The Consortium for Metabonomic Toxicology (COMET): Aims, Activities and Achievements

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A review for "Pharmacogenomics"

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Summary

The utility of metabonomics in the evaluation of xenobiotic toxicity has been comprehensively assessed by the Consortium for Metabonomic Toxicology (COMET), formed between five major pharmaceutical companies and Imperial College London, UK. The main objectives were to assess methodologies, to generate a metabonomic database using ^{1}H nuclear magnetic resonance (NMR) spectroscopy of rodent urine and blood serum and to build a predictive expert system for target organ toxicity. The analytical and biological variation which might arise through the use of metabonomics was evaluated and a high degree of robustness demonstrated. With the completion of studies on approximately 150 model toxins, the chief deliverables of a curated database of rodent biofluid NMR spectra and computer-based expert systems for prediction of kidney or liver toxicity in rat and mouse based on the spectral data have been generated, and have been delivered to the sponsoring companies. In contrast to consortia involving other omics approaches, the project with its relatively modest resources, has met and exceeded all of its targets, and was judged a resounding success by the sponsoring companies who are in many cases already enhancing and making use of the data in their in-house studies.

THE COMET PROJECT OBJECTIVES

The importance of post-genomic technologies for improving the understanding of drug adverse effects has been highlighted recently [1] and whilst there is already a comprehensive literature on the use of metabonomics [2] to investigate xenobiotic toxicity, and this has been reviewed recently [3], a rigorous and comprehensive evaluation would be of considerable value. To this end, a consortium was formed to investigate the utility of metabonomic approaches to the toxicological assessment of drug candidates. The main aim of the consortium was to use ^{1}H NMR spectroscopy of biofluids (and, in selected cases, tissues), with the application of computer-based pattern recognition and expert system methods to classify the biofluids in terms of known pathological effects caused by administration of substances causing toxic effects. The academic project was hosted at Imperial College, London, UK and involved subscription funding by, at the time, six pharmaceutical companies, namely Bristol-Myers-Squibb, Eli Lilly & Co., Hoffman-La Roche, NovoNordisk, Pfizer Inc. and The Pharmacia Corporation (now within Pfizer). The NMR instrument manufacturer, Bruker, provided access to new methodology and software and technical back-up.

The main objectives of the project were –

- Provision of a detailed multivariate description of normal physiological and biochemical variation of metabolites in urine, blood serum and selected tissues, for selected male rat and mouse strains, based on ^{1}H NMR spectra.
- Development of a database of ^{1}H NMR spectra from animals dosed with model toxins, initially concentrating on liver and kidney effects, and an associated database of meta-data on the studies and samples.
- Development of expert systems for the detection of the toxic effects of xenobiotics based on a chemometric analysis of their NMR-detected changes in biofluid metabolite profiles.

- Identification of combination biomarkers of the various defined classes.
- Testing of the methods to assess the ability of metabonomics to distinguish between toxic and non-toxic substances, and to assess the organ specificity of the predictive expert systems.

The classes of chemicals used and types of toxicity investigated were as diverse as possible to assist the validation of NMR methods for use in early "broad" screening of candidates for toxicity.

In this concise review, the background to metabonomics is summarized, the methods used in COMET are briefly described and results are used to exemplify the approach. An initial publication on COMET set out the project details and objectives, soon after its inception [4]. Finally, the achievements and the level of success are discussed.

Metabonomics background

Under mild toxic stress, cells attempt to maintain homeostasis and metabolic control by varying the composition of the body fluids that either perfuse them or are secreted by them. In more severe toxicity states, cell death leads to loss of organ function and more marked biochemical changes occur in biofluids due to loss of whole body homeostasis and metabolite leakage from damaged cells. Consequently, following either scenario there are characteristic organ-specific and mechanism-specific alterations in biofluid composition. Now it is possible to use a combined NMR spectroscopy/expert system approach to systematically explore the relationships between biofluid composition and toxicity and to generate novel combination biomarkers of toxicity [2,3]. The use of metabonomics in drug safety assessment has recently been comprehensively summarized in a book [5].

Within the last few years, with the development of high resolution magic-angle-spinning (MAS) technology, it has also become possible to obtain very high quality ^{1}H NMR spectra on small (~10 mg) samples of whole tissue with no sample pre-treatment, other than addition of a very small amount of D_2O to the sample. MAS involves spinning the sample about an axis at 54.7° to the magnetic field direction. This process removes line broadening caused by the heterogeneous nature of the tissue. This approach has also now been applied extensively to cells and tissues [e.g. 6].

The MAS NMR technique opens up many diagnostic possibilities since information on a variety of metabolites in different cellular environments can be rapidly obtained and specialized NMR experiments such as those to measure molecular diffusion coefficients can be used to probe compartmentation. Confirmation of biochemical composition can be obtained using standard high resolution NMR of both aqueous (protein free) and organic solvent extracts. This produces a comprehensive set of metabolic information that can be used in integrated metabonomics studies.

More recently, alternative analytical methods have been explored for use in metabonomics. One that is now gaining wider use is mass spectrometry (MS) with a prior directly-coupled separation stage such as liquid chromatography (LC) or even more recently, ultra-high pressure liquid chromatography (UPLC). Although this type of detector is less universal than NMR spectroscopy, it has a much higher inherent sensitivity and hence probable lower detection limits. It has recently begun to be applied in metabonomics studies [7].

A rigorous evaluation of metabolic differences caused by a pathological effect requires the use of multi-variate statistics and pattern recognition algorithms. Methods such as principal

components analysis (PCA) or partial least squares discriminant analysis (PLS-DA) can be used to classify the NMR-generated toxicity data in terms of toxin type and dose. Multi-dimensional metabolite trajectories can be constructed in order to visualize the biochemical time-course of the toxic episodes. More complex expert systems based on chemometric models in the multi-dimensional metabolic space can be constructed and used for class prediction [8,9].

Classification models can be interrogated in relation to the input data in order to identify key portions of the NMR spectra giving rise to classification. These parts of the spectra contain the biomarker signals. The spectra are then analyzed in detail to assign the NMR signals and identify the metabolites. If necessary, new biomarker metabolites which cannot be identified using multi-pulse and multi-dimensional NMR spectroscopy, can be separated and identified using solid-phase extraction-NMR-MS and HPLC-NMR-MS.

Project logistics and operations

After an initial meeting of interested pharmaceutical company personnel was held, a Steering Committee was formed from selected and agreed personnel from each company and Imperial College to oversee the project which was funded for 3 years.

It was important to define a set of compounds which gave reproducible and well defined toxic effects and demonstrate that the observed effects could be described using a common terminology across the many toxicologists involved in the companies, the university and in the contract research organisations (CROs) used by some companies. The standard operating protocols used by the companies and CROs were refined to keep unwanted biological variation to a minimum through detailed consideration of animal strain, food, adaptation to

metabolic cages, diurnal variation and animal stress. Thought was also given to the possibility of the presence of the dosed xenobiotic and its metabolites in the biofluids. In this way, the biochemical consistency of biofluid samples from the different companies was maintained at a high level. All aspects of the meta-data including histopathology reports and clinical chemistry results were also included in the database [4].

Over the lifetime of the project, it was intended that approximately 150 compounds (i.e. 25 per company) would be studied. The choice of compounds was determined by the Steering Committee with close attention paid to the interests of each company but balanced against the need to provide a useful resource for all participants. However, the primary selection criteria were based on the need to provide robust predictive models that covered an extensive range of metabolic space.

The in-life studies were carried out by the sponsoring companies and typically ten rats or eight mice per group were randomly assigned to control, low dose or high dose treatment groups. The high dose group, defined as a dose which exerts a clear toxic effect following a single administration, was chosen based on literature or in-house data where possible. An acceptable low dose was one which generated a threshold response. The selected doses for each study were supported by justification reports, also stored in the database.

Blood was sampled at 24 h, 48 h and 168 h post-dosing, and urine was collected into labelled containers maintained at 0±2°C, and containing sodium azide and then stored frozen at below -40ºC until analysis. Samples were collected over a period of 8 days which included a 1 day baseline collection. The usual range of conventional parameters was also measured.

The NMR spectra were measured at a ^{1}H NMR frequency of 600 MHz at Imperial College using a flow-injection process. Rat and mouse urine samples were prepared for NMR spectroscopy in covered 96-well plates as described previously [4]. All of the statistical analyses and expert system development efforts were also undertaken there. The methodology and results were transferred to the member pharmaceutical companies on a step-wise basis electronically. Training in applicable technologies was given to each member pharmaceutical company such that at the end of the project, each was capable of conducting studies in-house and was able to internally expand the database established through the consortium. The operations of the project and the ways in which they are interconnected are shown in the flow diagram, Figure 1.

Evaluation of analytical methods

For such a multi-site set of studies it was important to assess the inter-site biological variation in the samples and to consider the analytical reproducibility of the techniques used for obtaining the metabonomic data. NMR spectroscopy was the analytical method of choice as it is known to provide highly reproducible data for replicate samples and it allows high precision and accuracy. The sample preparation and NMR measurement protocols were devised taking these requirements into account. During the first period of the project, a detailed examination of the analytical and biological quality control was undertaken with each company dosing the same often-used model liver toxin, hydrazine [10], to rats using an agreed, standard protocol. The NMR spectroscopic quality control was further tested by a procedure whereby a comparison was conducted of Imperial-measured and company-measured NMR spectra. These studies showed that the analytical variation between measurement sites was very low with a coefficient of variation below 2% [11]. Moreover for

samples produced using the same toxin at different company sites, the dose level effects were easily distinguished from any inter-site variability [11].

Given the availability of thousands of control urine samples for both rat and mouse, the project provided an excellent opportunity to develop an understanding of the biochemical variation in these fluids by building statically-based models of control urine. These models were continuously evolved as more samples became available. The control model successfully detected outlier samples known to be deviating from normal control behaviour based on clinical chemistry measurements, and also successfully detected early time point deviations in animals for which a full time course was not available. Early fault detection such as this was then used as an integrated part of the fully automated expert system in order to discard abnormal samples at the earliest possible stage.

The database of NMR spectra and meta-data

The project has now been completed and results on a total of 147 studies have been reported. Chemometric models were derived for each study on the basis of three classes, namely control, low-dose and high-dose animals. Information was reported electronically to and from the sponsoring companies, including protocols, both for dosing and sample collection, histopathology reports and clinical chemistry data, NMR spectral data, lists of compounds studied and reports issued.

In order to effectively manage the large quantity of data generated by COMET, both the NMR spectra themselves and the associated meta-data, in this case comprising clinical chemistry measurements and histopathology, the COMET data was organised as a relational database. The careful standardization of the study design in the COMET project allowed an extremely

simple database design. The database was organised in a hierarchy descending from the each of the 147 studies as the root element. Each study is associated with a cohort of animals that in turn are the source of a series of samples. Each sample type (urine, serum and tissue), has its own table in the database which records both the details of the NMR spectra and the relevant meta-data collected for that sample type.

The use of a relational database greatly simplified cross-comparison of animals or samples between studies within the project. Advanced queries could be entered, based on any combination of criteria in the meta-data. This facilitated the easy selection of the most relevant set of samples for any analysis across the COMET dataset, allowing detailed examination of only the most relevant samples. Examples include the simple exclusion of animals with high variability in selected clinical chemistry measurements prior to dosing that may otherwise obscure analysis, or the generation of very large aggregate control groups based on such parameters as predose-weight or age at the study start point.

Development of a toxicity prediction expert system

One of the primary goals of the COMET project was to develop an expert system which could identify animals presenting abnormal urinary profiles and use these to automatically predict the main organ of effect for liver and kidney toxins. This was achieved through the multistage data modelling pipeline illustrated in Figure 2. Initial standard pre-processing of the NMR data of each urine sample involved reduction of the spectra to a small number of integrated bins, replacement of spectral regions obscured by peaks from dosed compounds and their metabolites [12] and normalization to a constant total intensity. Next, a PCA-based model of normal urine was constructed with profiles from control animals, using an iterative approach to obtain a set of 4023 spectra which represented the normal metabolic variation within the

COMET control population in a robust manner. Samples from dosed animals were tested against this normal model and those from time points where more than half the profiles were abnormal were input to the next stage of modelling. Data from the 2056 samples passing this step were subject to two novel scaling procedures developed as part of the COMET project – SMART [13], to align the temporal metabolic changes seen in multiple dimensions in animals with different basal metabolic profiles and magnitudes of response, and VAST [14] aimed at down-weighting the effects of a number of hyper-variable metabolites. These scaled data were then used to build a probabilistic model based on novel software termed CLOUDS [15] which compared the multidimensional distribution of profiles from each pair of treatments. Finally the CLOUDS similarity indices were interpreted using a simple set of rules to predict the toxin class (liver or kidney) of each treatment when compared to all others.

When tested on an 80-treatment subset of the total COMET database, the resulting expert system identified the main organ of toxicity in agreement with the histopathology gold standard in over half the cases. For most of the remaining treatments prediction was not possible and this was due to a combination of inconclusive histopathology, the fact that NMR peaks from the dosed compound or its metabolites obscured significant regions of the NMR spectra or in a few cases because of genuine class overlap, usually caused by a toxin causing both liver and kidney dysfunction. In only 5% of cases did the system predict the wrong type of toxicity, a low error rate that was shown to be statistically significant. Furthermore, in a 'blind study' test, two treatments from the database were independently selected and dosed at lower levels to that of the original studies. The toxicity types of these treatments were successfully identified by the expert system in both cases, confirming the reliability and practical use of the approach.

An assessment of the COMET project

Single markers of toxicity are unlikely to be of value in the detection of subtle lesions that are the most problematic in primary toxicology studies on novel drugs. Pattern recognition and multivariate statistical analyses of NMR spectra therefore offer a realistic prospect of identifying novel combination biomarkers of toxic effect for use in toxicological screening at the discovery/development stage. In COMET, the scope of the NMR/pattern recognition approach was broadened and NMR-based expert systems suitable for "high throughput" *in vivo* toxicology screening were developed. The development of the spectral and meta-data databases is expected, through continued data mining, to generate new markers of drug toxicity *in vivo* and further the understanding of the systemic toxic mechanisms and biochemical effects of novel drugs. The output of the consortium has facilitated the development of valuable new approaches to *in vivo* drug toxicity screening at the drug discovery stage and in the investigation of more subtle lesions emerging for specific compounds during the drug development stage.

This success of the project at the metabolic level on 147 studies can be compared to the corresponding efforts in toxicogenomics where a large consortium has been examining the variability caused by the use of different platform technologies and as well as the analytical variability within a given technology using a small number of well characterized toxins. This evaluated and compared biological and gene expression responses in rats exposed to two model hepatotoxins - clofibrate and methapyrilene [16]. *In vivo* studies for both compounds were conducted in two laboratories using a standard experimental protocol, and RNA samples were distributed to 16 laboratories for analysis on six microarray platforms. Gene expression results demonstrated reasonable agreement between laboratories and across platforms. Discrepancies in expression profiles of some individual genes were said to be due to platform differences and approaches to data analysis rather than to biological or inter-laboratory

variability. Despite these discrepancies there was overall agreement in the biological pathways affected by these compounds,.

In another experiment, animals were treated at one facility, and RNA was distributed to five different sites for gene expression analysis [17]. A preliminary evaluation of the number of changes in gene expression found large differences between the five different sites, but this did not prevent a correct determination of the biological effect, and despite some variability, it was concluded that robust gene expression changes were consistent between sites. A further study compared samples produced by the same platform by comparing high-density gene expression data sets generated on two sets of RNA from methapyrilene experiments conducted at two different sites using a single gene-chip platform and analyzed at seven different sites [18]. Although the site effects were significantly large in the analysis results, they appeared to be primarily additive and therefore could be adjusted in the statistical calculations in a way that did not bias conclusions regarding treatment differences.

The situation with regard to toxicoproteomics is beginning to be assessed [19], but this approach is likely to suffer from even higher inter-site and inter-platform variability and be rather time consuming and not suited to screening studies. Thus it is likely to remain a technique for targeted analyses when a hypothesis of mechanism has already been formulated.

The published studies on the utility of toxicogenomic approaches (transcriptomics, proteomics and metabonomics) in screening for toxicological mechanisms and evaluation of dose response effects have been reviewed recently [20]. This supported the use of transcriptomics to screen in a targeted manner for specific toxicological mechanisms for which there is an *a priori* hypothesis, but pointed out that in some areas, such as mutagenicity

testing, toxicogenomics appears to have limited value. It was suggested that targeted transcriptomics might be valuable for screening for specific mechanisms of toxicity considered to be irrelevant for assessing risk to humans, which would help to reduce the need for detailed testing of some chemicals. An integrated approach was suggested where data from more than one toxicogenomic approach could be used as an adjunct to conventional toxicology to assess dose-response in toxicological tests. An outline preliminary proposal for use by regulators was suggested, although it was agreed that more data are required before this could be formally used in a decision-making process.

Although this suggests that transcriptomic analysis using microarrays holds promise for toxicity classification, the use of different microarray platforms, protocols, and data analysis tools might hinder comparisons between studies. To improve the ability to share and compare data, a standardized set of microarray reagents and reproducible protocols to simplify the analysis of liver gene expression in the mouse has been published. The web-accessible database was also linked to chemometrics tools that include clustering methods and database search algorithms. The whole resource, known as EDGE, is publically accessible [21, 101] and might serve as a prototype resource for the sharing of toxicogenomics information and be used to develop algorithms for efficient chemical classification and hazard prediction.

Outlook

The US Food and Drug Administration is evaluating metabonomics as a tool for leveraging the development of new medicines [102] and the NIH has a road-map approach for the investigation of metabolic profiling methods [103]. One of the limiting factors that will slow down the integration of the various “omics” approaches is the lack of standardization of data

formats and reporting methods, although this is now being addressed across all of the omics, and particularly for metabonomics [22-25].

Given both the increasing numbers of publications from different laboratories in which metabonomics has been used to probe xenobiotic toxicity and the interest that the pharmaceutical industry has shown through the COMET project and by other means, it is clear that metabonomics will pay an increasing role in drug safety evaluation. It can thus be expected that metabonomics will enter the mainstream activities of pharmaceutical companies and environmental agencies for identifying toxic compounds and their mechanism of action. At least for the pharmaceutical industry, this should lead to a reduction in the attrition rate of compounds failing at later stages in the R&D process.

HIGHLIGHTS

- Highly cooperative group of industry and academic

FIGURE CAPTIONS

Figure 1. Operational flow chart for the COMET project. CRO: Contract research organisation. LIMS: Laboratory information management system. DBMS: Database management system.

Figure 2. Component activities in the COMET expert system operation.

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Figure 1

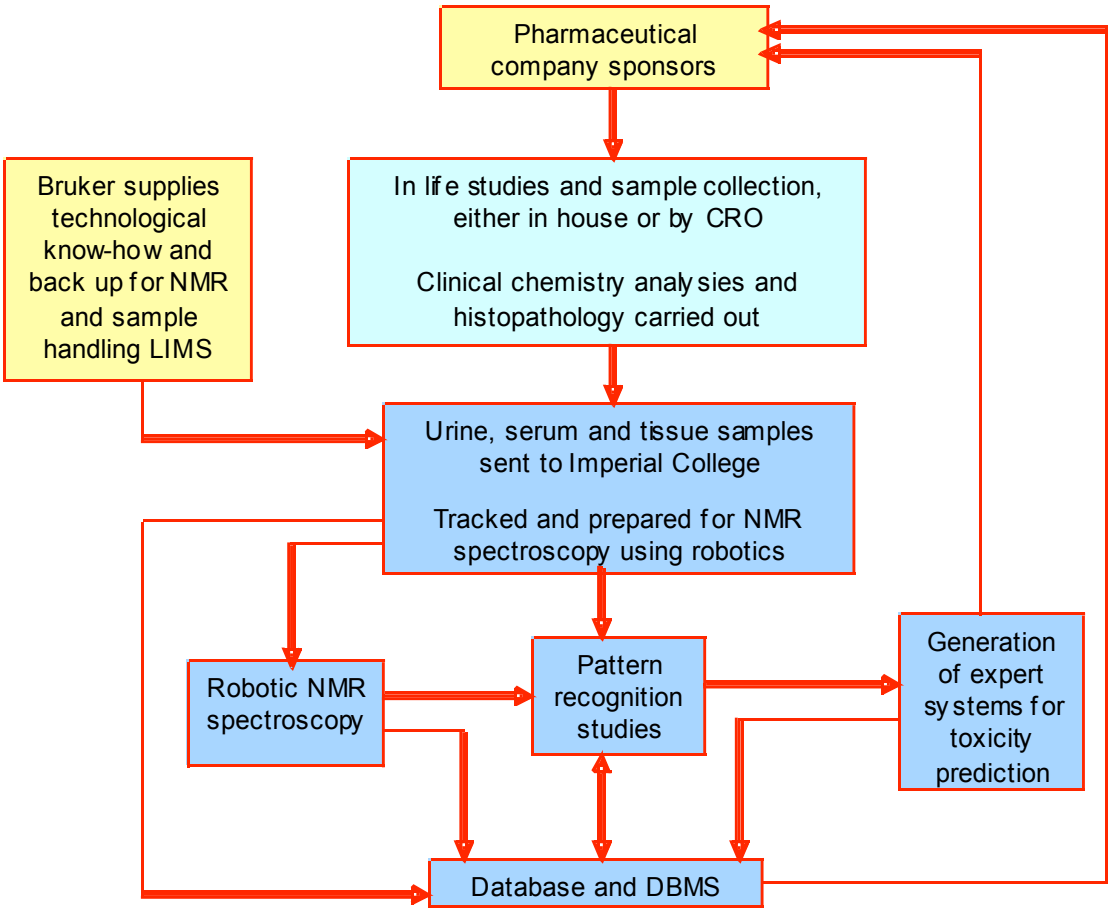
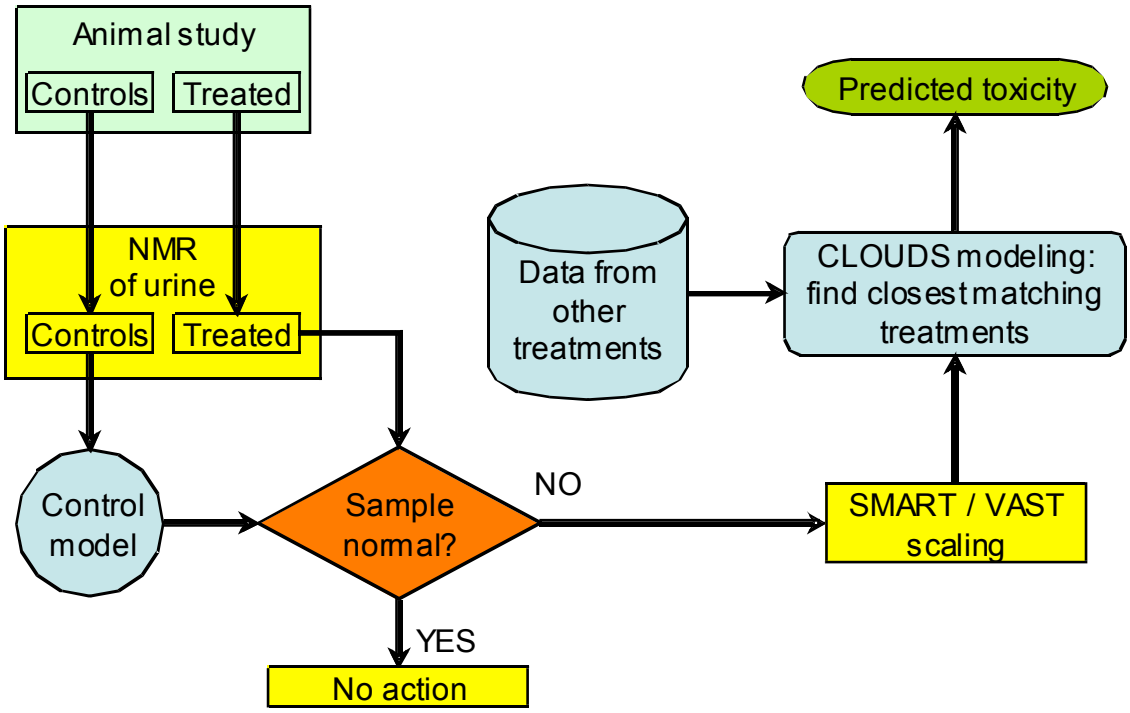

Pharmaceutical company sponsors
Bruker supplies technological know-how and back up for NMR and sample handling LIMS
In life studies and sample collection, either in house or by CRO
Clinical chemistry analysies and histopathology carried out
Urine, serum and tissue samples sent to Imperial College
Tracked and prepared for NMR spectroscopy using robotics
Robotic NMR spectroscopy
Pattern recognition studies
Generation of expert systems for toxicity prediction
Database and DBMS

Figure 2


Animal study
Controls
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NO
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No action
Data from
other
treatments
CLOUDS modeling:
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treatments
Predicted toxicity
SMART / VAST
scaling