Session 6.3
Computational toxicology

Lecture

Computational modelling of biological systems: Implications for use of laboratory animals in toxicological testing and research

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Living organisms from single cells to people can be thought of as "biological machines" – feedback control systems following genetically-determined developmental programs and in adulthood focusing on homeostasis and reproduction. Systems engineering principles that define the control circuits in man-made machines are also applicable to living systems. In fact, striking parallels exist between control circuits in complex machines and in biological cells and tissues (Carlson and Doyle, *PNAS 99*, Suppl. 1, 2538-2545, 2002). Regulatory networks exist at all levels of biological organisation – molecular, cellular, tissue and organism – and a systems engineering approach to characterising their structure and function appears to be possible. We can ask if and when computational models will be ready to replace laboratory animals in toxicological research and testing. First, however, we should recall a cardinal rule of computer programming – garbage in – garbage out. In other words, a robust, predictive computational model of a biological system must be based on a sound understanding of that system. The rate-limiting step in the development of these models is the rate of our progress in understanding the relevant biology. Although a revolution is underway in the study of basic biology it will be some time before we can draw inclusive circuit diagrams of living cells and tissues. Today's computational models are thus incomplete and are not suitable replacements for laboratory animals. Computational models do, however, have important roles to play as adjuncts to classical toxicological methods. Three dimensional modelling of protein structure, for example, can be used to screen chemical structures for binding behaviours potentially associated with toxic effects. Physiologically-based pharmacokinetic models help to ensure efficient experimental design and thereby refine animal use. The ongoing, rapid development of new biological understanding and the explosive growth of computer hardware and software technologies guarantee that the role of computational modelling in toxicology will expand continually. While these developments will not, in the foreseeable future, eliminate the need for laboratory animals, they will lead to significant refinement and possibly to reduction of animal use.

Although this work was reviewed by EPA and approved for publication, it may not necessarily reflect official Agency policy.

Lecture

TIMES skin sensitisation model: Development and validation

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The Tissue Metabolism Software (TIMES) was used to facilitate the interface of skin sensitisation model which incorporates skin metabolism and consider the potential of parent chemicals and/or their activated metabolites to react with skin proteins. The training set comprised 634 diverse chemicals classified as significant, weak and non-sensitisers. Since skin sensitisation potential depends upon the ability of chemicals to react with skin proteins either directly or after appropriate metabolism; a metabolic simulator was constructed to mimic the enzyme activation of chemicals in the skin. This simulator contains 203 hierarchically ordered spontaneous and enzyme controlled reactions. The covalent interactions of chemicals and their metabolites with skin proteins were described by 83 reactions falling within 39 alerting groups. For some of these groups specific (Q)SARs were utilised to determine stereo-electronic characteristics that might enhance or inhibit activity. The present skin sensitisation model was able to predict correctly 80% of the significant sensitisers, 34% of the weak sensitisers and 72% of the non-sensitisers. A set of 96 chemicals tested for skin sensitisation and not used in the training set were used for external validation of the model. The model predicts the external data fairly well if a model domain was determined based on the concept of the mutual influence amongst first or second neighbour atoms in a molecule. In this case, the correctness of predictions was either 71% or 87% depending on how strict the domain was defined. The correctness of predictions was reduced to 52% if the model domain was ignored.

Lecture

Implementation of new molecular endpoints in a validated method: A case study of data collection and statistical assessment

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The process of validation is an integral part in the establishment of new toxicological methods. A crucial part of this process is based on the application of adequate biostatistical methods.

Especially when new technologies shall be applied within existing test systems, qualified procedures for comparisons of different endpoints and their results are essential.

In this study the results of a joint project by ZEBET and German pharmaceutical companies to improve the Embryonic Stem Cell Test (EST) were taken as example for the biostatistical evaluation of new endpoints.

The validated endpoint of the EST consists of the microscopic analysis of cardiomyocyte differentiation after application of reference substances. Additionally, molecular endpoints assessing gene expression by flow cytometry (FACS) and real-time-PCR (RT-PCR) were selected. Ten reference substances with different embryotoxic potentials (non, weakly and strongly) were tested by microscopic analysis, FACS and RT-PCR.

Differences between substance effects and test methods for ZEBET (Microscope/FACS) and for Schering (Microscope/RT-PCR) were assessed with univariate analysis of variance (ANOVA). The inter-laboratory reproducibility was determined using results obtained with the validated endpoint. The already validated biostatistical prediction model was applied to the new endpoints and the predictive power assessed.

It could be shown that the occurring variance in the test results is mostly caused by reference substances and only to a minor part depends on the applied method or the conducting lab.

In conclusion, new endpoints may be implemented in a test system if they do not differ significantly from the validated ones and the results lead to the same predictive outcome.

Poster

Strategy for (Q)SAR evaluation of chemical genotoxicity

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There are approximately 20,000 existing chemicals that should be evaluated urgently for human health effect but only 10% of them have been, more or less, evaluated. It is, however, not realistic to perform toxicological tests on all chemicals. In 2003, the law concerning the examination and regulation of manufacture/import of new/existing chemical substances in Japan had been amended, and became effective last year. The (Quantitative) Structure Activity Relationships (Q)SAR approach has also been recommended for consideration as an aid in risk evaluation. We have validated commercially available (Q)SAR systems (DEREK, MultiCase, and AdmeWorks) employed widely in overseas regulatory agencies, by using data on more than 200 chemicals registered for existing chemicals in Japan. These chemicals had been tested under GLP compliance, thus the quality of test results could be considered sufficient for the learning dataset. We evaluated the (Q)SAR systems individually using existing chemicals with genotoxicity data and using also the database published by Kirkland et al.. An *in silico* evaluation flow was constructed, combining these systems after filtering with molecular weight of the chemical. We obtained satisfactory outcomes of evaluation of Ames assay results applied to the proposed flow.

Poster

The combined use of (Q)SARs and *in vitro* testing methods for creating intelligent testing strategies for local effects – potential and current limitations

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In the EU, for both animal welfare and economical considerations, the future policy for the risk assessment of chemicals (REACH proposal) relies on the use of both (Q)SARs and *in vitro* testing methods.

Consequently, so-called intelligent testing strategies that are based completely on non-animal testing and/or *in silico* prediction methods have lately received increasing attention. In the past 15 years, activities at the BfR in this field have focused on both the validation of *in vitro* methods at ZEBET and the development of valid (Q)SARs for the prediction of the presence or absence of substance-related adverse effects.

The work of Ingrid Gerner and colleagues at the BfR to predict/exclude local effects such as skin/eye irritation and corrosion or skin sensitisation has led to a set of physico-chemical exclusion rules and structural alerts that have been submitted to the European Chemicals Bureau for external validation in early 2005.

The potential and possible limitations of these rules and alerts in combination with well-established *in vitro* testing methods to be used in the frame of animal-free testing strategies for local effects are discussed. Both components could complement each other to close data gaps and extend the applicability domain of such a testing strategy. Future work needs are addressed.

Poster

Optimisation of pyrogen testing in parenterals according to different pharmacopoeias by probabilistic modelling

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The rabbit test to detect pyrogenic contamination in parenterals is crucial to ensure patient safety. The pharmacopoeial tests in Europe, the US and Japan are based on the fever reaction of rabbits, but differ in their experimental design and in their algorithms to assess contamination. Employing an international reference endotoxin, fever can be induced in rabbits. Data from 171 rabbits built the base for probabilistic modelling of the fever reaction and for the comparison of the pharmacopoeial tests. The rabbit fever reaction could be modelled as a function of the amount of injected endotoxin (per kg body weight) by linear regression. Combining the pharmacopoeial algorithms of the rabbit pyrogen test with the developed model allowed analysis of differences regarding test results and animal consumption. This showed that the assessment of pyrogenic contamination strongly depends on the respective pyrogen test stipulated by regulations. Additionally, the approach was used to develop a new experimental design. Two specific versions of this design resulted in a reduction of the number of animals used by about 30% while the safety of the test was maintained. A need for harmonisation is evident, allowing optimisation of the experimental design, which promotes animal welfare.

Lecture

Prevalence and test interdependence: Pivotal parameters in the design and validation of testing strategies

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Testing strategies in toxicological hazard assessment are usually defined by the sequential use of existing knowledge, chemical information, *in silico*, *in vitro* and *in vivo* approaches. As stand-alone alternatives, replacements to animal tests seem to be achievable for only a few toxicological endpoints. Thus, a combination of *in vitro* and other tests applied in a strategic manner is needed to replace or at least optimally reduce *in vivo* testing. Besides animal welfare, such a strategic approach needs to accommodate safety and economical aspects. These three factors can be optimally addressed only when the prevalence of the toxicological effect of concern and test interdependence are analysed and incorporated. As pilot cases, the prevalence according to the current *in vivo* test for skin irritation (<10%) and eye irritation (15-20%) for the applicability domain of new chemicals were assessed by employing the European New Chemicals Database. Predictive values of alternative tests, calculated with such prevalence estimations, enable an optimised test assessment and strategy design, e.g. in low prevalence situations they demand a strategy first focusing on the correct identification of non-toxic substances. To highlight this, we modelled two strategies proposed for eye irritation with three steps each based on assumptions of tests' predictive capacities and test interdependence. Consequences for test development and the validation process are discussed. We conclude that information on prevalence and test interdependence is essential for the design of testing strategies in which the objectives of safety, animal welfare and costs have to be balanced.

Poster

ECVAM Key Area "Biostatistics and computational toxicology": Summary of ongoing activities

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The ECVAM Key Area "Biostatistics and computational toxicology", established in 2005, was set-up in order to address the growing analytical demands of rapidly developing validation studies and concepts. Anticipating an increasing number of validation exercises, this key area shall support their design, conduct and analysis. Emerging fields in validation such as retrospective approaches or testing strategies bear new statistical challenges. Furthermore, this key area shall serve as a contact point for validation efforts dealing with computational approaches, e.g. (Q)SARs or expert systems. One of its first activities is to build up a network of experts especially addressing the emerging challenges, e.g. retrospective data analysis, evidence-based approaches to toxicological problems and decision making aspects, foreseeing a first meeting at the end of this year. However, two recent publications (Hoffmann et al., 2005, *Regul. Toxicol. and Pharmacol. 41*; Hoffmann and Hartung 2005, *Tox. Sci. 85*) constituted first achievements demonstrating ways to introduce evidence-based approaches into validation. A feasibility study on more efficient validation study designs and a case study highlighting statistical aspects in the design of testing strategies are just finalised. Regarding actual studies, the major achievement was the integral statistical supervision of validation projects on *in vitro* pyrogen tests (Hoffmann et al., 2005, *J. Immunol. Methods 298*). These were accompanied by a thorough analysis of the corresponding animal experiment (Hoffmann et al., 2005, *J. Endotoxin. Res. 11*). Ongoing validation activities involving this key area are *inter alia* studies on skin and eye irritation, cell transformation assays and acute systemic toxicity.

Lecture

Role of *in silico* methods in alternatives strategy

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To balance limited possibilities to generate experimental data, safety assessments will increasingly rely on data from the non-test methods. *In silico* based information may supplement available experimental test data making it possible to reach a conclusion with more certainty on an endpoint of concern, or it can be used to replace experimental test where there is no test data available. The non-test methods include SAR and QSAR. SAR can be applied for read-across/analogue/chemical category identification while QSAR for a quantitative endpoint prediction. Formal derivation of SAR is very similar to QSAR development. (Q)SARs (QSARs and SARs) can be developed for both *in vivo* and for *in vitro* endpoints. Validated (Q)SAR models of *in vivo* data may potentially lead to replacement. Feasibility to develop reliable (Q)SARs depends on an endpoint. For example NOEL for systemic toxicity is not a well defined endpoint from modelling point of view as it represents already interpreted data coming from a suite of endpoints and inherently "noisy". Further some of the *in vivo* endpoints such as eye irritation elicit substantial intra chemical variability due protocols used. Quality of QSAR models of such "noisy" data is limited. QSAR for *in vitro* endpoints can be built to further reduce costs and time. These models can likely achieve higher predictivity compared to QSARs for *in vivo* endpoints because they will be based on a higher quality data, will model simpler (in terms of biology) endpoints, and there will be a better mechanistic knowledge about the endpoint.

Poster

Realistic approaches to sharing data and knowledge

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Lhasa pioneered the sharing of knowledge about mechanism-based structure-activity relationships by computer, and many commercial and regulatory organisations have contributed to the knowledge base. Information can be shared in sufficient detail both to make predictions about potential toxicity and metabolism, and to support and justify the predictions to a user, without disclosing commercially sensitive material. More recently, projects run by the International Life Sciences Institute in Washington, Lhasa, and a consortium in the USA, have explored ways of encouraging the sharing of toxicological data with some success. This talk will discuss how issues that have constrained data and knowledge sharing are being addressed. The indications are that data and knowledge sharing will become established as ways to make research more productive, to save cost, and to reduce the need for animal experiments.

Poster

Development of topical formulations with corticosteroids utilising physicochemical and *in vitro* methods

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Introduction: The objective of this work is to define a practical sequence scheme for a fast and reasonable development of semisolid formulations without testing on animals. For this purpose four active compounds were chosen from the corticosteroids group: Betamethasone valerate, clobetasol propionate, hydrocortisone and mometasone furoate. Two OECD-markers for *in vitro* percutaneous absorption (OECD 428): caffeine and testosterone were taken additionally as controls. The first step summarised information on physico-chemical properties of the substance. Physico-chemical profiling of the model drug Hydrocortisone are here presented.

Experimental methods: The solubility was estimated by nephelometric analysis. The saturation solubility was measured by the shake flask method (32°C, KRB buffer). The partition coefficient was determined in experimental systems by measuring octanol/water partitioning also by the shake flask method. The Immobilised Membrane Partition Coefficient (KIAM) and protein binding were determined by HPLC.

Results: Table 1 Summary of parameters determined for Hydrocortisone; Molecular weight 362.47 g/mole; CAS Number 50-23-7; Molecular formula C21H30O5; UV maximum 242 nm; pKa none; Log P 1.56; Protein binding 49%; Solubility (nephelometric) >202.46 µg/ml; Saturation solubility 333.00 µg/ml; KIAM 7.56.

Discussion: Hydrocortisone is a very slightly soluble compound. The data summarised in Tab. 1 indicate that Hydrocortisone is highly permeable. This model drug is a moderate lipophic substance with approximately 36 times higher affinity to the lipophilic octanol phase than to the water phase. The KIAM value correlates well with log P. The *in vitro* drug release profile of the test drugs from semisolid formulations has to be determined in outgoing studies.

Poster

3D-Quantitative Structure-Activity Relationships (3D-QSARs) to predict binding to the human oestrogen receptors α and β: Use in risk assessment

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Quantitative Structure-Activity Relationships (QSARs) are increasingly being used to predict the effects of a diverse series of toxic endpoints. As part of these efforts there has been considerable interest in predicting whether a compound will cause endocrine disruption. In particular there has been much success in using tiered computational approaches for the prediction of endocrine disruption. These start with simple and easily applicable structural rules and go through to more complex receptor binding studies. The present study has investigated the use of a 3D QSAR approach, Comparative Molecular Field Analysis (CoMFA), to model the relative binding affinity of 99 compounds to the human oestrogen receptors α and β (hERα, hERβ). The binding data were obtained from the literature (Malamas M. S. et al., 2004, *J. Med. Chem. 47*, 5021-5040). The study indicates that for compounds known to bind to hER, relative binding affinity (RBA) can be predicted accurately. Such predictions could form part of an integrated strategy to assess whether a compound has the potential to be a significant endocrine disruptor.

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Poster

DEREK for Windows: A computer system for sharing toxicological knowledge

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Computer systems have an important role to play in reducing the use of animals in toxicity testing. This includes providing mechanisms of dissemination whereby maximum use can be derived from the results of tests which are necessarily carried out to avoid repetition of the same or similar experiments. In some cases, however, it may not be possible to share either the exact nature of the chemical tested or the results of the test because of commercial sensitivity. In such instances, it is often nevertheless possible to share the general conclusions of the study. Knowledge of this type can be stored and utilised in a toxicological expert system such as DEREK for Windows.

DEREK for Windows is a knowledge-based expert system designed to predict the toxicity of a chemical from its structure. The knowledge base is composed of alerts, example compounds and rules which each contribute to the predictions. The alerts define chemical environments which are associated with a particular toxicological endpoint such as genotoxicity, carcinogenicity or skin sensitisation. Each alert is based on toxicity data for specific compounds together with other relevant information, including mechanistic understanding where this is available.

Examples will be presented to illustrate how proprietary toxicity data contributed by DEREK for Windows users have been used in conjunction with published evidence to derive and refine alerts without compromising confidentiality.

Poster

A pre-validation of *in vitro* photo genotoxicity tests and the effort to find a useful statistical prediction model

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Two *in vitro* tests – the Photo Micronucleus Test (PMNT) and the Photo Comet Assay (PCA) – were evaluated to proof their ability to identify the photogenotoxicological potential of chemicals. Thirteen photoactive substances were tested under blind conditions in three to five laboratories in the years 2002 to 2004 in a ring trial coordinated by the Federal Institute for Drugs and Medical Devices (BfArM), Bonn, in two independent runs.

Statistical methods used in this validation study will be discussed: (1) The definition of useful toxicological endpoints and the handling of the data as a first and very important step in the process of the statistical analysis. (2) The repeatability and reproducibility must be given for the test to be of practical use and has to be assessed.

For the interpretation of the results, "individual" prediction models were developed by all of the participating laboratories: (3) Comparison of the experimental findings with the classification of photogenotoxicity shows the sensitivity and specificity of the tests. A 2x2-contingency table proved to be most useful for this purpose. (4) Despite the small number of laboratories, the limited number of replicates, and chemicals used, an attempt is made to develop a general prediction model.

Lecture

The role of electrophilic reactivity in Quantitative Structure-Activity Relationships (QSARs) for toxicity

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There is great interest in the use of Quantitative Structure-Activity Relationships (QSARs) to predict toxicity. QSARs and *in silico* approaches have traditionally been applied most successfully in toxicology to predict endpoints, such as narcosis, where potency can be related to passive, steady-state, effects such as accumulation at the site of action. However, many toxicities (e.g. mutagenicity, sensitisation and numerous others) relevant to risk assessment are not elicited in this manner and are brought about by the covalent interaction of the xenobiotic with a biological macromolecule. Many of these mechanisms are electrophilic in nature, and are considered to constitute *reactive toxicity*. Predictions for such compounds and endpoints have been rather poor. There are a number of reasons for this, most notably the failure of QSAR descriptors to parameterise electrophilicity adequately. To address this problem this study has investigated chemical reactivity as quantified experimentally by reaction with the model nucleophile glutathione (GSH). The compounds chosen to study were olefins conjugated to a carbonyl group. There are inherently electrophilic and convey the potential to act by Michael-type nucleophilic addition. The measured reactivity parameter ($React_{GSH}$) endpoint was then related to acute toxicity assessed in the 40 hour *Tetrahymena pyriformis* population growth impairment assay. A high quality linear relationship was observed between toxicity and reactivity for compounds most of which act via Michael addition. This approach is successful and may have possible applications to other toxicity endpoints.

Lecture

Comparative analysis of gene networks at multiple doses and time points in livers of rats exposed to acetaminophen

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Gene interaction network analysis using microarray data sets has been developed to quantify systematic changes of gene expression after chemical exposure. In this study, methods based on Bayesian networks for identifying and quantifying linkages between genes was applied to detect differences in gene expression interaction networks between multiple doses and time points. Seventeen (17) genes were selected from the gene expression profiles of microarrays from livers of rats orally exposed to 50, 150 and 1500mg/kg acetaminophen (APAP) at 6, 24 and 48 hours after their exposure. The selected genes are related to three biological categories that are associated with response to acetaminophen; apoptosis, oxidative stress and acetaminophen-influenced genes. Gene interaction networks between all 17 genes were identified for the nine dose-time observation points by the TAO-Gen (Theoretical Algorithm for identifying Optimal Gene interaction networks) algorithm. Using k-means clustering analysis, the estimated nine networks could be clustered into two clusters, the first consisting of the low and middle dose groups, and the second consisting of the high dose. The analysis suggests that the networks could be segregated by doses but within doses, was consistent over time of observation. The networks formed by these two clusters were quantified to calculate the probability distribution for the strength of the linkage between any two genes in the networks at different times, suggesting that there are different molecular mechanisms between lower doses and high dose or different time of APAP. The approaches shown here could provide predictive information to understand high- *versus* low-dose mechanisms of toxicity.

Poster

Developing a QSAR framework to predict the toxicity of electrophiles: Findings of an expert workshop

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The potency of chemicals which has reversible interactions with macromolecules is often much lower than that of similar chemicals which can bind irreversibly to proteins and DNA. Direct acting electrophiles and pro-electrophiles may react covalently with cellular nucleophiles. Depending on the nucleophile involved, reactive toxicity can be expressed by a variety of endpoints from cytotoxicity to sensitisation and genotoxicity. There are many mechanisms by which electrophiles can interact with nucleophiles, and the molecular descriptors required to model these reactions are complex. The First Annual Knoxville Reactivity Workshop was held in Knoxville (May 2005) to initiate the development of a modelling framework for reactive toxicity. Beginning with soft electrophiles, a new assay using glutathione (GSH) as a model nucleophile was evaluated for its ability to rank chemicals as skin sensitisers, aquatic toxicants and respiratory irritants. Reactivity data from the GSH new assay were found to be related linearly to *Tetrahymena pyriformis* toxicity. These data suggest a direct relationship between toxicity and reactivity for esters and amides predisposed to undergo a SN2 displacement reaction with soft nucleophiles. These results further suggest that the GSH assay will be of assistance in determining the chemical domain of reactivity with the thiol nucleophile. In addition, QSAR methods to predict GSH reactivity, using structural rules for the different reaction mechanisms, were evaluated. Finally, research implementation plans were made for the development of other model nucleophiles that can serve as a surrogate for the many important molecular initiating events in the toxicity pathways of reactive chemicals.