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Review

ALTERNATIVE IN VITRO TOXICOLOGY: THE PRESENT AND FUTURE IN ARMENIA

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Abstract

The fundamentals and basic principles of alternative *in vitro* toxicology are discussed in this review. The concept of 3Rs (Refinement, Reduction, and Replacement) is described in brief. The legislative and regulatory backgrounds to shift from animal research to testing in experimental cell systems *in vitro* (cell cultures) are considered. Advantages and disadvantages of toxicological *in vivo* and *in vitro* models are discussed. The modern paradigm of toxicological testing *in vitro* and the strategy of its implementation are presented. The prevalent techniques (including validated ones) for determination of substances' cyto- and genotoxicity are briefly described. The results of authors' investigations in the field of the *in vitro* toxicology are given. The current state-of-the-art and prospects of alternative *in vitro* toxicology in Armenia are discussed.

Keywords: review, toxicology, *in vitro*, alternative methods, Armenia.

The term "alternative" emerged following the publication of Russell and Burch's book "The Principles of Humane Experimental Technique" [*Russell W., Burch R., 1959*]. According to authors, proper experimental design should consider methods that refined techniques to lessen pain or distress, reduced the number of animals necessary for a particular test, or replaced animals with non-animal models, such as *in vitro* cell cultures. The concept of *Refinement, Reduction, and Replacement* is now well known as the "Three Rs" (3Rs), and methods which incorporated the 3Rs are considered alternative ones [*Balls M. et al., 1995 b; Stephens M. et al., 2001*].

The strong incentive for development of 3Rs and alternative testing concepts was the global animal right movement. It aims preventing cruelty, abuse, suffering of animals, and their exploitation for human purpose (see: *The Universal Declaration of Animal Rights*). Public opinion strongly objects laboratory experimentation in animals. Awareness of moral obligations of the community in the animal welfare field, social activity of associations for animal protection, campaigns of demonstrations against vivisection have forced governmental and standardization bodies to revise the policy of laboratory animal use in the science and industry. Our country has a short story of independent social life, and the animal right movement does its first steps in the science [*Gasparyan G., Aroutiounian R., 2005*] and education [*Vardapetyan A., 2007; 2008*]. Nowadays we have an opportunity to rebuild toxicological studies to modern standards and disseminate 3Rs principles in the research area and in the general public of Armenia. The reduction of animal experiments in biomedical testing should be an initial goal.

In modern toxicological research, there has been a significant move-away from animal studies, largely due to the development of novel molecular technologies and cellular models. In recent years the considerable progress has also been made in developing and validating 3Rs alternatives for toxicity tests. To reach international consensus with both the scientific and regulatory communities, an agreement was reached on the validation process of toxicity test procedures [*Abdulla E. et al., 1995*]. The Organization for Economic Coopera-

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tion and Development (OECD) [*Balls M. et al., 1995 a*] and Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) [*Botham P. et al., 1995*] have developed and provided criteria for regulatory acceptance of methodology (see also: *Bruner L. et al., 1998; Purchase I. et al., 1998*].

Today we are witnessing an exciting paradigm shift in toxicology, a moment when non-animal techniques are going a mainstream. In safety evaluation, drug discovery, and product development, the use of *in vitro* tests has become common. Consistent with the US Government Principles on the Use of Animals in Research, Testing, and Education [*Interagency Research Animal Committee, 1985*], and the US Public Health Service Policy on Humane Care and Use of Laboratory Animals [*Public Health Service Policy, 2002*], *in vitro* basal cytotoxicity test methods should be used where appropriate before testing is conducted in animals. These techniques should be considered as a part of a weight-of-evidence approach to estimate the starting dose for acute oral *in vivo* toxicity test methods. For some types of substances, this approach will reduce the number of animals needed, and in some testing situations, the approach may also reduce the number of animals that die or need to be humanely killed.

The widespread use of alternatives is already being implemented by several large multi-national companies. In the pharmaceutical area, such a highly influential institution as the US Food and Drug Administration (FDA) acknowledges the substantial limitations of animal testing [*BioCentury, 2005*]. Animal testing of cosmetics will be eliminated in Europe within two-three next years [*Explanatory Memorandum, 2004*].

In 2007, the US National Research Council (NRC) issued a report on the future of toxicity testing that suggested that the time is ripe to develop a new toxicology paradigm not based on animal testing. The basic proposal of the report is to re-orient testing to the molecular level, rather than observing phenotypic responses of whole organisms, to shift "from the current apical endpoint whole-animal testing to cell-based testing" [*Toxicity Testing, 2007*].

There are substantial reasons to shift. The imperfection and limitations of animal testing are obvious and comprehensible. More than 20 years ago the report of the Royal Society (UK) identified the intrinsic shortcoming of *in vivo* toxicology, the problem of species-specificity of tests: "... it is rarely possible to be sure that the animal model properly represents the relationship in man" [*Risk Assessment, 1983*]. Other citations of the same sort: "… for the determination of the human lethal dose, the LD_{50} in animals is of very little value" [*Zbinden G., Flury-Roversi M., 1981*]; "... even if the LD_{50} could be measured exactly and reproducibly, the knowledge of its precise numerical value would barely be of practical importance, because an extrapolation from the experimental animals to man is hardly possible" [*Lorke D., 1983*]. Traditional animal toxicology is laborious and time-consuming; it requires large quantities of a product tested. Animal testing is difficult to adapt to modern trend for high-throughput screening technologies [*Vanparys P., 2002*] that ultimately put obstacles in the way of drugs and chemicals mass screening.

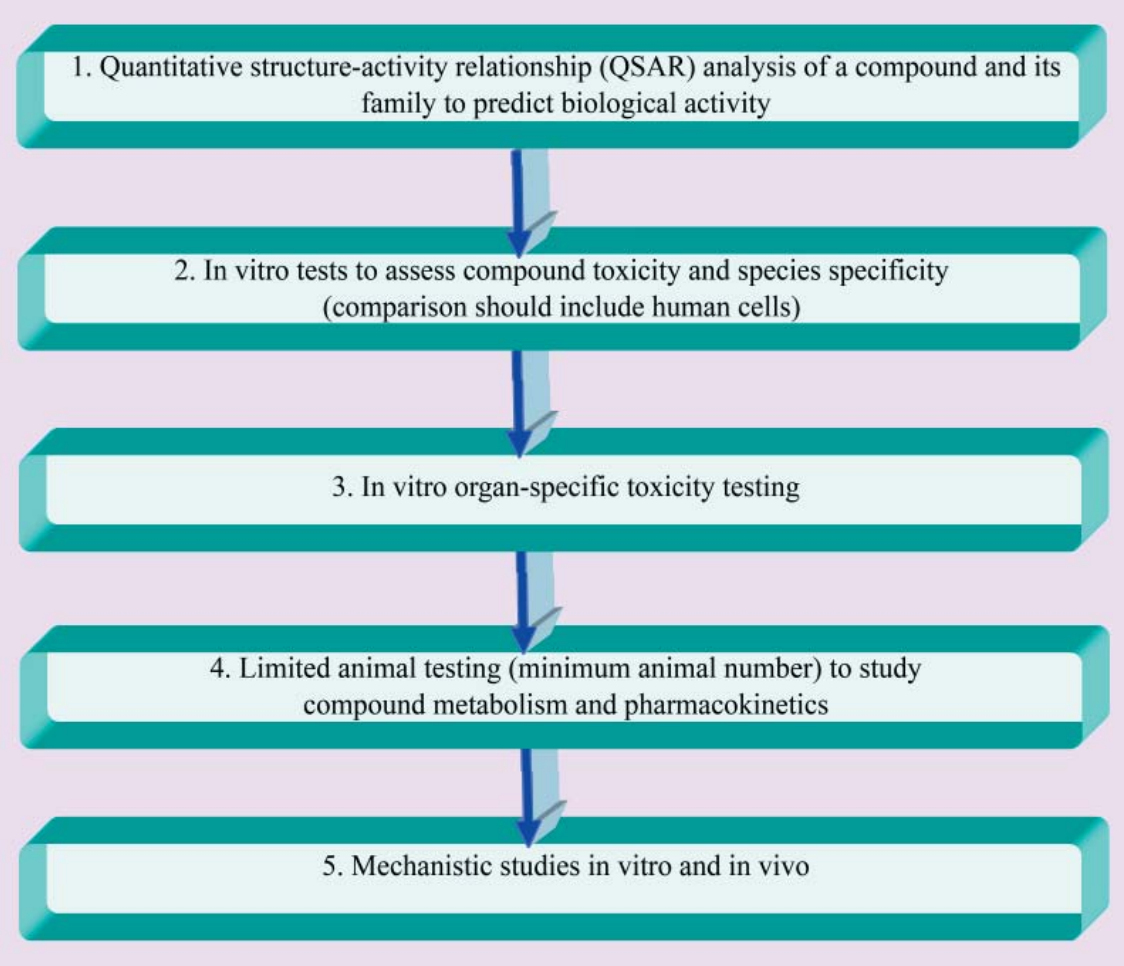
It is a necessity to use novel scientific tools to demonstrate safety and effectiveness of new products faster, with more certainty, and at lower costs [*Critical Path, 2004*]. A tool envisaging further development seems to be cells *in vitro* [*Huang R. et al., 2008*]. A wide range of cell types can be cultured, including those from a variety of tissues and from several species. These benefits are very useful, because they enable some measures of target organ and species–specific toxicity to be studied in culture. If human cells can be used, this can minimize problems with inter-species extrapolation [*Drug Testing, 2007*]. Shortly, the advantages of *in vitro* tests are that they are quick, inexpensive, and allow studying specific mechanisms of action.

In 1990, the US Center for Alternatives to Animal Testing (CAAT) has recommended a toxicology decision paradigm that can be now considered as a routine one [*A Report of the CAAT, 1990*]. It consists of the following main issues (Scheme).

In Armenia, there is an obvious problem in the risk assessment and revealing biological activity of new chemicals, potential drugs, food additives, environmental pollutants, etc. Only few research groups perform toxicological investigations. Is this sketchy schedule (Scheme) feasible in our country and, if yes, at what extent?

Scheme.

Toxicological paradigm of CAAT.



1. QSAR analysis. Chemical characterization would provide data such as a molecule's physical and chemical properties, probable routes of exposure, possible routes of metabolism, and likely interactions of the compound and its metabolites at the cell level. Much of this information would be obtained using various computational methods, including QSAR models, which predict biologic activity on the basis of molecular structure. In Armenia QSAR analysis is put into research practice at the Institute of Fine Organic Chemistry of Scientific-Technological Center of Organic Pharmaceutical Chemistry. Results of a structure-activity study of newly synthesized porphyrins were recently published by Armenian researchers [*Tovmasyan A. et al., 2008*].

2a. *In vitro* tests to assess compound toxicity. Our joint team at the Yerevan State University and the Institute of Molecular Biology of Armenian NAS is the only Armenian research group that uses animal cell cultures in toxicology. Now we have a collection of several human cell lines derived from various tissues (liver, blood, kidney, breast, cervix, bone, urinary bladder, and astroglia). We also culture normal embryonic human, chicken, and rat fibroblasts.

The cytotoxicity of soluble compounds and biocompatibility of insoluble ones (used in medical devices and as implants) is determined at our Laboratory by the following techniques (Table 1).

The cytotoxicity of compounds is routinely evaluated in dose/effect experiments (compound concentration/cell viability). The endpoint of all the tests is the determination of IC50 value (test compound concentration inducing 50% inhibition of cell viability). IC50 obtained can be extrapolated into LD50 values for compound acute toxicity *in vivo*. Regression formulae are used to weigh up the starting doses for single dose oral application in rats [*Wind M., 2008*]:

Table 1.

Cytotoxicity and biocompatibility *in vitro* techniques applied at the Yerevan State University

Tests	Cell functions and structures affected	References
Vital dye (trypan blue) exclusion	Cell membrane integrity	*Strober W., 1997*
Flow cytometry	Cell membrane integrity	*Ross D. et al., 1989; Flow Cytometry, 2000*
Neutral red (NR) uptake	Endocytosis and storage of vital dye (NR) in cell lysosomes	*Test Method Protocol, 2003*
MTT	Mitochondria	*Mosmann T., 1983*
Biocompatibility, elution test	Cell culture morphology	*Wallin R., Arscott E., 1998*

$$\log LD50\ (mmol/kg) = 0.439 \log IC50\ (mmol/L) + 0.621,$$

or

$$\log LD50\ (mg/kg) = 0.372 \log IC50\ (\mu g/mL) + 2.024.$$

Knowing the LD50 value, one can identify to what class of toxicity the investigated compound belongs (Table 2).

In vitro studies of genotoxicity provide important tools to enhance our understanding of chronic hazard of chemicals of interest and to predict these compounds' effects on humans. It is widely accepted that the vast majority of carcinogens can be detected with some confidence using the battery of *in vitro* and *in vivo* short-term tests for genotoxicity [*Ashby J., 1991*].

Table 2.

Classification of toxic compounds (see: *A Guide to the GHS, 2006)*

Toxicity classes	LD50 values
I	$LD_{50} \leq 5$ *mg/kg*
II	$5 < LD_{50} \leq 50$ *mg/kg*
III	$50 < LD_{50} \leq 300$ *mg/kg*
IV	$300 < LD_{50} \leq 2000$ *mg/kg*

The strategy for testing genotoxic activity includes a limited number of well-known and partly validated test systems. A battery approach is reasonable because no single test is capable of detecting all genotoxic effects. A unified approach for genotoxicity testing of pharmaceuticals has been developed [*Genotoxicity, 1997; Muller L. et al., 1999; Genotoxicity Testing, 2008*].

In our routine research we apply a set of techniques to study the chronic effects (genotoxicity) of compounds (Table 3).

The battery of tests listed (Table 3) was earlier used by us to evaluate the genotoxicity of new porphyrins' derivatives and known anticancer preparation cisplatin (cis-DDP) [*Hovhannisyan G. et al., 2004; 2005a; Arutyunyan R. et al., 2005a; Gasparyan G. et al., 2007*].

The applied set of techniques listed above (Tables 2 and 3) meets international standards for preliminary *in vitro* evaluation of compound toxicity and risk assessment.

2b. *In vitro* tests to assess compound species specificity. These tests include the study of compound cytotoxicity for cells derived from various species (the cells are desirable to be from the same tissue) and the comparison of obtained toxicity indices (e.g., IC50's). Human cells should be necessarily included. It is a way to learn interspecies differences in cell sensitivity to the compound of interest.

Earlier we have applied this approach to investigate species-specific cytotoxic activity of water extract of medicinal plant oleander [*Hovhannisyan N. et al., 2007*]. This extract was demonstrated to be toxic for humans (Jurkat, K-562, L-41, and HT-1080 cell lines) and primates (green African monkey COS-7 cell line) cells but not for rodent (rat PC-12 cell line) cells *in vitro*. These results agree with data obtained earlier in animal experiments [*Pathak S. et al., 2004*].

3. *In vitro* organ-specific toxicity testing. If a toxin acts in a specialized organ system in a whole animal, it may not produce a toxic effect by the same mechanism in cultured cells that are derived from tissue different from the target organ. For example, a neurotoxin that acts by a neuroreceptor-mediated

Table 3.

Genotoxicity *in vitro* techniques applied at the Yerevan State University

Tests	Cell structures and molecules affected	Endpoints	References
Chromosome aberration induction	Mitotic chromosomes	Heritable chromosome damage	*OECD Guidelines, Test № 473*
Micronucleus induction	DNA integrity in post-mitotic cells	Heritable chromosome/ DNA damage	*Parry J., 1998*
Comet assay	DNA lesions (reparable ?)	Early (acute) DNA damage	*Tice R. et al., 2000*
Ames test	Gene	Gene mutation	*OECD Guidelines, Test № 471*

pathway in animals would be expected to produce toxicity by a different mechanism in 3T3 or NHK cells, which are derived from fibroblasts, and skin cells, respectively. This type of testing includes the study of compound cytotoxicity for human cells derived from various tissues. For instance, we succeeded to reveal that blood cells (K-562 cell line) are more sensitive to cytotoxic action of new Ag- and Zn-porphyrins than cells derived from kidney (Cos-7 cell line) and prostate (DU 145 cell line) [*Gasparyan G. et al., 2007*].

4. Limited animal testing (minimum animal number) to study compound metabolism and pharmacokinetics. Both cell death and animal death may be produced by the same mechanisms, such as disruption of membrane structure or function, inhibition of mitochondrial function, disturbance of protein turnover, disruption of energy production, etc. [*Gennari A. et al., 2004*]. At the same time, the disadvantage of *in vitro* tests is that the homeostatic mechanisms and pathways found in animals are absent. *In vitro* models often represent the acute or short-term effects of the toxicant, while *in vivo* models include longer-term-treatment effects and the influences of biological integration [*Tiffany-Castiglioni E. et al., 1999*]. It can be stated also that animal and cell culture systems are different with respect to how a substance is delivered to the cell and how it is distributed, metabolized, and excreted. After oral administration, the toxin is absorbed from the gastrointestinal tract which involves the passage of membranes. Then the toxin may be metabolized and excreted. In a cell culture system, only membranes of the target cell and cellular organelles must be passed. No absorption and distribution by other cellular systems occurs. The excretion from the cell culture milieu cannot take place because of the absence of excretory system. Meanwhile, it is very important to study ADME (this abbreviation is an acronym for Absorption, Distribution, Metabolism, and Excretion) characteristics of chemicals as they are critical to compounds success or failure as medicines. That is why the *in vitro* testing should be followed by animal experiments. Results obtained *in vitro* should be used as predictive ones to minimize the number of both experiments *in vivo* and animals used.

5. Mechanistic studies *in vitro* and *in vivo*. Experimental cell systems *in vitro* are of exceptional value in mechanistic studies. They are ideally suited for investigations of the molecular, cellular and physiological mechanisms of chemically induced toxicity. There are substantial activities in using *in vitro* systems to advance mechanistic understanding of toxicant activities, and the use of human cells and tissue to define human-specific toxic effects.

We have been applying *in vitro* techniques to evaluate the cell death rate and discriminate necrosis and apoptosis (by morphological analysis of cells, DNA gel-phoresis, and flow cytometry) [*Hovhannisyan N. et al., 2007; Babayan N. et al., 2008*]. It should be noted, that apoptosis is almost impossible to be quantified *in vivo* due to problems of heterogeneity and the short half-life of an apoptotic cell. Moreover, we have been estimating the effects of substances on the cell proliferation and cell cycle rate (by flow cytometry) – parameters hardly measurable in a whole organism. Using the acellular comet assay modified version based on the treatment of DNA after lysis [*Kasamatsu T. et al., 1996*] we have demonstrated that Co-metalloporphyrin is able to interact directly with DNA, inde-

pendent of other possible cytotoxic effects [*Arutyunyan R. et al., 2005 b; Hovhannisyan G. et al., 2005 a;b*].

The future of alternative *in vitro* toxicology is not difficult to predict. Its main pathways are high-throughput human cell and cell-line assays, which progressively replace all animal tests in this area. The missing enzyme systems will be incorporated into target cells by genetic engineering [*Kirkland D. et al., 2007*]. Compounds would be further evaluated in "targeted testing" and new ways of identifying metabolites *in vitro* rather than in animals would be developed. New dose-response models would be based mainly on mechanistic *in vitro* assays, and extrapolated to humans [*Langley G., 2007*]. Today a tremendous creative energy is focused on advancing new techniques and technologies to replace animals in chemical and drug hazard identification and risk assessment, including the prediction of ADME characteristics. Some of these tools are based on cells, tissues, and sub-cellular components, ideally from human sources; some are "virtual" tools, harnessing the power of modern computational systems.

Thus, there are reasons to assert that in our country the alternative in vitro toxicology takes its earliest steps. For development of alternative *in vitro* toxicology in Armenia both the time and investments are needed, particularly, conditions and facilities for *in vivo* and *in vitro* research that will be in line with international standards and will meet demands of Good Laboratory Practice [*Seiler J., 2005*] and Good Cell Culture Practice [*Hartung T. et al., 2002*]. It seems more realistic and feasible to promote the alternative toxicology. The prerequisites to move on to this goal already exist. At the same time, Armenian researchers have a lot to do to join this mainstream in modern toxicology.

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