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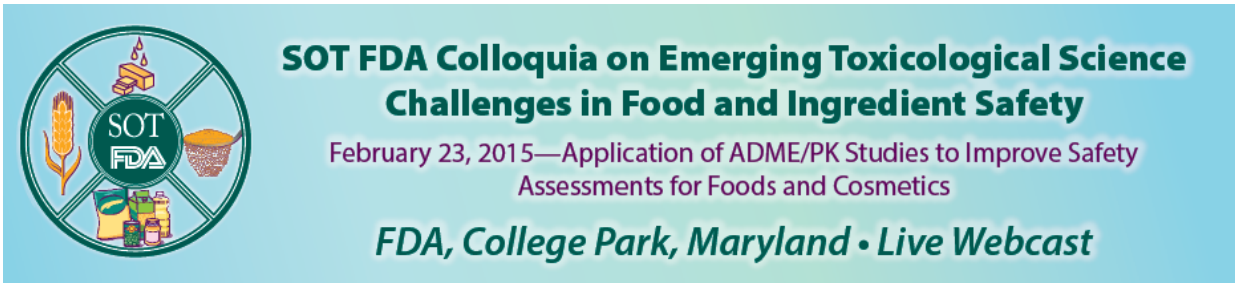

Application of ADME/PK Studies to Improve Safety Assessments for Foods and Cosmetics

Schedule

8:00 am-8:10 am	**FDA Welcome and Overview** Dennis Keefe, FDA, CFSAN, College Park, MD
8:10 am-8:15 am	**Welcome from SOT** Harvey Clewell, The Hamner Institutes for Health Sciences, Research Triangle Park, NC
8:15 am-9:05 am	**Overview: Value of ADME/PK Studies in Safety Assessment** Harvey Clewell, The Hamner Institutes for Health Sciences, Research Triangle Park, NC
9:05 am-9:55 am	**The Importance of ADME/PK to Inform Human Safety Assessments Based on Animal Studies: Example with Furan** Greg Kedderis, Independent Consultant, Research Triangle Park, NC
9:55 am-10:10 am	**Break**
10:10 am-11:00 am	**The Role of ADME/PK in the Extrapolation of *In vitro* Toxicity Results to Equivalent *In vivo* Exposures: Where it Started with the Acrylamide Example and Where We are Now** Bas Blaauboer, Institute for Risk Assessment Sciences, Utrecht University, Utrecht, The Netherlands
11:00 am-11:50 am	**Consideration of ADME/PK in Safety Assessments for Engineered Nanomaterials: Example with Silver Nanoparticles** William Boyes, US EPA/NHEERL, Research Triangle Park, NC
11:50 pm-12:30 pm	**Roundtable Discussion: Issues for Use of ADME/PK in Safety Assessments for Foods and Cosmetics** Harvey Clewell, moderator All speakers

8:00 am-8:10 am FDA Welcome and Overview-Dennis Keefe, FDA, CFSAN, College Park, MD

Harvey Clewell
Good morning. We are going to begin. I want to welcome you to this Colloquium concerning Emerging Toxicological Science Challenges in Food and Ingredient Safety that was put together by the SOT and the FDA. I am the chair for this particular session of the colloquium. The welcome from FDA will be given by myself -- will be given by Dennis Keefe. Timely, very timely.

Dennis Keefe
Good morning all. My name is Dennis Keefe, I am the director of the office of food allergy safety here at FDA. It's my pleasure to welcome you to the second of a series of SOT/FDA colloquium on toxicological sciences, challenges and food and ingredient safety. This is held in the Wiley Auditorium here at FDA. We have a webcast. It's very timely and appropriate that we are in the Cindy Harvey Riley Auditorium, given that Harvey Wiley was one of the leaders on food safety and food ingredient safety, the ancestor of our office of food additive safety. We have a long history in terms of working on food ingredient safety and it goes back to Dr. Wiley. FDA Center for Food Safety and Applied Nutrition is partnering with the SOT to provide these series of four half-day training sessions that focus on cutting-edge toxicological science. These sessions are intended to provide a well-grounded scientific foundation to inform the work of FDA scientists and to stimulate dialogue with the scientific community on issues of importance to the agency. These colloquia are open to the public and offer the public an opportunity to hear the same information that the agency hears. I want to stress that these colloquia are not intended to offer a public forum to discuss or make recommendations to the agency on regulatory issues. They are intended to provide a forum to learn about the latest toxicological science.

Today's session is the second of our four colloquia and it is titled Application of ADME NPK Studies to Improve Safety Assessments for Foods and Cosmetics, featuring the following distinguished scientists: Dr. Harvey Clewell, Dr. Greg Kedderis who is an independent consultant, Dr. Blaauboer from the Institute of Risk Assessment Sciences Utrecht in the Netherlands and Dr. William Boyes from EPA.

The next colloquium is scheduled for April 14 of this year. It will be on immunotoxicity and food and ingredient safety assessment. This will be held also in the auditorium.

I want to make one-a couple of administrative announcements. First for those in the auditorium, there are restrooms up in the hallway as you came in you can utilize during the break. There is a public cafeteria. You have to go outside and reenter the building. There will be cards passed out for those would like to submit questions for the panel discussion at the end of the meeting. There will not be time to answer all the questions. Likely we won't have time to answer the questions but we like to have a lively discussion during the panel session.

It is my pleasure to introduce Dr. Harvey Clewell from the Hamner Institute and Dr. Clewell who will be our moderator today.

8:10 am-8:15 am Welcome from SOT– Harvey Clewell, The Hamner Institutes for Health Sciences, Research Triangle Park, NC

Thank you. It is my honor to be able to welcome you here on behalf of SOT. The Society of Toxicology has worked with the FDA in putting together this colloquium. I just wanted to point out before we begin that the materials from the first colloquium are available now online and so if you want to download those you can use the link that is shown on this slide.

Our schedule this morning is going to be three talks followed by a break and then two more talks followed by a discussion period. I will introduce each speaker in turn. I do want to thank the organizing committee for helping put together this program. You can see here all the people who participated in the decision process and it was long and complicated.

I will begin by giving just an overview of the subject. The value of ADME/PK studies and safety. I will begin by discussing what we mean by that.

8:15 am-9:05 am Overview: Value of ADME/PK Studies in Safety Assessment-- Harvey Clewell, The Hamner Institutes for Health Sciences, Research Triangle Park, NC

Harvey Clewell: First we'll talk about what I prefer to call biokinetics. This study of the movement of a chemical in a biological system and I will give examples of the application of this. In the past it was mostly a matter of doing safety assessments based on *in vivo* data, usually animal data, and so for many decades we have been applying biokinetics that way. Then I will switch to the present and future movement towards alternative testing and how biokinetics continues to play a role, but a somewhat different role. Biokinetics is a term which is more neutral, which is an alternative to the more commonly used terms pharmacokinetics or toxic kinetics which imply that the kinetics of the chemical somehow relate to other effective or toxic dose which Paracelsus taught us long ago is not the case. I prefer to use the term biokinetics although I will sometimes fall into pharmacokinetics because this is a new awakening for me. But the ADME of course refers to some of the processes of biokinetics, the absorption into the biological system, the distribution through the blood and other processes to the various tissues, metabolism primarily in the liver but also in other tissues including testing from kidney, lung and excretion of a given chemical which can be by either active or passive processes in the bile or in the urine. So all of these are the processes that explain the behavior of a chemical over time kinetically. For many years, partly because of limitations of computer capabilities for computational methods, classical compartmental modeling was used almost exclusively and in recent years non- compartmental analysis, which is a way of trying to avoid a hypothesis you might say and just let the data speak for itself, is one way of putting it. These descriptions were useful and FDA continues to use this kind of information supplied by particular pharmaceutical companies to describe the nature of a data set. So I can tell you how quickly a chemical rises to its maximum, how quickly as it cleared of the biological system, the problem with it is that it is difficult to say how that information related, if it is in a rat how that relates to human. Those processes are different. So the maximum concentration in time for clearance would be very different in the human and you don't really know how to predict that. Some time ago, decades ago, chemical engineers in particular began applying a method called physiologically-based pharmacokinetic modeling or PBPK modeling where you use a compartmental description but the compartments are constrained to be consistent with the physiology of the system. So you use tissue volumes, blood flows, you have a physiological structure, where the blood that goes through the intestinal tissues, goes down to the liver rather than communicating directly with the central blood compartment and so those kinds of details are actually extremely important for predicting the clearance of many chemicals. What you end up with, hopefully, is a framework upon which you can -- a test hypotheses and do extrapolations across species and across routes of exposure. So the whole idea is to be able to simulate the *in vivo* transport processes in a realistic system.

This diagram puts together the various uses that have been made of this kind of pharmacokinetic modeling for many years now. As I said, if the safety of a chemical relies on data from animals, usually what you have is a dose that was associated with or not associated with an effect in the animals. Except for drugs, you typically only known something about the concentration to which the person is exposed. You may have a blood sample, biomonitoring data that tell us you what the

concentrations are in a human, so you have the green box in the animal and the red oval in the human. And the problem then is to be able to compare those. The two ways of doing that are either to use pharmacokinetics on the animal studies to predict what the blood concentrations were in that animal study associated with the toxicity because unfortunately even today when studies are done toxicity studies are done in animals it's extremely rare for the people to conduct a study to measure blood concentrations in the animals at the various doses. And there are some who do that quite faithfully, particularly 3M and Dow Chemical come to mind, where they understand the importance of considering pharmacokinetics at the same time that one does those effects studies. But it is rare. The other possibility is to try to infer from the biomonitoring concentrations that are taken from human subjects what the exposure could have been that would produce that. I coined the term “reverse dosimetry” some years ago, many years ago, because you are actually trying to go from the inside out. It is just like dosimetry where you have a dose and you try to predict with the tissue concentration is except backwards. That is a process that can be performed and then you can actually compare across the green boxes instead of across the internal ones. For those of you who aren't familiar with physiologically-based pharmacokinetic modeling, this diagram shows a schematic of what a model can look like. They of course are tailored to the -- the spoke models, they are tailored to the characteristics of the chemical.

This shows a model that would be appropriate for a chemical which was not metabolized but was inhaled like fluorine. Something that you would use as an anesthetic. You have a lung compartment, the blood flows from the lung compartment to the rest of the body and the liver gets about 25% of the blood flow. And then all of the blood again goes to the Venus pool and back to the lung. You'll notice the heart isn't actually given it's physiological place, it is just one of the rapidly perfused tissues in this description. The level of detail or level of accuracy you require for these models depends on their application and what you're trying to determine, what health effects you are going for, what the characteristics of the chemical are and what is important for its kinetics. And so there is not a one model fits all situation. But the purpose of the modeling is to try to define the relationship between an external measure of dose as in administered dose or air concentration and an internal measure of an exposure or dose at the tissue level that hopefully is more directly related to the biological effect. And being able to do this in both the x-bar mental animal and human allows you to then predict the human concentration or dose that would produce the same target tissue dose as in the animal studies where effects were seen. That is the idea.

First example is from methylene chloride. Methylene chloride in the 1980s was a major concern for almost every agency, and it was interesting that a number of different approaches were used in the risk assessments. And about that time, Mel Anderson and I were at Wright Patterson Air Force Base and we were developing a model for methylene chloride because it was a fun thing to do. And methylene chloride and its colleagues, the brief moment of fame, Brummell chloromethane made excellent tools for investigating in a rat and we are happy doing that. Then the EPA came out with a risk assessment for methylene chloride it didn't consider our modeling, which at the time was unpublished. But we convinced the Air Force to let us brief EPA about our modeling, and that actually generated a national Academy of Science meeting where they reviewed the question of is PBPK modeling worth doing? They decided yes it is. Then eventually produced this model you can see here. There is a new wrinkle in this model compared to what I showed you on the previous slide. We have compartments for the perfused tissues which is like the kidney and the brain, and don't have a fat compartment because methylene chloride is lipophilic so you tend to have higher concentrations in the fat so you need to keep track of the time course for loading and unloading. You have to have that compartment separate. These slowly perfused tissues are primarily muscle and the liver. And the liver now has two metabolic pathways because that was what we had determined in the studies in rats, that there is both oxidative metabolism by [Indiscernible] and a conjugated metabolism by transferase and then because the interest in this case was lung tumors for methylene chloride when given by inhalation, we added a compartment which was our way of treating the lung tissue compartment to allow for generation of a reactive metabolite in the lung.

And this model, after a lot of controversy, tempest in a teapot, was used by EPA and OSHA and Health Canada, FDA did not use it at that time, but everybody had different notions about how they would use it if asked and I will show you that in a moment. As it turned out, this PBPK model predicted much lower risk in humans compared to the felt approaches and those related to the scaling across species particularly of liver blood flow which limits metabolism, but also the differences in the rate of metabolism or the metabolic capacity in the animal and the human.

For a while people saw PBPK modeling as a way of reducing risk estimates across the board. It is not at all true. In most cases if you are talking about oral exposures, the modeling will say that the human is at higher risk because of slower clearance. It is actually a fairly neutral objective approach which can go either way. Being at Wright Patterson I had access to people doing all kinds of strange things including operational research so we talked with some people in the zero are department about the fact that we had all these people with different ways of using or not using the PBPK model for a risk assessment or methylene chloride and they put it into this decision tree structure. So what this does is show you that it's not simple to add science to a risk assessment. I like to quote Gary Flamm who was a director at [Indiscernible] back in the 80s. He said you have to take the science and take it as far as it can go. And then clearly state where the science stops and how the science policy came in and what you have done with that. In this case it is still the same thing. Incorporating the PBPK model even though you have incorporated the science there will still be uncertainties that have to be addressed by science policy decisions and so the original or the previous EPA risk assessment used applied dose rather than publish the PBPK model in their defense. The EPA of course by policy uses body surface area extrapolation from animal to human and then use the applied dose in the humans. That gave them a risk about 3.48e-6. When we ran the PBPK model, using the model we did not use a body surface area science policy adjustment. We let the model decide the relationship, which in effect means body weight scaling, using the PBPK model and the glutathione transferase pathway, the modeling actually identified that it was the glutathione pathway that the oxidative pathway that produce the important reactive intermediate associated with the tumors and so then we got a number of 4.38e-8. Two orders of magnitude lower. The EPA cleverly split the difference. They used the model but they put back in the body surface area scaling, and used the pathway and make up 5.55e-7. That actually was what was on IRIS then, and that actually generated a controversy that went on for several years, about should you or should you not use the science policy scaling when you actually incorporate real objective measure of the animal human equivalents. And then the IRIS for vinyl chloride, they did not use the body surface area scaling. This illustrates the problem of the risk assessor trying to determine when you fold in science, how does that affect what you used as a fault approach or science policy in the past? The FDA got a number of 2.75e-7, which is very similar to EPA number because they by default used body weight scaling across species.

Those are just to illustrate the dilemma of the risk assessor or the risk to use new technologies. The second example is methylmercury. It's another chemical that is greatly loved by CFSAN? and over the years -- in this case there was a bit of a battle between EPA and FDA over the right number for the RFT for methylmercury, EPA was adamant it was 0.1 micrograms per kilogram per day. FDA wasn't so sure that was the biblical number, so this is a model that we developed, which has a lot of compartments and it's really not the point here to go through them all but we had data for all these compartments because a pathologist in Japan had measured methylmercury concentrations in all the tissues and a large number of cadavers so we had actually data that we wanted to use so that's why we broke out some of the compartments. We also had to have a brain compartment, which has diffusion limitation because of the blood brain barrier and the only reason methylmercury gets into the brain is because it looks like an essential amino acid, it’s fairly clever in the way. And we also had a placenta and fetus because of the effects on the fetus, and the key for modeling methylmercury is inorganic vapor. Which is then trapped in the brain and the fetus what I wanted to show you with this and I talked about the reverse dosimetry. This is the first time I actually did that. I was working with Kenny Krumpet at the time, a run-on statistician who told me

what I was doing was there. Or correct as he would put it. What this shows is remarkable data from Iraq during the days when they had the poisoning episode. There was seed grain that was used to bake bread. It wasn't meant to be made into bread. It was meant to be seed that you sowed and it had a fungicide in that that was metabolized, I think in the environment to methylmercury and so people started having tremors and paraplegia and things like that. And so they recognized that there was a problem. This is from a woman who was pregnant, and they were able to reconstruct when she was pregnant which is the bar down here. They knew from when she gave birth an estimated time when she became pregnant. They also knew for that region in which the woman lives, when the seed grain became available in the region of Iraq and when they told people -- alerted people to the fact that they should not use of any more. We had an estimate of the duration of exposure and the relationship of that exposure to the time of pregnancy. The only thing we really need to do was to estimate what was the exposure. If you assume it was in bread we assume it was a fairly constant exposure on a daily basis, and so we just ran the model with the information about when the exposure started, an estimate of what would be the exposure that would produce the hair concentrations that were in the mother. When the mother was taken to the hospital they cut off some of her hair and hair grows at 1 centimeter per month and you can measure the mercury in each centimeter segment and reconstruct what the concentration in the hair was and from the hair to blood ratio, between here and blood of methylmercury, you can ask me blood levels. And so we were able then to predict the blood concentrations of the mother once she was in the hospital and you can see that is this curve here and the symbols are the measurements once she entered the hospital, the measurements of her blood as the mercury was cleared from her body and we predicted with the model also what the fetal blood concentration would have been at birth, and that is the line here and these are the measurements of fetal blood at birth. And so the model did a remarkable job of predicting the blood levels in the mother and the infant, and all we estimated was the exposure based on the hair concentrations in the mother. This is an example then of reverse dosimetry of determining a human exposure from biomonitoring data.

One of the things that was also of interest about methylmercury was how much variability there would be in a safe exposure level. That is because the clearance of methylmercury in different individuals varies considerably, and so we were able to put that into the physiological-based model. The variation in tissue weights and blood flows and also the blood-hair partitioning coefficients Kenny Crump did a global analysis of the variability of the uptake of methylmercury into hair, and we ended up predicting this distribution of the ingestion rates. The ingestion rate in micrograms per kilogram per day across a population associated with the exposure in the NOAEL? (derived by US EPA) Iraqi incident. And for comparison the EPA RFD was 0.1. That's because they added uncertainty factors but as you can see it dances about an order of magnitude total, a factor 0.2 on this side. And ATSDR actually used the ratio of average to do is most sensitive individualize a determining uncertainty factor in there MRL. What is an MRL? FDA residue, minimum risk level. So this was another kind of application of the biokinetic modeling. The final example of this more traditional approach is perchlorate -- this is some were traded with Mike Bolger, CFSAN, before he retired where he actually used the FDA food basket to determine the perchlorate concentrations in various foods, and then with CDC we determined they had 2-day recall, food inventories, or estimates from a population where they measured perchlorate in the urine. We used the model to try to see whether the estimates based on food recall and the FDA food basket were consistent with estimates based on the urinary concentration data from the population, Ben Blounce from CDC had conducted that study. And what you see here is the red line which is the individual measurements put in order from low to high across that study group. And then the blue dots are when we ran in Monte Carlo the estimates of the model from the food inventory, and you can see it actually captures reasonably well the population distribution of the estimated exposures. What we concluded was we could at a population level show that the food inventory data did match with the biomonitoring data, but it did not match up very well person to person because we couldn't reproduce the exact characteristics of any given individual so we are creating random individuals and comparing them with one real one.

Time to do a quick switch from the way things happen to the way things ought to be. I work with Mel Anderson and get pretty maniacal working with Mel. It all started with Bas Blaauboer who you will hear shortly. He converted both Mel and I to believing in this vision. And it was good timing because Melvin participated in the NAS committee that wrote the document “Toxicity Testing in the 21st Century: A Vision and a Strategy.” It was a real departure in terms of what they proposed. Switching from *in vivo* assays with animals to *in vitro* assays in human cells. And it was like a galaxy long ago and far away. In the future time not too far away, they expected that we could switch entirely to that. And the question is how long the transition will take. But one of the characteristics of it is the idea of perturbations of specific biological signaling pathways which unfortunately have been named toxicity pathways. They are normal cellular signaling pathways. Just wants one descriptive have been associated with toxic outcomes. And one of the issues, which is still being discussed, is how to define adversity at the cellular level. What is adverse to trying to do it at the cellular level is that it is going to be challenging. And then the key element is to assess the dose response *in vitro* response and equivalent *in vivo* exposures. That is extrapolation and that is what I will focus on now that that is the ADME/PK equivalent in the new paradigm.

It is eight years after the NAS document, as Dr. Phil would say, so how is that working for you? Tox 21 was actually coming up at the same time that NAS book was being written. And originally both Tox 21 and ToxCast had a different goal. Their goal was profiling and prioritization to predict animal studies and not human health effects. Predict animal studies. The animal studies were the gold standard. To relate animal results to humans. Prioritizing with the gold standard and assist in risk assessment, which of course if you are familiar with the EPA cancer guidelines. *In vitro* studies, mechanistic studies into the risk assessment document and never a few to gain. As a dose response study into a calculation oblivious to all mechanistic studies. What I considered to be an extremely telling study was done by a friend of mine, Rusty Thomas, who was at the Hamner, worked with folks that [Indiscernible] to evaluate the predictive power of Toxcast phase one and they determined it was no better than QSR. QSAR. That is not very satisfactory and I've seen other analyses by other QSAR people that showed the same thing. Adding in Toxcast data to QSAR does not improve it and actually nobody is very satisfied with QSAR. We aren't there yet. To his credit, Rusty then moved to EPA to try to see if he could make things work better. So he is now at EPA trying to make ToxCast more predictive. If anyone can do it, he can.

Meanwhile, Mel Anderson has taken it upon himself to try to implement the NAS vision if no one else will, and so we are at the Hamner, working on these issues of assessing it adversity *in vivo*, *in vitro*, and then using computational assistance biology pathway modeling to determine what would be the appropriate *in vitro* point of departure and then using *in vitro* and *in vivo* extrapolation to estimate the equivalent *in vivo* point of departure. I am focusing on the IVIG are working on the computational pathway modeling. And this is a Rusty Thomas future. Eyebolt of that because most people biokinetics is nice to have. In the days of animal studies then you could ignore it and use the folds and goodbye. That actually there is a non- equivalency or if you have any of these draw results it is in terms of media concentration and if you want to go to *in vivo* equivalent he wanted terms of dose or concentration in the air or something which is in completely different units. 'S you have to do some sort of conversion to get to the *in vitro* assay to get to the equivalent *in vivo* human exposure. And develop the term QIVIVE for trying to actually be accurate as one doesn't consider free concentration as opposed to just nominal concentration, and be more careful about if you're going to use it in risk assessment. So this shows the elements of the approach, we don't know how to predict the rate of metabolism of chemicals at this point, so using QSAR we have to use *in vitro* metabolism studies. We can use QSAR for a good deal of other things like partition coefficients and identifying likely target tissues. And then we used the biokinetics model, both to interpret the *in vitro* toxicity assays because the chemical in there is being in some cases metabolized if the cells are metabolically active. Another cases it is evaporating or adhering to the side of the plate of the plastic or to the cellular membrane. It is the nominal concentration is not

necessarily informative. And biokinetics model also allows us to estimate the human *in vivo* equivalence.

As it happened, once Toxcast was first working through their phase one, they came and talked to us, Rusty Thomas and I, about the kinetic issues, and we impressed them with what we felt was the importance of considering *in vivo* clearance and trying to estimate the relationship between the bioactive concentrations *in vitro*, their term, and the equivalent human exposure, and they agreed. And said that they hope someday they would be able to look into that. Rusty was able to get the American Chemistry Council to fund us to do the dosimetry at a cellular clearance and plasma protein binding which allows you to estimate renal clearance, and then we provided that reverse dosimetry. Some agencies now called this reverse toxicokinetics, RTK. Has the advantage of being summarized well in an acronym. So the Toxcast people were ecstatic. We were going to be able to do work that they didn't feel they could take on themselves. This shows an example of what you get. This is actually -- I looked at human studies. We looked through all the phase one chemicals and found all of these that had human studies of kinetics. I used the steady-state concentration for repeated exposure and then we used our simple *in vitro* metabolism, and the fraction unbound to compare with the *in vivo* derived values. And one thing we found was that a typical drug company approach of assuming that clearance is only a free compound doesn't work as well as the more traditional environmental assumption that all of the chemical in the blood is available for metabolic clearance.

But we found that in most cases, the predictions were pretty good, and in cases where they weren't, we could use that term and it was because of either active reabsorption in the kidney or pre- systemic elimination in the intestine. And so the fish in the study, Toxcast remora 398 *in vitro* assays and calculated AC50, and then we did the *in vitro* to *in vivo* extrapolation to get an oral equivalent those and they compared with estimates that they made of human exposures. And what was an eye-opener for them was when they saw that compounds with the same AC50, these are all compounds that happen to have the same lowest effective level, that the oral equivalent those could vary by almost two orders of magnitude, here from 0.26 to 7.5. That is in differences of *in vivo* clearance. If it clears slowly you don't need to be exposed to it as something that is expose quickly. It is a no-brainer when you put it that way. Impress on you that it's really important for these kinds of studies. And so if you actually look at the bioeffective concentration data, you find that almost all the chemicals have about the same bioeffective concentration within an order of magnitude or so. If you look at the equivalent *in vivo* exposure considering the differences in how rapidly they are cleared *in vivo* it is a magnitude difference for the same compounds. As it is important to incorporate this even if it's uncertain, even if your estimate of the *in vivo* clearance is uncertain you are better off considering it and leaving it out, for prioritization, as well as for risk assessment initiations.

One of the key limitations in current *in vitro* testing that is going to be difficult to do anything about is metabolism. Animals nicely do metabolism for you. Not always the same way as humans but they do have metabolic activity and in many cases it is similar if not exactly the same as humans. But *in vitro* assays are much more variable, and the Toxcast Tox21, they are all really primarily designed to detect parent chemical toxicity. Those of us who have worked in the environmental toxicity business for a long time know that all of the people we seem to work with, it is their metabolites that are toxic. You'll miss those unless you happen to have an *in vitro* assay that has the right metabolic capabilities. And so this is a real problem that some people are working on. And particularly, repeated exposure toxicity is very often from a metabolite. So it's difficult to imagine how you're going to be able to do in particular chronic long-term repeated exposure toxicity using high throughput testing. There may be a need to actually allow for some medium throughput testing that allows you to spend a little bit of time on chemicals to determine whether they really are toxic or not.

These are some examples of some popular chemicals where its metabolism is responsible for the critical toxicity and so you would miss it if you used the wrong *in vitro* assay. This is an approach that [Indiscernible] was the first author on this. This was a transatlantic think tank meeting. We talked about how would we do risk assessment under the *in vitro* only paradigm. And there is actually a lot of efforts going on now to rejuvenate risk assessment to make it more up-to-date. This is one of them. The main difficulty is in determination of biokinetics behavior. This means determining metabolism and other factors related to the biokinetics of a compound before doing the *in vitro* test. And so that's a challenge to be able to maintain throughput and I know that NIEHS is looking at that problem now. And then having a concentration response modeling in order to do the *in vitro* and *in vivo* extrapolation. And the -- a lot of the work that we are doing at the Hamner now is trying to be able to estimate *in vivo* dose response curves from *in vitro* concentration response which involves both the *in vitro* and *in vivo* extrapolation and also computational systems biology pathway modeling. And we're going to have a course in September on this at the Hamner for those of you who are interested. We will look at both the PBPK modeling, IVIVE, and CSDP modeling.

This is an example of work I've done with arsenic. You can see the dose response for interactions of arsenic with T genes in the growth regulation of oxidative for stress, DNA damage something an immune response. You can actually use genetic information, this is different individuals, different symbols for individuals. Look at not only the dose response for the interactions of a chemical but also how it varies from individual to individual and this is getting easier and easier to do with the advent of the induced for pluripotent stem cells. Rusty Thomas put together a proposed risk assessment approach using *in vitro* data as much as possible and having *in vivo* studies as a backup when necessary. It's fairly complicated and I will not go through it now but I recommend that you look at Thomas 2013, listed among the references on the last slide.

But one of the things that is going on now is people are starting to think ahead to if we were going to use *in vitro* data as a base for risk assessment how would we use that? There are new challenges that have to be dealt with. One of the things that Rusty has spent a lot of attention on is to determine whether genomic studies can actually predict the effects of chemicals compared to *in vivo* data. And the data shows a comparison of *in vivo* and *in vitro* oral equivalent doses, which shows that the *in vitro* data tends to be conservative compared to *in vivo* data. The chemicals that they can compare.

And there is a second tier in the Thomas paper that looks at following up with *in vivo* studies for problem chemicals where you can't resolve everything *in vitro*. And this is another comparison showing how will a benchmark dose based on transcriptional pathway responses, genomic responses actually, predicts the lowest typical benchmark from a traditional toxicity assay. So this works remarkably well. This is what Rusty Thomas did when he was at the Hamner. Thank you. [Applause] And the references are in the talk if you want to look at them. Questions?

Audience Question:

Answer: It is problematic for a complex system to pick out any element and expect that it works independently. I think that is something that you would have to take the whole as it were and so I do think that you have to look at the interactions between the parameters. They are running a Monte Carlo analysis, allow the parameters to vary within a reasonable distribution. It is the only way to make that comparison correctly. And there are a lot of trade-offs between Metabolic pathways where one pathway has decreased activity and then another pathway consumes more of the material an action is SIMSIP is a great and a huge number of [Indiscernible] SIMSIP to go through a lot of those principles.

Audience Question: This is great. Thank you for your presentation. But I'm still saying the same [Indiscernible] extrapolation, animal to human without realizing the species difference in metabolism and pathway .

Response: It's in there. I didn't have time to get into that, if you want to come to the course at Hamner we go over that the absolutely. There were significant differences betw rat and uman in therm sof enzymes that metabolize biochloride and so just 2E1 in human but all 2C16&17 examples by metabolize and the animal data was I would like to say they should call PK modeling metabolism models to do it properly and you have to rull out things that might be true, like active transfer which can differ ifyou look at the PFO8, reabsorption in the human but not in the mouse. It is definitely not simple. And has to be done with a collection of metabolic data in the different species.

Audience Question: That makes the Toxcast system, understanding where humans fit in without understanding what are the metabolic pathways, what are the active metabolites in humans, taking the overall approach doesn't work, shouldn't work.

Response: I happen to agree with you and so does Mel Anderson that is what we are doing, we call TT21C, we're trying to develop assays for particular toxicity pathways that use human cellular data and human both in terms of response and in terms of metabolism and then we try to put together a suite of assays that will work for a particular endpoint or concern. It is not as potentially high throughput I think eventually there will be a meeting around this, the high throughput testing. Toxcast can be used to flag things and to prioritize which is what they really originally had in mind for to do. But it may be instead of prioritizing for the *in vivo* test in an animal you might be able to prioritize for *in vitro* testing in human cells to answer the questions of human toxicity. That would be our goal.

Audience Question: You are talking about the mercury poisoning episode in Iraq. I wasn't aware of that. I was well aware of the benzene poisoning episode in 1953. What year did the methylmercury happen?

She can answer that (referring to female in the audience).

Response: I was just interested because it sounds like Iraq has had multiple grain poisoning episodes.

What happened there, they treated the rice. I think they did it with the methylmercury underside. And they put a water-soluble food coloring and let the people know that shouldn't eat the red rice. And they found that they could wash it off.

So, they thought it was no longer the red rice. And they fed the rice to their cattle and sheep. Very terrible. It would have to be in the 70s. I was a grad student at Rochester. That was the group that took the human blood samples.

It is tough to live in Iraq for many reasons.

I think we should go to the next speaker.

I would like to introduce a friend of mine. Greg Kedderis, preceded me at CIIT by many years, I remember visiting him, he was an expert in metabolism an PBPK modeling I think his case study of furan is thoughtful research trying to answer questions about the toxicity of the chemical and how pharmacokinetics works in.

9:05 am-9:55 am The Importance of ADME/PK to Inform Human Safety Assessments Based on Animal Studies: Example with Furan--Greg Kedderis, Independent Consultant, Research Triangle Park, NC

Thank you, Harvey and good morning, everyone. I'm going to talk today about some studies on furan. I have no conflicts of interest. The work was conducted as Harvey said when I was on staff at CIIT and it was funded by CIIT core funds, which is basically a pool of funds that is then applied to research programs decided internally with some external guidance.

What is furan? Why do we even care about it? It is a prototype of a large group of chemicals. A lot of furan containing chemicals provide flavors and aromas in various types of food. Not furan itself, but furan containing chemicals, Found in canned foods and in coffee. Used in some synthesis and some chemical synthesis. It has also been found in things like smog, cigarette smoke, and even spaceship air. I mentioned that because to get this research funded, I had to do quite a sales job, and they did find it in the Mars spacecraft. Why would you find this in the environment? It is actually a semi-aromatic molecule. So this is really a stable rearrangement product. And that will come into play later.

What was interesting, and NTP did a bioassay, and I believe some of it is thinking was the parent compound of the whole large series of furan containing compounds. They found it was fairly potent carcinogen in rats and mice. And the doses are noted here. At the same time, in [Indiscernibl] they had developed an assay for DNA repair -- excision repair and genotoxic -- chemicals that interacted with DNA, that the more in some way repaired. And when they looked at furan, having some preliminary results from the bioassay, they found that there was no DNA repair, excision repair whatsoever but what they did see in bioassay doses were huge patches of perforation and there was hepatotoxicity taking place under the bioassay conditions. At the same time [Indiscernible] who was at NTP at that time did the supporting genetic disposition studies with furan *in vivo* and once again it was a remarkable result. If you're following carbon-14 and these molecules he didn't find any DNA-binding whatsoever but extensive protein binding. From a point of view of hepatotoxicity there was hepatic necrosis taking place under the bioassay conditions. From a more interesting point of view it is a mid-zonal hepatotoxic and it begins in zone two and spreads into zone three. This hepatotoxicity requires bioactivation and creates sustained cell proliferation in response.

So in my laboratory we were looking for compounds -- our hypothesis was the furan was driving cell proliferation. We didn't understand how that was happening. I will give you insights later. And the actual objectives here are to understand the relationship between administered dose and the dose and the liver. Bioactivation transformation kinetics both *in vitro* and *in vivo*. And hepatocytes and going back to *in vivo* you -- the *in vivo* study. Where *in vivo* studies. This was done in collaboration with Dr. Mike Artis who was at CIIT at the time. We developed a PBPK dosimetry model as shown in the diagram and we had metabolism taking place in the liver. This was described in [Indiscernible] and we wanted inhalation with this keenly volatile compound. Inhalation was clearly the route of exposure because there are all kinds of conflicting studies in the early literature because people try to do *in vitro* assays with this molecule and it basically evaporated. You really couldn't get any kind of meaningful results. With whole rats enclosed in a chamber in our study, so we have the rats in a chamber of known volume, and expose them to an initial concentration and measure the changes in the timber concentration over time. And we use that data to inform our PBPK model. We estimate of four bioactivation and that we did an experiment where -- technically quite challenging where we made predictions with the model and then try to take tissue samples from exposed rats under the same conditions for four hours in a closed chamber and that we took them out of the chamber and as quickly as we could harvested blood and liver samples and put those in closed vials and measured the furan concentrations. What is shown here on these graphs is the predictions of the model, the data points are the actual data that we measured and there is some variability here, but it's really you notice it gets bigger later time

points. It is really from evaporation of furan. This was a fairly reasonable job of a model in predicting both liver and blood concentrations. At the same time underlying this, I had a hypothesis that freshly isolated hepatocytes could be a quantified reasonable model for chemicals that were primarily metabolized by the liver *in vivo*. Think about this hypothesis obviously if there was a lot of metabolism in extra hepatic tissues than hepatic metabolism by itself is not going to describe that.

What we did to study furan was this is a cartoon that I drew, but it actually physically we had to come up with a system to study this. What we used were 25 Erlenmeyer flasks with a screw top, and a silicone membrane so we could sample the headspace concentration. Then we developed a two-compartment model. Measured in air and in the media and the media that contain inactivated cells. 'S and predictive partitioning between the headspace and the medium, and then in the medium, the hepatocyte suspension. The disappearance inside the medium would be due to metabolism. When we used this kind of model, this is the kind of data that we would get from isolated hepatocytes suspensions. These are different starting concentrations of furan and we measured this over time. The lines are the fit of the model to this data, and little crosses are the actual data points.

To extrapolate, the *in vitro* rates to *in vivo*, at least for these studies, the whole principle really came from enzyme kinetics, which is what one of the areas of expertise I've gained from my thesis work, and enzyme catalyzed reactions are directly proportional to the total enzyme that is in the system. Simple little equation of the parent velocity. So what is the total enzyme? Proportional to the number of cells either in the liver or the cells that you're using *in vitro* so we know there is approximately 130 million hepatocytes per gram. The actual numbers vary slightly between species so what else you need to know, the overall enzyme metabolism and most enzymes for many cytochrome P450 reactions follow Michaelis-Menton kinetics. That is where the PBPK model comes in. And we did tabulations. These are the numbers we saw from our studies *in vivo*. There is a fairly low and this is metabolized exclusively by P450. And the values are similar when we extrapolate that to our average 250-gram rat. So this looks good but what is more impressive is when we take -- this is now a chart, this is not the same chart that I showed you before. But this is the same type of chart. This is the closed chamber data from *in vivo*. And in this case the lines are the predictions of the model using the genetic parameters that we determine from hepatocytes suspensions *in vitro* and it fits the data very well. This opened the door to then doing what we were really trying to get at and that was not so much rats the rats, mice, and humans. This table summarizes some of the studies that we did. And you can see that the mice also metabolized furan very rapidly. And we managed to obtain at this time, back in the 1990s. We obtained fresh human liver samples and prepared human hepatocytes suspensions. At the time I was associated with Duke University Medical Center and Dr. Randy Journal helped us obtain these livers. Basically, we had three human samples and we were dumbfounded there was a huge difference. In Vmax here. And oddly enough, these humans, two or three, both lost their lives in car accidents that involved alcohol. A very well-known inducer and that is the explanation the human Vmax value between the species and this individual variability.

We started with his kind of information, ask a more general questions. What is the dosimetry of furan to the target organ after a four-hour exposure to temperature million? We chose that as a reference concentration. When we look at that and this table shows rats, mice, and humans using genetic parameters from previous tables, we are defining a lower dose here as inhaled minus exhaled. Here the absorbed dose is actually pretty different. And that leads to quite a different exposure of the liver to furan metabolites, which take into account both the physiology as well as the biochemistry and the bioactivation. It turns out that this type of difference and absorbed dose is pretty will predicted by [Indiscernible]. The human dose is nine or 10 times lower than a mouse does. Four times lower than for the same external exposure. And that leaves us a new(?) question. So what is the dose? Barely the exposure concentration not be does. In our simulations here we exposed three species to the same external concentration and got three different internal doses.

Clearly as a mentioned there are differences in size. Bringing in various metabolic rates. We could measure the difference in P450 rates. At least in mammals, smaller animals have a higher breathing rate. That is going to mean your inhaled gas into the animal more rapidly. And other things need to be considered. When it reached that saturation point you are -- your metabolism can only go so fast.

We wanted to look back again about the consequence of this interindividual variability seen in three human samples. What is the actual delivery of furan to the liver? These are simulations, using the same parameters. We look at the blood concentration of furan and between the three species it's really quite similar. And they have different liver perfusion rates. We calculated a rate of furan oxidation for the three samples. The thing to note in this table is that the perfusion and rate of furan to the liver is at least an order of magnitude lower than the rate of oxidation or metabolism once it gets to the brink. Clearly there is a blood flow here that is essentially suppressing the effects of the interindividual variability due to exposure to agents, or inhibiting agents.

In this chart is another simulation, looking again, using human genetic parameters, I just took the lowest Vmax and simulated and unrealistic induction Temple. And there is only one line, but two sets of data points, Vmax and 10 times Vmax, and this is a functional concentration of furan for four-hour exposure and basically there is no -- you can get a 10-fold induction of P450 and entirely metabolized to the richest liver essentially has no effect.

The practical effect when you back out from a risk assessment point of view is to suppress that obvious interindividual variability. That is pronounced with cytochrome P450 and now we have that hepatocytes were accurate models for the bioactivation of furan and we wanted to develop in an *in vitro* system where we could study the mechanisms involved in furan. That is critical -- one of the first things that happens. The toxicity is going to lead to cancer. The first thinking about this, the usual approach and it's very easy, to do these kinds of *in vitro* experiments and come to the conclusion that the systems don't work. You have to think about what are you trying to do! If you are trying to generate tables and tables of data to publish somewhere, you can do that. But if you want to generate data that has a context of *in vivo* relevance, I think there are a few things you need to consider. This is just a thought slide. With orally administered hepatotoxic things like chloroform, acetaminophen and furan, a lot of the scenarios look like this. You administer the chemical, the concentration goes up to [Indiscernible], and these compounds tend to be rapidly metabolized and the majority of metabolism excretion is over in six to eight hours. Where is the toxic effects usually are not manifested until the next day. If you look at the livers of these animals, you don't see growth of hepatotoxicity 12 to 24 hours. Because of that, than was a case of furan we had our PBPK model and we can start asking questions about dosimetry comparing *in vitro* with *in vivo*, and that is what is shown in this table. Looking at both the high bioassay dose an actual macro genic dose, vary hepatotoxicity those and some of the *in vitro* doses we were looking at and you can see probably the important column here is the amount metabolized and this is just micromoles and four hours. And you can see that some of these *in vitro* concentrations are giving similar amounts of toxic metabolite as we are predicting would be happening *in vivo*. What we did was essentially exploit the metabolic capability of freshly isolated hepatocytes and the longevity of the cells placed in culture and develop what we call the suspension culture approach. We incubate the hepatocytes suspensions with the toxicant and these concentrations and incubation times are guided by the PBPK dosimetry model and after the incubation we place the cells in culture to express toxicity at 24 hours. That is shown in this chart here, where the cells are exposed to furan here, then they are washed, placed in fresh media, and allowed to attach to college and plated petri dishes and cultured for 24 hours, and the measurements are here. The exposures are back here, no more furan, and we make the measurements here. You can see in our chart this is looking at viability as a function of incubation time. We are getting both time and concentration dependent cell lethality of furan, and we can identify from this experiment no effect level of one micromole. You can see two micromolar we start having a fax.

Again, I don't have the time to show you the detail of all these subsequent studies as I did in the initial development of the system, but we showed that there is no adverse effect level is one micromole *in vitro*. And that bioactivation required bioactivation by cytochrome P450. We could prevent lethality, we could prevent [Indiscernible] depletion with P450 inhibitors and all the studies we did we constantly went back and forth between *in vitro* and *in vivo* to make sure we were using *in vitro* conditions that reflected what was happening *in vivo*. So this was the initial thing, cell lethality, that has been noted *in vivo*. What we found was that very marked it area of ADP depletion. And this would indicate mitochondrial dysfunction. This is in the paper where *in vitro* and *in vivo* we isolated mitochondria from treated hepatocytes, treated rats, we looked at oxygen uptake and found that what was happening as there was an irreversible uncoupling of phosphorylation and that was very early about that happened *in vitro* as well is *in vivo*, follow the same dose response curve. We keep using P450 inhibitors as our tools. We are using final him it is all as our preferred inhibitor *in vitro* because that is not specific, very specific for particular isoforms and it's a very potent and dead and inhibitor. And it is not very toxic to cells. *In vivo* we could use another tool, we did this *in vitro* that is a suicide inhibitor. It binds to P450 and gets metabolized and destroys the active site and wipes out the P450. We could pretreat animals and isolated hepatocytes. Treat hepatocytes but it takes time for this to happen. It is more effective if we treat the animals. The ATP depletion and uncoupling was prevented by P450 inhibitors.

What are some of the consequences? Clearly we have uncoupling and ADP depletion. Any process like the calcium pump that maintains calcium concentrations in the cell, going to be compromise. Calcium can go up and that can activate -- that is part of the signals that are involved in cell death activation of protease is an and nucleus. And we have found that there was an activation that led to DNA double strand breaks. In hepatocytes as well as in livers *in vivo*. This is the mechanism that the summary of the mechanism here where you really started from the beginning here. External exposure, looking to bioactivations for this metabolite. And this is the kind of thing, it is very reactive, it will react preferentially. And this leads to irreversible uncoupling and all of this has a no effect level. Then we hypothesized as I say that the decrease in ATP leads to endonuclease activation that could lead to DNA double strand breaks if you get way too many of those. Along with a lot of other damage to the cell you get cell death. That is leading to the cell proliferation. You can get repair of these double strand breaks and survive and maybe nothing happens or you could get erroneous repair and if that happens that could be a way to propagate mutation events. Without a direct interaction of the chemical with DNA. And so we had done some subsequent studies that remain unpublished. But it leads from a risk assessment point of view of what is all this information and how do we take this into account? I have said that -- this is not a statistical conclusion. This is the mechanism of toxicity. It observed no adverse effect level. *In vitro* and *in vivo*. Why not use that information in risk assessment? There is clearly, I hate to say the word, there is a threshold. Below that threshold, the adverse effects aren't happening. There may be some other things happening. That everybody has ignored our work of conclusions. Health Canada has continued to do studies with furan. I think they have similar concerns to the FDA of the occurrence of furan in canned foods and so on. And they have done -- they did a series of 90-day studies and began using oral lavage and corn oil. I didn't highlight before, but they identified no adverse effect levels in both rats and mice. I just want to say something at this point about the [Indiscernible] oral lavage a formal them. That is not an appropriate route of exposure for two reasons. Oral lavage, that is a bolus, that is not the way -- that's the way you drink shots of whiskey. Harvey and I were thinking about this last night, what is the equivalent of giving a rat an oral lavage of corn oil? It would be drinking like a juice glass full of corn oil. Everyday. I don't know who would be able to do that?

An interesting follow up by the NTP, this was just published a few years ago and this was a study that was in the works for quite a while and then they basically did -- came back and did another bioassay where they looked at lower concentrations to try to see if they could identify a no effect

level in a two-year bioassay for furan carcinogenicity. They actually found one, they found an extremely nonlinear increase in both toxicity and tumors and no adverse effect 5 milligrams per kilogram and I will come back to that in just a minute. Back to the mechanism, what are some of the things we can use or what would furan be doing if it's not directly interacting? Aside from actually killing the cells, what about the other things? There are several recent studies and I think these are collaborations between Integrated Laboratory Systems and Health Canada looking at both global gene expression and I highlighted here this, and off our 2 family of genes that are critically involved in inflammation, and thought to be a part of oxidative stress pathways but inflammation is huge component of carcinogenicity because it occurs at all the dose levels, and in the bioassay.

The other thing that they found that I think is really intriguing changes in what is now called this used to be called nonsense. Now they call it non-coding RNA. These are RNA that have regulatory functions, they are coded by messenger RNA and they don't actually code for proteins but they have regulatory effects on cells. And so these may well be potential epigenetic markers to consider not just for furan but for chemicals working through inflammation, working through these non-genotoxic mechanisms.

Let's get back to NTP data because I think this is important. I display that data from their publication here. And so these pink squares here are the total adenomas and carcinomas. And the dark diamonds are carcinomas alone. These are our controls. And B6 three -- [Indiscernible] there is always something for controls. You can see that really nothing is really starting to happen until about 2 milligrams per kilogram. This is a lifetime bioassay. And then you get a markedly nonlinear increase in this. So here once again it is not just my conclusion from our biochemical work, you've got bioassay data here showing we use that threshold word again, but there are clearly levels of furan that do not produce excess tumors.

There's another problem of furan when you try to consider the context of our meeting here, I call it problematic dosimetry of furan in foods. When I started working on furan I thought this must be a natural product found in all these things. There has been some pretty good work published recently through the USDA, Dr. Fan and others have found that the furan is formed in the processing of foods. It is formed by heating foods in closed containers during pasteurization processes and it's also -- he looked at irradiation of foods and it's also formed in a radiation. He did a series of studies and showed it is formed from sugars, ascorbic acid, and from fatty acids and good fatty acids. And the thing that I think is extremely intriguing is what is not found -- if you take apples or vegetables on things and make slices, thin slices of fresh, it is not there. Him clearly, the exposure -- potential for exposure from eating prepared foods but this is a compound with a volatility that is similar to Diablo either. This stuff is -- as you are heating -- it's very hard to imagine what the dose of furan would be from eating foods that they are heating to eat. There may be some there, but it's being produced in variable amounts. The other concern, this is a concern, infant food. It's all little cans and emphasize the sweet potato food, which I'm sure babies really love that, and the concern is here's this event and the diet is pretty much this little canned vegetables and stuff. But the thing that I think needs to come into play here is infants do not have the total P450 capacity of adults and they certainly do not have the metabolism of adults. And so there are data in the literature on this. We could take somewhat of a more quantitative approach to that kind of thing. I haven't tried to simulate infants in this particular -- with this particular chemical and model. If they cannot buy or activate the furan, I'm not sure that anything significant is going to happen. In our animal studies were we had pretreated animals with Neil Banzel try is all to wipe out the P450 and that we dose them with furan, those animals acted like they were drunk. They were wobbling wrong, they look like animals on phenobarbital and according to the Merck index, I have never inhaled furan vapors, didn't want to see what it's like, but according to the Merck index, furan vapors have a narcotic property. Without it was amusing or confirmatory of this anecdote that when we stop by our activation rats the rat appears to be intoxicated. It doesn't get liver toxicity from -- once he does not have a P450.

I will end with just acknowledging the people I work with. Mike Curtis who helps with the initial *in vivo* modeling. My two postdocs. My technical staff, and especially the families of the liver donors. We couldn't have done this kind of work without people donating tissues. I will stop there and be glad to answer any questions. [Applause

Audience Question: [Indiscernible - low volume] thank you for the very interesting presentation. I have a question on hepatotoxicity with respect to furan. On one of the earlier slides, you had a reference to protein binding and how that contributes to toxicity. Looking at the data that you have shown I would believe that it is the plasma protein binding of metabolite. That contributes to toxicity.

The parent compound is inert, it is a semi- aromatic molecule until something happens, it is just -- [Indiscernible]

Do think there is a chance that this plasma protein binding of the metabolite to be a limiting step or has not been shown here?

Kedderis: There is extensive protein binding that has been shown and a follow the radioactivity studies. Clearly, irreversible uncoupling that we found we did not measure protein binding but I'm sure that it's a consequence of an interaction with the [Indiscernible] was some component of the transport chain. It is a difficult molecule but we are handling this and bags of a person handling it that way. We were not -- we inquired about getting radioactivity, I think Tom may have synthesized that molecule the carbon-14 molecule himself. 'S he did those studies in the early 90s with the C14. We did not have access to that and when I thought about the potential for contamination and so on with radioactivity, I didn't think we needed to go there. So I am inferring that the protein binding is responsible for some of those effects. Clearly there could be other effects on the cell. I'm sure the cell toxicity, not just one push one button, press two buttons, there is clearly a myriad of things going on all at the same time but when you start doing things like depleting ATP, in the first hour, that seems like a critical event.

Audience Question: Just a follow-up on your theory of the ethanol being what was responsible for the differences in the human sample. Have you tried dosing your mice or rats with alcohol?

Kedderis: I didn't have time to show that. I should have had that on the slide. We used acetone as a means to them to use cytochrome P450. We did studies with phenobarbital and that have a slight effect, but it was really the acetone that made a huge induction for the metabolism.

Audience Question: We will talk about the uncoupling of [Indiscernible] and ATP depletion and hepatocytes. Given that most cancer cells are thought of using aerobic glycolysis for energy metabolism, have you ever looked at the effect of furan untransformed has had on carcinogenic cells and see if it has similar effects on --

Kedderis: We have not done that and I am not aware that anyone has done that. People have looked at furan although not -- certainly not looked at the uncoupling. I don't think so. People have looked in transformed models, mostly like *in vivo* mouse and kinds of things.

Audience Question: The reason I thought it would be interesting to look at it if furan has an effective killing off normal cells is through uncoupling [Indiscernible] and let's say you had a very limited effect on cancer cells or cells that are grown under aerobic conditions would do if that is way to select for those populations of cells not using the [Indiscernible] as major metabolism. That would lead to selection of cells.

Kedderis: I see what you are driving up but the transformed cells may have also had a decrease P450 content and it is not the furan, then it is the furan metabolite. You have to really -- know what the metabolic capability of the cells were and I think they tend to be lower than normal cells.

Harvey Clewell: Thank you. [Applause]

I believe now we have a break coming. I want to apologize for not telling those who are listening over the phone or web that you should ask questions using the Q&A box. You will know better than I do what I mean. Do we have any questions? Let's keep those postcards coming, folks. We will take a 15- minute break. Do you want to make it 20? We are starting at 10:10 so we are actually ahead of schedule. See you at 10:10. Please be back probably at 10:10 so we can continue to be on schedule.

9:55 am-10:10 am Break

Welcome back. We are ready for the second half of the session. And I would like to introduce one of the leaders in the development of alternative live animal testing for quite a number of years now, Dr. Blaauboer has been leading the area of biomimetic and metabolism in the development of alternative methods. He recently retired from the University of Utrecht, so he is the Emeritus Chair on alternatives at the Institute for Risk Assessment Sciences. He is also editor of *Toxicology In Vitro* which now has an impact factor of 3.26, coming up, it shows that people are paying more and more attention to *in vitro* methods so that is a good thing. So, Dr. Blaauboer will talk about the role of ADME/PK an extrapolation of -- *in vitro* toxicity results exposures and he will take you back to kind of the beginning of things with acrylamide and then bring you up-to-date where we are now.

10:10 am-11:00 am The Role of ADME/PK in the Extrapolation of *In vitro* Toxicity Results to Equivalent *In vivo* Exposures: Where it Started with the Acrylamide Example and Where We are Now--Bas Blaauboer, Institute for Risk Assessment Sciences, Utrecht University, Utrecht, The Netherlands

Thank you very much, Harvey. Thank you very much for me being able to speak to you today, it's an honor. I am, as Harvey was pointing to, I would like to bring some history in this talk, not only because the two letters that are in front of my name now, Emeritus, mean that I am history.

But, I really hope that I can contribute also in the future a little bit to this field. So also, I have to declare that there was no specific interest, conflict of interest in what we are doing, all of the research has been carried out at Utrecht University and partners. I do not claim that under the partners are not in this -- industry partners but that's how it is, we work together, and how it should be.

An overview of my talk. I like to come up with the development of how we are trying to introduce risk assessment schemes on the basis of nonanimal methods. That's more or less the work that I have been doing over the last 35 years. And, one of the first thing that I was involved with that was, I think, a good story to tell is the ECITTS program of acrylamide, amongst other compounds. And so, I will dig a little bit deeper into the acrylamide story, and then, I will come up with a number of other examples of integrated testing schemes, other examples of reverse dosimetry, and I like to start with a number of possibilities of fees, the limitations of this, and some of the pitfalls that I have when you four.

In vitro toxicology. This is one of my favorite slides. You know, *in vitro* means in glass. You can see it in toxicology, because the mouse, you can see its eyes, it has drunk all of the whiskey in the glass.

I think this is what we are talking about. Of course not. We're talking about other things when we're talking about *in vitro* things. Probably, we should talk about in plastic. And, I would like to start this story by telling you about the history of the group of friends from different European countries, but call themselves the European Union Research Group for Alternatives in Toxicity Testing. Michael Bowls, Andre [Indiscernible], I think also in the beginning, Marcello [Indiscernible] were members of this group. Eric Varland, not to forget. And I joined the group I think in the beginning of the 90s, and one of the most crucial things I experienced with this group was a workshop that was organized by Eric in Sweden in 1981. At that time, we had a little stories about the huge amount of compounds that needed to be tested, and all kinds of high production volumes, teens. -- Schemes, letter. And there was a lot of pressure on animals at the time, and as a group we decided that -- am I doing that? And, okay. We, as a group of friends, decided that it would be probably helpful if we could come up with a sort of, let's say, parallel testing strategy. So, if all of the data would come, and fortunately, many of these data are not coming up because all these are interrelated, etc. And so we said, well, if these data are coming, this *in vivo* data, and we could also look at all of the other schemes, like the NTP, for instance, we could probably come up with a parallel scheme and see how we could predict on the basis of *in vitro* design -- data on the basis of other things.

And, we said we would try to probably come up with prediction systems *in vitro* for as many endpoints as possible, or practical. And, from the beginning, we concluded that it was absolutely necessary to include biomimetic factors. Actually, a little story in between, this group of friends, I was working at the time with hepato-science, like Andre, so somebody had to cover the field of hepato-toxicity, and what was left was a biokinetics. So, just by coincidence, I dived into the field of biokinetics and I'm stuck with it, I'm afraid.

So, we said, well, what methods are available? At that time we had a lot of methodologies that were focusing on neuro- hepato-, renal-, immuno-toxicity as well. And then we said, how can we use these as well with the kinetics, to come up with good predictions of toxicity *in vivo*? That was the starting point of it. Well, this is one of the publications out of this. And we said that I think as a conclusion, it was the introduction of the scheme. I think it was the first time we use the phrase integrated toxicity testing schemes at that time. As we said, from the information produced, which contributes to the establishment of a scientifically based and efficient toxicological assessment based on the knowledge of mechanisms of toxicity and kinetic parameters. We talked about hazard assessments, I think it was much better to talk about risk as well, because we were trying to predict doses.

We started the program just by selecting a problem. And we said, well, we know a lot about neurotoxicity, one of the groups involved was the group of Eric Varlow, and later taken over by [Indiscernible] in Sweden. And, they had a good *in vitro* testing strategy. We selected a group of compounds, and we performed *in vitro* neurotoxicity testing, trying to find the most sensitive parameter of this. And then, we used the lower effect concentrations as a point of departure for reverse dosimetry and on the basis we take later the *in vivo* dose that would result in the effect observed in the *in vitro* systems, more or less like Harvey described to you early this morning. The acrylamide case was chosen as an example. You know the story of acrylamide, I think the *in vivo* data are well known, it is causing specifically toxicity at the peripheral neural system, and hind legs are involved in the first place at higher doses, etc. We ignored the genome toxicity of the compound at the moment and just focused on the neurotoxicity. Could we predict the neurotoxicity of this compound? And, this is what we found as the most sensitive parameter in a series of neurotoxicity testing systems *in vitro*. This is the so-called [Indiscernible] cell system, human, which if you tested

in the *in vitro* system and start making extensions of neurites, and I think that's what they called differentiation, right? And what happens if you expose these cells to acrylamide you see concentration and exposure time dependent in the decrease in the number of neurites personnel, and people more clever in mathematics could come up with, let's say, a formula that runs out of this which is, giving the relationship between the concentration and the time and effect. And, on the basis of this, we built the kinetic dynamic model. You see, I'm from Europe, I spell modeling with two L's.

This is a kinetic model, it's very simple. It takes into account the uptake and possibility to measure risk, and we took the distribution of it, just because this is a water-soluble compound and the volume of distribution is 0.67 of the body weight. Right? That's what happens with water. We had *in vitro* data on the metabolism, so we could, more or less, describe the concentration in *in vivo* situations on the basis of this over the course of time. And let's say this formula came out of this advance curve from the other end, and, this is the concentration affecting the relationship like what you see here, there is a lower amount of effect. And, we just talked about the effect of concentration, more or less the lower effect, if you lower the dose, your first the symmetry and I should use the mouse. And calculate the dose that is responsible for this amount of effect, and to come up with predictions of lower effect. We took literature on acute measurements, and took studies on people that were studying starting effects, which is just being able to [Indiscernible] I'm of course joking about it, that study. Interesting even how we could extrapolate this way out of the exposure, the time of the *in vitro* system, and 72 hours. Up to 90 days, and you see that there is a very good prediction of the *in vivo* effects, just on the basis of this simple calculation that you can do. I think this is the first example of *in vitro*/*in vivo* extrapolation, and we made many of them, some of them were very successful, like this one, and some of them are less successful, for instance, the one that was on [Indiscernible], we were way out, by that time we were way out, and we now know why. Because then, if you find failures, you can use your physiological knowledge, you can use other things to see where you make the failures. And then, to predict better on the basis of your extended knowledge. So, this is how it works, more or less. On the basis of this, we developed a sort of, let's say, general scheme in which this type of activities could go on. We need to build kinetic models. On the basis of, as much as possible, *in vitro* data. And you can do that. On the basis of QSAR or even better QSPRs, you can predict most of the, let's say, passive diffusion processes operating. And, the drawbacks are those processes that are active, then we have, as you can see, it's in progress. We can see many things, not only in the uptake, but also the expiration, that is the role of transporters in these processes are better understood and can be implemented in PK modeling is all. So, the basis of *in vitro* kinetics, also on metabolism data, like record shelving in the earlier talk this morning, we could probably build a kinetic model. At which time predicts the target tissue concentrations in the system. And of course, for the time being, we have to compare it with the kinetics of an *in vivo* system, however it is, how good it is, and how well it predicts the tissue concentration. On the other hand, we have the possibility to do *in vitro* data on the dynamics, and I'll tell you a little bit more about it in the rest of the talk, later on. And from there, together with the target tissue concentration you can predict the dynamics, and finally you can predict the system toxicity and for the time being, we need to compare this with *in vivo* systemic toxicity data. As long as we don't have enough trust in these systems, we need to do this. In the future, hopefully, will be that we can leave out this part of the scheme so, what we have is *in vitro* experimental data on the left hand, the modeling part is in the middle, and the evaluation or validation, whenever you want to call it, part, is on the right-hand side. Liz's edits end here 4-15-15

From this, this the attention of the health Council, this program. And, they formed a committee, and I was a member of the committee, together with [Indiscernible] Crewes and Victor Hahn, probably people that you know, and they came out with an interesting report in 2001 that you can find on the website, if you go to this website, which describes what we would say toxicity testing approach. And I showed you a number of figures out of that publication, this is one of them. You cannot read it, that's why I am large part of it. And, you can see here, what would be introduced were a number

of issues here. We introduced, knowing the property of the compound. Does that give the rights to, let's say, first signs of where you need to look? And the other side is, from the beginning of the compound, exposure scenarios, maybe on the basis of this, you can conclude that the relevant potential exposure is probably in the range of the TTC concept. Which then, probably, will stop your whole scheme. If there is a relevance potential exposure that is relevant in terms of toxicity, then you go on. I cannot show you the whole scheme, let's look at in this publication, the next part of this scheme, again, you cannot read. And the last part of it, you see that both of the basis we take into account looking at these dynamics, *in vitro* methodologies, etc., and on the basis of this you could probably come up with a scheme for which I described, and then come up with this. So, this is more or less a stringent scheme that is as flexible as well, because you have ins and outs, decision points that are very helpful, I think, in terms of the way in which system -- systematic integrated testing schemes will be built. And then you can see it probably works for such things as simple as acrylamide. And, then we have a more complex system. One of the studies that I was involved in, and which resulted in a PhD work of [Indiscernible] together with [Indiscernible] University, a complex system, it was of reproductive toxicity of glycol ethers. For this, Johan used the stem cell culture and used the mouse is outlines, and, you can see here, the development of the system that was used in his *in vitro* measurements, and this is the so-called cultures which develop as a hanging drop of cells, so, if you put these cells together, they just sediment in the bottom of the drop. In which then means that you get embryonic bodies. We culture these and if you put them in a culture plate, then if you do the right sticks with them, you can have them differentiated in either cardiomyocites which are actually dosing together, if I was more clever with the computer, I could show you this, in this figure here. Or, to the normal cells or to osteoblasts, etc., there are number of possibilities here. I think that we should, I think Harvey also mentioned this, the work with stem cells especially induced the stem cells, has a huge future in this field. I will come back to it later, because it involves the other programs that I was about to describe to you that we also used. So, how does this work? So, we built a model for this, and mind you, we're talking about compound here that need to be metabolized before it was acting as a reproductive toxicity compound, and, these are the alcohols that come out of this. So, you can see here, they metabolize -- computer, this is the PBPK model for the compounds, and these are the ones that metabolized. What we did was, we equated the law concentration of the mother, with the concentration in the fetus. And, that is, later on, we could confirm that by measurements. That were found in the literature. So, the input is the effect on the embryo stem cell, the test, and then if you use the reversed a symmetry, either to the oral exposure, or to the [Indiscernible] -- Anheuser dust inhalation exposure, you could come up with exposures that would be responsible for the effect. Well, we have to check the kinetic model, that is what we could do, in *in vivo* data with my. I think it was nice, I'm not sure. And here, you can see a study that was done with repeated dose exposures of the glycol ethers for, let's say, hours per day, and this is the predicted exposure, the measure concentrations are very well following this. Four the [Indiscernible] and for the compound. So that works. On the basis of this, Johan Louisse benchmark approach in the *in vivo* system, and with the help of reversed symmetry -- dosimetry to a response relationship, so the whole carboys translated. And, here, you can see, .2 different approaches, houses fits -- how this fits with the *in vivo* data, on the embryo toxicity of these compounds. For two of the glycol leaders. So here again, and attempt which really shows you the value of this disordered approach. A couple of warnings. And also, Harvey talked about this, one of the things that is probably well absorbed in the use of systems, like the program is that the use of the call nominal concentrations in *in vitro* systems. By critics *in vitro* are essential. We also talked about it because you have the possibility that your compound evaporates out of the system and very fast, other things that are happening is that they stick to the plastic, and, you could also have a completely different binding to proteins, because the protein compound is different from what you see *in vivo*. So, what actually is important to do is to measure preconcentration, because that's easier, than, for instance, the concentration in the cells, but which would actually like to know is the concentration of the time. So, how do we do this? Well, there is a publication impressed that you can find it for quite a long time, because it's part of a special issue in toxicology. This work will come out soon. *In vivo* /*in vitro* extrapolation and in our Institute, there is a publication, a good

overview of this, in which he compares the different situations. If you talk about the nominal concentration, you just take the amount of a compound that he put in the system, divided by the volume that is for normal concentration. You can also measure the total median concentration, or the freely available concentration, you can tear the black dots are what are directly measure. You could measure the cell concentration, or, the target concentration. So you need to know what you measure. You need to know how to do the measurement. Well, for the free concentration, you have the number of nice tools now in the form of what we call solid phased micro extraction. I cannot spend much time on this because of time, but that is absolutely a possibility to do. It does not make your system larger, but we need to have scientifically sound analysis, not just figures, right? The other thing is that you can measure a lot of things in *in vitro* systems. All kinds of curves you can make. It is up to us, as toxicologists, to pick those dose effects out of it that are really relevant, and even then, if we pick one, then we have the possibility to make a distinction between no effect and a little bit effectiveness, probably through the adaptation processes. And, the reverse effect spirit and here again, we need to come up with good solutions for this. This is not solved, yet. We are not in the possibility to come up with all of the data answers that are given with the possibility to do this. It's easy if you are having an effect and use this as a point of departure, and, well, take, let's say, safety factors into account, again, but that is not what you want. We should probably be much more precise and have the possibility to do this with all of these systems. I have seen a range of very good publication, among this, Dr. Handler, that are focusing on stress pathways and how they are influenced, what are the key events in these stress pathways? What are the molecular initiating effects? How does this result in well-defined biomarkers that you can pick out of the systems? And use it. The other thing that I have to warn you about is that *in vitro* systems lack the possibility of the feedback loops that you see *in vivo*. Here again, I think there is a challenge for people that are mobile , because what you see then, as a possibility, is to use the *in vitro* data, and see what is the result of one *in vitro* system, seeded into a computer model, as in effect on another *in vitro* system, and come up with a scheme on how this would work in the entire physiology of the organ -- organism. That's the future. But, for others to do. Emeritus, right?

Will then have the point of departure, as I said, it's quite easy if you have, let's say, a point of departure that is probably related to a lower effect level or a real effect level. It is, I think, possible to come up to more precise things in this area to the lower effect. You have seen this, this scheme, I think, is extremely important. It comes from a publication that we did as a group that is called the transatlantic think tank on toxicity testing. Let's see, Thomas Harden is a member, Marcel Lyst, and bring together groups of people in the workshops, and this is the result of such a workshop. And here, you see, let's say, the extension on the schemes as I showed you before. Talk about exposure assessment. Maybe that is not a risky evaluation. And know your literature. On the basis of structural properties, you could probably read across these other compounds that are giving you a risk evaluation that is possible. But, much more important is that these structural properties probably will ask to give you help in the selection of the proper *in vitro* testing that we use. Don't forget the evaluation of the biogenetic behavior of the compound, also in selecting this. From this, you can come up with *in vitro* concentration response curves, then you have to do the modeling, and then you have a point of departure for QIVIVE. That is, more or less, what we came up with as a possibility. And, this is practical, today, we can use it on many examples. We can even include the variability. Because, if you have a point of departure, then you have that point of departure, and other physiologically based variability that we know of in the human population that you can use the risk assessment. So, you can come up with probabilistic risk assessments, also on the basis of this, and I think Harvey show that a little bit already.

But one more issue that I would like to raise. many of these things that you have seen the literature on *in vitro* systems is short exposure. So, one of the challenges is repeated doses. One of the programs that I was involved in which is quite successful, and there is a special issue of toxicology *in vitro* coming up on this, was to predict IV program, *in vitro*. Which was a systematic approach on

exposure to up to 14 days of *in vitro* systems, taking into account the kinetic compounds of *in vitro*, and all of the possibilities that what we would have in finding the dynamics of the compounds.

A couple of words. What is the difference between acute and repeated dose toxicity? And, I think the -- there are a number of possibilities. One of which is the concentration of the test compound might change. You can have accumulation over time, which we found in our project, number of examples from this. You could see that there is a changing of the metabolic rate, and I think that Greg also showed that the metabolism might change in your *in vitro* system a lot. Which is also reflecting what is happening in the *in vivo* situation in certain cases, but you need to know this. I skipped this because of the time. This is one of the publications that came out of this. It's published in Proteomics, but this shows you an example of one of the compounds, and it is on the cyclosporine aid. Here you see the kinetics, this is one of the compounds in which the concentration is high and have a repeated dose, then you have a accumulation in the South. And, that describes exactly what is happening in the *in vivo* situation, and the box around it are related to the block concentrations that you could find off of such dosing. So, the cellular concentration is coming up, and that is predicting what's happening with the toxicity of these compounds after repeated doses. Use it.

Another project that we did, and this was a program that we together with the Netherlands, and this was a program that we did with a sponsorship of the long-range initiative. The idea behind this is, how far can we get in having a look at the QSARs of compounds, the structural activities of the compound, and, *in vitro* data, and IV/IVE to come up with predictions of the toxicity. So, we use the systems like DEREK, TOPKAT the OECD toolbox to predict what would be qualitatively the endpoints. We found that if you compare this with the *in vivo* data, we found that we missed a lot. Because we did not take into account the metabolism. So, we could also come up with predictions of the metabolism of these compounds using Meteor, which is related to DEREK, or again the OECD toolbox and then feed these models back into systems like DEREK, TOPKAT, etc., and then have a mouse -- much better choice for your *in vitro* systems. The next thing we did was by literature data on *in vitro* systems as we were predicting the toxicity of these compounds and we are now in the process of doing the IVIVE, and I continue that those compounds were spot on. For other compounds, we are way out. And now it is going to be interesting, why are we play out? So, this is a more simplified system, also, difficult to interpret. And over the next two days, I'm afraid, Harvey. But, this is a project. Well, coming to the end of my talk, conclusions. If you develop, let's say, nonanimal based into beta testing systems, you need to take into account the *in vitro* bio kinetics. What's happening with your compound in the *in vitro* system? And especially for the Toxcast people, do this, please, it helps a lot. And of course, the kinetics are also interesting in the *in vitro*/*in vivo* extrapolation. What happens, then, is that you can build integrated with very much -- image importance are also -- you can show what you are doing. I once described, let's say, the old *in vivo* data as pushing something into rats and financing the end effect, and what is in between is a black box. What we have to do with these *in vitro* systems is use the real meaning of the word *in vitro*, transfer it. Probably, I would say, that we can also make optimal use of all of the typical, biological and physiological knowledge, to build the PBBK models to come up with QSARs for certain effects, but the cellular biology which is developing hugely over the last decades, not to forget the systems biology and the "omics," as we have. And, I've seen lately a lot of examples of this coming up. It's not for nothing that, let's say, the impact effective toxicology *in vitro* matter that much. And, we have to do this. We have to deal with so many compounds but we cannot do this with the animals anymore. Not from a point of practicality, not from the point of view of ethics, not from the point of view of the fact that I think that we can put much more science in and the result is that we use less animals. Thank you very much for your attention.

Clewell: Thank you, Bas. Questions?

Audience Question: Thank you, Bas, as usual, wonderful presentation. I just want to discuss something with you. I think the emphasis on high screening the number of chemicals may not be the right approach. I think that maybe for the environmental chemical that is important, but not for tracking development, where you go in one candidate on a time. I think that the really important approach is really understanding how to predict accurately human toxicity of a substance.

Response: I agree with you, but, let's say the answer is not with high throughput for the end result, but it is probably a very good tool to come up with, let's say, priorities to test more high priorities than others. That is, I think, what is meant from the beginning. But, what you see is that people are using these data and try to interpret them in a way that is probably not optimal.

Audience Question: Right, yeah. Just one more thing, and then I will walk away. We have *in vitro* and animal data *in vivo* that do not translate into humans. Yeah? And how, for instance, what is toxic in animals are non-toxic and humans, and vice versa. I think we should really concentrate on those examples and put our energy to it, and then ask the question, how do we, using *in vitro* systems, especially human *in vitro* systems that allow us to better interact animal data or better use *in vitro* data.

Response: I agree with you completely. And can see especially in *in vitro* systems, we see the use of stem cells from human origin, and you can see, well, you can build the PBBK models on the basis of what is happening in the human, so that's what's happening and I think that is the future.

Audience Question: So, I want to talk a little bit about the extrapolation down from the range where we see things in our experiments to actual exposures. And the data that you showed on acrylamide and some of the early glycol data, I noticed that the exposure is more in the millimolar or hundreds of micromolar range, and I'm wondering when you try to extrapolate down lower, curves, it did not seem like much was going to be happening in the low micromolar range. And I'm wondering, which endpoints do you use to extrapolate which you decide are not usable to extrapolate further down to the ranges where we really want to know things are happening, where people are exposed, especially in food and contaminants.

Response: I think for acrylamide, the *in vitro* data in millimolars, but let's say here, we're talking about, let's say, this is quite low here. I would say. So, we dared to do these extrapolations in the lower doses. I think so. But, you are right, the glycol leaders, if you look at the concentrations that are responsible for the effects of the metabolites in these cells, we're talking about many molars. And probably, -- millimolars, and this is probably talk about the action of these compounds because they influenced the internal pH of these cells which is probably the basis of the toxic effect as wel .

The other thing is that there endpoints were a longer exposure is a valid way to extrapolate down into the range, and other endpoints.

Yeah.

Where they are not. and a think it's worth inking about when you report out the data like this, which kind of end. you're dealing with.

Yeah. And this is also related to, let's say, the predicted mechanism of action again. For instance, in the case of the glycol leaders, desk leaders and we decided that, let's say, that the best *in vivo* parameter to take into account is the peak concentration of the compound, whereas in another example in the same study, we did it with slated acid, we need to go into another area of the curve because the effect is much more related to the accumulation of facts, also, which is also the case, by the way, in acrylamide. So, thinking, that's important.

Do we have any Q&A? On the Internet? Any other questions? Thank you, Bas.

Should I just stay out of this?

I'm learning. All right, our final speaker for this morning -- oh, that's not the one. Oh, did you -- oh, no, you are right. Sorry, sir. Okay.

Our next and final speaker is Dr. Bill Boyes from the EPA national health and environmental effects laboratory, research laboratory. He's had a long and productive career at EPA, establishes himself, in particular, as an expert on neurotoxicity of solvents, looking at more acute effects. And, I understand that he also worked with acrylamide at some point. But, most recently, he's turned his attention to everybody's favorite new threat, engineered nanomaterials. And so, he's been doing some really nice work at EPA on silver nanoparticles and he has agreed to talk more broadly about the challenges associated with doing safety assessments on nanomaterials, and how can index is still an issue.

11:00 am-11:50 am Consideration of ADME/PK in Safety Assessments for Engineered Nanomaterials: Example with Silver Nanoparticles--William Boyes, USEPA/NHEERL, Research Triangle Park, NC

Thank you, Harvey, for this part of my checkered past. And thank you to the FDA and SOT for facilitating my participation today. I'm happy to be here. I think what you're going to hear from me as a less-developed stories and heard from others because nanomaterials are really just entering into this type of consideration for looking at biodistribution and *in vitro* to *in vivo* extrapolations, and so there are a lot of issues that we're still going to have to solve. Let me go ahead and say, I was asked to say that there are no financial conflicts of interest and also because I work at EPA have to say that what I am saying is not EPA policy.

So what is now technology? There's a whole new science and technology creeping things on a very small scale, nanoparticles are typically defined between one and 100 nm, and it is one dimension part of being in that range, so, a nanometer is 10 to minus 19 eater, so if you think of the beach bopping about a meter, the Earth is about 10 to the seven 7 m, so . the other way, it gets you to the scale of what we're talking about below those, something that are very small, 1000-fold were 10,000 fold. Why are we doing this? Well, because as Richard said, there is plenty of knowledge, a lot of interesting science and applications for small size materials, when they get that small, they have different and unique properties, many of which are very useful for a variety of products. One of the more important principles is that you get a very small mass per surface area ratio, large surface area per mass, if you say it the other way around. So, since most reactivity happens at the surface of these particles you can get very active materials for using a very small amount of them. And so, that is one of the things that is most interesting. But, they have a variety of other applications as well. This is a graph from a recent report by the Pres.'s Council of advisors on science and technology, showing the anticipated growth in our economy, and the world economy, basically, due to nano-enabled products, and you can see that the curves are increasing, hopefully exponentially, and, people expect this to be a major driver of economic development. Both in Europe, the US, and Asia and throughout the world. So, these things are coming and they are coming in a big way. And we need to be ready to evaluate them, so that we can achieve the benefits of these products without causing undue risks. And that's the challenge that lays before us. another thing from the Internet, this was a couple of years ago when I do this, but, this is a website that has commercially available products for manufacturers that may want to incorporate them into their products, and just to show you the variety of materials, carbon-based, nanotubes, fullerenes, graphene, particles made of elements like silver which we are going to be talking about, silver, compounds, metal oxides, complex compounds with materials with different surface characteristics, the quantum dots, etc. And so, large numbers of listings of silver or different products and

formulations. so, it's really an enormous and Brownfield, and trying to keep up with this is not going to be easy. So again, they have a high surface area per mass which relates to high reactivity. There is concern among toxicologists at this high reactivity could lead to high or inadvertent toxicity. If you have a highly reactive particle in contact with biological tissues. Early on, we thought that their small size could enable them to distribute widely, flowing right through potential barriers or other tissue barriers, it turns out, this may not be as big of a concern because they are also very sticky, they adhere to services, to the cells, and so they are not as bioavailable as we thought early on, although some are, and if you're using these as drug development vehicles which has impacted the research, they are trying purposefully to get to cross biological membranes to get into tissues than normal drugs would not. So, another challenge for the toxicologist is that -- [Background noise] I'm having the same issue. Evaluating these materials requires tools that a traditional toxicologist has not had in terms of physics and engineering and other techniques that we typically don't carry around. And so, it requires a multi-investigator, multidisciplinary approach to evaluate them in a realistic way. So, these drugs are developing rapidly and it's going to exceed our capacity to test these materials in a conventional way. It's not me. Okay, I thought I was standing too close to something. People logging off?

Anyway, random development of these materials requires an approach from the toxicologist, and so we are going to have to consider alternative approaches *in vitro* to *in vivo* extrapolation and pharmacokinetics, pharmacodynamics are going to be really important in evaluating this new technology coming forward. So, I think of nanomaterials as a perfect example for what was being considered in the publication that Harvey mentioned, toxicology testing in the 21st century. They are growing so fast, we can't test them one by one, we need new approaches. And so, we are thinking that their behavior will likely depend on their unique and inherent chemical and principal properties. And so, we can understand those and how those cause the materials to interact with their environment, then we can become productive. And so, transformative science will rely on the development of predictive models, based on how we think that these materials will interact. With the environment, and with the tissues in a biological system. So, we're not there, yet, but this is the goal, become productive in these transformative models. And, I'm going to have a sip of water.

So for the past couple of years I have actually been coordinating EPA, and so wanted to do a little bit of time on our intramural program before we get more detailed in the pharmacokinetic issues. So, some of our goals are to develop test systems that are adequate for evaluating in a materials, identify their current critical parameters that determine their behavior and determine how these behaviors influence their biological activities in an adverse outcome path scenario. So, we are interested in the effect on the environment, as well as in the tissues. So, we have a component of our program, looking at release of materials, and their fate and transport from products, and for this, we have taken a lifecycle approach where you look at the pristine manufacturing, engineered nanomaterials, how they get put into products, how those products get used, and where they end up at the end of their life cycle. Do they release nanomaterials in the process? And importantly, what happens to them? Are they transformed? These are small, highly reactive molecules are very unlikely to stay in their original state in a complex environment. And so, what a person or organism in the environment get exposed to is likely to be different from a particle that came out of the box. How do we understand the transformations, and what do they mean in terms of their activity? And then, once you get into either a human or ecological system, we are interested in understanding them at a molecular and subcellular level, with a variety of different potential outcomes and the organ and tissue level. Or for populations of ecosystem species. What happens to potential populations dynamics if we are attracting individual numbers like that, on the population. So, more focused and Nano silver, silver has a lot of really exciting uses, and I've listed a couple of misuses, in my terms. Most of these are based on antimicrobial properties, and silver is a very effective antibiotic, it's effect against viruses and fungi as well, and so, many of these applications are based on it having antimicrobial functions. Fabric coatings, inserts and socks so that they don't smell that when you wear them, surface coatings for areas that you want to keep free of microbes, like, let's

say bathroom coatings, services and things in the kitchen counters, things like that, great disinfectants, children's products, I mentioned plush toys already, chew toys, other things that children may be coming into contact with, electronics, probably not because of the antimicrobials, household appliances, there was a product of clothes washers that had silver fragments in it, to disinfect your wash, EPA -- disinfectants for FDA, medical wrappings and devices are a big use of nanosilver to prevent infections in the past hospital settings, primarily, also used in food packaging's and food supplements, considered, and then, on the misuse side, on the Internet there's a whole range of people that are selling homeopathic colloidal silver for you to eat, drink, inhale, put on your skin, supposedly to keep you healthy, FDA, to your credit, has been on the record since 1999 sing these products are not safe and effective as used, and they can have substantial side effects, the most dramatic of which is argyria, if you eat enough silver or drink enough or put it on your skin, it will actually turn your skin blue. There are some really amazing Internet videos if you go on YouTube and look for blue man, some of it is a dance troupe, some of it is a guy who took a lot of these and actually died his skin and cornea of his eyes blue.

Other side effects, absorption of drugs can be impaired and possible toxicity to multiple organ systems, liver, kidney, and nervous system, in particular.

So, a little bit about the EPA program, as a city are looking at -- as I said we're looking at release of products, I copperheads of assessment of these, just a few papers as examples, so, we looked at the release of silver from children's products, this is a collaboration with the Consumer Product Safety Commission. So, what we found was that silver does come out of these products, primarily when they come into contact with liquids, that can help with silver ions, including sweat and some of the most important factors. Fabrics, plush toys, like teddy bears and things like that, and spray products were most likely to cause exposures, and in most cases, the silver ions dissolved into ionic forms and that facilitate the exposure to children. But, overall, the levels of exposure were fairly low. So, probably, even though we could document exposures the concern was not that great. Silver T-shirts, they transformed in the wash. So, when you wash them in a detergent, particularly the chlorine-based detergents, you can change the silver in the fabric, it forms silver chloride, silver sulfate, phosphates, ionic species, it's a complex assortment of silver, pre-and post-wash, so, any risk assessment of the change in materials during uses going to be complicated by the relate -- the really large number of silver species are present. By the way, there's a list of all of the researchers, if you're interested. And the low pH in stomach fluid will alter silver nanoparticles and cause them to form silver fluoride, primarily. They can also cause them to aggregate so that you have different sized particles upon exposure to them. Let's see. So, this study looked at HepG2 cells and tissue culture and did a genomic and protein analysis on them, the effect of them, they showed up of silver nanoparticles and silver nitrate which would be a pure source of silver ions activated the same cellular express response pathways and inflammatory pathways. They found that smaller sized particles were more potent than larger particles, this was often reported in *in vitro*, and we'll talk about that later. And so, because they saw the same stress response and inflammatory pad activated, the near collision was that the influence of nanosilver, probably to regraded silver ions. In contrast, this study of Daphnia aquatic species showed exposure to nanosilver, looking at Janine -- gene expression profiles, they look to nanosilver from ionic silver, and they came to a completely different conclusion that the nanosilver had a unique toxicity from that expressed silver on. They also had different toxicity outcomes than the pathways activated by nanoparticles tended to involve protein metabolism and signal -- whereas silver nitrate was more downright related in development of sensory systems. So these two illustrate the ongoing debate about whether there is a argument for silver particles, whether silver particles act as a time released capsule for silver ions and other mechanisms. So, in 2012, the EPA published this comprehensive environmental assessment that looks at a lot of issues, you know, it's a nice reference if you want to look it up, it's available on the EPA website. They tend to describe more things that we don't know and then things that we do know, but it is a well-considered evaluation of the literature.

Right now, I'd like to dive down a little bit into the pharmacokinetics of silver. There was a group in Switzerland, Bachler, I think, is how you pronounce his name, who has developed a very nice PBPK model of silver and publish it in the open access journal nicely so that I could have access to their graphics for the talk today. So, they developed models for ionic silver and nanosilver, and for humans and for rats. And interacted the PBPK models together through dissolution and precipitation. There are papers that show that silver ions can auto conglomerate in the body to form silver particles, and they validated them with toxiokinetic data for literature, and inhalation exposures. This shows a structured model, for ionic, silver and now nanosilver, there likely December there are a couple of important differences. They model ionic silver going into the brain but not coming out because of the blood barrier, whereas they had a two-way pathway for nanoparticles over, and for nanoparticles, they featured the mononuclear phagocyte system where the phagocytes will involve particulate matter including silver and close if there, or facilitate ingestion in the crisis all. And there's no component like that in the ionic silver model.

So, simulations against data from the literature, these are rats treated orally for 28 days, and, the data from -- is in the letter squares, the model in the darker squares, and so, what you're seeing is that the model fairly accurately replicates the data from the animal studies. A different study here, looking at silver nanoparticles , again, the model looks pretty good at the different tissue distributions, if they model this data without the formation of silver nanoparticles from silver ions, when the rats were treated with cellular acetate, you can see that the model does not work very well but, when they allow the silver to reform into the silver particles in the model, then it gets a much better fit for the data.

They also modeled data from humans, with the human model, these are data from deceased human volunteers, tissue donations and the exposure for these people would have been simply through their diet. You can see the levels here are much lower than for the other people. And again, you get a fairly good distribution for the model predictions, against the measured values in human tissues. These two patients were burn victims that were treated with silver nitrate to prevent infections in their burn sites, and so, the levels are very high but again, the model does a good job of predicting that. And, for occupational workers with inhalation exposure to silver, again, the model has shown that.

So, some interesting things that came out of the modeling exercise, they found that particle size, service charge and coding really did not have much of an influence on distributional. And, they postulate this was due to oxidization -- optimization of the plasma, opson proteins which happens naturally because of the attraction of the particles for the proteins, and the functions to help either take particulate matter to cells where it can be absorbed for nutrients, or to have been excreted through the [Indiscernible] system. But, these opsons form a corona around the particles that can stabilize them in their suspensions. They found it was more likely for silver nanoparticles to be formed as an insoluble salt particle, primarily silver sulfide through interactions that have to be dissolved into free silver ion, and free silver ion is likely to be toxicity dust toxic in species. they found no significant evidence in the model for dissolution and that was a major factor in the *in vivo* studies as we talked that. and then finally, they found that the mononuclear phagocyte system was a really minor component of the human exposure, primarily or principally at the lower levels of exposure. So, kind of interesting results from the model.

So moving now into some more *in vitro* models, there is a lot of things that you have to consider with nanoparticles, so, there is important to have good physicochemical characterization of the particles, both as they come out of the box and as they are present in your immediate suspensions. Most of these are not readily soluble, and so, they form suspensions and not solutions and suspensions can be very unstable unless you take good efforts to develop stable suspensions. Largely, this can be done by adding serum proteins that will stabilize the material, and other

factors, depending on your application. It's important to characterize the stability of the suspensions over time, sometimes if you don't take care of it and you dump a bunch of non-soluble particles into your media, can you can watch the silver out. So, it can be a very unrealistic type of exposure. And particles tend to conglomerate, they stick to each other and other things, and so, what you put into the media is not what would be present in the media after some period of time, due to either agglomeration or transformations that happen naturally in that suspension. And finally, many *in vitro* assays rely on luminance detection, fluorescence detection or colorimetric assays, particularly MMT assays as a standard used for *in vitro*, and because you have these optically dense particles in the suspension, you can interfere very greatly with your detection of these light-based signals. And, if you add in a concentration -based fashion, you can more and more particles, you get more and more interference, so it's important to evaluate your assays to see whether or not you are getting interference due to the presence of the particles. But a lot of people don't do that. Those metrics. -- dose metrics. We've had some discussions on this already but with particles it becomes more complex. Most of the studies report on mass concentration, micro grams per milliliter, when you add it to the well, some people are reporting it as master service area, because of the ability of the particles to settle out and it is important how wide the well is, so how much material you are adding per service area of the well. Some people are recording the particles and number of particles that are added or that get exposed to the cells, those are looking at the surface area of the particles, which is likely the reactive surface, so they are the dose of particles is the meters squared per gram but you have to adjust that for the volume of media that you are adding it to. And then, those are all applied dose, but as we've seen, I'm going to talk about this more in a minute, the delivered dose is important. How much of the material that you had actually gets to the cells, and do estimated with the model or mention it? So all of these considerations are on top of the normal *in vivo*/*in vitro* extrapolations, [Indiscernible] being a good surrogate for the tissue that you are interested in.

We have heard about ToxCast, and they have evaluated a number of materials, or merely the ToxCast is interested in standard materials but they have evaluated whether you can see that these approaches are applicable to engineered nanomaterials. so, what they have done is to look at a variety of nanomaterials, and put them into the ToxCast assays, they selected a range of materials and sizes and structures, and then, they are trying to evaluate these as a function of class types of nanomaterials. So, this analysis is still underway, but I do have some to show to you. These are some of the nanomaterials that were tested, five samples of silver in the nano size, and two of the dispersion media, larger silver and microsized, and one that was ionic silver salt. We also looked at asbestos, gold, nanotubes, since cerium, copper, silicon, titanium and Zinc, so large number of these, a large number of outcomes that range from low effects at DNA, RNA, all the way down to whole animal studies in zebrafish. So, a lot of range of outcomes. And so, this is the data [Laughs] I hate it when people show heat maps. But anyway, in this map, blue is more active, yellow is less active. Along the top. Are the different materials that were tested, gold, silver, these are dispersion media, copper, etc., and each one of these along the bottom is the individual designation for that particular part. and these are groups of the outcome, we show stress, oxidative stress, immune responses either go up or down, and in the tissue model, so basically what you can see here is that the activity is in this region, here and here, and these are associated with silver, nanoparticles, copper nanoparticles and zinc nanoparticles. These are the three vendor tend to be soluble and generate toxic elements, the others are less soluble. so briefly, the bioavailability of these materials was in the one to one hundred microgram per will arrange -- range of dosing, and some are more active than others, and the rest of the data is still being analyzed.

So, I'm going to talk a little bit more about some *in vitro* studies, how my doing for the time? Good? So, in our labs, my lab, we are looking at particle size and coatings, trying to look at how you measure cellular uptake and distribution, the measures of cellular dose, so for reasons not related to silver, we're looking at this human derived retinal pigment epithelial culture, APRE-19 cells, so we spend the particles in the culture media so it has proteins to stabilize it for 24 hours and then

evaluate uptake in toxicity. So, these are the particles that have evaluated, 10, 50 and 75 nm silver covered coated with PVP or Citrate, the supplier of nano composites noted that the scale bar is different. These are well-characterized circular spherical particles. This is a simple cytotoxicity assay, not a sensitive measure, just if the cells were alive or dead, shows would want to depict for 10, 50, and 75 nm particles. The smaller particles tend to be more toxic. And, this is based on micrograms per mil, added to the tissue culture. There's also a difference in the coating, it's not as dramatic, especially for the smaller particles, but for the larger PVP tends to be larger particles, The paper on cytotoxicity shows the particles. So, small particles were more toxic.

What you see is reflected light and the particles are very reflective, they appear in this kind of micrograph as white spots. Photograph and superimpose them and some are blue and [Indiscernible] is green. And what you can see is that those cells take up the silver nanoparticles, interesting there is very little near the cells on the bottom of the dish, that far away from the cells you can see silver grains deposited on the bottom. The cells are accurately taking out the silver, transporting it from the periphery, sometimes you see them lining up along a track that apparently pulls them into this nuclear region which contains the goal G and the endoplasmic reticulum. I also like to show this slide because they happened to catch this long process. Vindication, early striking picture and you can see the silver taking up in this use, in this process as well. Slightly different intensity shows the concentration dependency, the control slide with no silver, and then three, 10, or 30 micrograms per mill added to the suspension. You can see as you go up, the white is the reflected light from the nanoparticles. You can see that there is more and more in the higher cells. And again you can see it collecting right around the nucleus, not inside the nucleus and you can see it trafficking in from the edges. Again, very little between the cells on the bottom of the dish. The light reflecting properties have led us to look at them through psychometry, so if you're not familiar with psychometry you liberate the cells from the bottom of the tissue culture plate and put them into single file, there's a digital light that shoots across the new can measure light that come straight across or it scattered to the side, side scatter them forward scatter and you could measure fluorescence and the number of other things. What we find is that cells that have incorporated these reflective nanoparticles tend to scatter more light. And so what we see is the increase in side scatter any decrease in forward scatter. Much more sensitive. Interesting the silver we also see the fluorescent signal that comes off of the particles that is not in the wavelength of the laser that is admitted. The silver particles are absorbing the light and then readmitting it at different wavelengths in this we think is due to a surface plasma -- [Indiscernible - low volume] this shows you some of the side scatter data. This is the number of cells that would show this amount of side scatter. These are control cells and then increasing concentrations of silver nanoparticles. You can see nice dose-related increase in the signal. This is the fluorescence, the far red plasma neck residence. In higher concentrations there is a very large signal coming off of the cells and it is much greater than the increase in side scatter. That was concentration. This is related to particle size down here. A function of concentration so you can see that the small particles reflect less light than the larger particles. And this is a well-known physical phenomenon. The amount of scatter you are seeing in the side scatter signal is determined both by particle size and by [Indiscernible] and looking at the relative increase, it's much greater when you look at the red fluorescence, the service plasma and residence for higher concentrations. A very sensitive signal to detect. We heard a little bit about tissue dosimetry and this is really important for nanoparticles *in vitro*. If you added particles of different sizes to the media, what we would find is the larger particles are more influenced by sedimentation then all -- are the smaller particles. Him it was particles tend to stay in suspension and affected by diffusion primarily so what you have is a differential sedimentation based on the density of the particles, and the small particles also tend to conglomerate so they can form larger particles. So what you have is a complex dosimetry and in [Indiscernible] lab,Teegarden developed the IST be, I think it is *in vitro* him and dosimetry and they have combined laws of sedimentation with Stokes Einstein equation diffusion and predicted based on particles size and density that diffusion and deposition of particles into the media. And then Harvard and [Indiscernible] has

number papers where they have taken this model and furthered it to -- for particle density. Agglomeration and things like that.

We were interested in trying to see if we could use our side scatter signal that I showed you earlier, which is really just a measure of reflective light. If we could translate that in some of -- a dosimeter in some sort of massive silver so we did an experiment where we took cells growing in culture or just media, but silver nanoparticles, this is 20 nanometers. But different concentrations and then we split them, half of the cells went to centrifuge and then some went to flow cytometry and some of the other cell went to the ICP MS where we could measure the quantitative silver. With ICP MS. And so what we found looking at the massive silver. As a function of the attic concentration. So for 20 nanometers silver we have 90% or so this is percentage, this is mass, 90% of the silver is still in the media, 24 hours after treatment. Very little of the silver has gone down into the cell. 475-nanometer silver, you still get more media silver and silver in the cells, that the percentage is less. This corresponds to what we were just talking about, the larger particles are more likely to settle into the self. But if you're counting on your attic concentration of the media, you can vastly Ms. Estimate the dose because 90% of the small particles maintains in the media and the cells never see it. That is an important lesson. I think I have already said all of this. Basic message is the dose added to the [Indiscernible] is very different, very significantly from the actual dose of the cells exposed.

So comparing the doses now, remember we talked about different dose metrics might be used. And I'm only looking at these cell pellet, not the media so comparing 20 nanometers in the grain to 75-nanometer silver in the blue. So if you look at it as a function of the mast that you added to the culture, you can see that much more of the large silver is settling into the cell layer. You're getting a much higher dose of the large diameter cells. However, if you calculate particle number because there are so many more particles of the smaller cells you see you get much more exposure to the small than the large. Exactly the opposite, same data, just how you calculate your dose. Surface area, which many of us think is going to be the ultimate best predictor, looks like particle number but not so severe. There's still a lot more surface area in the small particles than the large particles, even though you have a larger mass. If you are comparing two different particles for example using this measure of dose, you might consider that one of them had less toxicity, have less -- let's see most of the large silver you would come to the exactly the opposite conclusion. Less material here and would be more toxic. Depending on how you calculate your dose, and remember wanted to ask if we could use flow cytometry as a substitute for measuring the dose. This is because flow cytometry is very efficient and if you have access to a machine and it is very rapid and my lab, ICP-MS. Other analyses are expensive. We have a lot of use for capacity see have to wait a long time to get tissues that. If we could use flow cytometry as a surrogate, it would make it much more efficient. What I've done here is to measure flow cytometry side scatter, against different measures of those absorbed those with ICP-MS. For the micrograms in the cell you can see that there is a different relationship for the large and the small particles but in each case it is only a relationship. And so you could use the side scatter as a surrogate for the concentration in the cells and as a function of nanoparticles number, although here the difference is very dramatic. To the -- [Indiscernible - low volume] and so what we can conclude from this is however you measure the dose, if you understand the relationship between side scatter and that particular particle, the mass in the cells, you can easily use side scatter concentration of the dose. So I think I have said that. And rapid and expensive expanding panel materials alternative testing data and genetic information is going to be increasingly important how we evaluate these new materials. Dosimetry models critical for evaluating those data. Nanoparticles versus silver ions. Silver I thought related where they appear to cause different toxicities. Someone open. One of these theories is that silver nanoparticles maybe accessible to compartments silver nanoparticles might be getting an acting as a Trojan horse to release the toxic the member in the set encoding. There is data like that weather and I wanted to leave you with a [Indiscernible] thanks to these people Kaitlyn Sarah long-term

associate laboratory technician, did the Toxcast and Bob Zucker -- Bob Zucker thank you very much. [Applause]

Two slides of references. Questions?

Audience Question: One of the problems we've had with chemicals know you have introduced a lot of other possible metrics that need to be compared to have any recommendation potency it see assessments set of metrics?

Response: The issue is not resolved in the literature. The literature were they have a lot of data and experience with air particles. Many of those people have moved into engineering particles. Surface area. Across different doses. If I had to make a choice the surface area is the most important parameter. The trouble is that most of the data are reported as [Indiscernible - low volume] you have to have some good way to [Indiscernible].

Audience Question: And do you have any recommendation if you are looking at something not the same materials, nano and nominal thinking of substituting in now a chemical that is not the same as how you go about trying to compare which looks better on the risk trade-off?

Response: We did have a group that is studying lifecycle applications, the nanomaterials and other products in sustainable chemistry environment. It's not just the relative toxicity, but it's the whole lifecycle of the material in terms of which does a cost to produce it or the side effects, what are the implications for down the road. It's a very complicated answer, and I don't have any good suggestions for you.

11:50 pm-12:30 pm Roundtable Discussion: Issues for Use of ADME/PK in Safety Assessments for Foods and Cosmetics--Harvey Clewell, moderator All speakers

Harvey: Any other questions? Thank you all. [Applause] Could the other speakers come up? Who will go to our discussion session. Now we are dependent on you. To raise issues. I will start things off with two that came in from the Internet. And let everybody jump for the ball as a toss it out. Him first general question from the peanut gallery is the role of pharmacokinetic information for dose selection in toxicity studies has been overlooked. What you recommend for the use of PK information in the design of toxicity studies? Excellent. In the design of toxicity studies using pharmacokinetic data for the design.

Bas: I think it's extremely important to start thinking about possibility of the kinetics in terms of for instance the absorption. And we were thinking about this decision tree for coming up with safety of cosmetics. And why would you want to do a study on systemic toxicity with cosmetics if the general absorption is zero? As an example in which you can have genetic considerations to influence the way in which you study [Indiscernible]. I think we can learn a lot from: Narrowly considerations with regard to the possibility of kinetics. That will lead you to the proper study designs. Especially as I showed in my talk, if you are focusing on *in vitro* studies, the choice of *in vitro* studies is also driven by the kinetics. If you are having a compound that is not able to go through the blood brain barrier, the let's say the first choice would not be working with the cells. This type of consideration, is that the answer to the question?

Greg: I think another consideration here is certainly in drug development, usually you are starting with a series of molecules, at least in the [Indiscernible] paradigm that have biological activity. And at least from a preclinical trials point of view, you usually want to get genetic information, because if a molecule is rapidly metabolized and cleared very rapidly, it's not going to -- is going to fail as a drug. You need to know that up front. Similarly molecules have the right kinetic properties to allow

delivery of there [Indiscernible] and usually the kinetics is taken into account to inform looking at toxicity studies which are usually done at some later point in development of selected candidates. And so there is another example of how you could use this and at least during the time when I worked at [Indiscernible] and many other companies did the same sort of thing, a lot of encouragement of using *in vitro* systems because she really could allow data using to rats versus [Indiscernible]. The whole idea was really trying to get the most information.

William: I can have a couple of answers. Both of them are that the firm code kinetics in talk studies so one of the things I didn't get to talk much about was absorption and [Indiscernible] of these nanoparticles. Silver is one of the ones that does dissolve so part of the clearance process with silver is dissolving into the ions -- [Indiscernible - low volume] there isn't much excretion of silver and if you look at the insoluble nanoparticles, titanium, [Indiscernible], it looks like the clearance of those particles is even less. So one of the concerns is that even though absorption may be low and excretion may be low, so that over a long period of time would give you the possibility of bio kinetic [Indiscernible]. That is something that some of us are beginning to suspect and I have not seen a lot of experimental validation of that particular risk. It's been so difficult to do those long-term bioaccumulation studies. That is one answer. The other answer I was reminded of the short-term kinetics and so before he did nanoparticles I -- [Indiscernible - low volume] and we developed the ability to measure neurophysiological function during exposure to vapors, things like toluene. And in every case what we found was that the amount of change in neurophysiology was related to the acute on board concentration of the material in the brain. And if we hadn't been testing during the exposure, we would have missed that because those compounds are rapidly cleared. And so traditional toxicity study, where he exposed the animal to the big chamber and you close the chamber down, wait till it is clear and you take the animals out and move them down the Hall. Of the time you ever get to testing, the test material is completed, it looks like there's no affect. There was a very strong acute impairment that [Indiscernible]. They cannot ask there were key in trying to figure out.

Harvey: And I will have my own pace which is the [Indiscernible] has the crown protection chemicals committee looked into how to improve toxicity testing in animals and they actually recommended that a 28 day study with from a coat Connecticut endpoints should be done first him so that you can look for [Indiscernible] chemical and determine the relationship between the administered dose and internal dose. And I know Jim Buss at Dow Chemical has been suggesting that's the important thing to do for many years. It generally doesn't catch on because analytical chemistry is expensive but it is something that I think it's recognize that it would be a good thing, most people are just reluctant to actually do it.

[Indiscernible - low volume]

Audience Question: Additional comments Re: pharmacokinetics and food safety?

Bas: And food safety you have of course a number of specific problems. One of them is let's say the matrix of food. In which let's say the influence of this matrix on the possibility to have systemic exposure is an important issue. But further on, if we are looking at food safety as long as you know something about this the internal exposure and it doesn't matter what type of compound we are talking about. It's probably apparently especially in the area of food exposure scenario, and in the [Indiscernible] with regard to the internal exposure that I see is a specific issue with regard to [Indiscernible].

Harvey: It depends on the paradigm you're talking about. The old paradigm animal -based or the new paradigm human *in vitro* based but with the old paradigm, the big question is if you have an item that accrued contaminant or ingredient that you are basing the toxicity on animal studies, differences in the metabolic parents first pass at elimination of the chemical it would really change

between the high doses of animal studies in the low doses at which the human ingests the food and also human animal differences. *In vitro* metabolism is of itself very informative for determining to what extent you can actually in first human risk from the animal study. And the same way of course with the new paradigm that you have to know what the actual exposure target tissue is the level of food in this media concentration you have to have pharmacokinetics and as I said for the new paradigm kinetics is essential

William: For nanoparticles -- There is a strong if you ingest a silver mounted nanoparticles chloride which is less soluble You at information things get more complex,

Harvey: and has a disadvantage of us in wrong. Anything else on the and I will let you do that

Audience Question: Call me a dreamer, with the dioxins we came up with dioxin equivalence. Has anybody attempted cumulative PBPK or the Max? Mixtures?

Harvey: We have looked at that late and that is one where you can use *in vitro* potency data on inhibition of testosterone production and then use information on the *in vivo* clearance of the different compounds into a cumulative risk assessment based on total inhibition of testosterone. There are a few cases where we have looked at [Indiscernible] and what is the organophosphate everybody looks at? So it is possible to look at these things. It hasn't been done much. It is mostly been case studies demonstrating it would be possible but of course it requires a good deal of data collection or even new data production in the laboratory and able to be able to do a cumulative PBPK type description but you need metabolism information.

Audience Question: Just more numbers and more math?

Harvey: It is data limited. That it actually is a much better answer. It depends on how important the chemical is in terms of public safety. In the case of phthalates, it seems worth the trouble because of the level of rubble concern but I wouldn't say was something that you would continually expect to continually apply. This is an interesting question. I will just read it. Are there any chemicals which are task sicken humans which have not been discovered in humans first. I suppose that begs the question of the questions. If they have been discovered in animals. Just not observe them in humans. We actually years ago at ICS comparing animal and human evidence for carcinogenicity. And found very good correspondence. There were a lot of carcinogens effects in humans. They don't occur. What stood out was arsenic and there was no animal evidence. To do animal studies that could get a fax but to some extent I'm not sure I'm not sure to answer this question we know the ones with human evidence

Greg: Where there chemicals toxicity pathway I can think of a good example there certainly are a lot of chemicals that are toxic where there are different pathways between animals and humans. I've done at work on a [Indiscernible]. That involves euro toxicity. The disposition of that compound that is metabolized thing. Bite [Indiscernible] conjugation and -- [Indiscernible - low volume] at species differences in those pathways. And from the animals that humans did the Mrs. Based on cellular metabolism and kinetics.

Harvey: Initially we thought this is interesting. They humans detoxified certainly going to predict genetic studies of that in PBPK model compared rats things balanced out the DoD found -- detoxification overall very similar. It makes me think about is there others at Hamner that are working on drug-induced injury modeling of that. And I was involved early on when they were first putting together the metabolism modules. And we looked at acetaminophen and then add a couple of other drugs with classic cases of apparent species sensitivity differences, and in that case there is actually nice publication, I can't remember it is either [Indiscernible] or somebody. We were actually able to resolve species differences in toxicity of either the rat or the mouse being more

similar to the human solely on the basis of metabolism differences. And so that's usually the first place to look. There are enough differences in metabolic pathways between the rodents and between the rodents and with humans that often it is just that a particular reactive metabolite is or is not produced at significant levels. At a similar dose. And there's no reason to expect there couldn't be similar differences in the pharmacodynamics and so certainly as we explore more of the pathways I remember mouse skin cancer for P H. It activates the pathway in the mouse that the human does not respond with. And so as I hinted before, I have spent the last 30 years explaining away animal results because of the differences between animals and humans so that those a problem if you have an animal -based assay and farmer has been spending a lot of money, bless their hearts, some of it comes to Hamner, to look at this question because they get tired of getting the red flags in the animal studies them and having something fail post marketing surveillance because it causes liver toxicity. And so as they say, a good part of that turned out to be due to metabolism and recently there was some other publication the same group, [Indiscernible] group, showing that transporter differences and transport activity you can get accumulation of bioassays that eventually reach toxic levels and that actually is consistent with the fact that there are some forms of [Indiscernible] to take a period of time to develop, you don't see them right away. I think all this points to the fact that the idea of trying to go through an *in vitro* system of human cells has a really big built-in advantage that if you see something it is relevant and then was just a matter of trying to cover -- the hard part is covering the landscape of things that one is concerned about. And when I talk in particular with developmental toxicologist they of 57 different things that can go wrong, more than that in a developing fetus him I want you to be able to have it and *in vitro* equivalent to everyone of them.

Bas: Friends of mine working in the Pharma industry, talk about specific compounds of course, it's much more useful than we know. It has many of the data that they find in differences in let's say animal metabolisms and him and Saul -- [Indiscernible - low volume] don't even reach the literature because it is [Indiscernible] compound.

Harvey: Never know.

William: I've often heard questions like that in the context of the futility of traditional [Indiscernible]. We haven't really been effective of preventing human toxicity episodes. Why do we continue to exist? I think as [Indiscernible] just said, there are a number of compounds in development. Please that get killed before they get the market, because they show up specific [Indiscernible]. Part of the chemicals that were in the Toxcast system have a non-toxic pathway. Something that they were trying to [Indiscernible]. Also an outline I think of pesticides. Would certainly be in that. Him carefully developed and comprise to have toxicity to mammals that would be picked up. And not ever being expressed. Some of them possibly [Indiscernible]. And I have one more thing that comes to mind. In an airborne system, the [Indiscernible] acts in some ways like asbestos. Insoluble, as fireable fiber and that can produce lesions in the long and in some cases that look like normal [Indiscernible] similar inflammatory [Indiscernible] response in the lungs of animals but not aware that there is a confirmed case of similar lesions in humans, although there was a [Indiscernible] industrial hygiene was not very good at it did appear that there were some pulmonary fax. And now that they have been evaluated to that level, I think there certainly have been successes -- [Indiscernible - low volume] in the measure. A lot of the issues when they get well identified -- [Indiscernible - low volume]

Harvey: Any other -- yes? Or of the *in vitro* studies to complement or replace *in vivo* studies. Some of the issues that are brought up when using [Indiscernible]. For example, for a particular cell line were determined empirically or historically. Not always accurately reflect cells that are growing in our bodies. Especially for problematic as we often -- grower cells in atmospheric oxygen which is much higher level of oxygen themselves, most of our souls and higher oxygen could translate to higher levels of reactive species which could have an effect on metabolism itself. Related to that is

this idea of selection. When you -- primary cells you grow them in tissue culture and by the time you do your studies, not always sure that the composition of the population really accurately reflects the composition of the organ in your study. The question I wanted to ask the panel is as you move forward towards moving more *in vitro* systems. To see a need for really assessing how accurately the *in vitro* conditions are for optimizing those conditions were less reflect what might be going on.

Greg: I think the worker showed you today, we had a fairly specific purpose in mind and I was to try to re-create *in vitro* systems that mimic him *in vivo* situations. I think it's perhaps overambitious to say that we are going to have an entire liver in a file or [Indiscernible] or anything like that but certainly we anticipated some of the problems and at least with hepatocytes, again I am sure you know that you can put hepatocytes in an isotonic buffer and they will still on for a few hours. But what they're doing, they are auto lazing. They have lost all the signals to maintain permanent stasis and they are basically degrading themselves. I alluded to some of the things before the early work of people making conclusions in the system. It's not very useful and that really was -- don't have a little bit of Sistine in the media and don't [Indiscernible] in 15 minutes. Those kinds of considerations where we developed him our system. is the medium of choice because it was really designed for keeping hepatocytes alive, healthy, and liver like. The problem then with the longer cultures, there are a number of problems there where him you since guess you get differentiation of cells into other phenotypes and a lot of that differentiation appears to depend on not just the culture medium, and the various components of that medium that are providing feedback inhibition for these are Telesis pathways but also the configuration of vessels and so in a sense, technologically, the data I showed you which was generated in the mid to late 90s, we were not dealing with some of the issues that are trying to be dealt with now, about three D cultures, flow through, bioreactors, I've had experience with bioreactors, Harvey has, it's very difficult to get these bioreactors that actually work reproducibly for any length of time. Certainly, the whole arguments, there are all sorts of pros and cons. Then when you get to other types of cells, we had recently example of years back done some studies with naphthalene trying to compare animal responses to human responses. And how we were doing that was with primary cells and so we were able to prepare from mice hepatocytes, we were able to prepare might send rents hepatocytes, that the allele souls and one sections. And we were able to get -- we felt at the time, and I still feel this way, we had our best chance of mimicking what might be happening in the whole animal by using freshly isolated preparations. As opposed to immortalized cell lines which I will not comment on that for the moment, so we felt good about it, but getting for example human nasal epithelial cells, they are commercially available, they are considered primary cells because they are not immortalized but you don't give very many of them from the biopsy and you have to grow. You have to -- we had to do that because we couldn't get enough cells from one donor to do an experiment. We got cells from various donors and then we grew them several double length so we had enough to do our experiments but that was a concern and I don't know the answer to how they changed or not. Certainly, when we isolated those nasal epithelial cells from animals and look at them under the microscope, you have a lot of reassurance that this is a good so prep. Humans, those human cells -- it was very hard to do that. And then when we look at the data, the data were somewhat unremarkable. In some cases the human tissues, human nasal epithelial cells were more sensitive than the animal cells. But we don't really know why. It wasn't because -- there might have been some kind of metabolism in those cells but we couldn't -- at least at the level that we could look at we couldn't see. Let me wind up with immortalized cell lines. That's not a feature of normal tissue. So I think when we are talking about *in vitro* systems that are going to be relevant for humans and for interpreting animal data in relation to humans, I think we really have to keep that in mind. I'm afraid I think that's a huge pitfall over Toxcast. It's not -- the attempt was him all the silence we've got intestinal the same stuff in them and then click the data and see what shakes out as opposed to saying let's develop cell systems that actually has characteristics that we know are useful to generate useful data. So there may be some systems that are in the Toxcast screen that are useful. I'm not sure [Indiscernible] cells are that useful. Those are cells that have been shown as

you passage them, they change and so the [Indiscernible] cells today are probably not the same cells from 1990s or 1980s. You look at the data that is in the literature, it is hard to know can you reproduce all of that data? So I am very cautious about -- having said that, immortalized cells are tools and let's use them. But keep it in the context that these aren't normal cells.

Bas: I agree with you completely that you have to be careful with the systems you are using. Relevance in terms of let's say the normal physiology reflects and I think a lot of optimistic talk about the use of newer systems. Steroids, all the three D systems. Bioreactors as you call them. Flow-through systems throughout. I think there are a lot of things going on there. I am fully optimistic about the possibilities to use stem cells. I think especially in the field of longer-term well directed depreciation studies with the cells. They give you the possibility to [Indiscernible].

Harvey: At the risk of overstating and I think we are in a revolution on *in vitro* methods right now. The bioengineers are creating these tissue cultures that reproduce the pulmonary action and free concentration, I would be happy. But there is a lot of work on multiple tissues on a plate, multicellular tissues being able to have cell/cell signaling. Having production of realistic without light production. All of this is a high level of activity right now, there is a horizon 20/ 20 funded project being reviewed in Europe right now. Many of which are on these more realistic more *in vivo* relevant tissue type systems, and how to interpret the data that comes from them. So your are absolutely right, that is the key question that needs to be addressed in the good news is a lot of good are addressing it right now.

Audience Question: [Indiscernible - low volume] had a factor that into the equation while performing such pharmacokinetic modeling?

With furan, fish

I am asking what their role of and Truro hepatic circulation is an understanding and how do you include that into the modeling while you are simulating or extrapolating?

Greg: It seems with furan, that's actually -- there's not and throw hepatic circulation, but one of the types of hepatotoxicity that produces a high doses is -- homeostasis. And I have alluded to this zone to zone three transition and that was observed in studies were increasing dose over time, seems like the initial, but it's not clear to me, I don't know anyone knows why it would be the target. Blood comes in and blood goes out. Should the day progresses to zone three. Some zone three hepatotoxic of, they are Neil -- near where the biliary tree could desk next to the [Indiscernible]. So it's certainly when you have a chemical, a drug, a food, that has [Indiscernible] conjugation for example as a metabolic pathway, that should be a trigger for you to at least him examine the question of illumination of the vial and the possibility of and throw hepatic circulation. Not food additives, previous work I've done with hepatic compounds. That was the unique mechanism of toxicity where we get oxidation of these compounds on the first pass through the liver, the conjugation or other types of conjugation giving them a large enough molecular weight, solubility excreted in the vial, they get into the gut, conjugate is cleaved, they come back as -- the nature group gets reduced in the come back to the liver as more reactive aromatic the bio activated [Indiscernible]. And it appears to be generally how these things end up producing liver cancer. But you could model this, and what I was going to say is this information historically we've got most of this information from animal studies. Where did you would have --

Harvey: I don't think at this point we are -- I know how to do hepatic research relation model without *in vivo* data. I think it's a level of complexity beyond current capabilities. You have to consider that bacteria and to a large extent it will remain them. And so chemicals like that if they were sufficient interest of my require *in vivo* testing for that possibility. Or you could just a

concentrations below where anything happens. I am in favor of the latter. We need to move along. I will answer the next question myself because it is in my sweet spot.

Audience Question: Your [Indiscernible] model seem constructed new using normal physiological parameters how do you justify that models the constructed this constructed can produce taxing effects that are known by mechanisms. Affect physiological function.

Harvey: Excellent question we don't assume word nor that fact and if you -- we've done modeling where we look at the interaction of the chemical and the health effects and then the health affect causing change in function and that modeling back down to compound and pick things out here at the FDA has been doing some really nice studies on that so if you want to see, looking at for example liver function, effects on liver function and so it is also model a bowl. It's not easy but it can be done and if it's something where you are talking about a clinical situation certainly worth the effort. Him I think it's the same answer to all the questions on pharmacokinetic modeling. You get as complicated as you need to him and they really simple Toxcast, and if you get somebody were you say a concern based on QSAR, you might have to look further and so it is tailoring the analysis, the experimental studies, to the characteristics of the compound I think is really the bottom line. Here is a good one for [Indiscernible] in general,

Audience Question: What steps will you suggest to take to improve if an IVIVE approach fails? You mentioned some of the predictions were way off.

Bas: That's an interesting one, because sometimes you're looking for the unknown then. And in a number of cases, we found out that we missed out an important parameter. In the study that I refer to one of the outliers was [Indiscernible]. And the prediction of [Indiscernible] and kinetics, we missed out an important factor that this type of compound is [Indiscernible] at least in male rats. Transported by the [Indiscernible]. Which is not taken up into your model. You don't know this. That was one of the reasons that we missed out. And so you try to find solutions in this way. Other examples are for instance that the metabolic parameters as measured *in vitro* were inadequate. We had to improve on this. We just found out that the data in the literature were not good enough to be able to put it [Indiscernible]. And on the other hand, let's be fair. Some models are useful. Look at all models. [Indiscernible]. George box.

Harvey: Yes, George box. Any other comments?

Audience Question: Last question I have is not a fair one for this colloquium. What are your recommendations for PK study design for NDA submission? I didn't think we were doing drugs. Okay. That's the trouble with acronyms. New dietary ingredient submission. What kind of TKA studies would you -- what kind of biokinetics studies would you say -- would you recommend for a new dietary agreement -- ingredient in order to try to start beginning to look at safety?

Bas: In the new paradigm if you are talking about *in vitro* -based studies I would start with trying to find a [Indiscernible]. In the first place and also try to be able to have realistic exposures scenarios in relation to this. And the results in appreciable amounts of compounds in the system, then you have to be aware of -- you had to build on this and make more -- etc.. Build on the whole model.

Harvey: I think the next step that would be looking at metabolic clearance. Very rapidly cleared that it is against him is unlikely there will be very much a must the clearance that causes the toxicity in the liver. Him but are those are the keys noted. Absorption in clearance. It is 11:30 which is what we are supposed to them. If someone has a screamingly important question, seeing none, thank you for your time and attention. [Applause]

[Event Concluded]