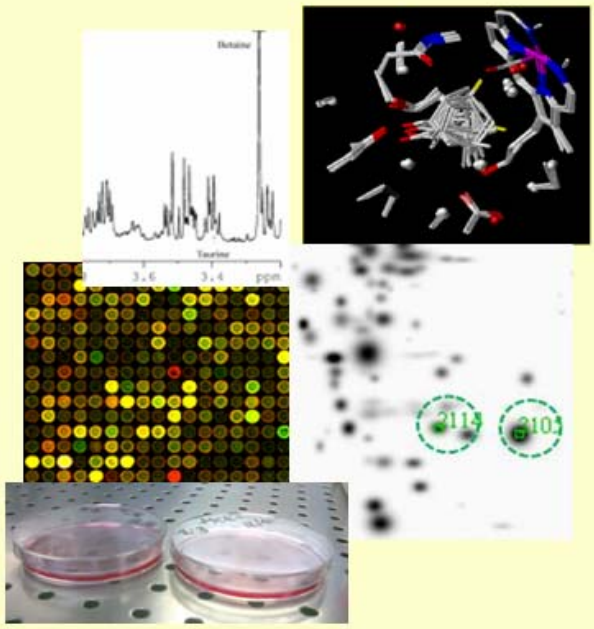
Toxicity Testing in the 21st Century and Alternative Methods



Workshop organized by

Italian Platform on Alternative Methods
European Consensus-Platform for Alternatives

Milan (Italy) - November 26th, 2010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Toxicity Testing in the 21st Century and Alternative Methods

Milan, November 26th, 2010

Toxicity testing is posing increasing challenges to health care, industry, and research communities, as well as the society as a whole. The complexity of issues at stake is matched by the recognition of theoretical, operational, methodological, and economical constraints that may hamper the efficacy of current procedures in toxicity testing. Likewise, the same constraints have been a powerful drive for the development of new methods, technologies, and scientific approaches to tackle difficulties and overcome bottlenecks.

The importance of understanding the possible adverse consequences of human and other living systems exposure to new agents, the recognition that individuals are exposed to complex mixtures of toxic compounds in the real world, the implementation of the REACH regulation in the EU, are just a few examples of the challenges that demand further developments in toxicity testing at many levels.

Initiatives are ongoing worldwide to support the advancements of our theoretical and technological tools for the evaluation of the hazards posed by the large number of existing agents. One such initiative, representing a key contribution owing to its wide scientific perspective and societal oversight, is the report entitled "*Toxicity testing in the 21st century - A vision and a strategy*", issued by the US National Research Council.

Building up on recent advances in bio-medicine and biotechnology, the report describes a transformative paradigm shift in toxicity testing, "...(1) to provide broad coverage of chemicals, chemical mixtures, outcomes, and life stages, (2) to reduce the cost and time of testing, (3) to use fewer animals and cause minimal suffering in the animals used, and (4) to develop a more robust scientific basis for assessing health effects of environmental agents." (Report, p. 3).

In order to contribute to discussions aimed at supporting thinking, research activities and action in the area of toxicity testing, the Italian Platform on Alternative Methods (IPAM) and the European Consensus-Platform for Alternatives have organized a workshop entitled "***Toxicity Testing in the 21st Century and Alternative Methods***".

The topics of the workshops will be approached in three sessions, to structure the major contents identified for such an initiative, as described in the programme outlined below.

The opening session will be devoted to setting the frame of the workshop, by presenting key elements of its scope and contents. This session will then include an introduction by the Presidents of IPAM and ECOPA, a presentation of the report issued by the US NRC, the description of challenges and opportunities for alternative methods in toxicity testing, as well as the bottlenecks of toxicity testing within the perspective of alternative methods.

The first session will be devoted to the core scientific/technological issues, with presentations of tools available for the paradigm shift in toxicity testing. The talks will then focus onto the use of most recent technologies and approaches of systems biology in toxicity testing (transcriptomics, proteomics, metabolomics, as well as *in silico*/computational approaches).

The second session will be devoted to selected existing cases of toxicity testing.

A round table will complete the workshop, and will be aimed at gathering key stakeholders, to discuss perspectives and difficulties in implementing the paradigm shift in toxicity testing approached in the workshop.

The workshop is intended to meet the interests of the scientific community at large, the industrial world (both providers and end-users of technologies), as well as decision-makers and political consultants.

Scientific and organizing committee

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Franca Fassio, IPAM
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Luca Nava, IPAM
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Program

Opening Session (9-11.00)

9.00-9.20 - President IPAM – opening speech

9.20-9.30 - President ECOPA – opening remarks

9.30-10.00 – John R. Bucher (National Toxicology Program; National Institute of Environmental Health Sciences, National Institute of Health, Research Triangle Park, NC, USA) - *Toxicology in the 21st Century, Transforming Environmental Health Protection*

10.00-10.30 – Thomas Hartung (Johns Hopkins University, Dept. Environmental Health Sciences, Center for Alternatives to Animal Testing; Baltimore, MD, USA) - *Challenges and opportunities for alternative methods in toxicity testing in the 21st Century*

10.30-11.00 – Emanuela Corsini (Università di Milano, Milano Italy), - *The major bottlenecks of toxicity testing within the perspective of alternative methods*

coffee break 11.00-11.30

Session 1 – Systemic approaches to toxicity testing (11.30-13.30)

11.30-12.00 - Jürgen Borlak (Medical School of Hannover; Fraunhofer Institute of Toxicology and Experimental Medicine; Hannover, Germany) – *From Mice to Man? Replacing meaningless animal studies through sophisticated alternative testing methods*.

12.00-12.30 – Gian Paolo Rossini (Università di Modena e Reggio Emilia; Modena, Italy) – *Proteomic approaches in toxicity testing: learning about toxicity pathways and their functioning*.

12.30-13.00 - Richard Currie (Syngenta Jealotts Hill International Research Centre, Bracknell, UK) - *Toxicity pathway identification through data fusion of metabolomic with transcriptomic data*.

13.00-13.30 – Emilio Benfenati (Istituto di Ricerche Farmacologiche "Mario Negri", Milano, Italy) - *How reliable are in silico methods for toxicity prediction?*

lunch break 13.30-14.30

Session 2 – From in vivo tests to high throughput in vitro tests (14.30-16.10)

14.30-14.50 - Horst Spielmann (Faculty of Biology, Chemistry, Pharmacy; Freie Universität Berlin, Germany) - *The transition to a toxicity pathway-based paradigm for chemical safety assessment in a EU dimension*.

14.50-15.10 - Aldert H. Piersma (Laboratory for Health Protection Research; National Institute for Public Health and the Environment RIVM; Bilthoven, The Netherlands) - *Towards animal-free alternative testing strategies in developmental toxicology*

15.10-15.30 - Hector Keun (Department of Surgery and Cancer; Imperial College London; London, UK) - *Metabolomics and alternative models to animal testing*

15.30-15.50 - Maurice P. Whelan (Institute for Health and Consumer Protection, European Commission Joint Research Centre, Ispra, Italy) - *Toxicity testing in the 21st Century – moving from principles to practice*

15.50-16.10 - Joachim Coenen (Merck Serono, Darmstadt, Germany) - *In vitro prediction of side effects of newly developed pharmaceuticals*

coffee break 16.10-16.30

Round table - *Toxicity Testing in the 21st Century: A toxicologist meets the four areas of the platforms for alternatives* (16.30-18.00)

Chair: IPAM

Speakers: Prof. Corrado Galli (Italian Society of Toxicology), Odile de Silva (Oréal, Paris, France), Troy Seidle (Humane Society International, London, UK); Romano Marabelli (Italian Ministry of Health, Roma, Italy), Prof. Jose V. Castell (Universidad La Fe, Valencia, Spain)

Workshop end 18.00

TOXICOLOGY IN THE 21ST CENTURY, TRANSFORMING ENVIRONMENTAL HEALTH PROTECTION

John R. Bucher, Ph.D., DABT

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Tox 21 is a consortium effort between 4 US Federal organizations with the goal of jumpstarting the practice of toxicology by transforming its tools and approaches. In 2004 the NTP issued its Roadmap for the 21st Century, calling for creation of a more predictive science focused on a broad inclusion of target-specific, mechanism-based, biological observations. Taking advantage of advances in automated high throughput screens (HTS) established by the pharmaceutical industry, along with computational tools capable of integrating and analyzing massive datasets, the participating agencies recognized the potential to study the interaction of thousands of chemicals with "biological space". The NIH Chemical Genomics Center (NCGC) was established in 2004 providing state of the art robotic assay capabilities and chemical libraries comprised of hundreds of thousands of substances. The EPA established its National Center for Computational Toxicology (NCCT) in 2005, and started its "ToxCast" program focusing on providing fingerprints of pesticide actives in 2007. As a response to the 2007 NAS report "Toxicology in the 21st Century", the NCGC, NIEHS/NTP, and EPA established Tox 21. FDA joined in June of this year. The 4 agencies are coordinating efforts to select chemicals and assays, develop analysis tools and integrative databases, and interpret the findings through additional targeted testing approaches. These can include additional HTS assays on different platforms, or assessments in zebrafish, *C. elegans*, or in traditional rodent models. The hope is that the creation of biological profiles for activation of "toxicity pathways" before designing more traditional toxicology studies, will result in far more efficient safety assessment studies, and result in fewer surprises once a chemical is in the public domain. Current areas of emphasis for assay development involve general stress responses as an integrative measure of toxicity resulting from a wide variety of mechanisms, along with nuclear receptor activation, as a measure of cellular perturbation that may result in altered differentiation, development, or responses to exposures to other agents. Preliminary results are very promising and suggest that these tools will allow toxicologists to bring a whole new dimension of information to the table very early in the process of the search for adverse effects.

CHALLENGES AND OPPORTUNITIES FOR ALTERNATIVE METHODS IN TOXICITY TESTING IN THE 21ST CENTURY

Thomas Hartung

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A mechanistic toxicology has evolved over the last decades, which is effectively relying to large extent on methodologies which substitute or complement traditional animal tests. The biotechnology and informatics revolution of the last decades has made such technologies broadly available and useful.
Regulatory toxicology has only slowly begun to embrace these new approaches. Major validation efforts, however, have delivered the evidence that new approaches do not lower safety standards and can be integrated into regulatory safety assessments.
Political pressures especially in the EU, such as the REACH legislation and the 7th amendment to the cosmetic legislation as well as the revised laboratory animal welfare Directive from 2010, further prompt the need of new approaches. In the US, especially the NAS vision report for a toxicology in the 21st century and its most recent adaptation by EPA for their toxicity testing strategy have initiated a debate how to create a novel approach based on human cell cultures, lower species, high-throughput testing and modeling. Currently there are prospects for a reauthorization of the Toxic Substance Control Act, which might become a large testing program for old chemicals in the US.
The lecture summarizes the lessons learned from the development, validation and acceptance of alternative methods for the creation of a new approach for regulatory toxicology. Beside the technical development of new approaches such as systems toxicology, a case is made that we need both conceptual steering and an objective assessment of current practices by evidence-based toxicology.

THE MAJOR BOTTLENECKS OF TOXICITY TESTING WITHIN THE PERSPECTIVE OF ALTERNATIVE METHODS

Emanuela Corsini, Corrado L. Galli

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At present, assessment of chemical-induced toxicity to a greater extent relies on the use of animal models. The use of whole animals, however, presents many secondary issues, such as high costs, ethical concerns, but it still represents a valid tool in the risk assessment process. Furthermore, due to the new policy on chemicals (REACH), in the European Union, *in vitro* methods (in silico as well) are regarded to play an important role in the near future as complementary methods.
Several concerns on the predictive capacity of alternative models exist. More robust evidences must be provided before a greater and extensive use of alternative methods in all scientific fields and in particular toxicology can be identified. Limitations of in vitro methods include:

- technical limitations, such as chemical solubility and stability, reaction to plastic;
- relevance of the in vitro endpoints and mechanisms with the in vivo adverse effects (only mechanistic relevant endpoints should be explored);
- metabolic competence of the in vitro system is often very limited, and since the biokinetics of a compound, including its metabolism, can greatly influence its toxicologic properties, pharmacokinetics need to be considered when interpreting results from in vitro models;
- due to interspecies differences, which may cause false positives or false negatives when screening compounds for adverse effects on humans, human cell systems should be preferred;
- systemic interaction, as the interaction/interplay between neuroendocrine and immune function are loss. Therefore, only direct citotoxicity can be identified in vitro;
- chronic, reproductive effects cannot be yet tested.

Considering all these limitations, a battery of in vitro assays seems at present the most appropriate way of providing the added value of the alternative approaches.

FROM MICE TO MAN? REPLACING MEANINGLESS ANIMAL STUDIES THROUGH SOPHISTICATED ALTERNATIVE TESTING STRATEGIES.

Jürgen Borlak

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Drug induced liver injury (DILI)is the leading reason for drug removals and restrictions, and remain a challenge to the industry and regulatory authorities. Such toxicity is frequently doseindependent and not related to the pharmacology of the drug. Also, idiosyncratic adverse drug reactions (ADRs) usually cannot be predicted during preclinical or clinical drug development. The inability of animal models to detect these events may lie with interspecies differences or the lack of accurate disease models. Furthermore, clinical trials are often underpowered to detect rare events such as idiosyncratic ADRs. In my presentation I will focus on mechanism of DILI and the possibilities to detect early signs of liver toxicity through the application of toxicogenomics to cultures of metabolically competent hepatoctyes.
Indeed, the lesions learned from the 20 drug removals from the market over the last ten years have been very instructive. As a case study I will focus on Trovafloxacin, a fluoroquinolone antibiotic targeted against bacterial DNA gyrase and topoisomerase IV. This drug is not the only drug of its class to be associated with such idiosyncratic toxicity.
Postmarketing surveillance of other fluoroquinolones (e.g., temafloxacin, and grepafloxacin) revealed serious ADRs as well, including potentially lethal liver- and cardiotoxicity, associated with their use – but not identified during drug development, and therefore resulting in withdrawal. Taken collectively, DILI may arise via several mechanisms and gene expression profiling of cultures of metabolically competent hepatoctyes helps to pinpoint location and pathways perturbed by drugs and chemicals. Studies with primary human hepatocyte cultures provide important information on a mechanism of liver toxicity, and may have been used to predict the excessive hepatotoxicity of trovafloxacin, and of other drugs.

PROTEOMIC APPROACHES IN TOXICITY TESTING: LEARNING ABOUT TOXICITY PATHWAYS AND THEIR FUNCTIONING

Gian Paolo Rossini, Gian Luca Sala, Giuseppe Ronzitti, Mirella Bellocci

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The characterization of toxicity pathways has become a major task of toxicity testing, aiming at a description of the full chains of events participating to cellular responses to toxicants in biological systems. The classical reductionistic approach does not appear to suffice when approaching molecular processes at a system level, and proteomics is being increasingly used for this kind of studies. This presentation will be devoted to studies on microalgal toxins using cell lines and tissues, as an example of natural compounds altering basal cellular functions. Investigations based on differential expression proteomics have been aimed at the characterization of the mode of action of toxins, the prediction of toxicity of biological materials in the environment, as well as the detection of contaminated samples by biomarkers of effects exerted by toxins. Modules of toxicity pathways are emerging from these studies. A critical analysis of existing data indicates some major issues requiring further investigation for a better comprehension of these pathways and their interactions. The distinction of artifacts from robust biomarkers appears a critical issue, and the characterization of molecular features of relevant proteins could contribute to overcome difficulties. Owing to the extreme complexity inherent into the characterization of toxicity pathways and their interactions at a system level, some consensus should be reached among investigators to identify a few model systems for a concerted effort. Furthermore, the efficacy of integrating systemic and reductionistic approaches is highlighted as a means to support the development of quantitative, predictive models of toxicity pathways.

TOXICITY PATHWAY IDENTIFICATION THROUGH DATA FUSION OF METABOLOMIC WITH TRANSCRIPTOMIC DATA

Richard A. Currie[1], Claire L. Waterman[2], Denis V. Rubtsov[2], Hiroaki Watanabe[3], Domingo Salazar[1], Stephen Muggleton[3], Jayne Wright[1], Julian L. Griffin[2]

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The recent US-NRC report "Toxicity Testing in the 21st Century" proposed a new paradigm for toxicity testing and risk assessments: moving away from animal testing at arbitrarily high doses to *in vitro* tests for perturbations in human "toxicity pathways" at doses that are realistic for the risk assessment being performed. Traditionally, *in vitro* assays applied to human health risk assessments have been most successful when used in the context of an understanding of mode-of-action (MOA). In these cases *in vitro* tests are designed to model key events in the MOA hypothesis. The report recognised this reality and also recommended a sustained research effort to define human relevant toxicity pathways by increased understanding of underlying molecular mechanisms. The application of "omics" technologies has been suggested as one way to build this knowledge. Using statistical and signal processing techniques we fused and integrated data from histopathological; clinical chemistry; microarrays for mRNA, and metabolomics of liver, plasma and urine from a panel of rat liver-carcinogens and -non-carcinogens. These analyses identified perturbations to key pathways that may drive carcinogenesis. The application of systems biology tools such as Inductive Logic Programming allows us to formulate new mechanistic hypotheses of toxicity pathways, in the context of existing molecular biological knowledge, for subsequent testing and validation. The application of this process shows how understanding *in vivo* toxicities may be used to generate knowledge of toxicity pathways. By applying this knowledge appropriate *in vitro* assays, a prerequisite for the vision of 21st century toxicity testing, may be developed.

HOW RELIABLE ARE IN SILICO METHODS FOR TOXICITY PREDICTION?

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In silico methods are those which use computer to make the prediction of the property of interest. They include the so called (quantitative) structure-activity relationship (Q)SAR. REACH requires, for a correct use of the QSAR model, that "the substance is included in the applicability domain of the model". Thus, the acceptance of the model is not given a priori, but in relation to its appropriate use. It means that the same model can be accepted if used for a substance, but can be not accepted if used for a second one.

Within the CAESAR platform (http://www.caesar-project.eu) we developed a freely available tool to assess the applicability domain (AD), through quantitative and visual ways. This free, fast tool is based on: Chemometric check, Fragments for outliers, Similarity index, Prediction Concordance, Prediction Accuracy, and Uncertainty of the prediction. Thus, the CAESAR's tool is based not only on the chemical information, as the typical AD tools, but also on toxicity results. This tool proved to discriminate cases where QSAR can be applied, and cases were there are problems. In this way, the user can know if the use of the model is reliable or not. Examples will be given, where the models should not be used.

THE TRANSITION TO A TOXICITY PATHWAY-BASED PARADIGM FOR CHEMICAL SAFETY ASSESSMENT IN AN EU DIMENSION

Horst Spielmann[1], Monika Schäfer-Korting[1], Vivian Kra[1]l, Emily McIvor[2], Troy Seidle[2] & Greet Schoeters[3]

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Conventional approaches to toxicity testing and risk assessment are often decades old, costly and low-throughput, and of dubious relevance to humans. These factors have prompted leading scientific bodies to call for a transition to a 21st century paradigm, including a move away from apical outcomes at high doses in whole animals, and toward a mechanistic understanding of the source-to-outcome continuum between xenobiotic exposure and adverse health effects. Such a shift will require a robust understanding of the cellular response/toxicity pathways which that can lead to adverse effects when perturbed; appropriate in vitro systems to study chemical interactions at key targets along a pathway; and computational systems biology models to describe the "circuitry" underlying each pathway as a basis for creating biologically realistic dose-response models. The AXLR8 project aims to support the transition to a toxicity pathway-based paradigm for quantitative risk assessment and will: 1) organize a series of annual workshops to map research progress, gaps and needs in the FP6/FP7 program on alternative testing strategies. 2) Provide a range of tools and opportunities for enhanced interdisciplinary and international communication, coordination and collaboration in order to maximise the impact of available resources. 3) Work to streamline regulatory acceptance procedures to provide for the uptake of validated 3Rs methods, including a smooth transition to 21st century systems as they become available. 4) Produce annual progress reports on the state of the science, including recommendations on priority research and funding targets, in order to ensure a prominent role for European science in this rapidly developing global research area.

TOWARDS ANIMAL-FREE ALTERNATIVE TESTING STRATEGIES IN DEVELOPMENTAL TOXICOLOGY

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The current system of risk assessment of chemicals is complex, very resource-intensive, extremely time-consuming, and requires millions of experimental animals. Modernization of this process is only feasible through alternative integrated testing strategies incorporating more rapid, cheap and ethically less controversial test methods. Reproductive toxicity testing requires over 60% of all animal testing in REACH. In spite of extensive research, alternatives accepted in regulatory reproductive toxicology are lacking. Current alternatives require appropriate definitions of predictability and applicability domains. This situation will only change if new approaches are explored. This presentation will survey novel developments towards innovating risk assessment in reproductive toxicology. Integrated testing strategies need to be built, making optimal use of existing and alternative tests in a tiered and battery approach. Optimization of testing should be informed by retrospective analyses of existing and newly developed databases, to define the critical end points to be tested among the wealth of end point parameters usually studied in reproductive toxicology. Alternative assays need to be developed in a fashion that is tailored to the specific questions to be asked in terms of hazard identification. Application of the various omics approaches could lead to a more specific assessment of chemical effects. Wherever possible, human based biological material such as established embryonic stem cell lines should be employed to facilitate extrapolation of testing results in the risk assessment. Taken together, the challenges are multiple and complex, and they merit extensive research investments in order to reach the necessary innovation in terms of enhanced efficiency, reduced cost, and increased level of scientific knowledge within the risk assessment process.

METABOLOMICS AND ALTERNATIVE MODELS TO ANIMAL TESTING

Hector Keun

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Metabolic profiling (a.k.a metabolomics or metabonomics) is a holistic approach to the study of metabolism that is now an important element of systems biology and biomarker discovery. Perturbations to metabolism can reveal both the presence of pathology and the endogenous pathways that play a role in its development. In the generation and evaluation of alternative models, normal metabolism is a critical phenotype that is often lost in vitro. While xenobiotic transformation is of obvious important, correct and reproducible endogenous metabolic behaviour will also make a significant impact on the validity of in vitro findings. This lecture will review the potential of metabolomics to contribute to the development of successful alternatives to animal testing.

TOXICITY TESTING IN THE 21ST CENTURY – MOVING FROM PRINCIPLES TO PRACTICE

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The United States National Academy of Sciences report entitled "Toxicity testing in the 21st Century – A vision and Strategy", published in 2007, has set the scene for a major paradigm shift in the way we determine potential hazard of chemicals and evaluate the risk they might pose for human health. There are many important facets to this vision, but a key component is the anticipated shift away from animal testing to an integrated alternative approach based primarily on vitro assays, complimented by computational methods. At the heart of the strategy is the concept that ultimately, toxicology can be deconstructed into a comprehensive set of toxicity pathways that describe the mode of action of any chemical agent, from the first significant molecular event in vivo, to an observed pathology. Coupled with that is the hypothesis that pathway activation is triggered on exceeding a target-tissue dose threshold, and that the dose will dictate which specific pathway will ultimately result in an adverse outcome. Such principles, if taken seriously, should have a major impact on the way we approach in vitro testing, bearing heavily on assay design, chemical selection, experimental design, data processing, and interpretation of results. This talk will describe in practical terms how our high throughput in vitro testing programme has been influenced by exploring the Tox21c strategy, and will reflect on where the alternatives field is headed if as a community we commit to achieving the vision.

IN VITRO PREDICTION OF SIDE EFFECTS OF NEWLY DEVELOPED PHARMACEUTICALS

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Pharmaceutical companies use animals for the purpose of discovery and development of pharmaceuticals. National and international regulatory legislation (e.g., ICH-Guidelines) mandate in-vivo studies to be performed during the drug-development process.
The use of in-silico and in-vitro assays is a crucial step to predict side effects of development candidates to select the best, most efficacious and safest candidate for regulatory drug-development. By selecting the most promising candidates before these compounds become subject to in-vivo studies, animal studies used for regulatory purposes can be limited to the minimum required by law.
While it is not likely that in-vivo studies can be completely replaced, substantial efforts are made to reduce the number of animal studies by developing and performing batteries of in-vitro and in-silico studies to select the most promising candidate before the conduction of mandatory regulatory animal studies.
Besides candidate selection and mechanistic approaches, the highest potential for a reduction of animals used in pharmaceutical development lies with the global harmonization of the legislation. Global harmonization is crucial to further reduce, refine and eventually replace some in-vivo studies currently required to be performed for the global drug-development process. Worldwide acceptance of harmonized development standards will result in a major reduction of animals used in drug-development.
This workshop presentation will briefly summarize the different phases of the non-clinical development process and will provide a personal and subjective estimate where replacement of in-vivo studies may be possible in the 21st century to replace in-vivo studies in predicting or explaining side effects of drug candidates.