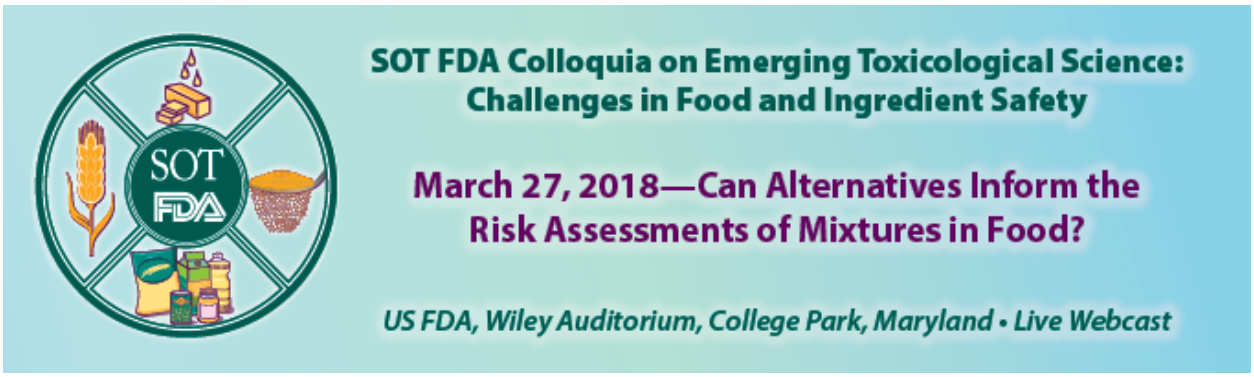

Real Time Captioning

Note: this is not a transcript

Schedule

8:30 AM–8:35 AM	**Welcome from FDA** Conrad J. Choiniere, Director, Office of Analytics and Outreach, CFSAN, US FDA, College Park, MD
8:35 AM–8:45 AM	**Welcome from SOT and Speaker Introductions** Suzanne Fitzpatrick, CFSAN, US FDA, College Park, MD
8:45 AM–8:50 AM	**Why a New Approach is Needed** A. Wallace Hayes, University of South Florida College of Public Health, Tampa, FL, and Institute for Integrative Toxicology, Michigan State University, East Lansing, MI
8:50 AM–9:30 AM	**Can High Thru-put Assays/Tox 21 Inform Hazard Identification?** Michael J. DeVito, NTP, Research Triangle Park, NC
9:30 AM–10:10 AM	**Proposed *In Silico/In Vitro* Approach for Botanical Mixtures** Catherine Mahony, Procter & Gamble Technical Centres Ltd, Surrey, UK
10:10 AM–10:30 AM	**Break**
10:30 AM–11:10 AM	**Non-Mammalian *In Vivo* Models: *C. elegans* As a Model System to Inform Hazard Identification** Piper Reid Hunt, US FDA, Laurel, MD
11:10 AM–11:50 AM	**Extrapolating New Approaches into a Tiered Approach to Mixtures Risk Assessment** Mike Dourson, Toxicology Excellence for Risk Assessment, Cincinnati, OH
11:50 AM–12:50 PM	**Roundtable Discussion** Moderator: A. Wallace Hayes All speakers

Welcome from FDA and Overview
Conrad J. Choiniere, Director, Office of Analytics and Outreach, CFSAN, US FDA, College Park, MD

Good morning, welcome to the SOT FDA Colloquia on Emerging Toxicological Science: Challenges in Food and Ingredient Safety. I direct an office in here. Just out of curiosity, how many people in this room are toxicologists? Okay, I don't think I've ever been in a room with as many toxicologists. I am not a toxicologist, nor do I play one on TV. I have been at the FDA for 15 years. I have a PhD in economics. I work on the other end of things—consumer behavior, consumer perceptions— different side of the perspective than you guys work. In my time here at the FDA, I have grown an appreciation for what you all do.

And 2003 -- in 2009, I took a break from foods, I went into tobacco products. That is why really started to get a better appreciation of what you all do. One of my roles was to be a lead reviewer for tobacco product applications. We had a manufacturer that had products that have fairly low levels of carcinogens. They wanted an exemption for the warning. They wanted to market a product without this warning. So as a lead reviewer, it was my job to basically oversee the scientific review and then take all of this scientific information from the reviewers, epidemiology, toxicology, sociologist and stitch it altogether in one coherent review and make a final recommendation. I don't know if you guys have ever dealt with this … can be like a needle in haystack. I found myself talking to my colleagues in toxicology quite a bit and that is when we spent a lot of time talking about the dosages for these carcinogens with the reality there was not sufficient evidence to see that there is a threshold. That there was a level below which these carcinogens would not have a cancer-causing effect. That really helped clarify my mind in the right way to go here.

Fast forward a couple years, I came back in 2016 and in my role here, one of the jobs that I had was risk manager. As the risk manager, I had to look at the toxicology, you guys provide that first inkling of whether there is potentially a problem out there. To take that information and combine it with the other information that we have, and we can see how broad is this problem, how big of an issue do we have here? What are the tasks that I'm dealing with right now is that I'm getting a team of risk managers and looking at the medals that we have in our food supply, lead, cadmium, the list goes on…They are naturally occurring in many ways, but they are not necessarily things that you want in your foods, right? We're trying to figure out how we manage this problem, how do we mitigate that problem and how big of a problem do we really have? In looking at our data, one of the things I have come to a realize is oftentimes you do not have these metals…find these metals in isolation. Arsenic, Cadmium, so I find myself talking to my toxicology colleagues to see what the sign says about the effects when you have these mixtures of metals and foods -- in foods? It turns out, science has not gone to the point where we can determine this. These are all of the new terms that I'm learning.

I am really glad that you guys are here today to figure it out. We have four hours, I think that we could do it. I think that this is great topic that you guys are all tackling, and I look forward to hearing what you have to say about it. I'm probably going to try to join the first few sessions. I'm glad to see you all here. I wish you a good day and good discussion for today, thank you.

Welcome from SOT and Speaker Introductions
Suzanne Fitzpatrick, CFSAN, US FDA, College Park, MD

I'm here on behalf of both the FDA and the SOT on behalf of the colloquium series. This is a partnership with the SOT to present high quality, future-oriented, cutting-edge talks on toxicology findings. We're partnering this with SOT, but this is also open to FDA employees and the public. We are not talking about regulatory issues, we're talking about sites here. It's well-within both our mission and the mission of the SOT to support and develop toxicologists and expand our reach and impact globally. This is webcast worldwide. This is the fourth one that we have had. We have one in June on biotechnology. As I said before, we have a very global audience. We have people participating worldwide from 70 different countries. This is an organizing committee, composed of both SOT and FDA people. With that, I will introduce Wallace Hayes and we will get started. Thank you all for being here.

Why a New Approach is Needed
A. Wallace Hayes, University of South Florida College of Public Health, Tampa, FL, and Institute for Integrative Toxicology, Michigan State University, East Lansing, MI

For those of you that are here in the audience, welcome and for those of you who are on the webcast, welcome. I think that we are going to really delve into something that as toxicologists, we kind of put aside for many, many years. And that is mixture and alternatives. We're going to try to bring them together in this section as we talk about why a new approach is needed. This is my declaration that we need to put up. When we think about mixtures, what we are really finding ourselves with are the real-life circumstances that we find ourselves in. We are exposed, simultaneously to many, many things. Not just in our food, but in the air that we breathe and the water we drink. Everywhere that we go, we are being exposed to low levels of mixtures. You can see in our air, and our water, most relevant to all populations are the facts that we are exposed to mixtures. Our good friends at EPA have to be concerned about mixtures at Superfund sites. At the FDA, when you think about it, that is all food is. It is a mixture, a mixture of many different types of chemicals, including those that unfortunately get in through natural contamination or through the processing process with a variety of those. We have to think about all of those. It is a real-life situation and it is a challenge for us. As toxicologists, we struggle with understanding one chemical, much less two or three chemicals. We get to thinking about the hundreds of chemicals that are in our food, we have a real challenge.

I will only take a minute and remind all of us of this very simple word RITE. It means Risk is equal Toxicity Times Exposure. If something is in a food item, literally millions of people are exposed to this by eating their food. If you see a rattlesnake in a cage, it is a hazard, but it is not a risk. However, if you are walking along and you see that snake is out in the woods and you get bitten, you now have exposure and you have the risk. This is a hazard and too often we see documents coming out of places that people assume are risks. It is not a risk for this highly toxic fish until this young lady happens to eat it. When we move out of that idea Rite, and think about foods, particularly of the chemicals that we are seeing in the market, we pull out ingredients we say do good things. Why not just use the whole botanical? We need to begin to understand that these botanicals are a mixture and may cause or have the potential to cause problems. Keep in mind aggregate risk, cumulative risk and multiple exposures. That is

really what we're talking about, we are being exposed to multiple things from different areas over time. It's how life works…with this question of how we handle these various types of risks.

In addition to that, typically we can assume that when things are mixed together, they are additives. Those of us who come out of a formal toxicology background, we understand that individual actions, but need to at least to think about the interactions of the chemical mixtures. This is a very, very simple framework and we used to say that the worst enemy of a toxicologist, in addition to the lawyer, is the chemist. But the chemist can help us as we think about these very complex biological and botanical sources. If they can identify what is there, and understand his compounds of concern, and we are able to sort out whether we have sufficient data to address the issue and we may build to move forward by just looking at the chemistry. Here is the program. We have four phenomenal speakers that are going to be sharing their experiences in time with us. We're going to start out with Mike from the NTP at the research triangle Park. Catherine will follow. And then Piper. And finally, Mike will close out the session and bring it all together. Begin now to think about your questions. Let's have some really thoughtful questions to think about. With that, I will turn the microphone over to Mike, and he is our first presentation.

Can High Thru-put Assays/Tox 21 Inform Hazard Identification? Michael J. DeVito, NTP, Research Triangle Park, NC

I'm going to be talking about high thru-put assays/toxicology 21. I want to remind you of the challenges that we are facing in toxicology and hazard assessment. I want to talk to you about toxicology 21 versus two's toxicology cast. Thinking about how we can evaluate the mixtures in Tox21. So, let's talk about the mantra of why we have Tox21. There are too many chemical mixtures and commercial mixtures. When you think about the chemicals, pesticide formulations, there are just a lot of things that we are exposed to. Mixtures. There are too many co-exposures. We are actually exposed to mixtures of mixtures. We cannot use traditional methods to test our way out of this.

How do we move forward? This is something that a lot of the regulatory agencies worry about. And a number of these organizations in the early 2000's started to realize that traditional testing approaches were unsustainable in evaluating the challenges. In 2004, the NTP came up with a roadmap to address this issue. In 2005, Tox21was initiated with NTP, in CTC. The difference between Tox21 and traditional screening is the use of lots of chemicals, somewhere between 9000-10,000. It is screening 70-80 biologicals. Tox Cast is looking at a smaller amount of biology, so they complement each other. They provided division and strategy and then in 2010, the U.S. FDA joins Tox21. Joining forces to try to move the … field forward.

I want to talk about the Tox21 approach. With Tox21, we are screening about 75-100 different pathways. We include zebrafish in alternative species. We are using smaller libraries. We may use liquid handling stations. They are smaller libraries. We can handle 100 chemicals in a couple of assays a week. With these assays, instead of just screening everything, we have a hypothesis. Why are we screening this library? There is…are complementary approaches. Mixtures in risk assessment. I just want to remind you some of the approaches that we take to deal with mixtures. One is, the whole mixture approach. What this requires is toxicology data on hold mixtures. The problem is, we usually have more than one mixture. One of the things that we're trying to do is develop approach. That is something that I will talk about later. The other way to do that is component-based. This requires toxicology data for individual chemicals

within the mixture, perhaps relative potency factors. You have these two different approaches. They both have equal numbers of successes and challenges. Just to bring up, some of you may not be familiar with sufficient similarity. It refers to a mixture that is very close in composition to the mixture of concern. Such that the differences in the components and their proportions are small. We believe that one mixture will be representative of Texas -- toxicity of the other major -- mixture. What is sufficient and what is similar? We do not have real good consensus on either of those words. I will give you an example. When we are working with statisticians, they looked at two chemicals and said “Aha, they are different.” Their definition of sufficiently similar, at the exact same dose that produces the same response. When we saw it, we said that they are different by a relative potency factor, so this is not trivial.

So, I think that I want to go into the first case study and that is evaluating dose addition in Tox21. So, with Tox21, one of the things you want to do to be most efficient is to make sure that every well has a chemical. We realized that one of our plates was about 200 chemicals short. So, they said Mike, why don’t make up some mixtures. So, what we did, we looked at the estrogen receptor from phase 1 of Tox21. There were 10, we chose eight. We made up 67 mixtures of these 18 chemicals. Then we made mixtures of them. One of the things that we are asking is, how far away do we go, how many chemicals do we put in these mixtures? All of these individual chemicals for phase I and phase II, including the mixtures, but there were one on two different assays. We used phase 1 to design the study. Keep that in mind. Here's the list of chemicals.

There is a story here. There are challenges in hypothesis testing in Tox21. One of them is you're not going back. This better be right, because we’re not repeating it. So, think about Tox21 in the library as a ship, it leaves ports. You are either on the ship when it leaves port, or if you are on the dock, you're not getting on the ship anymore. The ship, once it leaves the port is not coming back. We’re not going to stay, we are going, nobody's coming back on. So, you have one shot. The problem we had is that there actually inconsistencies between phase I and phase II. About half were not and phase II. Phase I, with one concentration and lease one well -- in at least one well. So, what happened is if you were next to this, and it was really bright and really high and your sample that may not have been estrogenic, turns out looking like it. We fix that and phase II. We did things in triplicate we move things around on the plate the other challenge was you have to get it right. Phase 2, Zearalenone was in maximum responses. We needed to have dose-response data. We had a real challenge dealing with this because it was in almost every mixture. But, I will show you the results. Out of it is okay, right? The other thing to think about is, we do actually have some actual estrogen there, but we are testing it was non-estrogenic chemicals. So, kind of like making lemonade. This is what I want to show you. Okay, on the X axis, what we did is fit a dose-response to each mixture. The y-axis is the prediction from the individual chemical data. So, the y-axis is the predictions of what the mixture should’ve done. In the X-axis is the fitted prediction of what it actually did. The green is our estrogen chemicals. And the blue is our estrogen agonist mixtures. What you can see --- is doing a really good job of predicting almost all the mixtures. The only problems with these really high ones, one of the challenges with the AR mixtures, we are getting 5-10 of the maximal dose. Predicting that has a lot of uncertainty, as you can see. I am not sure that we are really off, it is just that I would not call these estrogens. It is just a little blip of the response. Just a little background…one of the challenges of Tox21 is that background is really low usually. So you can get a bump over control pretty easily. I think that we did pretty well for most of the mixtures, given some of the challenges that we had. I think that you might build to test but you have to come to grips with the fact that there really no good studies, so you have to hope for the best. Now to sort of change gears, one was an opportunistic study.

NTP is studying a lot of botanicals. This is just sort of a list of the ones that we have completed. We spent a fair amount of time. Challenges with botanicals. There are many constituents, there are multiple active constituents. There's also a large and identified fraction. And there is variability across the marketplace. Their differences in raw material due to source, season, …. processing, manufacturing. Here are some pretty pictures that I forgot show you. There are also slight differences in plants. They may be the same species, but there may be slight differences in different strains. For us, our primary challenge is to test articles flexion and relating animal doses to human intake. Secondary challenges are extrapolating findings to other botanicals on the other botanicals on the market. Identifying active constituents, so we do not spend a lot of time on, but we know that there are a lot of drug botanical interactions. So, we took botanicals through Tox21 we take advantage of this really, but we did it this time. It was part of phase 2 and we used a select number of botanicals in a select number of assays. We did not run every assay with it. Here's a list of the botanicals that we ran through. You can see that some of the samples that we ran are in multiple products. Some of them are just one, like olive oil. We ran a fair number of them. We ran them through nuclear receptive assays and some of the stress response assays, genotoxic assays.

What we were hoping is that the approach would give us some idea of the biology of these dietary supplements and what they do. One of the challenges is, not everything is soluble in this apparently. We would put it in and try to get it to dissolve, there were some challenges there. But, hopefully, we thought that we would see something in it that might be useful. So, some things worked out pretty well. We did a couple of extracts, they did nothing, but at least they group together, right? That is good to know. Resveratrol -- it was -- goldenseal root powder had some biological -- activity, but when we ran it through, we saw that it had a lot more activity and it did not look like goldenseal root powder. In Ginkgo, not all of them had the same thing. One of the things that I will go over in more detail is the GB results. We see a lot of estrogen receptor responses. When you get into the pure chemicals, you start getting disruptions. We have a lot of toxicity in these cells. So, they seem to have pretty consistent responses across the different lots. So, one of our challenges is, while these results are kind of interesting is extrapolating it out is a bit challenging. One of our real challenges is, we are looking at one assay at a time. What is the right biology? That is something that we are struggling with. But we can actually test these. We do see levels of activities *in vitro* that seem to correspond to levels of activity *in vivo* -- alive -- live. So, I want to go over the 51. We decided to go a little bit deeper with *Ginkgo biloba*. One of the botanical associations suggested that …. -- our *Ginkgo biloba* was not theirs. We do not know how well these differences in chemistry relate to biology. Without that we would go out and go deep in this. We brought about 20 different *Ginkgo biloba* extracts. We brought the gold standard *Ginkgo biloba* and we compared them. Did we learn anything by adding the biology to them? This is our sufficient similarity example. We are actually trying to figure out is our *Ginkgo biloba* that we tested out the same?

Why is this important? It lets us actually think about our GBE and how it relates to the variety that are on the market. So, I'm going to give you an idea of some of the chemistry challenge that we saw. We have some of these that look like *Ginkgo biloba.* There was actually something that you could see. There was a substance that you can measure. Then you have another, this is dirt +1. You go from this word Ginkgo Biloba, to dirt, to dirt +1. I don't know why they do that. Here are some of our others. We actually did get a number that had *Ginkgo biloba* in it. Every once in a while, that slips in that looks a little much like a dirt. So, we can do a quantitative comparison. Now what? I have a nice grouping. There are couple. They take the pill and grind it up and they can actually go with the pure 761. So, what you say about these things? The groupings come out with different ratios and different constituents. That is how

they are grouped, but it still makes it difficult to extrapolate. So, we took these 20 different lots and we did the chemical analysis. We looked at the nuclear receptive assays, we threw some of them in *in vitro* rat studies. So, the mission is complete on a chemical analysis. The studies are done, I managed show you how we interpreted it. So, any suggestions would be useful. If we looked at our primary human hepatocyte data, at the end, we looked at the gene expression. What you can see is, here is our sample, that is good. They kind of work together. Here is our GBE 761 and some of our test articles. That is good. GBE W, is the pill that we ground up and that actually looks a little different than the GBE peer product. And we had another group of GBE that are closed chemically but looked differently biologically. So now, a real challenge is trying to combine this information. So, we put all of the information together and we had a hierarchical clustering. We kind of put things together and think about where they came out in this sort of statistical approach. We did a similarity judgment. Mixtures in the same group as a reference are considered similar. Mixtures *in vivo*-- this is a work in progress.

When you look at this, we can come up with the different markers that we looked at. We looked at chemical analysis coming from here up. This is the primary hepatocyte -- I forgot what version this is, we had a similarity score for the different responses. You can see, groupings of these different chemicals based upon how we described what is similar. We had chemical and biological similarity. This helps us understand which ones we may actually come back in group as sufficient similarities when we do our toxicology study.

Just to give you an overview, there is a clear difference between the extracts samples that resemble the reference sample and other high-quality samples reference material. The findings are relatively consistent across *in vitro*. The untargeted chemistry and human hepatocyte data were judged to be the most informative and cost-effective combination of determining sufficient similarity. We actually looked at and said, we have liver tumors in rats and mice, we think that this is related to some sort of receptor activation. Lo and behold, the chemistry and biology seem to work well up in predicting that.

So, I think that the way we are looking at this is force case study three. It's been the more that we know about chemical composition, active constituents, and biological effects of a mixture, the easier it is to determine sufficient similarity of other, related mixtures. When the active constituents are unknown, and we are trying to test an unknown biology, we may run into problems. This is really a challenge with some of the mixtures. And so, we need to think about how to connect the biological assays to the toxicities that we think we are observing and use that as our screens for individual botanicals.

Just to give you a higher-level summary. HTS -- HTS can provide screening level information on biological activity. We can get, some of the challenges are going to be extrapolated. I think that we could do well on individual chemicals *in vitro* and *in vivo*. I think with the mixtures, it becomes a little bit of a challenge, but I do have hopes for that. I think that some of our advances might allow us to do that. So, while the individual chemists -- there is no zero any longer. I think that technology will help us with mixtures. I tend to focus on moderates screening. I like it better because I am a control freak and I can control that number one. Number two, it becomes more hypothesis-based and can more easily be replicated. I am on the ship and the ship is going to go where it goes. I think that, what we have shown is that combining chemical and biological data can in the hands our ability to implement sufficient similarity approaches. I think that is kind of where we are going to have to go. This was just a ton of work by a large number of people. Particularly at NTP, our chemists, they are really needed on these projects. We had the office of dietary supplements, and NCATS. Questions?

Audience Question: I am wondering what cell lines you have explored?

DeVito: Where we're going now is phase 3 of Tox21. What we have done is come up with the S 1500+ and it is about 300 genes. We have been looking at this cell line, when the cell lines are doing steroids. They have stem-like properties. When you grow them in 3-D, they have almost as much metabolic activity as hepatocytes. At about day 10, they are outside, and the hepatocytes are inside. I do not know what it means, but I think that it is cool. I also think that it means that it is replicating some sort of biology. But that is where we are going and hopefully it will get us to the unknown biology.

Audience Question: Did I understand correctly, that you said that your ginkgo was causing liver cancer, and if that is correct, how do you explain the fact that ginkgo has been used around the world for 100-200 years and we don't seem to have human problems?

DeVito: I put that in a couple of ways. One, it is probably through receptor activation. There's challenges of extrapolating those modes of actions to humans. The other is, it is uncertain with the botanicals, how we kind of know their long-term toxicity and safety. When you think about it, there are very few studies looking at those effects, so most people think that they are safe and anything they see, they are not going to attribute to the Gingko. I am not saying that they are toxic, I am not saying they're safe. I am saying that we really have not studied the product very well. I think that's why these things get nominated. I think that's what we are looking at Tox21, so when we have these challenges, we have seen these carcinogens happening pretty frequently. I think that will help us get there.

Audience Question: Does the FDA, EPA, or any federal agency have a regulatory position on Tox21 yet?

DeVito: I'm not aware of that at the moment. But what I am aware of is our effort with the ----. We're going to be screening them in collaboration with the EPA. With divided the biological world with certain assays. That data will be handed off to regulators in a way that we think we can help them to interpret. We're also working with some of our partners at FDA to make sure that the chemicals that they are interested in are included in this. We will see how they do with that because they are not getting by. We are not doing multigenerations on 75 chemicals in the foreseeable future. So, this is the *in vitro*/ Tran12, *in vivo* in the cells. So, one of the things there we're also doing with that, renal reuptake, we're trying to figure out what the chemicals are in the cell. When we are saying that it is equivalent, we will at least know if that is true and it is not we will know how to scale it. So, it is not going to be true.

Audience Question: Just for clarification question. You showed the slides showing about 20-30 botanicals, did you test the mixture or the active ingredient?

DeVito: Both. Depending on the botanical. We tested Resveratrol. We end up screening them. Some of them are botanical and some of them are dirt. It does not matter which botanical we purchase for the study, we end up with the same chemistry problem. That is one of the ways that we went into such detail with the Ginkgo Biloba.

Audience Question: Before we spend lots of money on a bioassay, how can we incorporate biology to sit back and think about sufficient similarities? What is our test going to represent

that is on the market? The reason that I am asking is that there is one chemical that I am interested in. I'm wondering if the result will be published in?

DeVito: On that slide, it is either completed or in progress. I don't know off the top of my head, when that is, hopefully for your sake it is completed. If it is in progress, I have no idea. It could be in the pipeline, it could be, I have no idea right now. But if you contact me, I will try to figure that out.

Proposed *In SilicolIn Vitro* Approach for Botanical Mixtures Catherine Mahony, Procter & Gamble Technical Centres Ltd, Surrey, UK

Thank you for the invitation. Thank you to the head of security for permitting me access. I am Dr. Catherine Mahony. If he had not yet noticed, I work for Procter & Gamble. That had nothing to do with why I was not permitted access, it was really my British accent. The conflict of interest means that Procter & Gamble provided financial support. Okay, so really my presentation today is going to focus on two main parts. The constituent's characterization *in silico* approach. I want to explain why we're doing this. We are really aiming through our botanical safety research program to provide toxicologists with tools and strategies to enable them to better predict two variables -- toxicology and risk. This is driven by an increasing consumer desire. It also presents a challenge…is everything that is natural safe? I have to ask whenever we go into a new skincare shop.

Anyway, the challenges that botanicals represent three main categories. First, is the question of identity and possible adulteration in just the fact that we are dealing with complex chemistry. There are number of gaps in they toxicology literature. We've also seen limitations with that data. Finally, there is this question of herb drug interactions. In the supplement arena, that I am heavily focused, we are likely to be taking more medications and prescriptions. Really for today, the art is these challenges that I'm going to be focusing on. So, in Procter & Gamble, we have taken a tier-based approach to testing botanical safety. Underpinning this is the assurance of identity and quality. Suffice to say, they go hand-in-hand with the botanical safety assessment. At the lowest level, at the lowest tier, where we have low exposure, we may be faced with data gaps. We are working to develop a threshold of toxicological concern approach for the whole botanical mixture. There's a publication, Hoadley, coming out later this year. This tier 2 level, based on significant human use history. Here, we need to consider potential delayed effects, things like carcinogenicity….reproducibility, as well as target populations may change. We must also consider the potential for co-use with other medications.

And then at this tier 3 level, this is where I am focusing for this presentation. It is where we base the safety on the in-depth characterization of botanical constituents and evaluation of toxicity. So, this is our tier 3 approach. This is the approach that we will take when the data is insufficient from tier 1 and tier 2 ... Where we are particularly assessing higher exposures or toxicology data that are lacking or differing extractions. This is a paper that was published last year. It was really an approach that was derived through necessity when we started to work on tier 3 botanicals. We have now progressed to 40+ botanicals in this way. This approach continues to be quite useful. At a really high level, the approach is to collect existing data, generate the new data, analyze that data and safety tools and decide ways in which to go next. Let's look at the data details. When we are starting to gather information, when we are looking at the plant species and the method of extraction, we are building assessments using reliable sources of information. We expect a positive assurance of safety. Just an absence of evidence

is not going to cut it for us. We are looking for evidence by support and having this evidence, we can evaluate the botanical. If we have adequate support, the botanical can carry on the R&D journey. Because we have identified this. Often, these gaps will be because we see a difference in the systems that are used in the preparation of the botanical extract. We may have questions about natural variations. Maybe the target audience has changed who may have traditionally use the botanical, or perhaps at a higher exposure than what was traditionally gone before. There may be that there is a real scarcity of safety data. So, in this case, we look to obtain a sample that we can progress through our classification and identification decisions. It is useful in this regard that can help identify this. We are really looking at obtaining information at the qualitative and quantitative level. The toxicologists can say lower limits of detection for the chemist and we could do that knowing that at that intended dose. For example, we have a good data set, but we are lacking developmental data, we can use the TTC, --So another -- as they get started, they are already using the advanced multi-vector approach. Using this to make an identification of components in botanical mixtures simultaneously with eight quantifications. What this provides is constituent information where the structure and the dose is described. So, then we can start to plow our way through that information. For some cases the structures are not known, or only partially, according to our decision tree. We can go straight to asking questions about the relative vent TTC level. And then if we are, we can exit those ingredients from the decision tree. If we are not, we can work quickly to make a decision to reduce the dose or see if we require additional information. We have gotten better and better at identifying what are in these mixtures.

So, for those constituents, the next question that we are asking is whether they are commonly found in diet. Here, you have to be a little bit cautious about how you qualify dietary intake. For us it means a dietary exposure that would be fortified by a genetically diverse and sufficiently large population. If you can affirm that it is found in the diets, the dose you are accessing, you can use that in the decision tree. If not, we can ask if there is adequate toxicology data for each. If you can derive a sufficient margin of safety, it could be on the decision tree, but if not we are in that *in silico* tool. First, we would look at the whether there would be sufficient margins of safety from the decision tree. Or we can use expert systems and expert opinions to help inform mode of action, structural, and decide which class we can put these constituents in. Having been at the threshold of toxicological concern, we can exit it from the decision tree, or we decide that we need to reduce the dose, so we can use the thresholds to set the level. Or do we need follow-up to generate additional data? A lot of the questions that we are needing to generate, the first is, the sorts of scenarios that were used in this *in silico* approach is for benchmarking, constituent safety between the diets and the botanical supplements. It is really representative of what is found in the diet. Or using this type of analysis where we profile the botanicals, so we can bridge safety data between the different methods of botanical preparation. These two probably represent about two thirds of the cases of the botanicals that we have looked at. For those cases that we cannot use this benchmarking a profiling approach, we can use the *in silico*, using that tool to set what would be an acceptable dose. Finally, at the end of this, where you have that detailed characterization, it can be really helpful in informing the design of any follow-up safety study.

So, just a summary of this EC ID *in silico* approach. It starts with early vetting the scientific literature and defining the questions that you're trying to ask. And then following on with the advanced multi-detector analytical characterization approach to identify and quantify simultaneously the identity. And then the process starts information through a decision tree to either close safety data gaps and form supportable exposure levels, or to identify any additional safety studies. Another way you can think of it is a focused approach for detecting possible bad actors in botanical extracts.

So, this is a case study, that is not in the paper, but I'm going to share with you. It kind of provides a nice segue. I really encourage you to take a look at that paper because there are a number of case studies described in there that illustrates the scenarios that I have mentioned. The artichoke leaf is traditionally used for the relief of digestive disorders. It is the leaf coming from the base parts and not the flower head. There is a history of use, but we identified a deadly gap for developmental toxicity. We put this through the decision tree and found for the most part, that the constituents were food like. But there was this faction, at the very end from that decision tree that we could not address through any other means. When the dosage is 13 milligrams a day, we could not support using that.

This is where we are now moving to some of the *in vitro* testing approaches, particularly ones that we are going to use to inform at a mode of action level. Customized Cerep panel. By lack of response on developmentally relevant targets or by functional. The first question that we are asking when we are thinking about what an appropriate *in vitro* assay would be is to understand what developmental actions are. What they did is calling it a dart ontology project. What they did is look at structural features of chemicals associated with developmental outcomes. They had Harry focus on developmental toxicity. Basically, they derived 19 major categories and multiple subcategories. Things like iron channels, neurotransmitters, and a number of different enzymes.

So, we took this information into design of the Cerep assay. This started with the safety screen and we added to it, our dart mode of action. At the time that we finished adding to it, we had some eight steroids in retinoic acid receptors and 32 neurotransmitter receptors and transporters. We have tested a number of botanicals in the platform either because of their dietary use or the assumed lack of development on reproductive toxicity or because they have a pharmacology that might preclude use in pregnancy. Which has a concentration based on a multiple of measured CMAP of an active marker compound, we do that with the thinking that we see nothing on the higher dose, we need to follow up.

So, this is a picture of some of our data and there are a few things to draw your attention to. One is that some botanicals and others do not. This is just a closer look at some of the data, and back to the artichoke leaf study. In this case, we saw receptor activity that was consistent with traditional use, which is the relief of digestive disorders. But we did not see a response on developmentally relevant targets. So now we start to have things that we can bring in, not just history of use, but ... The other observation that we made in this activity is that when we run these experiments, we need to follow up with a subsequent experiment. Also, what the IC50 value would be. We could for the… and metabolize. What is interesting is, when you look at the scale, you can start to contrast the botanicals versus endogenous hormones, contraceptives and so, when I think about this sort of data, I think about how the more information you have of this kind, the more you can start to inform potential potency differences. And you are able to separate what a food-like activity is versus other activities.

So, the other approach that we are using is this connectivity mapping approach. Gene expression in connectivity mapping. It was a paper initially published in toxicological sciences and ensure that you can group chemicals based on mode of action using connectivity mapping approach. Essentially, it's on the gene expression profile for chemicals, you look for function analogs. To look for chemicals that have similar gene expressions. This is an example the gene expression profile showed that it was similar to others. So, it makes sense. We have taken this approach now for a number of botanicals. In doing so, we are using four different ones, which we think are providing a rich information.

So, we get our gene expression data for botanicals and do what? This is an example where St. John's wort. The gene expression profile with this, when we put it through, gave close association with antipsychotic drugs. When we look at the molecular target for those, with all that, they too have serotonin activity. They also had activity for dopamine and estrogenic receptors -- addressing -- receptors. If you knew nothing about the motive action for St. John's wort, you might think that it will be related to this. Indeed, it is used to treat depression. The number of chemicals that we have worked in this way, and the predictions actually seems to be a meaningful approach for this.

Back to the dart question that we are sorting and trying to address here…how can we use the sort of data to inform our dart? This is an illustration of a way that we may be able to go to compare active concentrations. So, if you know the concentration that you are affecting gene expressions and you can relate to that to the compound in your botanical, you also know the inhibitory constant. This is a key receptor for functional analog. Then you can contrast these together for relative potency. If you furthermore have a developmental adverse effect level for your functional analog, in this case, you can start to develop this based on the relative potency to give us this adjustment for potency which then you can contrast to what would be the therapeutic dose of the botanical. So, when we did this for St. John's wort, we had a margin of just over one and -- 1. With a margin of 1.6, you're probably going to develop -- follow-up.

Patient clinical data using in that patient is not recommended. The mode of action approach is really about characterizing biological activity through these *in vitro* approaches which we think will be a combination. The gene expression conductivity mapping approach can help identify functional analog it has a broad of biological coverage but is not a complex analysis. Then on the other hand, we can provide a better focus on the target, but it does not matter what biological focus and we are working further at the moment to utilize the data that we are getting from these two platforms to see how we might use it for different purposes. These are comparisons and concentrations to assess relative potency, but I think there are a couple things that we need to do to take that further, probably tighten the dosage to get more on the effect as well. Try to elaborate how that is the key receptor coming from a botanical mixture so that it may be converted into that value. And finally, I guess encourage the results that we have so far, we are now exploring how we might extend these panels for greater coverage systemic modes of action, not just developmental and reproductive toxicity.

So, homestretch now, conclusions and outlook. And hopefully, this is what you are taking away. Constituent level characterization, and the approach can really help inform the botanical safety assessment. I was to address gaps in the toxicological data or to refine support exposure data levels and inform the design of further follow-up safety studies. These high content, high input *in vitro* approaches, these methodologies, they can inform and motivate action or show us a lack of response on toxicologically relevant targets. They can use them to help make some decisions. And finally, botanicals are challenging, yes. But good science takes good time and money. So, just a couple of papers here if you are interested more in some of those, publication by Karen Vedder Mullen, where we took the analytical and *in silica* approach to safety assessment of the mushroom ingredients. And, Jason Lytle who is sitting in the back there actually. Hands waving. And, this is our seminal paper on the *in silica* approach using the constituent level characterization. So, just finally, to acknowledge the number of people involved in this work and it's really a growing team now, and on the *in silica* side, it was Jason Lytle and Tim Baker. I would say partners in crime in the early conception of this approach. And, the botanical mode of action, dart project, Karen Vander Mullen, who led some work and our colleagues at European who wanted to follow up with them for more details, and

George who lead on the gene expression on the CMAP slide, so with that I want to say thank you for your attention.

Audience Question: Are there studies looking at the effect of dietary and topically applied botanicals. On clinical outcomes?

Mahony: Yes, I think, the work that we have done looking out and informing, that is tallying right well with clinical outcomes. To me, actually, this was this was a big moment as I have worked through a number of these botanicals in this way. Using the Cerep data, using the gene expression, gene mapping approach. But it is now starting to make sense what a possible mode of action of a botanical could be. That is natural with tallying with the clinical outcome.

Audience Question: Skin exposure. (webcast question was not displayed/read out loud)

Mahony: We have really predominantly worked with this in the dietary supplement contexts. We just started to utilize the tool now, towards beauty care botanicals. That's something you have to think about with the bioavailability through the skin. I think the tool still works in the same way and you can still process through the same decision points, but you probably need to pay closer attention to that bioavailability question as a different route of exposure, right?

Audience Question: PXR Induction (webcast question was not displayed/read out loud)

Mahony: Oh yeah, this other question here, we did not have PXR are in our Cerep, because it's not a developmentally relevant target, but this is some of what the other botanicals have I think, towards other toxicity endpoints, then we probably would start to see data. I have seen that approach for St. John's wort and hinted at the PXR link. There may be some other liver targets.

Audience Question: (webcast question was not displayed/read out loud)

Mahony: Yes, we did. We used human's liver S9. We do a pre-incubation as opposed to a post-incubation and it's actually something that we are going to be working a bit further to develop the mess but on because we had a little bit more interference in our last round of testing that I would've liked to have seen so we are going to see how we can clean that up.

Non-Mammalian *In Vivo* Models: *C. elegans* As a Model System to Inform Hazard Identification
Piper Reid Hunt, US FDA, Laurel, MD

Today, I am presenting my own work as well as the work of others, telling you what I think that means for predictive toxicology. These statements do not represent FDA positions. Additionally, while I have no competing interests, I do think that even the fact that I am a person who really thinks it's fun to watch worms crawl around and try to figure out what that means for various endpoints and modes of action, that qualifies me as a major nerd. So, while I don't have any competing interests, I do have a definite incentive to convince everyone that they are not beyond them and you really can understand how they might be useful to predict the toxicology. Also, importantly where they might not be useful, so that it has fit for purpose models. Before I go into my talk, I just want to talk a little bit about the office of applied

research and safety assessment. We have wonderful areas of toxicology there. We have people working to develop new cell-based situations for the new toxicology products. All the people out there in TV land who are from White Oak or anywhere there, we are just around the corner.

The main points that I hope you take away today, are a couple of details about *C. elegans* biology and how those will relate to predictive toxicology and how they might hold us back from using this particular model from toxicology. In this case we want to use another model which is more appropriate. I'm going to be comparing this model to a couple other different models, as well as talk about some established methods. Some of the methods that I have been using myself and the utility of this model for looking at mixtures.

So, the *C. elegans* worms, the adults are just visible by eyes; if a new adult was only six eggs inside of it, it's approximately a millimeter long. What is great for the *C. elegans* model for toxicology is that the cuticle is transparent, they are hermaphrodites. So here we have an unfertilized. It gets fertilized as it moves through the through the uterus. It can be visualized in real time. So, alterations in timing of various events can be watched in the live worm. There are many transgenic strains that are easily available. We don't have to start from scratch. This particular one is green fluorescent proteins. The protein is produced in muscle cells. It is translocated directly to the nucleus. This particular gene begins to be expressed just prior to hatching, so you can see it in the eggs.

So, many of these tools are available making this model quite useful. Here we have a transgenic and the transgene it contains is the promoter. It is attached to green fluorescent protein. This particular transgene is highly responsive to activated stressors, so the worms are not green at all. In the absence of oxidative stress --- and they turn green quite quickly in the presence of oxidative stress. They can be used to monitor modes of action. As I said this isn't the only transgenic that is out there. You can just go to the CDC, you can buy them, and you can look at various pathways of toxicity, and different transgenic worms. So here is an example of that. We are looking at two transgenic worms, one that has a heat shock protein and the other that has the oxidative stress marker that I have showed you. Looking at four different types, of arsenic, to organic, to inorganic, we can see with our heat shock protein that responds to physical stressors. That only with the very toxic arsenic, oxide, and sodium arsenate, which all of these are the same concentration by the way, you see this slight increase of a heat shock protein which has this concentration that arsenic oxide is slightly toxic. At the same time, we have a tenfold increase in our oxidative stress response marker, whereas just in the million cells we see that there is very little response at the same concentration for these two other organic compounds. What's really key for using the *C. elegans* model for predictive toxicology is this was done in a single day by an undergraduate in my lab who trained with me for three months. So, these types of methods can be used and done using standard liquid culture techniques that most labs have standard pipettes for handling cells. Here is an example, of increasing concentrations of sodium arsenate. And before I talk more about this I just want to point out that this data is not all over the place like it looks with these air bars. All of these low points are from one experiment. All the top ones are from another experiment. We get really nice dose of responses. The thing about this is they are very responsive to their nutrient status, to their temperature, we could go a long way towards quality control for our cultures. You can only maintain your lab temperatures within a certain range and so, these changes represent differences between individual experiments, but our dose responses are quite nice.

Here we are looking at sodium arsenate. I chose concentrations for lead based on similar effects on larvae growth. So, we had approximately 15% reduction in larvae growth. So, looking at lead acetate, we showed concentrations that would match that toxicity for larval growth. So, we see as expected, in Mammalia systems, that it definitely is, and oxidative stress are. It certainly isn't anywhere near as strong as sodium arsenate and when we mix those two together. We see that our oxidative stress is not really different than sodium arsenate alone.

So, this is a type of mixture assessment that we can do with *C. elegans* in one day. There are many pathways that are conserved between *C. elegans* and mammals. This pathway is an example. Before we even knew that these pathways were involved…they eventually won a Nobel Prize for this discovery because it was found that they had found the same systems. It can actually replace the Ced-9 gene with full functionality in *C. elegans* that are mutants for the Ced-9. Their cells are post mitotic so it would appear that this model is not really a great model for carcinogenesis because they do not develop tumors. But, if you look at the cellular level, the individual pathway is much conserved. The trial endpoint may not be the same at the level of pathways and pathways disruption, the function is conserved between *C. elegans* and humans. ATP synthesis. Not only that synthesis and the respiratory chain but also what genes are encoded in the nucleus and what genes are encoded in the mitochondrial DNA. This is really important for aging and metabolic disorders.

Many elements of this machinery were first discovered in *C. elegans* and what's neat about the model, is not the cell three homologue that is translocated into the nucleus by similar signals that are either external to the cell, or internal to the cell. What you can do with worms, is you can actually watch this GFP signal. This is making it ideal for watching the response to this particular pathway. Many pathways that are involved in human development are conserved. This is a cell culture plate, the reason I have this picture here is because I want to make the point that *C. elegans* can be maintained with the same techniques as cell culture, except you do not need a CO_2 incubator as long as you have a temperature-controlled incubator. In the space of a single rat cage, you can stack maybe at least 20 plates here. If you have 1000 worms per … you can look at 10 different compounds in the space of one single rat cage. Importantly, many of the assessments can be done in a single day. The larval growth assay can be done by a by a researcher with an undergraduate degree and you can have data within a week.

So, while *C. elegans* lack eyes, they do not have a skeletal system, but, many of their responses are similar to that of Mammalia systems and it’s certainly because they have intact reproductive systems. While they do not have an endocrine system per se, they do have tissue to tissue signaling. Their neuromuscular junctions are nearly identical to ours; they use serotonin and dopamine the same way I do and you do. And so, that makes them a good intermediate between cell-based assays. At the same time, while this has been used since the 1960s to study development and to study patterning, to study cell to cell interactions, they have not been used for toxicology for very long and so, validation studies are really needed so that we can determine where can we use *C. elegans* to predict human toxicity and where can we not, because it's really important to determine it for purpose.

So, in comparing *C. elegans* to zebrafish, zebrafish embryo model which is an excellent model. If you want to look at skeletal development, zebrafish have pack six and just for us, that controls their eye development. You are going to need to stick to a model that has a skeleton, eyes, a heart, and lungs. *C. elegans* does not have any of those things. On the other hand, they develop from an egg to egg-laying and that is only in three days. It's important for the food safety and individuation that they are an oral toxicity model versus the zebrafish embryo which

isn't absorbed as well. Looking at the toxic substance control act, if anyone else was like me that was at SOT, everyone was talking about section 4H and how we reduce testing on invertebrates, but also we make sure that these invertebrate models are providing information that is as good or better than the tests that we are using. So, that leaves us to animals like *C. elegans* which are invertebrates. Just looking at this older study that was done looking at behavioral toxicity, they used toxicity as an endpoint. They were looking at 13 organic phosphate pesticides and looking at motility, they found a significant correlation when they ranked the effects on *C. elegans* motility for those pesticides to rat and mouse responses. Additionally, for eight of those 15 councils, they also assessed them for activity, and of the eight that they tested, I have the activity in mammalian systems, seven of those also had the same activity in worms. So, not only are we able to look at and endpoint, but which is, in organismal and point of movement, we can also look at a response. That is a mode of action such as activity of essentials.

Looking at different assays, this is an egg viability assay and they are looking at 57 different chemicals with mammalian reproduction affects. They found that while the sensitivity of this particular egg viability assay was low, the specificity was high, and while it did not detect all of the mammalian active chemicals, the positive productivity was also quite high. Additionally, when they looked at the activity, they found many similarities between the responses, and when they looked at toxins with intermediate effects, and they did a knockdown of P450 compounds, they transferred those from intermediate toxicity to high toxicity, so again, we have a conservation of mode of action.

This is an assay that was a major study that was done by Wendy Boyd and Jonathan Lederman. They were looking at the phase I and phase 2 libraries of ToxCast. They were comparing *C. elegans* larval growth data in outcomes in rabbits and rats. They found a 58% concordance for the entire library of chemicals. When they looked at *C. elegans* versus either rabbit or rat, they found 52% concordance. So, we have a 6% drop in productivity for an assay that can be done by one person in four days versus a study in rats, that everyone here knows can take a year and would cost potentially $1 million and at least five people. Additionally, I just wanted to point out, that this particular larval growth assay … specificity and much higher sensitivity and what had not been done yet, is to look and see if we could take the egg viability that the previous group did, which had high specificity but low sensitivity and combine it with this assay and project better human response. These are the types of things that have yet to be done.

This particular assay was done with a machine called a flow cytometer. So, if you have an object and it passes by the laser—the amount of time it takes to pass the laser is called the time of flight— and it's an indication of size. Additionally, you can measure optical density with that. In the previous assays that I showed you, we were using this machine because it also detects resin levels. So, one of the things that you can do, and that we have done is this larval growth assay, so we were looking at nano silver toxicity. When we were looking at nano silver toxicity and we were looking at ways to assess nano particle toxicity, we know there is a huge growth curve, especially, if you're used to buying your chemicals from Sigma and trusting that they are going to be just like you expect them to be. Nano silver work is not quite at that level yet, so we were working very carefully with our manufacturer and we were testing each batch for toxicity. We were getting really consistent results and all of a sudden, we got a new batch and it was way more toxic. We then went back and spent a great deal of time and ended up finding out that their supplier contaminated their source of citrate buffer, with the toxin. So the beauty of this test is that it's completed in four days, we didn't know what we were looking for, and we were able to find out something about this product, and then we were able to go back

to the manufacturer and they spent days and weeks to find out what was wrong but at least they knew something was wrong. So, up to now, we have been looking at gene expression, we have been looking at motility, and now we're looking at effective concentration for an endpoint of depth.

Here we have the *C. elegans* as human cells, if you incubate them with a premium I-9, they fluoresce, the dead cells fluoresce, and we were able to show that ranking in *C. elegans*, for different concentrations corresponds to rat oral IC_{50} rankings, as well as with these wide-ranging differences. When we use …, likely we would not have this perfect correlation of ranking, but if you look at not toxic, mildly toxic, very toxic, we are pretty sure that we will get similar results. So, here is an assay from another group which did something very similar to ours. And they also found that the correlation between the affective, the IC_{50} in *C. elegans* correlated to the rat with IC_{50}. That was only after they excluded the highly acidic chemical, so I want to point out that this is a liquid culture. And this restriction also applies to zebrafish assays and it also applies to cell-based assays that the sell you. The solubility and temperatures affect the outcome. So, we got these lovely correlations, I should say they got these lovely correlations with rat and mouse, only after excluding the chemicals that reduced pH to below three.

We have here, cadmium induces intestinal abnormalities in mammals. We can treat them with cadmium and look in a microscope and see the cells on the attack intestines which correlate to the abnormalities, so before we can show you how to assess this with the compass, I want to look quickly at mercury. Here we have low concentration mercury. You see a slight increase in auto-fluorescence, which indicate damage of the cellular level. At this low concentration of mercury chloride, which is when we quadrupled that concentration, we see the auto fluorescence sites and we see an apparent decrease in size, as indicated by time-of-flight, with our machine and also a decrease in optical density, whereas is concentration, those two values are approximately the same. When you look at cadmium, suddenly this non-toxic 7.8 parts per million is a very large difference between optical density, which you can see here. Not only can you look at this quite quickly, you don't even have to look in a microscope. You can run it through this machine and immediately see that this difference is indicating that there is something wrong. But optical density, looking at the different larval stages, this is a capital one larva. And between each one of the larval stages, *C. elegans* goes through a process. This worm is quite straight, one of these things is not like the other. It is not eating, it is not moving, it goes through this process for about an hour where it detaches itself from its cuticle, wiggles out of the cuticle, and then goes back to eating and moving. So, this happens between each larval stage, each time it sheds its cuticle.

Using this I thought, how can I use this knowledge of *C. elegans* biology to develop a new assay for developmental neurotoxicity? This is a narrow tracker and it detects population motility. So, if worms are in wells, each time a worm wiggles past because of the beam interaction is then recorded. And the reason that I was so excited to try this assay is that this machine costs 5% of what the copious costs. So, if I was successful, I could not only have a great new assay, but also labs that can't afford a copious could potentially be working with this new assay. Sure enough, it did work. So, here we have the first larval stage. They stopped moving, they shut the cuticle, they start eating and moving again into the second larval stage. That is what this looks like, so we went and checked, taking my copius images, and sure enough, a six larval stage looking here, at 27 hours at the peak of this, what appears to be the second larval stage, again, by looking at that biology at the second stage, so we were able to confirm that this assay really is detecting the development of *C. elegans* and it corresponds to the stages.

So, what can we do with this assay? So, we know that low levels of mercury cause hyperactivity both in *C. elegans* and in children. That can be affected by nutrition status especially omega-3 fatty acids. We want to show that new levels of mercury and we show that mercury acetate has two micrograms per milligram, and when you see this increase in peak height, that is an increase in activity and when you see a light shift of the peak that is an indication of developmental delay. And so, as we increase three micrograms per mill, four micrograms per mill, you see increasing levels of hyperactivity and increasing levels of developmental delay. When we went up for four micrograms per milliliter to six micrograms, we lost our hyperactivity and we got increasing developmental delay. We know that Mercury causes reduced weight gain in developing rodents, and you can imagine if you look at this time point right here, these worms are still at their second larval stage. While they are too small for us to measure their weight, you can imagine there are quite a bit smaller and so, this and point corresponds to weight gain in rodents. Looking at higher concentrations, when we first saw this data we were like yes, this is hypokinesia. Looks like this was lost in synchronous development. And so, in doing quality control, as we move forward with this new assay, we are going to be looking at how high the peaks need to be relative to the depth of the valleys. So, before we can say that we have lost synchronous development, however, going back to hypo Canadian mammals, when you look at adult *C. elegans*, we see there is a clear dose response. There is this hyperactivity in the first hour to which we believe is an escape response, and so, that hyperactivity is dose responsive and over time they show hypomotility.

So, again showing with some endpoints we can directly correlate the mammalian response to *C. elegans* responses. So, going back, looking at over three, no, we did this one five times, you can see it is very small. We have a significant reduction in growth at three micrograms per mill, of Mercury acetate, which when we did the same experiment with the older technology, which again this machine costs way more than this machine. The micro tracker, the lowest concentration was 10 micrograms per milliliter, so increased sensitivity, lower cost, that is what we are always looking for. Looking at arsenic exposure, which also causes hyperactivity in rodents and human children, especially at 100 micrograms per milliliter, is way higher than our concentrations for hyperactivity, in Mercury. We did definitely see the developmental delay and hyperactivity with arsenic.

Multiple studies have shown hyperactivity with arsenic and hyperactivity at low doses, arsenic with rodents. Hyperactivity at higher doses. So, we were also able to show that at 200 micrograms per mill, we see this low, high peak. This is actually the third larval stage, so developmental delay is over 10 hours and there's decreased activity. Again, corresponding to the mammalian response. So, I feel pretty confident at this point, we have only tested five different things with this new assay, that we are reliably detecting developmental delay. The changes in activity peaks are somewhat more variable. We are working on standardizing the number of worms per well. So that we can more precisely tracks this, but this data is from three separate assays, where this moved, I will just tell you that for the third larval stage, you can see clear significant increases in activity at 100mL and decreases at 300 micrograms per mL. So, confident about the developmental delay we are still looking at the smaller air bars, looking more consistent results. But, it's definitely promising for our neurotoxicity assessment. This is a symposium on mixture assessments, and so again, I just want to stress as hard as I can, this is only taking four days from start to finish. We are looking at Mercury at four micrograms per milliliter, arsenic at 60 micrograms per mL. Together they cause a clear increase in a developmental delay. Also, a decrease in peak height. So, if we look, at the developmental delay, the clear differences from the control— this is a comparison of two controls. I know that for motility, we can get up to 5% change from one control to the next.

That's what we are looking at decreasing. The changes in motility can be quite different at least at this concentration. When we have looked at these concentrations, we saw that the shifts were different. The reproducibility was quite a bit higher. So, we think definitely we can detect developmental delay, maybe we can detect the neurotoxicity as well.

And so, moving forward, we are planning to do a validation study, with this assay, in the hopes that we can detect and see if we can use this assay to separate neurotoxins from developmental neurotoxins. Separate developmental toxins from developmental neurotoxins, that is the goal of our validation study and that's what's coming up next. So hopefully I have convinced you that *C. elegans* assays are rapid and inexpensive and that's what we need from mixture assessments. There have been many correlations that have been demonstrated, they are not good for everything. We need more studies to look at demands of applicability and choose your model fit for purpose. But for the things that the model is good for, it's really good for and it's fast.

Audience Question: As you expressed your results in a concentration manner, have you thought to think towards how those *in vitro* doses would correlate to *in vivo* exposure?

Hunt: That is a great question, thank you. I think that we will not be able to do that. But, what we have been able to do is show that ranking corresponds, and so once we have enough data, we may be able to say, okay, well here are these components that have been found in contaminated wells. And we know that these already have known effects, they already have known pharmacokinetics and they rank like this. From and point X. Therefore, I think eventually we will be able to use that ranking to predict doses in humans. I don't think that we are ever going to be able to directly predict the dose from *C. elegans*.

Extrapolating New Approaches into a Tiered Approach to Mixtures Risk Assessment
Mike Dourson, Toxicology Excellence for Risk Assessment, Cincinnati, OH

Okay, now for something really different. First of all, I want to thank Susie Fitzpatrick for inviting me to this conference, it's good to be back. I have been to one of these prior and I found it delightful. I do really mean this is going to be something that is quite different than the prior discussions. For all the folks in the audience that do risk assessment, you will understand why, because there is a lot of good science here. Risk assessment is taking this good science and other people's good science and trying to make some sort of decision for human or ecological risk. Because we get problems like the BP Horizon disaster where you get oil into the Gulf and then you have to make a decision, you have to advise your managers, which oil dispersant do you use? Gosh, you need to give me an answer by noon today, what time is it? Oh, 50 minutes, right? You have to advise on the basis of what? On the basis of whatever you had. Flint city had led in the water. The lead levels as I understand, I could have this wrong were 26,000 parts per billion. That's a lot higher than EPA safe level of 15 parts per billion. Okay, so what other metals are people exposed to in the food, there was not only the lead problem which we have solved right now, but the national question is being exposed to other things. How do you advise? So, these are the kinds of questions that risk managers need to answer and risk assessment people need to address somehow. So, what I'm going to do is I'm going to step through some of this, and fortunately we do have some guidelines, and so we will

step through the guidelines a little bit. Then, I will try to see where some of this wonderful research fits. So, I am going to press this button. Oh, there it is. I don't have any complex of the subject. No one is paying me to do this, I have been paid by a lot of different people, to work on risk assessment and chemical mixtures in particular, primarily the U.S. EPA. I'm going to use their guidelines and also the U.S. agency for toxic substances and disease Registry. There is also a lot of work out of the European commission. It's not that we have not been thinking about chemical mixtures for a long time, we have been as a group. Even goes back to the early 1950s, 1960s, the American conference of governmental industrial hygienists has an equation for how to deal with chemical mixtures.

Okay, so after this, in one sense, mixtures in food is easy, it's just an easy question because it is a mixture, right? You can have all sorts of things and food, here is your thing about coffee. You can't quite read this, this is from Mr. Di Justo. You have cockroach, you have antioxidants, you have bacteria, rotting meat odor. 20 to 30 of them are carcinogens. The focus of this lawsuit was the chemical and coffee, and the question is, is coffee harmful? Here in California perhaps it is, I'm not sure what the result of the lawsuit were, but they wisely stepped me away from testifying, or not, I don't know. They decided to go a different way perhaps.

But, as toxicologists, we have done studies on coffee. There was one study in Swiss mice, they were fed instant coffee up to 5% of their diet, which is the max. You cannot go higher than 5% because it disturbs the nutritional balance of the animals. They found it was anti-carcinogenic. It was not negative. It was anti-carcinogenic. Now, was that because the mice are running around all the time? Who knows. They might not have even had exercise, when we opened the study, it's a little bit back, but the results were confirmed in rats in 1984, so it was the study beforehand and, in that study, the rats, that's all they got to drink ever, was fresh brewed coffee. Okay? That's pretty cool. Anti-carcinogenic. So, is coffee harmful? Well, the toxicologists would say no. It's not harmful. Does it have chemicals in it that can cause cancer? Yes. Part of the idea backup, or however we want to do that, is we get the steal slides from people.

So key definitions. Aggregate risk is a single compound. Cumulative risk, or mixtures risk, are multiple compounds or stressors. The EPA made a distinction between chemical mixtures in 1986 in 2001 I think was the update and the added stressors. Actually, they have been really good adding the idea stressors and we have very good people that have been mentioned before that are leading the charge down there. I have been to some meetings we have talked about chemicals and stressors together. That's the idea of cumulative risk it's going to be chemicals and stressors. It's mixtures what you are going to add some non-chemicals there. Multiple exposures, okay, we have a definition there. Again, as Dr. Hayes said, this is really how life works, so when you talk about mixtures and food, we have been talking about you know, food as a mixture.

From a risk manager point of view, you need to advise your management on what to do about food. You cannot say stop eating potatoes because of the acrylamide, or the only way you can eat potatoes is to boil them because then it might form. People are looking for ways to eat potatoes to not have it form as much. Or they can say we need to emphasize golden fries. Why golden fries? You don't cook them as much. You don't produce as much acrylamide. You don't have to label it. Right? That's been a big push for golden fries. Now, my better half is a lawyer who loves to do oven fries. The ones that she likes the best are the ones that are not golden. I keep trying to advise her that is the way. When you go back to the 60s or 50s for occupational, they had an equation and it really talked about additivity. We are going to take chemicals and we are going to treat them additivity. They did have things like synergism. And

we all have examples of synergism. When we were toxicologists, we were given a study where we did LD50's in rats and we gave them one pesticide and another pesticide and of course, they were being baited by the professors, right? Then we gave those, and nothing happened, then we gave the two pesticides together. And of course, you turn around and the rat died, right? We were all in shock. Did we do wrong doses? The professors all knew that and that's what they were teaching us. You also have antagonism. So, why is it that the coffee has so many carcinogens and mutagens and you get anti-cancer effects? Well maybe it's the antagonism. Maybe the concentrations are not sufficient to really cause the cancer to begin with and after all, coffee is mostly water.

Potentiation and correlative effects. So, in 1983, the national economy of science comes out with decisions that are risk assessments for the general government managing the process and they had this idea of assessment versus management and laid it all out, and then the EPA and the FDA and other groups started putting together this idea of guidelines. This is the slide that we use for all our managers. It is very simple. It is very effective. We got a mixture. And you test the mixture of your concern. You want to find out if EDTA potatoes are bad for you, do an experiment with potatoes. Or a sufficiently similar mixture. I was there for a 60-day detail. We had file drawers full of PCB levels in fish. Dr. Clark said we need to do something with all of this data—there were nine files full of data. I said, what do you want to do? I knew what the PCB levels were in the fish and it was all well laid out and I went to the integrated risk information systems and I knew that we had some PCB reference doses that are acceptable daily intake, to use the former word for different mixtures. The problem was, they were not the same as what we are finding in the fish. The PCBs in fish are weathered. I said, Dr. Clark, these mixtures are not the same. Well, EPA came out with their idea of mixtures of concern, or sufficiently similar. He said to me, are they sufficiently similar? I said I do not know how to judge, as is well I do, I said yeah, well, he says they are. I said well, Dr. Clark how do you know that? He says because I said so. And that was the manager's response to dealing with the problem. What's the problem? I have a loss of data and I need to answer this question. You are going to tell me. You got a PCB input reference dose and it's how close? It's close enough for me. The result is no decision at all and we are not doing that.

It turned out that chemicals were in the lakes due to one site location where they had taken marine oil that they had put PCBs in and PCBs were good to prevent fires and oil causes fires. You put the PCBs in, you have less than a risk of fire, so fire on a ship, that's not a good thing. There was some good here but as a result of all of this dumping of oil, there is a lot of PCB contamination in the Great Lakes. We remove the PCB from the Great Lakes every time you eat perch and things like that of the Great Lakes. That's how we get rid of it. Okay. I am not saying do not eat perch, they are very good.

So, what has the EPA done? They have done an expansion of the simple, so that was the manager side, this is the slide that we all kind of look at. Ask yourself are data on exposure, does fonts and mode of action available and acceptable? Do you have data on the whole mixture? Well, if you've got that, then you are going to do the home mixtures approach. Then somewhere on here is the line, it does not matter. There it is. Okay. So, you really want to do data. We have risk assessments of date is on the whole mixture, we have gasoline which is a mixture. Then we have PCBs that are mixtures. I'm sure they have examples of this, data on similar mixtures, probably. We have some of those decisions as well. And groups, group of similar mixtures, what you have, you have ways to approach this. When you have the toxicity on the mixture, or similar mixture, of concern. Most of the time, you do not have it. Most of the time, you are stuck with components. I think this is what Dr. Hayes referred to earlier by saying, we have kind of pushed at this. It's hard to test them. Once you test the mixture after

months, and you get into the environment, it is weathered material. It's not the same mixture. Do I have to do this all over again? Dr. DeVito is right, we do not have time or money for this. So, it's easy to stay and take components of toxicity, we do this all the time. We have reference doses and we can do this. So what EDTA would say, is take this. You have the exposure, the ADI, and you have hazard quotient. You have hazard questions that are less than one and you need the exposure less than the ADI. But the point is, you add them together and if you get a hazard index over one, you might have a problem.

So what EPA has done is they added them altogether and that they have a problem, then you get a little bit better and you say well, maybe we should just add chemicals together that have the same toxic effects or similar modes of action. So they go in a triage and they kind of narrow it down. This is what they are doing now is they're saying okay, if we have similar toxic effects and similar modes of action, then we are going to do dose addition. We are going to treat the chemicals as they are replicants are substitutes from one to the other. Now, if you have the same toxic effects, but it is an independent mode of action, you do a response addition. So instead of adding the X axis, you are adding the Y axis. If you have similar and different effects similar to modes of action, then you do the interaction. You just treat the chemicals as if they are separate. At those sites, you get people who had everything together. They had a problem of the hazard index, okay fine, then subdivide the chemicals and then go into the same toxic effect, independent modes of action, you start to think that way. It has a very nice way of approaching how you do the independent addition. He's got some publications in the mid-90s that will give you directions on that. The European commission also has the similar kind of construct and I'm not sure with this, but a little bit, they are almost all the same here.

You have significant human exposure, likely, well no. While you don't need to do anything, well this is again for the manager, no exposure, no risk. We have to get that to our managers. You know, it's the same here, noted a significant exposure to the ecosystem, then there is no risk, if you do have significant exposure, is the information on the mixture composition available? If we do not have a safe dose, a TTC, a threshold of technological concern, and the lowest one out there is.15 micrograms per day which is about 10 fold maybe 100 fold higher than the LD50 for that toxin. More in that range. So, when you have an exposure that's less than 1.5 micrograms per day, you do not have a problem. According to the TTC construct, then of course you have different instructors and different levels of TTC and I think the highest one is 90 micrograms per day. The point here is, you do have similar kinds of thinking to the US EPA and for the next part of the talk I'm going to go through some of the research that has been presented here and I am going to fit it perhaps poorly. We will see. We will see how the authors and our prior speakers' response.

Into the structure of U.S. EPA framework. This is from the risk point of view. This is what I need to be able to do. I need to be able to take this information and advise a senior manager somewhere, what to do because he has to do something. At those sites, if you do not make the evaluation, the chemical is not even considered. Is that a public health protective? I don't think so. That's what we do right? There is a big push to consider, you know, to get safe doses or TTC's for all chemicals. Okay. Let's go forward. It's not going forward sir. Do it again. Oh, there it is. Okay. You have seen this conclusion; high throughput screening can provide screening level information on biological activity. Moderate throughput screening, libraries of smaller libraries that can do hypothesis testing. Oh, even better, right? Risk persons say yes. We get all these epidemiology studies and they are good hypothesis generating studies. They are great at that but we don't necessarily draw conclusions just yet on each thing. Okay, combined chemical and biological data to enhance the ability. Okay, fine. Similarity

approaches, so we can use prototype mixtures that have sufficient toxicology and I believe, there is a fingerprint somewhere that Dr. DeVito said I thought was perfect. We can fingerprint the mixture. I'm not quite sure what that means but it sounds good. I can convince somebody a manager, that, no fingerprinting the mixture, ma'am. Then they will say okay, good, go for it. I've got other things to do. Okay.

So, where did Dr. DeVito's, where did his work come in? You can test and fingerprint the mixture. So, you can take one particular oil disbursement in the Gulf and you can do a mixture on it and get some sort of heat map. I'm sorry Dr. DeVito. It's like a fingerprint, right? Then you can take another oil disbursement that had the same fingerprint or not, or if it had something different and you can start to say well, wait a minute. This fingerprint looks somewhat innocuous compared to others. And I suspect that something like that was done for the oil disbursement and picking the one that went over the Gulf of Mexico. There was some background done on this, I just don't know the details. If you happen to have a mixture that has known toxicity like gasoline, you can fingerprint that gasoline and compare it to other fingerprints. Does that help you? It seems like it would help. I'm not quite sure how yet. But that's just his idea of the fingerprinting and to me, the high throughput screening, the most effective use of it is in chemical mixture assessments. We need to keep working this about how. I'm not sure yet.

Of course, the high throughput, you can do mode of action and see different steps, regulated or not. So, that will help you. Many years ago, we had this, lead paper, and we are saying oh, the acrylamide is causing thyroid tumors in rats. Due to a dual mode of action, we are getting criticized for this dual mode of action, we sent it into a journal and got a comment back from the FDA that had just published a paper and said well, you know, you are wrong, and here is why. It was very nice of the person to send us the paper, because it was not even published yet and it was like well, okay, work on that one, why don't you. Well, it went to one of our colleagues and I said what do all of these regulated genes mean, and he was stepping me through all these things and I said oh, this thyroid, and I was like wait a minute, what this paper is showing me is something that we have associations with thyroid homeostasis, or I'm not even getting the right word that are different from mutation or the kinds of genes that were regulated if we had damage through the mutagenic activity. It looks like it was supposed to do a mode of action for the review that remained anonymous of course. We used that as a way of convincing the editor that they should probably publish our paper anyhow. The point here is, the high throughput was trying to gather modes of action and that is something that risk assessment people can actually contemplate because we are starting to do that.

Okay, now Catherine's, she had a different kind of, two different kinds of things. What she is basically saying is with this constituent characterization you have the literature. You have analytical detection that is advanced. You go through a decision to resolve questions. And you use this TTC approach for closing safety gaps. So, the TTC approach has been around for quite some time. In fact, I think it was an FDA concept many years ago when they were doing, taking food and finding there were lots of chemicals in potatoes. Not just starch, right? So, do we worry about all of those small levels of all these constituents in the potato or not? So, they came up with, it was not a TTC, it was called something else. I am apologizing. My FDA colleagues would probably remember. Threshold regulation, okay. And it was a certain level, I'm not quite sure where it was, but it was in the range where the TTC is now. It was a 1.5 microgram per day. It could have been. The TTC approach that I think most people are focusing on now was an effort about two or three years ago internationally at the World Health Organization. The FDA was a big player in that. Dr. Jacobs was, I think, one of the lead FDA

scientists over there and as a result, we are now having a way to go forward with the stress of ontological concern and it makes sense to me as a risk assessor.

So, in this game that the EPA is using, where does Catherine's data fit? It seems to be fitting very nicely in this area of dose addition. So, you have a TTC which is a dose, microgram per day, which you can put into kilograms, body weight, if you want to go that way. That's fine. In addition, you have the assay and restriction and you can read across to a dose or a dose response. These other alert tools, you can use the hazard index for mixtures. I don't think you can use it for response addition, because it does not give you a dose response curve. I'm not so sure about the interaction-based hazards. Of course, you can use TTC down here if you know the chemicals are different. You know, you just separate the chemicals into respective differences. And you can within a group do the TTC approach, or they end up individual chemicals. You add them individually. Now the logic behind that, from a risk perspective, is of the chemical that comes into your body and different modes of action and difficult effects that are presumably a threshold at certain levels and you are below. A separate chemical comes in and hits the different targets and different modes of action and your level is below that. Then you do not have a problem with the combined exposure. That is the logic behind this particular way of going forward.

Dr. Catherine Mahony also had other approaches. She talked about the CMAP and there was further work going on for this purpose where I thought this particular thing fit. It was in the mode of action, understanding of the fingerprints of the mixture, so it seems to me that they could use this in characterizing. Her examples were not quite that but characterize a whole mixture. Was it one particular chemical from the one extract? I think you could do this for chemical mixtures as well. It does not have to be a mixture of concern. It could be a stress factor. In light fashion, would we use Dr. Mahony's. We are going to go to the next slide. It does not matter. You can use Dr. Mahony's same particular thing here to talk about modes of action for component-based toxicity. So, that's what that next slide says bid we can go on. We can go to the next slide. It just shows that. Okay.

Alright, so now Dr. Hunt's information. I am looking at this and saying I did not know what *C. elegans* did before. I am thinking well, really, the last time I really studied this, I was a student out in West Africa and we were working with a medical doctor. They were trying to find a liver worm. That's the only thing I ever knew about *C. elegans* and I was not even close. Not even close, right? But, the more I got into this lecture, I am thinking okay, mice and rats are not very good, 58%, 52%, that's pretty good. As good as you know, a mouse or rat. I had this idea, that the neurotoxicity and we have the various lar base ages, and you can see the delays that makes biological sense. Yeah, you would expect hyperactivity in the beginning. Maybe for a variety of reasons, and then, you get a delay, where maybe, hyperactivity afterwards. And so, characterizing the thing for similar chemicals made a lot of sense to me. The real payoff was when we did these combined mixtures. Again, if you characterize this for chemicals that are already lowly characterized, it is nice. This is the whole point demonstrated for the model, but it's a mixture payoff. That was really helpful. What's really helpful here, I guess, let me see the next slide and I will do kind of a wrap up.

There are a lot of things, urgency where the *C. elegans* assays would work in both capacities. It would be easy to do this. You can take the different types of gasoline or whatever and you can do this pretty quickly. To see if there are differences in toxicity with gasoline, not that people care about that, but you have an *in vitro* well-defined risk assessment for gasoline. So, if you had a foodborne kind of mixture that you were concerned about, as well as characterized *in vivo* for the assessment, then you can take that and do similar mixtures and get pretty good

responses. You can also do this for component assessments as well, so you do not just have to limit it to mixtures, but you can do it for components.

I got to thinking alright, I am a risk assessment person. Which one would I pick? If I had to pick one, which one would it be? Of course, most are going to say what a silly question. You would not pick one, you would say just bring it all on. I want all of it, right? That's what the risk person is going to say. I am not quite sure, right? I'm not sure how we are going to use it, but if Dr. DeVito put the assay and Dr. Mahony with the CMAP and the *C. elegans* are all leading the same way, I'm going to feel more comfortable going to the senior manager and saying okay, we don't need to spend money on a $90 assay or, a type II assay for sure. But if we have to make a decision right now, within short order, which is often the case, I'm going to feel comfortable advising you to do this if all of these three things are in my toolbox. Because, what I do not want to do, no risk assessment person wants to do it. Okay, we got type II assays and we are going to make a decision and then if something goes south, badly, that would not be good. It's not good for public health and it's not good for the whole development of these alternative methods. Bring them all on. What I would do is suggest taking five chemicals of interest to the FDA and say okay, we are going to work all three of these, or four actually. The point is, that's what I would suggest to the risk person and I think that's sadly the end of the talk. Yes, here is the summary. Okay. It's the rule of course. It's not the exception. Dr. Hayes is right, we have kind of pushed it off because I feel really comfortable doing individual chemical assessments. We have pushed it off a little bit, maybe kind of embraced it. We have guidelines, alright? But, can we improve them? Of course. Then of course, new methods offer significant improvement and I would just say that we should maybe test them together. I don't see doing this individually. Let's work together on that, so thank you very much.

Roundtable Discussion
Moderator: A. Wallace Hayes
All speakers

Audience Question: (Indiscernible-low volume) risk numbers…so, I think the mode of action, people in academia are pushing yet in the analysis in the program are anywhere else. The mode of action information, technology information, all Department of technology, have not been used by either the EPA or other federal agencies in terms of calculating doses. Cancerous but noncancer research. So, my question is, I think you emphasized several mixture guidelines which you and I are both familiar with. 150 from all chemicals in gasoline, they use the benzene as a marker. As the most, ---- is the lowest level among all of the compounds you can think of. So, my question to you, how far is this information, that is coming from many other places, how can the information with the height of technology, if it can only be effectively used with different modes of action in the picture. Can you shed some light on that?

Response 1 (DeVito): So, we are in a sort of brave New World. I will look at it from the sort of the NTP perspective. Historically what has happened is one chemical was nominated to us. What we do is we studied to death that chemical and right when we are about to come out with the report, a substitute comes up. Then we start the whole process over again. So, now, with these newer technologies, these newer capabilities, I look at it from my lab. When you give me a chemical, I am going to turn that into a 14 to 28 chemical study because, that is what fits on a level plate and it takes the same amount of energy for my staff to develop a chemical as it does from 14 to 28 depending on how many doses I want. So then, I began thinking about everything that I read across approach, here is what you want me to study, here is the structural analog, here are the substitutes as we go along, that is the kind of data we are going

to be generating. We will go *in vivo* with maybe that prototype that has been nominated but we will be able to put things in different classes and groupings and sit back and ask how well does the one chemical that we have evaluated what does it represent in this class of 14 to 28 chemicals? Are there other prototypes we should have put *in vivo* and made follow-up with some shorter-term studies like that? So that when asked the question about this chemical, as we moved down, generating all of that *in vivo* data when the substitutes begin coming on the market or people are thinking about it, they can make more public-health-based decisions on which substitute actually comes to mind. Which is the most biologically active chemical? For drugs and botanicals, we have this really mixed bag of you want something that is biologically active, but it better be really specific on one end point, we do not want it to be this sort of, just hitting a broad range of biology. We would like to have some chronological specificity. I think these approaches allow you to get as Michael described, I want all the information on everything you can get me and that is kind of what we are bringing to the table. It will be less of the bioassay. Another way to think about it is when I'm done with a two-year bioassay in the mouse and there is clear evidence or carcinogenicity in a rat or a mouse, I would say it is probably carcinogenic in people. There has got to be a cheaper way to get to the probability and I think that is out of the mines that we have to be thinking about is not yes, no, but probability. What is the probability of something as carcinogenic, and how do we get to that probability and where do we decide to regulate? Those are the discussions we are going to be having in the next 5 to 10 years versus yes, no, in a rat or a mouse. I think we have to change our mindset into clear categorization and when you're thinking about probability and that is not going to be trivial. I'm still trying to figure it out for myself, so, anyway.

Response 2 (Dourson): Just a different perspective. First of all, as you all know, the EPA's guideline for mixtures has been around for a while. Also, the guidelines for cancer in 2005 that you were a part of. When those guidelines specifically say a chemical causes cancer, what is the first question you ask when you discover that? What is the mode of chemical action. Sorry. The question is how does the chemical cause cancer? It seems to me the high truth for the bioassay is going to be, sorry, do we still have a problem? That one is off. Did you turn that off? Okay. It seems to me that the high throughput is going to give us a better understanding of the mode of action that will allow risk assessment people to pick or sort among the different ways to do dose-response extrapolation. It's not going to allow us to do the response extrapolation necessarily, but it will give us an understanding of motive action where we can make that judgment. Now, maybe in the future, we can even use hi to input to give us a point of departure and the despond's, there has been some good papers. He came out with a paper that had quite a bit on that. It was with *in vitro* doses and the benchmark doses were comparable and reassuring and they were not offset by a whole bunch. So, I think mode of action, you are right, it's where we should be, as risk assessors.

Audience Question: To all presenters, the systems all require expert judgment to design experiments and interpret results. Every expert has bias. How do you create a system which accounts for individual bias and still delivers a transparent, reproducible result?

Response 1 (Mahony): I certainly will make a first offering. I think it relates somewhat to the previous discussion. The more that we can do to categorize mode of actions and adverse outcome pathways, then the more visible and transparent the situation is. Then we can show, I think kind of illustrating the need where test systems fit in terms of applicability relative to that kind of web of toxicity mechanisms.

Response 2 (Hunt): I just think that this question applies to all of science. Not specifically for this topic. The more we have established protocols for setting up a system, within which we

make a judgment, and the more we can make those protocols transparent, the more we try to have those protocols standardized. You know, even in medicine, they are doing check risks now and showing how much they can improve standard of care just by really simple checklists making sure that every patient with lung disease has their bed tilted, really simple things. So, the more we can standardize these methods for making these judgments, the better off we will be throughout all fields of science.

Response 3 (Hayes): As a journal editor, one of the things I struggle with continuously, is convincing people that their methods section needs to be sufficiently detailed so that others can reproduce their work. Quite often I find that people will submit papers that say, I carried out these experiments by modifying John Doe's methodology. What are the modifications? We need to have the information in papers that are prevalent.

Response 4 (DeVito): I have two points. One is, Piper actually presented something to me that was really compelling. It was that rats and rabbits predict each other's behavior 50% of the time and we are really confident about rat and rabbit toxicology data. For reasons that I really think we have to get off of this gold standard that a rat, a mouse, or any individual species, actually is trooper humans. It is truth for the particular species. We have tested it and actually it is truth for that strain. Maybe not the entire species. So, we have to get off of that to be analyzed. The other question about, you know is transparency. All of our raw data goes out on the web. It's all available. We told you what we did and here is the data, we have analyzed it, and interpreted it in our way. Good luck to you. You can do it yourself, you can analyze it completely different, and we think that is best. There probably isn't one way to analyze different data strains and that's what we are getting to. The more available the data is, the better analysis will be interpretation and what it has in the future.

Response 5 (Dourson): Okay, so I don't want people to confuse passion with bias. Many of us are passionate about what we do, and that is great. Passion is good. Biases are inevitable I believe, and so if the public or anybody for that matter is concerned with bias of a particular thing, the way to draw that out is to bring a group of people together and balance the biases. Avoid the conflicts of interest but balances the biases. That way you get this vetting process which is good. I have seen people say well, you are biased. Not necessarily me because what. When I look at it, it's just passion. You want people to be passionate about their work.

Response 6 (DeVito): Can I just add one more comment on this? One of the things I think is helpful is NTP and another organization that is developing system review, and why I think that's important is when you look at the paper and systematic reviews, is the methods. Were they blinded? How did they get the dose? Did they actually describe what they did? I think we are going to see in response to that comment is people are going to look and say well I want my paper to get an A. How do I get the A? Oh, here's the check list. I think when we take those approaches it will be a lot more transparent and we will get a lot more consistency from literature.

Audience Question: I am curious if presenters could comment on whether you think the regulatory paradigm to needs to shift to fully utilize these results. From my limited experience, I have some understanding of how decisions are made on chemicals on a chemical by chemical or substance by substance basis and maybe on a product. If you are kind of fingerprinting different substances and trying to pick the one with the most optimal characteristics, how does that play out if only one chemical company can leave you with one fingerprint? Does that make sense or is that an appropriate question for this panel? I was thinking with arsenic and lead together, they do worse. We already know they are bad. Probably the arsenic is coming from

one source and the lead is coming from another so how does that help us dealing with two agents agent that might be introducing these chemicals into a particular matrix? Does the regulatory paradigm have to shift?

Response 1 (Mahony): To your first question, I am a European. I'm still a European for the time being, so I am working in a regulatory environment in the cosmetics field where animal testing is in fact banned. The regulatory paradigm has already shifted I would say in that regard and yes, I think it needs to shift further. What I mean by that is we had this question of prediction versus protection. In a regulatory context, you may be trying to predict the outcome of a particular toxicity study to protect a rat 90 day, no well, but what good is that for protection? I think that is the question we need to start having. If my purpose is to make a human safety assessment the question needs to shift more toward protection of the regulatory goal versus protection. That is just my personal view.

Response 2 (Dourson): I would agree the regulatory process is shifting. Over in Europe, the first time you test a beauty product on an animal is when your teenage daughter puts lipstick on. That is the first time the gets applied to a human. It is shifting, and I am hopeful the methods that are being used are adequately determining the safety of the products being put on the market. Here is an example of perhaps the cart before the horse, you don't get anywhere that way, but we can be hopeful and if we have a good example in good fidelity and safety in Europe and maybe it will help us take the next step. It is hard for me is a risk assessment scientist to advise a senior manager on the basis of high throughput data only. I n fact I won't do it because I'm not comfortable. That doesn't mean I can't learn. I am learning even today, and maybe a bevy of these tests will help me be comfortable enough to make an advice to go a certain direction. The regulatory area is shifting because we are talking about this. This is really good.

Audience Question: (Inaudible)

Response 1 (Mahony): I would defer to piper for a study of the legislation in that regard. I don't believe it is not classified as a vertebrate.

Response 2 (Hunt): The question is what is an animal and what isn't? Legally, as far as I know in this country, *C. elegans* is not considered an animal in terms of GLP studies. Every biologist, myself included find that incredibly painful because obviously they are animals because legally here, they are not animals, but I don't know about the EEC.

Audience Question: It comes down to narcissism, right? What is an animal and what is not wax going on from that, I was going to say Dr. Dourson, when somebody gives me data and says such and such is a carcinogen, my next question is how much am I exposed to? I think mode of action is spending a billion dollars and not finding out the answer. That's pretty much been the case, isn't it? I have been trying to do mechanisms of carcinogenesis my whole career and it just doesn't work very well. It is not a good cost-benefit, but the risk is what is really important. That is the bottom line. What is the risk to me? If the risk changes because of the mode of action, I understand that if you are not very confident in being able to do that, that is sort of a dry hole, but we can rank risk on some basis and try to make some judgment about all of these chemicals. The range of potencies of carcinogens that we know is nine orders of magnitude. While we are messing around with these small numbers, whether it is two or four, we have nine orders of magnitude to work with. I presume toxicity is in that same range, and we can do relative risks. They are not going to be perfect but if we want to deal with 700,000

chemicals or whatever, we need to do a relative risk type of approach or I don't think we will be a will to deal with it.

Response 1 (Dourson): Ron, I agree with your comments and let me add a little bit to them. This idea of the FDA came out with de minimis risk, we're not going to worry about it. It is too small to worry about it. The law does not do with trifles or something like that. The TTC concept is similar. If we have an exposure to chemical a chemical is less than .15 micrograms per day, it is a trifle not to be considered even if it is a carcinogen. There may be some exceptions to that, and I'm sure there are but I just don't know them. If we can get across to our public that life is chemistry, everything we touch, everything is chemistry, dihydrogen monoxide is a neurotoxin. That is its critical effect. It is a flame retardant and endocrine disruptor. If you drink too much of this, that is endocrine disruption. We need to get across to our public that chemicals are toxic, and that is a safety assessment.

Audience Question: I agree entirely with you and I think that is the way we go. Everything we are exposed to is a chemical substance. It's all about defining the levels we can live with, and we have to live with them. Oxygen is very toxic, but we wouldn't be very good if we didn't have been the oxygenated pathways. This is ranking everything with respect to how much harm can it possibly do to us, and what levels are safe? What are the levels that are safe of everything we do, eating food? That is a little different approach, but I think it may be a much better approach.

Audience Question: If we have to test food mixtures, considering the numerous macrobiota in the intestines that have their specific growth requirement, for example, the probiotics, how do we compensate for their growth in alternative methods? I was like to end on a high note here.

Response 1 (Dourson): I would like my colleagues appeared to take a crack at that question.

Response 2 (Mahony): I don't have any answers, but I do think it is one of the next challenge in the new approach methodologies to understand the role of the microbiota and that is probably going to be some role…right? The absorption, distribution and metabolism. There are some tools out there. It would be really interesting to work on a case study that brought that kind of thinking into play, but I think it is a challenge we're going to have to wrap our heads around.

Response 3 (Hayes): I would agree it is going to be a challenge but isn't that just another mixture we have to think about as we move forward? The microflora?

Response 4 (Mahony): You are more bugs than you are human cells, so it is not a mixture to be discounted. It is subject a huge variability.

Response 5 (Hayes): Just like all the ones on the skin.

Response 6 (Hunt): I would like to add to that, models like *C. elegans* and zebra fish don't have intestinal mucosa like humans do. I'm not sure these smaller alternative models are going to do anything to even approach the answers. They do have their own microbiome, but that doesn't mean the way that microbiome interacts with their intestine is the same. We are going to need to give really creative to try to answer that question because I'm not sure the systems we are working with now are competent to answer it.

Response 7 (Mahony): Not to get into details necessarily, but I do think there are some systems out there that can be used in conjunction with microbiota models. At the end of the day, you have to first define the question you are trying to address to understand what models you need to assess and provide some answers where it's all about understanding the question generating a hypothesis.

Audience Question: My name is [Indiscernible] and I live here in Maryland. My comment has to do with the mode of action. If you're looking the way of the assessment guidelines by EPA, you're looking at an adverse outcome which is an over-toxicity if you want to call it. Unless you understand -- that is the biomarkers, the genomic markers and the -- markers and the other markers of exposure, and if you don't have one event predicting the next event in the next event, except in the case of leukemia, if you understand the mechanisms of the toxicity of leukemia and then you have the pathways. I think the dose response information is available for this particular marker and the next marker and the next marker, you can have an approach that I can predict using this. This is outcome pathway. Then you can use the mode of action information effectively. Otherwise we have a problem just describing the mode of action. We describe the mode of action. We rarely use the mode of action information except for understanding but actually we rely upon the rats and mice epidemiological data. Unless we understand this is the outcome pathway and the mode of action to it, I think we have a long way to go using the information in risk assessment.

Response 1 (Dourson): This is a great comment, let me give you an example from a years ago when I talked about a series of regulations of genes at different concentrations of formaldehyde in rat and nasal tissues. I'm not going to get the concentrations right, this was a lecture I didn't want to go into to because they did understand it but have parts per million the salad just sat there. It is being stressed and it doesn't respond and doesn't regulate anything. At the production of additional protective enzymes. I'm thinking that makes sense. If I was a cell, I would make enough protective enzymes I need plus a little more. It takes energy to turn on the machinery to make more enzymes. That they have parts per million of formaldehyde, the cells. It's there because it has enough protective enzymes pause a little more. At 2ppm it has to turn on protective enzymes. At 5ppm we turn on repair enzymes. I am thinking I'm at this lecture because I'm going to learn something today. At 10ppm the cell turned on pop ptosis. The seller saying I'm so damaged I can prepare myself anymore and I'm going down. The war thought about it is this description makes a lot of sense to me as a biologist and I can use this information in doing my dose response assessment or something along those lines. I'm with you 100% on mode of action but we need to use this a lot more. I have a challenge for FDA, but I can hold it until you are ready. (Go ahead.) I got to thinking as I put this final presentation together using everybody's information. Thank you for your slides, it made it easy. It seems if FDA had a chemical mixture of concern, you probably have dozens, but one that was either all in food or water or drugs or herbals, it seems to me you could take the five or six chemicals or the Sentinel chemicals of the mixture and test them in multiple processes and work that as a collective. Sort of like what they're doing now, like for endocrine disruption, there is this group doing all these things around this in addition to the two-tier bioassay. Is that something FDA is contemplating maybe in the future?

Response 2 (Hayes): It is certainly something you have asked them to think about. As we begin to wrap up our session, if I understand correctly, we are in the Wiley building. That seems to be extremely appropriate since we are talking about alternatives. As I think back in the history of the FDA, Dr. Wiley had the first alternative to animal testing. He had his poison squad in which he brought his gang of bringing folks to lunch every day and began to feed them on Monday and additive, a little bit more on Tuesday, Wednesday and Thursday and if

everything was okay on Friday, the additive could be approved. Unfortunately, we can't do that anymore, so we have to look for other alternatives and that is what we have been talking about today. I want to thank our panel of Michael, Michael, Catherine and piper for giving excellent talks. I want to thank Suzi for putting this together and to thank Betty for making sure everything worked like it was supposed to work, even to the point that she chased down slides that came in at the lastminute and somehow arranged through Suzi to get one of our speakers here. The organizing committee for all of these sessions, you have their names listed here and we want to thank them. Especially we want to thank you in the audience and you folks out in the web where ever you are, thank you, very much and again, help me think our panel for their talks. Have a great day.

[Event Concluded]