Workshop 5.15
In vitro metabolism: applications in pharmacology and toxicology

Lecture

The use of genetically modified V79 cell lines for the investigation of species differences in the metabolism of chemicals

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In risk assessment of chemicals – due to the lack of human data – extrapolation from the results in animals to the human situation has to be performed. Qualitative and quantitative differences in metabolism play an important role for species extrapolation. CYP2E1 is an enzyme, which plays a critical role in the metabolism of many industrial chemicals. In order to determine species differences in CYP2E1 dependent metabolism of chemicals, a cell battery consisting of V79 cell lines stably expressing CYP2E1 from rat (V79r2E1), man (V79h2E1) and mouse (V79m2E1) has been established. The cell battery has been characterised with regard to enzyme kinetic properties towards the CYP2E1 model substrate chlorzoxazone (CLX) and towards the mutagenicity of N-nitrosodimethylamine (NDMA). Species differences in vmax values of CLX hydroxylation (V79r2E1: 130, V79h2E1: 60 and V79m2E1: 40 pmol/mg protein/min) and in the mutagenic effect of NDMA could be observed.

In order to extend the investigations on species differences in CYP2E1 dependent metabolism, acrylamide has been selected as a further compound. Acrylamide is activated by CYP2E1 to the ultimate genotoxic metabolite glycidamide.

Currently, a LC-MS-MS methodology for the quantitation of acrylamide and glycidamide in cell protein obtained after culture of the respective cell lines is being developed. After incubation of the cell lines expressing CYP2E1 from rat, mouse and human with acrylamide, the parent compound and glycidamide will be quantified and thus, information of probable species differences in CYP2E1 dependent metabolism of acrylamide will be obtained.

Poster

ECVAM Key Area Toxicokinetics: Summary of ongoing activities

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Toxicokinetic information is crucial to extrapolate *in vitro* toxicology data to *in vivo* situations, and to select concentrations to be tested *in vitro*. An ECVAM workshop on toxicokinetic prediction using physiologically-based biokinetic models is in preparation. Following a workshop on blood-brain barrier (BBB) permeability, an ECVAM Task Force was set up and a feasibility study to evaluate various *in vitro* BBB models is planned. ECVAM is involved in the study of absorption barrier models: gastro intestinal barrier (*in vitro* model validation studies), skin barrier (alternative tests for percutaneous absorption have obtained regulatory acceptance at EU and OECD level in 2004).

Studying the fate of compounds in *in vitro* toxicology test systems (*in vitro* biokinetics) is most promising for their correct interpretation. An ECVAM Task Force on metabolism and toxicokinetics was established and a workshop on metabolism in *in vitro* tests was held in January 2004. A workshop on *in vitro* biokinetics practices is currently in preparation. ECVAM *in vitro* biokinetic research looks for correlations between cellular uptake, intracellular distribution, metabolic pathways, interaction with biomolecules of compounds, and their mechanisms of action and toxicological effects, using advanced spectrochemical and radioanalytical techniques.

Furthermore, in the context of the 7th Amendment to the Cosmetics Directive a detailed document was elaborated by expert consultation, including a chapter on toxicokinetics. This provides a basis for further work necessary to replace, reduce and refine the use of animals through alternative tests and strategies under development, before validation at the European Union level.

Poster

HTS for human cytochromes P450

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A high-throughput system was developed for cytochromes P450 based on a 96-well-plate technology with a build in sensor for measuring oxygen consumption as a measure for metabolism of drugs. Several drugs were applied to check for stability of the system, reproducibility and reliability. The system can be applied particularly in the early phase of preclinical development to yield metabolism data relevant for humans, e.g. metabolic stability, enzyme profiling, and kinetics to support an efficient selection of lead compounds. The system is available on a non-exclusive licensing base.

Lecture

The drivers for *in vitro* technologies in drug development

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There is widespread concern about increasing cost and time in drug development due to several attrition factors causing poor pharmacokinetics, unforeseen toxicity, and lack of efficacy in humans. In order to improve the situation, a wide range of *in vitro* technologies are being implemented in the drug development process and substitute for animal studies from early stage of target identification, defining better lead compounds, up to preclinical studies, which yield data with higher predictive value for humans.

The *in vitro* technologies currently applied result from various areas, e.g. molecular biology, bioanalytical chemistry, computational chemistry, statistical sciences, biochemistry, and automation engineering.

Examples are given for checking on metabolism related problems with genetically engineered V79 cells expressing specific human cytochromes P450, and the implementation of a HighThroughPut system in the early screening phase of drug development to yield information on metabolic stability of drug candidates which may support the lead compound selection process.

Drug development is considered as the most promising area for installing alternative methods.

Lecture

Metabolic activation of pro-teratogens *in vitro*

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Introduction: Many substances are not directly toxic but must be enzymatically activated to reactive metabolites. Our previous studies with valpromide (VPD) show teratogenic effects in mice that were not found *in vitro*. The development of metabolic systems which can be incorporated within *in vitro* techniques is, therefore, a critical step for the use of *in vitro* methods in toxicology. Ideally, the metabolic system should express all relevant enzymes, because during screening of many substances it is *a priori* not known which enzyme(s) may be involved.

Methods: S9-mix is an easy to handle metabolic system and standard method. Two approaches are tested: pre-incubation and extraction compared to direct cultivation of S9 with target cells. Target cells comprise F9-teratocarcinoma cells and D3 mouse embryonic stem cells, as both proved very useful for detecting teratogens. Their capacities for proliferation and differentiation are monitored.

Results: Known metabolic *in vivo* differences can also be observed *in vitro*: VPD is metabolised by human S9-fraction to teratogenic VPA, whereas with rat S9-fraction VPA is further metabolised. The treated target cells show effects depending on the concentration produced via metabolism. A major problem was encountered: direct product concentrations resulting from S9-metabolism are comparatively low, so a concentration step is needed.

Discussion: Although S9-mix could be used as a metabolic activation system, it is difficult to obtain physiologically relevant data. Enzymes and cofactors are not present in physiological concentrations. Further studies will be done with freshly isolated hepatocytes, which are considered to be the better model for metabolic activation.

Lecture

"Issue-driven" drug metabolism screening and the value of human based *in vitro* models

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Early assessment of absorption, distribution, metabolism, excretion, and pharmacokinetic (ADME/PK) properties of new chemical entities is nowadays occurring at nearly every stage of the discovery process, from lead identification to lead optimisation. A main reason for this is that biologist and medicinal chemists have recognised the value and importance of optimising on drug-like properties in addition to potency and selectivity. Although ADME screening is a necessity, there is more and more awareness that the ADME screening should be rational ("issue-driven") and should be flexible enough to address the specific needs of each project. This approach requires a careful balance of *in vivo* studies and *in vitro* high throughput screening and a careful balance of screening *versus* prediction.

Despite the improved human-based *in vitro* ADME screens and the ever-growing evidence of significant species differences with respect to drug metabolism and excretion, animal models are still commonly used within drug metabolism departments of pharmaceutical industries. Area's such as allometric scaling to predict human pharmacokinectics, enzyme induction and toxicity evaluation, still rely heavily on animal testing in spite of the numerous examples of misleading results.

Currently, one of the major challenges in drug discovery is to accurately predict drug-induced adverse reactions and the role of drug metabolism. This is emphasised by the several examples of drugs withdrawn from the market because of adverse reactions due to metabolic activation. Presently, many *in vivo/in vitro* tools are in the exploratory stage, including computation approaches and experimental assays to predict drug induced adverse reactions. At this moment, it is unlike to believe that a single assay will be able to predict the potential risk of reactive metabolites and (like for many ADME/PK parameters) a combination of several assays and an integrated approach is required to obtain the best prediction and to minimise the risk for late stage attrition.

Lecture

Metabolic activation for *in vitro* systems

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A significant portion of substances are not directly toxic, but must be activated to reactive metabolites which exert their toxic potential by reacting with constituents of the cell, especially proteins and nucleic acids. These compounds include polycyclic aromatic hydrocarbons (e.g. benzo(a)pyrene), cyclophosphamide, aflatoxin B1, Vitamin A and acrylamide, and their metabolic activation pathways are well established. For most compounds it is unknown if metabolic activation plays a role. *In vitro* systems presently can only determine the toxicological potential of the parent drugs, and not that of potentially toxic metabolites. The development of metabolic systems which can be incorporated within a particular *in vitro* technique, is therefore of high priority. Ideally, the *in vitro* system should express all the relevant enzymatic activities because during screening of large number of substances it is *a priori* not known which enzyme(s) are involved. Also, the metabolic system must be compatible with the *in vitro* assay. Different metabolic systems appear suitable for such a task, and main emphasis is placed on the liver preparations as liver exhibits in most cases the highest amount and complexity of metabolic enzymes:

- S9 (liver 9.000 x g) preparation as used in the Ames test
- Hepatocytes or liver slices
- Genetically engineered cells for expression of relevant metabolic enzymes

The possible advantages of using hepatocytes for activation are several fold: (1) human cells can be used to avoid species differences; (2) they contain enzymes and cofactors at physiological levels; (3) high activity of both phase I and phase II enzymes are present. These metabolic activation systems are now being developed for incorporation into *in vitro* systems for development of robust testing systems which can be transferred to other laboratories.

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