An Expert Roundtable Discussion on Mitochondrial Toxicity

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Applied In Vitro Toxicology: Special Issue on Mitochondrial Toxicity

An Expert Roundtable Discussion on Mitochondrial Toxicity

Mitochondria play critical roles in all cell types. They are involved in processes such as calcium storage and signalling, cell proliferation, apoptosis, steroid synthesis, immunity, energy storage and production. Inherent to these vital functions, mitochondria represent frequent targets for toxicity and disease. A plethora of toxicants are indeed known to affect mitochondria. Furthermore, more than 200 clinical pathologies have been associated with impairment of mitochondrial functionality, including cancer, inflammatory disorders, neurodegenerative diseases and diabetes. Both structural defects and functional deterioration may underlie mitotochondrial toxicity, and as such form key events in various adverse outcome pathways. Not surprisingly, mitochondrial toxicity is increasingly gaining attention by toxicologists and therefore was the central theme of this expert roundtable discussion.

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Dr. Philip Hewitt
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Dr. Harmut Jaeschke
University of Kansas Medical Center-USA

DR. MATHIEU VINKEN: Thank you all for joining our panel today. My name is Mathieu Vinken and I am the European Editor of *Applied In Vitro Toxicology*. The idea of today's panel is to discuss the importance of mitochondrial toxicity.

Now before getting into the discussion, I welcome you all to briefly introduce yourselves

DR. YVONNE WILL: My name is Yvonne Will. I am based in Connecticut currently. I have spent the last 16 years at Pfizer, really pioneering mitochondrial toxicity testing in industry.

DR. HARMUT JAESCHKE: This is Harmut Jaeschke with University of Kansas Medical Center in Kansas City. My interest is in drug hepatotoxicity and especially acetaminophen toxicity, and I've done a lot of work on mitochondria over the past 20 years.

DR. KEN WALLACE: This is Ken Wallace with University of Minnesota, and my interest is in mitochondrial toxicity as well, focusing somewhat on Adriamycin and the cardiac, but also looking at the molecular biology of mitochondrial biogenesis and turnover.

DR. PHILIP HEWITT: This is Phil Hewitt. I am at Merck in Germany. I am head of the investigative toxicity group here so my expertise is with *in vitro* and organ toxicity. I also have some experience with maitotoxin.

DR. MATHIEU VINKEN: Thank you. My main expertise is in the field of liver-based *in vitro* modeling and also mechanistic modeling, so I am really into these AOPs—adverse outcome pathways. Mitochondrial toxicity is a key event that pops up in a lot of these AOPs. This is one of the points that we will touch upon during the roundtable discussion.

So, the first question for our panel is: How indicative is mitochondrial toxicity for organ-specific toxicity? It seems to manifest as a generic event in a lot of toxicities, so how specific would that event be?

DR. PHILIP HEWITT: I could begin as I perhaps come to that question from a different perspective, before you jump into real details or mechanisms. I would say with my limited experience and also working in industry, within the investigative tox groups here in Europe, that there are not very specific target organs.

I get the impression that it is linked to certain target organs, like liver, kidney or heart, mainly because they are the most studied and perhaps we will find more target organs coming up as we study them in the future. So, I think it is not very specific, apart from

when you start looking at metabolism, maybe the liver is the main target only because of exposure and the metabolism the liver causes in the toxicity in mitochondria. I put that out there first as my generic response, before anyone jumps in with very specific cases, which are linking the mitochondria to a specific organ tox.

DR. YVONNE WILL: I agree with Phil. The organ toxicities studied mostly, at least in pharma, were due to attrition in the clinic and so, when you do the statistics, you do see that the majority is liver and heart. That association has been made; it has been extensively studied.

And then there were a few exceptions to the rule when you think about cerivastatin with muscle toxicity, there has been some neurological. So, I would agree with Phil that the most studied are the ones that really attributed to severe toxicity or death. But, if you look more into the academic field, you do find that it is applicable to any organ toxicity, starting really with sperm.

DR. KEN WALLACE: My impressions are that interfering with mitochondrial function is a universal event. It has the potential of occurring in almost any tissue and a major factor that determines organ-specific toxicity first is pharmacokinetics, including the metabolism, as you already mentioned. If it is not delivered to that tissue for pharmacokinetic reasons, through transporters or perfusion or metabolism, then it will not have a prominent effect in that tissue, so that would be the first thing, pharmacokinetic ADME kind of a consideration.

The other thing is you will see the type of adverse effects, but only those that you are looking for. So, in the heart, you might see it as a contractility or rate, cardiac function. In the liver, if you have a model system that depends highly on cholesterol synthesis or steroid hormone synthesis or whatever, you will see it there. But if your experimental model system does not have a prominent or a very high activity in those metabolic pathways, you may not see it. Basically, you are only going to see it depending on the experimental model and what you are looking for.

DR. MATHIEU VINKEN: That is a really good point, Ken.I agree, and we will be coming back to this when we talk about *in vitro* toxicity. Regarding the first question, on the organ-specific features, any comments left from Harmut, for example?

DR. HARMUT JAESCHKE: The only thing I would add is it depends on the cell as to how many mitochondria are available and how relevant the mitochondria really are for the function. For example, the liver has a lot of mitochondria, exposed to a lot of the chemicals in high concentration. That is why it is important. Other organs may not quite be as exposed as well as they do not have as much mitochondria. They are not as relevant, depending on the mitochondrial function directly.

One thing we have to be careful to argue is mitochondrial toxicity being the primary cause of cell dysfunction, versus an indirect effect. If it is an indirect effect, I think

mostly every cell type would probably be affected by that. If you start killing the cell, mitochondria will go down, too. The question is—is that a primary cause of it.

I think in testing and also in the pathophysiology, whether you want to target mitochondria or protect mitochondria against an insult, it is a big question if that insult really causes cell toxicity by initially targeting mitochondria or as an indirect effect. That will definitely be a question when you measure or try to analyze these things *in vivo*, what is the cause versus an effect.

DR. PHILIP HEWITT: I think you have hit the nail on the head there, as to how you actually define that and how you actually study that. And if you look at that question—is mitotoxicity indicative of organ toxicity?—I would probably change my answer to say 'yes.' It is not organ-specific, there are too many other factors involved, like you described, and the different mechanisms where you might be hitting the mitochondria, which is then maybe driving the organ-specific toxicity.

DR. KEN WALLACE: I would like to agree with what Harmut said—is the mitochondrial dysfunction a cause or consequence—it is important to distinguish between those two.

DR. MATHIEU VINKEN: Exactly. And this brings us to the second question, which more or less relates to this. How would you define or how would you consider mitotoxicity within the concept of AOPs. Ismitotoxicity a mode of action or a

mechanism of action?. Would you consider mitotoxicity as a mechanism of action or an AOP on its own?

DR. HARMUT JAESCHKE: I do not want to halt the discussion too much but I think it is a key event in an adverse outcome pathway. I do not think you can just put mitochondria out there by itself. It has to be really considered in the context of the pathophysiology; and it comes back to the cause or effect question. My vote would be it is a key event or it can be a key event by itself. Mitotoxicity is not an AOP by itself.

DR. PHILIP HEWITT: How I see it is I completely agree that there are so many different mechanisms and events that lead up to that or what would be the consequence of mitotoxicity, not that is an AOP itself. And it is involved in so many different things that it is a very important key event in many AOPs probably.

DR. YVONNE WILL: Yes, so in my laboratory, and others as well, we have studied hundreds of drugs for their mechanisms of toxicity and we sadly came to the conclusion that really the majority of drugs have multifactorial toxicity. The only drug I can think of that was probably rather straightforward as the purest mitochondrial tox, where you could pinpoint the mode of action, would be phenformin.

But, when you look at drugs, for example, inducing liver injury and there is enough out there in the literature now showing that most of them either have a BSEP (bile salt export pump) inhibition, a metabolite formation, another transporter, ER stress test, that is the

other thing. I have to agree that, 10 years ago, I would have said that mitotox, you know, is driving the toxicity but I think it is just part of that equation for really 99% of drugs.

DR. MATHIEU VINKEN: The reason why I brought this question up is because people in the AOP field really would like to make or establish an AOP on mitochondrial toxicity. I fully agree with you that it is rather like a key event in different AOPs than an AOP as such. According to you, would it make sense to make an AOP on mitochondrial toxicity?

DR. PHILIP HEWITT: I would be interested to see someone try.

DR. KEN WALLACE: I have a fundamental concern with this question. First of all, I agree that if mitochondrial dysfunction is part of an AOP, it is a key event. Certainly, it is not the end result. And the reason is that you may have mitochondrial dysfunction measured by whatever means you wish to measure it, ROS production, decreased ATP, mitochondrial biogenesis, whatever measure you choose to use and label it as a mitochondrial toxicity. It may or may not result in an adverse outcome.

DR. MATHIEU VINKEN: Correct.

DR. KEN WALLACE: My belief is that mitochondria are highly resilient and they are able to withstand a variety of different changes in environment. They adapt to this and so a lot of the changes that we report as mitochondrial toxicity are, in my opinion,

compensatory in order to avoid an adverse outcome. So, if I had my way, I would discourage all of us from using the word mitotoxicity and use 'altered mitochondrial function' or 'mitochondrial dysfunction' or whatever. The term 'mitotoxicity' itself implies adversity and, in my mind, it does not necessarily result in an adverse outcome.

DR. YVONNE WILL: I really like that, Ken. I think that is important for us, in this introductory chapter, to talk about. I am smiling as I listen to everybody because, for me and all of us, we have done this for a long time and we had a lot of learning from thinking about toxicity to admitting that it is most likely multifactorial, to Ken talking about the dysfunction, the adaptation, the lack of true toxicity. This is wonderful that we can talk about the learnings we have had in the last 10 years doing some of this more systematically.

DR. HARMUT JAESCHKE: I would fully agree with that. The mitotoxicity also implies somewhere that the chemical hits the mitochondria directly and, in many cases, mitochondria are a secondary target during the process of toxicity.

The issue with mitotoxicity, as Ken pointed out, implies a little bit something different what actually the involvement is of mitochondria in cell injury or cell death in addition to the adaptation issue. So, that is in addition to the question of adaptation. I fully agree with Ken; biogenesis, mitophagy and other things, the cell really tries to respond to an insult and depending on, of course, if it is a chronic insult or very acute, can or cannot successfully mitigate some of the dysfunction.

During cell death mechanism, the mitochondria can be involved very central to it but it may not be the direct hit. So, mitotoxicity implies that and I think what Ken suggested, mitochondrial dysfunction, or something like that, is probably the better term here to be used.

DR. MATHIEU VINKEN: I fully agree with that. Thank you, Harmut. Maybe we can move to the next set of questions, which is more about the *in vitro* testing of mitochondrial toxicity. Let us talk about *in vitro* testing, which brings me to the question: How relevant is the testing of mitotoxicity *in vitro* for the *in vivo* situation,? What are the most important biomarkers? What are the prominent techniques and *in vitro* models that are used these days, ?

DR. YVONNE WILL: I can share what we have learned. Again, I like to talk about learnings these days. So, there was a 2019 paper that we published and it shows that you really cannot do that. You can certainly assume that mitochondrial toxicity was organ toxicity, that you can do.

And one of the things you will see very soon is there is a special issue on liver injury that Eric Blomme from AbbVie is the editor of, and there are numerous papers in there where people have developed what they call a matrix, which is combinations of the mechanisms that could lead to a liver injury. And mitochondria are certainly part of the assessment and contributes.

If you assign arbitrary severity scores, so you say anything less than 30 times Cmax or whatever gets a score of 10 and everything over it gets a score of 5, you will get a predictivity towards adverse outcomes or organ toxicity. However, what we do know is that a simple *in vitro* assessment alone will not tell you which organ will be affected.

So, unless you have other very organ-specific contributing factors, such as an inhibition BSEP for a liver and you include that measurement, you will not even get close to predicting where it might happen. But there are several papers out there where the lack of predictivity towards organ toxicity is being demonstrated as well as the contributing factor matrix assessment.

DR. MATHIEU VINKEN: If I understand correctly, basically this would mean that the selection of the biomarkers and the *in vitro* models is to be seen on a case-by-case basis?

DR. YVONNE WILL: Yes, so an *in vitro* model alone will not tell you what organ toxicity you will experience.

We have made progress, especially in the liver space by knowing the additional factors and using computational and modeling approaches to actually build the relationship from *in vitro* to *in vivo* outcomes. And, like we said earlier, it is, at this point, a contributing factor, not per se a driving factor.

DR. PHILIP HEWITT: You could also say that you cannot predict the organ but an *in vitro* mitotox assay per se will also not predict organ toxicity mechanisms, or potentially additive mechanisms—a positive *in vitro* assay does not guarantee a positive *in vivo*.

DR. YVONNE WILL: That is correct, Phil, yes. And that is why there are a couple of papers coming out very soon on at least showing that progress has been made with liver, where once you do know the additional mechanisms, you can establish an *in vitro* to *in vivo* correlation.

DR. PHILIP HEWITT: But that does not mean the basic mito dysfunction screening *in vitro* assay is not worthwhile, especially in the pharmaceutical industry when you do it early enough and to screen out those compounds that have very potent effects on the mitochondria. Why would you continue with those generally? So, I think it is not like you should be picking and choosing assays. Some of these screening assays have a lot of utility still.

DR. YVONNE WILL: Oh, absolutely. You brought up a really good point, Phil. Within industry, there is a lot of learnings of how property space can drive certain toxicities just as it can drive positive ADME properties. So, yes, there is definitely a need, even in the absence of any kind of predictivity, to try to make sure you have better chemical matter. There is no doubt about it.

Ten years ago, we screened thousands of compounds, and then we learned. We learned about property space, we learned some of the structural alerts. Now, this has shifted to more of an *in silico* approach, where you know that you might be in a space where you do not want to be and that is when you do somewhat targeted testing.
Some companies that have embraced this early on, they can use a lot more *in silico*. The same is true for BSEP with respect to liver; it is the same thing. So some companies that have done a lot of the work can apply more of the *in silico* approach and then can go in with more targeted testing.

DR. MATHIEU VINKEN: Thank you. Harmut, you wanted to add on that?

DR. HARMUT JAESCHKE: No, I want to ask a question first. When you talk *in vitro* assays, are you talking about using isolated mitochondria and exposing a drug to that, or do you talk about cell lines or primary cells being exposed to the drug? That would be a fundamental difference.

DR. YVONNE WILL: Yes, correct. I can only speak for pharma, and every pharma company has a bit of their own testing paradigm. Some companies do isolated mitochondria, knowing the caveats, and then go into, for example, glucose/galactose model system or primary hepatocyte. Mathieu and Phil probably know under Horizon 2020, there was a big initiative in Europe, where they validated a particular glucose/galactose liver model for the prediction of liver injury. So, every company has a

little bit of their own flavor of what they do, knowing what the caveats are of the cell system, as well as the isolated system.

DR. HARMUT JAESCHKE: From my perspective, and it is more from an academic perspective, but what I see also in the literature is that no, you can really bark up the wrong tree by exposing isolated mitochondria to any chemical. That can lead you in a completely wrong direction. I would definitely propose using isolated cells as a minimum to do that. Isolated mitochondria, is to some degree, very questionable. I do not know what Ken's perspective is?

DR. KEN WALLACE: I see this as basically two different questions we are asking. One is more on the industrial side or regulatory side. From pharma—if I may take the liberty even though I am in academia—I would imagine that you set up a mitochondrial screen and the objective there is to screen out any chemical that has a potent effect against the mitochondria, so you are very willing to withstand false positives.

What you do is you take a sensitive model system, and it might be isolated mitochondria in this case, and you screen for a potential effect that the chemical may have on mitochondrial function. Then you compare the concentrations required to that, which might be achieved clinically.

Using isolated mitochondria, however, will not reveal any effect that your chemical may have on transporter systems or the citric acid cycle or fatty acid transporters or

metabolism, so it is limited in that sense. It is one question for the clinical application or clinical trials. To me, it is a different question in academe and as Helmut, you were getting towards, is to look for relevance.

You try to pick a model system that may be most relevant for extrapolation to the *in vivo* model. Take a primary hepatocyte, for example, and it has all these components that are active, including drug metabolism, drug transporters and intermediary metabolic pathways and such. There, you would avoid the isolated mitochondria and you would use a model system that is closest to *in vivo* that you can. But to me, it is a totally different question if you are asking for clinical screening.

DR. HARMUT JAESCHKE: I would argue a little bit against that. For us, for our screening, my experience was most companies are using HEPT-2 cells or cell lines. Only very few are still using isolated mitochondria. But, at the latest stage in development, when the molecule goes nearer to clinic, we would want to have a better mechanistic understanding what is going on and so we want those better maybe primary cell-driven assays, that are more relevant for academia, we would also want to see those as well.

DR. KEN WALLACE: Right, I do not mean to discount that. Again, I am in academe so I am speaking out of my field, but I would say the *in vivo* cell model would be like a stage 2 process.

DR. YVONNE WILL: That is correct, Ken. There is a tier 2. What industry does, they

know what their issues have been. They know what drugs have failed in animals or in humans and so they have put in place what they saw the most relevant.

And from there, if there are hits in whatever these assays are for that particular company, there is a tier 2, which looks more academic, very much to what you guys are trying to do. Can we have a better model? Can we go in with better biomarkers? Can we do gene expression? What is the best model system, absolutely? Just not when you have hundreds or thousands of compounds.

DR. MATHIEU VINKEN: So, we have been talking about the industrial relevance of all of this, but what about regulatory relevance to testing mitochondrial toxicity *in vitro*? Do you think that would be of interest to regulators?

DR. YVONNE WILL: Some of us have made that our lifetime dream and hope that that was the case. It has happened when you look at the transporters. There is an understanding how inhibition of the liver transporters can lead to adverse events and there is at least some guidance documents from the FDA.

Again, the learnings we had in the last 10 years, and mostly in the liver injury space, it has been with the exception of phenformin, probably the contributing factor. As such, I do not think that you have any case to push it as a regulatory mandate. I think the most you can get is an acceptance that it can be a significantly contributing factor and if you

have more than three or four mechanisms of toxicity, that you would have to show some certain biomarker requirements to monitor it in the clinic.

DR. PHILIP HEWITT: I personally am in the early discovery, not really going into clinic, but I would say the question is also quite slightly wrong. It has a relevance to regulatory. I mean, if you understand the mechanism of your drug as you go into clinic and it may involve mitochondrial dysfunction, and you have data to show it, you should submit it. And the regulatory agencies will highly appreciate it, but it should not be regulated by the agencies to say you need to provide this information.

Whether assays themselves or the data your generating needs to be qualified or validated also is up for debate. Normally, from my very limited experience, they are quite open for these types of assays and not fully validated.

DR. KEN WALLACE: I agree with what has been said. Any type of mitochondrial evidence you have would be supplementary to support observations that are made in a more robust model system.

DR. HARMUT JAESCHKE: I would agree with that.

DR. MATHIEU VINKEN: Okay, thank you. What do you all think the future will hold for this field? What are the main obstacles? What should be prioritized from the technical or conceptual point of view?

DR. YVONNE WILL: I do know what the issue is for pharma and that is to get from hazard to risk prediction. So far, we have not been able to do that, which hampers the uptake in the teams of saying, 'look, you have a 10 micromolar hit and multiple mitochondrial assays; you need to find a new molecule.'

So far, we have mostly only been able to show as a hazard and not as the risk. In the liver, there has been progress to get to more of a risk assessment but it is based on retrospective analysis of compounds we do not make anymore. Compounds with many structural alerts, metabolite formation, BSEP inhibition, ER stress, you name it, they have it all.

Generally speaking, pharma does not make these types of risky toxic compounds anymore. People ask for the relevance to the current portfolio but I would say this is really the biggest issue. You cannot get uptake if you do not know what the risk is.

A good example would be hERG. And even that has been revisited in the CiPA initiative but, for the longest time, there was an acceptance that a certain potency in hERG would lead to adverse events, and people believed that and people adopted it and it became regulatory mandate. That is what I know where pharma is struggling, that there is no risk assessment.

DR. PHILIP HEWITT: I can only agree. At Merck this is where I see the main

problem, the acceptance of the assays. We need to generate our own database of experience with these early assays and with the mechanistic assays like Seahorse. Then you can start persuading teams to use it and then, hopefully, we have some nice publications coming out on how to perform risk assessments.

But that is, to me, the key hurdle at the moment. We know we need it. I think pharma has realized that this is an important key mechanism of toxicity, but how we do it and how we present it to regulatory agencies is still unclear. How you mount that in the clinic as well, I also have no experience how you would do that.

In the future, we will definitely more *in vitro* testing and I am hoping improve in using maybe more primary cells rather than cell lines. Maybe the 3D models, I know that Seahorse—I do not know whether you do that at Pfizer, Yvonne, whether you use spheroids, that is an improvement. All these things need to come in and be tested and where models like Zebrafish *in vivo*, stroke *in vitro* assays. We have no experience whether that is better for predicting. I do not know. Do you have experience, Yvonne?

DR. YVONNE WILL: No, and I think one of the handicaps in all of this is that with the exception of liver, we do not actually have enough compounds to start modeling. You need certain numbers and certain outcomes to actually start modeling risk and we do not have that. So we would love to do this for KIT in testicular but how many compounds do we really have even in the large companies like Merck or Pfizer. There have been efforts

at times under IMI and others to try to push forward joint studies trying to get to this risk but it has not really come to fruition, I would say.

DR. MATHIEU VINKEN: Thank you for that. Ken, any ideas or suggestions for the future of mitochondrial toxicity testing?

DR. KEN WALLACE: I think that Yvonne summarized my major concern, and it goes back to using the term mitotox, distinguishing between risk and hazard. There is a value in doing both types of testing but I think encouraging our colleagues to be very discreet in defining whether it is a hazard identification or a risk assessment based on the model systems that they are using.

DR. HARMUT JAESCHKE: I agree, but on the other hand, I come from a slightly different angle compared to industry. If you want to look at the pathophysiology, you normally start with a disease or an injury or toxicity, and then you go into the mechanism. From that, mechanistically, based on the importance of mitochondrial injury in the pathophysiology overall, it is very important.

This is a different kind of approach compared to what industry is trying to do. You have a compound, you have no idea how safe it is and you want to test if there is any issue when you go into people with that. And so, the testing and the way you approach it is I think completely different.

DR. YVONNE WILL: Yes and no, Harmut, because that is how it started, right? Drugs attrited and people started trying to figure out why and that is how mitochondria were identified and transverse identified, reactive metabolite. So that is kind of where it started was the acceptance that, yes, every drug we ever attrited would flag in these effects. So, I think both exist. It is just the realization of it in industry resulted in establishing cells that spurred an *in vitro* testing cascade.

There is something else I'd like to point out. What is very important is to continue the partnership between industry and academia; it is because of people like Ken, Harmut and Hilmi who are helping us to understand the nuances, and all the different mechanisms and the adaptations. That was not even something we talked about until recently.

I think it would be nice to continue to encourage the relationship between academia and pharma because without academia, there is no way that pharma will put any more money into basic research. Those times are over. The partnership needs to be there and that is why efforts like Horizon 2020 or IMI are beautiful, because both partners can be involved and only if you have that partnership, you are going to advance the field.

DR. MATHIEU VINKEN: I fully agree. As an academic, I can only echo this. This kind of collaboration with industry is really valuable, especially in such a challenging field. Are there any other comments or closing remarks?

DR. PHILIP HEWITT: I have one question, which is on the response from different species. As a toxicologist, that may be an important question to address or maybe it is obvious that there is no species difference between rat, dog, and human in this field?

DR. YVONNE WILL: Indeed, you bring up a mouthful there. Interestingly enough, there has only been one study, that was published out of Ann Arbor about 15 years ago, where somebody took a compound and tried to put it into a rat, dog, monkey, and human mitochondria. And the problem with the study itself was, how do you actually know, first of all, to make proper mitochondria from all these species. What is your benchmark? Though, other than that study, there is really not much out there. But it is a very important point, there is no doubt about it.

DR. KEN WALLACE: I think that is a great question and, as Yvonne said, it is one that is going to be very difficult, if not impossible, to answer because of the technical complications of isolating mitochondria or, for that matter, primary cells from different species of animals and from different tissues.

But, I think Harmut would agree that even though one may propose that there are interspecies consistencies in mitochondrial effects, if they are primary, when it comes to the mitochondrial effects that are secondary to other disturbances, those would carry a whole host of interspecies differences. We have seen that as far as framing the molecular response, gene expression, transporter abundance and things like that. I think there is a

huge opportunity for interspecies differences in mitochondrial effects that are secondary to a primary effect of that chemical.

DR. MATHIEU VINKEN: I fully agree. That also comes back to the earlier question of *in vitro* mitotoxicity testing for *in vivo* adversity. At the end of the day, of course, we are looking for adversity in people and many *in vitro* tests are with some animal species. I think the relevance of the species and how we can extrapolate that is of utmost importance. And, in the past, a lot of the failures come from kind of the blind assumption that what I do in the rat applies to the people. That is something that really needs to be much more paid attention to.

DR. YVONNE WILL: Actually, it is probably going to be much, much more complicated than that. Years ago, when we were trying to think about this, we took Hamner mice strains and they were supposed to mimic genetic diversity.

So, we took four of these and we found mitochondrial haplotypes far away from each other on the phylogenetic tree and we put a drug in, and yes, they all had a different dose response curve. And if you read the discussion, we had to really throw our arms in the air and say, well yes, it is very different. We do not know why; we could not really find particular mutations. At the end of the day, you even had to ask which mouse do you want to be—the mouse that did not respond or the mouse that did response.

It is probably going to be much more complicated, considering haplotypes and your

mitochondrial makeup, your reserve capacity, your age, your sex, your lifestyle. I think until you get also a combination of really understanding system toxicology in the mitochondrial sense, you will not get to that risk assessment.

DR. HARMUT JAESCHKE: I think you can make a case here to really push mechanistic studies in the animal model or in *in vitro* system, and looking at biomarkers, at least for certain diseases, that are also already happening in people.

The good news, and one of the reasons why I am working with acetaminophen, is because it is so satisfying that we can use biomarkers, we can compare directly—for certain mouse strains, it very much mimics what happens in people. However, if you go into a rat, it is totally irrelevant.

If you really look at the more detailed mechanistic aspects, you can really then make the connection between an animal model or an *in vitro* test, or the relevance of it. Now, I recognize that is possible if you make an effort with a single drug or a single disease. When you are dealing with hundreds or even thousands of compounds in industry, you have to be more superficial.

One way to approach that issue of species variability and all this kind of thing is by looking at biomarkers in people. Go back to your *in vitro* system and find out what it showed you as a comparison. It goes back to the relevance of *in vitro* testing for the *in vivo*. It is a major problem as well as an opportunity to really improve things.

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