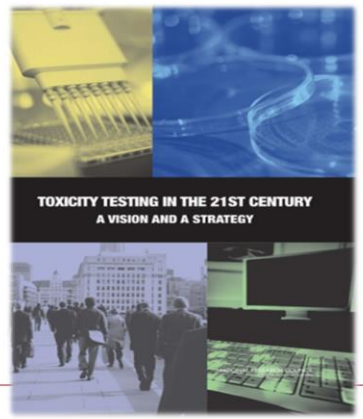
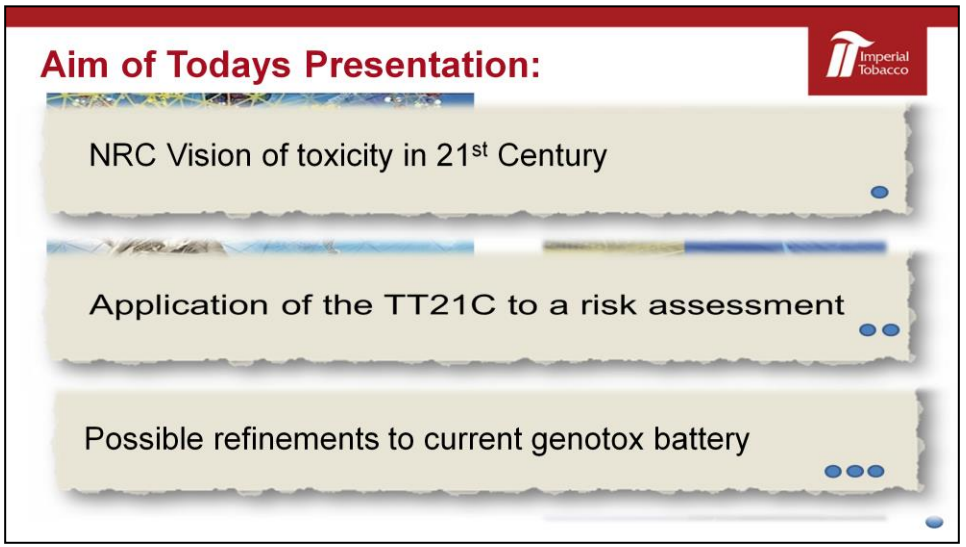
The CORESTA Recommended In Vitro Test Battery alongside the NRC Vision for Toxicity Testing in 21st Century.

Liam Simms

Québec October 16th ST80

Aim of Todays Presentation:





Thank you it is a pleasure to be able to speak to you all today. As the aims of the congress are to look to the future, the aims of my talk are to briefly discuss the future of toxicology at a basic level. Firstly I will focus on the National Research Council vision of 21st Century toxicity testing**; I will then talk through **an example of the application of the TT21C approach to risk assessment; a very brief description of an AOP, how all the data generated in mechanistic, toxicology studies are put in to context and **and finally I will propose some potential refinements to the standard genotoxicity assays

I must add these are my views of where the science of toxicology is heading to in the future and do not reflect those of CORESTA.

NRC (2007): Toxicity Testing in 21st Century: A Vision and a Strategy

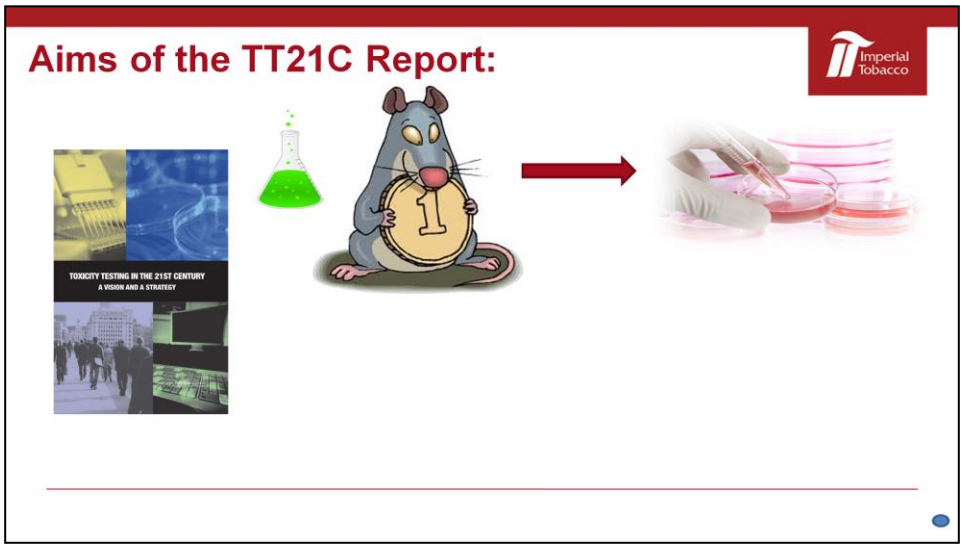
- **The committee identified 4 key areas**:
- '**Depth:**' *providing the most accurate, relevant information possible for hazard identification, and dose response assessment.*
- '**Breadth**': *providing data on the broadest possible universe of chemicals, endpoints, and life stages*
- '**Animal welfare**': *causing the least animal suffering possible, and least numbers of animals used*
- **'Conservation**': *minimizing the expenditure of time and money on testing and regulatory review*

National Research Council Toxicity testing in 21st Century: A vision and a strategy http://www.napedu/catalog/11970.html

The Environmental Protection Agency in the USA recognised the need for a comprehensive review of toxicity testing strategies due large numbers of chemicals in the environment that had no toxicology data associated with them. In 2007 The National Research Council (NRC) provided guidance on new possible directions in environmental toxicity testing, incorporating new technologies such as genomics and computational systems toxicology. The National Research Council (NRC) is the working arm of the United States National Academies, which produces reports that shape policies, inform public opinion, and advance the pursuit of science.

The committee identified four key areas identified were: Depth, Breadth, Animal welfare with a focus on human cell lines, high through put technology and conservation

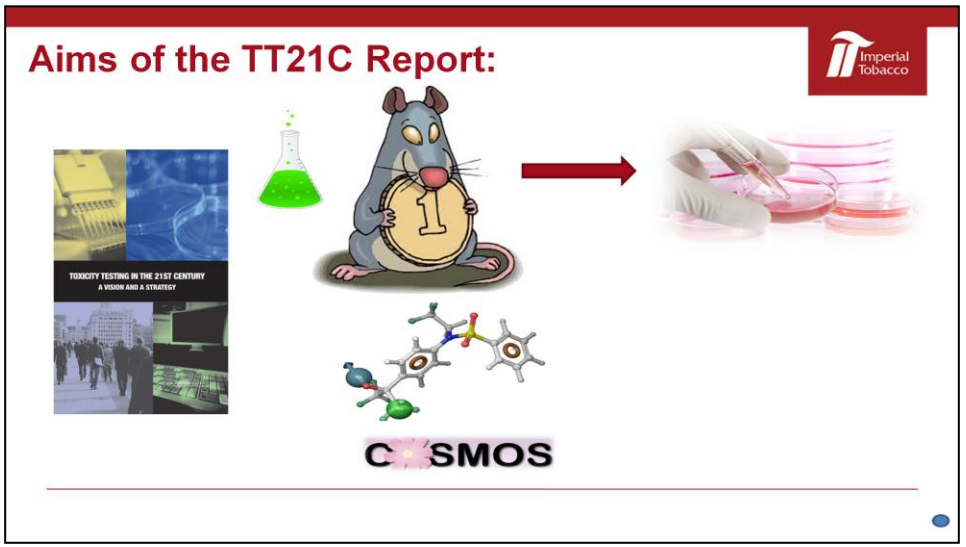
In Europe there is specific legislation that restricts the use of animals, whilst others increasing the need for hazard data.. In Europe The European 7th Amendment, there is now a ban on the testing of all cosmetics on animals. REACH (Registration Evaluation restriction and Authorisation of chemicals), demands data on chemicals for Hazard assessment.



**So what are the Main aims of the Toxicity testing in 21st Century

** Move away from the idea of high dose toxicity animal tests as the ‘gold standard’ vs low dose exposures seen in humans, essential paradigm shift, identify areas of low exposure that are safe, rather than trying to extrapolate findings seen at high doses in animals to humans.

Use human cell lines exposed at the levels of expected human exposure



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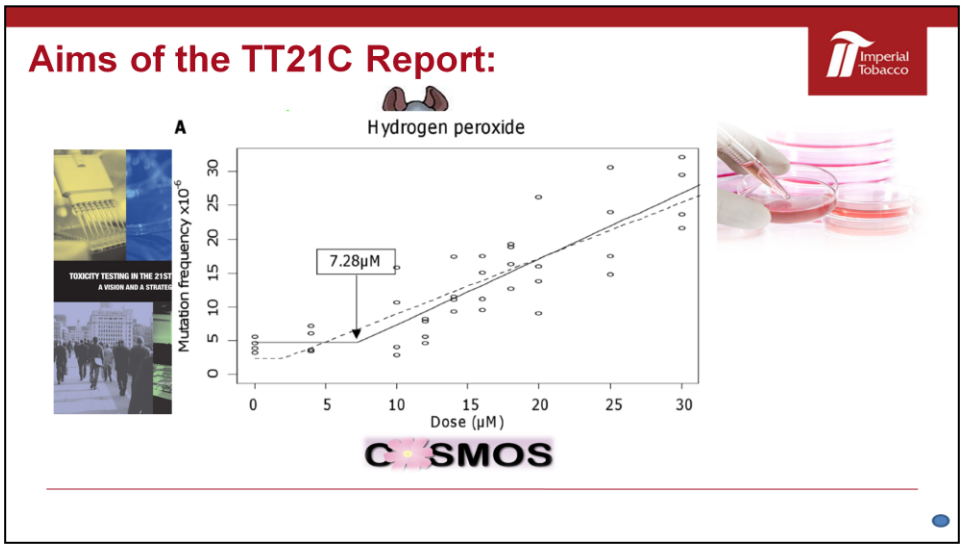
** Increase the use of in silico models to estimate or predict possible toxicological properties of compounds



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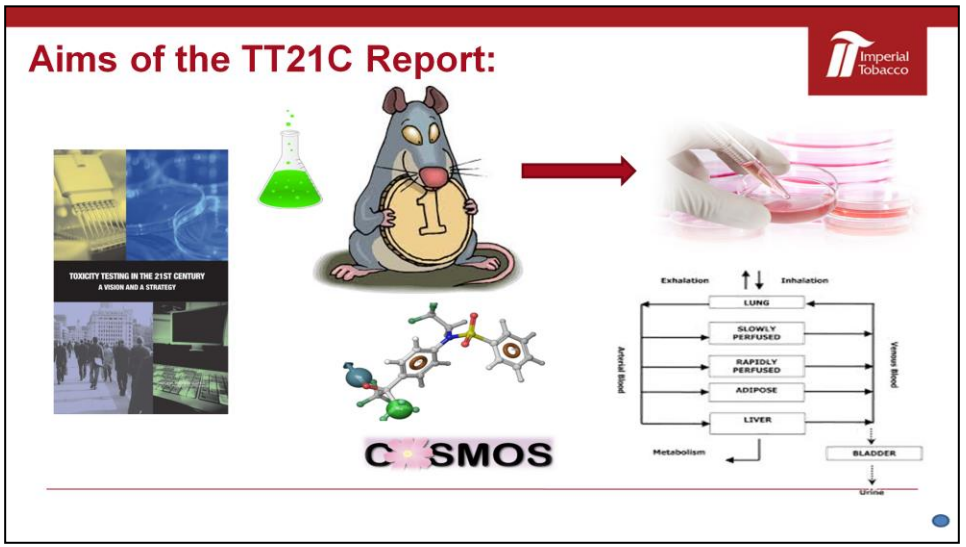
** Increase the use of in silico models to estimate or predict possible toxicological properties of compounds

Identify toxicity pathways (normal signal pathways) that may be perturbed (disrupted) by chemical exposures, critically at what concentration does this occur in relation to human anticipated exposure? Using high through put screening.



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**Use of Physiologically based pharmacokinetic (PBPK) studies to extrapolate from in vitro to in vivo.

The ultimate aim to replace whole animal testing.

Pro's and Cons of Integrated Testing Strategies (ITS)

Strengths of ITS such as high throughput screening (HTS) allow for an

- Ability to prioritise chemicals for screening
- Reduce animals testing

However, **limitations** include lack of prediction for :

- Chemically induced disease associated pathways
- Metabolism
- Interactions between different cell types
- Tissue-cellular interactions
- Chronic exposure

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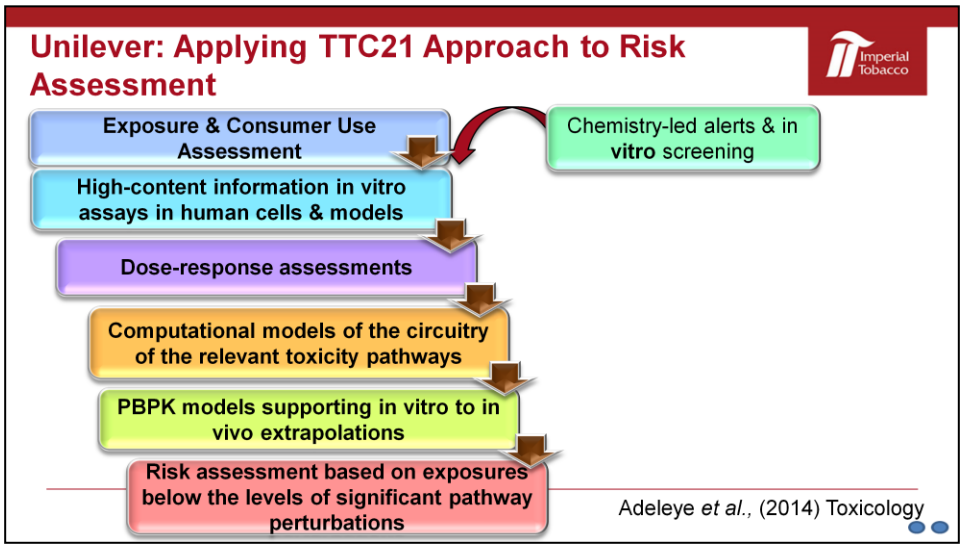
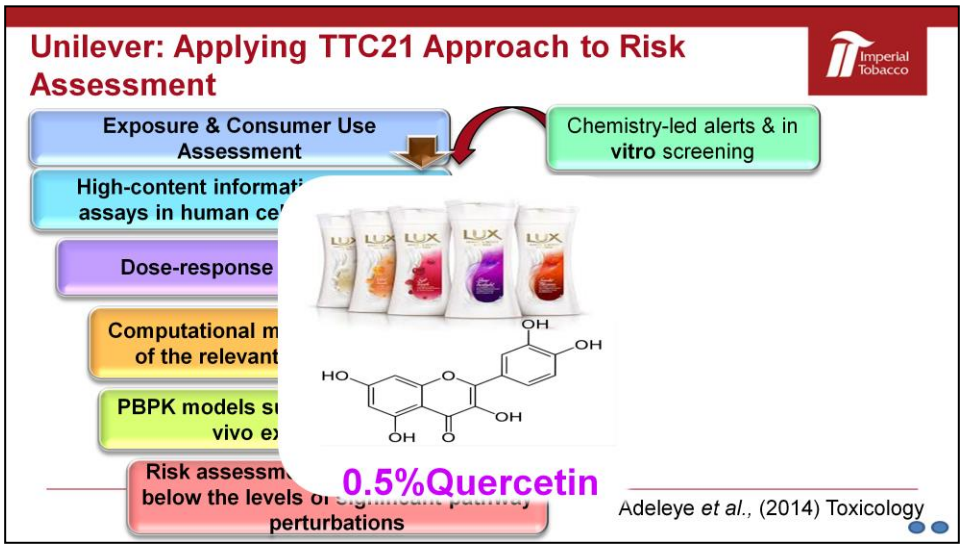


As highlighted at the start I will now talk you through an example of the application of the TTc21 to a risk assessment.

Unilever defined the key elements of the TT21C vision in to a set of studies outlined on the slide.



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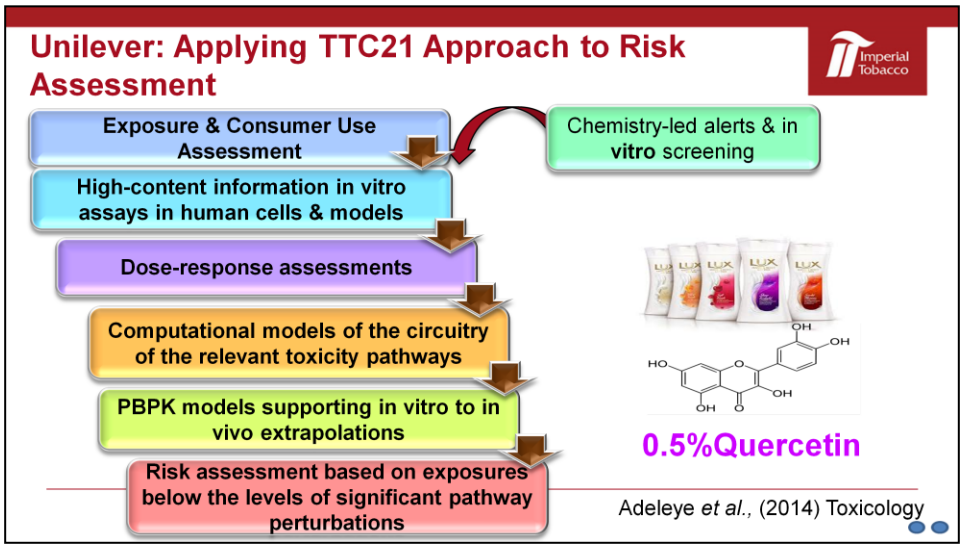
** The question posed was would the use of 0.5% Quercetin in a body lotion cause a significant pertubation in DNA damage/p53 pathway responses if used by a consumer.



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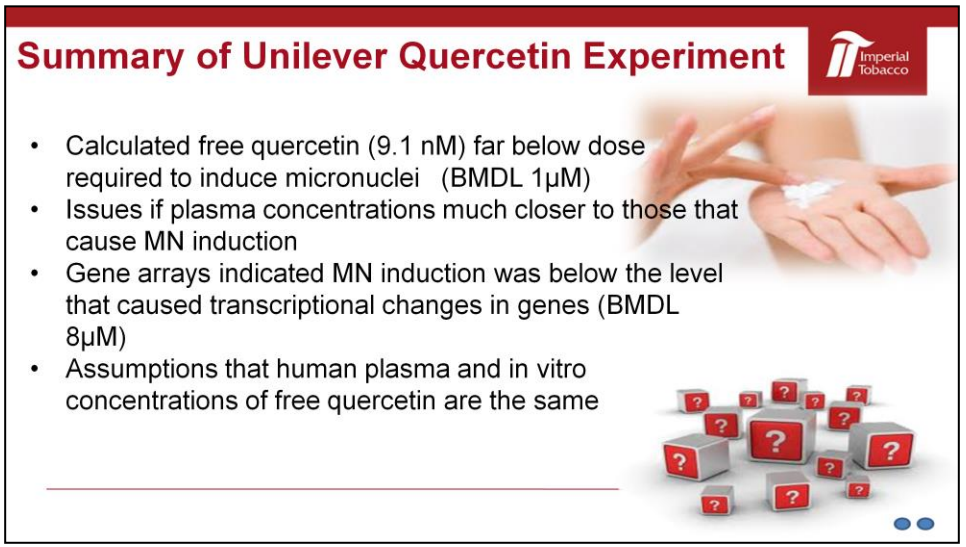
**Could quercetin could be sufficiently understood to construct aTT21C risk assessment without the need to use rodent carcinogenicity study data. The data used included

Eighteen point dose response curves were generated using flow cytometry and imaging analysis to determine the concentrations that resulted in significant perturbation. Total quercetin concentration in the in vitro systems was compared to the predicted total quercetin concentration in plasma and tissues of exposed humans.

The summary results are on the next slide.

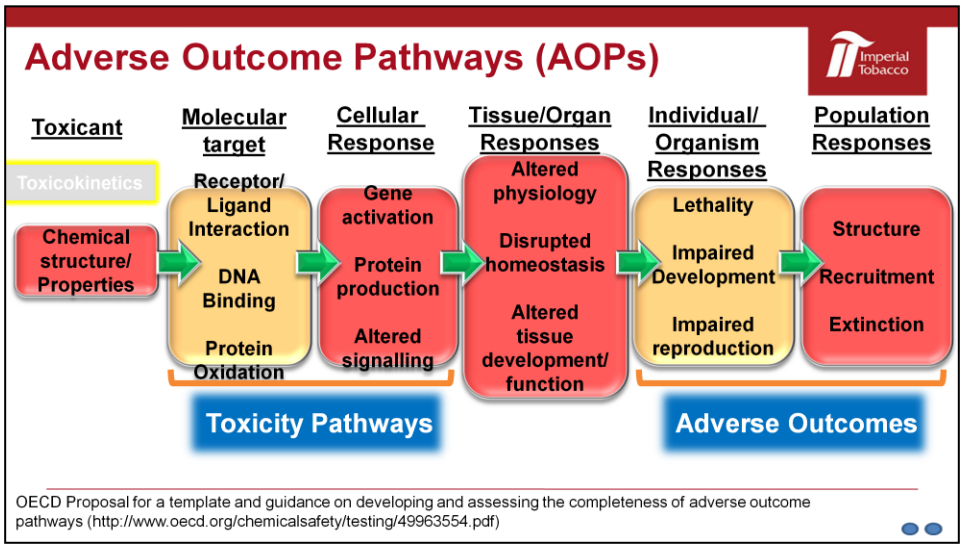


**It was calculated that the plasma concentration of Quercetin was many orders of magnitude below the dose required to induce in vitro micronuclei (1uM BMDL) BMDL (95% CI around the BMD value) the perturbed network.

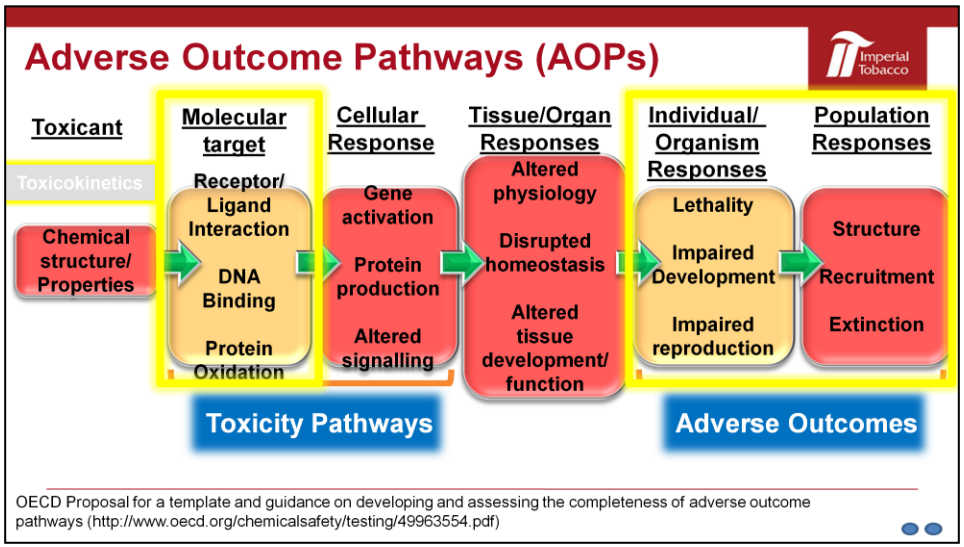
**There were if the plasma concentrations were much closer to those that cause MN induction, my question is how will systems be integrated to give a more quantitative approach?

**Gene arrays indicated MN induction was below a level that caused transcriptional changes in genes (BMD 12 µM).

**Assumptions that human plasma concentrations and in vitro concentrations of free Quercetin are the same is an area for refinement, depending on the compounds in question binding to e.g. possible binding to plastic etc may occur, different protein concentrations in vitro vs in vivo.



Adverse Outcome Pathway (AOP) methodology is one approach to provide a framework for information from toxicogenomics, bioinformatics, systems biology and computational toxicology, to be collected and rationalised. The AOP is key for the ttc21c vision to be able to predict the effects of seen pertubations in biological systems with the likelihood of adverse health effects in humans.



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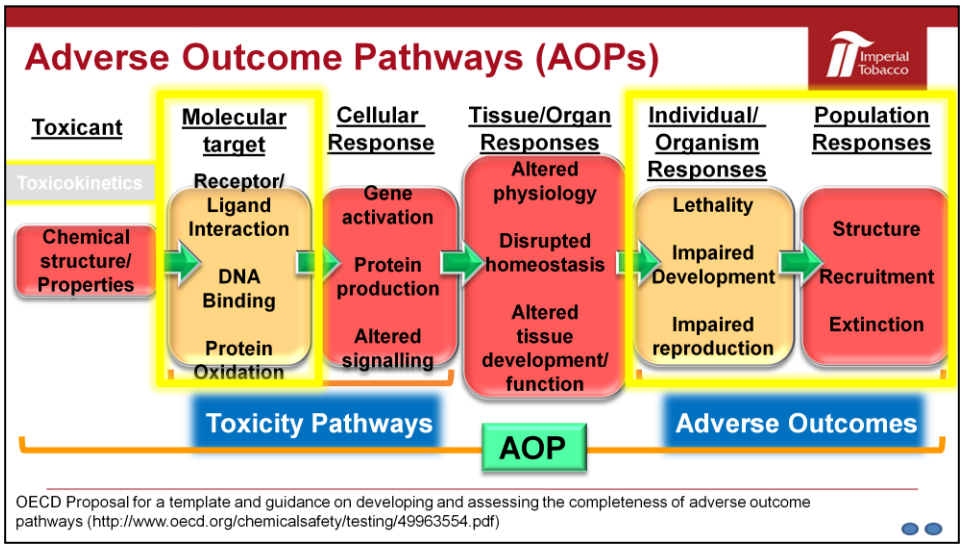
** Each AOP begins with a Molecular Initiating Event (MIE) in which the chemical inter-acts with a biological target, leading to a sequence of events across different levels of biological organization (subcellular, cellular, sub-organ, organ, individual and population) and resulting in an adverse outcome **. Toxicity pathways help define steps leading from the MIE to the adverse outcome.



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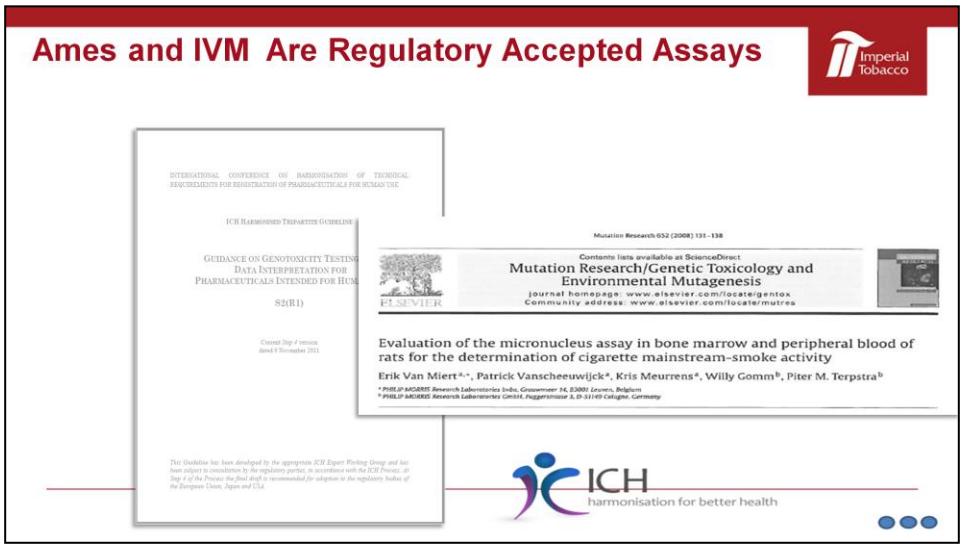
An AOP is a description how a system fails and a tool to bring systems biology thinking into mainstream biomedical and toxicological research. It provides a practical scientific framework and language to facilitate dialogue between scientists, and between scientists and regulators.

Finally I will talk to you about some proposed refinements to standard genotoxicity assays. For ingredients added to tobacco there are no recognized standards for evaluating their effects upon the toxicity of mainstream smoke.

As you are aware from previous talks standard assays for genotoxicity and mutagenicity assessments exist and are recognised internationally. ICH (Nov 2011).

Imperial tobacco does not test products on animals, so would not conduct an in vivo micronucleus assay, typically used by pharma to confirm a positive result.



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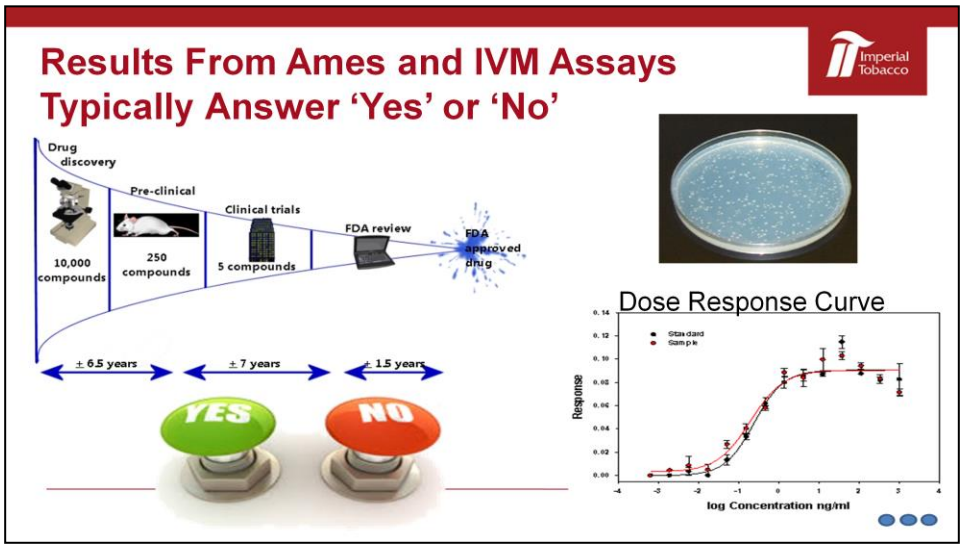
Imperial tobacco does not test products on animals, so would not conduct an in vivo micronucleus assay, typically used by pharma to confirm a positive result. **Van Miert (2008) reported no response for cigarette smoke the in vivo micronucleus assay for Sprague Dawley rats, exposed for up to 90 days duration. This indicates a poor sensitivity of the assay to cigarette smoke or poor penetration of cigarette smoke in to the bone marrow.



In Pharmaceutical testing, is compound X genotoxic or not**, these assays can also be used when looking at complex mixtures to see if does the addition of ingredient X or a product modification change the inherent genotoxicity when compared to control/reference product. The current genotoxicity assays are geared up to predict carcinogenicity studies in rodents. **Hence for more relevance to humans, Human lung S9 could be used in the Ames assay. The genotoxicity tests are, however, exceeding well studied and standardised and could be key to delivering the vision of 21 century toxicity testing.

An Imperial tobacco work programme has applied a modified Ames and IVM assay, using repeat experiments to construct dose response curves ** to assess the effects of adding ingredients to cigarettes on the overall mutagenicity of the cigarette smoke condensate. Each commonly used ingredients were tested on its own in the Ames and IVM assays. Neat data for most of the 250 or so ingredients data in the Ames and especially the IVM assay was lacking. Groups of ingredients were then added to the reference cigarette at a low (the maximal usage across the product portfolio) and a high level (5 x the MUL where technically feasible or 3X where it was not technically feasible to do so). The linear portion of the dose response curves were compared between the reference and modified product. The summary for some key ingredients are presented on the next slide.

CSC: Addition of Ingredients Does Not Increase Mutagenicity/Alter Histopathology

Ingredient	Ames* (Neat)	IVM* (Neat)	CS Condensate (Mul 5x Mul) Ames and IVM Assays
Cocoa	−	−	Control ≥ Low ≥ High
Ethyl vanillin	−	+	Control ≥ Low ≥ High
Glycerol	−	−	Control ≥ Low ≥ High
Licorice	−	−/+	Control ≥ Low ≥ High
Menthol	−	−	Control ≥ Low ≥ High
Vanillin	−	+	Control ≥ Low ≥ High

*Testing of Neat ingredients
Cigarette smoke Condensates tested at Maximum Use Level and 5x MUL

These ingredients were chosen as they are common ingredients

Neat data is the genotoxicity testing of the ingredient on its own. This data is from work conducted 2002-2005. In both the Ames and the IVM assays the addition if ingredients lead to either no difference when compared to the control product or lead to a significant reduction. The general effects of the addition of ingredients on tobacco was to dilute the tobacco and reduce its mutagenicity per mg/tar. This general finding is in agreement data in the scientific literature from other Tobacco companies. In 13 week rat inhalation studies, apart from very isolated incidences, all the scientific papers found no differences in histopathology between rats exposed the control cigarette and ingredient cigarette exposed rats.

There are High Rates of False Positives in Rodent Cell Lines

False positives	Rodent cell lines						Human cell lines					
	V79		CHO		CHL		HuLy		TK6		HepG2	
Pthallic anhydride	Neg	Neg	Neg	Neg	1480	1380	Neg	Neg	575	725	1481	Neg
2,4 Dichlorophenol	8.5	8.6	27	23	145	155	Neg	187	Neg	Neg	Neg	Neg
Curcumin	4	6	12	17	14	19	35	35	10.5	16	Neg	Neg
Ethyl acrylate 3+21hours	16	20	32	20	39	Neg	38	50	25	20	Neg	96
Ethyl acrylate 24+0 hours	1	4	Neg	Neg	7	Neg	Neg	Neg	Neg	Neg	Neg	Neg
1,3 Dihydroxybenzene (resorcinol)	196	261	480	444	307	319	Neg	Neg	Neg	Neg	Neg	614
p-nitrophenol	Neg	Neg	587	950	1043	750	Neg	Neg	Neg	Neg	Neg	Neg
Eugenol	13	10	19	50	219	180	Neg	Neg	Neg	160	Neg	Neg
Propyl gallate	5	8	13	10	24	32	Neg	Neg	4	16	Neg	Neg
Sodium xylene sulfonate	1562	1666	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	1481
Tertiary-butylhydroquinone	21	20	60	89	55	25	Neg	Neg	Neg	Neg	119	Neg
D,L-menthol	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
0-Anthranilic acid	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
Ethionamide	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
Urea	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg

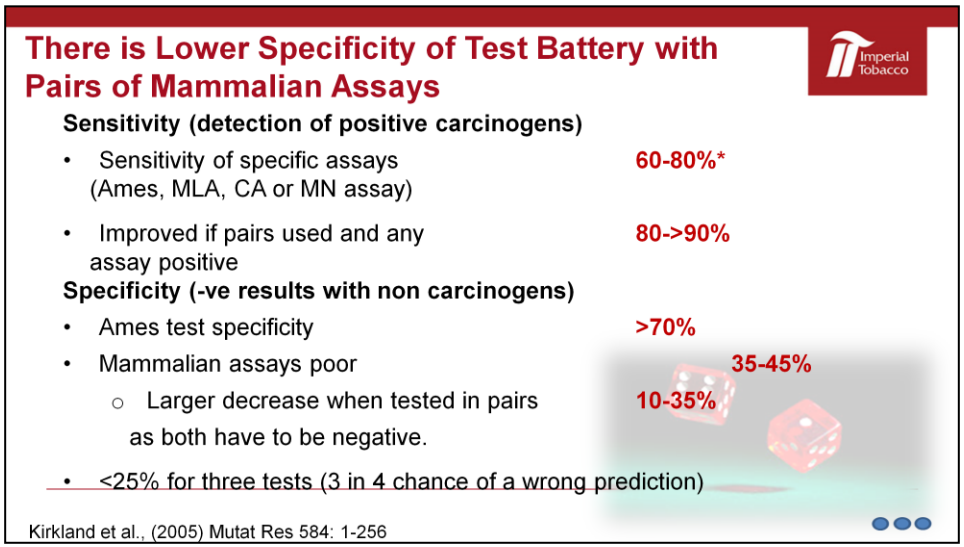
Positive for micronucleus induction (toxic)
Negative for micronuclei (toxic) — Neg
Negative for micronuclei (non toxic) — Neg

From Fowler et al., (2012) Mutat Res 18: 742(1-2) 11-25.

A current concern with in vitro mammalian cell genotoxicity assays is a high rate of positive results. Fowler et al., compared several rodent cell lines (V79, CHL, CHO) with p53-competent human cell lines peripheral blood lymphocytes (HuLy), TK6 human lymphoblastoid cells, and the human liver cell line, HepG2. The authors compared in vitro micronucleus (IVM) induction following treatment with compounds that were accepted as producing misleading or "false" positive results, from the scientific literature in in vitro mammalian cell assays.

False positives from indicated as red on the heat Map, with the correct prediction being either yellow or green. A p53-deficiency in many of the rodent cell lines has been indicated as a possible key factor for this poor predictivity. The p53 protein is crucial in multicellular organisms, where it regulates the cell cycle and, thus, functions as a tumor suppressor, preventing cancer.

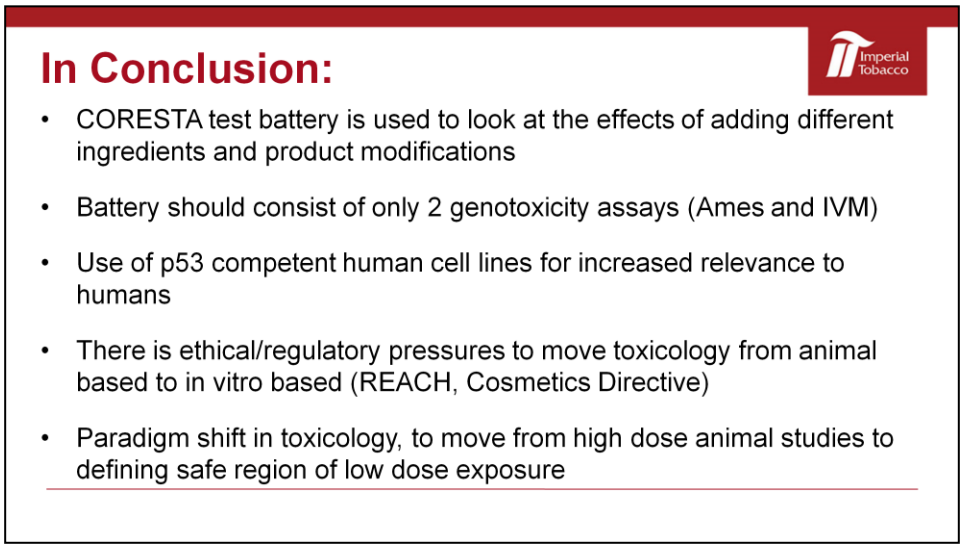
The rodent cell lines (V79, CHO and CHL) were consistently more susceptible to cytotoxicity and MN induction than p53-competent cells, and are therefore more susceptible to giving misleading positive results. This also correlates with some of the ideas of the 21st century of toxicology that advocates the use of Human derived cell lines in an in vitro based testing strategy.



In vitro genotoxicity testing needs to include tests in both bacterial and mammalian cells, to be able to detect gene mutations, chromosomal damage and changes in numbers or size. Kirkland et al compared a database of genotixicity studies 962 chemicals, looking at the sensitivity (ability to detect a carcinogen) of different genotoxicity assays and the ability to not detect non carcinogens (specificity) of both individual gentoxicity assays and when multiple assays were put together as a battery of tests.

Almost all rodent carcinogens and in vivo genotoxins were detected by an in vitro battery comprising Ames+IVM. Only four chemicals emerged as potentially being more readily detected in MLA than in Ames+MN..

** A significant advantage of a single mammalian cell assay is a reduction in the number the misleading positive requiring follow up work (specificity). There was a marginal increase in sensitivity if a third assay was added e.g. MLA was added, however there was a significant reduction in specificty. EFSA recently agreed that the small increase in sensitivty was not worth the loss in specificity of adding a second mammalian test to the test battery.



**CORESTA battery is used to look at the effects of adding different ingredients to the products and the genotoxicity assays being well understood may be key to delivering the goals of TT21C.

**An ideal test battery for genotoxicity consists of 2 assays (1 Bacterial and 1 mammalian) that covers all the required end points. The addition of extra assays is not complimentary and increases the chances of having a false positive result.

** With the preferential use of human derived cell lines in the future reduces some use of uncertainty factors in risk assessment to extrapolate from rodents to humans, at the levels humans are actually exposed to.

**There is regulatory pressure to move toxicology from animal based to in vitro based (REACH, Cosmetics Directive) to cover the needs for hazard data that can not be met by animal testing alone. This is an essential shift in the toxicological assessment paradigm.

**21st Century toxicology is still in its infancy and will take many decades to deliver fully.

21st Century Toxicology is Still in its Infancy and Will Take Decades to Fully Deliver

For this and other scientific research please see the Imperial Tobacco Science website
http://www.imperialtobaccoscience.com/