Innovative Approaches to Reduce Animal Testing

Replace whenever possible, reduce through refinement and mechanistic understanding

Prof. dr *ir.* Bennard van Ravenzwaay

Inaugural lecture upon taking up the post of Special Professor in Reproduction and Development Toxicology at Wageningen University on 2 May 2013

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Dear Rector Magnificus, dear colleagues, dear friends and family,

Toxicology has been a part of human society long before there was a written history. It is not too difficult to imagine that human beings in search of food will have encountered occasional intoxications. Evidence of the use of herbs and plants for medical purposes has been found in nearly all cultures, with or without a written history. In fact throughout the ages, those who practiced the use of natural medicine have generally taken a relatively high place in any society. Knowledge about beneficial and toxicological effects of naturally occurring substances (most likely complex mixtures) must have first been obtained from human practice. Without the advantages of the written word, such knowledge has been passed from one generation to the other by oral narrative.

I think most of us are also aware that this knowledge of toxins has also been put into practice for good and for bad and even for political purposes. For instance, the philosopher Socrates was put to death by forcing him to drink a cup of poison hemlock extract in the year 399 BC. For those who are interested; the most noted of hemlock toxins is Coniine. Coniine disrupts the functioning of the central nervous system by blocking the neural signals to the muscles in a manner similar to the Amazonian dart poison curare. This results in an ascending muscular paralysis which eventually affects the respiratory muscles causing death by suffocation.

Where there is bad, there usually also must be good. Venom based cures are mentioned in Sanskrit text in the second century B.C. and Mithradates VI of Pontus was known to use an anti-dote which supposedly saved his life twice on the battle filed. This anit-dote known as Mithridate, contains as many as 65 ingredients. It was among one of the most complex, highly sought-after drugs during the Middle Age

and Renaissance. Legend has it that Mithradates fortified his body against poisons to such an extent that when he tried to kill himself, he could not find any poison that would have an effect. After loosing the final battle against Rome, the recipe of it was found in his cabinet and was carried to Rome.

More recently some cases of intentional human poisoning have been recorded that would have suited a James Bond movie. In 1978, Georgi Markow, a Bulgarian dissident writer, was pinched in his thigh, presumably by a needle hidden in an umbrella, in London. He noticed a small red welt had formed at the site of the sting. That evening he developed a fever and was admitted to a hospital where he died three days later. The cause of death was poisoning from a ricin-filled pellet. In 2006 Alexander Litvinenko, an ex-officer of the KGB, went to hospital complaining of signs of poisoning. In the next days his health deteriorated rapidly. Initially it was thought to be a case of Thallium poisoning, urine samples only a few hours before he died demonstrated great quantities of radioactive Polonium-210 in a London hospital. Apparently, London is a dangerous place to live.

On the other hand, not all attempted poisonings are successful. For example, the attempted assassination of Ukrainan president Viktor Yushchenko with TCDD is quite revealing. This case is interesting because it demonstrates a misinterpretation of the toxicological data from animal studies. In the popular press, TCDD (the most potent of the dioxins) is often referred to as a mega poison. TCDD indeed has a significant toxicological potential, but in terms of lethality, it is far less deadly to humans than guinea-pigs. Victor Yushchenko survived the attack on his life although the characteristic acute effect of dioxin poisoning, chloroacne, affected his appearance. (Details of the above mentioned cases were obtained from Wikipedia).

The first documented animal experiments to detect toxic effects also date back to Greek times. Aristotle was amongst the first who performed and reported on in vivo animal experiments. In this context I will be using the word “in vivo” for studies involving animals and “in vitro” for studies involving cell based systems; such studies are also referred to as replacement or alternative testing methods. Considering that humans have existed as a species for more than 200,000 years, it can be said that we have survived reasonably well without animal studies for more than 99% of our “species time”. The number of animal studies, however, has gradually increased in the last century. In fact, there is a positive correlation between the number of animal studies and the gross social product of a country (see figure 1).

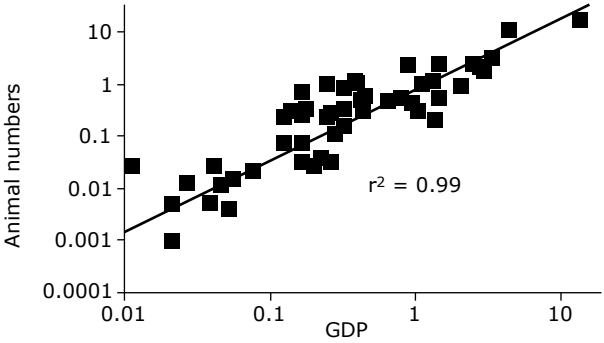

Figure 1. Correlation between the number of animal studies performed in a country and its gross domestic product (GDP)

In the previous century new chemicals were developed at high speed as a result of the industrial revolution and scientific progress, particularly for pharmaceuticals, and these chemicals were often put on the market without much toxicity testing. It should thus come as no surprise that the history of economical development and toxicology has been anything but smooth. There are a number of well-known cases were previous knowledge of the toxicological potential of compounds would have prevented human and environmental disaster. We only need to think about the effects of DDT and Thalidomide, to understand why the catalogue of studies that need to be performed has grown (which each disaster) over the years. At the same time, ethical concerns for animal welfare have also been rising, necessitating the development of alternatives to animal studies.

The first (and still one of the best) of these alternative approaches to animal research were proposed by Russell and Burch in 1956 (Russel and Burch, 1956). They described a concept generally referred to as the 3Rs: Reduction, Refinement and Replacement. The beauty of this concept is that it approaches alternatives to animal testing not only from the replacement point of view (i.e. *in vitro* only), but also encourages other methodologies. Among these is reduction, the idea that studies can and should be done with the least number of animals necessary. Similarly, I believe that the concept of reduction also means that the number of animal studies performed during product development should be limited to the minimal number required to do proper hazard identification and risk assessment. I will get back to this point little later.

Refinement was originally intended to indicate any new *in vivo* type of study using methods that would be less harmful or stressful for the animals. One example of a

successful refinement is the development of the mouse local lymph node assay (LLNA), in which the test substance is applied to the ears of a mouse, as an alternative to the maximization test, where it is injected under the skin of guinea-pigs (OECD test guideline 429). I propose to extend the concept of refinement to also include methods which have recently become available and provide a wealth of new data without the need to do additional animal studies. These new methods are often referred to as 'omics sciences and consist of transcriptomics (the study of gene expression, by measuring messenger RNA), proteomics (the quantitative determination of proteins in organs and tissues) and metabolomics (the quantitative determination of naturally occurring small molecules in the body (see figure 2). 'Omics data can be obtained from any animal study and provide a (sometimes overwhelming) wealth of information. Thus, these 'omics data now allow for an insight into the modes of action of a substance and thus for a better interpretation of the animal study. I believe that with this improved information situation we will be able to do fewer studies, each using fewer animals. I refer to this concept as "reduction through refinement". Again I will give an example of this concept a little later.

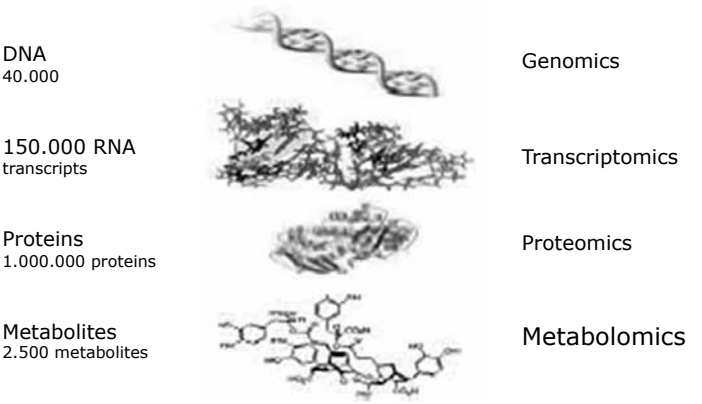

Figure 2. The science of 'omics is the study of a complete suite of biomolecules

So now let's take a closer look at replacement, i.e. *in vitro* studies, now. The first, and considered by many to be the main advantage of *in vitro* studies is the reduction of animal testing . This ethical aspect is continuing to increase in importance; as exemplified by the fact that the Netherlands has a political party called "Party for the Animals" (partij voor de dieren) or by the fact that the German constitution was amended in 2002 to include provisions for animal welfare.

A second often mentioned advantage is that *in vitro* studies are better suited for a targeted approach, or to address very specific questions which are often far more

difficult to address in an *in vivo* study. While this is true, this advantage turns into a disadvantage when we look at the fundamental question toxicologist are trying to address from a holistic point of view. There are two aspects which determine the toxicity (hazard) of a compound. (1) The toxicodynamics, that is what the chemical does with the body. This is the part that sometimes can be better studied in vitro. (2) The toxicokinetics, that is what the body does with the chemical. This part of the equation is often not included in in vitro models. What the substance does to the body is also referred to as ADME (absorption, distribution, metabolism and excretion). Therefore, if we go for replacement of animal studies by in vitro studies we must also address the kinetics. In my engagement here in Wageningen we will indeed look at both parts of this equation. Here in Wageningen we are developing mathematical computer models, based on biological evidence, which translate *in vitro* concentrations into *in vivo* values. Without such models it will be very difficult to use the results of *in vitro* testing beyond a simple yes or no answer. And yes or no is not enough to perform the necessary risk assessments in toxicology. Therefore I believe that the research work here at Wageningen University will be an essential tool in the enhancement of scientifically sound replacement of animal studies, and I am proud to be part of that effort.

A third advantage of *in vitro* studies is that they use less test substance. This may sound somewhat trivial, but is, in my opinion, currently by far the largest advantage of *in vitro* studies. Why? Larger amounts (in the Kg range) of totally new chemicals and in particular (pharmaceutically) active ingredients usually are only produced in later stages of product development (following scale up of synthesis). The greatest misuse of both financial and animal resources is related to projects which fail during late stages of development. Here, I am particularly thinking about the development of new active ingredients for pharmaceutical and agrochemical products. In both cases, development costs far exceed 100 million euros and use up to 4000 animals, with rats making up the largest portion. Imagine if all this money, and all those animal lives were spent for nothing because during the last stages of development unacceptable effects are observed. Let me be clear, one of the most important roles that a toxicologist has in society is to ensure that disasters, like those seen with DDT or thalidomide, are prevented. In the past we may have been over-optimistic, you may even say careless, about the advantages of some pharmaceutical products without taking a closer look at their risks (see figure 3). So we need good regulation and we need good, reliable and relevant toxicological studies.

However, there is also a risk that we use all of the aforementioned resources, and erroneously conclude that there is a serious risk and subsequently stop the development of a very valuable product to society because of toxicological findings. I have

no doubt that aspirin would not have been developed by pharmaceutical companies if its toxicological potential would have been evaluated according to the methods and paradigms that we use today. Aspirin, sometimes referred to as the 8th wonder of the world, would not have made it to the market because it causes malformations in developmental rat studies, and few companies if any today, would have dared to try and market such a product.

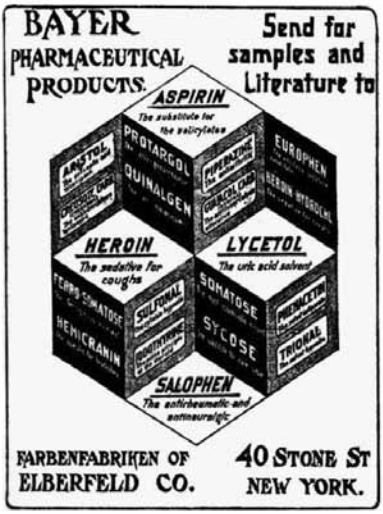

Figure 3. Asprin, the 8th wonder of the world, together with what would now be perceived as inappropriate 19th century adverting of an illicit product

So what has this all got to do with the advantages of *in vitro* toxicology? It's simple. We would like to know as much as we can about a new compound, as soon as possible. However, even a relatively small *in vivo* study, such as a 28-day toxicity study in rats, may require up to 200 g of test substance. Furthermore, at this stage of early development, companies are usually not only working with a single compound, but with many sharing a particular desired effect. It is not possible to synthesize 200g of material (which costs more than its weight in gold) for so many chemicals, to see it being devoured by rats within a month – only for one single test. So what is the solution? Behind all (severe) toxicological effects there is a mechanism, a mode of action. If we can develop the appropriate in vitro test, this would allow us to identify these effects at very early stages of product development. For example, the Ames test, an *in vitro* method, uses bacteria to determine if a compound may cause mutations. It may not be a perfect predictor for genotoxic carcinogens, but if a compound causes mutations do you really do want to invest significant resources in its development if you have alternatives that do not cause such effects?

At BASF, we have developed a yeast based assay which detects compounds with receptor-mediated endocrine effects (Kolle et al. 2010). With current EU legislation indicating that compounds showing endocrine effect may not be suitable for registration, why run the risk investing in such a compound if you have alternatives? And the fact is: early in development industry usually has alternatives. So, knowing the toxicological hazard at an early stage helps to select those compounds which cause the least harm and have the best chance of passing the regulatory tests. *In vitro* tests help tremendously in this selection process. I can say with certainty that this type of early screening with *in vitro* tests has reduced animal testing far more than the *in vitro* tests which now can be used as complete regulatory alternatives to *in vivo* testing.

Finally, it is often mentioned that alternative methods are less expensive than animal testing. Unfortunately, this is only partly true. Let me give you two extreme examples: The aforementioned yeast assay to determine endocrine effects is approximately 7 times less expensive than the *in vivo* equivalents, (the Hersberger and Uterotrophic assays) and avoids the use of 120 animals per compound! However, the *in vitro* alternatives to skin and eye irritation testing that discriminate between severe irritants, irritants and non-irritants are 2 – 3 times more expensive than the animal test using rabbits. I am very glad that we can now avoid this *in vivo* test, but from a purely financial point of view, the *in vitro* studies were a step back rather than a leap forward. Therefore, the conclusion of the economics of alternative methods is: it depends *(see: www.estiv.org/docs/Report ESTIV2012.pdf)*

So how do you develop alternative methods? One way to do this is to look at correlations between tests. I will give a few examples: We test for acute toxicity in rats, daphnia (water fleas) and fish. If we compare the outcomes of such studies for a set of compounds, there is absolutely no correlation between the acute toxicities seen in rats and those found in fish or daphnia (see figure 4).

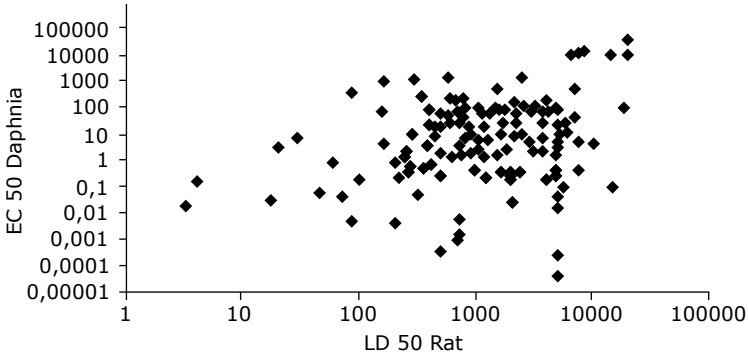

Figure 4. Absence of a correlation between acute toxicity in rats and daphnia

There is, however, a correlation between the two aquatic organisms. It may not be perfect, but as daphnia are not more similar to fish than rats are, it does indicate that the environment apparently plays an important role. If we now take one further step towards an alternative method and consider the use of fish eggs, we can determine a correlation coefficient of $R^2 = 0.83$, a rather good correlation, between the acute toxicity endpoints in fish and fish eggs (see figure 5).

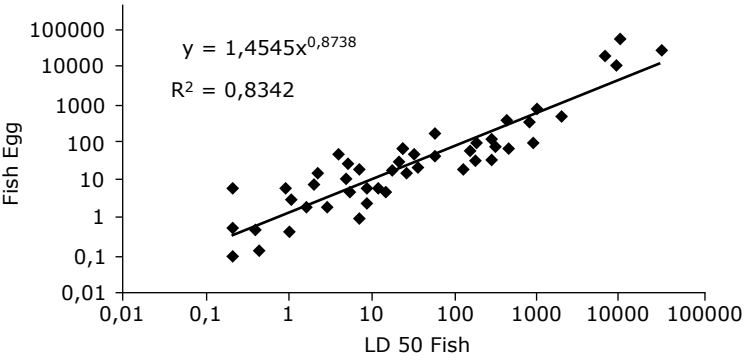

Figure 5. Fish eggs are a useful model to predict acute toxicity (lethality) in fish

This correlation can be further increased by taking into account some physico-chemical properties. As a result, fish eggs have become one of the most important alternative methods for reducing animal testing. Why? Toxicity studies are not only performed for chemical development, but also used to monitor environmental quality. Because of fish eggs, many thousands of fish are not used in water quality studies, in particular determinations of the quality of the effluent at waste water treatment plants.

Before I continue to show you how we try to address the development of alternative methods to replace more complex toxic effects, let me pour you some water in this good alternative wine.. Over the last few years we have seen good progress in the regulatory acceptance of 3R methods. We have new methods with OECD guideline status in which skin and eye irritation testing, formerly performed in rabbits, can be replaced by *in vitro* methods. However, approximately 15 years passed between the invention of the method and its regulatory acceptance a very long innovation time. Fortunately, the pace of regulatory acceptance of 3R methods is gaining momentum and we have seen the innovation time decrease to approximately 7 - 8 years. Last year, the OECD guideline 443 – the extended one generation study - was adopted. I take pride both in having been a member of the larger International Life Science Institute (ILSI) team which helped with the scope of this new study, and in having performed and published the first validation study with this guideline (Schneider et

al. 2011, Fegert et al. 2012). This study is an excellent example of reduction through refinement! The extended one generation study uses up to 1,000 (!) animals less than the original 2-generation study, while maintaining sensitivity. Within the context of REACH, the European chemical legislation, this new protocol can contribute more to reduced animal testing than sum of all other alternative methods currently available.

But sometimes we are too optimistic concerning the contribution of alternative methods. For many years, cytotoxicity studies have been used as guidance to select the starting dose for the acute oral toxicity tests, normally the first *in vivo* study to be performed. We have evaluated the contribution of the cytotoxicity test to the correct dose selection in comparison to expert opinion and to a fixed dose procedure. It turned out that expert judgment outperformed the other two options, but that even a fixed dose as a starting point was better than relying on the cytotoxicity test (Schrage et al 2011). In my opinion, the reason for this failure is because a post-validation evaluation of test method performance is only rarely implemented, even though the data are (freely) available. We prefer to look for new horizons rather than looking back on the quality of the ships that we have built. Again, let me be clear, this does not only relate to *in vitro* studies, the absence of post validation exercises is also a problem in animal studies. For a decade, industry has been required by the USA environmental protection agency (EPA) to do developmental neurotoxicity studies, which come with a price tag of 750,000 € and use approximately 1,000 rats. Very few, if any, of these studies have resulted in significantly new knowledge that was used in the risk assessment process. And there are more animal studies of questionable relevance to which we will come later.

Most of the *in vitro* studies developed and validated so far have related to relatively simple endpoints for which often only a yes / no answer need be given, like skin or eye irritation and genotoxicity. How do we tackle more complicated toxicological endpoints? Most likely the road to success will be in first describing the so called "adverse outcome pathway", that is understanding the process which causes an effect, and then designing a series of *in vitro* studies that address key steps in this pathway. Let me give you a successful example. Skin allergy used to be tested in guinea pigs, which received multiple injections with the substance, followed by an exposure to the skin and evaluations of skin redness. This study was redesigned to now consist of a single application of the test substance to the ears of a mouse and then evaluation the immunological response in the lymph nodes of these animals. Remember the 3Rs, this is a clear refinement.

But we can do better than just refinement. Let's look at the process of skin sensitization (see figure 6). Step (1): a chemical needs to penetrate the skin – we have

an *in vitro* study (OECD 428) for this. Step (2): the chemical needs to be able to react with proteins in the skin – there a chemical reactivity assay for this (the Direct Peptide Reactivity Assay, developed by Proctor & Gamble Company). Step (3): the chemical must be able to react with skin cells (the keratinocytes) – Givaudan & BASF have developed assays. Step (4): the cells of the immune system in the skin (dendritic cells) must be able to recognize the altered proteins and be activated – there are different assays to test this effect.

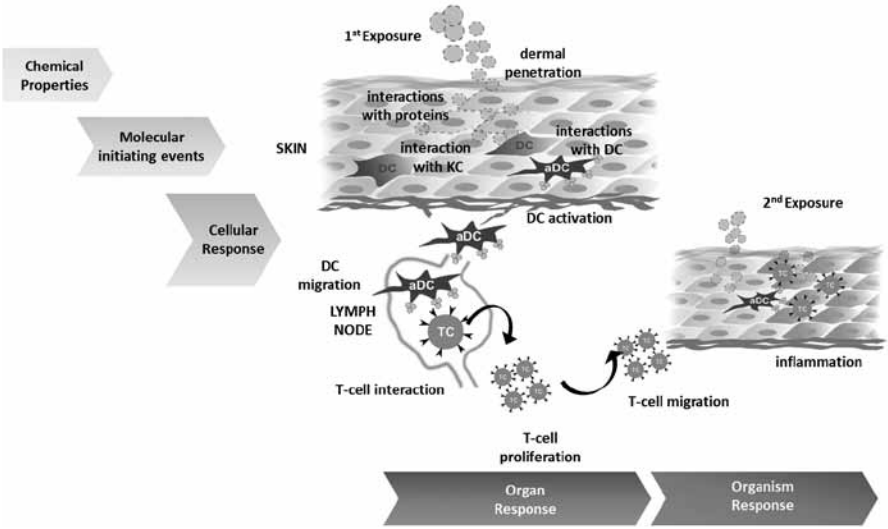

Figure 6. Physiological process of skin sensitization (adverse outcome pathway)

We then evaluated the individual and combined performance of these tests and tried to set up a testing strategy that gives the best predictive outcome for skin sensitization. We have evaluated more than 50 substances of which the allergenic potential in humans and animals has been established (it is a rare case in toxicology to have extensive well documented human data). Our data so far indicate that the *in vitro* testing strategy outperforms the animal study in predicting human skin allergens. I do remember my own words, we will need to keep track of the performance of the strategy (the post-validation evaluation), but at this stage I can say that we have found a way to identify skin allergens *in vitro*, which should reduce future need for animal testing (Bauch et al, 2012).

One of the most important endpoints in toxicology are those of reproduction and developmental toxicity, which include infertility, malformations, and retarded development. Not only is this true in terms of the consequences, but also in the number of animals necessary to perform tests, as well as the financial resources

required for new compound development. Reducing the need to rely on animal testing for reproduction and developmental toxicity is the topic of my research work here in Wageningen. Reproduction toxicity is a very complicated topic and it would be foolish to believe that we can simply replace the animal study by a single *in vitro* method. We will need to carefully evaluate existing methods for their performance and applicability domain (the chemical classes or modes of action for which an assay has a good predictivity). We will also need to try and break down the processes which can cause developmental and reproduction toxicity, in particular malformations. This will allow us to better understand the mechanisms involved, so that we can design *in vitro* studies following the adverse outcome pathway in a way analogous to that previously described for the process of skin sensitization.

First steps have already been made. For a compound to directly interact with the fetus it must first pass a barrier, the placenta (like skin allergens first need to penetrate the skin). Last year we started here in the toxicology department in Wageningen the first tests with an *in vitro* placenta model to evaluate its accuracy in predicting the rate of placental transfer of a range of chemicals. This work has shown that the BeWo transwell model is an adequate in vitro model to mimic placental transfer of a series of selected model compounds and these data are now described in the first paper of my PhD student Hequn Li working on this BASF funded project (Li et al, 2013). We are now validating this model with a new set of chemicals for which the in vivo placenta transfer data are provided by BASF and the *in vitro* studies are performed here at Wageningen University (see figure 7).

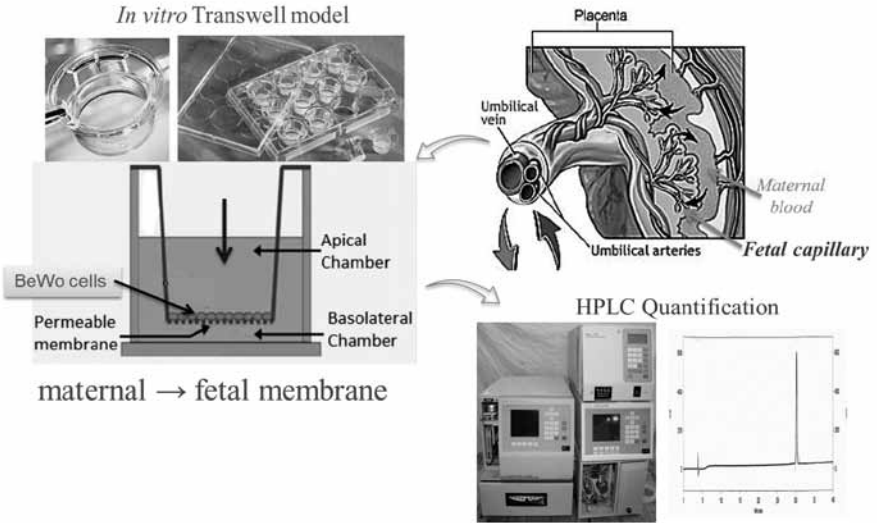

Figure 7. Schema of the BeWo assay for placental transfer

Two *in vitro* models to determine embryo toxicity (the induction of malformations) are currently being validated experimentally, and a few others are under evaluation. I hope that in the course of time we will be able to build a tool box of *in vitro* assays which can be used to assess embryo toxicity. With knowledge about the applicability domain of each individual assay, we should be able to set up a testing strategy for different compounds, particularly if their modes of action are known. Therefore, one of our research strategies is to look at groups of compounds and determine which assays are best suited to the generation of information (including data from 'omics sciences) which then can be used for a general approach to the identification of embryotoxic compounds. The task of producing a strategy for the *in vitro* measurement of developmental and reproduction toxicity is not a piece of cake and will require a significant effort and a little bit of luck.

With the following example, I would like to demonstrate that knowledge of the mode of action is important in the development of alternative methods and that this goes hand in hand with the design of a testing strategy. Chemicals interacting with the hormone system are often referred to as endocrine disruptors. I do not particularly like this expression because it suggests massive changes and deregulations, whereas in reality some of the endocrine effects are actually quite subtle. The European Union has decided on a political level that chemicals demonstrating "endocrine disruption" should be more strictly regulated or even banned. Interestingly, there is currently no definition of what exactly constitutes endocrine disruption. Given the consequences and financial risk involved in developing a new compound and finding out that your latest darling is an endocrine disruptor which needs to be abandoned, you can image that companies would like to know as soon as possible about potential endocrine disruption effects.

The current focus is on compounds that affect steroid hormones, in particular the male and female sex hormones, testosterone and estradiol. There are two specific animal studies to address this question, the Hershberger assay (OECD 441) and the Uterotrophic assay (OECD 440). Without going into too much detail it can be said that there are three main mechanisms which can cause chemicals to have endocrine effects: (1) receptor mediated effects (2) effects on hormone synthesis, (3) interference with hormone metabolism. I mentioned earlier that for receptor mediated effects there is a simple yeast based assay. This has been validated with more than 100 compounds with known activity and a predictive outcome of > 90 % (Kolle et al, 2010).

For hormone synthesis there is also an *in vitro* assay using an adrenal cell line which produces steroid hormones. By combining these two systems we are able to detect the majority of compounds with endocrine disruption activity (see figure 8). What is

lacking is the effect of substances on the metabolism of hormones and, as indicated earlier, the kinetics and metabolism of the compound itself. To take these parameters into account, we have proposed to use information from one of these 'omics sciences, metabolomics.

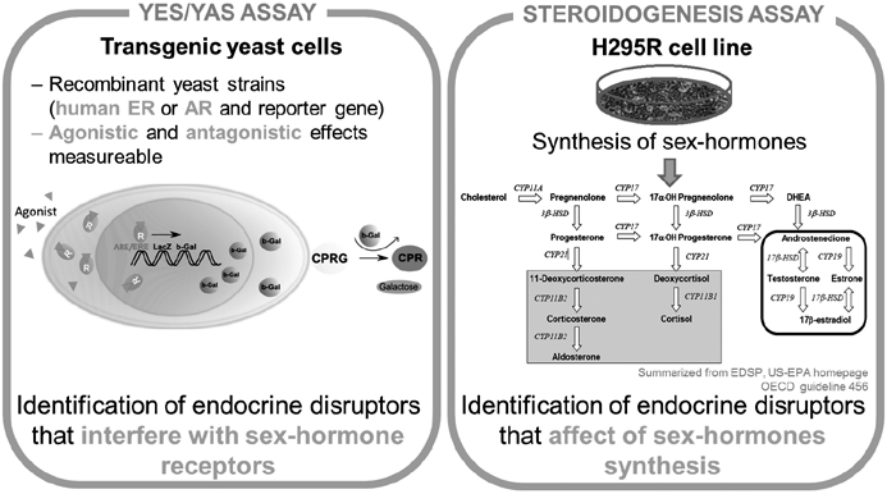

Figure 8. In vitro assays for the identification of compounds with endocrine effects

Metabolomics is the analysis of the relatively small molecules (< 2,000 dalton) that are natural components of living organsims. We have built up a metabolome database using blood samples. The reasons for this are simple: blood is easily accessible by relatively non-invasive methods and flows through nearly all organs. The idea is that chemically-induced toxicity, as well as diseases, will result in a particular fingerprint of metabolome changes in the blood. Identification of specific patterns of change can then be associated with a particular mode of action, using reference compounds, should also enable us to detect these modes of action in new compounds (van Ravenzwaay et al 2007). We have now established a database with the metabolome patterns of more than 500 chemicals and their toxicity profiles. Combining the metabolome information with the known toxicity of these reference compounds, we have established more than 100 specific metabolome patterns that are associated with particular toxicological effects or mode of action (van Ravenzwaay et al 2012). Among these specific patterns are those toxic consequences of compounds with endocrine effects.

Thus, the two aforementioned *in vitro* studies for the identification of endocrine effects work as an excellent first screen to filter out those compounds that have a clear endocrine active profile. For those compounds that are "negative" in these

assays (i.e. that do not show in vitro endocrine effects) we move forward in the development of a product that one day may go to the market. If all other aspects (performance, economics, investment required, ecotoxicological profile) are favorable, we proceed with *in vivo* testing (Kolle et al, 2012).

For an agrochemical this means that we embark on a series of up to 40 different toxicological studies necessary for regulatory evaluation and registration as a new active ingredient. In one of the first of these studies in rats, we take a small sample of blood and perform a metabolome analysis. With this, we are able to identify up to 100 different modes of action including a number of effects associated with endocrine disruption. As we are also now dealing with *in vivo* studies, we have automatically included additional modes of action as well. These modes of action include interference with hormone metabolism, for which there is as yet no *in vitro* test, as well as all other aspects of kinetics and test substance metabolism, which are still hard to account for in *in vitro* assays. Since we apply metabolomics in studies which are already required by regulatory authorities, we do not have to use any additional animals. So what we now have for the identification of endocrine disruptors is an effective two tiered approach, an *in vitro* screen for early detection and an omics approach to an *in vivo* system which *refines* current testing, resulting in an overall reduction in animal testing (see figure 9).

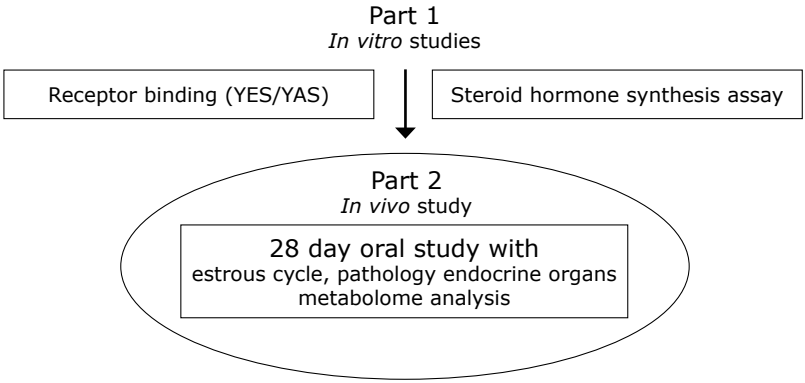

Figure 9. A two-tiered testing strategy for the identification of compounds with endocrine effects

As indicated earlier, up to 40 different toxicological studies may be required for the development of new active ingredients. Out of these only a few will eventually be needed for compound registration with the authorities. The problem is: at the start of the toxicological testing program, we do not know what the critical effects will be. Consequently some studies will be highly relevant while others will not contribute to the risk assessment of the compound in question. This would seem to be unavoidable,

however, taking into account the rather low efficiency of this process, it is worthwhile to consider whether we can do better. And indeed, we can do better.

The best and simplest alternative to an animal test is to not do the test at all. Is this possible without losing essential information for risk assessment? In a retrospective analysis of the regulatory relevance of the agrochemicals study set, both the study duration and the relevance of the model species (rats, mice and dogs) was assessed (Doe et al, 2006). I would like to highlight one particular finding here, and that is the use of dogs in this set of studies. Several authors have concluded that the use of dog as a non-rodent species provides important information for the risk assessment process. For about 30 – 50 % of all investigated agrochemicals (depending on the database), 90-day studies in dogs provided a lower no observed adverse effect level (NOAEL) than comparable studies in rats (Spielman and Gerbracht, 2001). Thus, the 90-day dog study provides essential information and needs to be performed.

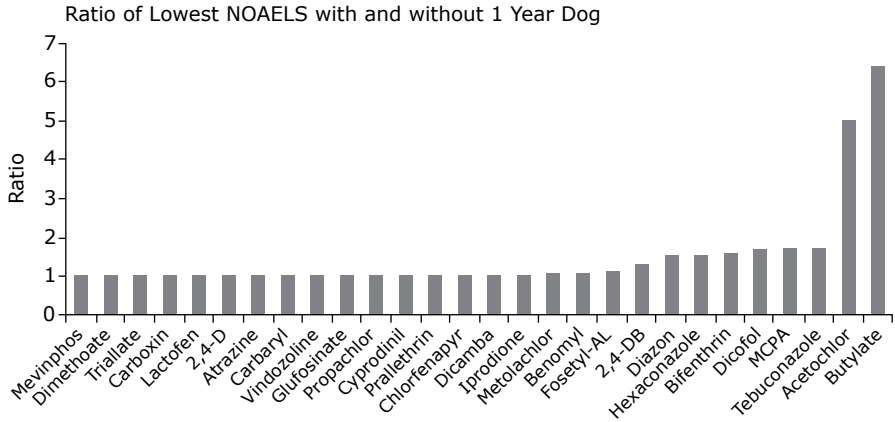

Figure 10. The 12-month dog studies do not provide any additional information necessary for risk assessment

However, the performance of a 12-month toxicity study in dogs has also been required for global regulatory acceptance for the last 30 years. Thus, quite a large number of 90-day and 12 month dog studies have been performed, providing an extensive database. These data were then used to address the question of whether there is any additional value added by a 12 month dog study, over and above that of the 90-day study. Spielman and Gerbracht (2001) found no clear difference in sensitivity between 3-month and 12-month studies. In this report, the distribution of the ratios between the lowest observed effect levels (LOEL) of the subchronic and chronic studies (insecticides, herbicides, fungicides) also did not reveal significant differences between the outcomes of these studies. Doe et al. (2006) as well as Kobil et

al. (2010) evaluated sensitivity based on no adverse effect levels while looking at the impact on the regulatory outcome if the 12-month dog study would not have been performed. In this endeavor, they examined the lowest no adverse effect level of the standard set of 4 systemic toxicity studies (90-day rat, 2-year rat, 90-day dog, 1-year dog) and compared the resulting lowest NOAEL from these studies with and without consideration of the 12-month dog study.

Using a similar approach, we found that for only two compounds the no adverse effect level from 12-month dog study was more than 2-times lower than the one obtained from the other studies (see figure 10). For one of these, there were confounding factors, which made an evaluation difficult. It was therefore concluded that the 12-month dog study does not provide essential data for risk assessment. One reason for the lack of increased sensitivity with the longer exposure time in dogs may be related to their life expectancy relative to duration of treatment. In rat studies, the extension of exposure from 3-months to 1 year (chronic) or 2 years (cancer studies), takes the duration of exposure, relative to life expectancy, from 12 % to 50% or even 100 %. In dogs, the 3-month study is equivalent to about 2% of life expectancy, the 12-month study not more than 8%. Therefore it is plausible that chronic and old-age effects are noted in rat, but not in dog studies (see figure 11).

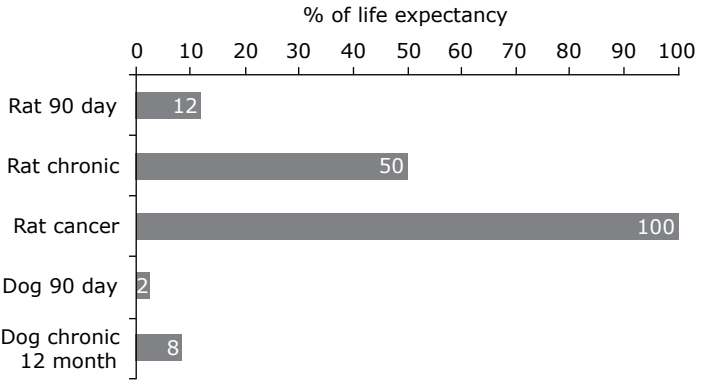

Figure 11. Study duration as a percentage of the life expectancy of the test species

The good news is that these evaluations and conclusions have found their way into the regulatory arena. In the EU the 12-month dog study is not an absolute data requirement anymore, and the USA-EPA has also indicated that this study does not necessarily need to be performed. However, the world is much larger than the EU and USA and we need global acceptance, before a data requirement can be completely eliminated. In Brazil the discussion about the 12-month dog study as a regulatory requirement has started; we hope that Japan will follow soon. Such retrospective

assessment, would increase the efficiency of regulatory testing and reduce the number of animals used, and therefore, should become a standard procedure.

In addition to the elimination of irrelevant studies from the list, we can also focus on the right testing strategy in order to reduce animal testing for the development of new active ingredients. How can we do that without previous knowledge of a compounds toxicological profile? The answer can be found in short-term *in vivo* studies, as well as in a broad set of *in vitro* assays. Such studies can provide significantly more data on different endpoints in toxicology than we get right now by simply following a check-box approach. I believe that toxicologists would then be able to target subsequent testing to those endpoints which are relevant for the compound in question, and consequently waive (i.e. provide a rational for not doing a particular study) those studies which do not address the toxicological profile of the compound.

Today the focus of most of the regulatory studies still is phenomenological and descriptive rather than trying to understand the toxicological profile of a compound. However, times are indeed changing! With development of new *in vitro* studies (e.g. those mentioned for endocrine disruption) and with the increased use of 'omics technologies in toxicology, we are entering a new frontier which is much more mechanism-based. It is exactly this understanding of the toxicological profile of a compound that is essential for a credible targeted-testing design. Consequently, this would require a series of *in vitro* and short-term *in vivo* studies which would indicate not only the general toxicological profile of a compound, but would also aid in understanding which modes of action are playing a toxic role for the compound in question.

For this, it would be necessary to agree on the types of *in vitro* and short-term *in vivo* studies needed (which could include analysis using 'omics sciences (transcriptomics, proteomics, metabolomics), *in vitro* determination of receptor interaction and a good understanding of the kinetics and metabolism of the substance. Toxicity testing could then be focused and targeted. Such a tiered and targeted toxicity testing approach has been proposed more than 7 years ago by the International Life Science Institute (Doe et al. 2006). At that time, however, 'omics sciences and *in vitro* assays to determine mode of action (or adverse outcome pathways) were far less developed then they are now. Understanding this underlying toxicology and using these data as the basis for targeted testing should be one of the major tasks of the toxicological community. As exemplified by the "toxicology in the 21st century" paradigm of the US National Academy of Sciences and the USA-EPA tox-cast program, we are well underway in this direction.

In conclusion, I am optimistic that we can achieve a reduction in animal testing via a multi-layered approach. We should continue to assess the standard study package for its regulatory effectiveness and eliminate those studies that are not useful for compound registration. We should also continue with the innovation of alternative, *in vitro* methods that can replace animal studies. This should include the development and use of *in vitro* studies to identify modes of action during early stages of compound development. Such innovations can also be used to terminate development of compounds that do not have a chance of being accepted by regulatory authorities. Inclusion of 'omics approaches in short-term regulatory studies can be used to understand mode of action in an *in vivo* situation; then, an understanding of all these modes of action can be combined into a single targeted approach for toxicity studies.

With all of this said, what makes work at Wageningen University so attractive to me? A good team, a long tradition of doing excellent toxicological work, and a unique expertise in the development of so called physiologically based pharmacokinetic (PBPK) models has been developed here. These mathematical models are necessary for the transformation of an *in vitro* concentration, usually expressed as µg/ml, into a dose that would conventionally be used in an animal study (mg/kg body weight). For risk assessors, these conventional values are essential; without them it will be close to impossible to replace animal studies used for systemic and reproduction toxicity. Jochen Louisse from the toxicology group at Wageningen University has already demonstrated that such a PBPK approach is feasible (Louisse et al. 2010).

With my work here at Wageningen University, I hope to contribute to the development of *in vitro* approaches to developmental toxicity. I my teaching assignment I will pay special attention to reproduction toxicity as well as alternative methods in general and in particular those related to developmental toxicity. We are in the process of designing a second PhD project, which will include a transcriptomic approach to developmental toxicity. In this project we want to investigate the effect of chemicals on the expression of genes which play a role in embryonic development. In addition we will take a look at the contribution of nuclear receptor mediated effects. We are digging in deep to better understand, at a molecular level, how chemicals can cause developmental toxicity. With this understanding I think that we will be able to rationally design alternative methods that will contribute to reliably identify developmental toxins. I hope that I will be able to give the students a flavor of how the results of basic science can be turned into assays that will have an immediate application and impact on chemical- and pharmaceutical-industry. Within the scope of this work there are several opportunities to work together with other groups at Wageningen University, and I am looking forward to new collaborations.

The ultimate goal is to completely replace animal studies but this will be a long and winding road, with uncertain outcome. I hope that I have been able to give you a feeling of what can be achieved *now*, how animal testing can be reduced and how an understanding of toxicological mechanisms is key to the development of new and better alternative methods.

Now in closing, I would like to express my gratitude to all of the people that have made it possible to help me stand where I stand right now.

I would like to thank Mr. Rector Magnificus for demonstrating confidence in me and accepting me as a new Professor at Wageningen UR. I would also like to thank Prof. Raul Bino for his trust in me and the stimulating discussions we have already had in such a short time. I am particularly grateful to Jan Koeman, for two reasons. Firstly, he was my first teacher in toxicology and aroused an interest in the science that has shaped a good portion of my life. Secondly, because he sent me off to the Germany Cancer Research Institute (Deutsches Krebsforschungszentrum – DKFZ) in Heidelberg for a six-month internship. Well, that was nearly 29 years ago and I still live in the Palatine region. At the Cancer Research Centre I met Dr. Henk Tennekes, a former Ph.D. student of Jan Koeman, my future mentor. He introduced me to the mathematical aspects of cancer research and biology. I stayed well beyond the intended six months; in the end, I did my Ph.D. research there in collaboration with Wageningen UR, and so I too became a Ph.D. student of Jan Koeman. In Heidelberg, I had the good fortune to be able to work with many German scientists who have contributed significantly to the early investigations of the biology and molecular aspects of cancer. I am in particular grateful to Prof. Werner Kunz, who received me with open arms and helped to find my way in the Institute as well as in Heidelberg.

Having just finished my experimental work in Heidelberg, I found myself, to my own surprise, being offered a job at the toxicology department of BASF, Ludwigshafen. My great thanks and appreciation to Prof. Hans-Peter Gelbke, who had sufficient faith in me that I would be able to survive in the chemical industry as a 26 year old, not-quite-yet-Ph.D. scientist. A very special thanks to my first boss at BASF, Dr. Volker Schulz, who introduced me to single-engine aviation, one of my passions, and who has given me the opportunity to start any introduction of myself with the words: “I am a true flying Dutchman”.

The first opportunities to start teaching were given to me by Prof. Gerd Eisenbrand in Kaiserslautern and Prof. Rolf Schulte-Hermann in Vienna. With hindsight, I am now happy to have seized these. With Prof. Dieter Schrenk, we completed the initial courses and turned these into a master’s program in toxicology at the University of

Kaiserslautern, receiving official acceptance of this curriculum by the federal German state Rheinland-Pfalz.

I am honored to be able to expand my work and teaching in academia here in Wageningen, and key to this, without any doubt, is Prof. Rietjens. I first met Ivonne in 1981, during a course in organic chemistry, then in 1983 in the toxicology department where she was doing her Ph.D. research. We met again, only two years ago, when we started to discuss the possibility for me to work here at Wageningen University.

When I look back on how the toxicological science has affected me, I suppose that there are three scientific fields that I will be associated with. Firstly, the potential endocrine effects of chemicals; I serendipitously stumbled into this theme while trying to explain a number of observations of a compound that turned out to be an anti-androgen. Due to the mechanistic toxicology studies we initiated, I met Dr. Leon Earl Gray of the US-EPA who continued and expanded this work and eventually set the stage for what is now generally referred to as "endocrine disruption". Secondly, alternatives to animal studies; at BASF we have focused our "academic" research over the last 10 years on the development of alternative methods. Finally, I have enjoyed witnessing the rise of the use of metabolomics in toxicology. I am more than grateful to the amazing teams at BASF in Ludwigshafen and my dear colleagues at Metanomics in Berlin who have achieved more than I could possibly have hoped for. Also very big thanks to all the other colleagues in the Experimental Toxicology and Ecology and Product Safety departments at BASF; I am proud to work with all of you.

And now, the van Ravenzwaays in Altrip, Germany: to Claudia and my children Kimberly and Valerie. All of you have seen me go to many business meetings and congresses over the entire world. Although I like to travel, it was always best to get back and see you again. To my young ladies, you brighten up my life simply by just being with me.

Then to Milena, I am so glad that you stepped out of your Colombian world and into mine, changing it in your South-American way, adding exotic flavors and lots of salsa.

Finally, I would like to thank Henni, my mother, for all she has given me. I moved to Germany the year my father died. Ever since then, she has seen more than her fair share of the Dutch and German rail- and motorways on the way to the Palatine region where I have been living. Thank you very much for your love and support over all these years.

I have said,

References

Bauch, C., Kolle, S.N. Ramirez, T., Eltze, T., Fabian, E., Mehling, A., Teubner, A., van Ravenzwaay, B., Landsiedel, R. Putting the parts together: Combining in vitro methods to test for skin sensitizing potentials. Regulatory Toxicology and Pharmacology, 63, 489 – 504, (2012).

Doe, J.E., Boobis, A.R., Blacker, A., Dellarco, V.L., Doerrer, N.G., Franklin, C., Goodman, J.I., Kronenberg, J.M., Lewis, R., McConnell, E.E., Mercier, T., Moretto, A., Nolan, C., Padilla, S., Phang, W., Solecki, R., Tilbury, L., van Ravenzwaay, B., Wolf, D.C., A tiered approach to systemic toxicity testing for agricultural chemical safety assessment. Critical Reviews in Toxicology, 36, 37 – 68 (2006).

Fegert, I., Billington, R., Botham, P., Carney, E., Fitzgerald, E.,Hanley, T., Lewis, R., Marty, M.S., Schneider, S., Sheets, L.S., Stahl, B., van Ravenzwaay, B. Feasibility of the extended one-generation reproductive toxicity study (OECD 443). Reproductive Toxicology, 34, 331 – 339 (2012).

Kobil, W., Fegert, I., Billington, R., Lewis, R., Bently, K., Bormann, W., Botham, P., Stahl, B., van Ravenzwaay, B., Spielmann, H. A 1-year toxicity study in dogs is no longer a scientifically justifiable core data requirement for the safety assessment of pesticides. Critical Review in Toxicology, 40, 1 – 15 (2010).

Kolle, S.N., Kamp, H.G., Huener, H. A., Knickel, J., Verlohner, A., Woitkowiak, C., Landsiedel, R., van Ravenzwaay, B. In house validation of recombinant yeast estrogen and androgen receptor agonist and antagonist assays. Toxicology in vitro, 24, 2030 – 2040 (2010).

Kolle, S.N., Ramirez, T., Kamp, H., Buesen, R., Flick, B., Strauss, V., van Ravenzwaay, B. A Testing Strategy for the Identification of Mammalian, Systemic Endocrine Disruptors with Particular Focus on Steroids. Regulatory Toxicology and Pharmacology 63, 259 – 278 (2012).

Li. H., van Ravenzwaay, B., Rietjens, I.M.C.M., Louisse, J. Assessment of an in vitro transport model using BeWo cells to predict placental transfer of compounds. Submitted to Archives of Toxicology (2013).

Louisse, J., de Jong, E., van de Sandt, J.J.M., Blaauboer, B.J., Woutersen, R.A., Piersma, A.H., Rietjens, I.M.C.M., Verwei, M. The use of in vitro toxicity data and physiologically based kinetic modeling to predict dose-response curves for in vivo developmental toxicity of glycol ethers in rat and man. Toxicological Sciences 118 (2010) 470 – 484.

Schneider, S., Kaufmann, W., Strauss, V., van Ravenzwaay, B. Vinclozolin: a feasibility and sensitivity study of the ILSI-HESI extended one generation Reproduction protocol. Regulatory Toxicology and Pharmacology 59, 91 – 100 (2011).

Schrage, A., Hempel, K., Schulz, M., Kolle, S.N., van Ravenzwaay, B., Landsiedel, R. Refinement and reduction of acute oral toxicity testing: a critical review of the use of cytotoxicity data. ALTA, 39, 273 – 295 (2011).

Spielman, H., Gerbracht, U. The use of dogs as second species in regulatory testing of pesticides. Archives of Toxicology, 75, 246 – 250 (2001).

van Ravenzwaay, B., Coelho-Palermo Cunha, G.,Leibold, E., Loose, R., Mellert, W., Prokoudine, A., Walk, T., Wiemer, J. The use of metabolomics for the discovery of new biomarkers of effects. Toxicology Letters, 172, 21 – 28, 2007.

van Ravenzwaay, B., Herold, M., Kamp, H., Kapp, M.D., Fabian, E., Looser, R., Krennrich, G., Mellert, W., Prokoudine, A., Strauss, V., Walk, T., Wiemer, J. Metabolomics: A tool for early detection of toxicological effects and an opportunity for biology based grouping of chemicals—From QSAR to QBAR. Mutation Research, 746, 144 – 150 (2012).

Prof. dr *ir.* Bennard van Ravenzwaay

'Many of the in vitro toxicological studies have not been sufficiently validated to determine their applicability domain, even less have gained regulatory acceptance. Major advantage of in vitro testing today is the early identification of significant hazards in compound development and reduced and targeted animal testing. Replacing complex animal tests may be achieved by a battery of in vitro test addressing the adverse outcome pathway in question. Kinetics models are needed to translate in vitro results into in vivo values.'

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