Is this Ingredient Safe?

A Guided Educational Tour

Conceptualized and prepared by

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The Booklet

This booklet aims to guide the interested reader, who is not necessarily an expert in the field, through a virtual 'case', in which non-animal testing tools and methods are combined to answer the question of whether a new compound (e.g. a new ingredient in a shampoo, called chemical X) used in a defined exposure context is safe for the consumer.

The booklet summarizes a *Guided Tour*, which was developed for the final symposium of the SEURAT-1 Research Initiative, on 4 December 2015 in Brussels. The tour was organised as a parkour of subsequent stations addressing the different steps required to answer a safety assessment question.

We will use symbolic pictograms to indicate the content of the different stations

and separate them from background information, that clarify key terms used in the content to non-experts.

The SEURAT-1 Conceptual Framework

The SEURAT-1 'Conceptual Framework' provides an overview about the guiding principles of the tour. It outlines the 'table of contents' of the whole exercise. This framework was developed within the SEURAT-1 Research Initiative and sets out a structure to guide assessors through the various risk assessment steps, with the aim to combine existing knowledge with information from predictive, non-animal testing tools and a stated protection goal.

Throughout the following pages, each step outlined in this conceptual framework will be explained in more detail. It is important to understand that the particular case, to which the framework will be applied, defines the content. Therefore, applying this framework implies that:

- the case and, in particular,
- the protection goal of the exercise

must be defined as a first step.

The SEURAT-1 Conceptual Framework

Hypothesis generation regarding how compound X causes toxicity

Pieces of evidence and initial considerations

- Purpose of the assessment
- Exposure context
- Expert knowledge and judgement based on existing evidence / data

Type of toxicity

General adversities | **Organ specific adversities**

Uptake and fate of compound X in the body

Toxicokinetics

TTC

Concentration in the blood that is likely to cause toxicity

Toxicodynamics

- Many biological targets
- Specific targets present in many cells / tissues / organs

Toxicodynamics

- Target organ: full assessment based on Adverse Outcome Pathway

Calculate the amount a person needs to uptake to reach that blood concentration

Quantitative *in vitro* to *in vivo* extrapolation

Evaluation

Overall assessment

Result

Improve assessment if necessary

Use of prediction for pre-defined purpose
with consideration of acceptable uncertainty

The Case

Here we define the case, to which we will then apply the conceptual framework outlined in Station n°1.

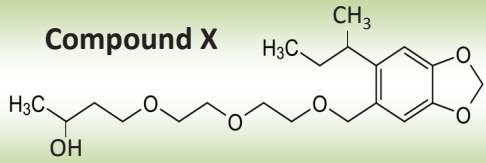
It provides important contextual information such as the structure of the chemical and the amount at which it will be applied in a product. In addition, it clearly defines the protection goal (note that the goal is to assess the safety of compound X in a particular use scenario!) and summarises the type of information required to answer this safety assessment question.

With this background information we can now go through the conceptual framework. We will restrict ourselves in such a way that we only allow 'alternative', non-animal methods in case new data on compound X is required.

The Case

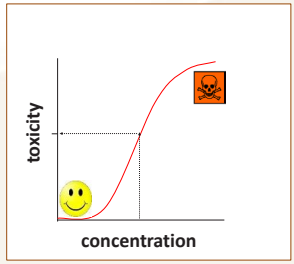
A new formulation of a shampoo has been developed which contains 10% of a new ingredient, entitled 'compound X'.

Compound X



The question:

Is this compound and the concentration at which it is used safe for the consumer?



Information required to answer this question:

- **Exposure scenario** (dermal, rinse-off product)
- **Amount** of compound X taken up into the body
- **Hazard identification**, (i.e. the intrinsic toxic properties of compound X)

The Threshold of Toxicological Concern

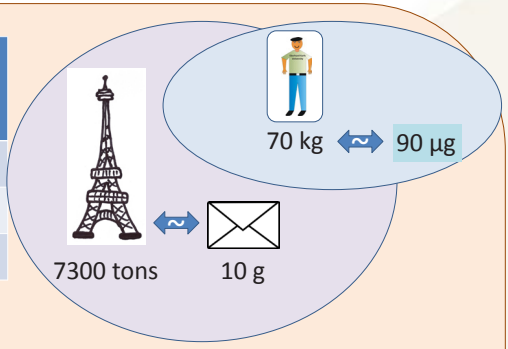
Before we use any other data, we first check if the exposure scenario alone provides sufficient information to assess the safety of compound X in this application scenario. This brings us to the 'threshold of toxicological concern' (**TTC**) concept, which allows us to waive all other investigations (see arrow on the right hand side of the conceptual framework, Station n°1).

The exposure to the skin and potential local effects have also to be considered (but is not part of this exercise). If this exposure level is expected to be extremely low, the TTC concept may be applicable. Of particular interest is the amount of compound X penetrating the skin barrier, reaching the blood stream and thus becoming available to the internal organs. This concept was initially developed for oral uptake, but within SEURAT-1 it was extended to include cosmetic relevant chemistries and to take account of availability via dermal uptake versus oral TTC.

What is TTC?
(Threshold of Toxicological Concern)

The Threshold of Toxicological Concern (TTC) was created by looking at a large collection of compounds and determining whether different classes of chemicals (called Cramer classes) cause toxicity below specific (and very low) doses. If exposure of a compound is below the TTC, then it is considered to be of low concern.

Classification	(µg/person/ day)
Cramer class I	1800
Cramer class II	540
Cramer class III	90



Most existing toxicity data is measured using oral dosing, although exposure to compound X will mainly be through contact with the skin. It is generally accepted that there will be a greater systemic exposure from the oral route. Calculations can be made regarding dermal exposure based on the amount of product applied, the frequency of application, the site(s) of body exposure, the length of exposure and the ability of the chemical to penetrate the skin. If, following dermal exposure, it appears that there will be a blood concentration approaching the TTC, then further data (e.g. on skin permeability) in addition to an expert opinion may be required or the exposure reduced.

Existing Data

Let‘s assume that compound X exceeds the threshold of toxicological concern and that, hence, it must be further investigated. The next step entails pulling together all available toxicological information of compound X. However, compound X is an entirely new chemical which has never been synthesized and investigated before. Therefore, no information regarding its toxicological properties is available. We can, however, search for structural analogues and try to predict the toxic properties of compound X with the assistance of ‘**read-across**‘.

What is “Read-Across”?

Read-across starts with structural similarity.

- Results from toxicity studies of one or more compounds (the ‘sources’) are ‘read-across’ to another compound (the ‘target’).
- Read-across assumes that the chemical structure and physico-chemical properties can be used to determine similarity and hence allow for toxicity prediction.
- A mechanistic hypothesis is required that connects the related structures with known (toxicological) processes.
- Strategic considerations, templates and workflows developed in SEURAT-1 were recently published.

Contents lists available at ScienceDirect

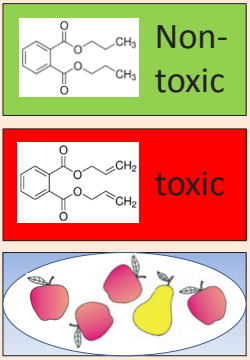
Regulatory Toxicology and Pharmacology

journal homepage: www.elsevier.com/locate/yrtph

Regulatory Toxicology and Pharmacology 72 (2015) 586–601

A strategy for structuring and reporting a read-across prediction of toxicity ☆

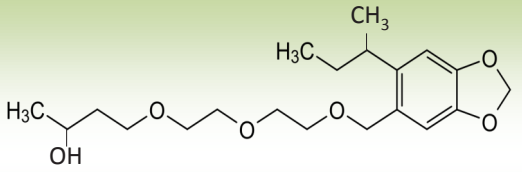
T.W. Schultz [a], P. Amcoff [b], E. Berggren [c], F. Gautier [d], M. Klaric [b], D.J. Knight [e], C. Mahony [f], M. Schwarz [g], A. White [h], M.T.D. Cronin [i,*]



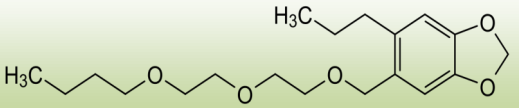
Caution! Read-across requires expert knowledge. Similar structures can sometimes have very divergent toxicities. However, results obtained by *in vitro* experiments can support a read-across argument.

Can We Apply Read-Across?

Compound X is a hypothetical new chemical. Currently there is no publicly available information on compound X.
Compound X, however, was developed from a compound known as piperonyl butoxide (PBO) and has therefore structural similarity to this compound.



Compound X



PBO

Piperonyl butoxide, Wikipedia

- There is information on the toxicity of PBO in rodents.

Compound X is structurally close enough to PBO. We can therefore consider performing a 'read-across' from PBO to compound X.

What is Known about PBO?

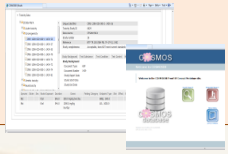
Literature mining:

- At higher doses, PBO is a liver toxicant in rodents.

- At a very high dose it produces liver tumors in rodents by a non-genotoxic mechanism (i.e. not directly damaging DNA).

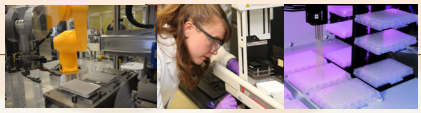
COSMOS database:

- Highly relevant for cosmetic related ingredients.



EPA ToxCast results:

- PBO affects several important cell signaling pathways potentially relevant for liver toxicity.



In summary:

- The available information suggests the liver as the target organ of toxicity from PBO. Therefore, our further investigations with compound X are focused on the liver.

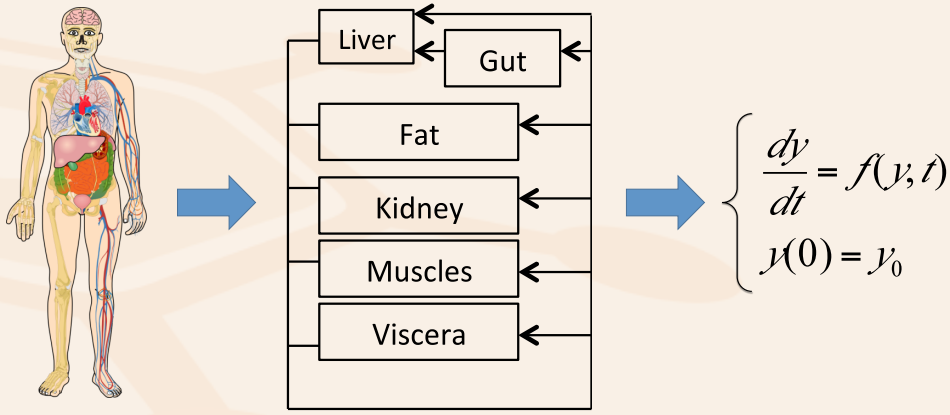
Toxicokinetics of Compound X

We have now completed the first steps of the conceptual framework: based on the close structural similarity of compound X to piperonyl butoxide, we assume that we can 'read-across' from PBO findings to compound X. We then collect all available information on PBO from the available literature and database sources. This allows us to define the type of adversity and to identify the liver as the expected target organ of compound X.

According to the conceptual framework, we now need to estimate the concentration of compound X in the presumed target organ – the liver – following dermal exposure. For this, we apply physiological-based pharmacokinetic (**PBPK**) models. These are used in the first instance to instruct the experimental researchers about the concentrations to apply in their cell culture systems.

PBPK-models

- Physiological-based pharmacokinetic (PBPK) models are applied to predict blood concentrations of test compounds which are then used to define concentrations in cell-based assays.
- *'In silico'* tools are used to predict the skin penetration of compound X and similar compounds to evaluate blood concentration.



How Do We Determine Toxicity *in vitro*?

We now have all of the required information to commence the experimental work. According to the conceptual framework, the next step concerns the investigation of the toxicodynamics of compound X.

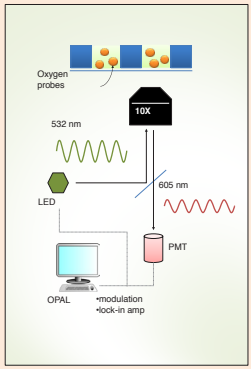
One of the first questions that needs to be addressed is to define whether the test chemical causes ‘non-specific toxicity‘ (defined, for example, in a yes-or-no answer as cell death) or whether it is acting via a ‘specific toxicity‘ mechanism (defined, for example, as interaction with a specific receptor).

Various tools are available to answer this question...

How Do We Determine Toxicity *in vitro*?

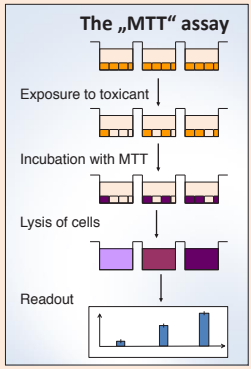
Tools to determine toxicity in the various *in vitro* cell culture models.

- Methods to determine 'non-specific' toxicity are available by measuring, for example, cell viability or oxygen consumption.



Test for oxygen consumption

Modified after: Prill *et al.*, Arch. Toxicol. 2015



Cell viability assays (e.g., MTT-test)

- The use of **'omics'** technologies allows for the identification and separation of non-specific and specific toxicities.

- Biomarkers for toxicity can be identified. The concept of so-called 'adverse outcome pathways' (**AOPs**) has been very helpful in this respect.

What is Omics?

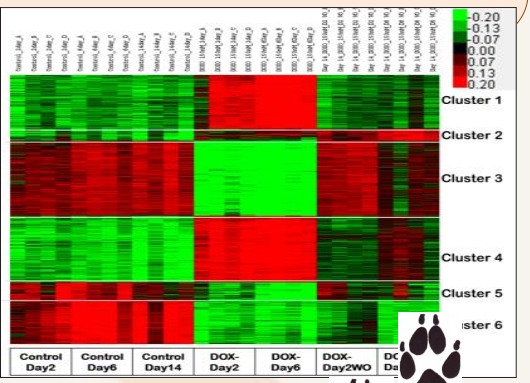
Gene Expression Arrays

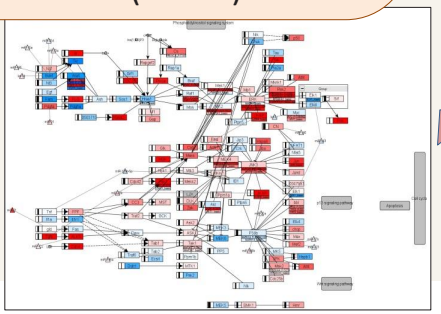
Images courtesy of Affymetrix, Inc

Array design | Hybridization | Signal detection

- The experiments investigate whether there is a change in the genes activated in cells when exposed to a chemical at given concentrations for given time periods.
- In this way we can detect characteristic „ ‘footprints‘ produced by toxicants.

From these ‘footprints‘, we identify the normal cellular processes that have been affected to develop a hypothesis on the mechanism(s) underlying toxicity as well as the concentrations at which effects occur (or not).



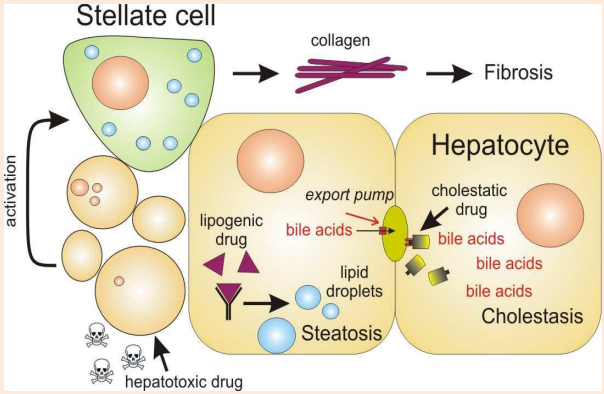


There are many ‘omics‘:
Metabolomics
Proteomics
Transcriptomics
…….

What is an AOP?

The **A**dverse **O**utcome **P**athway (AOP) describes the sequence of events from the exposure and the molecular initiating event (i.e., the interaction of the toxic compound with a cellular target molecule) to adverse effects at the organ and organism levels and finally at the population level. The AOP is a formalised way for scientists to describe a mechanism of toxicity.

- SEURAT-1 has developed AOPs for three major types of liver toxicity: Cholestasis, Steatosis and Fibrosis. Specific biomarkers allow the detection of these toxicities.



For additional information on these AOPs see the AOP-Wiki at https://aopwiki.org/

Considerations for Selection of *in vitro* Models

The selection of appropriate biological models to assess the toxicological properties of a compound *in vitro* represents a daunting task. Several new models were developed within the SEURAT-1 Research Initiative, exploring the capabilities of stem cell-derived cell systems, as well as combining different cell types in culture systems in an effort to mimick the complex structure of the liver ('liver-on-a-chip'). The selection of appropriate test system(s) depends on the question that needs to be answered.

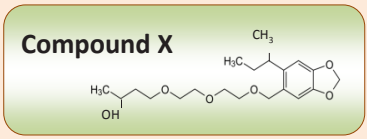
Chemicals are not always toxic *per se*. They can be deactivated or transformed to toxic metabolites in our body by metabolic conversion that predominantly occurs in the liver.

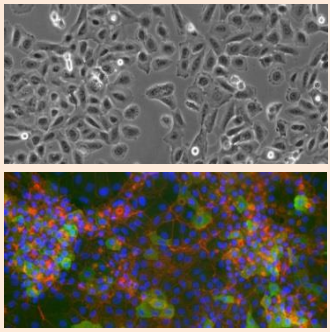
The enzymes that catalyse these reactions are mainly present in the hepatocytes of the liver. However, they are not well preserved in hepatocytes taken into culture and, thus, information relating to metabolism and clearance is important to ensure the selection of the appropriate *in vitro* model.

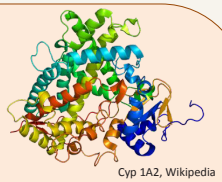
How is Compound X Metabolized in the Liver?

Metabolism is a normal cellular process and may detoxify a compound and assist in its excretion. However, some compounds may form toxic metabolites, especially in the liver, and this possibility should be considered.

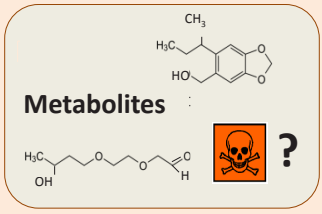
- *In silico* tools: Predict the metabolite pattern formed in the liver. However, the models are not able to predict the concentration of the metabolites.
- Therefore, metabolite patterns can be studied through the incubation of **human liver-like cells** such as HepaRG cells with test chemicals.





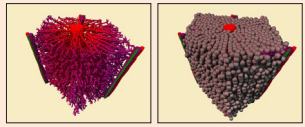

Cyp 1A2, Wikipedia

Activating and deactivating enzyme



Which Biological Models Simulate Liver Function?

In the experimental design, we need to carefully select the appropriate biological model that is fit for purpose. Metabolic capacity is often an issue. We have…

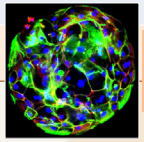


3D liver model

- Human (cancer-derived) cell lines
- Primary cells (human)
- Human iPS cell-derived differentiated cells
- Human organoids (cultures of mixed cells that reflect the composition of an organ or tissue)

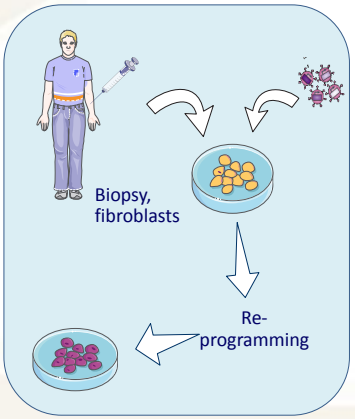
SEURAT-1 has developed several highly valuable 3D- and multi cell-type culture systems.

Cell type	Advantage	Disadvantage
Human hepatocyte-like cell lines	Easy to cultivate	Often decreased metabolic capacity
Primary human hepatocytes	Full capacity for metabolism of xenobiotics	Difficult to get; rapid loss of differentiated phenotype in culture
Human iPS cell-derived hepatocyte-like cells	High potential for toxicological screening	Still in an experimental phase
Human organoids	Very close to *in vivo*	Still in an experimental phase



Organotypic culture

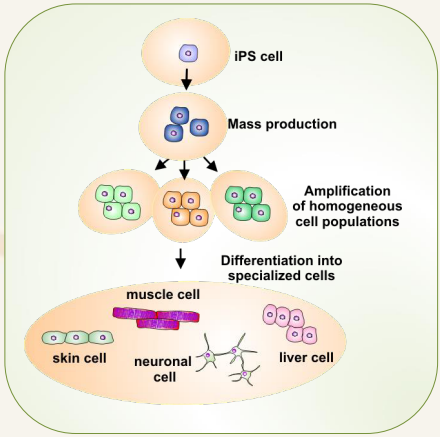
What is an iPS Cell?



Induced pluripotent stem (iPS) cells are produced by treating specialised cells (cells in the body that have matured to take on specific functions such as skin cells) with a special mix of proteins that causes them to revert to an unspecialised (more primitive) state.

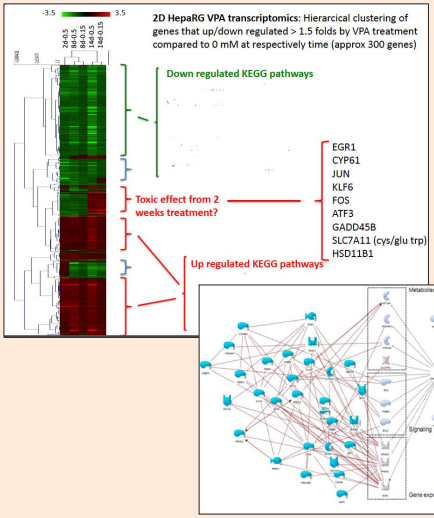
Through this 'reprogramming', the iPS cells acquire two key attributes of stem cells:

- an unlimited capacity of self renewal and
- the ability to become any type of cell in the body – the capability to be pluripotent.



Beginning Experimental Studies Using *in vitro* Systems

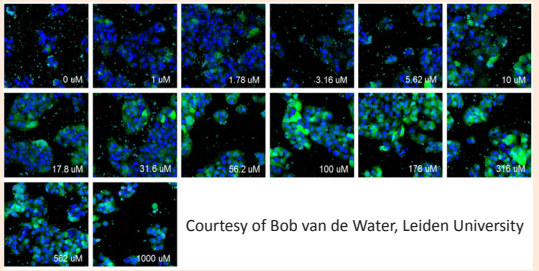
Omics analysis for the identification of pathways



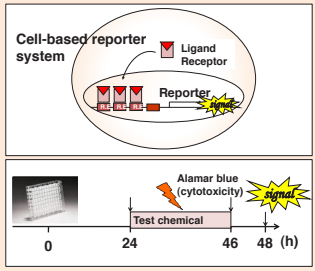
Changes in the expression of different genes are selected as indicators (biomarkers) of disruption in certain important cellular pathways.

Using the knowledge about which pathways are disrupted, custom test systems can be created using sophisticated cellular engineering techniques (so-called 'reporter systems').

Pathway-specific reporter cell lines (reporter systems)



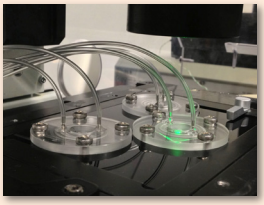
Courtesy of Bob van de Water, Leiden University



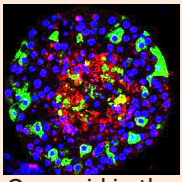
Cells can be made to glow or change colour when a pathway is activated by incorporating genes from a jellyfish into the cell's genome.

Complex *in vitro* Systems and *in silico* Tools

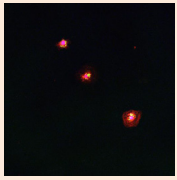
Bioreactors are needed for co-culturing different cell types for the investigation of certain responses in adverse outcome pathways that require cell-cell interactions. An example is the late response in the fibrosis AOP with the interaction between hepatocytes and stellate cells.



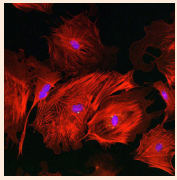
Courtesy of Hebrew University, Jerusalem, Fraunhofer IZI



Organoid in the bioreactor

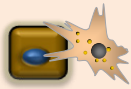


Quiescent stellate cells



Activated stellate cells

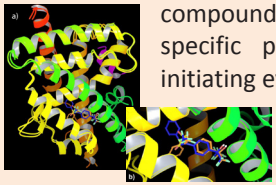
Hepatocyte and quiescent stellate cell



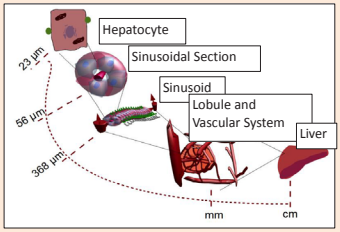
Hepatocytes respond to injury

Activated stellate cell, collagen formation

Computational toxicology is the use of mathematical and computer-based models to predict the potential toxicity of a chemical, using knowledge of toxicity pathways combined with relevant chemical (e.g. structure) and biological (e.g. omics) data. The computer-based models are developed as multi-scale models to enable insights into the mechanisms through which a chemical causes toxicity. Computer models can also provide an estimate of the distribution of a compound throughout the body.



Computer model of a compound bound to a specific protein, as the initiating event in an AOP.



Multi-scale modelling to predict toxicity

Data Management

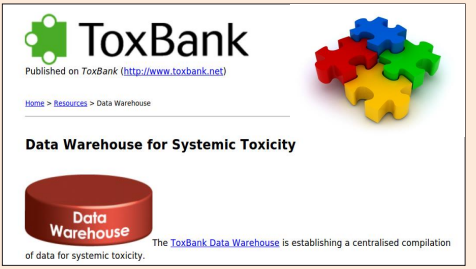
Infrastructure is required to compile and analyse the experimental data, including those coming from high-throughput ‘omics‘ experiments. Here we are talking about ‘big data‘. The overall goal is now to predict the dose that is expected to cause toxic responses in the target organ, in our case the liver.

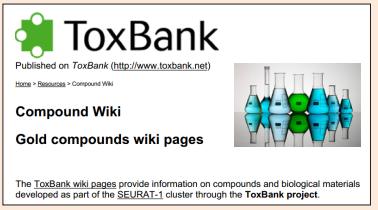
The data should ultimately become publicly available as a resource for the safety assessment studies of other compounds.

The SEURAT-1 Data Warehouse

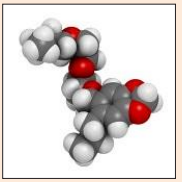
All experimental data needs to be collected and stored for a combined (meta-) analysis.

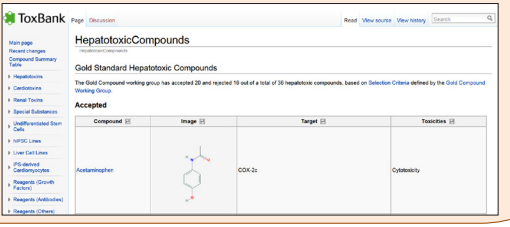
The SEURAT-1 ToxBank project has developed a data warehouse, where both protocols for tests (Standard Operating Procedures) and test results are stored for retrieval and analysis.





An expert group has selected “gold compounds”, which operate through known AOPs, as reference compounds representing certain types of toxicity to demonstrate the relevance, reliability and reproducibility of the *in vitro* test systems so that a new test chemical with a presumed type of toxicity can be evaluated with confidence.





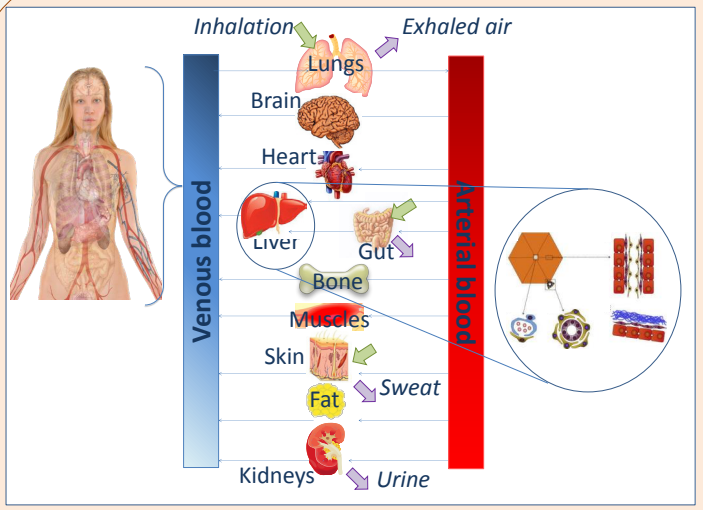
SEURAT-1 Guided Educational Tour, Station n°9

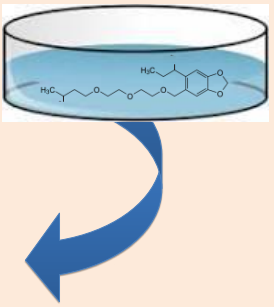
Quantitative *in vitro* to *in vivo* Extrapolation

In order to calculate the critical dose that will cause a toxic effect in the target organ, we again use toxicokinetic models in an approach which is called *in vitro* to *in vivo* extrapolation. Results obtained from *in vitro* experiments can usually not be taken without further treatment to predict the toxicity of a compound in an living organ or an entire organism. This is due to the fact that *in vitro* test systems reduces the biological complexity of a living organ. Hence, consistent and reliable extrapolation procedures are required to predict *in vivo* effects from *in vitro* concentrations.

Quantitative *in vitro* to *in vivo* Extrapolation

The concentrations at which compound X produces toxic effects in our *in vitro* models need to be converted into concentrations that are present in the body such as blood and liver concentrations. This is not straightforward but powerful computer models, called PBPK models, exist that simulate the movement, metabolism, and excretion of a compound from the body. Ultimately, models can be used to determine the amount of a compound a person would need to apply e.g. to their skin or ingest in order to achieve a toxic concentration in their blood or liver.





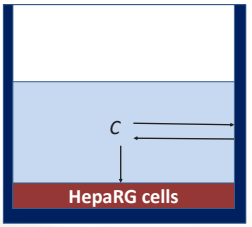
Use *in vitro* concentrations as input parameter (initial concentration) and calculate *in vivo* organ concentrations over time.

Overall Assessment

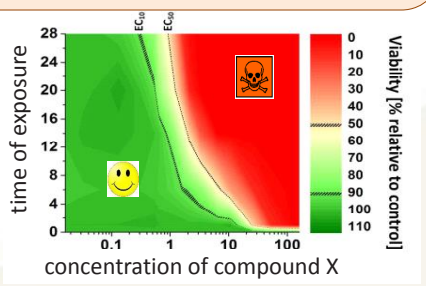
In the overall assessment, we can then define ‘margins of safety‘, i.e. concentration ranges of compound X in the exposure scenario at which we do not expect toxicity. We can then compare these margins of safety with the concentration at which compound X is present in the product in order to answer the safety assessment question raised at the beginning of the exercise, namely whether compound X used in a defined exposure context is safe for the consumer.

Overall Assessment

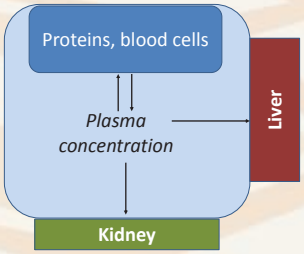
From the *in vitro* experiments, we are able to predict at what concentration of compound X liver toxicity is to be expected. Toxicity will increase as a function of concentration and time of exposure. The green area in the figure below shows the safe *in vitro* exposure scenario.



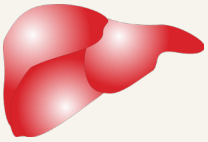
In vitro **chronic exposure**



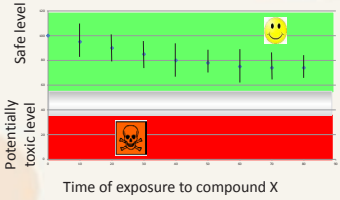
We then use the toxicokinetic models described above to extrapolate the *in vitro* concentration causing toxicity to the systemic amount of compound X a person is exposed to. In our present 'case' we then need to extrapolate to the amount of compound X a person would need to apply to their skin in order to achieve a potentially toxic response.

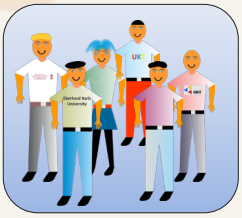


In vivo **toxicity prediction**



Population toxicity prediction





Remaining Uncertainty

Finally, we need to assess the variabilities and uncertainties associated with the data and the models. Each assessment, whether based on animal studies or non-animal methods, needs to assess the variabilities and uncertainties associated with the data and the models. Characterising variability and reducing uncertainty increases the confidence, transparency and understanding of the assessment. Addressing variability and uncertainty can inform safety assessors about the reliability of results and guide the process of refining the safety assessment as necessary. With that, the conceptual framework as outlined in Station n° 1 is completed.

Remaining Uncertainty

The predictions we make based on our *in silico* and *in vitro* results inherit a number of uncertainties. These uncertainties are associated with the choices we make in each step of the process and may include those from the original 'read-across' with PBO, the choice and interpretation of our *in vitro* models, the conversion of the *in vitro* toxic concentration to the amount applied to the skin, and potentially more sensitive individuals that may not be captured in our experiments. These uncertainties are addressed by building-in additional margins of safety into the overall assessment, to ensure that ultimately the population is adequately protected.

Coming back to the question:
Is this ingredient safe?

Most likely YES!

However, neither with or without animal testing, can we reach 100% certainty. If innovation brings new products to market, a certain risk unavoidably remains. However, new technologies based on human cells make safety assessment more human relevant, thereby minimising the need for the “classical“ bioassay with experimental animals.

Notes

SEURAT-1

SEURAT was announced as a strategy of the FP7 Health Theme by director Dr. Manuel Hallen on the occasion of the EPAA Annual Conference in 2008 in line with the Comissioners G. Verheugen and J. Potocnik. The long term strategic target is defined as „Safety Evaluation Ultimately Replacing Animal Testing“ (SEURAT).

SEURAT-1 is the Research Initiative launched by the European Commission and Cosmetics Europe with a total funding of EUR 50 million from 2011 to 2015. It is called „SEURAT-1“, indicating that more steps have to be taken before the final strategic target will be reached. SEURAT-1 developed a long term research strategy and building blocks needed for the development of new non-animal test systems in the field of repeated dose systemic toxicity for the innovative assessment of human safety.

The European Commission, together with Cosmetics Europe, launched the Research Initiative ‚Towards the Replacement of in vivo Repeated Dose Systemic Toxicity Testing' in order to develop a sound research strategy leading to the long-term target of ‚Safety Evaluation Ultimately Replacing Animal Testing' (SEURAT). The Research Initiative was called ‚SEURAT-1' to indicate that this is a first step in a specific area addressing the global long-term strategic target SEURAT. One of the primary objectives of SEURAT-1, which was active from 2011 – 2015, was the development of innovative tools and methodology that can ultimately support regulatory safety assessment.

The applicability of the developed tools and methods was demonstrated on the occasion of the final SEURAT-1 Symposium ‚Painting the Future Animal Free Safety Assessment of Chemical Substances: Achievements of SEURAT-1', which was held on 4 December 2015 in Brussels. On this event, a so-called Guided Educational Tour was implemented which is summarized in this booklet. The purpose was to show how the available tools and methods can be integrated to support regulatory safety decisions at the end of the project's lifetime in 2015.