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Review

In vitro testing of basal cytotoxicity: Establishment of an adverse outcome pathway from chemical insult to cell death

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ABSTRACT

In this paper, an *in vitro* basal cytotoxicity testing strategy is described for new chemical entities that lack any pre-existing information on potential toxicity. Special attention is paid to the selection of the cellular system, cytotoxicity assay and exposure conditions. This approach is based on a newly proposed generic adverse outcome pathway from chemical insult to cell death that consists of 3 steps, including initial cell injury, mitochondrial dysfunction and cell demise. The suggested strategy to consider *in vitro* basal cytotoxicity as a first step in evaluating the toxicity of new chemical entities can be placed in a tiered strategy that could be continued by evaluating more specific types of toxicity.

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1. Introduction

Evaluation of safety is a prerequisite *prior* introduction of new chemical entities onto the market. Historically, animal testing has formed the basis for such risk assessment exercises. Driven by scientific and ethical constraints, and initiated more than 3 decades ago, however, there is a clear tendency worldwide to increasingly address animal-free methods for this purpose. This has been reinforced by a number of legislative changes over the past few years in the European Union, imposing a ban on animal testing for particular groups of chemicals, *in casu* in the cosmetics field (EU, 2003; EU, 2009). This has been followed by other parts of the world, such as in Norway, Israel, India, New Zealand and the state of São Paulo in Brazil (Laquieze et al., 2015). In response to this ubiquitous matter, the scientific community has been urged to develop animal-free methods for evaluating the safety of chemicals,

Abbreviations: AO(P), adverse outcome (pathway); ATP, adenosine triphosphate; DMSO, dimethylsulfoxide; DNA, deoxyribonucleic acid; KE, key event; LDH, lactate dehydrogenase; MIE, molecular initiating event; MTP, mitochondrial permeability transition; MTT, [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide]; NADH, nicotinamide adenine dinucleotide.

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including *in vitro* and *in silico* assays, being a research area that is gaining *momentum*. Interestingly, this has triggered a paradigm shift from classical toxicology, focusing on apical endpoints for toxicity in animal models, to predictive toxicology, relying on information on mechanisms of toxic action (NRC, 2007; Vinken, 2013).

A major tool adopted in predictive toxicology is the adverse outcome pathway (AOP) framework, which refers to a conceptual construct that portrays existing knowledge concerning the linkage between a direct molecular initiating event (MIE) and an adverse outcome (AO) *via* a number of key events (KEs) at a biological level of organization relevant to risk assessment. AOPs can serve several purposes pertinent to non-animal chemical risk assessment, such as read-across methods, integrated approaches to testing and assessment, quantitative structure-activity relationships or the elaboration of prioritization strategies (Vinken, 2013, 2015). In fact, AOPs embody a number of proposed frameworks for the implementation of animal-free safety testing of chemicals. Such frameworks typically start with exposure assessment, physico-chemical profiling, read-across and biokinetic evaluation, all which dictate the subsequent selection of *in vitro* biomarkers and corresponding assays (Blaauboer et al., 2012). For many new chemical entities, however, such pre-existing information may be scarce, which thus impedes targeted establishment of an *in vitro* testing battery. In the present paper, a strategy for setting up basal *in vitro* cytotoxicity testing of such data-poor chemicals is outlined. This is based on a newly proposed generic AOP from chemical insult to cell death.

2. Development of a generic AOP from chemical insult to cell death

Basal cytotoxicity refers to the ability of a chemical substance to damage living cells, in particular by compromising functional and structural features related to general cellular housekeeping. Being a rather comprehensive term, it is not surprising that the pathways leading to basal cytotoxicity are quite generic (Eisenbrand et al., 2002; Ekwall et al., 1995; Schoonen et al., 2009). Nevertheless, a tentative AOP to basal cytotoxicity could consist of 3 consecutive steps. The first step (*i.e.* the MIE) involves initial cell injury caused by the parent chemical and/or its metabolites. In the second step (*i.e.* the KE), a mitochondrial dysfunction takes place as a consequence of the primary insult. This ultimately leads to cell death in the third step (*i.e.* the AO) (Fig. 1). Each of these steps will be discussed in the following sections.

2.1. Initial injury

Chemicals can cause direct cell injury through a variety of mechanisms, which may involve a single specific event, such as altered activation of an ion channel (Gennari et al., 2004; Schoonen et al., 2009) or a receptor (Gennari et al., 2004; Houck and Kavlock, 2008). However, a generic AOP from chemical insult to cell death should preferably encompass more general processes that instantly disrupt cellular homeostasis (Fig. 2).

A first mechanism in this respect is disturbance of plasma membrane integrity. A prerequisite for performing cellular functionality includes appropriate physical segregation between the extracellular environment and the cytosol, which contributes to selective passage of substances between both compartments. This is accomplished to a large extent by a solid double phospholipid layer. Damage to this plasma membrane induced by chemicals can occur in a number ways, of which accumulation and binding to the phospholipid bilayer, a process called narcosis, is a prominent one (Escher et al., 2002).

A second mechanism relates to interfering with subcellular architectural organization. In order to maintain homeostasis, cellular functions are restricted to specific organelles within the cell, such as the nucleus, where genetic material is stored and processed, or the rough endoplasmic reticulum, taking care of protein synthesis. This so-called compartmentalization may be compromised by chemicals, thereby jeopardizing overall cellular functionality (Eisenbrand et al., 2002; Schoonen et al., 2009).

A third mechanism involves directly negatively affecting cellular energy supplies, in particular by targeting mitochondria. Thus, chemicals may uncouple the mitochondrial respiratory chain, inhibit adenosine triphosphate (ATP) synthesis, damage mitochondrial deoxyribonucleic acid (DNA), interfere with the replication of mitochondrial DNA or decrease the synthesis and stability of mitochondrial transcripts (Jones et al., 2010; Pessayre et al., 2010).

2.2. Mytochondrial dysfunction

Mitochondria are considered as the fuel stations of the cell. In this context, pyruvate, produced from glucose through the process of glycolysis, is taken up by mitochondria and is transformed to acetylco-enzyme A. In a parallel pathway, fatty acids bound to acetylco-enzyme A enter the mitochondrion, where they are split by successive beta-oxidation

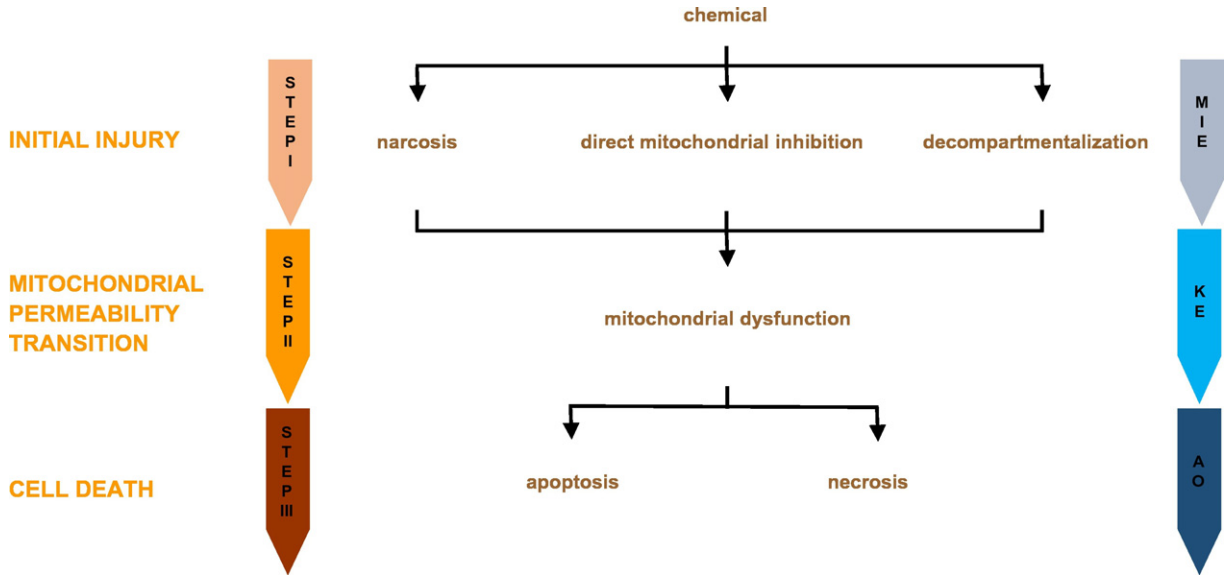

Fig. 1. Generic adverse outcome pathway from chemical insult to cell death. The first step or the MIE involves initial cell injury, whereby the parent chemical and/or its metabolites cause narcosis, directly impair mitochondrial function or induce decompartmentalization. In the second step, which is a KE, a MPT process takes place as a consequence of the primary insult. This ultimately leads to cell death by apoptosis or necrosis in the third step, being the actual AO.