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Practical use of the Virtual Cell Based Assay: Simulation of repeated exposure experiments in liver cell lines

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ABSTRACT

The Virtual Cell Based Assay (VCBA) was applied to simulate the long-term (repeat dose) toxic effects of chemicals, including substances in cosmetics and personal care products. The presented model is an extension of the original VCBA for simulation of single exposure and is implemented in a KNIME workflow. This work illustrates the steps taken to simulate the repeated dose effects of two reference compounds, caffeine and amiodarone. Using caffeine, *in vitro* experimental viability data in single exposure from two human liver cell lines, HepG2 and HepaRG, were measured and used to optimize the VCBA, subsequently repeated exposure simulations were run. Amiodarone was then tested and simulations were performed under repeated exposure conditions in HepaRG. The results show that the VCBA can adequately predict repeated exposure experiments in liver cell lines. The refined VCBA model can be used not only to support the design of long term *in vitro* experiments but also practical applications in risk assessment. Our model is a step towards the development of *in silico* predictive approaches to replace, refine, and reduce the *in vivo* repeated dose systemic toxicity studies in the assessment of human safety.

1. Introduction

In order to reduce and eventually replace the use of animals for predicting toxicity in humans, models based on concentrations that cause effects *in vitro* will have to be developed, taking into consideration both toxicokinetics (TK) and toxicodynamics (TD). The characterisation of the concentration that produces an effect (whether this is a perturbation of a molecular pathway or an apical toxic endpoint) is necessary at two levels: first, for *in vitro* experiments since "nominal" concentrations do not correspond to real concentrations to which cells are exposed (Gülden et al., 2001; Groothuis et al., 2015); and, second, for extrapolating a dose for human toxicity assessment, since for the assessment of a hazard of a chemical compound, we need to know the true concentration in the target organ. Normally, concentration-response curves in *in vitro* experiments represent the total amount of substance added to a microtiter-plate well and not the dissolved (free) concentration that corresponds to a bioavailable fraction, which could induce an effect (Groothuis et al., 2015; Kramer et al., 2015; Hamon et al., 2015). However, these curves (often characterized by their potency parameters, *e.g.* IC50 values) do not properly reflect the actual concentrations, which induce an effect at the site of action of the chemical in the cell (particular receptor or enzyme). One possible improvement, already suggested and demonstrated by several research groups (Gülden and Seibert, 2003; Heringa et al., 2004; DeBruyn and Gobas, 2007; Kramer, 2010), would be to design *in vitro* experiments taking into account the toxic (but bioavailable) concentration in the medium which corresponds to the free dissolved concentration. In order to introduce a correction within the *in vitro* experimental set up our group previously developed the Virtual Cell Based Assay (VCBA) model (Zaldívar et al., 2010; Zaldivar Comenges et al., 2011; Zaldivar Comenges et al., in press), which can be considered an integrated modelling approach to improve the characterisation and analysis of *in vitro* cell-based assay data.

The VCBA was originally built using the Matlab platform and was applied to study the toxicological effects of chemicals on cells, as assessed by High Throughput Screening (HTS) and High Content Imaging (HCI) using single dose exposure conditions (Zaldívar et al., 2010; Zaldivar Comenges et al., 2011; Zaldívar et al., 2012). The model consists of ordinary differential equations whose solution allows the calculation of the dissolved concentration of a chemical over time, both in the plate and in the cells. The mathematical modelling of HTS and HCI experiments serves not only to predict experimental results (*e.g.* cell viability) but also to simulate the dynamics of several processes that are not easily measurable but which can be of toxicological relevance. These processes include chemical losses due to evaporation or adsorption onto plastic, as well as the effects of chemicals on cell growth and survival.

The integrated modelling approach of the VCBA thus consists of:

- A fate and transport model
- A cell partitioning model

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– A cell growth and division model
– A toxicity and effect model

This mathematical model takes into account the fate of a compound in the *in vitro* cell-based model, based on the partitioning between (i) the plastic wall, (ii) headspace, (iii) serum proteins, (iv) lipids, and potentially the compound dynamics within the cell. This is driven by a series of dynamic mass balance equations based on the compound physico-chemical properties and partitioning. The gas phase was included to allow, in a future version of the model, the possible losses and cross contamination between the 96 wells in the TC plates. The model is coupled with a cell growth model and a toxic effects model (Zaldivar Comenges et al., 2011). The cell growth model is a typical population model, described in terms of continuous ordinary differential equations, which ignores population structure by treating all individual cells as identical. Matrix population models (Caswell, 1989) integrate population dynamics and population structure and they are very useful when the life cycle is described in terms of size classes or age classes. In the case of the HepG2 cell line model, the appropriate description corresponds to a four stage-based approach, each stage corresponding to one of the four cell cycle phases: G1, S, G2, and M (Hartwell and Weinert, 1989). On the other hand the HepaRG cell line does not proliferate (it remains in phase G1). Furthermore the VCBA takes into account the Dynamic Energy Budget (DEB) approach, by Kooijman and van Haren (1990) showing changes in lipid contents and size of the organisms. The introduction of DEB models into matrix population models was already used by Lopes et al. (2005), Klanjscek et al. (2006); and Billoir et al. (2007). Following this approach, the chemical is taken up by the cell and it partitions instantaneously over three compartments: one aqueous fraction and two non-aqueous fractions: structural component (proteins) and the energy reserves (lipids). The direct toxic effects of a chemical concentration were related to the internal (intracellular) concentration of the toxicant with the no-effect concentration (NEC) for survival, and the kr, the killing rate of the toxicant (Zaldivar Comenges et al., in press). The combined model enables simulations of the true concentrations causing biological perturbations in cells, over time, given the nominal concentrations applied in a microtiter plate well.

In order to simulate the long-term (repeat dose) effects of chemicals, there is a need to develop a tool which can predict, in an efficient and reliable way, the intracellular concentration within the cell after multiple exposures. The following steps were applied in order to perform the repeated exposure simulations. First, for caffeine, *in vitro* experimental data from two human liver cell lines, HepG2 and HepaRG, were obtained. Second, the single exposure experimental cell viability data were used to optimize the VCBA (kr and NEC values). The third step after model optimisation was to simulate the time-dependent response and compare the predicted results with *in vitro* data found in literature or achieved experimentally in house; this can be considered as a validation step of the VCBA for caffeine. In the fourth step, using HepaRG cells, concentration response curves were tested experimentally and simulations for amiodarone under repeated exposure conditions were performed.

Thus, the VCBA was implemented into a KNIME workflow as described in Fig. 1, and an extension was made to simulate the repeated exposure, as illustrated in Fig. 2.

We describe the development of an open source VCBA methodology for simulating the long-term (repeat dose) toxic effects of chemicals, including substances found in cosmetics and personal care products, in *in vitro* (liver cell) systems. The approach is based on the previously developed VCBA model (Migita et al., 2010; Zaldivar Comenges et al., 2011; Zaldívar et al., 2012) which is re-coded and re-implemented in the open-source KNIME platform. The usefulness of this tool in simulating the repeated dose toxicity of selected compounds is illustrated with reference to two case study compounds: caffeine and amiodarone.

2. Materials and methods

2.1. Chemicals and supplies

HepaRG cells were obtained from Biopredic International (Rennes, France) and stored in liquid nitrogen. William's E medium, L-glutamine, penicillin/streptomycin and trypsin-EDTA were purchased from Invitrogen (San Giuliano Milanese, Italy). HyClone Fetalclone III serum was from Thermo Scientific (Pittsburgh, USA). Caffeine (58-08-2; purity set with HPLC was 99% as reported from supplier), bovine insulin, hydrocortisone hemisuccinate, and amiodarone hydrochloride (19774-82-4; purity by TLC > 98% as reported from supplier) were purchased from Sigma-Aldrich (Milan, Italy). Tissue culture treated 96 well clear bottom black polystyrene microplates were acquired from Corning (Pero, Italy). Fluorescence staining was performed with Hoechst 33342 (Invitrogen).

2.2. Cell culture and differentiation

Differentiated HepaRG cells exhibit many characteristics of primary human hepatocytes, including morphology and expression of key metabolic enzymes, nuclear receptors, and drug transporters (*e.g.* morphology) and are metabolically competent, thus express relevant phase I and II enzymes. HepaRG cells were cultured in William's E medium supplemented with 10% HyClone Fetalclone III serum, 1% L-glutamine, 1% penicillin/streptomycin, 5 μg/mL insulin and 50 μM hydrocortisone hemisuccinate. The cells at passage 18 were seeded at a density of 4×10^6 cells in 150 cm^2 flasks and the medium was refreshed every two days. After two weeks, 1.7% DMSO was added to favour the cells

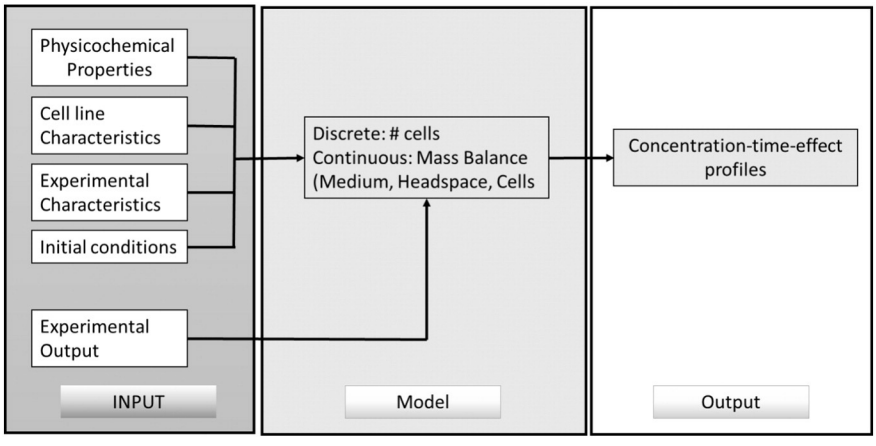

Fig. 1. A graphical representation of the general setup of the automated VCBA implemented into KNIME platform; which consists of three zones: input, model, and output.

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