

# Evaluation of Some *In Vitro* Tests to Reduce and Replace the Sub-acute Animal Toxicity Studies

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**Summary** — The toxicologic and carcinogenic potential of chemicals is usually determined through a sequence of acute, sub-acute (14-day), sub-chronic (90-day) and chronic (two-year) studies in rats and mice of both sexes. The US National Toxicology Program (NTP) does not conduct acute toxicity studies. Dose levels for 14-day toxicity studies are typically estimated from information in the literature, if available. The toxicology information obtained from 14-day studies is used in the selection of doses for 90-day studies. The protocol for 14-day studies consists of five doses and control groups and five animals per group of each sex and species, resulting in the use of 120 animals per study. At present, in addition to refining the current testing protocols, the NTP is evaluating the potential for *in vitro* test methods to partially or completely avoid the need for 14-day toxicity studies, especially for chemicals where the dermal route of exposure is used. The *in vitro* assays used were the EpiDerm™ bioassay to estimate dermal irritation, the neutral red uptake (NRU) bioassay to estimate systemic toxicity and the primary rat hepatocyte cytotoxicity (PRHC) assay to estimate hepatotoxicity. The purpose of using these assays was to assess their potential for predicting relative *in vivo* toxicity and to support dose selection decisions for 90-day studies. In general, based on these limited number of studies, the EpiDerm and NRU tests were predictive of the responses observed in *in vivo* studies. However, a larger comparative database is needed to derive definitive conclusions regarding the value of *in vitro* tests in the prediction of *in vivo* effects.

**Key words:** *in vitro* testing, sub-acute animal toxicity, systemic toxicity.

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## Introduction

The US National Toxicology Program (NTP) toxicity and carcinogenicity study designs seek maximum toxicology information on chemicals, while following the philosophy of the Three Rs: using a reduced (optimum) number of animals, replacing animal with non-animal methods where possible, and refining animal procedures to minimise discomfort (1). The NTP has evaluated more than 500 chemicals for carcinogenicity in rodents. In general, the chemicals selected for carcinogenic evaluation by the NTP are studied in a sequence of sub-acute (14-day exposure), sub-chronic (90-day exposure), and chronic (two-year exposure) studies. Most of the studies are performed in Fischer 344 rats and B6C3F<sub>1</sub> mice. The results of 14-day toxicity studies are mostly used to select the dose levels for the subsequent 90-day studies. The purpose of the 90-day studies includes identification of target organ(s) lesions, similarities and differences in sensitivity between species and sexes, and determination of the shape of the dose–response curve. The toxicology information obtained from these studies is useful in risk assessment of non-cancer adverse effects. Also, the data from 90-day studies are the primary information source used for selecting the high dose for two-year studies. The two-year chronic toxic-

ity/carcinogenicity studies are performed at three dose levels, plus a control, using 50 animals/sex/species (2, 3).

The NTP ceased performing single-dose acute toxicity studies in 1980. The dose levels for 14-day toxicity studies are estimated from information in the literature, if available. Otherwise, the maximum dose level is selected based on the previously established criteria (2, 3). If sufficient information is available on the toxicity of chemicals being studied, 14-day studies are not performed. In such instances, the dose levels for 90-day studies are selected from the available information in the literature. The objective of this paper is to describe our efforts regarding the refinement of 90-day study designs to reduce the need for 14-day data and to evaluate some *in vitro* tests that might be used to replace or refine 14-day animal studies.

## Methods

### 14-day toxicity study protocol outline

As previously mentioned, the primary objective of the 14-day study is to provide information to select doses for a 90-day study. Usually, the route of exposure chosen is the same as expected for

**Table 1: Outline of National Toxicology Program (NTP) 14-day toxicity study design**

Parameter	Animals	Species	Sex	Group size	Total
Test group	5	2	2	5	100
Controls	5	2	2	1	20
Total					120

*Exposure duration: 14 days. Toxicity endpoints: survival, clinical signs of toxicity, body weights, selected organ weights, gross pathology, histopathology on selected organs.*

humans. The information from 14-day studies includes effects on target organ(s), sex/species differences, dose–response relationships, microscopic lesions, and a no adverse effect level. All of this information is used for dose selection in 90-day studies. However, when enough information is available in the literature, 14-day studies are not performed.

The usual protocol (Table 1) for the 14-day studies consists of six groups (five dose and one vehicle control) of animals of each species and sex, with five animals per group. The animals are observed two times daily, at least 6 hours apart, including holidays and weekends, for clinical signs of pharmacologic and toxicologic effects, as well as for moribundity or death. Animals, whose condition makes it unlikely that they will survive until the next observation, are sacrificed based upon criteria established by the NTP, immediately necropsied, and tissues retained in formalin for possible histopathologic evaluation. Body weights and organ weights (liver, thymus, right kidney, right testis, heart, brain and lungs, as well as other organs, as appropriate) are determined for all animals surviving until the end of the study.

A complete necropsy is performed on all exposed and control animals. All tissues are preserved in formalin. If histopathological examination is required, these tissues are trimmed, embedded, sectioned and stained with hematoxylin and eosin for microscopic examination.

### Reduction and refinement efforts

The NTP is actively trying to reduce the number of 14-day toxicity studies by refining 90-day study designs, so that preceding 14-day studies are not required. Also, the NTP is evaluating some *in vitro* tests that might give enough information to reduce the need for 14-day studies for some chemicals. A brief description of these efforts follows.

## Results

### Refinement of 90-day study design

The top dose levels for 14-day toxicity studies are estimated from information in the literature, when available. The remaining four dose groups are generally spaced by a factor of two, that is  $1/2$ ,  $1/4$ ,  $1/8$  and  $1/16$  of the top dose. The same spacing regimen is often used for 90-day studies. A sample of 60 studies from the NTP database of over 500 chemicals was analysed to determine if a wider spacing (three-fold versus two-fold) between dose levels in 90-day studies would eliminate the need for a preceding 14-day study (4). Derivation of maximum tolerated dose or minimum toxic dose (MTD) for chronic toxicity and carcinogenicity studies was used as an endpoint for this analysis. The results of the analysis suggest that the NTP need not perform 14-day studies routinely and, instead, could proceed directly to 90-day studies, using a three-fold spacing of the dose groups. It was concluded that wider spacing of dose levels in 90-day studies would not affect interpretation of the toxicology data or the selection of MTD for carcinogenesis studies. Furthermore, the wider dose range of 1–81, instead of 1–16, in five treatment group studies would have a much better chance of achieving a no observed adverse effect level (NOAEL), a value often used for setting safe exposure levels for non-cancer endpoints (5).

### Evaluation of *in vitro* tests

The NTP is developing a comparative *in vitro* and *in vivo* toxicity database on selected chemicals that might help reduce or replace animals in future toxicity studies (6). At present, development of the database is limited to the chemicals that are studied by the NTP by dermal route of exposure only. In that context, the NTP has tested the widely used industrial chemicals, diazoaminobenzene (DAAB), trimethylolpropane triacrylate (TMPTA), pentaerythritol triacrylate (PETA), diisopropylcarbodiimide (DIC), dicyclohexylcarbodiimide (DCC) and 2-

chloropyridine (2-CP), in a battery of *in vitro* assays and in 14-day dermal toxicity studies, using both sexes of B6C3F<sub>1</sub> mice and Fischer 344 rats.

The 14-day dermal toxicity studies were performed as outlined in Table 1. Male and female B6C3F<sub>1</sub> mice and Fischer 344 rats (3–4 weeks old) were used in these studies. For DAAB, TMPTA, PETA and 2-CP dosing was performed using a constant concentration of the test agent, varying the volume to adjust for dosage. For DCC and DIC, dermal doses of 0.3ml for rats and 0.1ml for mice were administered while concentration varied. All chemicals were administered in ethanol. Animals were dosed for 12 days (excluding weekends). The toxicity endpoints studied were: clinical signs of toxicity, moribundity and death; body and organ weights (liver, lung, right kidney, right testis, heart and thymus); complete necropsy on all treated and control animals; and histopathologic evaluation on organs or tissues that show gross evidence of treatment-related lesions, plus corresponding tissues in control animals (hematoxylin and eosin). The major findings from these studies are summarised in Table 2.

The *in vitro* assays used were: the EpiDerm™ bioassay to estimate dermal irritation, the neutral red uptake (NRU) bioassay to estimate systemic toxicity and the primary rat hepatocyte cytotoxicity (PRHC) assay to estimate hepatotoxicity and the systemic toxicity of chemicals that need to be biotrans-

formed to exhibit toxicity. The purpose of using these assays was to assess their potential for predicting *in vivo* toxicity and relative potency. A summary of results from these studies is presented in Table 3.

## Discussion

The key findings from *in vivo* and *in vitro* studies are compared in Table 4. Both the EpiDerm and NRU tests were effective in identifying skin irritants and most systemic toxicants, respectively. However, only the EpiDerm test ranked the chemicals according to results observed *in vivo*. Compared to *in vivo* rodent studies, the NRU test over-predicted PETA and TMPTA toxicity, and underestimated DIC and DAAB toxicity. DAAB was not accurately detected by the NRU test, but was identified as a liver and systemic toxicant by the PRHC assay. The response that was observed in the PRHC assay more accurately reflects the toxicity of DAAB, because it is biotransformed to two toxic intermediates, benzene and aniline. The PRHC assay predicted TMPTA, PETA and DIC to be liver toxicant, but these compounds were not toxic to the liver *in vivo*. However, since the route of exposure for the *in vivo* studies was dermal, it is not surprising that the *in vitro* tests did not give better predictions. This was especially true of the NRU test,

**Table 2: Summary of *in vivo* study results in rats and mice**

Chemical	Doses for 14-day study (mg/kg)	Major target organs
DAAB	0, 12.5, 25, 50, <b>100, 200</b>	Skin, site of application (SOA), thymus, lymph nodes, spleen, haematopoietic system, liver
TMPTA	0, 12.5, 25, 50, 100, 200	Skin (SOA), thymus
PETA	0, 12.5, 25, 50, 100, 200	Skin (SOA), thymus, kidney
DCC	MR 0, 3, 8, <b>26, 72, 212</b> FR 0, 4, 12, <b>35, 104, 320</b> MM 0, 7, 23, <b>64, 188, 566</b> FM 0, 9, 29, <b>81, 240, 728</b>	Skin (SOA), kidney, heart, thymus, liver
DIC	MR 0, 20, 60, <b>231, 692, 2121</b> FR 0, 26, 82, <b>208, 821, 2418</b> MM 0, 41, 113, <b>384, 1150, 3444</b> FM 0, 49, 144, <b>466, 1528, 4219</b>	Skin (SOA)
2-CP	0, 6.25, 12.5, 25, 50, 100	No effects

2-CP = 2-chloropyridine; DAAB = diazoaminobenzene; DCC = dicyclohexylcarbodiimide; DIC = diisopropylcarbodiimide; FM = female mice; FR = female rats; MM = male mice; MR = male rats; PETA = pentaerythritol triacrylate; TMPTA = trimethylolpropane triacrylate. Numbers in bold represent lethal doses.

**Table 3: Summary of *in vitro* study results**

Chemical	EpiDerm™ (EC50; µm/cm²)	Systemic toxicity (NRU50; µM)	Hepato- toxicity (MTT50; µM)
DAAB	++ (0.482)	+ (75.09)	++ (7.71)
TMPTA	+++ (0.228)	+++ (5.03)	+ (21.06)
PETA	+++ (0.268)	+++ (3.29)	+ (21.29)
DCC	++ (0.436)	+++ (6.01)	+++ (2.62)
DIC	+ (0.990)	+ (220.29)	++ (5.43)
2-CP	- (11.502)	- (25308.10)	- (17957.75)

+++ = severe; ++ = moderate; + = mild;  
- = negative.

Values in parentheses represent EC50, NRU50 and MTT50 values.

2-CP = 2-chloropyridine; DAAB = diazoaminobenzene; DCC = dicyclohexylcarbodiimide; DIC = diisopropylcarbodiimide; PETA = pentaerythritol triacrylate; TMPTA = trimethylolpropane triacrylate.

where the correlation would be expected to be best with intraperitoneal exposure, better with oral, and least good with the dermal exposure. Thus, decisions on the appropriate *in vitro* test needed to address chemical-specific endpoints, and other refinements in this *in vitro* battery are necessary to better address the range of endpoints derived from the 14-day dermal toxicity studies.

**Table 4: Comparison of *in vivo* and *in vitro* study results**

	EpiDerm™ Dermal irritation		NRU Systemic toxicity		PRHC Liver and systemic toxicity	
	<i>In vivo</i>	<i>In vitro</i>	<i>In vivo</i>	<i>In vitro</i>	<i>In vivo</i>	<i>In vitro</i>
DAAB	++	++	+++	+	++	++
TMPTA	+++	+++	++	+++	-	+
PETA	+++	+++	++	+++	-	+
DCC	++	++	+++	+++	+	+++
DIC	+	+	+++	+	-	++
2-CP	-	-	-	-	-	-

+++ = severe; ++ = moderate; + = mild; - = no effect.

2-CP = 2-chloropyridine; DAAB = diazoaminobenzene; DCC = dicyclohexylcarbodiimide; DIC = diisopropylcarbodiimide; NRU = neutral red uptake bioassay; PETA = pentaerythritol triacrylate; PRHC = primary rat hepatocyte cytotoxicity assay; TMPTA = trimethylolpropane triacrylate.

## Conclusions

The NTP is actively trying to reduce the number of 14-day toxicity studies by refining 90-day study designs so that preceding 14-day studies are not required. Also, the NTP is evaluating some *in vitro* tests that could be used prior to performing 90-day studies to identify target organs and/or potential systemic toxicity, thus reducing the need for 14-day studies.

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