused together into the chip by a syringe pump at a flow rate of 5 µL/min, where they mixed continuously with the nano-LC effluent set at 500 nL/min. The system is currently applied for the screening of snake – and cone snail venom, which contain neurotoxic peptides with high affinity to the AChBP. These valuable compounds, when identified, can then be used for further pharmacological research to e.g. brain and muscle related nicotinic acetylcholine receptors and maybe as lead compounds for drug discovery as biopharmaceuticals.

32. ACUTE ADMINISTRATION OF CORTICOSTERONE REDUCES SEIZURE-INDUCED ARBORISATION OF DOUBLECORTIN EXPRESSING NEURONS IN HIPPOCAMPAL DENTATE GYRUS

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Status epilepticus (SE) causes an increase in adult neurogenesis in the dentate gyrus. Adult born neurons are able to form connections and integrate into the network. Neurons born after SE migrate ectopically and make aberrant connections, which may underlie emergence of recurrent seizures and cognitive impairment.

Stress is one of the most self-reported triggers for seizures. The correlation between stress and epilepsy however, is not well understood. Previously our lab has shown that the glucocorticoid receptor (GR), critically involved in the stress-system, controls the correct integration of the newborn neurons in the hippocampus. This leads to the hypothesis that stress-induced glucocorticoids, via activation of the GR might be involved in

aberrant integration of newborn cells after SE. By administration of high concentrations of corticosterone we have mimicked an acute stressor.

C57BL/6J mice were pre-treated with corticosterone 30 minutes before treatment with kainate (KA). Repeated doses KA were administered until SE occurred. After 3 days, brains were processed for immunohistochemistry while spatial memory was tested 5 weeks after SE in a second set of animals.

Newborn neurons are identified by the expression of doublecortin (DCX). After three days, we show an increase of DCX positive cells after kainate-induced SE, but if animals were pre-treated with corticosterone this increase was prevented. We observe a similar finding for dendritic complexity of newborn cells where corticosterone reduce the seizure-induced increase in dendritic arborisation. In our behavioural studies we did not observe a change in hippocampus-dependent learning and memory consolidation. However, we do see a significant change in learning strategy after KA administration that was exaggerated by corticosterone administration. Together, our data suggest that the GR is involved in seizure-induced aberrant integration of adult born neurons with consequences for hippocampal learning strategies.

33. POPULATION PHARMACOKINETICS OF MIDAZOLAM FROM PRETERM NEONATES TO ADULTS, A MATURATION MODEL

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A previous investigation showed major impact of critical illness on midazolam clearance, however no influence of age-related changes on the cytochrome P450 (CYP) 3A4/5 mediated clearance of midazolam was found in children between 1 month to 17 years of age.¹ In this analysis we aimed to develop a maturation model for CYP3A4/5 enzyme activity using midazolam clearance as in vivo probe, for preterm neonates from 26 weeks gestational age (GA) onwards to adults.

Pharmacokinetic data after intravenous midazolam were obtained from 6 previously reported studies. Subjects were 20 healthy male adults (20-31 years)², 18 pediatric oncology patients (3-17 years)³, 18 pediatric intensive care (a term) patients (2 days to 17 years)⁴, 23 children after elective major craniofacial surgery (3-23 months)⁵, 24 preterm neonates with respiratory distress syndrome (26-37 weeks GA and 0-1 days postnatal age (PNA))⁶ and 32 non-ventilated preterm neonates (26-33.5 weeks GA and 3-11 days PNA)⁷. Population PK modeling was performed using NONMEM v6.2. In a systematic covariate analysis, the influence of PNA, GA, postmenstrual age, body weight (BW) and PELOD score (organ failure) was investigated.

Upon inclusion of preterm neonate datasets, BW proved a significant covariate for clearance in a two-compartment model. The influence of BW was best described using an allometric equation with a BW-dependent maturational exponent (BWME): $BWME = Coeff_1 \times BW^{exp2}$, in which BW is body weight, BWME is the exponent for body weight in an allometric equation, $Coeff_1$ is the coefficient of the exponential function, $exp2$ is the additional exponent of the allometric function. It was shown that BWME gradually changed from 0.84 in preterm neonates to 0.44 in adults, with $Coeff_1$ of 0.8 (CV of 9.1%) and $exp2$ of -0.141 (CV of 32.1%). BW was also linearly correlated with midazolam central volume of distribution.

A maturation model for midazolam clearance from preterm neonates to adults has been developed showing that CYP3A4/5 activity matures in (preterm) neonates up to 5-10 kg of body weight. Thereafter, maturation slows down resulting in minimal increase between 10 and 81 kg of body weight.

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34. APPLICATION OF STOCHASTIC DIFFERENTIAL EQUATIONS TO DISEASE PROGRESSION IN A NEUROPATHIC PAIN MODEL IN RATS

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In nonlinear mixed-effects modelling the variability may be decomposed into an inter-individual, an inter-occasion and a residual component. Stochastic Differential Equations (SDE) can be used instead of Ordinary Differential Equations (ODE) to further decompose the residual variability into system and measurement noise. The former may reflect true physiological fluctuations in the biological system, whilst the latter encompasses measurement errors and other unexplained sources of variability.

The aim of this work was to evaluate the feasibility of using SDEs in a model of Neuropathic Pain in rats, as compared to the use of Ordinary Differential Equation.

Neuropathic Pain was induced in rats by Chronic Constrictive Injury (CCI), i.e. applying loose ligatures around the sciatic nerve. Paw Withdrawal Threshold (PWT), measured in grams, was used as a measure of response. For the current analysis only placebo data were available. PKPD models were built with both SDE and ODE. Subsequently, the models derived were used to perform simulation in order to compare performances of ODE and SDE. Several scenarios were also used to determine the impact of different sampling schedules on the predictive value of the models. Data analysis was performed in NONMEM v7. R was used for data manipulation, statistical and graphical summaries.

Despite the increased complexity, the use of SDEs does not seem to capture time dependent changes in CCI-induced allodynia. The results of the simulations suggest that limitations in the sampling scheme may contribute to the limited performance of SDEs.

Our findings demonstrate that SDEs are a valuable tool in modelling of disease progression. However, their usefulness in capturing time-dependent oscillations in system-specific parameters is very sensitive to sampling frequency.

35. PARALLEL *IN SILICO* APPROACHES IN THE QUEST FOR PARASITE-SELECTIVE DRUGS

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African sleeping sickness and Leishmaniasis are infectious diseases endemic to developing countries. Some treatment options have remained unchanged for over 60 years. Unfortunately this is not the result of success, so much as neglect, by the pharmaceutical industry. The treatments are expensive, some require iv infusion and may last weeks, furthermore those in treatment risk severe or even fatal side effects. Novel treatments are long overdue.

Phosphodiesterases (PDEs) were recently identified as a new target to tackle these neglected infectious diseases. High throughput screening provided a lead compound, which unfortunately has a higher affinity for human PDEs than for the parasite PDEs. Ligand based optimisation has been able to equalise the affinities, however achieving high selectivity for the parasite PDEs has proven elusive.

New approaches are now being tried to identify highly selective compounds targeting just the parasite PDEs. The *in silico* efforts in this regard involve both ligand based and structure based screening of large libraries, built-up of either fragments or larger drug like molecules. This “many nets in many oceans” approach should maximise the chance of success.

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The barrier function of the skin is provided by the stratum corneum (SC), the outermost layer of the skin. The SC mainly consists of dead cells (corneocytes) surrounded by an extracellular lipid matrix. The corneocytes are almost impermeable for compounds. As a consequence the transport of substances applied onto the skin is mainly directed along the intercellular lipid regions.

Ceramides (CERs), cholesterol (CHOL) and free fatty acids (FFAs) belong to the major lipid classes in SC and form highly ordered crystalline lipid lamellae which are crucial for a proper skin barrier function. The lipids in healthy human SC form two lamellar phases: the short periodicity phase (SPP) with a repeat distance of approximately 6 nm and the long periodicity phase (LPP) with a repeat distance of around 13 nm. However, in diseased skin, like atopic eczema (AE), which is often characterized by a reduced barrier function, the lipid organization as well as the lipid composition may be altered.

In order to study the lamellar organization in SC we used small angle X-ray diffraction (SAXD) of SC isolated from 4 mm skin biopsies from the ventral forearm of uninvolved (no visible signs of AE) skin. The lipid composition was studied in SC that was harvested by tape stripping close to the region of the biopsy. Tapes number 6 to 9 were extracted, pooled and analyzed by liquid-chromatography (LC) coupled to mass-spectrometry (MS).

We observed drastic changes in CER composition in AE patients that coincide with drastic changes in lipid organization, as compared to healthy subjects. In addition, the results show also an increased variance in both the lipid organization as well as the CER composition for AE patients. Whether these changes in lipid organization and composition correlate directly to an impaired skin barrier function will be subject of future studies.

37. ORP8 DEFICIENCY IN BONE MARROW-DERIVED CELLS REDUCES ATHEROSCLEROTIC LESION PROGRESSION IN LDL RECEPTOR KNOCKOUT MICE

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Oxysterol binding protein related proteins (ORP's) are involved in the transcriptional regulation of sterol metabolism. ORP8 is highly expressed in macrophages and is reported to downregulate ABCA1 expression and cellular cholesterol efflux to apolipoprotein A-I. Our lab also found that ORP8 was upregulated in collar-induced atherosclerotic lesion development in murine carotid arteries. However, the role of macrophage ORP8 in atherosclerotic lesion development is unknown.

LDL receptor knockout (KO) mice were transplanted with bone marrow from ORP8 KO mice and C57Bl/6 wild type (WT) mice. Subsequently, they were challenged with a high cholesterol and high fat Western-type diet (WTD) to induce atherosclerosis.

Serum levels of triglycerides in the VLDL fraction were 56% higher ($P=0.02$) in the ORP8 KO transplanted mice ($1836\pm 161\mu\text{g/ml}$) compared to the WT transplanted animals ($1171\pm 166\mu\text{g/ml}$). No significant effect of ORP8 deficiency was found on atherosclerotic lesion development after 6 weeks WTD feeding. After 9 weeks, however, a difference in lesion size and lesion composition was found. Lesions in mice transplanted with ORP8 KO bone marrow ($5.53\pm 0.53\times 10^5\mu\text{m}^2$) were 20% smaller ($P<0.05$) compared to WT transplanted controls

($4.44 \pm 0.30 \times 10^5 \mu\text{m}^2$). In addition, ORP8 KO transplanted mice showed a significantly higher ($P=0.03$) percentage of macrophages in the lesion ($48\% \pm 1.9\%$), as compared to total lesion size, than the WT group ($42\% \pm 2.4\%$).

Deletion of ORP8 in bone marrow-derived cells, including macrophages, reduces lesion progression after 9 weeks of WTD challenge, despite increased amounts of circulating triglycerides in the VLDL fraction.

38. A RECEPTOR FOR THE STRESS HORMONE CORTISOL MODULATES RESILIENCE TO MAJOR DEPRESSIVE DISORDER IN FEMALES

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A fundamental question in mental health research is which factors can tip the balance from vulnerability to resilience. Clearly, genes and stressful environmental factors play a role. Here we focus on the mineralocorticoid receptor (MR), which mediates effects of the hormone cortisol in the brain, facilitating appraisal of stressful information, emotional arousal and behavioural response selection.

We tested whether common MR gene variants are functional *in vitro* and whether they associate with the risk for depression. Three common MR gene variants could be identified that affect MR expression and functioning *in vitro* and are linked to variability in activity of the neuroendocrine stress system (the hypothalamic-pituitary-adrenal axis) *in vivo*. Genetic association analysis showed that one of these MR gene variants, that results in the highest MR expression, associates with heightened dispositional optimism in one study group, with less thoughts of hopelessness, rumination and fewer diagnosis of depression in a second study group and with a lower risk for depression in a third and large depression cohort study. All effects were restricted to women, which is interesting considering the two times higher prevalence of depression in women. Moreover, results from an association study among a large group of depressed patients indicated interaction between antidepressants and MR genotype.

To conclude, common and functional MR gene variants resulting in altered receptor expression may confer inter-individual variability in resilience to psychopathology in women. We propose the MR to be an important target for antidepressant treatment with the MR genotype providing guidance for selecting a specific antidepressant and dose.

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39. MOLECULAR INTERACTION FINGERPRINTS AS A POST-PROCESSING TOOL FOR DOCKING-BASED VIRTUAL SCREENING

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Molecular Interaction FingerPrints (IFPs)^[1] are more and more used for post-processing the results of docking simulations. IFPs are a very simplified way to score docking poses based purely on interactions of the ligand with residues in the active site. This simple representation of 3D protein-ligand interaction information is the strength as well as the weakness of IFPs; the simple approach can allow underrepresentation of interactions, but it can also easily focus on the key interaction features.

Using the Directory of Useful Decoys (DUD)^[2] as input data and PLANTS and GOLD as docking methods, a lot of docking poses were generated for both the ligand and decoys of each of the 40 targets. This resulted in ~6 million docking poses which were analyzed and refined by IFP post processing. The application of data mining techniques to the generated data highlighted interesting correlations and possibilities for general and target-dependent optimization of virtual screenings using IFPs.

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40. DISSECTING THE BINDING POCKET OF THE IMIDAZOLYPYRIMIDINE CXCR2 ANTAGONISTS:

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The chemokine receptor CXCR2 is involved in different inflammatory diseases, like chronic obstructive pulmonary disease, psoriasis, rheumatoid arthritis and ulcerative colitis and therefore considered an attractive drug target. Different classes of small CXCR2 antagonists have been developed. Recently, we have pharmacologically characterized the distinct binding sites of low molecular-weight synthetic compounds at the chemokine receptor CXCR2 (de Kruijff et al., 2009 JPET 329:783-790). In these studies we further dissected the binding pocket of the imidazolypyrimidine CXCR2 antagonists by making use of non-primate CXCR2 orthologs that show high homology to one another.

We found that these orthologs show differential affinity for the imidazolypyrimidine antagonist (compound I). Chimeras containing the human and baboon CXCR2 sequences were useful to localize the domains of the receptor that determine affinity of compound I for CXCR2. Furthermore, using site-directed mutagenesis and in silico modeling we propose two different binding modes of compound 1 at human CXCR2 in the trans-membrane region. These results open new possibilities in the structure-based design of allosteric modulators of CXCR2.

41. AUGMENTED ATHEROSCLEROSIS IN MICE LACKING BOTH MACROPHAGE ABCA1 AND APOE

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ABCA1 protects against atherosclerosis by facilitating cholesterol efflux from macrophage foam cells in the arterial wall to extracellular apolipoprotein (apo) A-I. In contrast to apoA-I, apoE is secreted by macrophages and can, like apoA-I, induce ABCA1-mediated cholesterol efflux. Yet, the combined effect of macrophage ABCA1 and apoE on lesion development is unexplored.

LDL receptor knockout (KO) mice were transplanted with bone marrow from ABCA1/apoE double KO (dKO) mice, their respective single knockouts, and wild-type (WT) controls and were challenged with a high-fat/high-cholesterol diet for 9 weeks.

In vitro cholesterol efflux experiments showed no differences between ABCA1 KO and dKO macrophages. The serum non-HDL/HDL ratio in dKO transplanted mice was 1.7-fold and 2.4-fold ($p < 0.01$) increased compared to WT and ABCA1 KO transplanted mice, respectively. The atherosclerotic lesion area in dKO transplanted animals ($650 \pm 94 \times 10^3 \mu\text{m}^2$), however, was 1.9-fold ($p < 0.01$) and 1.6-fold ($p < 0.01$) increased compared to single knockouts (ABCA1 KO: $341 \pm 20 \times 10^3 \mu\text{m}^2$; apoE KO: $402 \pm 78 \times 10^3 \mu\text{m}^2$, respectively) and 3.1-fold increased ($p < 0.001$) compared to WT ($211 \pm 20 \times 10^3 \mu\text{m}^2$). Moreover, when normalized for serum cholesterol exposure, macrophage ABCA1 and apoE independently protected against atherosclerotic lesion development ($p < 0.001$).

Combined deletion of macrophage ABCA1 and apoE results in a defect in cholesterol efflux and, compared to ABCA1 KO transplanted mice, elevated serum total cholesterol levels, thereby inducing a more dramatic and significant increase in atherosclerosis.

42. NIACIN REDUCES HEPATIC AND PLASMA CETP LEVELS BY DIMINISHING LIVER MACROPHAGE CONTENT IN CETP TRANSGENIC MICE

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Background and purpose

The anti-dyslipidemic drug niacin lowers plasma levels of pro-atherogenic lipoproteins in mice. Only recently, it has been shown that niacin also lowers the hepatic expression and plasma levels of pro-atherogenic CETP. The aim of the current study was to investigate the mechanism underlying the hepatic and plasma CETP-lowering effect of niacin in mice.

Experimental approach

Transgenic mice expressing the human CETP transgene (CETP Tg) were fed Western-type diet with or without 2% (w/w) niacin for 4 weeks. Bone marrow-derived macrophages were obtained from CETP Tg mice and incubated in the absence or presence of niacin *in vitro*.

Key results

Niacin does not directly regulate CETP expression in bone marrow-derived macrophages *in vitro*. In CETP transgenic mice, niacin reduces plasma (V)LDL-cholesterol and hepatic cholesterol level. Niacin also reduced plasma CETP mass and hepatic CETP expression. Importantly, niacin decreased hepatic expression of CD68 and ABCG1, which are specific markers to assess hepatic macrophage content. There was a significant correlation between the decrease in hepatic CETP expression and the reduction of hepatic macrophage content. Furthermore, niacin treatment attenuated inflammation in liver, as shown in decreased expression of MCP-1 and TNFalpha.

Conclusions

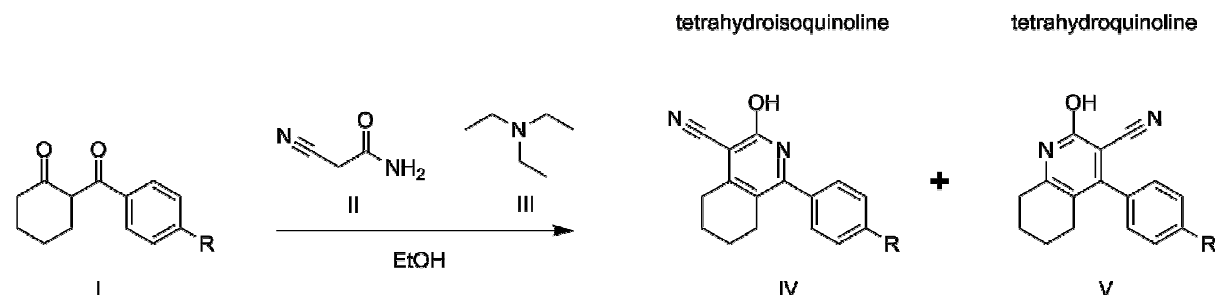
Niacin does not alter CETP expression directly in the macrophage, but attenuates liver inflammation and macrophage content in response to its primary lipid-lowering effect, which leads to a decrease in hepatic CETP expression and plasma CETP mass.

43. ON THE REACTION OF *PARA*-SUBSTITUTED 2-BENZOYL CYCLOHEXANONES WITH CYANOACETAMIDE

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In the synthesis of a more complex heterocyclic system, we explored 3-hydroxy-1-phenyl-5,6,7,8-tetrahydroisoquinoline-4-carbonitrile (IV) as an important intermediate. The reaction of substituted 2-benzoyl cyclohexanone (I) with cyanoacetamide (II) and triethylamine (III) in ethanol at room temperature has been reported to lead to the formation of 3-hydroxy-1-phenyl-5,6,7,8-tetrahydroisoquinoline-4-carbonitrile (IV) (1). However, depending on the nature of the *para* substituent on the phenyl ring, formation of regioisomeric 2-hydroxy-4-phenyl-5,6,7,8-tetrahydroquinoline-3-carbonitrile can compete (V).



No molecular determinants for the regiochemistry have been described. Therefore, we undertook efforts to gain more insights in the mechanism of this reaction. We investigated the effect of different para-substituents and their influence on the observed ratio between formed tetrahydroisoquinoline and tetrahydroquinoline products. For the characterization and quantification of precursors and products we used an array of techniques, including GC-MS and 2D NMR. We postulate that the electron-donating or -withdrawing properties of the para substituents directly determine the ratio of product formation.

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44. DEVELOPMENT OF SMALL pDNA-CONTAINING LIPOSOMES FOR DNA VACCINATION

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Purpose

DNA vaccination is based upon the administration of antigen-encoding plasmid DNA (pDNA), and subsequent production of the antigen, to which an immune response is elicited. DNA vaccines can induce both humoral and cellular immune responses and have several benefits over the current vaccines like the possibility of using multiple antigens in one vector, higher stability, better safety and their potential capability of offering protection against intracellular infectious agents like HIV, malaria and tuberculosis. One of the main disadvantages of DNA vaccination is the low potency in humans, which may be related to poor delivery of pDNA to the appropriate cells and/or degradation of pDNA by endonucleases. This can be overcome by using particulate delivery systems, such as liposomes, to formulate the pDNA. The purpose of this research was to develop small pDNA-containing liposomes for DNA vaccination.

Methods

Liposomes composed of egg L- α -phosphatidylcholine (EPC), 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE) and 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP) were prepared by the dehydration-rehydration method and were sized by extrusion. Liposomes were analyzed by DLS and electron microscopy, DNA content and integrity by a PicoGreen assay, UV spectroscopy, and gel electrophoresis, respectively. The encapsulation efficiency was determined by separating non-encapsulated pDNA from the liposomes with a membrane filter, followed by a PicoGreen assay.

Results

Sizing by extrusion (2x400 + 4x200 nm) of large pDNA-containing liposomes (diameter ~460 nm) resulted in liposomes with an average particle size of 145 ± 7 nm (mean \pm sd), a narrow size distribution (as reflected by a polydispersity index of 0.15 ± 0.02), and a zeta potential of 54 ± 2 mV. Electron microscopy showed that liposomes were unilamellar after extrusion. DNA recovery after extrusion was $48 \pm 8\%$ of the initial DNA content, of which most was encapsulated inside the liposome ($91.1 \pm 0.3\%$). The pDNA was intact after sizing by extrusion and protected against DNase degradation. Reduction of the liposome size could also be achieved by sonication and extrusion through 100 nm filters, but this resulted in a low DNA content and/or pDNA damage.

Conclusion

The dehydration-rehydration method, followed by an extrusion cycle of 2x400 + 4x200 nm enabled us to create well-defined small pDNA-containing liposomes that can be used for DNA vaccination.

45. EPIGENETICS OF STEM CELL RENEWAL AND DIFFERENTIATION IN CYTOTOXICITY

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Embryonic stem (ES) cells have been shown to possess globally open and bivalent chromatin structure. Bivalent areas show both active and passive histone modifications, which enables quick activation or repression of different genomic areas or specific genes. This enables sensitive responses of stem cells to different developmental and environmental cues. Better understanding of toxicity in stem cell differentiation and pluripotency is of great importance and may help defeating ageing related diseases and prevent embryotoxicity.

Our goal is to gain insights into the role of epigenetic modifications in chemically induced stem cell differentiation. Also, suspects are rising that epigenetic modifications are involved in the initiation of epithelial mesenchymal transition (EMT) in development and cancer metastasis.

First, a number of compounds are analysed in an embryonic stem (ES) cell assay for effects on renewal, differentiation and cellular status (apoptosis, cell cycle, proliferation). A selection of these compounds are further analysed by a semi-high throughput qRT-PCR for a set of early differentiation markers to elucidate the cell fate. Next, we will assess the involvement of epigenetic modifier genes in chemically induced differentiation and toxicity by a high throughput gene silencing study. Finally, the most prominent findings will be analysed in detail and extrapolated to the behavior of cancer stem-like cells. Findings of the project may include potential targets for therapeutic intervention in ageing related diseases and cancer treatment.

46. THE ROLE LIPID PLAYS IN THE BARRIER PROPERTIES OF THE SKIN

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The lipids in the outer layer of the human skin referred to as the stratum corneum (SC) plays important role in the skin barrier function. They can form two lamellar phases, the long periodicity phase (LPP) and short periodicity phase (SPP) with a predominantly orthorhombic lateral packing. The main lipid classes in the stratum corneum are ceramides, cholesterol and free fatty acids. So far, 12 different subclasses of ceramides have been identified in human SC.¹ In the absence of ceramide EOS (one subclass of ceramides), formation of the LPP is drastically reduced.² This most probably results in a reduced skin barrier function and demonstrates that, ceramide EOS is crucial for the formation of LPP.²

The aim of the present study is to obtain insight in the relation between skin barrier function and the lipid organization in the SC. In order to examine this, we developed the stratum corneum substitute (SCS).³⁻⁴ SCS is a lipid membrane casted on a porous support. The lipid organization is very similar to that in SC. Previously synthetic ceramides were used to prepare SCS. However, as there are differences in lipid phase behavior in mixtures prepared with natural and synthetic ceramides, in the present studies we decided to use natural ceramides.

The lipid organization of human SC is very similar to that of pig SC. Because pig skin is readily available, ceramides were extracted from pig SC and separated by column chromatography. SCS were prepared using the isolated pig ceramides, cholesterol and free fatty acids and the phase behaviour determined by X-ray diffraction. When using mixtures of isolated pig ceramides and cholesterol, both the LPP and SPP were formed with a hexagonal lateral packing. Addition of free fatty acids changed the packing from a hexagonal to orthorhombic one. Therefore, the lipid phase behavior in the SCS is similar to that in human SC. In future, the SCS will be used to study the effect of lateral packing and lamellar phases on the SC barrier function using diffusion studies.

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47. HIJACKING OF HUMAN GPCRS BY THE KAPOSÍ'S SARCOMA-ASSOCIATED HERPESVIRUS ENCODED GPCR ORF74

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Herpesviruses have pirated and modified host genes coding for G protein-coupled receptors (GPCRs) for their own benefit. These viral GPCRs (vGPCRs) are expressed on infected host cells, and display highest sequence similarity to the family of chemokine receptors. Since chemokine receptors coordinate migration and activation of immune cells, it is believed that vGPCRs can take over immune cell control to evade antiviral responses,

resulting in a widespread and life long infection. For a long time, GPCRs have been considered to exist and function as monomeric entities, however, accumulating evidence indicates that GPCRs can physically interact with each other. Importantly, heterodimers can exhibit altered functional characteristics compared to monomeric GPCRs, including receptor trafficking, ligand binding and signaling.

We show that vGPCRs heterodimerize with human GPCRs, and hypothesize that vGPCRs hijack cellular communication by physically affecting the functional characteristics of human GPCRs.

48. CHARACTERIZATION OF THE FIRST IRREVERSIBLE ANTAGONIST FOR THE HUMAN HISTAMINE H₄ RECEPTOR

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The majority of endogenous and synthesized ligands bind reversible to their cognate GPCRs. In this study we designed and synthesized an aminopyrimidine that was hypothesized to form a covalent bond with the human histamine H₄ receptor (hH₄R). Both radioligand binding, functional and site-directed mutagenesis studies were used to confirm the pharmacological properties and irreversible character of this compound. In addition we tested the affinity of this compound for other histamine receptor family members.

This irreversible ligand will be a valuable tool for future hH₄R structure and activation studies.

49. ONCE DAILY POPULATION PHARMACOKINETICS OF LAMIVUDINE IN HIV-INFECTED CHILDREN

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The use of antiretroviral (ARV) drugs in children imposes careful considerations regarding dosing frequency and patient compliance. There may be considerable benefits for both children and caregivers if dosing frequency can be reduced to once daily for all drugs used during the course of therapy. The objective of this investigation was to assess whether once daily dosing provides similar exposure to lamivudine (3TC), as compared to the recommended b.i.d. regimen in HIV-infected children between 3 months and 12 years old.

Simulation scenarios were explored using a pharmacokinetic one-compartment model previously developed to describe drug disposition in the paediatric population. In the model body weight was found to have an effect on clearance and volume of distribution. The simulated population consisted of a cohort of 180 patients, aged between 3 months and 12 years old. 500 replicates were simulated, in which the use of solution and tablets were considered taking into account the wide age range. To ensure appropriate representation of different age groups, the WHO weight-for-age tables were used as reference for the correlation between age and body weight. Systemic exposure (AUC) and maximum peak concentration (C_{max}) were derived as primary parameters of interest. In addition to median and percentiles, parameter distributions were also presented and compared to previous clinical trial data following once daily dosing. NONMEM VI was used to perform the simulations and R was used for the graphical and statistical summary of the results.

The simulations show that once daily dosing of lamivudine yields comparable exposure (AUC) to historical values observed in children on a twice daily regimen of lamivudine, as well as in adults receiving lamivudine once or twice daily, both for solution and tablet administration. Given the change in dose frequency, higher C_{max} are observed, but the observed values do not exceed tolerability limits.

Administration of lamivudine according to a once daily dosing regimen provides appropriate exposure in children aged from 3 months to 12 years. Our findings strongly suggest that the reduction in the dosing frequency to once daily may represent an improvement in treatment acceptability and adherence. Increased adherence may result in increased efficacy particularly in resource limited settings.

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The hippocampus is a major target of the stress hormone corticosterone (CORT) in the brain and has abundant expression of receptors for CORT, including the glucocorticoid receptor (GR). Upon CORT-binding, GR acts as a ligand-activated transcription factor. Using transcriptional-profiling, we have previously extensively characterized CORT-responsive gene profiles in the hippocampus. The aim of the current study was to complement the gene profiles by identifying genomic binding sites of GR in a neuronal setting.

We applied Chromatin Immunoprecipitation Sequencing (ChIP-seq) for GR and identified 1183 and 3673 significant GR-binding sites in in neuronally-differentiated rat PC12 cells and in vivo in rat hippocampus (HC) respectively. Among the GR-binding sites were several positive controls, such as binding sites upstream of Per1 and Ddit4. Interestingly, 30% (PC12) and 40% (HC) of these sites were located within genes. Screening the GR-bound regions for the presence of GREs revealed the presence of GRE-like sequences in 33% (PC12) and 58% (HC) of all significant GR-bound sites.

In addition, binding sites for the AP1 and MAZ transcription factors were significantly represented. Comparing the genes surrounding the GR-binding sites with known CORT-responsive genes showed a high degree of overlap. However, the extent of overlap with ChIP-Seq studies from literature based on peripheral tissues or cell lines was limited. The genes nearby the GR binding sites in PC12 cells and in HC were highly enriched for genes with a specific neuronal function. This reinforces the concept that GR-binding occurs in a tissue-specific manner and underlines the relevance of searching for brain-specific binding sites of GR. Since the identified GR binding sites control the expression of CORT-responsive genes in the brain they hold relevance to stress-related brain disorders such as depression.

This work was supported by grants from the Netherlands Organization for Scientific Research (NWO) 836.06.010 (MEERVOUD) to N.A. Datson, TI Pharma T5-209 and HFSP (RGP39). ERdK was supported by the Royal Netherlands Academy of Science.

51. DEPLETION ZONE ISOTACHOPHORESIS (DZITP): A NOVEL METHOD FOR FEMTOMOLE SEPARATIONS IN A NANOFLUIDIC DEVICE

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We report a novel method, called depletion zone isotachophoresis (dzITP), combining electrokinetic focusing and isotachophoretic separation of femtomole quantities of ionic analytes at the border of an ion-depleted zone. This depletion zone is induced by ion exclusion effects in a nanofluidic channel. Subsequent application of an electro-osmotic flow results in focusing of analytes that order themselves in distinctive separation zones corresponding to the respective ionic mobilities of the analytes present in the sample. We demonstrated this technique for picoliters of an academic mixture of FITC-labeled amino acids.

In contrast to conventional isotachophoresis our technique does not employ a trailing electrolyte, giving us a wider frame of mobilities to be separated. Sample injection is straightforward and the technique is quasi-static, meaning that separation emerges at a stable position which simplifies detection

We believe that this novel technique in combination with state of the art sampling and detection technology will allow us to analyze ultrasmall biological samples like single cells.

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High throughput cellular assays currently used in preclinical drug safety testing use two-dimensional tissue culture models that are poorly predictive of the effect of compounds in humans. This is principally due to the poor differentiation status resulting in aberrant drug handling and cellular response. To overcome this, three-dimensional cell culture models are being developed that mimic biochemical aspects of a real tissue and can maintain tissue function and homeostasis to a higher extent than monolayer cultures. In 3D cultures, cells form cysts and tubules, which are basic structural units of epithelial organs.

We have developed three-dimensional cell culture models for nephrotoxicity and hepatotoxicity testing. Cells in 3D undergo morphogenesis to form polarized cysts or tubular networks, a process which can be disrupted by toxicants. Furthermore, the response of a toxicant to a 3D epithelial structure differs from responses in 2D. The underlying mechanisms of these responses are poorly understood and may be determined intrinsically by specific genetic programs and extrinsically by cell-ECM and cell-cell interactions. Exploring the pathways activated in response to cell injury will give us more insights for prediction of toxicity, risk assessment and mechanism of action of various xenobiotics.

We are studying the effect of different classes of organ toxic compounds on tissue development, maintenance and function. The assay we have developed is implemented in a 384 well format for low cost and potential high throughput assays. In addition to conventional end points of cell apoptosis and necrosis we measure the changes in the organoid morphology in response to compound exposure, which is compared with gene expression data. By analyzing the set of compounds that share a common toxic phenotype and by comparing the molecular pathways they modulate with pathways modulated by non-toxic compounds we hope to establish links between pathways and particular adverse effects.

This high content analysis approach offers an alternative and potentially more predictive and qualitative means of monitoring effects of compounds *in vitro*. The availability of state of the art *in vivo*-like 3D cell culture system, robotics, data process and control software, liquid handling devices, high content image capturing (confocal) microscopes will allow us to identify the compounds that disrupt cellular function on a larger scale.

53. DNA VACCINATION AGAINST INTERLEUKIN 27 AGGRAVATES ATHEROSCLEROSIS IN LDL RECEPTOR DEFICIENT MICE

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Interleukin 27 (IL-27) interferes with T helper differentiation and can alter the outcome of autoimmune diseases. The goal of this study was to determine if IL-27 plays a role in atherosclerosis

To study the function of IL-27, spleen cells were isolated from untreated LDL receptor knock out mice (n=5) and stimulated with IL-27 *in vitro*. The secretion of cytokines related to TH17 and TREG was measured. A DNA vaccine against IL-27 was developed to induce auto-antibodies and neutralize IL-27 *in vivo*. The mice were vaccinated prior to the induction of atherosclerosis to study the role of IL-27 in atherogenesis. Atherosclerosis was induced in LDLR *-/-* mice by a high fat diet and subsequent collar placement around the carotid artery. The plaque size was measured in the aortic valve, aorta and carotid arteries. A FACS analysis was performed on the spleen and heart lymph node to detect changes in cell populations.

In vitro IL-27 inhibits the secretion of TH17 and TREG related cytokines significantly, indicating an important role in T helper cell regulation. Neutralisation of IL-27 *in vivo* increased the average plaque size in the carotids and aorta. The TREG population and B cell content were decreased in the spleen of the vaccinated mice. Under TH17 polarising conditions the TH17 population increased in the control group, whereas the TH17 population in the vaccinated group remained equal.

In conclusion, *in vivo* vaccination against IL-27 generated a more inflammatory environment, resulting in aggravation of atherosclerosis.

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We report on a novel, heaterless nano -atmospheric pressure chemical ionization (APCI) source for Mass Spectrometry (MS). Because of the positive effect of downscaling, the aerosol droplets generated by the APCI source are very small allowing ambient temperature and ambient pressure solvent evaporation. With the use of this novel approach reserpine was successfully detected using direct infusion. Moreover several peptides were detected with masses higher up to 2500 Da using direct infusion. This, to our knowledge, has never been reported yet (masses higher than 1000 Da are rarely seen using APCI). The heaterless configuration of the source is also important because it avoids sample/analyte degradation normally associated with APCI sources which operate at much higher temperatures (i.e. 250-450°C).

Electrospray Ionization Mass Spectrometry (ESI-MS) is widely used for analyzing samples in a qualitative and quantitative way. In the lab-on-a-chip field a considerable body of literature has been published detailing the fabrication of chip-based electrospray interfaces. However, although widely used, ESI is burdened by low ionization efficiency, ion suppression and is restricted to polar solvents. Surprisingly, much less scientific work has been published on the fabrication of other chip-based ionization methods such as Atmospheric Pressure Chemical Ionization (APCI) ¹. APCI is also very interesting since it suffers far less from ion suppression, has better ionization efficiency and can be used for non-polar solvents.

We have found that miniaturizing APCI to the very small scale does not only allow us to make a fully-operational APCI source, but also that the very small aerosol droplets generated by the source evaporate at room temperature allowing heaterless operation. This is a significant finding because it enables detecting molecules larger than 1000 Da. Also by avoiding the use of the high temperatures associated with conventional APCI (i.e. 250-350°C) thermally labile compounds could be detected.

Our nano-APCI source has a number of advantages including: simple on-chip integration (no needle as in ESI and no heater electrodes as in conventional APCI), low flow rates and room temperature operation which are interesting for the analysis of heat-sensitive compounds.

Further work is currently ongoing to further downscale the ion source to get better performance (i.e. smaller aerosol droplets). Also work will be done on the characterization of the nanoAPCI source. Micro-particle image velocimetry (μPIV) will be used to visualize the spray emerging from the micronozzle to optimize droplet emission process and the aerosol droplet size distribution.

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55. APPLICATION OF CYTOCHROME P450 BM3 MUTANTS FOR THE BIOACTIVATION OF CLOZAPINE AND STRUCTURAL CHARACTERIZATION OF CLOZAPINE GSH CONJUGATES

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Recently, a site-saturation mutagenesis library of 20 mutants with a different amino acid in position 87 has been created. In the present study we investigated the applicability of these mutants as biocatalysist for the production of reactive metabolites of clozapine. Clozapine is an atypical antipsychotic drug where formation of reactive metabolites is considered responsible for several adverse drug reaction (ADRs). Conjugation of reactive drug metabolites to GSH is an important detoxification mechanism that can be spontaneous and/or mediated by glutathione S-transferases (GSTs).

In our previous study we have shown that human GSTs play a significant role in the inactivation of reactive intermediates of clozapine. The screening of the library of 20 mutants in the presence of hGST P1-1 showed that the physical properties of the side chain of amino acid in position 87 have a major effect on the regioselectivity of clozapine metabolism. From this screening the mutant M11 87Phe, showing the highest production of clozapine reactive intermediates, was selected for the large scale formation of clozapine GSH conjugates. Six GSH adducts were produced in high levels, purified and identified by NMR.

This work shows that CYP450 BM3 mutants are very useful tools for the generation of reactive metabolites of drugs in order to enable their isolation, structural elucidation and toxicological characterization. These enzymes can be applied as novel tools in the development of safer drugs.

56. HIGH-THROUGHPUT siRNA SCREENING OF CANDIDATE EPITHELIAL – MESENCHYMAL TRANSITION (EMT) MODIFIERS

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Breast cancer is the most common type of cancer in women with high death incidence due to distant metastases, such as lung, bone or brain metastasis. Metastasis formation which starts with dissemination of tumor cells from the primary tumor is a complex and multistep process. During breast carcinoma progression, epithelial cells detach from the primary tumor, adhere to and invade surrounding tissue and basement membranes, intravasate into the vessels, disseminate and finally extravasate and adhere into distant organs.

These processes require dynamic cell plasticity and resemble epithelial to mesenchymal transition (EMT) of epithelial cells followed by a mesenchymal to epithelial reverse transition (MET) at the metastatic site. During EMT epithelial cells lose their epithelial characteristics and cell – cell attachments and acquire a mesenchymal – fibroblastic morphology with increased migratory and invasive capacity. Thus, EMT is believed to play a pivotal role in breast cancer invasion and metastasis.

Aim of this project is to identify genes involved in EMT of breast cancer cells in relation to cell morphology and migration, cell – cell junctions and focal adhesion (FA) dynamics. Therefore, a panel of 56 human breast cancer cell lines will be tested and a chemical / growth factor induced EMT – MET *in vitro* model will be set up. Then, an automated cell microscopy based analysis of EMT and FA dynamics will be established, followed by a high – throughput siRNA screen for cellular signaling and adhesion components in cancer cell EMT behavior. Candidate target genes' effect on migration and their requirement in EMT will be determined *in vitro* using live cell imaging and 3D migration – invasion models. In addition, candidate genes will be validated in an *in vivo* breast cancer mouse model and in relation to breast cancer patient cohorts.

As a compelling need for the development of improved therapies for breast cancer remains, the investigation of cancer cell EMT may lead to the development of novel therapeutic interventions for cancer patients. Since EMT is thought to be an early event in cell spreading and invasion, genes involved in EMT could also be useful prognostic markers for metastatic breast cancers.

57. SYSTEMATIC IMAGING-BASED RNAi SCREENING TO IDENTIFY SIGNALING COMPONENTS THAT CONTROL TUMOR CELL MIGRATION AND METASTASIS

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Despite improved treatment regimens of breast cancer still one third of the patients die from distant metastases. While cancer cell plasticity is essential in efficient cancer metastasis formation, a systematic analysis is required to define all the molecular determinants and signaling networks that underlie tumor cell plasticity.

Aggressive tumor cells have a dynamic cell migratory pattern that involves a high turnover of matrix adhesions structures, which consist of cytoskeletal structural components and adaptor proteins, the so-called integrin adhesome. The integrin adhesome itself is regulated by diverse signaling pathways dominated by (oncogenic) protein kinases and phosphatases. We search for cancer cell plasticity genes that define cell motility parameters using high-throughput siRNA-based screening. These genes are likely candidate components essential in metastasis formation.

A bead-based phagokinetic track assay was used to identify integrin adhesome components (library of 575 genes), kinases (library of 779 genes) and phosphatases (library of 198 genes) that modulate the plasticity of human lung carcinoma cells (H1299) and metastatic mouse mammary carcinoma cells (4T1). Cell migration was visualized by imaging bead-free tracks using automated autofocus-screening microscopy. Automated image segmentation and track analysis allowed the extraction of quantitative information of the migratory behavior. This

included track length and area, migration speed, persistence of migration and relative lamellar activity as measured by track roughness.

We identified 137 genes that affect cell migration (88 kinases, 7 phosphatases, 42 adhesion components). Different behavioral traits were observed: gene knock down that increases cell migration, knock down of genes that inhibits cell migration without causing increased cell-cell adhesion, and genes that block cell migration in association with a switch to a more epithelial phenotype. Interestingly, some genes modulate the lamellar activity without necessarily increasing cell migration speed. A strong overlap in migratory modulators was observed between both cell lines. Positive hits were correlated with patient breast cancer gene expression profiles and associated with metastasis free survival.

The most promising candidate metastasis genes are currently validated in *in vitro* 3D invasion assays and *in vivo* mouse orthotopic mammary tumor/metastasis models. With these approaches we aim to identify potential new targets for anti-cancer therapy.

58. DOUBLECORTIN-LIKE KNOCKDOWN AFFECTS ADULT NEUROGENESIS

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Members of the doublecortin family of proteins regulate the dynamics of the microtubule cytoskeleton in neurogenic cells. The best-characterized member of this family is doublecortin (DCX), a microtubule-associated protein that is crucial for correct migration of neuronal progenitor cells (NPCs) and an often-used marker for neurogenesis. We have recently reported the identification of a highly homologous protein, doublecortin-like (DCL) that is crucial for the formation of neocortex by regulating mitotic spindle formation of neuronal progenitor cells and by shaping the radial glia scaffold during embryogenesis. Therefore, in combination with DCX, DCL may control different aspects of neurogenesis. To study a possible DCL role in adult neurogenesis, we have mapped the DCL expression in the mouse brain by using DCL-specific antibodies and by using a genetic mouse model which contains an inducible and reversible short hairpin (sh)RNA construct that targets DCL.

Using Western blot analysis, we identified DCL expression in the two main brain areas with ongoing neurogenesis, i.e. the hippocampus and the bulbus-olfactorius. Consistent with a role in adult neurogenesis, we found DCL expression in the subgranular-zone of the dentate gyrus and in the subventricular- and rostral-migratory-zone of the bulbus-olfactorius. Interestingly, although we identified neuronal cells with only DCL or DCX expression, we observe extensive colocalization of DCX and DCL. *In vivo* DCL knockdown by doxycycline administration in food dramatically reduced DCX-positive dendrites traversing the granular cell layers, strongly indicating requirements of DCL-DCX interaction for proper migration of NPCs. We also will present evidence that DCL knockdown leads to cognitive impairment that may depend on both the hippocampus and bulbus-olfactorius.

Together, our data support the notion that DCL is a major regulator of migration of NPCs and pinpoint our inducible DCL knockdown mice as excellent models to study the biological significance of adult neurogenesis.

This work is a part of the TI-Pharma project T5-210

59. COMPUTATIONAL MODEL-BASED DIAGNOSTIC MARKERS IMPROVE CARDIOVASCULAR RISK PREDICTION

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Cardiovascular disease continues to be a major killer worldwide. Improved diagnostics contribute to early detection and timely treatment of this disease.

Current measurements of lipoproteins, the cholesterol-carrying particles in human blood, can distinguish up to 40 subfractions in a 'lipoprotein profile'. In medical practice, this information is much harder to interpret than the standard 'bad' LDL and 'good' HDL cholesterol. Therefore, we developed a computational model called Particle Profiler to interpret detailed lipoprotein profile data, and extract medically relevant information. The markers we derive are called 'lipoprotein metabolic ratios', which are different ratios of the metabolic production, lipolysis, and uptake processes that lipoproteins undergo.

We applied Particle Profiler to NMR lipoprotein profiles measured in 1981 subjects from the Framingham offspring cohort and calculated lipoprotein metabolic ratios. We applied stepwise cross-validated logistic regression to select classical risk factor variables and lipoprotein metabolic ratios that contributed to predicting 10-year risk for general cardiovascular disease.

Six lipoprotein metabolic ratios were consistently found to contribute to cardiovascular risk prediction after cross-validation. Adding the six selected lipoprotein metabolic ratios to a multivariate logistic regression model with the best performing classical risk markers and NMR lipoprotein derived markers, resulted in a significantly improved predictive power as measured by the 'area under the receiver operating characteristic (ROC) curve'.

Six calculated lipoprotein metabolic ratios significantly improved cardiovascular risk prediction above the best predicting 'classical' variables, similar to those included in the Framingham risk score. The improvement based on our diagnostic markers more than doubled the improvement obtained by adding total and HDL cholesterol to non-cholesterol risk factors.

60. MOLECULAR DETERMINANTS OF CHEMOKINE RECEPTOR CXCR7 REGULATION

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The chemokine receptor CXCR7 belongs to the superfamily of G protein-coupled receptors and recognizes two endogenous peptide ligands, CXCL11 and CXCL12. CXCL11 also binds to CXCR3, and CXCL12 to CXCR4. CXCR7 has been implicated in tumor development and progression, but the mechanism behind it remains unclear.

Here we report on the signaling and regulation of CXCR7, using several pharmacological assays, including radioligand binding, [³⁵S]-GTPγS accumulation, β-arrestin recruitment, immunocytochemistry and receptor internalization experiments. In summary, we show that ligand binding to CXCR7 does not result in classical G protein mediated signaling, but leads to recruitment of β-arrestin as well as clathrin-dependent internalization and recycling of the receptor. Moreover, this receptor regulation critically depends on residues in the C-terminus of CXCR7.

Our results suggest that CXCR7 is not a classical chemokine receptor, as it seems to be biased towards β-arrestin mediated pathways. Whether CXCR7 is directly involved in tumorigenesis, or indirectly by scavenging of CXCL11 or CXCL12, thereby modulating their availability to CXCR3 or CXCR4, still remains to be elucidated.

61. DEVELOPMENT OF A SOLVENT REMOVAL INTERFACE FOR ENHANCING TWO-DIMENSIONAL LIQUID CHROMATOGRAPHY TO AID METABOLITE PROFILING STUDIES

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New and fast emerging fields such as Metabolomics put great pressure on the resolving power, velocity and sensitivity of analytical chemistry techniques. Comprehensive multidimensional liquid chromatography (MDLC) is a promising tool for the separation and analysis of complex metabolic samples. However, solvents used for separation of compounds in the first dimension dramatically interfere with the separation efficiencies of compounds in the second dimension leading to a reduced resolving power and perturbation of data. Although several attempts have been reported in literature [1-3], a versatile interface that unlocks the true separation power for MDLC is not yet available to date.

Here, we present an LCxLC interface based on evaporation of the running buffer where sample is selectively concentrated and solvent incompatibilities & peak dispersion issues can significantly be reduced. Furthermore, the system is fully computer controlled, leading to a precise process control. We envision the use of this interface for detection and identification of (very) low concentration metabolites in complex biofluids or tissue samples, as well as to resolve metabolite classes (such as phospholipids) with only small structural molecular differences.

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62. BIODEGRADABLE PLGA NANOPARTICLES FOR PEPTIDE-BASED IMMUNOTHERAPY OF CANCER

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Synthetic overlapping long antigenic peptides covering both cytotoxic T lymphocyte (CTL) and T helper (Th) epitopes hold great promise for the immunotherapy of cancer (Zwaveling et al. (2002) J Immunol 169:350-358). A vaccine consisting of such peptides, covering the entire sequence of the HPV16 E6 and E7 proteins, has shown promising results in patients with HPV16-induced (pre)malignant lesions. However, these peptides have been emulsified in the mineral oil adjuvant Montanide ISA 51, which has some important limitations, such as: non-biodegradability; significant local side effects; poor control of release rate; lack of specific dendritic cell-activating capacity; limited scalability of production. Therefore, alternative delivery systems for peptide-based vaccines are highly needed.

In this project, poly(lactic-co-glycolic acid) (PLGA) biodegradable nanoparticles, incorporating long synthetic antigenic peptides, are being developed to optimize the delivery and potency of peptide-based therapeutic cancer vaccines. Therefore, a double emulsion/solvent evaporation technique was used, with dichloromethane (DCM) as solvent for the polymer.

First, we optimized the encapsulation of a model peptide, 24 residues long, covering CTL and Th epitopes of ovalbumin (OVA24), by adjusting process parameters, finally obtaining nanoparticles of approximately 400 nm and encapsulation efficiency circa 30%. Next, we successfully co-encapsulated a Toll like receptor ligand (TLRL) Pam3SCK4, in OVA24-loaded PLGA nanoparticles.

Co-encapsulation of antigen and Pam3SCK4 resulted in increased T cell activation and cytokine production when compared to antigen alone, showing that the combination of antigen, nanoparticles and an adjuvant is crucial to induce a potent cellular immune response.

63. HUMAN STRATUM CORNEUM LIPID ANALYSIS BY LC-MS AND ITS APPLICABILITY ON SKIN DISEASES

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An increasing number of people suffer from skin diseases like atopic eczema, with a current occurrence of over 15% in Western Europe. In recent years, several studies reported strong evidence of an impaired barrier function of the skin in patients with atopic eczema^{2,2}. It is known that the lipids in the outermost layer of the skin, the

stratum corneum (SC), are crucial for a competent skin barrier function, as the lipid matrix is the only continuous pathway in the SC³⁻⁴.

The SC lipids consist mainly of three lipid classes: Cholesterol (CHOL), Free Fatty Acids (FFAs) and Ceramides (CERs). Analyzing the stratum corneum lipid composition of patients suffering from atopic eczema is expected to lead to a better understanding of the impaired skin barrier function in these patients. For decades, the determination of the lipid composition was primarily performed using thin layer chromatography and/or gas chromatography, which resulted in tremendous knowledge of the lipid composition in stratum corneum⁵⁻⁷. Recently, more comprehensive methods are developed to analyze the stratum corneum lipids, like liquid chromatography coupled to mass spectrometry (LC-MS). This revealed the presence of additional lipid subclasses, in particular with respect to CERs⁸. However, robust and reliable analytical MS methods for analyzing skin lipids are few and far between, and no method is reported that analyzes all three main lipid classes in a single run.

Here, we describe a novel method for the combined analysis of the 3 main SC lipids (viz. CHOL, FFAs, CERs) by means of LC-MS, all analyzed in one dedicated run. We observed CHOL, as well as all known CER classes, as well as one new ceramide subclass which was fully identified. Concerning the FFA lipid class, to our knowledge for the first time, we could quantitatively determine the amounts of more than 15 different FFAs present in human SC using this LC-MS method. In total, the number of all lipid components exceeds 250.

In subsequent studies the method was used to compare the lipid composition in SC from healthy volunteers and of patients suffering from atopic eczema. We observed significant differences - with possible biological relevance - compared to normal human SC. Patients suffering from eczema showed significant lower levels of certain CER subclasses (CER subclasses with long acyl chains), a result that is supported by *in vitro* data.

Future research needs to focus on more profound studies for elucidating the biological background of all observed differences in SC lipids, but the method described here proves to be both robust and reliable for analyzing all these lipids in a quick, straightforward and very detailed manner.

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64. MASS SPECTROMETRY BASED TARGETED METABOLOMICS

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This project aims at screening and quantifying polar metabolites using targeted mass spectrometry (MS) approaches.

For this purpose, two multiple reaction monitoring (MRM) methods are being developed for analyzing 30 compounds covering various classes of metabolites:

i/ a Hydrophilic Interaction Liquid Chromatography (HILIC) - MS method for quantitative analysis of the metabolites of interest. HILIC chromatography is suitable for analysis of polar compounds by combining a polar stationary phase with an aqueous/polar organic solvent mobile phase, in which water is introduced to play the role of a stronger eluting solvent.

ii/ a Direct Infusion - Mass Spectrometry (DI-MS) method for rapid screening and semi-quantitative analysis of the target group without or with a limited sample separation step. Direct infusion of complex biological samples is hampered by the presence of large amounts of endogenous material that can suppress ionization of metabolites of interest. Thus, proper sample preparation protocols will be developed.

In both cases, a triple-quadrupole electrospray ionisation (ESI) MS is used MRM mode for analyzing the selected compounds, allowing for sensitive and robust and quantification.

The methods will be part of a medium throughput metabolomics pipeline employing on-line solid phase extraction (SPE) techniques for sample clean-up. Additionally, as both approaches can be implemented as complementary techniques in clinical settings, they should be compatible with non-invasive sampling techniques, e.g. Dried Blood Spots (DBS) in biological fluids (e.g. blood, urine).

65. INTERACTION OF MEF2 AND GR SIGNALING PATHWAYS AS A NEW DRUG TARGET FOR PSYCHOSIS

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Amphetamine use, both acute and chronic, results in symptoms that resemble many aspects of mania and psychosis. In inbred mice we showed that there are large interindividual differences in vulnerability to amphetamine, reflected by differential expression of Mef2 and GR target genes in the hippocampal CA1 area in the brain. Since both the stress system and Mef2 are involved in behavioral sensitization we set out to study *in vitro* whether an interaction exists between both signaling pathways.

Treating neuronally differentiated PC-12 cells with the synthetic GR ligand dexamethasone (DEX) we found that 1) GR-DNA binding is increased in the vicinity of Mef2-regulated genes, 2) expression of Mef2-regulated genes is DEX-regulated and can be blocked by the GR antagonist RU486 3) Mef2 phosphorylation, and hence Mef2 activity, is increased and 4) Mef2-DNA binding to validated Mef2 binding sites is increased.

The identified interaction between Mef2 and GR signaling pathways might represent a novel mechanism underlying psychostimulant induced sensitization and psychosis.

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66. OPTIMIZATION OF PROTEIN DIGESTION FOR THE CHARACTERIZATION OF DRUG-PROTEIN ADDUCTS

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Drug-protein adducts are suggested to play a role as mediators of Adverse Drug Reactions (ADR's). Their early detection and identification is crucial for successful drug development. Several Liquid Chromatography-Mass Spectrometry (LC-MS) based strategies have been developed for the analysis of drug-protein adducts, most of which depend on enzymatic digestion of the modified protein followed by LC-MS(MS) analysis of the resulting peptides. In this respect, it is crucial to choose the most effective protease and apply the optimal digestion conditions, such as buffer pH, temperature and digestion time.

In this study, an experimental design (XPD) approach was applied to the optimization of the digestion conditions for the enzymes trypsin, chymotrypsin and thermolysin. In comparison with the traditional one-variable-at-a-time (OVAT) approach, the use of an XPD reduced the number of necessary experiments considerably. Additionally, the XPD revealed significant interactions between the different conditions, which are neglected by the OVAT approach.

The digestion conditions were optimized for Human Serum Albumin (HSA) adducts. HSA is often a target for reactive drug intermediates due to its high abundance in the liver and the highly reactive free thiol located on cysteine-34. Therefore, the identification and yield of the peptide fragment containing the modified cysteine was used to evaluate digestion optimality in addition to the overall protein coverage. The found optima showed huge discrepancies to numerous published digestion conditions. A direct comparison was made between the XPD optima and literature of which the results are shown on the poster.

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Objectives: Recently, an optimal design technique was developed for the analysis of discrete variables [1]. We aimed to evaluate the feasibility of applying ED-optimality to screening of compounds taking model and parameter uncertainty into account. We illustrate these concepts using gabapentin as a paradigm compound.

Methods: The analysis consisted of two sequential steps: 1) model building & validation and 2) evaluation of a hypothetical screening experiment. Binary response in the logit space was used for optimisation of sampling times and dose levels under the assumption of known pharmacokinetic profile and expected potency range. Baseline/placebo effect, maximum effect and residual variability were assumed to be independent of treatment type and derived from historical data (n=45). We assumed drug potency (EC₅₀) to be the parameter of interest. Optimisation scenarios were based on feasibility, with limits for sample size, dose levels and sampling times.

Gabapentin concentrations were simulated for the selected range of doses and sampling times using a two-compartment pharmacokinetic model (V₂=0.18 L, V₃=3.8L, Cl=0.03 L/h, K_a=0.6 h⁻¹, Q=78 L/h, F=0.83). The estimated EC₅₀ was 198 ng/ml. Validation of the optimised design included simulation of response and refitting of the data to the same logistic model. For the prospective use of the method, optimisation factors were reassessed by testing a range of EC₅₀ values for a hypothetical compound with similar pharmacokinetic profile. Response profiles based on candidate designs were then simulated and data analysed using a logistic model. In addition to parameter estimates, dose response curves are also presented.

A Monte Carlo (MC) integration technique was used to integrate the FIM with Latin hypercube (LH) sampling. POPED 2.10/ MATLAB 7.9 were used for the optimal design. Simulations were performed in NONMEM 6.

Results: Our results show that optimal design concepts can be used together with logistical regression modelling to facilitate the screening of compounds in pain research. This approach reduced the uncertainty around the parameter estimates as evidenced by relative standard error values. Dose selection is essential for accurate parameter estimation (i.e., low relative standard error). Sampling windows ranged from 0 to 8.8 hrs post-dose. Relative standard errors in parameter estimates remain sensitive to group size, despite repeated sampling.

Conclusions: We show how optimality concepts can be used to assess drug potency in screening experiments using historical priors to support model parameter estimation.

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68. UNRAVELLING THE POTENTIAL SOURCE OF THE DECREASED BARRIER PROPERTIES OF HUMAN SKIN EQUIVALENTS

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The main skin barrier is located in the intercellular lipid regions of the outermost layer of the skin, namely the stratum corneum (SC). In a previous study we have shown that three of our in-house human skin equivalents (HSEs) have an increased permeability for benzocaine, which is indicative of decreased barrier properties. In order to determine whether the decreased skin barrier function of HSEs is caused by the culture conditions, we used full-thickness native human skin explants and expanded them under the same culture conditions that are used to generate HSEs. We investigated the barrier properties of the SC that was formed by the outgrowth of the explant and compared them to the barrier properties of the previously investigated HSEs and native human skin.

Our results show that the SC that is formed by the outgrowth of the full-thickness explant shows many similarities with the HSEs: 1) they show the presence of all barrier lipid classes that are present in human SC, although they have a relatively lower free fatty acid content than native human SC, 2) they contain saturated and mono-unsaturated long-chain free fatty acids in their SC, of which the latter is only present in minimal quantities in native human SC, 3) they show a less densely packed lateral lipid organization compared to human SC and 4) the SC lipids are organized in lamellar phases, similar to native human SC.

These results clearly demonstrate that the culture conditions have a strong influence on the skin barrier that is formed *in vitro*. Optimization of the culture conditions may therefore lead to improved barrier properties of HSEs. We hypothesize that the reduced free fatty acid level and the presence of mono-unsaturated long-chain fatty acids account for the predominant absence of the dense lipid packing in the SC of HSEs, resulting in decreased barrier properties. Therefore improvements of the culture conditions should be directed towards increasing the saturated long-chain free fatty acid content and reduction of the amount of mono-unsaturated free fatty acids. This will be subject of future studies.

69. PLAIN AND MONO-PEGYLATED RECOMBINANT HUMAN INSULIN EXHIBIT SIMILAR STRESS-INDUCED AGGREGATION PROFILES

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PEGylation has been suggested to improve the stability of insulin, but evidence for that is scarce. Here, we compared the forced aggregation behavior of insulin and mono- PEGylated insulin.

Therefore, recombinant human insulin was conjugated on lysine B29 with 5-kDa PEG. PEG-insulin was purified by size-exclusion chromatography (SEC) and characterized by mass spectrometry (MS). Next, insulin and PEG-insulin were subjected to heating at 75 °C, metal catalyzed oxidation, and glutaraldehyde cross-linking. The products were characterized physicochemically by complementary analytical methods. Mono-PEGylation of insulin was confirmed by SEC and MS.

Under each of the applied stress conditions, insulin and PEG-insulin showed comparable degradation profiles. All the stressed samples showed submicron aggregates in the size range between 50 and 500 nm. Covalent aggregates and conformational changes were found for both oxidized products. Insulin and its PEGylated counterpart also exhibited similar characteristics when exposed to heat stress, that is, slightly changed secondary and tertiary structures, covalent aggregates with partially intact epitopes, and separation of chain A from chain B. Both glutaraldehyde-treated insulin and PEG-insulin contained covalent and non covalent aggregates with intact epitopes, showed partially perturbed secondary structure, and substantial loss of tertiary structure. From these results, we conclude that PEGylation does not protect insulin against forced aggregation.

70. MICRO/NANO SAMPLING FOR LOCAL METABOLOMICS

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A nano/micro sampling device for local metabolomics is being developed. The main envisioned application is the sampling and analysis of interstitial fluid (ISF) from the skin. For this application, a device is being developed that uses microneedles to penetrate approx. 150 µm into the skin (i.e. into the viable epidermis) to sample ISF. Metabolites in the ISF are expected to better reflect local processes as ISF is not actively transported through the body as e.g. blood.

To be able to handle, efficiently stabilize and analyse the small sample volumes associated with microneedles, the needles are integrated with a microfluidic device. The microfluidic device will enable rapid sample stabilization, preparation and interfacing with the MS for sample analysis. Integration of the sampling needles on a microfluidic device will significantly reduce dead volumes and mixing associated with conventional couplings. Furthermore, this should allow for the ultra rapid sample stabilization and sample preparation as the sampled fluid can be directly mixed with stabilization agents on-chip. The modules are implemented in a very flexible

platform, so in addition to sampling from the skin the envisioned integrated device can in principle also be exploited in e.g. the analysis of a small droplet of blood.

A feasibility study for the construction of a nano/micro sampling device for local metabolomics in the skin and development and optimization of microfluidic chip production has been performed. The manufacturing and performance of microneedles for penetration of stratum corneum, sampling of interstitial fluid (ISF) and interfacing with microfluidic chip has been assessed and a production procedure of microfluidic chip in polydimethylsiloxane (PDMS), including monolithically integrated valves, pumps and electrospray emitters was developed.

The feasibility of sample analysis was assessed by performing MS analysis of epidermis lysate, performing ESI-MS from microneedles and from on-chip electrospray emitter of academic samples.

71. ENGINEERED P450 BM3 AS BIOCATALYSTS FOR REGION- AND STEREOSELECTIVE HYDROXYLATION

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Selective hydroxylation of non-activated C-H bond by conventional chemical synthesis still poses a challenge due to the lack of selectivity yielding a variety of side products. Therefore, enzymatic and microbial biotransformations for selective hydroxylation have gained importance over the years. Cytochrome P450 BM3 from *Bacillus megaterium* (CYP102A1) is a highly active monooxygenase that has been engineered both by site directed and random mutagenesis to catalyze the hydroxylation of a wide range of drug-like molecules.

In this work, the ability of engineered P450 BM3 to perform regio- and stereoselective hydroxylation of different substrates is being studied. P450 BM3 mutants that can selectively produce individual metabolites with high turnover numbers have been identified and the highly active and selective enzymes will be used to enable large scale production of hydroxylated variants in an efficient and low cost manner. Thus, engineered P450 BM3s are promising tools for selective hydroxylation of industrially relevant molecules.

72. FRAGMENT LIBRARY SCREENING REVEALS REMARKABLE SIMILARITIES BETWEEN HISTAMINE H4 RECEPTORS AND SEROTONIN 5-HT₃ RECEPTORS.

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Chemical genomics brings together diversity-oriented chemical libraries and information rich cellular assays with the intention of discovering novel hits for further optimization. A fragment library containing 1040 diverse compounds was screened against a wide variety of protein targets; these include an Ion channel (5-HT₃), a GPCR (Histamine H4), Acetyl Choline Binding Protein (AChBP) and a kinase (PKA).

It was shown that the fragment library does not contain promiscuous binders and, in general, different targets bind different fragments. However, the fragment hit set of two receptors, namely 5-HT₃ and H4, show remarkable overlap, illustrating similarities in ligand binding and indicating selectivity issues for 5-HT₃ and H4 drug development.

73. EXPLORING A NEW THERAPY FOR NEUROBLASTOMA: SILENCING OF DOUBLECORTIN-LIKE KINASE USING RNA-INTERFERENCE

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Neuroblastoma (NB) is the most common extracranial solid cancer in childhood and the most common cancer in infancy. Microtubule-destabilizing agents are used in the treatment of these tumors. However, resistance to chemotherapeutic agents and systemic toxicity make NB a difficult drug target.

In our previous work, we found that doublecortin-like kinase (DCLK) gene transcripts are crucial microtubule-associated proteins for correct proliferation and differentiation of neuroprogenitor cells. Gene expression profiling revealed a high expression of these transcripts in NB patients. Furthermore, these transcripts are endogenously expressed specifically in neuroblasts but are not found in other cell types. Suppression of DCLK by short interfering RNA (siRNA) disrupted the mitotic spindles in NB cells and gene expression profiling revealed numerous differentially expressed genes indicating apoptosis. Apoptotic cell death of NB cells by DCLK knockdown was further confirmed by several assays.

In addition, a synergistic effect inducing apoptosis by DCLK silencing and microtubule-destabilizing agents was detected in NB cells. We also found a significant correlation between DCLK expression and genes related with mitochondria activity in human neuroblastomas. Recently, we demonstrated the link between DCLK expression and mitochondria activity. Furthermore, we showed a successful delivery of siRNA targeting DCLK to NB cells by using specific peptide-siRNA conjugates.

In conclusion, silencing of the DCLK gene by siRNA interference is a novel potential therapeutic approach for NB with the promise of combining high specificity with fewer side effects. Peptide-siRNA conjugates might be the tool needed for specific NB delivery.

74. INTERACTIONS OF ORGANOPHOSPHATES WITH KERATINS IN THE CORNIFIED EPITHELIUM OF HUMAN SKIN

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There is an increased interest in a complete picture of contact risk and behaviour of organophosphorus compounds in the skin, since exposure via the skin is a relevant phenomenon not only from a military, but also from a civilian point-of-view.

The signs and symptoms of acute OP poisoning are an expression of the effects caused by excess acetylcholine (ACh), i.e. cholinergic syndrome. Their toxicity is not limited to the acute phase, however, and chronic effects have long been noted. Neurotransmitters such as acetylcholine (levels of which are affected by OP pesticides) are profoundly important in the brain's development, and many OP's have neurotoxic effects on developing organisms, even in case of low levels of exposure.

As a result of these acclaimed negative health effects of organophosphates, assessment of exposure to OP pesticides has gained a lot of interest during the last decades and from a military point of view, assessment of exposure to organophosphate-based chemical warfare agents is of pivotal importance for medical treatment of casualties. Methods for OP biomonitoring have mainly relied on determination of hydrolysis products, but in recent years various methods for assessment of exposure to chemical agents based on analysis of modified blood proteins have been developed. Though highly sensitive and well-accepted, a serious drawback of these methods is that invasive sampling methods are required in order to obtain suitable samples for analysis. Since for both OP pesticides and various chemical warfare agents the skin is a predominant route of entry, we hypothesized that proteins in the skin might represent an ideal source of unequivocal and persistent biomarkers

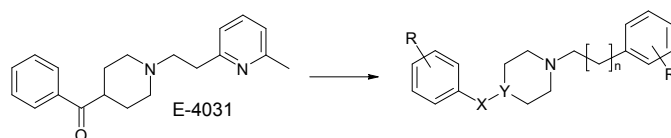
for exposure to these particular compounds. In human skin keratins are the most abundant proteins in the uppermost layer of the epidermis, the stratum corneum (horny or callus layer) and they have been shown to be prone to modification by various alkylating chemicals, including sulfur mustard, reactive metabolites resulting from naphthalene and various skin allergens.

We here present our first results on the covalent binding of a selection of relevant organophosphates to keratin proteins present in the thick cornified epithelium of human plantar skin (callus), in order to establish a firm basis for the development of future non-invasive methods for exposure assessment.

75. EXPLORING CHEMICAL SUBSTRUCTURES ESSENTIAL FOR HERG K⁺ CHANNEL BLOCKADE BY SYNTHESIS AND BIOLOGICAL EVALUATION OF E-4031 ANALOGUES

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In this study we followed a new approach to analyze molecular substructures required for hERG channel blockade. We designed and synthesized a series of analogues of E-4031, a potent hERG potassium channel blocker, and established structure-activity relationships (SAR) for their interaction with this important cardiotoxicity-related off-target.

Structural modifications to E-4031 were made by diversifying the substituents on the phenyl rings, changing the piperidine ring to piperazine and simplifying the carbonyl group to methylene. The analogues were evaluated in a radioligand binding assay and SAR data were derived with the aim to specify structural features that give rise to hERG toxicity.

Analogues of E-4031 with a phenyl group instead of 6-methylpyridine showed an increase in hERG affinity. Introduction of lipophilic substituents on the phenyl rings also increased the affinity, as was true for the reduction of the carbonyl group. The piperazine ring, however, led to a strong decrease in affinity.

76. YEAST AS A MODEL TO STUDY INTERPLAY BETWEEN CYTOCHROME P450 AND GLUTATHIONE S-TRANSFERASE IN DRUG TOXICITY

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Cytochrome P450s (CYPs) and glutathione S-transferases (GSTs) are known to play an important role in mechanisms leading to adverse drug reactions. Polymorphisms or absence of genes encoding these proteins, therefore, can have a severe impact on the individual susceptibility of patients to drug toxicity. Moreover, altered expression of human GSTs has often been implicated in increased resistance to anticancer drugs. However, little is known about CYP and GST interplay.

We aim to investigate concomitant CYP bioactivation and GST detoxification of drugs in a cellular context, using yeast as a model eukaryote. Bacterial CYP BM3 mutants, engineered to be capable of metabolizing drug-like compounds, were expressed in *S.cerevisiae*. Incubations of these strains with selected drugs showed the formation of drug metabolites and increased drug sensitivity. Human cytosolic GSTs alpha, mu and pi were also expressed and their activity was confirmed by the conjugation of glutathione to 1-chloro-2,4-dinitrobenzene.

Using this yeast model, involvement of CYPs and GSTs in drug toxicity (or resistance) will be studied both individually and combined, e.g. by monitoring cell growth, formation of protein-/DNA-/GSH-adducts, the formation of reactive oxygen species and by studying gene expression patterns. The suitability of yeast as a model system for CYP and GST interplay will be evaluated.

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For scaling propofol clearance between (preterm) neonates, infants, children, adolescents and adults, an allometric model with an exponent that matures with bodyweight (bodyweight dependent maturational exponent model, BWME-model) was developed leading to improved model performance compared to a $\frac{3}{4}$ allometric scaling model ^[1]. To explain this result, an in-depth study of the allometric exponents in different age combinations was performed.

We systematically selected two out of six studies comprising neonates, infants, toddlers, children, adolescents or adults ^[2,3,4,5,6,7,8] and performed a population pharmacokinetic analysis using NONMEM VI on each of these combined datasets. A three-compartment model together with an allometric scaling model for clearance was applied to estimate the value for the exponent of the allometric equation for clearance in every combined dataset.

The value for the allometric exponent for maturation in propofol clearance was found to vary mostly when the young age range (with bodyweights less than 6 kg) was included in the dataset resulting in estimated exponent values above 1. In older children (with bodyweights greater than 6 kg) and adults, the values for the allometric exponent were lower than 1. The allometric scaling exponents between the paediatric population and the adult population were in accordance with the exponent values that were identified in the BWME- model. However, the allometric scaling exponents of the combined datasets within the paediatric population were different from the results of the BWME-model.

Different allometric exponents for maturation in propofol clearance were identified depending on the included age-range, with values higher than 1 in young infants and values lower than 1 in older age ranges. Our findings confirm that allometric exponent changes across age-ranges and explain the fact that the $\frac{3}{4}$ allometric scaling model performs well in some of paediatric studies and fails in others.

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78. TREATMENT EFFECTS OF RIMONABANT AND A MULTI-TARGET APPROACH ON PLASMA LIPIDOMICS IN APOE*3 LEIDEN CETP TRANSGENIC MICE

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Obesity and its related diseases such as diabetes, cardiovascular diseases, dyslipidemia, are becoming a public health concern globally. Using lifestyle modification, diet and exercises intervention to control obesity have obtained sub-optimal results and limited long-term efficacy. Therefore, safe and effective treatments for obesity are in high demand.

Rimonabant, a selective cannabinoid-1 receptor antagonist for obesity treatment, targets on the endocannabinoid system, which regulates food intake, lipid and glucose metabolism in the central nervous system and peripheral tissues. Both pre-clinical and clinical studies showed that rimonabant intervention result in long-term maintained weight loss and optimizing cardio-metabolic risk factors including insulin resistance and lipid profiles. But its psychiatric adverse events including depression and suicidal attempts withdrew rimonabant's general application.

A Chinese multi-herbal formula (namely SUB885C in this poster) using multiple -targets approach to regulate body weight and dyslipidemia has long been used in China for treatment of metabolic syndrome, in particular early stage of diabetes type 2 in combination with obesity. SUB885C contains eight natural herbs: Fructus Crataegi, Folium Nelumbinis, Folium Apocyni, Flos Rosae rugosae, Radix et Rhizoma Rhei, Depuratum mirabilium, Thallus Sargassi, Honey fried Radix Glycyrrhizae. One SUB885C intervention study on male ApoE*3Leiden transgenic mice with pre-diabetes has already shown its control effect on insulin resistance.

The aim of the present study is to further evaluate the treatment effects of SUB885C on weight and dyslipidemia regulation. ApoE*3 Leiden CETP female mouse model was used to compare treatment effects of SUB885C, rimonabant and the placebo control on body weight and plasma lipidomics. After 2-week intervention, three treatments showed a separation trend in lipidomics pattern. Only rimonabant showed a significant weight reduction while SUB885C showed a wide range regulation on cholesterol esters, triglycerides, sphingomyelin and fatty acids and result in a prominent improvement in lipid profile.

79. REAL WORLD APPLICATIONS OF PROTEOCHEMOMETRIC MODELING

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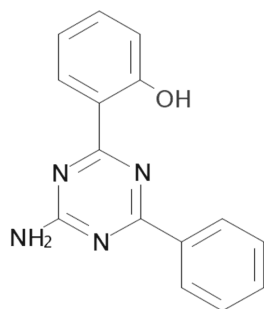
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The early phases of drug discovery often employ *in-silico* models to rationalize structure activity relationships and to predict the activity of novel compounds. However, the predictive performance of these models is not always acceptable and the reliability of prospective predictions – both to novel compounds and to related protein targets, where possible – is in many cases limited.

Proteochemometric modeling (PCM) ¹ attempts to remedy this situation by adding a target description, based on physicochemical properties of the binding site, to conventional, ligand-based bioactivity models. Our approach of PCM is based on Scitegic circular fingerprints on the compound side and on a customized feature based protein fingerprint on the target side which is based on a selection of physicochemical descriptors obtained from the AAindex database.

In this work we present a prospective validation on the four Adenosine receptor subtypes. Our model was trained on a dataset consisting of both human receptors and rat receptors. Protein descriptors were created by aligning the receptor's respective amino acid sequences, compound descriptors used were standard Scitegic Circular Fingerprints. After the model was created it was used to virtually screen and select 55 compounds from a database of approximately 800,000 compounds. Of these we identified 6 novel high affinity compounds on the A_{2A} receptor.

We conclude that PCM is able to reliably extrapolate the activity of compounds to new targets, on the datasets employed here and within the limitations of the training data provided. This ability makes it a useful tool to predict the activity of ligands on highly related proteins, such as in case of enzyme mutants or designing multi-target drugs.



5169-1142

Figure 1: One of the identified high affinity ligands

80. PREDICTION OF HUMAN BRAIN TARGET SITE CONCENTRATIONS: THE PRECLINICAL APPROACH USING MICRODIALYSIS AND IN SILICO MODELING

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To be able to predict CNS drug effects in human a more mechanistic understanding is needed on the individual contribution of mechanisms involved in brain target site distribution and ultimate drug effects. Moreover, insight is needed on the variability of such contributions, as a result of species, gender, and age differences as well as by disease state, diet, drug treatment, etc. So far, CSF concentrations are often used as a surrogate marker for brain target site concentrations during drug development. However, the use of CSF concentrations for the prediction of CNS drug effects is not that simple and straightforward, as a generally applicable relationship between CSF concentrations and brain target site concentrations does not exist.¹

The intracerebral microdialysis technique allows quantitative investigation of the processes that determine drug distribution into and within the CNS. This technique can be applied in multiple sites in the brain. With integrative cross-compare designed studies important mechanisms can be influenced in a well-controlled and systematic manner, for example specific inhibition of an efflux transporter. With the use of advanced mathematical modeling procedures the data obtained may be analyzed to dissect contributions of individual mechanisms in animals as links to the human situation.

Acetaminophen (i.v. infusion of 15 mg/kg in 10 min) was used as a model drug for passive blood-brain transport. Acetaminophen concentrations in brain ECF, CSF (from the lateral ventricle (CSF_{LV}) and cisterna magna (CSF_{CM})), and plasma were determined by HPLC and electrochemical detection. Quinidine (i.v. infusion of 10 or 20 mg/kg in 10 min) was used as a model drug for active blood-brain transport by P-glycoprotein (P-gp). The specific impact of P-gp mediated transport was studied by pre-administration of the P-gp blocker tariquidar (i.v. infusion of 15 mg/kg in 10 min). Quinidine concentrations in brain ECF, CSF, and plasma were determined by HPLC and fluorescence detection.

The acetaminophen concentration-time profiles for the brain ECF are similar to the plasma concentration-time profiles, whereas the CSF_{LV} and CSF_{CM} concentration time profiles are ~4-fold lower. For quinidine, important differences between CSF and brain ECF concentrations were found, being differentially affected by P-gp transport.

Differences between the brain ECF and CSF concentration-time profiles could be caused by: (1) the difference in surface area between the blood-brain barrier (BBB) and blood-CSF barrier (BCSFB); (2) The high turnover rate of CSF, causing a diluting effect of CSF; (3) the difference in expression and activity of active transport systems at the BBB and BCSFB.

Population-based PK analysis is currently being performed, using NONMEM[®], to reveal the impact of the passive and active transport processes on the brain ECF – CSF relationship.

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81. COMPLEMENT FACTOR C5A MODULATES VEIN GRAFT REMODELING AND ACCELERATED ATHEROSCLEROSIS VIA INTERFERENCE IN MAST CELL ACTIVATION

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We previously showed a role for Mast cells (MC) and C3-activation in vein graft disease (VGD). MC also express receptors for complement-factor C5a, by which they can be activated. However the role of C5a in atherosclerosis is still unknown. The present study examines the effect of C5a on MC activation in VGD and subsequent accelerated atherosclerosis in ApoE-KO mice.

C5a-induced MC-activation in vitro resulted in increased tryptase release of 15% (p=0.007). In murine vein grafts (n=3-4/time point) MC tryptase mRNA and protein levels as studied by rt-PCR and (immuno)histochemistry,

decreased after surgery and increased from 7 to 28d. C5a(R) levels increased after surgery due to influx of inflammatory cells, than decreased and stabilized further on.

To study the effect of MC- and C5a-activation on VGD, C5a and DNP (a MC-activator) were applied locally around the vein graft during surgery (n=10 ApoE-KO/group) resulting in significant increased vein graft-thickening (VGT) at 28d (79 and 36% resp. compared to control). This was accompanied by an increase in perivascular MC. Systemic application of C5aR-antagonist or Cromolyn (a MC-stabilizer) resulted in significant decreased VGT (40 and 22% resp.). MC were decreased by 40% and 24%. To assess the direct activation of MC by C5a, mice were treated with C5a and Cromolyn, VGT was decreased by 54% (p=0.0002) compared to C5a-treated mice, to the level of Cromolyn treated mice.

These data indicate an important role for the chemotactic potency of C5a on MC and their subsequent activation in VGD.

82. TOWARDS SUB-TYPING OF RHEUMATOID ARTHRITIS PATIENTS USING A QUESTIONNAIRE BASED ON A FUSION OF CHINESE AND WESTERN DIAGNOSIS

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Arthritis like diseases such as rheumatoid arthritis, osteoarthritis, fibromyalgia, systemic lupus erythematosus and many others affect billions of people worldwide and are a common cause of disability. Management of these diseases is generally based on reducing inflammation and pain using medication. The trial and error approach of assigning medication leads to much suffering of side effects. There is a great need for personalized medicine for treating rheumatic diseases.

Chinese medicine (CM) is a practice developed over thousands of years with a personalized approach to treat rheumatic diseases. In the present study a questionnaire is developed based on symptoms used in Chinese medicine diagnosis to characterize arthritis patients. The goal is to sub-type arthritis patients into sub-groups that will benefit from different treatment regimes.

In the first part of this study the questionnaire data from 40 arthritis patients were explored and compared with Chinese medicine theory. Network theory and mapping algorithms were used to visualize the connections between the symptoms and the relationship with Chinese syndromes. Personal symptom patterns are created to visualize differences between patients. The data show the wide variety of symptoms that patients suffer from.

Subsequently two Chinese medicine experts ranked the Cold and Heat status of all the patients. Cold and Heat are basic Chinese medicine concepts and easy to distinguish by looking at symptoms. The Cold pattern can be described as severe pain in a joint or muscle that limits the range of comfortable movement which does not move to other locations. The pain is relieved by applying warmth to the affected area, but increases with exposure to cold. In contrast the Heat pattern is characterized by severe pain with hot, red, swollen, and inflamed joints. Pain is generally relieved by applying cold to the joints. Other symptoms include fever, thirst, a flushed face, irritability, restlessness, constipation, and deep-colored urine. Our hypothesis is that Cold patients suffer from a more hormone related disorder while Heat patients have a more immune related disorder. Cold patients might therefore respond better to steroids and Heat patients more to biologicals.

The Cold and Heat ranking were used to develop a method to apply the questionnaire data to predict these rankings in future patients. Categorical principal component analysis revealed which questions are relevant for the Heat and the Cold variables. After validating this model further the questionnaire can be optimized for use in a clinical setting.

83. AUTOMATED HIGH CONTENT IMAGING OF CELL ORGANELLE MORPHOMETRY AND FUNCTION IN CELLULAR STRESS RESPONSES

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Current preclinical *in vitro* predictive toxicity models mainly focus on end point- and single time point- assays. These assays often lack mechanistic information content and are not specific enough to allow for predictive safety assessment of new drug entities. We anticipate that the specific subcellular perturbations, e.g. mitochondrial damage, endoplasmic reticulum stress or DNA damage, are underlying chemical injury responses.

In the current project a high temporal resolution high content-based toxicity assay will be developed using high throughput confocal microscopy imaging of specific organelle morphometry and function. Since drug-induced liver injury is a major concern, liver cells will be used. Transgenic liver GFP-reporter cell lines will be developed using BAC transgenomics approaches. Image analysis algorithms will be established that allow a multiparametric analysis of organelle function and morphology.

The specific objectives of the project are: *i.* to generate reporter cell lines for organelle specific morphometry and functional analysis; *ii.* to develop multiparametric image analysis algorithms for detecting morphological changes relevant for *in vivo* toxicity testing; *iii.* to evaluate drug compound class specific organelle stress responses; *iv.* perform imaging-based RNAi screening to identify the signaling components that define the drug-induced cellular stress-responses. We anticipate that at the end of the project we will have established an automated high content imaging model to predict preclinical toxicity based on cell organelle specific perturbations.

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84. INTEGRIN – ECADHERIN CROSS TALK IN CONTROL OF BREAST CANCER METASTASIS

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The purpose is to elucidate the mechanism that underlies integrin modulation of cell invasion and investigate how it controls breast cancer metastasis.

It was found that silencing of the Ecadherin gene was sufficient to induce a switch to individual cell migration and overexpression of Ecadherin in 4T1 sh-b1 inhibit metastasis. However, silencing Ecadherin in 4T1 is not sufficient for enhanced metastasis. These findings indicate that b1 integrins control Ecadherin mediated cohesion as well as additional pathways that together determine the efficiency of migration *in vivo* and metastasis. Ingenuity pathway analysis (IPA) software will be used to analyze microarray data by comparing wild type 4T1, two b1-shRNA lines, Ecadherin-shRNA line and the b1-shRNA lines in which Ecadherin expression has been restored. Which other parallel pathways are involved will be determined.

Constitutive shb1 constructs have been used to show that cells lacking b1 integrins already from the moment of transplantation form smaller tumors but colonize the lungs more efficiently. Conditional knock down or knock out models will be built to allow deletion at any stages in established breast tumors. A 4T1 cell line with conditional shb1 lentiviral construct will be generated. In the absence of IPTG (isopropyl- β -D-thio-galactoside), the expression of shRNA is repressed. After IPTG is present, b1 will be silenced by shRNA. These cells will be injected in mammary glands and spontaneous metastasis of the resulting tumors will be analyzed with IPTG addition *in vivo* at various timepoints. A conditional b1 knock out mouse model will be established. Mammary epithelial cells (MEC) will be isolated from the b1 homozygous floxed mice carrying Cre-ERT2 transgene. MECs will be lentivirally transduced with lentiviral vectors for stable expression of telomerase and for silencing of "p53" or other oncogenes in breast cancer cells (still need to be selected) to immortalize and transform these cells. Then these cells will also be transplanted into 3D matrices or into the mammary gland of immunodeficient recipient mice to test invasion and metastasis after deletion of b1 at different time points by tamoxifen treatment.

These two models would help to identify the target for integrins, either Zeb2 or mir200 family or other candidate genes in the identified network of miRNAs and Ecadherin repressors. Regulation in these systems will also be analyzed.

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Steroid receptor coactivator 1 (SRC-1) is a coregulator of the glucocorticoid receptor. It is involved in the regulation of basal expression of corticotropin releasing hormone (CRH) and in modulation of CRH expression by glucocorticoids in the brain. The *src-1* gene generates two isoforms which depend on the inclusion or exclusion of one exon. The two isoforms differ in their activities and distribution in the brain, with SRC-1a being more abundant in the paraventricular nucleus of the hypothalamus (PVN) and able to repress the *crh* promoter, whereas SRC-1e is more abundant in the central amygdala (CeA) and able to activate the *crh* promoter.

Antisense oligonucleotides have been shown to induce exon skipping in various animal models. Here, by injecting fluorescently labeled antisense 2-O'-methyl-phosphorothioate oligoribonucleotides (AONs) locally in the CeA of c57bl6 mice we sought to shift the expression ratio of the two isoforms in favour of SRC-1a. We first tested the effect of the AONs on the expression ratio of the two isoforms on a C2C12 cell line, where it shifted from 1:1 to 5:1 in favour of SRC-1a. Subsequently, we investigated the uptake and subcellular localization of AONs *in vivo* and their possible toxic effects compared with vehicle. We showed that AONs were taken up by CRH expressing cells in the CeA and localized in the cell nucleus. Furthermore, immunofluorescence for markers of astrocytes and activated microglia revealed no significant differences between AONs and saline control.

We expect that RT-qPCR will show an inversion of the mRNA expression ratio of the two isoforms in the CeA in favour of SRC-1a. We hypothesize that SRC-1 isoform switching in the CeA will result in lower expression of CRH. In conclusion, AONs shifted the expression of the two isoforms *in vitro* and did not produce any detectable toxic effects after local injections in the CeA, and is a promising approach to manipulate gene expression in brain.

86. RADIOLIGAND BINDING ASSAYS FOR THE HUMAN CHEMOKINE RECEPTOR CCR2B:
A COMPARISON OF A NATURAL (¹²⁵I-CCL2) AND A SYNTHETIC ([³H]INCB3344) RADIOLIGAND

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The chemokine receptor CCR2b is a G protein-coupled receptor that is primarily activated by the endogenous chemokine CCL2. It is involved in various diseases which are characterized by chronic inflammation and therefore several small molecule antagonists have already been developed. Despite a high target affinity, these antagonists show poor clinical efficacy. In order to improve the *in vivo* efficacy we aim to develop antagonists that have a long residence time on the receptor. Since the residence time can be determined with kinetic radioligand binding assays, we decided to select two different radioligands for validation. Here we compare the 8.6 kDa protein and natural agonist ¹²⁵I-CCL2 with the small molecule antagonist [³H]INCB3344 as radioligands for the human CCR2b receptor.

¹²⁵I-CCL2 and [³H]INCB3344 bind the human CCR2b with high affinity, having a equilibrium dissociation constant (K_D) of 0.068 nM and 1.8 nM, respectively. In kinetic assays we determined the association and dissociation rates of both radioligands. Next we compared the use of [³H]INCB3344 and ¹²⁵I-CCL2 for determining the affinity of four reference CCR2b small molecule antagonists. Three of the four compounds are structurally similar and displace both [³H]INCB3344 and ¹²⁵I-CCL2. The fourth compound displaces ¹²⁵I-CCL2 but did not interfere with [³H]INCB3344 binding to CCR2b. Hence, it may bind to a different (allosteric) site at the receptor than the other small molecule antagonists.

In summary, both radioligands are suitable for studying the binding behaviour of ligands on the CCR2b receptor, with [³H]INCB3344 being more convenient to work with. ¹²⁵I-CCL2 may be the radioligand of choice for the characterization of allosteric small molecule antagonists.

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The Center for Translational Molecular Medicine study “Circulating Cells” will screen 800 patients with indications for atherosclerosis. From these patients blood samples will be taken and analyzed for biomarkers indicative of atherosclerosis progression. Follow-up screenings will take place for a period of 5 years to determine the progression of the disease. The aim of this study is to characterize circulating T cell populations in patients with stable and unstable angina pectoris that may be indicative for progression of atherosclerosis.

Fluorescence activated cell sorting (FACS) will be used to characterize circulating T cell and monocyte populations using a panel of antibodies. The antibody panel will be used to monitor the distribution of various T cell and monocyte subsets. Using advanced database analysis software patients will be clustered according to their diagnosis and differences in T cell and monocyte subpopulations. According to results drawn from this first stage analysis the antibody panel will be further optimised and will eventually be used as a predictive tool for atherosclerosis progression.

The T cell and monocyte subsets will be further characterized by determining microRNA expression profiles. To investigate the implications of microRNA in atherosclerosis we will apply deep sequencing to different subsets of T cells and monocytes to identify microRNAs that are important in atherosclerosis disease progress. These data will subsequently be used to create a custom microRNA array to screen patients for microRNA expression in CD4, CD8 and CD14 positive cells.

Combining the data from the FACS staining and the microRNA array will provide new insight into the role of T cell and monocyte differentiation during the development of atherosclerosis and will result in an improved treatment regime for patients with atherosclerosis.