Current Considerations for the Effective Safety Evaluation of Drugs *In Vitro*

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Abstract: As the traditional *in vivo* tests are gradually shifting into *in vitro* tests, some new issues become apparent. This review discusses the essential considerations in endpoint selection, cell model quality, exposure concentration and linking factors between *in vitro* and *in vivo* studies in using *in vitro* models in risk assessment.

INTRODUCTION

Chemical safety is a major concern in modern society. The worries of health risks and environmental contamination require strict controls for the production of new chemicals. Drug safety is a particular concern as it is directly applied to human beings and animals. Risk assessment of chemicals is an essential part of pharmaceutical and chemical development and safety evaluation and toxicity studies are particularly important for drug development. Chemical risk assessment is currently undergoing a revolutionary shift from *in vivo*-based testing towards *in vitro*- and *in silico*-based testing [1-5]. Toxicological studies using *in vitro* models have dominated research reports in the literature for the past decade. However, some fundamental issues regarding *in vitro* models remain unresolved. These include (i) how to select endpoints for *in vitro* safety screening; (ii) what is the quality requirement for an *in vitro* model; (iii) how to decide an appropriate concentration range for *in vitro* studies; and, most importantly, (iv) how to link data derived from *in vitro* tests to the *in vivo* situation. This review will focus on these issues and explore a principle to frame *in vitro* and *in vivo* studies together.

1. ENDPOINT SELECTION: GENERAL TOXICITY AND SPECIFIC TOXICITY

General toxicity of a chemical is the fundamental information about its risk to human and animal health. Our body is a complex living system. There are more than two hundred different cell types in it [6] and about 20,000 genes in the cells encoding proteins to perform every function and action of life [7]. With the advances in life science and technology, toxicology has made great progress in understanding targets and mechanisms of chemical-induced adverse effects. It has been generally realised that chemical-induced adverse effects often show an enormous diversity. This implies an uncertainty that the adverse effects of a chemical can be identified using available tests based on current knowledge and strategy. No matter what advanced technology is introduced into toxicology studies, the fundamental priority information expected from drug safety evaluation includes two aspects: general toxicity and specific toxicity. Their relationship is a fundamental consideration in setting up strategies for drug safety evaluation. Although specific toxicity is often a cause of general toxicity, it proves difficult to identify. Thus the strategies for safety evaluation and target identification or mechanism elucidation are different. As shown in Fig. (**1**), when a toxicant is introduced into our body, its targets are often located at or below the cellular level. The cell is the basic unit of response to adverse effects. It is only when a normal cell function is affected that the higher levels in the system show functional alterations. Thus, we could introduce a concept, ‘effect-safety interface’, into the discussion. The cellular level can serve this interface because only when cells perform abnormal functions can our body ‘feel’ or detect it. For example, in the clinical trial stage of drug safety evaluation, volunteers, in fact, allow their own cells to sense any adverse effects which could then penetrate this interface and be detected. Therefore, the cellular level of response is the basis of safety evaluation. In other words, the main purpose of drug safety evaluation is to detect the adverse effects of a drug at the cellular level and above.

Here, general toxicity at the cellular level means general adverse effects on cell functions and behaviour rather than specific effects. Specific targets, in most cases, are relative concepts. For example, we often use terms like hepatotoxicity, neurotoxicity, nephrotoxicity, etc, to describe organ/ tissue specific targets. The affected tissues or cells are also called target tissues or cells. That description is specific enough for safety evaluation although it is not specific enough for direct target identification. The direct targets are often at sub-cellular and molecular levels. Target identification is a complex process and one compound may act on multiple targets. To assess the general toxicity, it is not necessary to know what specific targets might be acted on; if we do not know what the specific target is, we can still evaluate the fundamental safety of the compounds tested. As long as adverse effects disturb normal cell or system functions, it will alert the adverse risk.

We can explain why specific targets are not suitable for safety testing using the example of oxidative stress. Oxidative stress-induced cell damage is a multi-target event as

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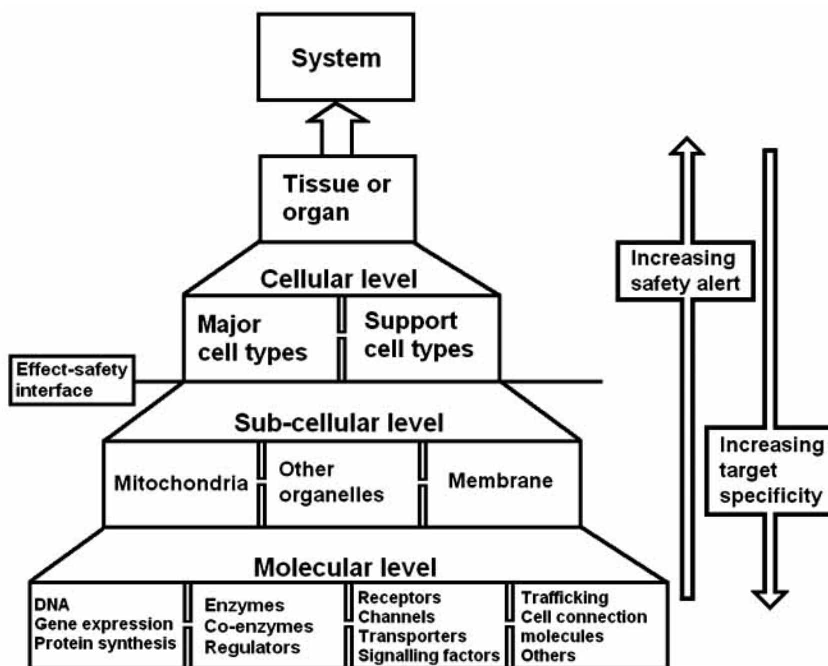

Fig. (1). Illustration of the effect-safety interface and target specificity. Targets of drugs or other chemicals can be tracked down to sub-cellular and molecular levels. Only when cell functions are affected can the system sense the effect and inform the risk. Thus the cellular level is the effect-safety interface.

shown in Fig. (**2**). Free radicals are main agents of oxidative stress. Reactive oxygen species (ROS) and reactive nitrogen species (RNS) generated by excessive nitric oxide (NO) are particularly concerned [8-12]. Fig. (**2**) shows the main consequences of oxidative stress reactions. ROS can damage many molecules directly but the oxidation of the [4Fe-4S] clusters in proteins by ROS has two main consequences: first, the protein (enzyme) inactivation; second, the released Fe^{2+} can drive oxidation of DNA *via* Fenton-induced OH radical formation. [13, 14]. Furthermore, electrophilic compounds or reactive metabolites, or an excessive production of free radicals can deplete antioxidants including glutathione (GSH), vitamins E & C [15]. This condition of excess of damaging oxidants due to lack of antioxidants, the free radical scavengers, cause oxidative stress. NO is soluble in both aqueous and lipid media and hence readily able to diffuse into plasma membranes and causes lipid peroxidation that can further form DNA adducts [16-19]. As a result, multiple targets can be attacked and a wide range of cell injuries can be incurred as shown in Fig. (**2**) [9,15]. Thus oxidative stress-induced protein dysfunction and DNA damage are unlikely to be limited to any specific protein or gene. Therefore, when an altered protein or gene is identified, it may not be a unique target because other proteins or genes might also be altered due to the nature of free radical-induced injuries. This implies that for oxidative stress-induced toxicity it is difficult to identify a single specific target. In other words, from the safety evaluation point of view, if we focus on some specific targets we may fail to detect damage elsewhere. In such cases, less specific or more general endpoints have more chance to detect adverse effects induced by the compound tested. Therefore more specific endpoints are not suitable for drug safety screening.

What endpoints are suitable for safety screening? As discussed above, endpoints at the sub-cellular or molecular levels can provide a fundamental understanding of mechanisms but limited information for safety risk assessment because different chemicals have different effects. At the sub-cellular level, mitochondria are special organelles which play a crucial role in maintaining cell functions because they are the major energy provider in cells. Mitochondria are often vulnerable targets of toxicants. When the respiratory chain is inhibited by free radicals or toxicants such as CO and cyanide or reactive metabolites, ATP production will be reduced or stopped. This can cause widespread dysfunction in cells or necrotic cell death. When the mitochondrial membrane is damaged by lipid peroxidation, it will increase permeability and trigger apoptotic or/and necrotic cell death [18]. Therefore, ATP, GSH (and GSSG) and enzyme leakage (e.g. LDH leakage, a commonly used cytotoxicity endpoint) are often used as endpoints to reflect mitochondrial function, oxidative injury and membrane damage respectively. These endpoints are less specific but reflect cell stress better than more specific endpoints and hence are better for safety testing.

The free radical injury theory can explain the phenomenon of multi-target involvement after exposure to some chemicals. After free radical attack, cells will eventually develop abnormal functions and behaviour. Therefore, at the cellular level, cell-performance-based endpoints can reflect general toxicity and better serve the requirements of safety screening of chemicals. Here, cell performance is defined as the ability of cells to perform their natural functions such as nutrient uptake, product release and other activities [20-22]. Cell behaviours include cell division, migration and cell-cell or cell-extracellular matrix (ECM) adhesions [23]. Cell func-

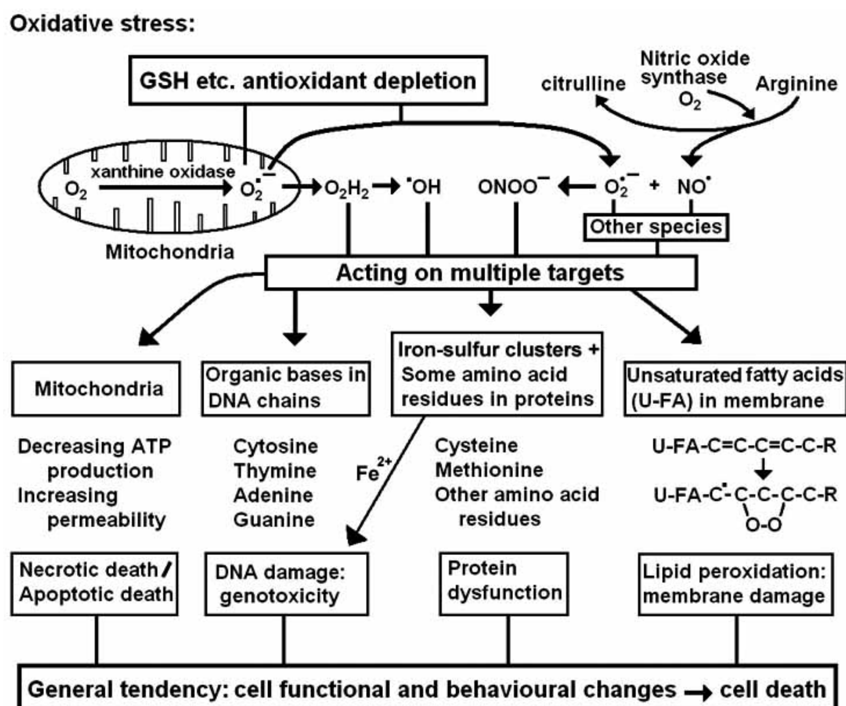

Fig. (2). An illustration of oxidative stress and multiple target injuries induced by free radicals. ROS and RNS are two main sources of endogenous free radicals. They can cause similar cell damages.

tional performance can be evaluated by examining nutrient and product changes in the medium. For example, the liver is a central organ in the transformation of energy-generating substances. When a cell is insulted, its energy metabolic function will be altered. Our previous work has shown in a liver spheroid model that glucose secretion, lactate release, galactose consumption and pyruvate uptake can generally reflect hepatic functional status and all these endpoints can be evaluated by detecting the changes of the components in medium outside cells rather than inside cells [20, 22]. They can give information about their functional situation and hence are regarded as performance indicators. Such endpoints, therefore, are suitable for safety screening testing of chemical-induced hepatotoxicity. Cell behaviour performance is a direct indication of cell general health. Currently used endpoints include cell growth doubling time [24] and the spheroid cell spreading inhibition test (SCSIT) including anchoring or detachment observations [21]. Abnormal or reduced cell behavioural ability is often seen after severe functional changes as previously reported [20-22] and hence indicates a more severe stage of cell damage than just functional alteration. Therefore, for safety screening testing of compounds, cell functional and behavioural changes after exposure to the compounds tested will provide robust information to inform safety risk no matter what the molecular targets or mechanisms might be.

In general, it should be clear that endpoints used for the risk screening testing of chemicals should be ideally reflect general cell responses to an unknown agent rather than more specific responses. As shown in Figs. (**1**) and (**2**), in endpoint selection, the more specific, the less chance to detect potential adverse effects simply because of the enormous diversity of potential targets at the sub-cellular and molecular levels. However, any effect that is significant enough to affect general cell functions should be detected by examining their performance changes.

Specific toxicity is the most complex part of safety evaluation. It is difficult to predict due to cell complexity, cell type variation, test model suitability and the huge varieties of chemical structure and physiochemical properties. Opposite to the screening testing of general toxicity, specific toxicity screening should identify specific targets. For example, phosphorous pesticides can inhibit acetyl-cholinesterase (AChE) that can break down acetylcholine rapidly after its action. When AChE is inhibited, acetylcholine will extend action time and cause neurotoxicity [25]; some antibiotics such as streptomycin can specifically cause hearing loss [26]. Many other chemicals may block or alter receptors and ion channels, enzyme catalytic centres etc and thereby act as stimulators, inducers, inhibitors or regulators. All these effects require specific means of detection. Some specific targets are difficult to identify until they have caused health problems and this often leads to a marketed drug being withdrawn from clinical use [2]. Such a withdrawal is often associated with a great economic loss. In addition, some long-term chronic effects may be a result of specific gene damage due to related gene mutation. Carcinogenesis is one such effect. Other gene mutations may cause dysfunction that may be not life-threatening. Theoretically, some protein dysfunctions are recoverable by synthesising new proteins and injured cells could be either repaired or replaced by new cells. Gene mutation, however, will cause permanent alteration of

cell phenotypes. Genomic and proteomic technology coupled with appropriate cell models will have unique advantages in mapping long-term chronic effects.

Specific toxicity is difficult to identify and still a bottleneck in drug development. Some new approaches such as qualitative structure-activity relationship (QSAR) of chemicals, genomic and proteomic technologies have shown great advantages in this area. QSAR can provide predictive information about the potential metabolism and biological action of a compound [1, 3]. Therefore *in silico* approaches can use accumulated structure-activity information of chemicals to make reasonable predictions that could guide appropriate testing [2]. Genomic and proteomic approaches offer the hope to understand specific targets and fundamental mechanisms of chemically induced adverse effects. This topic is beyond the scope of this review.

2. QUALITY REQUIREMENTS OF *IN VITRO* MODELS

Cells are the basic units of life. When cell function is altered, the system or body can reflect this change. Therefore, from a safety evaluation perspective, the cellular level is very important interface for effects of test agents as shown in Fig. (**1**). Thus *in vitro* cell models have particular value in the risk assessment of chemicals. However, there are some problems in using *in vitro* models. We must be clear what an *in vitro* model can do and what it cannot and understand what is the essential requirement for an *in vitro* model used for the risk assessment of chemicals.

First, we must realise that the differences between *in vivo* and *in vitro* models are enormous. A live animal or human being is an integral system of sub-systems. A sub-system has its specific histological architecture and carries out special functions. Cells in the body can either influence or be influenced by other cells or factors such as neurons, hormones, supply of energy substances, metabolic interaction, blood supply, and so on. In addition, the body has an immune system that plays a variety of roles in responding to foreign antigens and pathological processes. When a drug is introduced into the body, it undergoes administration, distribution, metabolism and excretion (ADME) processes. All these general 'built-in' structures and functions in one way or another can significantly modulate cell responses to drugs or other compounds. The complexity of *in vivo* systems, so far, cannot be mimicked by *in vitro* models. This means that we cannot expect *in vitro* models to reflect *in vivo* responses in full.

Then what can an *in vitro* model do? *In vitro* cell models are developed for different research purposes and applications. The models for toxicity study are expected to mimic *in vivo* cellular responses to adverse effects induced by the compounds tested. This requires the cells used in the model to possess essentially the same functionality as those cells *in vivo*. Primary cells and cell lines have been used widely for *in vitro* studies. *In vitro* studies have advantages in identifying targets and mechanisms, studying chemical metabolism, evaluating toxicity potential, achieving high throughput testing and time and cost efficiency. In addition, human primary cells can be used for testing to overcome the problem of species difference. The *in vitro* approach is also a promising way to achieve the goal of refining, reducing and replacing animal experiments (3Rs).

Second, cell model quality is an essential concern in the safety evaluation of chemicals. Both *in vivo* and *in vitro* models must serve the priority requirement in safety evaluation: sensing potential adverse effects. Drug or other chemical safety is not only a health risk but a regulation issue as well. Under current regulations from different organisations, no *in vitro* data are accepted for safety judgement except for skin tests [27, 28]. *In vitro* toxicity data have already dominated the current toxicology research reports in the literature over the past decade. One of the major concerns to judge *in vitro* data is the quality of *in vitro* models. The question is how to define the quality of *in vitro* models. The currently used cell types for *in vitro* models include primary cells and immortal cell lines. However, which cell type is better depends on several factors. A common issue for tumour cell lines is their phenotype drifting and differential functional changes compared with their original tissues [29, 30]. Theoretically, primary cells are the most relevant to *in vivo*. However, cell isolation induced functional alteration significantly jeopardises the application of some primary cell types. For example, primary hepatocytes show significant functional reduction in albumin synthesis and P450 activity after isolation [31-33]. For toxicity studies, the activities of phase I and II drug metabolism enzymes are an essential quality requirement because some chemicals exhibit toxic effects after metabolic activation or show reduced effects due to metabolic deactivation. In most cases, whether a cell model is sensitive or insensitive in responding to drugs mainly depends on its drug metabolizing activity.

A promising approach for maintaining a stable functionality of primary cells is three-dimensional cell culture. Liver spheroid culture is a good example. Primary hepatocytes in spheroids show clear functional recovery as indicated by albumin secretion, P450 activity, arginine take-up and nitric oxide levels, which can be maintained at a stable level for 1-2 weeks [34, 35]. This is significantly different from hepatocytes cultured in monolayers, in which hepatocytes loss some liver functions rapidly (within 24h) and do not show functional recovery afterwards [32, 33]. There are at least two factors affecting primary cell *in vitro* functions: first, cell isolation disrupts cell-cell connections and is a significant challenge to cells, which can induce NO synthesis in hepatic cells and alter cell functions significantly within 24 h after isolation [35]; second, the method of cell maintenance is crucial. In the *in vivo* situation, cell-cell contacts and communication are essential, whereas, in monolayer cultures, cells can only form limited cell-cell connections and communication. By contrast, cells in spheroid culture can establish better cell-cell connections and communication, which provides cells with a stable histological environment, limits cell division and maintains cells at the differentiation state to perform differentiated functions. All these factors are important in encouraging cell functional recovery and maintaining functions at a stable level for an extended period [34, 35]. In addition, some immortalized cell lines can also be cultured into spheroids and show improved functionality [36]. The spheroid model is definitely a better approach to

improve model quality and suitability for high throughput testing [21, 22].

3. IMPORTANCE OF A REFERENCE CONCENTRATION IN THE RISK ASSESSMENT OF CHEMICALS

In *in vitro* studies, a reference concentration can be defined as the exposure concentration that can cause a common distinctive effect. Referring to this concentration to design an exposure concentration range can give a rough guide to the status of cell functions as shown in Fig. (**3**). A biological response of cells to a chemical challenge often shows a great variety and does not always show linear dose-responses. In some cases, they exhibit a low-dose induction and high-dose inhibition response model, which is termed as hormesis or a U-turn response model [37-39]. In fact, most biological responses often show one of three response models: linear dose-response, threshold-linear dose-response and hormesis [39, 40]. The phenomenon of hormesis is particularly important in identifying drug effects and the tendency of adverse effects. Long-term or chronic effects of a chemical are often associated with a low level exposure and can usually be observed within a certain low concentration range of the chemical tested. In addition, functional changes are often the early event of response to a toxicant. An appropriate exposure concentration range of a drug tested is crucial to determining the altered cellular functions [22]. Higher concentrations could kill cells and provoke secondary changes that may not be directly relevant to the chemical effect. Thus conclusions from such research without an appropriate concentration range are less valuable because, for example, a high concentration of table salt can kill cells but we cannot classify table salt as a toxicant; the same also applies to drugs. For example, paracetamol is a commonly used anti-inflammatory drug but can cause hepatotoxicity when an overdose is taken [10]. Can we terminate it as a drug entry? We cannot, because at appropriate concentration, it is a potent therapeutic agent rather than a toxicant. Overdose or a higher exposure concentration may jeopardise a clear conclusion. Therefore, appropriate exposure concentration range should be taken into account in research, especially in *in vitro* studies. Identifying response models, such as threshold-dose response and hormesis, of cells to an agent tested at low concentrations is more meaningful than inducing any change at a lethal concentration.

To determine an appropriate exposure concentration range to identify effects of a chemical requires a reference concentration. In *in vivo* studies, LD50 and LC50 can serve this purpose. In *in vitro* studies, however, a wide concentration range (1-10000 µg/ml) is often used [41]. Although cell viability LC50 (the concentration of a compound causing 50% of cell death) and EC50 (the concentration of a compound causing 50% enzyme inhibition or reduction of tested function) can serve as reference concentrations, they often show a great variation from batch to batch or between laboratories. Their accuracy is often affected by many factors such as cell condition (medium, cell confluency, cell metabolic activity), cell counting skills, timing accuracy, intervals between concentrations, ratio of cell number and medium volume, and exposure time. Without controlling these factors, LC50 or EC50 from different batches of tests or laboratories will lose comparability even if using the same cell type. A better concentration reference system is required.

We have previously developed a new method to serve this purpose using a liver spheroid model called the *spheroid cell spreading inhibition test* (SCSIT) [21]. This method is based on cell behaviour activity. Cell membranes are involved in a series of behavioural activities such as migration, cell-cell connection, cell-matrix interaction, growth, etc [6]. This test can determine a minimum concentration which inhibits cell anchoring and growth, which is termed the *spheroid cell spreading inhibition concentration* (SCSI-C). The SCSIT was designed based on the changing sequence of cell health conditions. A cell from normal functioning to death undergoes functional changes and behavioural changes before death as shown in Fig. (**3**). Theoretically, cell death is the common aftermath after exposure to all toxicants no matter what mechanism might be involved. Functional changes often cover a wide range in both endpoints and exposure concentrations. It is inappropriate to use a single functional endpoint to represent cell health condition and difficult to define a single concentration to serve as a reference concentration. Cell behavioural change after exposure to a toxicant is a stage which could shift to the cell death stage. If a compound can kill all cells at 50 µM, we surely know that all concentrations higher than that are redundant concentrations and meaningless. SCSI-C reflects cell injured behaviour status which is a serious toxic effect and falls within the

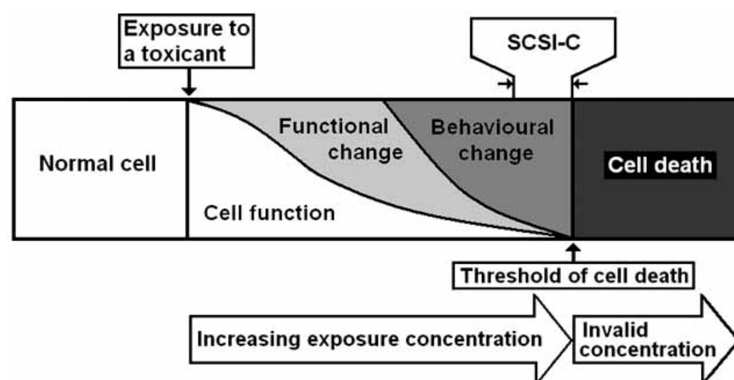

Fig. (3). A illustration of functional and behavioural changes of cells between normal and death after exposure to a toxicant. There are phase changes before cell death after exposure to a toxicant. SCSI-C falls in the phase of behavioural change.

boundary zone between cell functional alteration and death. Thus SCSI-C can significantly narrow down the variation range as a reference concentration. Scaling down this concentration, functional changes can be appropriately evaluated [21]. SCSI-C is high enough to inform safety risks and can also be used for comparing relative toxicity across different compounds or sensitivity across different tissues. As shown in Table **1**, the SCSIT can determine a single boundary concentration from low to high. The concentrations higher than the SCSI-C tend to kill cells and whereas at lower concentrations cells show a good linear dose-response functional change [21, 22]. The values of SCSI-C, the values underlined in Table **1**, represent the relative toxicity of the test agents. In addition, using SCSI-C as a reference concentration, the relative sensitivity of different cell types from the same species can also be compared to identify target tissues, provided the quality of cell models and tests are preserved.

In most cases, whether a compound is a drug or a toxicant is often determined by concentration. A drug choice is often a compromise between therapeutic effect and any minor side effects at the therapeutic dosage. Determining an appropriate concentration range is crucial in drug risk assessment. Moreover, if we want to make data inter-laboratory comparable, *in vitro*- and *in vivo*- linkable and relevant, a reliable and stable reference concentration is essential.

Generally speaking, cells show phase changes after exposure to a toxicant as shown in Fig. (**3**). The phase changes are concentration-related. When a wide exposure concentration range is applied, we should consider what purpose this concentration range is designed for and whether the higher concentrations could kill cells. With a reference concentration of the compound tested, an appropriate concentration range can be designed, functional changes can be appropriately evaluated, invalid concentrations can be avoided and data can be framed together. In some cases, biological responses of cells to a chemical challenge often show differential models and depend upon exposure concentration range. Some functional endpoints should be interpreted separately before and after cell death. The phenomenon of hormesis reminds us that within a certain concentration range, stimulation and inhibition are two opposite responses and its turning point may be a useful parameter to identify beneficial or adverse effects of some drugs. Therefore, an appropriate exposure concentration range is essential to design an experiment and make a clear conclusion and data more valuable. No drug is safe without considering dosage or concentration. The safety evaluation of chemicals is a concentration-related risk assessment. An effect referred to a reference concentration can make data repeatable and comparable. That is why a reference concentration is very important.

4. LINKING *IN VITRO* DATA TO *IN VIVO*: LINKING FACTORS

General or system response to a compound can eventually be traced down to target cells. Thus using cell models to identify target tissue is a reasonable approach. In line with this assumption, a variety of cell-culture-based *in vitro* models have been developed in the past decades. However, things are not as simple as we expected. There are some problems to overcome in developing and using *in vitro* models. In addition to cell quality, maintenance and cell type selection as mentioned above, how to link *in vitro* effects to *in vivo* response to compounds tested is the key issue. Without this linking, *in vitro* data is meaningless. As a result, some *in vitro* studies may end up wasting time and resources. Exploring a practical protocol to link *in vitro* data to *in vivo*, therefore, becomes an urgent and paramount priority. The key question is How?

As is well known, neurons do not suffer 'headaches', which means that we cannot expect data from *in vitro* models to mimic system responses as *in vivo*. As has been discussed in the first section of this review, the cell is at the effect-safety interface of a chemical; it is also the essential linkage between *in vitro* and *in vivo* studies. If we take *in vivo* cell responses as a reference, the differences between *in vitro* and *in vivo* responses could be estimated based on an appropriate principle. If the cell functionality of an *in vitro* model could mimic *in vivo*, the most valuable linking factor is the *exposure concentration*. If the concentration can be appropriately defined, *in vitro* data can be linked to *in vivo* for refining animal tests and reducing animal use. To achieve this goal, we need to clearly define and appropriately interpret three factors: reference concentration, effect and linking coefficient.

Table 1. SCSI-Cs of the Test Agents and their Relative Toxicity on Human Liver Spheroids

Chropromazine (µM)	**10**	**15**	**20**	**25***	**30**	**35**	**40**	**45**	**50**
Propranolol (µM)	10	15	20	25	**30**	**35**	**40**	**45**	**50**
Diclofenac (mM)	2	3	4	5	6	**7**	**8**	**9**	**10**
Valproic acid (mM)	2	3	4	5	6	7	8	**9**	**10**
Paracetamol (mM)	5	10	**15**	**20**	**25**	**30**	**35**	**40**	**45**
Galactosamine (mM)	5	10	15	**20**	**25**	**30**	**35**	NT	NT
m-Dinitrobenzine (mM)	100	150	200	**250**	**300**	**350**	**400**	**450**	**500**
Ethanol (mM)	200	300	400	500	**600**	**700**	**800**	**900**	**1000**

*: The bold value underlined in each row is taken as the concentration of spheroid cell spreading inhibition (SCSI-C).

NT: not tested.

The reference concentration is the key factor to link *in vitro* and *in vivo*. The question is what concentration can serve this purpose appropriately. As mentioned above, LD50 and LC50 are reference doses for animal tests. Cell viability LC50 (*in vitro* LC50), EC50 and SCSI-C are used as *in vitro* reference concentrations for *in vitro* studies [21]. The advantages of SCSI-C have been discussed above and it is used here as an example to interpret how an *in vitro* concentration can be linked to *in vivo* blood concentration.

An *in vitro* reference concentration should satisfy some basic requirements:

1. The endpoint should represent a common, seriously injured state of cells after exposure, which means no matter what toxicants are used and what mechanism might be involved, so long as the concentrations of the toxicants cause cells to produce a common injured state, they will have stable, universal, comparable value [21]. Cell death is definitely a common state after exposure to high concentrations of toxicants, but it is difficult to be defined precisely because cell tolerance to death has a big variation; that is why LC50 was initially introduced.

2. Quality of cell model should be well characterised, robust and stable.

3. The method for obtaining reference concentration should be simple, reliable, repeatable and easy to carry out to achieve high throughput testing.

SCSI-C represents cell behavioural change before cell death as shown in Fig. (**3**) and its variation is within a narrow range. It, therefore, is suitable to be used as a reference concentration. The cell type used in the *in vitro* model is regarded as the concerned target. A reference concentration is like a fixed point in geological measurement to locate position. Referring to this concentration we can estimate what health status the cells are at when cells are exposing to a concentration of a chemical. SCSI-C as an *in vitro* reference concentration cannot be directly extrapolated to a blood concentration. It requires a translation through a coefficient. As long as the concentration is linkable, the effect of a compound at a concentration referred to SCSI-C (e.g 2 × or ½ or ¼ of a SCSI-C) can be transferred to the *in vivo* situation. Through pharmacokinetic parameters, a linking coefficient could be worked out and the SCSI-C-referred concentration could be converted into a reasonable blood concentration and a recommended dosage. The linking coefficient can resolve the difference between *in vitro* and *in vivo* concentrations and responses, thereby linking *in vitro* data to the *in vivo* situation.

Linking *in vitro* data to *in vivo* is a complex task to fulfil because of the huge difference between the two testing systems. However, barriers must be overcome if we want *in vitro* studies to be interpretable *in vivo*. The factors of cell model, reference concentration, effects and linking coefficient are fundamental elements to frame the link between *in vitro* and *in vivo* responses.

5. SUMMARY

The fundamental issues in using *in vitro* models are discussed in this review. Endpoint selection, model quality, exposure concentration range and linking *in vitro* and *in vivo* studies are essential current considerations in using *in vitro* models in toxicological studies and safety screening testing. To put them into a single frame requires setting up a comprehensive principle to 'glue' them together. *In vitro* studies will not make sense to *in vivo* unless a way to link *in vitro* and *in vivo* data has been established. Without this effort, *in vitro* studies could generate too much information but may be not appropriately translated into *in vivo* response. As a result, such *in vitro* information may mean nothing to *in vivo* and hence has less value. Improving cell model quality such as cell spheroid culture or other three-dimensional cultures and introducing reference concentrations such as SCSI-C into *in vitro* studies will certainly improve data quality and the value of *in vitro* testing. Current advances in cell *in vitro* model development, technologies in biosciences and *in silico* approaches have begun to penetrate the barriers between *in vitro* and *in vivo* studies to make *in vitro* and *in vivo* data reliably interpretable.

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