Reduction and Refinement Alternatives: Where, When, and How?

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Summary

"In vitro testing" is the paradigm of the current era, and it is gaining increased acceptance from various research sectors, including regulatory science. The impetus that this subject has gained is credited to the awareness and understanding that there is enormous scope and value associated with in vitro alternatives. First, alternatives can reduce a great many of the ethical issues associated with the use of animals in research. Second, there are numerous in vitro studies currently available that are potentially competent to reduce, if not completely replace, animal experiments. Considering the number of chemicals that are added every year and the amount of testing that goes into assessing each of these chemicals, it is worthwhile to encourage the use of in vitro alternatives that may be money-saving, time-saving, and certainly "animal-saving!" Thus, it is a moral responsibility to wake up to the situation and adopt existing, established in vitro methods for chemical testing, as well as to devise newer methods for potential use in the future.

Keywords: in vitro genotoxicity, reproductive toxicity testing, 4Rs, GLP

1 Introduction

Safety evaluation studies are regulatory requirements to fulfill a risk assessment mandate. Almost all new chemical, pharmaceutical, and biotech products entering the market should be assessed for intrinsic toxicity by a battery of *in vivo* animal studies covering acute to chronic and special studies such as reproduction, neurotoxicity, and others. Although extrapolation of animal data to man is not always possible or easy, these animal data could be used to avoid the introduction of a potentially toxic product. In the case of new drug development, *in vivo* animal data are used to identify target organs, if any, that form the basis for clinical trials. In the case of new chemical development, *in vivo* toxicity studies are used to make risk predictions by deriving non-observable effect levels (NOAEL), which in turn will be used to predict maximum residue limits (MRL), acceptable daily intake (ADI), and acute reference dose (ARD). Complex *in vivo* metabolism studies also were conducted in large lactating animals to find out the distribution and accumulation of the active compound and its metabolite in edible tissues/fluid. Different countries have different guidelines and data requirements to register these products and efforts to harmonize these guidelines have met with only limited success. Consequently, there is often repetition of *in vivo* studies to meet the legal obligations in these countries.

2 Validated *in vitro* methods

Over the last 20 years, a number of validated *in vitro* methods have been developed as potential replacements for *in vivo* regulatory toxicity studies (Kandárová and Latašiová, 2011). The *in vitro* genotoxicity studies – micronucleus, chromosomal aberration, mouse lymphoma (gene mutation) studies, coupled with the classical Ames bacterial assay, are the most well-established battery of mutation studies, the results of which are widely used to draw conclusions on the mutagenicity of a chemical/drug/impurity.

3 Alternative methods that require fewer animals or no animals

New methods have totally avoided/replaced the painful and unethical *in vivo* studies in new chemical and drug development. The introduction of *in vitro* skin (OECD, 2004) and *in vitro* eye (OECD, 2009a,b) assays no longer require the use of rabbits. Use of artificial skin, biopsies, and bovine cornea from slaughterhouses are now used as alternatives for these tests. In addition, test guidelines have prescribed the conditions, such as pH, and previous information under which these studies can be limited, and therefore the animals' pain during the test could be minimized. Reduction of the number of animals has been successfully achieved for LD_{50} studies – from the classical multiple treatment dose studies to up and down / limit dose investigations, in which only animals of one sex, with as few as three animals, have been used in the experiment. The LD_{50} is the most important data requirement for classification of the product. In the case of agrochemicals, data from the acute oral LD_{50} is required in one or more rodent species. Classification of hazard is based on acute oral and acute dermal LD_{50} values from animal investigations. The revised guidelines of LD_{50} studies

(OECD, 2002a,b, 2008) have greatly reduced the number of animals used, and researchers employ statistical tools to predict LD_{50} values. This is the major achievement of the OECD group. In India, too, the Central Insecticides Board (http://www.cibrc.nic.in) banned the conduct of full-fledged LD_{50} studies as of 2005, recommending instead the use of LD_{50} studies with the revised OECD guideline using the limited number of animals to conduct the study.

Furthermore, the allergy skin sensitization study based on evaluations in Guinea pigs has been replaced with the mouse lymph node assay (OECD, 2010), which employs a minimum number of mice.

4 Extended one generation reprotox testing – Reduction alternative

Reproductive toxicity testing is expected to account for 90% of animals for REACH registration. So far, REACH has received more than 150 proposals with two-generation reproduction data. The International Council on Animal Protection (ICAPO) has called for immediate action by companies and regulatory authorities worldwide to replace the traditional two-generation animal test for reproductive toxicity with a new extended one-generation method. The new extended one-generation reproduction toxicity was accepted by OECD (2011) as the most viable alternative for the two-generation study. The extended one-generation test guideline is designed not only to evaluate reproductive performance but also to assess neurological, immunological, and endocrine integrity in the tested rat. Furthermore, in the OECD 416 two-generation reproductive toxicity study, 2600 rats are used , whereas in the extended one-generation study only 1400 (a reduction of 50%) rats are used and still produce the usual information. Piersma et al. (2011) proved that the one-generation reproduction toxicity study gives good information on the parameters assessed, and the second generation mating and offspring very rarely will provide critical information not seen in the first generation. With an estimated 2000 substances that have to be tested for reproductive toxicity for submission to REACH in the next 3 years, adoption of the extended one-generation reproductive toxicity study would mean a 2.8 million reduction in the number of laboratory animals used (EC, 2006). In August 2011, the OECD officially accepted the extended one-generation reproduction study protocol (OECD, 2011) in place of the existing two-generation reproduction study design. Although the exact *in vitro* alternatives for many *in vivo* animal studies may not be possible at this time, some of the replacement alternatives and refinements discussed above must be continued in order to reduce the number of animals in other regulatory studies (http://www.alttox.org).

5 Attention of Regulatory Authorities in India invited

Indian regulators still demand *in vivo* skin irritation, eye irritation, guinea pig based allergy studies, and two-generation reproductive toxicity study reports on products as dossier. It is time that we abolish these studies and accept the available alternatives, presented above, which are globally accepted. At least in the case of new registrants, repetition of such studies involving whole animals should be discouraged, and risk assessment should be determined with the already available *in vivo* data. Mere repetition of some of these *in vivo* studies must be avoided and discouraged, and a more logical approach must be taken. Registration guidelines should be modified to accommodate these practices. Now that India is a member of the OECD GLP system (http://www.indianglp.gov.in), Indian regulators are required to align their test guidelines to OECD guidelines and methods.

Some regulators do not accept test reports that are not GLP (Good Laboratory Practices) compliant, and, therefore, they will ask for repetition of studies that are not GLP. So, if all regulatory *in vivo* tox studies required are generated under GLP compliance, such test findings will be accepted across the world, thus avoiding repetition of studies and thereby reducing animal use. Since GLP ensures maintenance and experimentation of animals under a humane and ethical environment, the use of only GLP-based studies must be encouraged.

6 CPCSEA and 4Rs

Committee for the Purpose of Control and Supervision of Experimental Animals (CPCSEA) is a body operating under the Ministry of Environment and Forests of Government of India and is responsible for the welfare of animals under experimentation in India. CPCSEA has been actively promoting the concept of 4 Rs (Pereira and Tettamante, 2005) and playing a critical role in the reduction of animals and the promotion of *in vitro* methods in education, academia, and regulatory testing in our country.

7 Conclusions

The EPA recently launched a program to evaluate 10,000 chemicals for potential toxicity through a program called ToxCast (http://www.epa.gov/ncct/toxcast/). Its goal is to reduce expensive animal studies, turning instead to novel technologies to predict toxicity using stem cells and other non-animal methods.

Thus, research must be encouraged to help scientists search for more such alternatives, with the goal of reducing the number of animals in regulatory studies while still making accurate predictions of toxicity.

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