Theme V – Efficacy and Safety Testing of Drugs and Biologicals

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Session V-1: Pathways based assays in drug development

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Session V-1: Oral presentations

V-1-066

Development of functional human cell based cardiovascular construct for cardiotoxicity assessment

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Functional human cell based *in vitro* cardiac construct would benefit the present need for reliable *in vitro* model to replace animal biology based test systems in cardiotoxicity assessment (Guo et al.). Here the aim was to develop an *in vitro* cardiovascular construct modeling human heart tissue.

In this study, a vascular-like network formed by human adipose stromal cells/fibroblasts and human umbilical vein endothelial cells is used as a natural scaffold. Human embryonic/induced pluripotent stem cell-derived cardiomyocytes (CM) are seeded on top of the vascular-like network to form a cardiovascular construct. Vascular-like network formation, CM orientation and functionality as well as drug responses of the cardiovascular construct were characterized.

Results showed that the vascular-like network increases the viability and functionality of the cardiomyocytes. CMs were elongated and aligned with the vascular-like network and formed a synchronously beating cardiovascular construct. The electrical activity, calcium metabolism as well as response to adrenalin were shown to be normal in the construct.

In conclusion, the vascular-like network supports the orientation and contractile properties of CMs. Our results suggest that the developed cardiovascular construct has the potential to serve as a model suitable for toxicological and safety pharmacological testing of compounds targeting human cardiovascular system.

Reference

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V-1-229

Bioengineered organs-on-chips for modeling disease, drug safety and efficacy screening

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More than 30% of promising medications have failed in human clinical trials because they are determined to be toxic despite promising pre-clinical studies in animal models, and another 60% fail due to lack of efficacy. The challenge of accurately predicting drug toxicities and efficacies is in part due to inherent species differences in drug metabolizing enzyme activities and cell-type specific sensitivities to toxicants. To address this challenge in drug development and regulatory science, the NIH launched the Organs-on-Chips Program to develop alternative approaches that would enable early indications and potentially more reliable readouts of toxicity or efficacy, and provide suitable alternatives for animal testing. The goal of the program is to develop bio-engineered microdevices that represent functional units of the 10 major human organ systems: circulatory, respiratory, integumentary, reproductive, endocrine, gastrointestinal, nervous, urinary, musculoskeletal, and immune. The opportunities for significant advancements in the prediction of human drug toxicities require a multi-disciplinary approach that relies on an understanding of human physiology, stem cell biology, material sciences and bioengineering. This unique and novel *in vitro* platform could help ensure that safe and effective therapeutics are identified sooner, and ineffective or toxic ones are rejected early in the drug development process.

V-1-295

Fishing for teratogens: a consortium effort for a harmonized zebrafish developmental toxicology assay

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A consortium of biopharmaceutical companies was formed to develop a harmonized zebrafish developmental toxicity assay (ZDTA).In the first phase of this project, an optimized ZDTA method was developed to evaluate 10 known teratogens and 10 non-teratogens, which resulted in an overall predictive value of 88%. In this project, 38 proprietary pharmaceutical compounds from four companies were evaluated in two laboratories using the optimized ZDTA method in pond-derived or cultivated-strain wild type zebrafish embryos. Compound uptake analysis was also performed with all compounds. Compounds with confirmed embryo uptake of >5% achieved an overall predictive value of 82% & 75% at the 2 respective laboratories. Overall predictivity of the entire test set was 73% for one laboratory and 57% for the other when compared to their respective *in vivo* mammalian data. Low uptake (<5%) compounds classified as non teratogenic were re-tested up to 1000 μM, which improved ZDTA predictivity to 75% & 60%. When only logarithmic concentrations were considered (0.1, 1, 10, 100 & 1000 μM), ZDTA predictivity improved to 79% & 62%. Subsequent data analyses showed that technical differences rather than strain differences were the primary contributor to inter-laboratory differences in predictivity. Based on these results, the ZDTA is viewed as a promising screening tool for developmental hazard identification of pharmaceutical compounds.

V-1-670

Deciphering mechanisms of cisplatin-induced renal toxicity using a multi-omics-pharmacokinetics approach

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Cisplatin, a chemotherapeutic agent, works via formation of DNA adducts. The dose-limiting factor is nephrotoxicity, and due to accumulation and bioactivation, proximal tubular cells are highly sensitive to cisplatin.

Here, we used integrated omics (transcriptomics, proteomics and metabolomics) coupled to pharmacokinetics to identify mechanisms of toxicity induced by cisplatin. The human renal proximal tubular cell line RPTEC/TERT1 was treated repeat dose with subcytotoxic concentrations of cisplatin for up to 14 days.

Transcriptomic changes include activation of the p53, mTOR, AMPK, eIF2, and Nrf2 oxidative stress response pathways. Proteins changes could also be linked to mTOR and AMPK signalling, and DNA damage. Metabolomics revealed decreased L-carnitine levels, indicating an involvement of fatty acid metabolism which fits with decreased mRNAs of PPARalpha and BBOX1. Kinetic analysis showed a basolateral uptake and transport to the apical side. This fits with basolateral uptake of cisplatin via Ctr1 and OAT2, and its bioactivation outside the apical side of the cells.

This study demonstrates the power of integrated omics and biokinetics for uncovering deep mechanistic information pertaining to drug-induced adverse effects. It further shows how stable human cell culture-based models can be used for toxicity screening, moving an important step towards replacing animals in toxicity testing.

V-1-820

Assessment of current *in vitro* models at the protein expression level and correlation to the prediction of DILI

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Drug-induced liver injury (DILI) remains a burden to the public, the pharmaceutical industry and regulators. A key reason for this is due to our inability to accurately predict DILI at the preclinical stage using the model systems that are available today. It is therefore vital to fully understand what these current models are fit for as well as their limitations. This should then allow us to develop more innovative model systems that will enable mechanism-based predictions relevant to human DILI. To address this problem, in the MIP-DILI consortium we have carried out an extensive phenotypic characterisation of currently available *in vitro* systems, including primary human hepatocytes, HepaRG, HepG2 and Upcyte cells, at the protein expression level in order to determine their basal phenotype. In parallel, a multi-centre cytotoxicity study was also performed using these cell models and employing a combination of known hepatotoxins and non-hepatotoxins to determine their ability to predict DILI. The results from these studies will be used as discussion points to identify what our current *in vitro* models are useful for as we attempt to accurately model and predict DILI.

Session V-1: Poster presentations

V-1-011

Cardiotoxicity of captopril in chick embryos

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Captopril is one of angiotensin-converting enzyme (ACE) inhibitors and is indicated for initial therapy in patients with hypertension. ACE inhibitors are known to alter myocardial function manifested by electrocardiogram changes. With the recent concern for animal rights, experimental studies using mammals have been limited in number and methods. In order to develop alternative methods, we have studied the biological effects of drugs on the cardiovascular system of chick embryos using physiological techniques. The present study evaluated the effect of captopril on the heart in chick embryos.

Fertilized eggs of White Leghorns were incubated and investigated. Captopril was injected into the air sac of a fertilized egg. After injection with drug, the values of heart rate were measured. Electrocardiograms (ECGs) were recorded after the drug injection, and heart rate was determined from ECG wave cycles (Hiroyuki et al., 2009).

After the administration of captopril 0.5 mg/egg, the heart rate was not different compared with control. However, the heart rate was significantly decreased by the administration of 5 mg/egg, 10 mg/egg and 20 mg/egg captopril and arrhythmia was produced.

We have demonstrated that our recording system for ECG of chick embryos is useful for investigating the cardiotoxicity of captopril.

Reference

Hiroyuki, M., Iizuka, T., Okayasu, M. et al. (2009). *AATEX 14*, 954-957.

V-1-018

A zebrafish bioassay to evaluate gastrointestinal function

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Zebrafish are an important vertebrate model system with established and emerging applications for toxicology, drug discovery, and target optimization. Zebrafish fecundity, small size, and transparency through organogenesis, make them suitable for evaluating developmental toxicity of drug candidates, as well as safety assessment of cardiac, visual, and gastrointestinal (GI) functions. GI intolerance is a common preclinical finding and can be a serious safety concern in the clinic. Establishing a rapid, inexpensive, and predictive assay for GI transit would improve flexibility in drug screening and could have significant 3Rs impact as an alternative to conventional GI evaluation. Effects of test compounds on GI transit of fluorescent food were evaluated using fluorescence microscopy and spectrophotometry techniques. Results suggest strong agreement between the two measures and observed effects on GI function match known drug effects in humans. Results indicate significant inhibition of GI transit for atropine ($p<0.001$) and stimulation of transit for tegaserod and metoclopramide ($p<0.001$). We conclude that the measurement of GI transit time in zebrafish using a medium-throughput fluorescence assay can be useful for early drug candidate triage and may contribute to the reduction of GI safety testing required in more sentinent mammalian species.

V-1-019

Inhibitory effects of ambroxol on type A seasonal influenza virus infection *in vitro*

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Objectives: Mucolytic agent including, ambroxol and l-carbocisteine is known to have antioxidant and inflammatory properties with reduction of the release of inflammatory cytokines. L-carbocisteine also inhibits influenza A virus (IAV) infection by the reduced expression of the receptor on the human tracheal epithelial cells. Previous studies revealed that the protective effective of ambroxol, a mucolytic agent which has antioxidant properties and stimulates the release of pulmonary surfactant, against influenza-virus proliferation in the airway *in vivo* was investigated (1). To study whether ambroxol exerted anti-influenza A virus activity on MDCK cells *in vitro*.
Methods: To determine the inhibitory effect of ambroxol on influenza A virus infection, MDCK cells were treated with ambroxol and l-carbocisteine after/before influenza A virus infection.
Results: Ambroxol and l-carbocisteine treatment after influenza A virus infection had no effect on influenza virus replication, whereas, treatment with ambroxol and l-carbocisteine before influenza A virus infection induced distinct reduction in IVA replication.
Conclusion: These finding suggest that ambroxol may inhibit IAV infection via reduced expression of the receptor for influenza virus in the MDCK.

Reference

Hiroyuki, M., Iizuka, T., Okayasu, M. et al. (2009). *AATEX 14*, 954-957.

V-1-051

Electron paramagnetic resonance (EPR) spin labeling study of HT-29 colon adenocarcinoma cells after hypericin-mediated photodynamic therapy

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Aims: One of the alternative treatment methods for colon cancer is photodynamic therapy (PDT). Hypericin (HYP) derived from *Hypericum perforatum* is a strong candidate photosensitizer for PDT (1). Membrane fluidity of the cancer cell is related to cancer metastases (2). Presence of cancer cells increase the heterogeneity and also fluidity of plasma membranes (3). Our interest was focused on the biophysical status of plasma membranes in relation to HYP-mediated PDT.
Methods: HT-29 colon cancer cells were treated with 0.04, 0.08 or 0.15 μM HYP concentrations irradiated and examined (24 h). Cells incubated with 10^{-3} M 16-doxyl-stearic acid (16-DSA) spin label suspension for 60 min at 37°C. After centrifuguation the pellet was transferred to a capillary, EPR measurements were performed on a Bruker EMX-131 spectrometer at 23 and 37°C and repeated three times.
Results: The obtained spectra were evaluated by EPRSIMC program which provides the calculation of heterogeneous structures up to four spectral components with different fluidity characteristics. Generally, three spectral components were obtained. As the order parameters of the most populated components compared, an increase was observed with increased HYP concentration.
Conclusion: HYP-mediated PDT was observed to be effective on the fluidity of the HT-29 cells.

V-1-083

Nerve-muscle-cultures as an *in vitro* tool for pharmacologic testing

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Background: The *in vivo* mouse LD_{50} assay is routinely used for potency testing of botulinum toxins (Adler et al., 2010). However, this test is associated with severe pain and distress in animals and requires large quantities of mice (Bitz, 2010). Here we established co-cultures of spinal cord and muscle tissue as an alternative *in vitro* system for pharmacologic testing (Braschler et al., 1989).
Methods: Nerve-muscle-cultures were prepared from mouse embryonic tissue (C57/BL6J) and cultured for 21-24 days (Gähwiler, 1981). In these cultures spontaneous muscle activity was quantified in sham- and botulinum toxin-treated cultures for up to 3 days by video microscopy.
Results: Exposing the cultures to different concentrations of botulinum toxin A (1, 2, 5, 50 mouse units) reduced concentration-dependently the frequency of spontaneous muscle contractions.
Conclusions: The strength of the described *in vitro* assay is that spontaneous muscle contractions are monitored as read-out (Drexler et al., 2011). Thus, the pharmacological endpoint of botulinum toxin, which means a muscle relaxing effect, can be directly tested. This *in vitro* nerve-muscle-culture system might be a valuable tool for drug testing in the future.

References

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V-1-161

Use of upcyte® human hepatocytes to CYP inhibition and induction screening

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upcyte® human hepatocytes are "up-regulated" primary hepatocytes (PHHs) which have been driven into proliferation, whilst retaining important adult cell markers (Burkard et al., 2012). Phase 2 activities are similar to those in freshly isolated PHHs but CYP activities are equivalent to 5 day cultures. We have improved the upcyte® technology which resulted in higher basal CYP activities and greater responsiveness to inducers. For inhibition studies, upcyte® Hepatocytes were pre-cultured and CYP1A2 wells were induced using omeprazole. All other CYPs did not require pre-induction. The inhibitors were pre-incubated prior to the addition of substrates. For induction studies, upcyte® Hepatocytes were pre-cultured prior to the addition of inducers. The "Relative Induction Score" (RIS) prediction model was used to predict the *in vivo* induction potential. The RIS prediction model correctly identified the CYP3A4 potency of all compounds analyzed. These results support the use of upcyte® Hepatocytes for CYP inhibition and induction screening. upcyte® Hepatocytes are standardized such that the results are reproducible across experiments; this combined with the sheer quantities that can be generated from one vial of PHHs makes them a promising and unique alternative to PHHs for drug interaction screening.

Reference

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V-1-268

Using mode of action framework in prediction of liver toxicity

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The aim of the study was to develop and benchmark different liver toxicity Modes of Action (MOA). MOA, as inner layer of AOP concept, describes key events and processes starting with interaction of a compound with the cell towards biological response. MOA, as a representation of existing knowledge, concerns the linkage(s) between initial chemical binding, defined as the molecular initiating event (MIE), subsequent events on cell, tissue and organ level and biological outcome. In order to identify key events for which non-animal tests can be developed, MOA can be used, thereby facilitating mechanism-based, predictive toxicological assessments. Our focus was on 3 well studied liver toxicants and based on the existing knowledge, we created a computational model of biological pathways by manually annotating and processing molecular data from the literature from the

public domain (PubMed articles and FDA reports), describing cellular processes activated by these compounds. Moreover, we made all the data computable and applied newly developed bioinformatics analysis tools in order to group the compounds based on their structure similarity in addition to their MOA. Finally, for other similar liver toxicant and/or know biological downstream partners we were able to predict liver toxicity of these compounds and suggest possible MOA.

V-1-401

Trypanosoma cruzi: *in silico* and *in vitro* alternatives for identifying new antiparasites

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Identification of new antiparasites involve *in vivo* assays in the early phases. This includes Chagas disease, an endemic parasitosis caused by *Trypanosoma cruzi* (Chagas, 1909). Approximately 18 million people are currently infected, and 50,000 individuals die yearly (WHO, 2008). Only two drugs, nifurtimox and benznidazole, are available since 1970s, mostly due to toxicity issues. The objective of this work was to identify new antiparasites by using only *in silico* and *in vitro* alternatives. Therefore, we explored lapachone derivatives using cell culture and *in vitro* assays with *T. cruzi* infective forms, which revealed a potent biological activity for some of them (Ferreira et al., 2006). Molecules that are toxic for parasites and non-toxic for mammalian cells are then evaluated *in silico* and obtained data support the synthesis of candidate drugs (Da Rocha, 2013). This approach showed a structure-based relationship for safety and efficacy of some molecules. The *in vitro* method was developed by our group (Ferreira, 2006) using CBBR250 dye, whereas for the *in silico* approach we used free online programs. Using this strategy, we could select the best and most promising molecules hugely decreasing the use of animals on the pre-clinic steps. This process led to two patent submissions that are under evaluation.

References

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V-1-506 *

Antithrombotic potential of synthetic 1,2,3-triazoles evaluated through *in vitro* and *in silico* approaches

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Cardiovascular diseases, in which platelet aggregation and blood coagulation are involved, represent a major cause of disability and death worldwide. The current antithrombotic therapies have unsatisfactory results and may produce side effects. Therefore, alternative therapies have been extensively investigated. In this work, we evaluated, through *in vitro* experiments, the antithrombotic potential of a series of synthetic 1,2,3-triazole derivatives. Platelet aggregation was monitored on a Whole Blood Aggregometer (Chrono log 490 2D) using human platelet-rich-plasma (PRP). Coagulation assays were performed by Prothrombin Time (PT) and activated Partial Thromboplastin Time (aPTT) tests (Wierner Lab), on a Digital Coagulometer (Amelung KC4A). Results showed that some of the derivatives inhibited plasma coagulation on PT and aPTT assays as well as inhibited platelet aggregation on PRP induced by collagen and ADP. Theoretical toxicity studies using the software Osiris Property Explorer also revealed that these derivatives have low toxicity and a drug-score similar to commercial anticoagulant (warfarin) and antiplatelet (aspirin) drugs. In conclusion, triazoles are promising candidates for molecular modeling of new antithrombotic drugs. Moreover, *in vitro* and *in silico* studies should be firstly carried out as a trial, before the use of animal models, in the early studies of drug development.

** Supported by Young Scientists Travel Awards provided by ACT Germany and the German Foundation SET.*

Session V-2: Disease models *in vivo*

Co-chairs
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Session V-2: Oral presentations

V-2-077

The effect of additional rodent enrichment on local and systemic bacterial infection models, hematology, clinical chemistry, and serum cortisol

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Objectives: To determine the effects of foraging enrichment added to standard enrichment (SE) in a mouse skin (MS) (Fernandez et al., 2011) infection or rat endocarditis (RE) (Fernandez et al., 2012) model. Effects on hematology (H), clinical chemistry (CC) and serum cortisol (SC) were examined.
Methods: Animals were group housed into SE (mice, Nestlets™ ; rats, acrylic tubes) and additional enrichment (AE)(SE with autoclaved hamster food (mice) or sunflower seeds (rats)) groups. After 28 days, a group of mice or rats were euthanized and blood was collected for H, CC, and SC (rats). The remaining animals participated in a S. aureus MS or RE model. In both infection models, untreated animals were compared to vancomycin-treated animals.
Results: Additional foraging material had no apparent effect in either infection model with respect to bacterial load at the infection site or the efficacy of vancomycin. Additionally, H and CC values were similar for each group. SC in rats was lower in the AE group ($p<0.03$), suggesting that the animals did not experience additional stress from the added enrichment, and may have benefited.
Conclusion: Animals could benefit from the additional foraging enrichment to enable natural behavior. Clinical chemistry and hematology should be evaluated prior to implementing in a research program.

References

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V-2-246

Characterizing the gut microbiota as a way to reduce the group size in rodent studies

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The gut microbiota of animal models has a substantial impact on the expression of and the variation in the models (Bleich and Hansen, 2012). E.g., in models of type 2 diabetes the correlation between essential parameters and the gut microbiota composition is 30-40% (Ellekilde et al., 2014), while it in the oxazolone model of atopic dermatitis is more than 80% (Lundberg et al., 2012). Today, high throughput sequencing enables a full characterization of the microbiota based upon a non-invasive fecal sample. In animal experiments group size is calculated as 2 x Z-values (Significance + power)/(Average effect level/Uncontrolled variation) (Ellekilde et al., 2014). Therefore, it is possible to characterize the microbiota composition of individual animals in sensitive studies and thereafter turn the "uncontrolled" variation into "controlled variation" by incorporating the characterization in the data evaluation model. Alternatively, only mice with a gut microbiota coding for a strong expression of the disease could be used. We have previously shown that mice might be inoculated with tailor-made microbiota around weaning to achieve the immunological phenotype induced by the early life colonization (Hansen et al., 2012). A third approach might be to feed the mothers a microbiota-modulating diet that will induce a specific phenotype in their offspring (Hansen et al., 2014).

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V-2-465

Ischemic preconditioning against myocardial infarction: a systematic review of animal models

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Systematic review of available animal data has shortcomings in the design and quality of animal studies which may play major role in the poor translation of animal data to clinical benefit (Kilkenny et al., 2009; Van der Worp et al., 2010). For cardiac ischemic preconditioning (IPC), a protective strategy in which brief bursts of ischemia induce protection against myocardial infarction, several clinical trials in humans have not replicated the promising results reported in over 500 preceding animal studies (Hausenloy and Yellon, 2011; Ludman et al., 2012).

To identify factors hampering the translation of results from animal studies on IPC into clinical practice, we are performing a systematic review of all animal studies investigating the effect of IPC on myocardial infarction. We performed a systematic literature search in Pubmed and EMBASE. After study selection, study characteristics were extracted from the 556 studies which presented data on myocardial infarct size in an *in vivo* model. We performed meta-analysis of the impact of study design characteristics (e.g., species, IPC protocol used, co-morbidity and gender) and study quality indicators on IPC efficacy. Results of these analyses will be presented here for the first time.

The results of this systematic review will be used to optimize future, evidence-based, experimental animal studies and may inform the design of future clinical trials.

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V-2-502

From scientific failures to policy changes: moving away from animal use in research

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The ethics of using animals for research purposes has historically been a focus of attention, but there is an increasing body of literature calling the validity of animal models into question-historically considered the "gold standard." As a result, there is a growing community of scientists, legislators, regulators, policy makers and members of the industry that support strides toward replacing the use of animals in research and toxicity testing with more human relevant alternatives.

Critical examinations of animal use are also leading to policy changes. As one example, the Institute of Medicine, commissioned by the National Institutes of Health (NIH) in the United States, concluded that the use of chimpanzees in research is = largely unnecessary (Altevogt et al., 2011). Following this report, NIH announced that they will retire nearly 90% of their chimpanzees into sanctuaries.

Furthermore, several papers investigating disease models, including Alzheimers (Langley, 2014), asthma (Buckland, 2011), shock trauma and sepsis (Seok et al., 2013) have recently been published and have been instrumental in highlighting the lack of reproducibility and validity of animal models in the development of cures.

This growing body of knowledge and how it can ultimately lead to policy and regulatory changes, as well as how science is studied will be discussed.

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V-2-574

Murine models of human disease: why we must think outside the cage

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In the life sciences, *Mus musculus* is considered to be the quintessential human mimetic species suitable for studying human disease mechanisms and drug responses. Despite extensive murine-based research efforts, mechanisms of disease pathogenesis remain unclear and effective pharmacotherapeutics without adverse side effects remain elusive for many human diseases. Comparative data show major limitations of mouse models for many human diseases, notably type 1 and type 2 diabetes (Roep, 2007; Chandrasekera and Pippin, 2014) heart disease, inflammatory disorders (Seok et al., 2012), ageing (Demetrius, 2005), cancer, stroke, and neurological disorders (Cavanaugh et al., 2014). Immutable species differences have been identified at every level of biological action – from gene expression to whole organism – severely restricting the ability to reliably replicate human molecular mechanisms, pathophysiology, and treatment responses in mice. Despite evidence questioning the purported merit, murine models continue to form the cornerstone of biomedical research today. We review here the most noteworthy murine-human species differences that significantly impair translation and propose a step-by-step strategy by which researchers and funding agencies across the globe can begin to "demurinize" and, conversely, humanize the study of disease mechanisms and therapeutics. Given the extent of the intractable translational barrier, research and clinical advances require a paradigm shift, systematically incorporating human-relevant technologies that can improve bench-to-bedside success.

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V-2-606

Addressing threats to clinical generalizability in preclinical experiments: a feasibility study of sunitinib

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One possible explanation for attrition in cancer drug development is poor preclinical study design. In this exploratory study, we used sunitinib to evaluate relationships between preclinical study design and magnitude of effect sizes.

We identified all published preclinical experiments where sunitinib monotherapy was tested for tumour growth or survival for four clinical indications. We collected effect sizes as SMD and extracted experimental parameters addressing internal, external, and construct validity threats. Effect sizes were pooled by indication and exploratory subanalyses were performed on validity parameters.

We captured 113 eligible experiments collectively. Most experiments addressed threats to validity in a haphazard manner (e.g., 46%, 6%, 7% and 0% of experiments reported using randomized allocation, concealed allocation, blinded outcome assessment, and power calculations). Effect sizes by indication favoured sunitinib's efficacy for all four indications. The relationship between preclinical practices and effect sizes, as well as concordance with trials outcomes will be described.

Our study suggests the feasibility of using preclinical meta-analysis to explore factors that undermine valid clinical inference in preclinical research programs. We also uncover high risk of bias in cancer preclinical research. Ethical and policy dimensions will be discussed.

V-2-685

Combination of YM155, a survivin suppressant, with sorafenib: a new combination therapy for human hepatocellular carcinoma in patient-derived xenograft models

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Survivin is one of the most cancer-specific proteins, being up-regulated in almost all human tumors. Biologically, survivin has been shown to inhibit apoptosis, enhance proliferation and promote angiogenesis. This study was designed to investigate the efficacy of sepantronium bromide (YM155), a survivin suppressant, in combination with sorafenib using in four patient-derived xenograft (PDX) models of hepatocellular carcinoma (HCC). The four PDX tumor models (from two HBV-infection HCC tissues and two HCV-infection HCC tissues) retained their similarity of the origin donor characteristics of histology and biologic stability better than those established by HCC cancer cell lines. Our results demonstrated that YM155 combination with sorafenib inhibited the PDX tumor growth than sorafenib onlytreatment. By Immunohistochemistry assay, the expressions of cleaved caspase-3 (apoptosis marker), Ki-67 (cell proliferation marker), and CD31 (angiogenesis marker) all correlated to the antitumor activity of YM155 combination with sorafenib treatment. In comparison, the drugs treatment reduced serum levels of h-AFP. In summary, we established four HCC PDX mouse models that can serve as a useful platform for evaluating the antitumor activity of drug candidates. This result will provide the future clinical trial of the combination of YM155 with sorafinib in HCC therapy.

V-2-746

Refined models for GLP-conform translational studies in the preclinical safety assessment of cell, tissue or organ transplantation with human therapeutic biologicals

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Recent advances in regenerative medicine are based on the understanding that for the majority of pathological conditions there are three therapeutic solutions: replacement strategies using artificial materials and devices; the use of cells, tissues or organs for repair and substitution; the stimulation of the self-healing potential. Cell therapies and cell-scaffold combination products play a major role to deliver an effective therapeutic platform able to heal a variety of chronic, acute, and age-related diseases.

However, one major bottleneck to translate stem cell based science into therapies is the potential risk of some cellular products to induce malignancies *in vivo*. Regulatory agencies have responded to this risk and have introduced several preclinical safety standards. Nevertheless, the greatest hurdle to test the safety of cell therapies, cell-scaffold combination products and endogenous factors resulting from cell therapies in animal or alternative models is the lack of suitable models able to detect single cells that may develop into a tumour within a cell population of millions of "healthy" cells.

Here, we present an experimental and refined *in vivo* model that uses immune tolerance induction strategies to provide preclinical, GLP-conform safety studies for human cell therapies able to complement and validate *ex vivo* test systems.

V-2-950

Automated, continuous non-contact infrared monitoring system for assessment and stratification in models of infectious disease

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Introduction: Animal research is controversial but vital to understand disease mechanisms and to establish new drugs. These studies are burdening, labour intensive and their numbers are rising. We developed a system monitoring animals automatically, free of stress and manipulation.
Methods: The system with artificial intelligence and web access for remote control, recording temperature and activity profiles of multiple animals simultaneously by thermography was evaluated in models of infection/inflammation.
Results: In (semi-)lethal experiments, the combination of temperature and activity allowed prediction of death at least 7.7 h before this event. A clear discrimination between survivors and non-survivors was achieved. Antibiotic rescue was able to change expected outcome until a distinct degree of prediction. All animals undergoing infection demonstrated a characteristic temperature trajectory, indicating disease progression and severity, but also definition of disease phenotypes. The system allowed non-subjective decision making for euthanasia in survival analyses and pre-mortal tissue sampling from animals predicted going to death. It reduces harm and numbers of animals and enables humane endpoints.
Conclusions: Automated, infrared monitoring offers the opportunity to fulfil 3R-standards by reducing numbers and suffering of animals, improves quality of data by stratification and allows definition of a uniform state of a progressive disease.

Session V-2: Poster presentations

V-2-064

Comparative assessment of cytotoxic effects of garlic oil and 2,2′-dithio-bis(N,N-diethyl)ethanamine on human umbilical vein endothelial cells in culture

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It is known that garlic oil (GO) and its sulfur-containing components such as diallyl sulfide, diallyl disulfide and diallyl trisulfide exhibit a broad spectrum of anticancer properties, both *in vitro* and *in vivo* (Shukla and Kalra, 2007). However, the exact mechanism of effects of these substances is not known. Earlier, it was suggested that they can provide inhibiting effects on carcinogenesis by acting on endothelial cells and inhibiting neoangiogenesis (Xiao et al., 2006; Thejass and Kuttan, 2007a,b).

We have conducted a comparative assessment of GO and abiogenic disulphide 2,2'-dithio-bis(N,N-diethyl)ethanamine (DS) on viability of human umbilical vein endothelial cells in culture. Three methods were applied: the neutral red uptake assay, quantification of intracellular ATP and modifications of Mosmann method. We revealed that DS and GO have a similar cytotoxic effect upon the endothelial cells (EC_{50} ~ 0.6 mM). In addition, it was demonstrated for the first time that DS and GO at concentrations of 0.2 mM and more can serve as mediators of plasma membrane oxidoreductases activity, tetrazolium salts (MTT and MTS) being as the substrate. The mediator effect of DS developed more intensively, so we suggest it could possess the antiangiogenic and anticancer properties, not less or even more than those of GO.

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V-2-386

Trends in the use of genetically modified mice and their efficacy as a model for human disease

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There has been an overall decrease in the total number of animals used for scientific procedures in the past 40 years. However, there has been a consistent increase in the breeding and use of genetically modified animals, mostly mice, over the same period. Genetically modified mice (GMM) have become a routine model for researchers in a number of fields, yet many uncertainties and inconsistencies remain. Specifically, there is concern over the relative efficacy of GMM as an *in vivo* model for human disease and drug development. This work outlines the relative trajectory of GMM in research and questions their validity. Investigation into the scope of the areas of research, the factors related to the rise in numbers, and the varying mechanisms for genetic modification are made, as well as projections for where GMM research may be heading. The advantages and limitations of GMM are contrasted to determine the relevance and applicability of GMM. It is concluded that, although some GMM models do provide insights into areas of human disease research, the reliance and assumed ability to correlate results with humans may be overstated and requires significant further analysis.

V-2-903

Diet-induced iron deficiency anemia in rats – *in vivo* model

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Objective: The aim of the study was to induce iron deficiency and assess its bioavailability in rats.
Material and methods: During 28 days of the experiment 12 male Wistar rats received the deficit in iron AIN-93M diet and 12 male rats received a complete AIN-93M diet. After experiment blood was collected from the tail and haemoglobin, haematocrit and Fe concentra-

tion in serum were determined. After the Fe deficiency was detected the animals were killed and blood, heart, liver, spleen and kidneys were collected for analyses.
Results: It was found that level of haemoglobin, hematocrit, Fe in serum, Fe in the liver and Fe in kidneys significant decrease in rats fed with the iron-deficitdiet. Significant reductions were also recorded for MCV, MCH and MCHC values. No changes were observed in other analysed parameters.
Conclusions: Such an animal model was successfully established within a relatively short time. The applied model can be used for nutritional experiments for the evaluation of the bioavailability of iron.

Acknowledgments: This study was financed from research funds in the years 2011-2014 as a research project no 2011/01/B/NZ9/00130

V-2-931

Role of drug transporters in pancreatic adenocarcinoma therapy

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Recent studies indicate that expression profile of drug transporters contributes to the low chemosensitivity of patients with pancreatic ductal adenocarcinoma (PDAC; Mohelnikova-Duchonova et al., 2013a,b). We investigated mechanisms of action of paclitaxel and second generation taxane SBT-1216 in *in vitro* and *in vivo* models.

Effects of taxane drugs were followed by cytotoxicity test and flow cytometry in model cell lines *in vitro* and by real-time PCR with relative quantification of gene expression of 15 drug efflux ATP-binding cassette (ABC) and 13 drug uptake solute carrier (SLC) transporters in mouse xenografts *in vivo*.

IC_{50} for paclitaxel and SBT-1216 ranged from 15 to 65nM in BxPc-3, MiaPaCa-2, and PaCa-44 cell lines. Cell lines differed in induction of apoptosis and G2/M cell cycle block after exposure to both taxanes. Administration of SBT-1216 caused increase of gene expression of ABCB1, ABCB2, and SLC22A1 and concomitant downregulation of ABCC1, SLC29A1, and SLC29A2 in PaCa-44 tumor xenografts.

Our data shows that both taxanes are highly cytotoxic in pancreatic cancer cells but mechanisms of their action substantially differ. Considering recent data demonstrating activity of nab-Paclitaxel in PDAC patients (von Hoff et al., 2011; Awasthi et al., 2013), underlying mechanisms of taxane action should be further studied.

Supported by grants of Czech Science Foundation No.: P301/12/1734 and Internal Grant Agency of the Czech Ministry of Health No.: NT/14056-3.

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Session V-3a: Potency testing of human and veterinary vaccines

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Session V-3a: Oral presentations

V-3a-108

Replacing the NIH test for rabies vaccine potency testing: a synopsis of drivers and barriers

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Approximately 70% of animal use is utilized to demonstrate quality control of vaccines. Especially rabies vaccine potency testing, using the NIH challenge test, involves objections in terms of scientific relevance, animal welfare concern and costs. Several 3R models have been proposed to refine, reduce or replace this test. Some are formally incorporated into regulatory requirements, but actual regulatory acceptance and use by industry lags behind, raising the question concerning which factors influence this process. This question is answered by a combination of literature review, interviews and a survey among 50 rabies vaccine experts. The findings are analyzed using the multilevel perspective on technology transition, which distinguishes 3 levels of factors influencing innovation acceptance. At the micro level (where 3R models are developed and validated) the dis-advantages of, and fractional experience with, 3R models, scarce data sharing and demanding validation processes exist. The meso level (existing regulatory regime) encloses the barriers of the "gold standard", the lack of harmonization and the driving force of legislation stimulating 3Rs use. The macro level (the societal context) combines risk aversion an increased concern for animal welfare. Regulatory acceptance and use of 3R models requires dedicated stakeholder communication, cooperation and coordination at all three levels.

V-3a-147

The deletion of the target animal batch safety test from monographs in the European Pharmacopoeia and waiving possibilities at VICH level

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In the past, a target animal safety test (TABST) was indispensable for the release of batches of veterinary vaccines. A double dose of vaccine was injected into each of two animals. The batch passed the test when no systemic or local reaction occurred within 14 days after injection.

The need for release of each batch was questioned. The vague pass criteria and poor statistical relevance of the outcome called the test into question. Moreover it could not be excluded that the test produced wrong negative results.

As a consequence the TABST was deleted from the European Pharmacopoeia in 2013. Nevertheless, the TABST might still be performed due to the lack of international harmonisation. Lead by Europe, the International Cooperation on Harmonisation of Technical Requirements for Registration of Veterinary Medicinal Products (VICH) worked on the "Harmonization of criteria to waive the target animal batch safety testing for inactivated vaccines for veterinary use" and the relevant GL50 is in force since 1st April 2014. European manufacturers can now apply for a waiver of the TABST when exporting to the other VICH regions (Japan, North America) or countries following VICH. VICH is currently working on a comparable guideline for live veterinary vaccines.

V-3a-340

The consistency approach in lot release testing of vaccines

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Vaccine lot release testing is characterised by its extensive use of laboratory animals. Successes have been achieved in implementing Three R methods, but progress is tedious and time consuming.

A new paradigm in lot release testing of established vaccines is the consistency approach. This approach starts from the idea that subsequent lots of vaccine produced can be compared to a clinical/historical lot, which is thoroughly tested and has a well defined profile. The consistency approach has come into reach by improvements in production and control: optimised production processes, a tight protocol for in-process testing using innovative physico- and immunochemical techniques and a state-of-the-art quality monitoring system (GMP, QA).

Consistency testing may lead to a significant reduction in animal use since a narrow set of animal tests performed on each final lot, sometimes with questionable relevance, may be replaced by a battery of meaningful physicochemical-, immunochemical- and *in vitro* functional tests with enhanced capacity to measure equivalence with batches of proven safety and efficacy.

The paradigm of consistency is an interesting strategy for vaccine manufacturers as it might allow for a reduction in costs and shortening of testing period. This presentation will introduce the consistency approach and discuss advantages and limitations.

V-3a-462

Toward the replacement of the Rabies NIH potency test: International Working Group for Alternatives to NIH test

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Numerous studies have shown that immunization with the native immunogenic form of the rabies glycoprotein G results in the production of neutralizing antibodies and protection against lethal challenge. In human rabies vaccines, antigen quantification is used at final bulk stage, allowing definition of the vaccine final antigen content. Following a NICEATM, ICCVAM meeting, an EPAA project meeting in 2012 focused on gaps in technical knowledge of *in vitro* G antigen quantification methods and proposed solutions for the replacement of the NIH test. Regulators and manufacturers stressed that the NIH test should be replaced and emphasized that the current *in vivo* assay should not be used for correlation, since it is highly variable and therefore a concordance strategy should be followed. The ELISA methods under development should be able to discriminate between potent and sub-potent batches: *Concordance study*. An International Working Group was formed to coordinate a more harmonized approach of the alternative assay developments through the acquisition and distribution of a common set of rabies vaccines. A protocol was established to allow comparison of different ELISA methods. Results from these studies will be presented end 2014 at a Workshop on Alternatives to NIH to form the basis for an EDQM collaborative study leading to the replacement of the NIH test.

Session V-3b: Potency testing of human and veterinary vaccines

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Session V-3b: Oral presentations

V-3b-120

Residual live virus detection test for rabies vaccine for human use: a 3Rs proposal for the Brazilian pharmacopoeia test method

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Currently, the majority of rabies vaccines for human use are inactivated by betapropriolactone, an efficient and safe agent. However, it is necessary to demonstrate that inactivation was efficient, what can be tested in both animals and cell cultures.

WHO, used to recommend the mouse inoculation test, in at least 20 mice (WHO, 1980, 1987), but, since 2007, this test was deleted (WHO, 2007). However, testing in mice is still recommended in Brazilian Pharmacopoeia (2010) that preconizes for this purpose: A) the inoculation of 20 suckling mice and 20 adult mice; B) the test of viral amplification in cell culture alternatively.

This study, approved by FIOCRUZ ethical committee, evaluates: A) a reduction and refinement of the Brazilian Pharmacopoeia test for detection of residual infectious rabies virus by testing only ten suckling mice euthanized five days after inoculation and tested by the rabies immunofluorescence test; and B) a replacement by a cell culture test.

Results confirm the higher sensitivity of baby mice to rabies virus, that group provided equivalent titers to those obtained with adult mice observed for 21 days. The Cell Culture test also showed satisfactory sensitivity to detect the rabies virus when compared to WHO mouse test.

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V-3b-125

A cell line assay for in-process toxicity and antigenicity testing of clostridium septicum vaccine antigens

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Safety and potency are basic elements of the quality of vaccines. Clostridium vaccines must be free of residual toxicity and confer a protective immunity. Classically toxicity and potency are evaluated in an animal test; pass/fail criterion is death of the animal. The animals, usually mice, are basically used as the detector system.

Alpha toxin is the major potent toxin produced by the bacterium *Cl. septicum*. This toxin is toxic for VERO cells as well as for mice. This characteristic should allow the replacement of the detector system mouse by VERO cells. Cell line assays to replace mouse based assays for control testing of various clostridial toxoid vaccine antigens have been developed supported by an NC3Rs. In order to introduce the cell based assay in the regulatory framework a collaborative study under the aegis of the EDQM Biological Standardisation Programme, with the full support of EPAA has been initiated. Batches of toxin and toxoid from different manufacturers are tested by several laboratories from industry and authorities in parallel in mice and in VERO cells.

Successful completion of the collaborative assay should allow the replacement of the animal test in pharmacopoeias.

V-3b-197 *

Functional *in vitro* testing of the consistency of *Bordetella pertussis* vaccine production

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Batch release testing of vaccines is primarily based on animal models that are costly, time-consuming and sometimes of questionable relevance, while accounting for approximately 10% of the animal use. A new paradigm in batch release testing is the consistency approach, which is based on the integrated strategy of thorough process- and product-characterization and does not require animal testing. A panel

of physico-chemical assays has been developed, but these assays don't assess the capacity of the vaccines to induce immune responses. Therefore, we used several functional *in vitro* assays to study various aspects of innate immune activation by *Bordetella pertussis* (Bp) vaccines. To mimic inconsistent production, the production process was manipulated resulting in altered expression of proteins that are regarded as important for inducing protective immunity. Using ELISA and mass spectrometry, we demonstrated that the "inconsistent" vaccine contained significantly less of these proteins and a different LPS form than the "consistent" vaccine. In addition, the inconsistent vaccine induced lower innate immune activation compared to the consistent vaccine by different cell types: HEK293-hTLR4, MM6 and dendritic cells. The results of our study show that a combination of functional immunological assays can be used to asses Bp batch consistency *in vitro*.

** Supported by Young Scientists Travel Awards provided by ACT Germany and the German Foundation SET.*

V-3b-707

Consistency approaches for the replacement of *Diphtheria Tetanus Pertussis* potency assays

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For Tetanus and Diphtheria potency tests, the replacement of the challenge procedure by a single immunogenicity assay involving antibodies titration using non-animal methods, e.g., ELISA has been described in European Pharmacopeia for several years as an alternative for the release of combination vaccines. Pertussis activity is already assessed using a consistency test with an immunogenicity assay where the lot is compared to a reference vaccine.

The implementation of such immunogenicity assays for the routine control of vaccines is difficult and not always successful mainly due to the variability of these *in vivo* tests and their current design.

The limitations of current *in vivo* immunogenicity assays involving a reference vaccine and the alternative approaches based on unidose assays (Geometric mean titer – GMT – of antibody responses) as consistency tools will be presented. For Pertussis vaccines, the unidose assay with GMT read-out is already accepted in North America and described in WHO recommendations.

Reduction and refinement of the *in vivo* methods currently used for vaccines quality control are important improvements and their complete replacement with *in vitro* consistency assays will be the ultimate target for the future.

Session V-3: Poster presentations

V-3-057

Thermal imaging – alternative concepts for refinement and reduction of animal use for testing of biologicals

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Latest technical developments in high resolution Infrared Thermography (IR) provide further prospective opportunities for its use in biomedical research (InfraTec, VarioCam®, high resolution, technical specification). Here, we evaluated five potential refinement and reduction alternatives to long-established animal experiments. Experimental settings comprise (1) safety testing of veterinary vaccines, (2) the rabbit pyrogen test, (3) thermometry in guinea pigs, (4) investigation of oral ovalbumin allergy in mice and (5) tumor modeling.

Results: (1) IR-Thermography is well suited to assess local reactivity of veterinary vaccines causing significant increase of the surface temperature at the injection site. (2) Only pronounced pyrogenic reactivity can be detected by IR. (3) Thermography appears to be a suitable non-invasive tool to monitor body temperature in guinea pigs. Thermoanalysis of the tuberculin skin test did not yield promising results. (4) Thermal monitoring of mice to study clinical aspects of food allergy seems to be a promising application of thermal imaging. (5) Evaluating the tumor status by IR-thermometry promotes timely assessment of efficacious therapeutic intervention and provides the opportunity for early humane endpoints.

Conclusions: IR-thermography holds the potential to reduce pain and discomfort of animals involved in biomedical studies. Methodological limits are related to peculiarities of animal physiology like fur, skin thickness, and metabolic properties in small laboratory animals (Pascoe et al., 2007; Eckert, 2002).

This research project is funded by the German Federal Ministry of Education and Research (grant-number 0316009A).

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V-3-170

An animal-free batch testing method for alum-adjuvanted veterinary rabies vaccines

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Since many years efforts are ongoing at the Paul-Ehrlich-Institute to develop modern batch testing methods for vaccines and immunologicals according to the 3R concept. Here, we aim to establish an animal-free test method for the antigen quantification of alum-adjuvanted rabies vaccines. The challenge with alum-adjuvanted vaccines is to detach the adsorbed immunogenic components from the adjuvant and make them available to immunochemical quantification. To this end an electrochemical method was developed by which the antigens are detached from the aluminium salt matrix and simultaneously immobilized on a nitrocellulose membrane. In a second step the amount of desorbed antigen is determined using a quantitative immunoblot.

We can demonstrate that the method is highly specific. The antigens are near quantitatively desorbed and detected. Additional antigens present in the vaccine do not influence the results. The method is reproducible and suitable for all veterinary rabies vaccines currently available on the German market.

Together with additional tests assessing the quality of the adjuvant matrix this versatile and robust antigen quantification method holds promise to form an important component for a new animal-free test strategy for alum adjuvanted vaccines based on the consistency approach.

V-3-201

Notes on the validation of Vero cell test for the absence of residual diphtheria toxin

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Toxicity tests are mandatory during diphtheria toxoid production to verify the presence of residual toxin after formaldehyde treatment (Specific Toxicity Test – STT) and after six weeks at 37°C (Reversion to Toxicity Test – RTT). Once Brazilian Pharmacopeia (2010) does not provide for the use of alternative methods, every toxoid batch must be tested in guinea-pigs (500/year). The use of Vero cells as alternative has been described (Hoy and Sesardic, 1994) and according to the institutional QC activities modernization plan, observing the 3R's concept, we started the methods validation.

Based on the national rules (ANVISA, 2003) this work aimed to validate the Vero cell based RTT and STT in animal proteins-free medium. All the experiments were performed as previously described (WHO, 2013) with modifications: use of serum-free medium and synthetic trypsin; and identification of cytopathic effect by crystal violet staining.

Vero cells cultivated in SFM are 7.5 times more sensitive to diphtheria toxin than in 2% FCS supplemented MEM (Minimum Cytopathic Dose: 11.7×10^{-3} Lf/ml versus 1.56×10^{-3} Lf/ml). Interfering components in the toxoid (thiomersal and formaldehyde) had their concentration lower than the citotoxic concentration simplifying the test. Reproducibility and robustness were satisfactory. Therefore, the modified Vero cell test for residual diphtheria toxin can be considered valid according to Brazilian requirements.

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V-3-203

A serological assay to determine the potency of inactivated rabies vaccines for human use

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For potency determination of inactivated rabies vaccines for human use a classical mouse challenge test is prescribed. Groups of mice are injected intracerebrally with live rabies virus following immunization with serial dilutions of a test vaccine or a standard preparation. The potency of a vaccine is calculated on the basis of the survival rates. This test is imprecise, time-consuming and causes severe distress as animals develop clinical signs of rabies and finally die.

Based on the serological assay mandatory for veterinary rabies batch potency testing (Kraemer et al., 2009, 2013) we have developed a multi-dilution serological assay for human rabies vaccines. This test is performed by immunizing mice with different dilutions of either the test vaccine or the standard vaccine preparation. After two weeks blood samples are taken and tested individually for rabies virus neutralizing antibodies using a modified version of the rapid fluorescent focus inhibition test. The determination of the relative potency of a vaccine batch is based on the serum activities. This assay has several advantages compared to the mouse challenge test: The animals do not suffer from rabies as no challenge infection is necessary, the animal number is reduced, the test is more precise, less expensive, easier and faster to perform.

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V-3-249

In vitro characterization of adjuvant biological activity and safety

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The use of recombinant proteins as vaccine antigens requires the combination with adjuvants capable to overcome the low immunogenicity

of such antigens. Sanofi Pasteur R&D evaluates new adjuvants combining either emulsion or aluminum particles with or without TLR agonists. Such adjuvants are used to stimulate the innate immune system by triggering the secretion of pro-inflammatory cytokines, however the level of pro-inflammatory response induced by these adjuvants must be moderate to be well tolerated. To document and compare different adjuvant formulations, an *in vitro* assay based on the measurement of human pro-inflammatory cytokines secreted by a human monocytoid cell line, was developed and used as alternative to animal model to evaluate the adjuvant biological activity and safety. Thanks to this monocyte activation test, we documented the benefit of TLR agonist incorporation into emulsion.

V-3-321

Development of relevant serological methods for replacing *in vivo* seroneutralization tests used for determining potency of diphtheria, tetanus and whole-cell pertussis vaccines

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Multi-dilution challenge tests have been traditionally used for determining Potency of Diphtheria, Tetanus and whole-cell pertussis antigens in vaccines. In Latin-America, we use the classical WHO challenge method (Kendrick test) for pertussis antigen, while a variant developed at NIH and adopted by FDA, USA, based on *in vivo* toxin neutralization, has been used for Diphtheria and Tetanus toxoids for decades. However, both types of assays have been criticized in terms of variability, ethical and technical issues. As serological tests are more suitable to monitor the quality and manufacturing consistency than challenge assays, the present paper aims to show the progresses we have had in the development and implementation of serology for Tetanus, Diphtheria and Pertussis as alternatives to the seroneutralization assays. In all cases, validation processes were successfully performed for serology, including the demonstration of significant correlation against the routine *in vivo* seroneutralization assays. Vaccines with proven clinical efficacy were used for validating the relevance of antibodies in mice-serology for Diphtheria and Tetanus. Likewise, immunological functional tests were evaluated for sustaining the role for protection of the total antibodies in pertussis serology. Thus, we demonstrated the significance of using relevant serological methods as Potency testing instead of the challenge assays.

V-3-339

Study on the establishment of standard material for pertussis toxin and standard pertussis strain

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As the National Standard for the residual pertussis toxin (PT) testing of acellular pertussis vaccines in our current national lot release has characteristics of whole cell pertussis, PT having characteristics of acellular pertussis vaccines should be manufactured. And, as B. pertussis strain for use of modified mouse intracerebral challenge assay (MICA) is subcultured by every potency test, results of potency shows high variances. Therefore, establishment of Standards pertussis strain requires to improve quality control level of pertussis vaccines. To manufacture PT, the pertussis Tohama strain was cultured by optimized culture condition. PT was purified from the culture supernatant using various column chromatography. The final PT was prepared by optimization of large scale pertussis culture condition and lyophilization condition. Our study demonstrated that manufactured PT has specific characteristics of PT using *in vitro* both carbohydrate binding assay and enzymatic HPLC assay. Additionally, the potency of PT was obtained by histamine sensitization test (HIST) from co-work of 4 laboratories and was determined into 27.3 ng/dose (LD_{50}).

Strain 18323 of B. pertussis was used for MICA. The final Standard pertussis strain (200 vial) was manufactured and its viable bacteria count was 4x108 cfu/ml. The potency of final Standard pertussis strain was determined into 93 viable bacteria/0.025 ml (LD_{50}).

V-3-351

Determination of effectiveness of immunized chickens's Immuneglobuline Y against Salmonella infection in mice

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This study was aimed to determination of effectiveness of immunized chickens's Immuneglobuline Y (IgY) by inactive monovalan (*Salmonella* Dublin, *S*. Typhimurium, *S*. Kentucy or *S*. Anatum), bivalan (*Salmonella* Dublin, *S*. Typhimurium) and combined (*Salmonella* Dublin, *S*. Typhimurium, *S*. Kentucy and *S*. Anatum) salmonella vaccines. Chickens were immnized with inactive monovalan, bivalan and combined salmonella vaccines, three times at 21 days intervals. Immunized chickens's eggs were collected after last vaccination and IgY were purified. A total of 100 mice were challenged with salmonella strains by oral route. Then, IgY to mice were administrated by oral route after challenge. Mice were observed for occurence of morbidity and mortality. Also, feacal samples were analyzed to determination influence of IgY on spread of salmonella species by feaces.The antibodies titres in blood and eggs of immunized chickens were found to be high by ELISA than before vaccination and controls. Any cases of morbidity and mortality were observed in mice of groups. The numbers of re-isolation of salmonella strains were lower from internal organs and feacal samples of mice in all groups. In conclusion, IgY of immunized chickens were useful for protection against salmonella infections.

V-3-384

Study of an alternative pyrogen test for blood product

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Currently, the rabbit test is practiced as end-product pyrogen test for national lot release of blood derived products. However, the replacement of rabbit test needs to be considered due to the high consumption of animals and ethical issues. It does not coincide with international efforts for minimization of animal using test, also represented as 3R.

The aim of our study was to develop an alternative pyrogen test for final products of blood derived products. We established conditions and test method of bacterial endotoxin test (LAL test) for seven blood products. In addition, we conducted monocyte activation test (MAT) based on human fever reaction to study on the possibility of its application to five blood products.

Three laboratories collaborated to examine the validity of established LAL test method for 20 lots of each final products. As a result, we confirmed that endotoxin contents were less than the detection limits of each product respectively, and that recovery rate of the spiked endotoxin met acceptable standard 50~200%. Furthermore, data showing that MAT is capable of covering the total pyrogens and applicable to blood derived products.

However, further studies are needed to verify the propriety of these alternative pyrogen tests for other blood products.

V-3-458

Potency evaluation of Bothrops snake venom: a cytotoxicity assay as an alternative to the murine model

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Snakebites affects about 30,000 persons yearly in Brazil, and 90% (27,000 cases) are due to Bothrops genus. LD_{50} and ED_{50} mice assays are the official methods for potency evaluation of venom and antivenom. This work proposes an alternative Vero cell cytotoxicity assay for potency evaluation of Bothrops venom and antivenom. Moreover, this assay can evaluate the cytotoxic effect of B. jararaca venom and its main toxic components (metaloprotease, phospholipase A2) by using enzymatic inhibitors. When tested in the presence of Orthophenantroline (metalloproteinase inhibitor) inhibition of 50% Cytotoxic Dose (CtD_{50}) was achieved (from 4.07 μg/ml to 15.62 μg/ml) and with bromophenacyl bromide, (phospholipases A2 inhibitor), values of CtD_{50} increases from 4.09 μg/ml to 6.92 μg/ml. Therefore, we can conclude that this *in vitro* potency assay is sensitive to metalloproteinases and phospholipases. These results qualify *in vitro* Vero cell methodology, as candidate to an alternative method to the murine assay for potency evaluation of Bothrops venom and antivenom.

V-3-480

Evaluation of serological test for whole cell pertussis DTP vaccine

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Every whole cell pertussis vaccine batch must be potency checked by the mouse protection test (MPT) during lot release and other stages of manufacturing, for either single or combined vaccines (Brazilian Pharmacopeia, 2010). Painful and distressful, the method includes an intracerebral challenge, requires experienced operators, is highly variable and usually needs repeats due to invalidity (Xing et al., 2001). Serological methods assessing anti-*B. pertussis* mice (Matos et al., 2012) or guinea-pigs (von Hunolstein et al., 2008) antibodies against combined vaccines can reduce the number of animals for QC tests and avoid animal suffering. Furthermore, the idea of testing several combined vaccines components in the same animal series, lead us to this study. Here, we describe our efforts to find a suitable serological method by using different designs. Whole pertussis vaccine (VP), B. pertussis strain 18323 or purified pertussis toxin (PT) was used to coat microplates in an anti-*B. pertussis* ELISA. Guinea-pigs sera obtained 42 days after immunization in a strain 18323 coating ELISA, showed a better correlation (r=0.86) ELISA/MPT than the 28th day sera (r=-0.02), while VP coating with the 28th day sera resulted in an intermediate correlation (r=0.53). The use of PT avoided false positives, however, the correlation was fair (r=0.18). These data suggest an interesting approach needing further improvements.

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V-3-512

Optimisation of an *in vitro* assay system as an alternative to current murine histamine sensitization test for acellular pertussis vaccines

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The histamine sensitization test (HIST) is a safety test for batch release of acellular pertussis vaccines (ACV) which monitors residual toxic pertussis toxin (PTx) or reversion to toxicity of the toxoid. HIST is a lethal test requiring large numbers of animals and it is difficult to standardize. Therefore, there is an urgent need to develop an *in vitro* alternative to this test. An *in vitro* test system has been developed as a potential alternative to HIST. This test system examines both functional domains of PTx (enzymatic and carbohydrate binding domains) using a combination of enzyme coupled-HPLC and carbohydrate-binding ELISA. A previous international collaborative study demonstrated that the test system is transferable between laboratories and is suitable for differentiating three types of ACV products which sup-

ported its use as a potential alternative to the HIST. Further method optimisation has been made on ACV products spiked with PTx. A good dose response in the spiked vaccine samples were observed in the carbohydrate-binding assay. Here we report the optimised assay system with more data on various ACV products and suggest possible validation steps.

V-3-596

Development of a common immunogenicity assay on guinea pigs for the potency testing of multicomponent vaccines

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A serological potency assay on guinea pigs is now referenced in the European Pharmacopoeia for DTacP vaccine. This alternative assay will reduce the number of animals due to the ability to use the same animals for multiple antigens. We developed and validated according to Ph. Eur. requirements a common immunogenicity assay on guinea pigs with a multiplex antibody detection method. Concordance studies have been conducted on several lots using a multi-dilution assay design. A one-dilution assay design is preferable for routine testing in order to diminish the number of animals. Managing homologous reference on the long run is challenging, especially qualifying a new reference that is similar to the previous one for the response to several antigens. Therefore, we consider an alternative one-dilution assay design that would assess lot conformance based on the geometric mean antibody response to each antigen.

V-3-773

Avian encephalomyelitis live vaccines – alternative methods for batch testing

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Avian encephalomyelitis virus (AEV) causes server neurological symptoms in poultry worldwide (Calnek, 2008; Tannock and Shafren, 1994).

Control of AEV can be achieved by vaccination of breeder flocks during the growing period (Calnek, 2008). Most available vaccines contain a live, enterotropic, virulent virus strain and are administered via drinking water. The guidelines of the Pharmacopoeia require a titer determination for the final product- a titration in eggs with hatching of chicks or virus titration in cells (European Pharmacopoeia, 2012). The virus titration in cells is not established yet. Therefore, egg titration and the counting of chicks, that show clinical signs (tremor, paralysis), are the only titer determination methods currently used.

The objective of the project is to refine or replace animal experiments – according to the 3R principles – with a specific, sensitive method. Project one aims at refining the egg titration, by replacing the hatchout approach with antigen detection in the embryonic brain using an AEV-Antigen-ELISA. In project two, cellular aspects of AEV infection will be investigated in order to facilitate the development of a cell culture infectivity assay for vaccines.

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V-3-917

Refinement and reduction of 3R; *in vivo* imaging method can refine experimental procedures with less number of animals

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Among 3R, reduction of animal number is the shortest way to improve the animal experiment procedure for the aspect of animal welfare. Usually IACUC of each institute controls the number of animals for *in vivo* experiments to avoid excessive or unnecessary animal death.

In vivo imaging using molecular signals such as fluorescence or luminescence are already utilized as a routine procedure to diagnose many symptoms for clinics and to analyze cellular phenomena for experiments. Korea National Institute of Health is now developing an alternative method using molecular imaging tools to test safety/efficacy of vaccine/drug development using comparatively small number of animals. Bacteria including *E. coli* and *Francesella tularencis* were transformed with the plasmid containing dual-signal plasmid expressing fluorescence and luminescence simultaneously. Then, the pathogenic procedures including pathogen distribution, immune correlations were analyzed using the *in vivo* imaging methods without termination during infection. Finally, cellular or tissue level analyses were performed at the end of whole procedure after termination. The result clearly revealed the vaccination efficacy; tracing luminescence signals clearly showed the pathogen distribution and clearance in animals.

It suggests a new model for alternative methods, which can be potentially applied for safety/efficacy tests by refinement and reduction of animal usage.

V-3-949

The BINACLE (binding and cleavage) assay allows *in vitro* detection of active tetanus neurotoxin in vaccines

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Tetanus toxoid (inactivated tetanus neurotoxin) is an essential component of tetanus vaccines. In order to exclude a residual toxicity, safety tests in guinea pigs are prescribed for each toxoid bulk (Council of Europe, 2014a,b). Our aim is to replace these tests, which require thousands of animals annually, by an *in vitro* method.

Tetanus neurotoxin consists of two protein subunits: The heavy chain mediates the toxin binding and uptake by neurons, and the light chain cleaves the neuronal protein synaptobrevin, thus causing a spastic paralysis. We have developed an *in vitro* method for the detection of active tetanus toxin which takes into account the toxin's receptor binding capability as well as its proteolytic activity (Behrensdorf-Nicol et al., 2010). By employing this "BINACLE" (binding and cleavage) approach, false-positive results induced by partly inactivated or fragmented toxin molecules can be avoided. The applicability of this assay for the safety testing of toxoids has been examined in an in-house validation study and an international transferability study (Behrensdorf-Nicol et al., 2013, 2014). The results demonstrate that the method could allow at least a partial replacement of the prescribed animal safety tests. An overview of the current validation status will be given.

This project has been supported by the SET Foundation, Animalfree Research, and Doerenkamp-Zbinden-Foundation.

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Session V-4: Medical devices and biologicals

Co-chairs

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Session V-4: Oral presentations

V-4-044

The monocyte activation test (MAT) is able to predict the enhancement of the LPS-induced proinflammatory response caused by glucans

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Beta-(1→3)-glucans (BG) are considered Pathogen-Associated Molecular Patterns able to induce inflammatory responses although they are non-pyrogenic in rabbits (Roslansky and Novitsky, 1991). Fungal contaminations and cellulose-based filters can be the main sources of BG presence in parenterals. A few previous reports described high BG levels in parenterals; however its biological activity is not fully understood (Ohata et al., 2003; Nagasawa et al., 2003). BG seems to enhance the LPS-induced response in MAT when testing formulations of Human Serum Albumin (Perdomo-Morales et al., 2011). Therefore, this work attempts to study the influence of BG on the LPS-induced pro-inflammatory response in human whole blood and its implication for pyrogenicity test using MAT. Soluble BG were isolated either from *Saccharomyces cerevisiae* or from cellulose filters. Human whole blood was incubated with BG, either alone or concomitantly with LPS, and the releasing of IL-6, -1β and TNF-α determined. BG from yeast induced the releasing of all proinflammatory cytokines assayed. It was also found that both BG enhanced the LPS induced-cytokines secretion. We conclude that MAT improves predictability as pyrogen test as it can assess interactions between different pyrogens. Thus, MAT is not only able to detect most pyrogens as is already known, but it also may reflect their interactions.

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V-4-200

Establishment of a 3 R method for batch potency testing of bovine tuberculin

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The current batch test for tuberculins is an *in vivo* test: guinea pigs are infected with virulent *M.bovis*. Four weeks later flanks are shaved and serial dilutions of standard and test batches are injected intradermally. 24h later the size of the resulting skin lesions is measured. Here,

we attempted to establish an alternative *in vitro* method. To this end we sensitized guinea pigs with inactivated mycobacterial wet mass. Four weeks later peripheral blood mononuclear cells (PBMCs) were isolated, stained with the green fluorescent dye CFSE and stimulated with serial dilutions of WHO standard and test tuberculin. After 5 days loss of CFSE staining was determined by flow cytometry as a measure for antigen-specific cell proliferation. The potency for test tuberculins was calculated from the dose response curves of the standard and the test batches. The method is robust, sensitive and specific. It yielded reproducible results and it has a wider detection range and is more sensitive than the current skin test. In summary, the method has the potential to replace the most stressful part of the current potency test. It refines the read-out and reduces the number of animals required because more batches can be tested in parallel.

V-4-363

Proteomic analysis of tuberculin purified protein derivatives

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Rationale: PPD tuberculins are heat treated products derived from culture material of mycobacteria. Tuberculins reveal a delayed hypersensitivity in individuals sensitized to mycobacteria and are thus diagnostic tools. According to European Pharmacopoeia requirements batch potency needs to be tested in guinea pigs. Because of ethical concerns and poor reproducibility of these tests, there is an urgent need for *in vitro* alternatives. Therefore, we are evaluating mass spectrometry to characterize tuberculins and to assess batch to batch consistency regarding the presence and abundance of relevant proteins.
Results: So far, we examined PPD tuberculins from three manufacturers and the WHO International Standard. All PPDs passed *in vivo* potency testing. In total we identified between 19 (WHO standard) and 36 (Sample A2 & C2) proteins. A subset of 12 proteins was present in all PPDs, of which at least 7 appear to be relevant. 5 of these have been explicitly identified to be markers of tuberculin potency (Whelan et al., 2010; Stavri et al., 2012) and/or identity (Whelan et al., 2010; Stavri et al., 2012; Souza et al., 2012).
Conclusions: Mass spectrometry holds potential to assess batch quality and batch to batch consistency of bovine tuberculins and to further characterize the rather heterogeneous product group of tuberculins.

Funded by the German Federal Ministry of Education and Research 3R-methods to replace and refine legally required animal tests for immunologicals (0316009A – C).

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V-4-593

Towards a full-function molecular-based assay for botulinum neurotoxin

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Thousands of mice are used annually for potency testing of an ever growing number of products that use Botulinum Neurotoxin as the active ingredient. Here we present a first step in the development of a molecular-based assay to detect binding and translocation of Botulinum Neurotoxin type A (BoNT/A). It consists of a liposome (Giant Unilammelar Vesicles, GUVs) with a BoNT/A receptor (GT1b) on the surface. We demonstrate binding of BoNT/A to the GUVs using a fusion protein (eGFP-HcA) consisting of the BoNT/A binding domain (HcA) and Green Fluorescent Protein (GFP). Microscope images of prepared GUVs with HcA-eGFP show binding to vesicles containing GT1b. GUVs prepared without GT1b did not produce the same objects, so GT1b was necessary for binding of the eGFP-HcA. The next step is to demonstrate binding of a construct containing the BoNT/A translocation domain, in addition to the binding domain, to open the way for high-resolution confocal microscopy studies of translocation. When used with an automated sensitive luciferase reporter assay for the detection of BoNT/A proteolytic activity (van Oordt et al., 2013), the developed system has the potential to replace the mouse bioassay, measuring both the translocation and the enzymatic activity of the toxin.

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V-4-705

Incorporation of the 3R's into preclinical testing programs to support cellular and gene therapy product clinical trials that are regulated by US FDA

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In November 2013 the US FDA finalized the guidance document "Preclinical Assessment of Investigational Cellular and Gene Therapy Products[1]" that included a section providing recommendations on incorporating the 3R's in a preclinical testing program. Establishing a regulatory framework that is supportive of the 3R's in preclinical testing of emerging medical product areas such as cellular and gene therapies (CGT), presents a different type of challenge as compared to

[1] http://www.fda.gov/BiologicsBloodVaccines/GuidanceComplianceRegulatoryInformation/Guidances/CellularandGeneTherapy/ucm376136.htm

the use of accepted alternative methods in established medical product areas, such as vaccines or traditional drugs. There are no "gold standards" to enable validation of alternative preclinical models (both non-animal and animal) for testing the safety of CGT products. Therefore, to enable continued translational development of these promising therapies, the preclinical testing programs rely on the best available understanding of the product characteristics, putative mechanism(s) of action, and proposed clinical use. Thus, components of the 3R's are integrated in a preclinical testing strategy that is based on continually evolving science and technologies, to help define product benefit/risk elements applicable to human clinical trial design. The 2013 guidance provides examples of opportunities to address the 3R's in the development of preclinical testing programs for CGT products for regulatory submissions to US FDA.

V-4-885

The history of the mouse safety test for sera and vaccines

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At the end of the 19th century the invention of diphtheria antitoxin was an outstanding achievement in medicine. Large-scale production by an increasing number of companies soon required governmental quality control to avoid the release of batches with contaminations or low potency. The first animal safety test which was legally required was used to control the content of preservatives in the final product. A mouse safety test (MST) was suitable to detect phenol in high concentrations to avoid intoxication of patients. The MST became a standard test for all sera and vaccines developed in the following decades and became part of national pharmacopoeia monographs. When the World Health Organization started its work the MST became part of the general safety test. Today this safety test is still required in most national pharmacopoeias and international guidelines where it may be named abnormal toxicity test or innocuity test. However, over the decades the scientific basis to perform the MST has been lost because chemical-analytical tests have been developed to measure the phenol in medicines. Furthermore phenol is hardly used as a preservative nowadays. Therefore, the MST has lost its scientific basis and should be deleted from pharmacopoeias and guidelines.

Session V-4: Poster presentations

V-4-070

Magnetic resonance imaging – a way to reduce animal numbers for safety testing of veterinary medicines

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Safety-tests are a mandatory part of the licensing procedure for veterinary vaccines. Many experimental animals have to be killed to undergo a pathologic examination (1, 2). In order to reduce the number of animals, this study aimed at testing magnetic resonance imaging (MRI) as an alternative non-invasive method for evaluating local reactions in living pigs. During the study, 96 pigs (6 groups) were vaccinated into the left neck; each group was injected with a different commercial vaccine. At day 1, 3, 8, 15, 22 and 29 after vaccination, the animals were sedated and scanned using MRI. T1- and T2-weighted Spin-Echo sequences were used to examine the extent of local reactions. Imaging software was used to evaluate volumes of local reactions at both neck sides. Half of the animals of each group (n=8/group) were sacrificed for pathologic examination. A paired t-test showed highly significant differences between the left and right volumes of local reaction. Comparable results were found between histopathologic and imaging examination. The results demonstrate that MRI is suitable for safety-testing of veterinary vaccines in order to evaluate and monitor local reactions repetitively and to avoid euthanasia in pigs, which offers the possibility to reduce the number of animals needed.

This research project is funded by the German Federal Ministry of Education and Research (grant number 0316009B).

V-4-135

Pulmonary artery hemostasis: a model for surgical stapling and sealing

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Objectives: Pulmonary artery (PA) hemorrhage is a well documented adverse event in thoracic surgery, requiring appropriate animal testing to ensure medical device safety (Yano et al., 2013; Dunning and Walker, 2012; Craig and Walker, 1995). Thoracic anatomical constraints necessitate high animal usage with traditional canine/dog and ovine/sheep PA hemostasis models (Lacin et al., 2007; Izumi et al., 2007). This study aims to develop a novel porcine PA hemostasis model to reduce/refine animal testing of vessel sealing surgical devices.
Methods: Investigative surgery was conducted to improve PA access, integrating a Likert Scale for rating severity of hemorrhage. The model was validated for repeatability and reproducibility and screened for contributing factors. Animal usage requirements were compared to traditional animal models. Appropriate statistical analysis was preformed.

Results: Innovative surgical technique greatly increased PA exposure, provided ample device applications, and significantly reduced animal usage. Traditional PA models yielded 5 device firings/animal, while the newly developed model provided 25 firings. The Likert Scale successfully quantified severity of PA bleeding. External contributing factors did not affect hemostasis. Mechanical stapling and Energy sealing devices were successfully evaluated for hemostasis using the novel PA model.
Conclusion: A porcine PA hemostasis model was successfully developed that increased PA access, reducing animal usage by 5-fold. The model greatly increased device firing opportunities and allowed accurate evaluation of PA hemorrhage.

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V-4-196

In-vitro potency determination of botulinum neurotoxins A and B

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Botulinum neurotoxins (BoNTs) inhibit acetylcholine release from nerve endings, thus causing paralysis. This effect is broadly used for medical applications and in cosmetics. Because of the high toxicity of BoNTs, the potency of every produced batch must be precisely determined (Council of Europe, 2014a,b). This is currently done by a LD_{50} test in mice.

BoNTs are subdivided into two subunits. After the heavy chain recognizes its receptors on neurons, the light chain is translocated into the neuronal cytosol, where it specifically cleaves neurotransmitter release related proteins.

Botulinum toxins are structurally and functionally similar to tetanus toxin. For the detection of active tetanus toxin a sensitive and robust ELISA-based tool has been developed, relying on the two most relevant characteristics of the toxin: its ability for receptor binding and its proteolytic activity (Behrensdorf-Nicol et al., 2010). We are now generating a comparable test for the BoNT subtypes A and B as an alternative to the LD_{50} test.

Efficient binding of BoNT/B could be obtained using microplates coated with a synthetic receptor peptide in combination with ganglioside GT1b. The proteolytic activity of BoNT/B could be analyzed using a recombinant substrate protein and an antibody specifically recognizing the cleaved product. For BoNT/A, assay development is in process.

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V-4-256

Generation of neurons derived from stem cells and cell lines to develop cell based replacement assays for botulinum toxins and antitoxins

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Botulinum toxin is used therapeutically to locally paralyse muscles for clinical or cosmetic benefit worldwide. There is an increase in production of new countermeasures against several serotypes of Botulinum toxins.

Potency of active components in the therapeutic toxins for injection and for neutralizing activity of antitoxins still largely rely on animal models with LD_{50} end point which is associated with severe animal suffering and needs to be replaced (Sesardic, 2012).

Cell based assays show a great promise in replacing MLA, however the challenge of producing assays with high sensitivity and robustness for both applications has proved to be difficult (Sesardic and Gaines-Das, 2009).

Therefore, a number of different cells lines and endpoints have been utilised within this project. Differentiation protocols have been optimised using immortalised neuronal cell lines, mouse embryonic stem cells and human IPS cells to establish the sensitivity to the toxin through already established methods such as western blotting or neurotransmitter release (Pellett et al., 2010; Rasetti-Escargueil et al., 2011). The cells have then been applied to multi-electrode arrays system which has previously been shown to produce reproducible results (Novellino et al., 2011). Spontaneously active fully functional neural networks have been established and silencing of electrical activity observed after application of botulinum toxin.

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V-4-267

Pred-Immune; a novel integrative statistical approach for the prediction of the risk of immunogenicity of biologics

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Our aim is to develop a tool for the preclinical prediction of immunogenicity for biologics. Therefore, a statistical approach is presented that incorporates various data sources for the prediction of the clinical prevalence of the immunogenicity of biologicals.
The following steps were taken:

- Build a demonstrator database, Pred-Immune with 14 marketed biologics from 2 different categories,antibodies and recombinant proteins, i.e., antibodies blocking TNF-α, recombinant interferon-α (IFN-α) and recombinant IFN-β
- Incorporated data from preclinical animal models, physical chemical information and human immunogenicity data together with in-house generated *in vitro* data in a predictive model
- Describe the relationship between these parameters into a statistical model
- Using a demonstrator with 14 marketed biologics, we showed that when incorporating biologic class, biological stimulated dendritic cell CD86 expression, and molecular weight in one model, we obtained predictions that follow expected trends, showing promise for our integrated modeling approach.

Using this demonstrator database, we show it is possible to incorporate multiple sources of information, literature data, physical chemical data, as well as in-house generated *in vitro* analyses.

This model combines different assays, parameters and data in one model, generating a first step towards making a well-informed and structured choice of necessary information for immunogenicity prediction possible.

V-4-365

Photographic evaluation of skin lesions in batch potency testing of tuberculins – a reduction approach

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Rationale: Tuberculins are biological products derived from culture material of mycobacteria. When injected intradermally, tuberculins are capable to evoke delayed hypersensitivity in individuals infected by mycobacteria of the same species. Tuberculins are thus used for diagnostic purposes in human and veterinary medicine. To assess batch potency, compendial methods stipulate evaluation of skin lesions in previously sensitized guinea pigs (e.g., Monograph 01/2008 0536. Tuberculin Purified Protein Derivative, Bovine. *European Pharmacopoeia*, 8th edition). Apart from ethical concerns, the major drawback of this procedure is the poor reproducibility of test results. Here, we evaluated software assisted digital photography and infrared thermography as potential reduction alternatives to on-site manual measurements of lesions.
Results: Software assisted digital photography is suited to assess local reactivity in tuberculin batch potency testing. It allows for repeated measurements and thus helps to meet European Pharmacopoeia requirements for test validity in terms of precision of the potency estimate. It also facilitates documentation of test results in a QM environment. In contrast, infrared thermography did not reflect skin reactivity.
Conclusions: Software assisted digital photography is suited to reduce the number of test repeats and thus the number of animals used for tuberculin batch potency testing. Since covered by the current wording of the relevant monographs, immediate implementation is possible.

Funded by the German Federal Ministry of Education and Research 3R-methods to replace and refine legally required animal tests for immunologicals (0316009A – C).

V-4-461

Local lymph node assay on five medical devices including dental materials for alternative safety evaluation

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ISO 10993 is used for biological safety evaluation on medical devices such as dental materials. Recently 3R's concept and alternative methods has been adapted to the field of safety evaluation of medical devices with their frequent uses. Non-radioactive LLNA (OECD TG 442), representative alternative method for skin sensitization, have advantages that reduce animal uses and refine their suffer comparing with guinea pig methods (OECD TG 406). In this study, two LLNA (ELISA and noble flowcytometry method, FCM) were conducted to generate background data on medical devices. The noble metal alloys for dental casting (2 items), zirconia block for milling (1 item), biliary stent and vascular stent were selected. All test material was extracted according to "Accelerated Extraction Method" in ISO 10993-12:2012 and all extracts were evaluated in their appearance, pH and concentration of ions using ICP-MS. As results of two LLNA, 5 extracts have all negative responses in both LLNA. Otherwise, HCA (25%) that used as positive control were shown positive responses (SI≥1.6 in ELISA method and SI≥3 in FCM method). These results were same with previously guinea pig methods results.

This research was supported by a grant (13181MFDS636) from Ministry of Food and Drug Safety in 2013.

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V-4-599

An alternative micromethod to assess the procoagulant activity of *Bothrops jararaca* venom and the efficacy of antivenom

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The assessment of the capacity of antivenoms to neutralize the lethal activity of snake venoms relies on the traditional rodent lethality assay (LD_{50}), a time-consuming and expensive test that causes animal suffering and requires a large quantity of venom and animals. Validated *in vitro* tests are important for assessing the neutralizing capacity of immunized horse plasma and for the quality control of antivenoms. Considering the procoagulant activity of *Bothrops jararaca* venom in *in vitro* assays with mammalian plasma and the slow dynamics of fibrin formation in avian plasma, we propose a new coagulant assay. The clotting time was evaluated using the thromboelastometric profile of chicken plasma samples versus standardized doses of venom, either in the absence or presence of anti-bothropic serum (ABS). Ten nanoliters of ABS significantly neutralized the procoagulant effect induced by 0.3 μg of *B. jararaca* venom. Although it was not possible to obtain a direct correlation between our results and that obtained by the LD_{50} assay, this micromethod represents a highly sensitive technique for the characterization and quantification of procoagulant activity of small amounts of snake venoms and for the detection of specific antibodies against this activity using a minimal volume of antiserum.

V-4-717

Development of tridimensional murine and primary human osteoblastic cells models for biocompatibility studies

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A considerable number of animals is employed on biocompatibility studies of bone substitute materials. Cell culture models contribute to 3Rs through biocompatibility assessment by cytotoxicity, mineralization and differentiation assays. However, monolaminar bidimensional culture fails to simulate *in vivo* tridimensional cell-cell and cell-matrix interactions. This work presents trial of protocols of both MC3T3 cell line and primary human bone cells (HBC) to determine the ideal procedure and cell density to obtain spheroidal cultures and access its adequacy to material cytocompatibility tests. The sample included 0.5, 1, 2, 3, 4, 5, 7.5, 9 and 11x10^4 cells (n=15 per group) that were seeded on 24-well plates coated with agar, and incubated in agitation from 1 to 7 days.Cell aggregate morphology and quality was observed by Scanning Electron Microscopy (SEM), Confocal Microscopy and score methodology. Cell cytotoxicity was accessed by an adapted XTT assay. Higher cell numbers formed more stable spheroids. Handling and observation of spheroids was easier starting from 3x10^4 cells. Confocal microscopy and SEM revealed that cells within the core of the cell aggregate are viable. Aggregates were stable and presented good viability when employed on standardized assays, adapted for tridimensional cell culture, testing different ceramic, metallic and polymer-based biomaterials.

V-4-724

The application of rat hepatocyte aggregate for cytotoxity assay

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It has reported that the cellular functions, especially the metabolic functions, of 3D reorganized tissues convert locally because of their cell-cell communications or the slight difference in the culture environments (Landry et al., 1985; Waentig et al., 2011). Thus, the examination of the metabolic functions in 3D tissues should be considered their sizes to obtain physiological response for the application. This study examined the applications of rat hepatocytes aggregates in cytotoxicity assay to demonstrate the size-dependent physiological functions using mycotoxin and anticancer drugs.

The different-sizes rat hepatocyte aggregates were formed on the oxygen-permeable honeycomb microwells (Shinohara et al., 2013). Cytochrome 1A1, 1A2, 3A4 and 2C9 activities of rat hepatocyte aggregates were determined by P450-Glo™ Assay (Promega). Hepatotoxicity of Cisplatin and Aflatoxin B_1 were examined. The toxicity to human lung adenocarcinoma epithelial cell line (A549) of rat hepatocytes aggregates-mediated metabolites using CPT-11 were also tested.

The cytochrome assays and hepatotoxicity tests of rat hepatocyte aggregates demonstrated that medium size aggregates (52 and 88 mm) had more resistance to hepatotoxic chemical than that of other conditions. The results of cytotoxicity test using A549 cells exposed to rat hepatocyte-mediated metabolite of CPT-11 confirmed that 3D reorganization into certain size was important to obtain physiological response.

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V-4-727

Development of a mock circulatory system for evaluation of mechanical heart valves

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Purpose: Modelling of mechanical heart valve (MHV) flow dynamics is a challenging biomechanical problem and currently requires extensive *in vivo* validation. *In vitro* particle image velocimetry (PIV) techniques can be used to optimize MHV flow dynamics; however accurate pressures and flow rates are required to mimic the native heart. With newer valve technologies under development, PIV must be combined with accurate mock circulatory systems (MCS) to optimise MHV design and, therefore, reduce *in vivo* experimentation.
Methods: A MCS including the left heart, aorta and systemic circulation was constructed with a clear Perspex chamber to house mitral MHVs. Ventricular contractility, vascular resistance and arterial compliance were controlled respectively via a custom pneumatic controller, gate valve and variable Windkessel chamber. Haemodynamics were recorded and used to validate the MCS against the literature. The MCS was then used in combination with PIV to evaluate MHV flow dynamics.
Results and conclusion: The MCS successfully replicated the haemodynamics typically seen in healthy and failed human hearts. PIV evaluation of MHVs was successful and demonstrated the system's capacity to optimize MHV design before progressing to *in vivo* trials. Therefore, the combination of MCS and PIV used in this study should enhance MHV design and reduce the requirement for *in vivo* experimentation.

V-4-871

Applicability of monocyte activation test (MAT) in the routine of the quality control laboratory

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Some injectables are still tested by Rabbit Pyrogen Test (RPT), mainly biologicals. The aim of this study was to use MAT in hyperimmune sera (N=22) previously analyzed by RPT both considered negative and positive. Moreover, Zymozan spiked sample were also studied as non-endotoxin pyrogen. Sample were kept in contact with cryopreserved human blood. Saline solution that have passed the RPT were contaminated with different concentrations of Zymozan (from 1,000 to 5,000 ng/ml), kept in contact to cryopreserved whole human blood and in both cases, IL-1b release was quantified. These same concentrations were injected in rabbits in order to verify the fever response. MAT results were compared to RPT (sensitivity: 100%; specificity: 75%).This specificity can be explained due to a higher sensitivity of the MAT, which detected as positive samples that passed the RPT after repetition. It was also established that the 5,000 ng of Zymosan/ml was the threshold limit value for the RPT and MAT. These partial results strongly indicate that MAT may in the future replace RPT.

V-4-872

Monocyte activation test (MAT): identification of monographs that recommend rabbit pyrogen test (RPT) and bacterial endotoxin test (BET) as a kick-off for showing applicability of MAT

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MAT is thought to be a good replacement for RPT, however ICCVAM[1] stated that: I – MAT was not adequately evaluated to detect endotoxin in a sufficient number and range of parenteral pharmaceuticals and in no biological products and medical devices; II – there is no sufficient data that support the ability to detect non-endotoxin pyrogens; and III – it can be considered to detect pyrogen in human parenteral drugs on a case-by-case basis, subject to validation for each specific product. Products monographs in United States (USP[2]), European (Eur.Ph.[3]) and Brazilian (Braz.Ph.[4]) Pharmacopoeias that are recommended for RPT or BET are as following: RPT: 20 Monographs in USP, 37 in Eur. Ph. and 32 in Braz.Ph. BET: 619 Monographs in USP, 157 in Eur.Ph. and 48 in Braz.Ph., including drugs, biologicals and medical devices. Three products require pyrogenicity in the 3 Pharmacopoeias. Both RPT and BET are recommended by 6 Monographs in Braz.Ph. and 15 in Eur.Ph. and it is allowed to choose which test is to be applied. In Braz.Ph, most are biologicals, so, these products should be the first ones to be tested for the applicability to MAT since they are tested mainly by RPT.

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[1] Interagency Coordinating Committee on the Validation of Alternative Methods – ICCVAM (2008). Validation status of five in vitro test methods proposed for assessing potential pyrogenicity of pharmaceuticals and other products: ICCVAM. Test Method Evaluation Report. NIH, n. 8, p. 6392.

[2] United States Pharmacopeia – USP (2014). Edition 37-NF 18. Rockville: United States.

[3] European Pharmacopoeia 8.0 (2014). 8th Edition.

[4] Farmacopeia Brasileira (2010). 5. Edição. Brasília: Anvisa; 2010.

V-4-873

Assessment of pyrogenic contamination with lipoteichoic acid (LTA) in the monocyte activation test (MAT) and rabbit pyrogen test (RPT)

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LTA is a non-endotoxin pyrogen of great importance in the pathogenesis of sepsis. RPT is able to . all types of pyrogens but involves a great number of animals to be used. The Bacterial Endotoxin Test (BET) cannot fully replace RPT since it only detects endotoxins. MAT is sensitive to all types of pyrogens and is based on the same biological

mechanism responsible for the fever reaction in humans. ICCVAM has recommended its utilization for other pyrogens than endotoxin since its equivalence to RPT can be demonstrated. This work aims to evaluate the ability of MAT to detect LTA contamination. MAT used cryopreserved human whole blood and IL-1b release was measured. Rabbits were injected with different LTA dosis and the rectal temperature measured for 3 hours. The same curve was tested for both assays. Rabbit fever response was observed from 75,000 ng of LTA/kg and in MAT the limit detection was established in 50,000 ng/ml of LTA, or 5.41 UEE/ml. MAT showed to be more sensitive than RPT. Results suggest that MAT was efficient in detecting LTA and may contribute to the acceptance of this test by the Brazilian regulatory agencies and the replacement of animals.

V-4-946

Isolation of Shiga toxin-producing *Escherichia coli* (STEC) using magnetic beads

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In order to isolate pathogenic microorganisms from the natural environment, foods, or human-derived specimens, we commonly apply methods that utilize selection media or antimicrobial resistance. In cases where those methods are not applicable, desired microorganism for isolation was selected using laboratory animal passage. This research is an attempt to use magnetic beads for isolating STEC from mixed microorganisms in specimens. Monoclonal antibodies of Shiga toxin 1 and 2, which were excreted by STEC, were produced and then attached to the magnetic beads. Afterwards, STEC isolation from non-pathogenic *E. coli* was examined. In terms of sensitivity and specificity, STEC 1CFU and 10CFU couldn't be isolated but isolation ratio was higher than 80% at 10^2CFU. The isolation ratio of STEC from other enteric pathogens (*Salmonella*, *Vibrio*, *Shigella*, EIEC, EPEC, ETEC) was higher than 80% in average. In particular, *E. coli* O157:H7 strain EDL933 was isolated by over 90%. Such findings demonstrated that a magnetic bead-based method is effective in isolating pathogenic microorganisms. This method will reduce laboratory animal passage if it is applied to virus, *Borrelia* and *Rickettsia* that cannot be easily isolated by conventional methods.