Information on animal testing in the proposed regulatory regime for psychoactive substances

Stephen Manson

National Office Manager
New Zealand Anti-Vivisection Society Inc
PO Box 9387
Tower Junction
Christchurch 8149
(03)379 0093
stephen@nzavs.org.nz

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Introduction

In July 2012 the Ministry of Health (MoH) made available on its website copies of a Cabinet paper from the Associate Minister of Health Hon Peter Dunne to the Cabinet Social Policy Committee proposing legislation regulating the sale of low-risk psychoactive substances. On 2 July 2012 Cabinet agreed to the proposals in the document and the legislation is currently being drawn up. This legislation is in response to public concern over the non-restricted sale of “herbal highs” and “party pills”. The immediate government response was to introduce a temporary ban on the sale of these substances. This ban has been recently rolled over until August 2013. The legislation is intended to be before parliament by the end of 2012 and presumably be in place by August 2013 when the temporary bans end.

As part of the approval process for any substances under the regulatory regime the safety and risk is to be assessed and considered. The details of the testing regime aren’t to be included in the legislation but are to be set by the regulatory body that will be governing the approval process. This is to allow the testing regime to change without the need for amendments to the legislation. The MoH has though given some thought to, and done planning into, the nature of the testing regime. As human trials are to be used as part of the proposed testing regime the MoH is concerned with assuring that the products to be tested are safe enough to expose people to in these controlled human trials. The way that the MoH is planning on doing this is through an extensive round of animal tests. These tests are to be carried out on two species, one small and one large. Typically these are rats and dogs and it is these two species that are mentioned throughout the material from the MoH referenced in this report. The tests will require the animals to be forced to ingest the substance by the same route that the human users will be and then animals will be killed at the conclusion of the testing period so that their internal organs can be examined.

Our aim is to have a clause included in the final version of the legislation that would restrict the testing to non-animal methods only. As this report shows it is not only possible to do this but non-animal tests in the pre-clinical human trials give a much better indication of the possible effects of the drugs on the human subjects.

Source Material Information

This report covers a proposal for legislation and draws heavily on information provided by the Ministry of Health under the Official information Act 1982 (OIA). This material was requested by on 13 September 2012 and received on 10 October 2012. I received almost 600 pages of material, the majority of it being email correspondence between a MoH Senior Policy Analyst and various scientists and doctors that occurred during the development of the proposed toxicology testing regime. There was no material dating from after 31 May 2012.

The request under the OIA made in September 2012 was for "any and all correspondence and information relating to any consultations with toxicology and pharmacology experts during the preparation of the proposal to cabinet…" I will be following this up with another request under the OIA to get information on further developments in this area by the MoH, in particular to see if they have followed up on the information they have been provided by NZAVS to date.

Copies of all the material provided under the OIA have been scanned and can be as pdf files, simply contact me at any of the contact points on the title page of this document requesting a copy. The complete set of documents is available in five files totaling 17MB as well as this there are two separate files of just the final versions of the report that the proposed toxicology testing regime is based on and the MoH discussion document on the proposal that was sent out to various people for feedback.

Outline of the Proposed Testing Regime

The proposed testing regime is to be in four parts[1]:

- Pre-clinical toxicology animal tests
- Confirmation of general manufacturing quality requirements
- Human clinical trial
- Final risk assessment

It is the pre-clinical toxicology tests that are where the use of animals is to occur and is the one stage in the approval process that we are interested in and will be looked at in this report.

[1] Ministry of Health report 20120600 – pg. 2 of discussion document "Discussion on two proposals relating to the new regime for regulating low risk psychoactive substances in New Zealand"

Tests that will involve animals

The preclinical trials are to include acute toxicity, repeat dose toxicity testing, genotoxicity, toxicokinetic/pharmokinetic data and possibly tests for carcinogenicity and developmental toxicity. All of these tests will require the animals to be forced to ingest the substance in the same manner that humans will be. As smokable products are to be tested this will entail animals being used forced to inhale smoke over extended periods. When the author of the report that the MoH commissioned and based its proposal on was asked how this would be done they described a device where rats are confined in tubes to restrict their movement and have smoke pumped into them[2]. All the tests will be required to be carried out on both rats and dogs under the MoH proposal. The use of two species will be part of the basic methodology of all the animal tests. The only exception, which we are aware of, to the use of two species, will be in the one test in the genotoxicity section that specifies the use of rats. More detail on the four types of tests that will definitely be required follows.

Acute Toxicity

This refers to the potential adverse reactions to a single dose of the substance. There will be two types of tests carried out to fulfill this part. The first will require groups of animals being exposed to different doses of the substances, their reactions observed and then they will be killed to allow an internal examination. The second part is for the infamous LD50 test, this test requires sample populations of both species of animals to be given a dose of the drug in order to find the dose that kills 50% of the population of animals.

Repeat Dose Toxicity

This part of the animal testing regime requires the animals to be exposed to the substance being tested repeatedly over extended time periods. The MoH is talking about requiring studies lasting up to six months. The animals will be required to be placed into the devices that force them to inhale the smoke when it is for smokable products, or forced to orally ingest other substances, at multiple times during the day for the duration of these tests.

[2] "Reply to comments concerning the document Regulations Governing the Control of Novel Psychoactive Drugs – defining parameters associated with toxicity", 1 February 2012, name of author withheld by MoH.

When not in the device they will be being weighed, examined and have blood samples taken from. As well as observing any long term effects in the animals this part of the testing regime is used to discover the No Observable Adverse Effects Level (NOAEL). This is done by exposing the animals to increasing doses of the drug until the largest dose is found where there is no observable adverse effect.

Genotoxicity

This type of test is done to find out if the substance being tested can cause any genetic damage. The MoH proposal for this part is for the use of the tests that were required by the United States Food and Drug Administration (FDA) in a document produced in 1997[3]. One the tests that make up this battery of tests uses live animals, the rat haematopoietic cells (in vivo) test. In this test the animal is forced to ingest the substance over a prolonged time period and then the haematopoietic cells are examined for chromosomal damage. This test is fatal for the animals as these cells are found in the bone marrow.

Toxicokinetic/Pharmokinetic

These are the tests that look at how the drug is absorbed by the animal's body and then distributed, metabolised and finally eliminated by the body. These require the animals to be exposed to repeated doses of the drugs over the same time periods as the clinical trials (up to six months) while undergoing repeated blood, respiratory and cardiovascular tests. Again at the conclusion of the tests the animals are killed to allow examination of internal organs.

[3] http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/ucm074929.pdf

Ministry of Health's advice

Report defining toxicity approval requirements

The proposed testing regime is drawn from the sole report commissioned by the MoH on this. This report outlined only one possible testing regime, contained no options within it for consideration by the Ministry and contained no declaration of possible conflicts of interest. Best practice in policy development is for the agency developing the policy to obtain multiple proposals for consideration so they are best placed to implement the best option.

Peer Review

The report was also not peer reviewed. The report's author said in an email to the MoH on the 18th January 2012, two days after the first version of the report was written, that "*peer reviewing is very important*", and it is. Standard procedure in the peer review process is for two or three experts in the same field to review the piece in order to evaluate it and provide feedback. This is done to ensure that the piece being reviewed contains the best possible information and is accurate. The MoH asked one person to peer review the report. When asked, that person, Dr Paul Fitzmaurice from the Institute of Environmental Science and Research Ltd, replied with the one line email "*Happy to assist where I can. I know [name withheld] well; he is a good choice*."[4] This admission that he knew the author well should have required the MoH to ask elsewhere for an independent peer review but they did not. In further email Fitzmaurice says "*…I don't think I would add value by reviewing [name withheld]'s document*"[5] and didn't provide a peer review. The report the entire testing regime is based on was never peer reviewed.

Non-animal options considered by the Ministry

There were none. The OIA request made by NZAVS explicitly asked for *"…any information relating to the other options, if any, that were considered for the methodology of the testing*". No material relating to any consideration of any options was provided in response

[4] Email to Mark Heffernan on 16th January 2012
[5] Email to Mark Heffernan on 23rd February 2012

to this request so presuming that the MoH was fulfilling its requirements under the OIA no other options were considered in drawing up the proposal.

Non-animal options for the testing

The Physicians Committee for Responsible Medicine (PCRM) in conjunction with PETA's Regulatory Testing Division have provided NZAVS with a report commenting on the MoH advice that the proposed testing regime is based on. This report was co-authored by Amy Clippinger, who has a Ph.D. in cellular and molecular biology and genetics and has worked as a researcher at the University of Pennsylvania, and Kirstie Sullivan, who has a Master of Public Health in Toxicology from the University of Michigan. In the report they present multiple options for non-animal tests that can be used to meet the aims of the proposed legislation.

This advice follows in full under the heading "Advice received from the PCRM". This advice is extremely well researched and detailed and is attached so that it can be made available to show that despite the claim by the MoH that animal testing is essential this is not the case. While I have also carried out research along these lines, and there is some convergence with what the PCRM and PETA have put together, their work is more comprehensive so is presented here. It is pretty unequivocal in rejecting the use of vivisection, and describes the proposed MoH testing regime's use of animals as "indefensible".

This report from them is extremely valuable as it provides proof that any reasonable person can't deny that vivisection is unnecessary and gives poor information.

Advice received from the PCRM

Introduction

When NZAVS learnt that the Ministry of Health was planning on requiring the use of animal tests to approve "herbal highs" for sale in New Zealand, and that they had been led to believe that such animal tests were "required"[6], we sought expert advice to disprove what we knew to be an inaccurate statement by the Ministry of Health.

In order to this we first needed to know what had led the Ministry of Health to make such a demonstrably untrue statement. A request was filed under the Official Information Act 1982 for the foundations of that statement. The basis for the Ministry saying what they did was a sole report that was never peer reviewed. This report had the title "*Regulations Governing the Control of Novel Psychoactive Drugs – Defining Parameters Associated with Toxicity*" and we sent it to international experts in public health and toxicology for consideration. We received the advice that we present here in response to that report.

The advice that follows was written by Amy Clippinger Ph.D. and Kirstie Sullivan M.P.H.; Amy has a Ph.D. in cellular and molecular biology and genetics and several years of research experience at the University of Pennsylvania and Kirstie received her Master of Public Health in Toxicology in 2003 from the School of Public Health at the University of Michigan in Ann Arbor, her studies included environmental health exposure and risk assessment, pharmacology, carcinogenesis and environmental diseases, and pathology. Their work rebuts the claim from the Ministry of Health that animal tests are required and outlines multiple non-animal tests available for every part of testing regime that the Ministry is proposing that give more reliable and accurate results than the animal tests.

[6] Waikato Times on 11 September 2012

Comments on "Regulations Governing the Control of Novel Psychoactive Drugs – Defining Parameters Associated with Toxicity"

We are very concerned that in its current form, the *Regulations Governing the Control of Novel Psychoactive Drugs – Defining Parameters Associated with Toxicity* ("The Regulations") would lead to unnecessary animal studies being conducted. While it is stated in the Executive Summary that these drugs will be tested "in a similar process to that required for pharmaceutical drugs," the results of drug studies in animals are often not predictive for human health and can lead to dangerous drug reactions in humans (as will be described in more detail below). We recommend that non-animal testing methods be used exclusively to evaluate the health risks of these drugs.

In their current version, The Regulations require chemistry, manufacturing and controls information, preclinical toxicology studies, human clinical studies and post-registration surveillance. Required preclinical toxicology studies include acute and repeated dose toxicity testing, toxicokinetic, carcinogenicity, genotoxicity and developmental studies in animals.

We support the recommendation that chemistry, manufacturing and controls information of constituents should be the first step in the evaluation of any new product. Drugs should also be compared to similar drugs for which toxicity data already exists[1].

In vitro evaluation of products should precede controlled human *in vivo* assays. *In vitro* preclinical assays will identify particularly risky or toxic products that should not be tested in humans. At this point, prior to commencement of human studies, applications to test novel psychoactive drugs should be made publicly available for comments by interested persons.

When used in this manner, laboratory analysis and *in vitro* preclinical assays will be sufficient to predict particularly toxic drugs and preclude the use of animal tests. Clinical work and post-registration surveillance will always be required to unequivocally determine the safety of a drug in humans and to examine mental effects associated with use, such as dependence and withdrawal. As stated in Section 4.0 "Human Clinical Studies" of The Regulations, "*animal testing does not always predict performance in humans* and cannot therefore guarantee the safety of the drug in humans" – further supporting the elimination of any animal tests. For any products whose safety cannot be determined by non-animal tests alone, the ethical implications of inducing suffering and death in animals in the name of recreational drug use should be seriously considered.

Acute and Repeated Dose Toxicity

Psychoactive drugs may be ingested or smoked; therefore, the method of assessing toxicity may differ depending on the intended route of human exposure.

Inhalation Toxicity

Scientifically, numerous obstacles exist in gathering human-relevant results from animal tests designed to assess products that are inhaled (that is, herbs or substances that are smoked). First, it is not possible to make laboratory animals use products that are inhaled the way humans do. Second, inherent interspecies differences prevent meaningful extrapolation of animal results to humans. The respiratory system in humans is quite different physically and physiologically than the respiratory systems in the most commonly-used test species, rats and mice.

In this regard, studies with combusted tobacco products have shown that chronic bronchitis cannot be replicated in rodents and that the data are inconsistent as to whether inhaled tobacco smoke can induce tumors and cancers in animal models. A recent article written by a tobacco industry consultant reported that results from years of chronic cigarette inhalation studies in rats, mice, hamsters, dogs, and nonhuman primates do not show significant increases in tumor development and are "clearly at variance with the epidemiological evidence in smokers, and it is difficult to reconcile this major difference between observational studies in humans and controlled laboratory studies in five different species."[2] The major reasons for these discrepancies are the fundamental physical, metabolic, and physiological differences between animals and humans, especially with regard to respiratory anatomy and physiology.[3] In the same regard, psychoactive drugs that are smoked will face similar issues with interspecies extrapolation.

Given the physical and physiological differences and the methodological challenges presented by attempting to replicate the human smoking experience in animals, the human relevance of the data collected from animals in this realm is negligible and the suffering imposed on these animals unjustifiable.

Non-Animal Methods to Assess Acute Inhalation Toxicity

In vitro alternatives exist to assess inhalation toxicity of smoked products, including the reconstructed human tissue models described in Table 1. Tobacco industry scientists have concluded that "*in vitro* toxicology tests can be successfully used both for better understanding the biological activity of cigarette smoke… and for guiding the development of cigarettes with reduced toxicity."[4] Thus, these methods can and should also be applicable to novel psychoactive drugs that are smoked.

As an example, the Canadian government's federal *Regulations Amending the Tobacco Reporting Regulations* requires that manufacturers conduct three tests to assess the toxicity of their tobacco products. All of the required tests are *in vitro*, non-animal methods – bacterial reverse mutation assay, neutral red uptake assay, and the *in vitro* micronucleus assay[5]. These tests are widely validated and have been shown to effectively identify the

mutagenicity, cytotoxicity, and clastogenicity, respectively, of whole cigarette smoke as well as individual tobacco ingredients and compounds. If the Canadian government deems these *in vitro* tests sufficient to analyze the toxicity of cigarettes, they should be adequate to assess the safety of smoked psychoactive drugs.

Method Name	**Comments**
MatTek's (Ashland, Mass.) EpiAirway™ System	Consists of normal, human-derived tracheal/bronchial epithelial (NHBE or TBE) cells that have been cultured to form a pseudo-stratified, highly differentiated three-dimensional model closely resembling the epithelial tissue of the respiratory tract. EpiAirway tissues are grown on cell culture inserts at the air-liquid interface, allowing for gas phase exposure of volatile materials in airway inflammation and irritancy studies, as well as in inhalation toxicity studies. This system has been well characterized histologically and biochemically (cell markers) and in terms of biological response to known toxins and pharmaceuticals. See: http://www.mattek.com/pages/products/epiairway
SkinEthic Laboratories' (Nice, France) reconstructed human esophageal and alveolar epithelium models	These models use immortalized human esophageal (Kyse 510) or alveolar (A549) cells and are structurally and mechanically similar to MatTek's and also form epithelial tissue that histologically resembles cell layers of the human lung. See: http://www.skinethic.com/index.asp
Epithelix's (Genève, Switzerland) MucilAir	Epithelix's MucilAir is a three-dimensional model of the human airway epithelium which is made of primary human cells isolated from the nasal cavity, the trachea and the bronchus. This model mimics the *in vivo* tissues of the human respiratory epithelium. This model can also be used for repeated-dose studies because the cells maintain their characteristics for up to a year in culture. See: http://www.epithelix.com/content/view/5/6/lang,en/
Wyss Institute's Lung-on-a-chip	Lung-on-a-chip mimics the complicated mechanical and biochemical behaviors of a human lung. See: http://wyss.harvard.edu/viewpage/240/

Oral Toxicity

It is clear that the results of acute toxicity testing in animals are not relevant to human health considerations. The European Medicines Agency (EMEA) Committee for Medicinal Products for Human Use (CHMP) "Concept paper on single dose acute toxicity" presents the findings of a joint information-sharing initiative among 18 European pharmaceutical companies and contract research organizations seeking to establish the relevance of acute toxicity data to the drug development process[6]. In a striking consensus, these companies agreed that single dose acute toxicity studies are not useful for keeping unsafe compounds from reaching human trials and do not provide unique insights into the safety of a possible medicine. Additionally, a coalition of European pharmaceutical companies determined that regulatory decisions were almost never predicated on the results of acute oral toxicity tests[7], prompting the removal of the requirement for acute toxicity testing from the International Council on Harmonization (ICH) M3 guidelines for non-clinical safety studies for human clinical trials of pharmaceuticals[8].

With regard to whether acute toxicity testing is useful to predict the consequences of human overdose, Chapman *et al* report a consensus among representatives from poison centers, the pharmaceutical and chemical industries, and regulatory bodies that the information it provides is of little value[9]. This is partly because high doses of chemical substances often elicit non-specific effects in animals that have no relevance to incidences of human overdose. In addition, acute toxicity testing typically does not provide information on adverse and functional effects, target organ toxicity, and toxicokinetics that is considered by poison centers to be most useful. The authors conclude that better information for the treatment of poisoning could be obtained from tests that are already carried out as part of the regulatory process.

Furthermore, hazard classification often does not adequately predict human toxicity[9]. A study of outcomes of human poisoning cases with three organophosphorous pesticides, all categorized as class 2 (LD_{50} > 5 ≤ 50 mg/kg) by the Globally Harmonized System of Classification and Labelling of Chemicals, found significant differences in severity of symptoms and likelihood of death, despite having similar LD_{50} values from acute toxicity studies[10]. Even in cases for which hazard class has been reported to correlate with mortality, mortality rates are highly variable among substances within a class; in one study, mortality rates for seven compounds in class 1 ranged from 24% to 0%[11].

Non-Animal Methods to Assess Acute Oral Toxicity

There are several alternatives to oral toxicity testing in animals, including the Normal Human Keratinocyte Neutral Red Uptake (NHK NRU) Assay, the Balb/c 3T3 Neutral Red Uptake (3T3 NRU) Assay, the EvaTOX assay (currently awaiting acceptance from ECVAM to enter their validation programme), and Quantitative Structure-Activity Relationship (SAR) Models (Table 2). The results for immortalized 3T3 cells and primary NHK cells were similar in the validation study; however, the 3T3 NRU assay is more cost and time effective than the NHK NRU assay[12].

The United States Environmental Protection Agency (EPA) affirms that data from the 3T3 Neutral Red Uptake (NRU) cytotoxicity assay may be used in a weight-of-evidence approach

for determining starting doses for *in vivo* acute oral systemic toxicity studies but not for hazard category classification purposes. Recently, the ACuteTox project reported the results of its prevalidation of a tiered testing strategy using eight *in vitro* assays[13]. The outcome of this study reinforced previous results obtained with the 3T3 NRU assay, supporting its use to identify unclassified substances (LD_{50} > 2000 mg/kg) as a first step in a tiered testing strategy. In addition, a number of assays were identified that were able to flag substances as neurotoxicants and nephrotoxicants. These assays could be used to alert on tissue-specific toxicity for substances that are identified as toxic (predicted LD_{50} < 2000 mg/kg) with the 3T3 NRU assay. It was also concluded that the combined use of DEREK and METEOR software is likely to improve the ability to predict the toxicity of an unknown substance or its major metabolites.

TABLE 2: Non-Animal Methods to Assess Acute Oral Toxicity

Method Name	**Acceptance**	**Comments**
Balb/c 3T3 Neutral Red Uptake (3T3 NRU) Assay	OECD GD 129 (2010) and recommended to U.S. agencies by ICCVAM (2008) to estimate starting doses for oral acute toxicity	**Principle of the Test:** The NRU assays are based on the ability of viable cells to take-up and store the dye neutral red so that test substances that cause cell death and/or inhibition of cell growth will result in a decrease in the amount of neutral red retained by the culture. The *in vitro* 3T3 NRU cytotoxicity assay has been demonstrated to correctly discriminate non-toxic (those with an $LD_{50} \geq 2000$ mg/kg) from more toxic chemicals[14], and shows very good correlation with mammalian LD50 data at both extremes of the toxicity spectrum (i.e. very toxic and non-toxic)[13]. Both NRU *in vitro* assays (3T3 and NHK) are approved to determine starting doses of test substances for two acute oral toxicity test methods (the Up-and-Down Procedure OECD 425 and the Acute Toxic Class Method OECD TG 423). **Congruence with *in vivo* data:** 3 laboratories independently tested the ability of the 3T3 NRU assa distinguish between toxic and non-toxic chemicals wit 56 chemicals and obtained 92-96% sensitivity[15]. **Considerations:** Limitations to both NRU *in vitro* assays (3T3 and NHK): General differences between cell culture systems and animals create a difference with respect to how a substance is delivered and how it is distributed and metabolized within cells. Because animals must absorb the substance after oral administration, certain organs may not be exposed

		to the same amount of the substance or may not be exposed to the substance for the same length of time; this is in contrast to the direct addition of the test substance to cells in culture. Additionally, if a test substance only produces toxicity through a specialized mechanism in a specific cell type, the effect may not be observed in 3T3 or NHK cells. 3T3 and NHK cells have little to no capacity to metabolize xenobiotic compounds.
Normal Human Keratinocyte Neutral Red Uptake (NHK NRU) Assay	OECD GD 129 (2010) and recommended to U.S. agencies by ICCVAM (2008) to estimate starting doses for oral acute toxicity	**Principle of the Test:** See principle of Balb/c 3T3 Neutral Red Uptake (3T3 NRU) **Considerations:** See considerations of Balb/c 3T3 Neutral Red Uptake (3T3 NRU)
CeeTox's AcuteOralTox-LD50 *in vitro* screen		A recent collaboration between CeeTox and L'Oreal has resulted in the development of an AcuteOralTox-LD50 *in vitro* screen which combines several *in vitro* concepts to predict acute oral toxicity without using animals[16]. This screen considers both pharmacological and physical-chemical properties of a substance in addition to the CTOX Panel®, which is a multi-parameter, cell-based *in vitro* system for predicting acute systemic toxicity. Analysis of 76 substances demonstrated that 75% of chemicals in GHS categories 1, 2 and 3 were correctly classified and the sensitivity and specificity were 85% and 89%, respectively, at an LD50 threshold of 500 mg/kg. L'Oreal has already assessed more than 100 compounds using this assay, and a manuscript on the assay and results is currently in the process of being submitted for publication.
MatTek's EpiOral and EpiGingival models		For oral toxicity testing, MatTek's EpiOral and EpiGingival models consist of normal, human-derived epithelial cells that allow *in vitro* study of irritation, oral pathologies, and basic oral cavity phenomena. The cells have been cultured to form multilayered, highly differentiated models of the human buccal (EpiOral) and gingival (EpiGingival) tissues. Morphologically, these tissue models closely parallel native human tissues, thus providing a useful *in vitro* means to assess irritancy, disease, and other basic oral biology phenomena. These tissue models have been extensively studied. SkinEthic also offers models of reconstructed human

		oral and gingival epithelium. These cell systems have been well characterized in terms of histology, biochemistry, and biological response. See: http://www.mattek.com/pages/products/epioral
Quantitative Structure-Activity Relationship (QSAR) Models		QSAR models can be used to estimate the likelihood of toxicity of chemicals (for example, the combined use of DEREK and METEOR software can be used to predict the toxicity of an unknown substance and its major metabolites).

Non-Animal Methods to Assess Repeated-Dose Oral Toxicity

Differences in the activities of the liver are a major contributor to the species differences observed in the toxicity of chemicals and drugs. Several reviews of the ability of rodent tests to predict human toxicity have shown that they are only about 40-60% predictive[17,18].

TABLE 3: Non-Animal Methods to Assess Repeated-Dose Oral Toxicity (adapted from [19-21])

Target	Method Name	Comments
Liver	*In vitro* hepatotoxicity on human liver cell lines	One study showed 80% of 243[22] and another showed 100% ten[23] hepatotoxicants were detected using this method.
Kidneys	*In vitro* kidney cell lines	One study showed good prediction with *in vivo* data for 15 nephrotoxicants tested using this method[24].
Heart	*In vitro* heart cells	One study showed 81% of six[25] and another showed 97% of four[26] cardiotoxicants were detected using this method.
Nerves	*In vitro* neuronal cells test	Excellent agreement between *in vivo* and *in vitro* predictions for organophosphorus compounds[27].
Lungs	EpiAirway or MucilAir: *In vitro* lung epithelial cells	81% correlation with existing human data with 11 chemicals using MucilAir[28].
Immune System	CFU-GM (from bone marrow cells) *In vitro* human whole blood cytokine assay	Accurate prediction of *in vivo* results for five out of six substances tested for a pre-validation study; positive results for an additional 20 substances tested[29]. The *in vitro* results correlated well with *in vivo* data for 31 compounds tested[30]. 100% of 6 immunotoxic compounds were detected using this method[31].

	In vitro lymphocyte proliferation assay	
QSAR Computer Models	TOPKAT DEREK LAZAR	QSAR computer models can be used to assess repeated dose toxicity. TOPKAT was able to predict 30% LOAELs within a factor of 3, 60% within a factor of 10 and 96% within a factor of 100 for 393 chemicals tested[32]. LAZAR showed 89% accuracy within 1 log from experimental value[33].

Toxicokinetic Investigations

Animals have significantly different metabolism and physiology to humans. As a result, before *in vitro* ADME studies on human cell models were routinely used by the pharmaceutical industry, the failure rate of drugs in clinical trials due to poor prediction of ADME was 40% - now it is only 10%[34].

Non-Animal Methods to Assess Toxicokinetics

TABLE 4: Non-Animal Methods to Assess Toxicokinetics		
Endpoint	**Method Name**	**Comments**
Pulmonary Absorption (human lung epithelial for inhalation)	PRIT Air / Liquid Interface (ALI) culture and exposure system	The PRIT® ALI system uses membrane cultures of adherent cells or tissues and can be used to study inhalable substances. See: http://www.item.fraunhofer.de/de/forschungsbereiche/toxikologie-umwelthygiene/in-vitro-toxikologie/PRIT.html
Absorption	*In vitro* dermal absorption test	*In vitro* dermal absorption studies may provide information to characterize systemic absorption (through skin *or* other routes).
Absorption	Intestinal Absorption in Caco-2 cells	See: http://www.item.fraunhofer.de/de/forschungsbereiche/toxikologie-umwelthygiene/klinische-chemie.html
Distribution	Human-on-a-chip	Human-on-a-chip integrates multiple organ-on-a-chip systems to mimic the whole human body See: http://wyss.harvard.edu/viewpressrelease/91/
Metabolism	Liver-on-a-chip	The liver-on-a-chip is designed to mimic what happens in the human body and will be especially important considering how poorly animal studies predict human metabolism and human liver toxicity. See: http://spectrum.mit.edu/articles/features/liver-on-a-chip/
Distribution and Excretion	Mathematical physiologically-based toxicokinetic (PBTK) models	Mathematical physiologically-based toxicokinetic (PBTK) computer models consist of a set of physiological and chemical parameters that can predict the distribution and excretion of substances through the human body following initial input of information on absorption and metabolism. This information can be derived from existing *in vivo* or from *in vitro* assays. • 80% accurate distribution for 123 drugs within 2-fold error[35]. • 70% accurate for 19 drugs tested[36]. • 90% accurate prediction of renal excretion for 40 compounds tested[37]. • 88% precise prediction of renal clearance for 141 drugs tested[38].

Metabolism	*In vitro* assays on hepatocytes	Freshly isolated or cultured hepatocytes and subcellular fractions (e.g. microsomes) from liver may be used to study possible metabolites and examine local metabolism in a target organ. It may be useful to study the inhibition and induction of specific cytochrome P450 isozymes (e.g., CYP1A1, 2E1, 1A2, and others) and/or phase II enzymes by the parent compound using *in vitro* studies. Information obtained may have utility for similarly structured compounds[39]. A review of studies showed that hepatic clearance could be predicted using human liver microsomes[40]. *In vitro* assays using human liver cells were as predictive as animal tests for 50 drugs tested[41]. *In vitro* tests with PBPK modeling (SCHH-PBPK) were more accurate for humans than *in vivo* rat and dog assays[42]. Also see: http://www.item.fraunhofer.de/de/forschungsbereiche/toxikologie-umwelthygiene/klinische-chemie.html for examples: CYP profiling (microsomes), CYP inhibition screening (microsomes), CYP induction (primary human hepatocytes), N-acetyltransferase profiling (microsomes)

Genotoxicity and Carcinogenicity

The 2-year cancer bioassay in rodents has poor concordance between species (for example between humans and rats or rats and mice). Poor interspecies extrapolation can result from a number of different reasons, for example, different tumor types and mechanisms which are of little or no relevance to humans (described in more detail in PETA 2007[43]). This test is generally known to have serious limitations in its ability to predict human cancer risk[44,45]. In Europe, the most commonly performed carcinogenicity tests are the lifetime rodent bioassay[46] and combined chronic toxicity/lifetime rodent bioassay[47]. However, little attempt has been made to validate the lifetime rodent bioassay against human carcinogenicity[48]. According to Ennever *et al*[49], the sensitivity of animal bioassays is very high (all definite human carcinogens adequately tested were positive); however, the specificity is low. A survey of the US Environmental Protection Agency database to assess the human utility of animal carcinogenicity data showed the animal data were predictive for 42% of chemicals[44].

In 2006, People for the Ethical Treatment of Animals, US, (PETA US) analyzed the first 500 rodent cancer assays conducted by the US National Cancer Institute and National Toxicology Program (NTP) and found that these agencies judged approximately one in every seven studies to produce either equivocal evidence of carcinogenic activity or to be scientifically inadequate[43]. PETA US also analyzed the ability of one species/gender group (e.g., male mice) to predict the cancer risk for other groups of rodents (e.g., female rats) exposed to the same chemical and found that results in one species and gender frequently underestimated cancer incidence in the other species and genders, with the average false negative rate being 27.5 percent, but ranging as high as 40 percent in one case. With regard to false positives, the NTP has acknowledged that about half the chemicals it has tested have produced evidence of cancer in rodents[50-52] and reported that "two-thirds of the positive bioassays were positive only when the [maximum tolerated dose] was employed." The maximum tolerated dose is the highest-dose of a substance that will not shorten the animals' normal life span because of noncancer-related toxic effects and is often several orders of magnitude greater than typical environmental exposures. At these doses, cancer may result from nonspecific mechanisms such as increased cell proliferation.

Non-Animal Methods to Assess Carcinogenicity and Genotoxicity

Replacement of *in vivo* carcinogenicity testing can be achieved by employing a range of tests that assess both genotoxic and non-genotoxic effects. Table 5 describes the *in vitro* genotoxic assays that are accepted by the OECD and cell transformation assays (CTAs) that are in the process of OECD acceptance.

A number of well-established and regulatory-accepted *in vitro* genotoxic tests are available. Kirkland *et al* demonstrated that 93% of 553 rodent carcinogens were detected in at least one of the three most common *in vitro* genotoxicity tests (Ames-test, mouse lymphoma Assay and the *in vitro* micronucleus Test or Chromosomal Aberration Test)[53]. However, a caveat to the use of these tests is the relatively low specificity and high rate of misleading positive results, especially for tests measuring clastogenic effects (breaks in chromosomes, leading to sections of the chromosome being deleted, added, or rearranged) [20]. The combination of three *in vitro* genotoxicity tests as required by the European Scientific

Committee on Consumer Safety (SCCS) increases the sensitivity of the test battery (up to 90%), but the specificity (ability to identify non-carcinogens) decreased to below 25%.

Cell transformation assays (CTA) can detect both genotoxic and non-genotoxic carcinogens. These assays have been in use since the 1960s but have only recently been considered for regulatory use. CTAs rely on changes in cell colony morphology and monolayer focus formation. The CTAs are currently used for confirmation of *in vitro* positive results from genotoxicity assays and can be used in the weight of evidence assessment. Data generated by CTAs can also be useful where genotoxicity data for a certain substance class have limited predictive capacity (e.g. aromatic amines), for investigation of compounds with structural alerts for carcinogenicity or to demonstrate differences or similarities across a chemical category[20]. In addition, the tumor-promoting activity of chemicals can be investigated by the CTA.

The use of non-testing methods, including (quantitative) structure-activity relationships ([Q]SARs), grouping and read-across are an attractive means of filling data gaps in both hazard and risk assessment without requiring additional testing. (Q)SARs are mainly used for screening but also provide a means of filling data gaps in hazard assessment. Adler *et al* describe the status of (Q)SARs for carcinogenicity testing[20]. Most models are qualitative (SARs) and QSARs for non-genotoxic carcinogenicity are still in an early stage of development. Several (Q)SARs are available for predicting genotoxicity and carcinogenicity[54]. Freely available models in the public domain include CAESAR, Toxtree, OncoLogic, LAZAR and the OECD QSAR Toolbox. Commercial models requiring license fees include MultiCase, TOPKAT, HazardExpert, DEREK and ToxBoxes.

The threshold of toxicological concern (TTC) is a statistical approach used to establish a conservative default risk value based on worst-case assumptions about the chemical in the absence of data. It has regulatory acceptance as a risk assessment tool in the US for food packaging material and in the US and Europe to set acceptable exposure limits for genotoxic impurities in drugs. It has not yet been granted regulatory acceptance for use in cosmetics in Europe although the SCCS is conducting an on-going evaluation of the use of TTC for cosmetics.

Taylor *et al* describe an integrated testing strategy that combines the exposure-based threshold of toxicological concern approaches, with OECD accepted *in vitro* genotoxicity tests and CTA assays to replace *in vivo* carcinogenicity studies and provide a precautionary approach for consumers[19]. If the human exposure exceeds the TTC levels the Ames test, and one other genotoxicity test, should be performed. If both are positive it should be assumed that the chemical is a genotoxic chemical; if there is any doubt a CTA assay should be performed. Benigni and Bossa demonstrated that a tiered testing strategy, with inexpensive and fast tests in Tier 1 (e.g. the Ames test or structural alerts) and the Syrian Hamster Embryo (SHE) CTA in Tier 2, is able to identify up to 90% of carcinogens[55].

British American Tobacco uses the following *in vitro* assays to measure the cytotoxicity and genotoxicity of extracts of smokeless tobacco or the particulate phase of combustible tobacco smoke components: (1) the Ames test, to measure effects on single DNA bases (gene mutations) in bacterial cells; (2) the *in vitro* micronucleus assay, to measure structural

and numerical changes to chromosomes in mammalian cells; (3) the *in vitro* mouse lymphoma assay, to measure gene mutations and chromosome aberrations in mammalian cells; and (4) the Neutral Red cytotoxicity assay, to measure cellular viability[56]. The genotoxicity assays (Ames assay, *in vitro* micronucleus assay and *in vitro* mouse lymphoma assay) measure the ability of the chemicals to cause changes at different levels of the genetic material and each assay has different sensitivities (as discussed below). Therefore, when all three genotoxicity assays are combined, together with the cytotoxicity assay, they are able to detect most mutagens and cytotoxic compounds. The Committee on Mutagenicity (COM) "Guidance on a strategy for Testing of Chemicals for Mutagenicity"[57] currently recommends the Ames test, the *in vitro* micronucleus assay and the *in vitro* mouse lymphoma assay for the *in vitro* testing of chemicals. The Cooperation Centre for Scientific Research Relative to Tobacco (CORESTA) *in vitro* toxicology task force[58] recommends the Ames test and the neutral red uptake assay. Therefore, the battery of *in vitro* assays used by British American Tobacco meets the requirements of both COM and the CORESTA *in vitro* toxicology task force.

Table 5. *In vitro* genotoxicity and cell transformation assays that can be used in an integrated testing strategy to replace *in vivo* carcinogenicity studies (adapted from [19-21])

Method	**Regulatory Acceptance / Status of validation**	**Comments**
Genotoxicity Tests	OECD TG 471 (1997): Bacterial reverse mutation (Ames) test	**Principle of the test:** Identifies gene mutations (point mutations, base pair substitutions and frame shift mutations). **Congruence with *in vivo* data:** • 90% of rodent carcinogens detected when combined with MLA and MNT assays[53] • 77% accuracy on 368 chemicals[59] • The application of the Ames test to a large number of chemicals has shown that this test has a high positive predictivity for chemical carcinogens (around 80%)[60]. **Considerations:** Prokaryotic cells differ from mammalian cells in factors such as uptake, metabolism, chromosome structure and DNA repair processes. *In vitro* tests often require the use of an exogenous source of metabolic activation which cannot mimic entirely the mammalian *in vivo* conditions. The test may not be appropriate for the evaluation of certain classes of chemicals, for example highly bactericidal compounds and those which are thought (or known) to interfere specifically with the mammalian cell replication system (e.g. some topoisomerase inhibitors and some nucleoside analogues). For a full list of considerations see OECD 471.
	OECD TG 476 (1997): *In vitro* cell	**Principle of the test:** Identifies gene mutations (point mutations, base pair substitutions and frame shift mutations) and structural and numerical chromosome

	gene mutation test in mammalian cells (MLA)	damage in Mouse Lymphoma L5178Y cells. **Congruence with *in vivo* data:** • 90% of 553 rodent carcinogens detected when combined with MNT and Ames test[53] **Considerations:** See comments on Ames test regarding metabolism. False positive results may arise from changes in pH, osmolality or high levels of cytotoxicity when the test chemical is added to the medium. Assay does not detect carcinogens that act by non-genotoxic mechanisms. The assay may have low specificity. For a full list of considerations see OECD 476.
	OECD TG 473 (1997): *In vitro* chromosomal aberration test in mammalian cells (CA)	**Principle of the test:** Identifies structural and numerical chromosome damage in mammalian cells (i.e. clastogenicity and polyploidy) **Congruence with *in vivo* data:** • 85% of 553 rodent carcinogens detected when combined with Ames test and MLA32 **Considerations:** See comments on MLA. For a full list of considerations see OECD 473.
	OECD TG 487 (2010): *In vitro* mammalian cell micronucleus test (MNT)	**Principle of the test:** Identifies structural and numerical chromosome damage in mammalian cells (i.e. clastogenicity and aneuploidy) **Congruence with *in vivo*:** • 83% agreement on 113 chemicals in ECVAM validation study[61] **Considerations:** See comments on MLA. For a full list of considerations see OECD 487.
Cell Transformation Assays (to detect genotoxic and non-genotoxic carcinogenicity)	Syrian Hamster Embryo (SHE) pH 6.7 and pH 7 (OECD TG in preparation) Balb/c 373 (Currently undergoing validation by ECVAM) Bhas 42 (Validation	**Principle of the test:** Used for screening, clarification of *in vitro* genotoxic positive results, hazard identification, identification of promoters, chemopreventative activity and mechanistic studies. Exposure to carcinogenic chemicals results in an increase of morphologically transformed colonies, which are characterized by disorganized growth patterns and considered as an early stage in the carcinogenic process **Congruence with *in vivo*:** • The SHE (pH ≥7 and pH 6.7) correctly identified 100% of the 44 inorganic human carcinogens tested and identified 9 out of 11 organic carcinogens[62]. A meta-analysis performed by the OECD indicated that the three CTA assays have an overall sensitivity of 90% of class I (known) and 95% of class II (possible/ probable) human carcinogens[62] . • SHE has a concordance with the rodent bioassay ranging from 85% (SHE pH ≥7) to 74% (SHE pH 6.7)[63].

	ongoing by JaCVAM)	• ECVAM workshop found that 80-83% rodent carcinogens were detected on 213 chemicals[64]. • P&G study showed 85% agreement with rodent data with 56 chemicals[65]. • Pfizer study showed 89% agreement with rodent data with 19 chemicals[66]. **Considerations:** SHE cells retain the ability to biotransform xenobiotics. Future developments: An improved protocol has been developed for the Balb/c 3T3 method which allowed more reproducible results to be obtained. It should also be noted that the SHE assay uses embryos harvested from hamsters that are killed for this purpose. **Status:** A prevalidation study with SHE (pH 6.7 and 7.0) was organized by ECVAM to address issues of standardization of the protocols, transferability and reproducibility. The experimental work finished in 2009. The data demonstrated that the SHE protocols and the assay system themselves are transferable between labs.

Developmental and Reproductive Toxicity

Animal tests for reproductive toxicity take a long time and use many animals. In addition, a number of studies have shown that they only detect about 60% of known human reproductive toxicants[67,68]. The EU ReProTect project concluded that a battery of *in vitro* tests "allowed a robust prediction of adverse effects on fertility and embryonic development"[69], with a combined accuracy of 70 to 100% for ten test chemicals[70].

TABLE 6: Non-Animal Methods to Assess Developmental and Reproductive Toxicity (adapted from [19-21])

Endpoint	Test Method	Comments
Embryonic development	*Ex vivo* whole embryo culture test (WEC) Micromass test (MM)	An ECVAM validation study showed up to 80% accuracy with 14 chemicals (100% for strong embryotoxicants)[71].
	Mouse/human embryonic stem cell test (EST)	An ECVAM validation study showed 78% agreement for 14 chemicals (100% for strong embryotoxicants)[71]; another study showed 75% agreement with *in vivo* for 63 chemicals[72]; another study showed 88% accuracy for eight drugs[73].
Male fertility	Computer-Assisted Sperm Analysis (CASA)	This test was evaluated by two laboratories with more than 35 chemicals[70].
	Testicular fragment culture	82% correlation with *in vivo* data for 11 chemicals tested[74].
	Leydig cell test	Good correlation for 15 chemicals[70] and detected 100% of five endocrine disruptors[75].
	Sertoli cell test	Good correlation for seven chemicals in two different laboratories[70].
Female Fertility	Bovine *in vitro* (oocyte) maturation (bIVM)	Good correlation with *in vivo* results for 15 chemicals[76] and good correlation on eight chemicals when tested in different laboratories[77].
Endocrine Effects	Estrogen receptor alpha binding assay	High accuracy for ranking 12 chemicals as strong, weak or no effect[78].
	Estrogen receptor (ER) – transcriptional activation assay, MELN	High accuracy on 16 chemicals and good inter-laboratory concordance[79].
	AR CALUX reporter gene assay	74% agreement on an inter-laboratory study of 64 chemicals[80]; excellent agreement for 14 out of 16 in a pre-validation study[81]; 85% agreement with the animals test for 50 chemicals[82].
	Estrogen	100% of 28 estrogen receptors were detected[83].

	receptor transcriptional assay, LUMICELL-ER	
	OECD TG 455[84]: Stably transfected transcriptional activation assay (STTA) estrogen	80% accuracy for 46 chemicals tested[85].
	H295R steroidogenesis assays based on a human cell line	78% accuracy for testosterone effect on 18 chemicals, 88% for estradiol effect on 16 chemicals[86].

Conclusion

In 1997, the U.K. government enacted a nationwide prohibition on the use of animals for testing alcohol or tobacco products[87]. The Home Office stated that in making a cost-benefit analysis, it could not justify the use of animals, classifying these experiments as "morally or ethically objectionable".[88] Additionally, the internationally renowned UK Nuffield Council on Bioethics reported that the Home Office "issued a policy statement to the effect that, in making the cost-benefit assessment, these tests were no longer considered a sufficient benefit to justify any use of animals."[89]

In addition to ethical or economic considerations, the use of animals to determine the safety of novel psychoactive drugs is scientifically unjustified. Important differences in the anatomy and physiology between humans and other animals make relying on animal tests to predict human safety dangerous. And, as described above, there are numerous non-animal tests that meet or exceed the accuracy of animal tests for predicting human health hazards. The Canadian government only requires *in vitro* tests to assess the safety of tobacco products[5]. In a similar manner, the safety of novel psychoactive drugs can be determined using a battery of *in vitro* tests. Thus, for scientific, economic and ethical reasons, the use of animals for testing psychoactive drugs is indefensible and we hope that the government will use its authority to ensure that only human-relevant and humane non-animal testing methods will be utilized to assess the risks of these products and fulfill the data submission requirements.

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