View metadata, citation and similar papers at core.ac.uk

brought to you by CORE

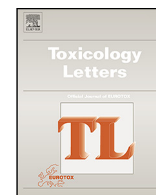
provided by Elsevier - Publisher Connector

Toxicology Letters 235 (2015) 96–106

Contents lists available at ScienceDirect

Toxicology Letters

journal homepage: www.elsevier.com/locate/toxlet



BK/TD models for analyzing *in vitro* impedance data on cytotoxicity

S. Teng [a], S. Barcellini-Couget [b], R. Beaudouin [a], C. Brochot [a,*], G. Desousa [c], R. Rahmani [c], A.R.R. Pery [a,d]

[a] *INERIS (METO), Verneuil-en-Halatte 60550, France*
[b] *ODESIA Neosciences, Sophia Antipolis F- 31076, France*
[c] *INRA Toxalim, Sophia Antipolis F- 31076, France*
[d] *AgroParisTech, UMR 1402 INRA-AgroParisTech EcoSys, Thiverval-Grignon F- 78850, France*

HIGHLIGHTS

- Mathematical models are proposed to analyze impedance data on cytotoxicity.
- The models account for both fate and effects of cosmetic ingredients in the system.
- We could describe the cytotoxicity over time of three cosmetic ingredients.
- Models calibrated on acute data failed to predict chronic data.
- *In vivo* toxicity could be predicted by coupling our models with kinetic model.

ARTICLE INFO

Article history:
Received 14 January 2015
Received in revised form 24 March 2015
Accepted 27 March 2015
Available online 28 March 2015

Key words:
In vitro modeling
Cell viability
Hepatotoxicity
Impedance
Acute to chronic extrapolation

ABSTRACT

The ban of animal testing has enhanced the development of new *in vitro* technologies for cosmetics safety assessment. Impedance metrics is one such technology which enables monitoring of cell viability in real time. However, analyzing real time data requires moving from static to dynamic toxicity assessment.

In the present study, we built mechanistic biokinetic/toxicodynamic (BK/TD) models to analyze the time course of cell viability in cytotoxicity assay using impedance. These models account for the fate of the tested compounds during the assay.

BK/TD models were applied to analyze HepaRG cell viability, after single (48 h) and repeated (4 weeks) exposures to three hepatotoxic compounds (coumarin, isoeugenol and benzophenone-2).

The BK/TD models properly fit the data used for their calibration that was obtained for single or repeated exposure.

Only for one out of the three compounds, the models calibrated with a single exposure were able to predict repeated exposure data.

We therefore recommend the use of long-term exposure *in vitro* data in order to adequately account for chronic hepatotoxic effects. The models we propose here are capable of being coupled with human biokinetic models in order to relate dose exposure and human hepatotoxicity.

© 2015 The Authors. Published by Elsevier Ireland Ltd. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

Abbreviations: ICCVAM, the Interagency Coordinating Committee on the Validation of Alternative Methods; ECVAM, European Centre for Validation of Alternative Methods; OECD, Organisation for Economic Co-operation and Development; ECIS, electric cell-substrate impedance sensing; RT-CES, real-time electronic sensing system; EC_{50}, effect concentration at 50% of the maximal effect; NOEC, no observed effect concentration; CI, cell index; NCI, normalized cell index; BK, biokinetics; TD, toxicodynamics; NEC, no effect concentration; GOF, goodness of fit; K_m, Michaelis Menten constant; V_{max}, maximum velocity; IVIVE, *in vitro in vivo* extrapolation; ADME, absorption – distribution – metabolism – excretion; PBBK, physiologically based biokinetics.

* Corresponding author at: INERIS, Model for Ecotoxicology and Toxicogy Unit, Parc ALATA, 60550 Verneuil-en-Halatte, France. Tel.: +33 3 33 3 44556850; fax: +33 3 44556605.

E-mail addresses: sophie.teng-etudiant@ineris.fr (S. Teng), s.barcellini@odesia-neosciences.com (S. Barcellini-Couget), remy.beaudouin@ineris.fr (R. Beaudouin), celine.brochot@ineris.fr (C. Brochot), desousa@sophia.inra.fr (G. Desousa), roger.rahmani@sophia.inra.fr (R. Rahmani), alexandre.pery@ineris.fr, alexandre.pery@agroparistech.fr (A.R.R. Pery).

http://dx.doi.org/10.1016/j.toxlet.2015.03.011
0378-4274/© 2015 The Authors. Published by Elsevier Ireland Ltd. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

1. Introduction

Since the 11th of March 2013, with the adoption of the seventh amendment to the European Union's Cosmetic (76/768/EEC), the marketing of cosmetics products tested on animals has been banned in the European Union. Alternatives to animal testing methods must therefore be developed in order to assess the risks of cosmetics.

Over the last decades, many *in vitro* assays have been developed in the context of replacing animal testing. However, as Adler et al. (2011) reported, repeated dose toxicity is difficult to predict, especially based on *in vitro* data alone, since it results from long-term repeated exposure to a chemical leading to the deterioration of cells or organs as a result of their interplay. As discussed in Prieto et al. (2006), the toxicity of the most affected isolated organ after repeated exposure can be assessed based on *in vitro* data provided relevant *in vitro* approaches are developed and kinetics is accounted for through modelling. Since the liver is the organ most frequently affected by chronic toxicity following repeated oral exposure to xenobiotics (Bitsch et al., 2006), the priority in the development of alternatives to repeated dose toxicity testing have focused on hepatotoxicity.

Although human primary hepatocytes are the gold standard models for xenobiotic metabolism and toxicity studies (Hewitt et al., 2007), they have limited life spans and show early phenotypic changes (Guillouzo, 1998; Guillouzo et al., 1993), which makes them unsuitable for long-term toxicity tests. Therefore, differentiated HepaRG cells (Gripon et al., 2002) would be good candidates for long-term toxicity testing, since they are stable for 4 weeks (Jossé et al., 2008) and maintain metabolic activities for 4 weeks (Gerets et al., 2012; Jossé et al., 2008; Kanebratt and Andersson, 2008). Nevertheless, for metabolism studies purpose, HepaRG cells and human primary hepatocytes expressed phase I and phase II enzymes, but at different levels (Aninat et al., 2006; Anthérieu et al., 2010; Jossé et al., 2008). Therefore, human primary hepatocytes may still be used for metabolism study.

Presently, neither ICCVAM nor ECVAM have yet validated any *in vitro* repeated toxicity test for systemic effects. However, a wide range of endpoints can be investigated through the recent development of *in vitro* assays such as label-free detection technologies for cell-based assays for monitoring long-term toxicity tests.

The first real-time, label-free cell substrate impedance sensing (ECIS) device emerged in 1984 (Giaever and Keese, 1984), allowing for real-time monitoring of cell dynamics. It was reported to be able to monitor cellular events. The system has been improved into a real-time electronic sensing system (RT-CES™) using impedance to monitor cell proliferation, cell spreading (Mitra et al., 1991) and, more importantly, cell viability (Solly et al., 2004).

With the development of this *in vitro* label-free cell-based monitoring system, an alternative to the classical static toxicity assessment could be considered. Indeed, classically after exposure, toxicity is evaluated at a predetermined point in time using a static statistical descriptor such as an EC_{50} (the concentration at which there is 50% of the maximal effect) or a NOEC (No Observed Effect Concentration). This has the disadvantage of not taking into account the variation of exposure concentration in the system as well as the time course of toxic effects, which, in contrast, becomes accessible in real-time data. Therefore, we propose a shift from a static toxicity assessment to a dynamic one.

In the present study, we monitored cell viability of HepaRG cells using impedance metrics. We propose here both the generation of long- and short-term data by exposing HepaRG cells to three hepatotoxic cosmetic related compounds, coumarin, isoeugenol and benzophenone-2 for 48 h (single exposure) and 4 weeks (repeated exposure) and the analysis of these data using a mechanistic model based on systems of ordinary differential equations which account for the time course of the effect.

We also address the issue of extrapolating from acute to chronic exposure by assessing the ability of models calibrated using acute data to predict chronic data. If short term testing could enable the reliable prediction of long-term *in vitro* toxicity, this would result in savings both in terms of time and money.

2. Material and methods

2.1. Cell cultures

Differentiated HepaRG® cells, purchased from Biopredic International (Rennes, France), were prepared from cryopreserved vial stock. Before seeding, an E-plate™ (a 96-well plate designed for impedance measurement) was coated with type I collagen at 67 μg/mL (Rajan et al., 2006), maintained at 37 °C for 4 h, then washed with 150 μL of Phosphate Buffer Saline solution (PBS, Gibco®) per well.

HepaRG cells were seeded with 65,000 cells per well in an E-plate™ with HepaRG Thaw, Seed, and General Purpose supplement™ (Biopredic International, Rennes, France) supplemented with 100 mL Gibco® William's E medium, Glutamax™ (Life technologies™). Cells were maintained at 37 °C and 5% CO_2 in an incubator. After 24 h, the cells were shifted to the differentiation medium with 1% DMSO (Sigma®, St., Quentin Fallavier, France) supplemented with 10 MUI/mL of streptomycin, 10 mg/mL of streptomycin (Life technologies™), 10% fetal bovine serum, 100UI/mL insulin (Eli Lilly, Indianapolis, USA), and 50 μM hydrocortisone (Sigma®, St., Quentin Fallavier, France). The medium was renewed every 2 to 3 days. When impedance measurements stabilized, after checking for cell adhesion and differentiation, which occurred on around day 7, HepaRG cells were exposed to the compound of interest.

2.2. Chemicals

The three compounds studied, coumarin (CAS no.: 91-64-5), isoeugenol (CAS no.: 97-54-1) and benzophenone-2 (2,2′,4,4′-tetrahydroxybenzophenone (THB), CAS no.: 131-55-5) were purchased from Sigma® (St., Quentin Fallavier, France). The solubility of the three compounds was checked in DMSO and in the differentiation medium, with no precipitation visible with the naked eye and a microscope slide.

Compounds were diluted in culture medium with 1% DMSO in order to obtain the targeted exposure concentrations while maintaining phase I and phase II enzyme activity (Aninat et al., 2006; Anthérieu et al., 2010; Jossé et al., 2008).

In addition, positive and negative controls (with no and high toxicity expected, respectively) were each monitored with differentiation medium with 1% DMSO and differentiation medium with 1% DMSO and 2% sodium-dodecyl-sulfate (SDS).

2.3. Cell exposures

In the short-term experiments, for the three compounds we tested, HepaRG cells were exposed on a single plate for 48 h. The selected concentrations, based on preliminary range-finding experiments were 8; 2.53; 0.8; 0.253; 0.08; 0.025 and 0.008 mM, respectively, for the three compounds.

In the long-term experiments, HepaRG cells were exposed for 4 weeks. The medium was renewed every 2 to 3 days. The selected concentrations were 8; 4; 2; 1; 0.5; 0.25; 0.125 mM, respectively, for coumarin and isoeugenol and 4; 2; 1; 0.5; 0.25; 0.125 mM for THB.

All available wells on the plate were allocated as follows: four and three replicates, respectively, were allocated to the positive controls in the short- and long-term experiments and for the highest concentration of coumarin and isoeugenol (8 mM) in the long-term exposure experiments; duplicates were allocated for each nominal concentration and negative control.

2.4. Impedance measurements

Impedance was measured using the xCELLigence™ system (ACEA Biosciences, Roche® Diagnostics). The system measures electrical impedance across inter-digitated micro-electrodes placed on the bottom of the 96-well cell culture E-plates® RTCA. The impedance measurements were displayed as Cell Index (CI) (Eq. (1)), which provided quantitative biological information about the cell population, cyto-morphological changes and viability over time (Atienza et al., 2005; Ceriotti et al., 2007; Ke et al., 2011; Solly et al., 2004). The CI was calculated using the following equation:

$$\mathrm{CI} = \max_{i=1,\ldots,N}\left(\frac{R_{\mathrm{cell}}(f_i)}{R_0(f_i)} - 1\right) \quad (1)$$

where N is the number of frequency points at which impedance is measured, R_{cell} and $R_0(f_i)$, respectively, are the frequency-dependent electrode resistance with and without cell at time point t (wells with 50 to 100 μL of PBS) (Solly et al., 2004).

CIs were normalized as described by Nawaz et al. (2014). Briefly, the CI value at time point t was divided by its value at the reference time point, *i.e.*, the last time before exposure of the compound to cells. In this way, the normalized cell index (NCI) value was set to 1 at the beginning of exposure. An NCI of 0 means that no cells are attached to the plates. An increase in the NCI is indicative of proliferation or spreading of the cells, whereas a decrease of the NCI is indicative of the detachment or death of the cells (Atienza et al., 2005; Solly et al., 2004 Xing et al., 2005; Xing et al., 2006). In the present study, we only considered the decrease in NCI in order to study the viability of HepaRG cells. A decrease in the NCI corresponded to a decrease in the cell population in the wells.

2.5. Data analysis

2.5.1. Model description

We proposed mechanistic biokinetics/toxicodynamics (BK/TD) models to analyze the data. They were based on different hypotheses regarding the fate of the tested compound in the system and its effects.

Regarding kinetics, we assumed that it could be described based on a simple linear one-compartment model described by the following equation:

$$\frac{dC_i}{\mathrm{dt}} = k_e \times C_e(t) - k_u \times C_i(t)$$

where k_e is the cellular elimination rate, k_u is the cellular uptake rate, $C_e(t)$ is the exposure concentration, and $C_i(t)$ is the intra-cellular concentration. The intra-cellular concentration can be scaled with $c_i(t) = C_i(t) \times k_e/k_u$. The scaled intra-cellular concentration then becomes:

$$\frac{dc_i}{\mathrm{dt}} = k_e \times (C_e(t) - c_i(t)) \quad (2)$$

We tested two hypotheses to account for a possible decrease in intracellular concentration: (i) a decrease due to unspecific binding, such as binding to the plastic or protein and evaporation phenomena (Eq. (3)), and (ii) a decrease in compound concentration due to cell metabolism (Eq. (4)).

$$\frac{dc_i}{\mathrm{dt}} = -k \times c_i \quad (3)$$

$$\frac{dc_i}{\mathrm{dt}} = -\frac{V_{\max,\mathrm{estim}} \times c_i}{K_m + c_i} \times \frac{N}{N_0} \quad (4)$$

where k is the unspecific decrease constant, $V_{\max,\mathrm{estim}}$ the maximum metabolism rate of HepaRG cell, estimated with the impedance data, K_m the Michaelis Menten constant, N the number of cell at time point and N_0 the number of cells at reference time. K_m was obtained from unpublished metabolism experiments on human primary hepatocytes performed at INRA Toxalim (Sophia Antipolis, France). The metabolism data are summarized in Table 1. We assumed that the K_m values are roughly similar between human primary hepatocytes and HepaRG cells and that the differences between the two would be chiefly attributable to metabolic rate.

To analyze cell population viability dynamics, we propose the following model, which correlated effects to intra-cellular concentration and accounts for the fact that no cell population growth is expected for HepaRG, as proposed by Pery et al. (2013):

$$\frac{dN}{\mathrm{dt}} = -b \times (c_i - \mathrm{NEC}) \times N \text{ if } c_i > \mathrm{NEC} \quad (5)$$

where N is the number of cells, b the killing rate, c_i the intracellular concentration, and NEC the No Effect Concentration, *i.e.*, threshold concentration below which there is no significant decrease of cell viability. Thus, as long as the internal concentration is below the NEC, there is no decrease in N.

We also accounted for the cell spreading phenomenon. Indeed, according to Xing et al. (2005), an early stage of toxic exposure can induce transient cellular spreading which leads to a transient increase in NCI. This phenomenon at subtoxic concentration has also been described by Solly et al. (2004) for A2780 cells exposed to an apoptosis-induction regent. Therefore, in addition to Eq. (5), we added another equation that accounts for transient toxicity-induced cellular spreading:

$$\frac{dL}{\mathrm{dt}} = \gamma \times (L_0 + \lim \times (c_i - \mathrm{NEC})) - L \quad (6)$$

where L is the surface of the spreading cells, L_0 the initial surface of the cells, γ the spreading rate and lim the spreading coefficient.

2.5.2. Parameter estimation

Calibration was performed using the variation of NCIs between two consecutive time points instead of NCI values, because we expected less correlation between successive variations throughout times compared to successive NCI values measured on the same population. The variations were weighted by the time intervals between two measurements. Time intervals were heterogeneous throughout the experiment. To account for this, and also to avoid long calculation times during analysis, we harmonized all considered intervals by considering only measurement times spaced out every 2 h and every 4 h for respectively single and repeated exposure. For long-term exposures, we

Table 1
Metabolic parameters obtained in primary human hepatocytes exposed for 24 h to coumarin at the concentration of 2.5, 5, 10 and 25 μM, isoeugenol at the concentration of 5, 10, 25, 50 μM and benzophenone-2 at the concentration of 2.5, 5, 10, 25, 50 μM. V_{max} and K_m are respectively the maximum rate of metabolism and the Michaelis–Menten constant provided by INRA Toxalim (Sophie Antipolis, France).

	Coumarin	Isoeugenol	Benzophenone-2
V_{max} (nmol/min/10^5 cells)	6.50×10^{-3}	0.295	0.324
K_m (μM)	1.6	7.9	34.19

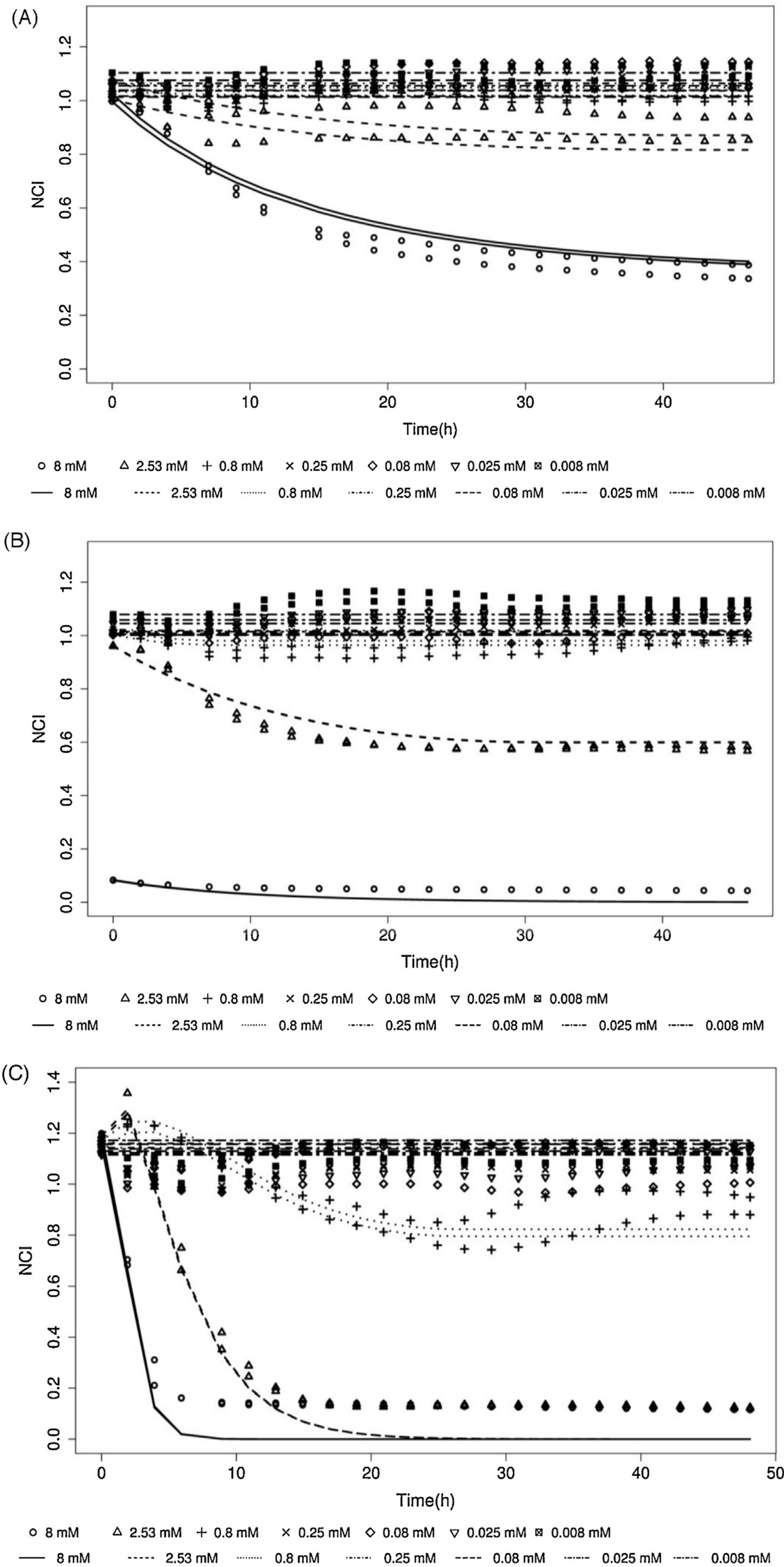


Fig. 1. Normalized Cell Index (NCI) of HepaRG cells subjected to short-term exposure (48 h) fitted by acute BK/TD models, for coumarin (A) with a BK model due to unspecific phenomena (Eq. (3)), for isoeugenol (B) with a BK model due to cell metabolism (Eq. (4)), and for benzophenone-2 (C) with a BK model due to cell metabolism and a TD model accounting for cell spreading phenomenon. Experimental data and model predictions are represented by points and lines, respectively. In order to improve the clarity of the figures, controls are not represented in the present figure but are shown in the Supplementary data.

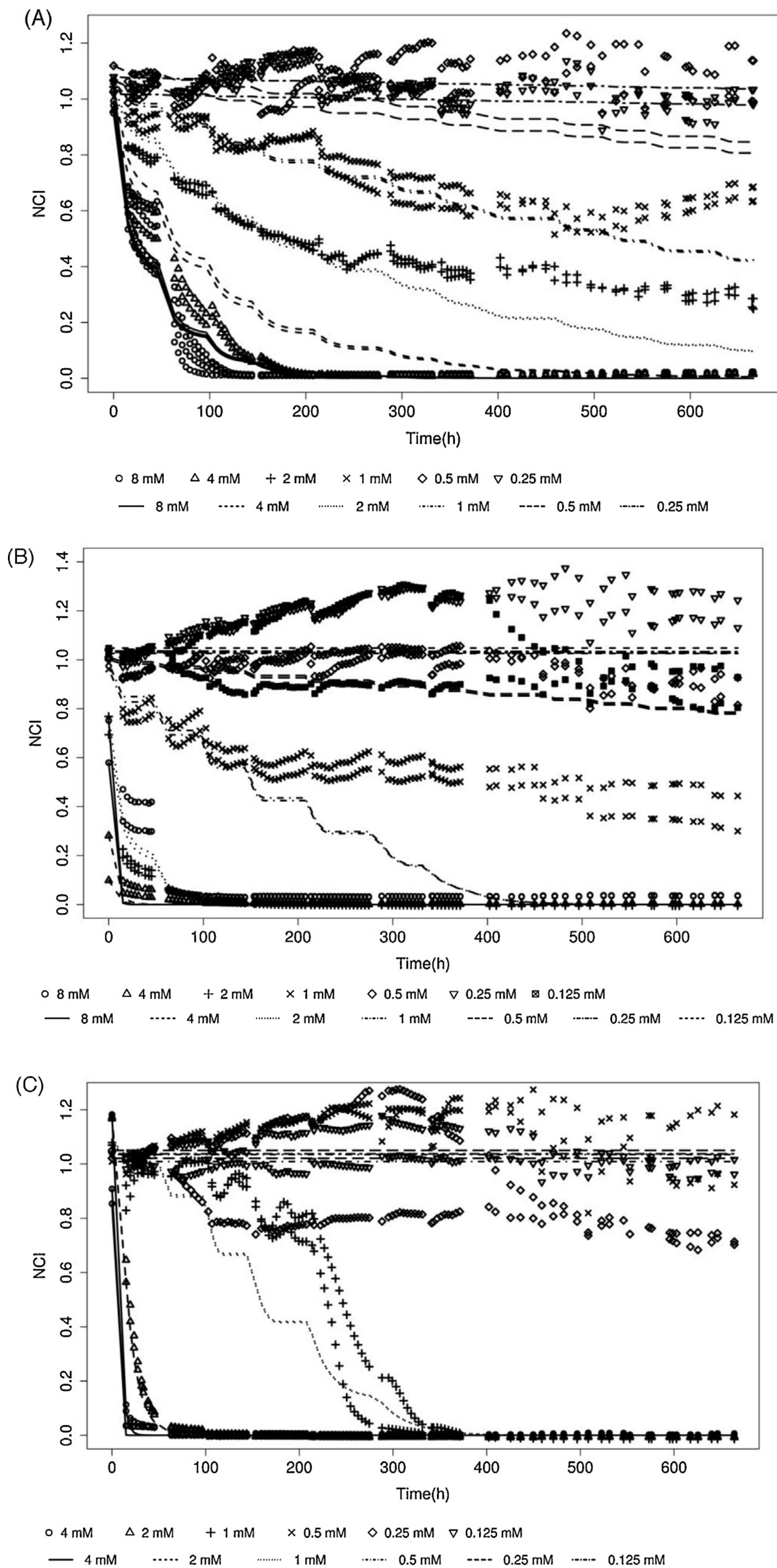


Fig. 2. Normalized Cell Index (NCI) of HepaRG cells subjected to long-term exposure (every 2 to 3 days) fitted by chronic BK/TD models, for coumarin (A) with a BK model due to unspecific phenomena (Eq. (3)), for isoeugenol (B) with a BK model due to cell metabolism (Eq. (4)), and for benzophenone-2 (C) with a BK model due to cell metabolism and a TD model accounting for the cell spreading phenomenon. Experimental data and model predictions are represented by points and lines, respectively. In order to improve the clarity of the figures, controls are not represented in the present figure but are shown in the Supplementary data.

observed an increase of the NCI for roughly 10 h after the following medium renewal. Since it was a reversible increase during less than 1 h, it was considered as an artefact which did not affect the cytotoxicity. This may also be due to the shortage of certain medium compounds during the medium renewal. The values in these intervals were removed from the dataset and from the data analysis.

We estimated the parameters' values together with their confidence intervals using the least squares method. Genoud's algorithm of Rgenoud package (Mebane and Sekhon, 2011) implemented in R software (R Development Core Team, 2013) was used for the model adjustment. 95% confidence intervals were calculated through bootstrapping with 1000 re-sampled datasets. Sampled datasets were built by sampling variations of NCIs between two time points in each of the replicates.

Once the BK/TD model had been calibrated for each compound after long- and short- term exposure, we checked the ability of the acute BK/TD model to predict long-term exposure and, inversely, the ability of the chronic BK/TD model to predict short-term exposure.

3. Results

3.1. Cell impedance data

Exposure of HepaRG cells to coumarin and isoeugenol induced a progressive decrease of NCI values as the tested concentration increased (Figs. 1 and 2). Regarding NCI of HepaRG cells following an exposure of 8 mM of isoeugenol, it dropped considerably at the first time of measurement after exposure. In contrast to coumarin and isoeugenol, benzophenone-2 showed a different pattern depending on the tested concentration. Indeed, for a single exposure, at the intermediate concentrations of 2.53 mM and 0.8 mM, a transient increase of NCI was observed.

3.2. BK/TD model after short-term exposure

Regarding the toxicodynamic component of the BK/TD model, for coumarin and isoeugenol, the cell viability was described with Eq. (5). For the third compound, benzophenone-2, we also had to account for the transient cell spreading phenomenon with Eq. (6). For all compounds, the best fits were obtained with very rapid kinetics (Instead of Eq. (2), internal concentration was thus assumed to equal exposure concentration).

For all compounds, we were not able to distinguish between the two fate models, either with a decrease due to an unspecific mechanism (Eq. (3)) or cell metabolism (Eq. (4)), only based on goodness of fit (GOF) (Table 2). Coumarin's and isoeugenol's NEC estimated by the model with a metabolism decrease were close to zero, whereas the NEC estimated by the model with a decrease due to unspecific mechanisms were not (*i.e.*, 0.445 mM for coumarin and 0.33 mM for isoeugenol). For benzophenone-2, both fate models, unspecific mechanism and metabolism, provided similar NEC value estimates (Table 2).

3.3. BK/TD model after long-term exposure

For two compounds, coumarin and benzophenone-2, there were differences in GOF depending on the selected fate model. For coumarin, the model with a decrease due to unspecific

Table 2
Parameters estimates from acute BK/TD model with different hypotheses relative to kinetics.

	Coumarin		Isoeugenol	
	Unspecific mechanisms	Cell metabolism	Unspecific mechanisms	Cell metabolism
k (h^{-1})	4.05×10^{-2} [3.84×10^{-2}–4.26×10^{-2}]	–	4.92×10^{-2} [4.64×10^{-2}–5.17×10^{-2}]	–
k_{met} (mmol/L/h/65,000cells)	–	0.283 [0.270–0.294]	–	0.113 [0.108–0.116]
NEC (mM)	0.445 [0.399–0.499]	0 [0–0]	0.330 [0.277–0.404]	500×10^{-4} [0–7.14×10^{-2}]
b (L/mmol/h)	6.40×10^{-3} [6.30×10^{-3}–6.5×10^{-3}]	6.00×10^{-3} [5.90×10^{-3}–6.10×10^{-3}]	1.60×10^{-2} [1.51×10^{-2}–1.71×10^{-2}]	1.32×10^{-2} [1.27×10^{-2}–1.41×10^{-2}]
lim	–	–	–	–
γ (h^{-1})	–	–	–	–
Sum of squares	0.0137	0.0131	0.0098	0.0098

	Benzophenone-2	
	Unspecific mechanisms	Cell metabolism
k (h^{-1})	3.70×10^{-2} [2.11×10^{-2}–5.12×10^{-2}]	–
k_{met} (mmol/L/h/65,000cells)	–	3.43×10^{-2} [2.12×10^{-2}–4.04×10^{-2}]
NEC (mM)	0.304 [0.248–0.443]	0.294 [0.234–0.438]
b (L/mmol/h)	0.146 [0.136–0.156]	0.140 [0.131–0.151]
lim	2.37 [1.61–3.30]	2.50 [1.94–3.40]
γ (h^{-1})	0.115 [8.89×10^{-2}–0.156]	0.100 [7.72×10^{-2}–0.130]
Sum of squares	0.0480	0.0485

Parameters estimated with the acute BK/TD models, with k being the elimination rate of the compound due to unspecific mechanisms, k_{met} the metabolic rate, NEC the estimated no effect concentration (threshold below which there is no effect on cell viability), b the killing rate by the compound. Coefficients relative to early stage of benzophenone-2 effects inducing transitory cell spreading phenomenon are lim, the cell spreading coefficient and γ the cell-spreading rate. The sum of squares corresponds to the sum of the squares of the differences between the variations of NCI simulated with the model and observed the variations of NCI of HepaRG cells exposed to coumarin, isoeugenol and benzophenone-2 for 48 h. Two hypotheses are considered regarding the decrease of exposure concentration: unspecific mechanisms or metabolism by the exposed cells. The confidence intervals are represented in brackets.

Table 3
Parametric estimates from chronic BK/TD model with different hypotheses regarding kinetics.

	Coumarin		Isoeugenol	
	Unspecific mechanisms	Cell metabolism	Unspecific mechanisms	Cell metabolism
k (h^{-1})	5.15×10^{-2} [4.94×10^{-2}–5.21×10^{-2}]	–	4.59×10^{-2} [4.16×10^{-2}–9.34×10^{-2}]	–
k_{met} (mmol/L/h/65 × 10^3 cells)	–	0.113 [0.107–0.118]	–	9.08×10^{-2} [6.19×10^{-2}–0.103]
NEC (mM)	0.147 [0.104–0.150]	0 [0–0]	0.305 [5.23×10^{-2}–0.331]	0.216 [0–0.292]
b	6.90×10^{-3} [6.50×10^{-3}–7.40×10^{-3}]	3.50×10^{-3} [3.3×10^{-3}–3.7×10^{-3}]	4.28×10^{-2} [1.78×10^{-2}–5×10^{-2}]	4.08×10^{-2} [1.59×10^{-2}–5.02×10^{-2}]
lim	–	–	–	–
γ (h^{-1})	–	–	–	–
Sum of squares	0.0132	0.0146	0.0102	0.0108

	Benzophenone-2	
	Unspecific mechanisms	Cell metabolism
k (h^{-1})	1.04×10^{-2} [9.00×10^{-3}–1.18×10^{-2}]	–
k_{met} (mmol/L/h/65 × 10^3 cells)	–	2.51×10^{-2} [2.16×10^{-2}–2.82×10^{-2}]
NEC (mM)	0.404 [0.395–0.414]	0.272 [0.256–0.291]
b	0.177 [0.173–0.182]	0.162 [0.158–0.167]
lim	2.370*	2.496*
γ (h^{-1})	0.115*	0.100*
Sum of squares	0.0126	0.0110

Parameters estimated with the chronic BK/TD models, with k being the elimination rate of the compound due to unspecific mechanisms, k_{met} the metabolism rate, NEC the estimated no effect concentration (threshold below which there is no effect on cell viability), b the killing rate by the compound. Coefficients relating to an early stage of benzophenone-2 effects inducing transitory cell spreading phenomenon are lim, the cell spreading coefficient and γ the cell-spreading rate. The sum of squares corresponds to the sum of the squares of the differences between the variations of NCI simulated with the model and observed variations of NCI of HepaRG cells exposed to coumarin, isoeugenol and benzophenone-2 repeatedly every 2 to 3 days for 4 weeks. Two hypotheses are considered regarding the decrease of exposure concentration: unspecific mechanisms or metabolism by the exposed cells. The confidence intervals are represented in brackets.
* corresponds to the value of the parameter estimated by the acute model.

mechanisms (Table 3 and Fig. 2) had a lower sum of squares compared to the model with a decrease of concentration of exposure due to cell metabolism. The NEC estimated by the model with a decrease due to unspecific mechanisms was of 0.147 mM, whereas the model with a decrease due to metabolism estimated it at 0 mM.

Regarding the benzophenone-2 BK/TD model, the spreading-related parameters could not be estimated with the long-term exposure data. Therefore, lim, the cell spreading coefficient, and γ, the cell spreading rate, were fixed to the values estimated with short-term exposure. The model with a decrease of exposure concentration due to cell metabolism better described the repeated exposure data than the model with a decrease due to a unspecific mechanism, based on GOF (Table 3 and Fig. 2). Unlike for these two compounds, there was no difference in terms of GOF between the two kinetic models for isoeugenol.

3.4. Predictivity of acute and chronic BK/TD models of respectively repeated exposure and single exposure data

Once the models for the three compounds were calibrated based on the acute data, we simulated their predictions for repeated exposure. Except for coumarin, the acute BK/TD model (*i.e.*, isoeugenol and benzophenone-2) over-predicted NCI at intermediate concentration of 2 mM for isoeugenol and 0.5 mM for benzophenone-2 (Fig. 3). Coumarin's acute BK/TD model did quite well in predicting the time course of the NCI at different concentration of exposure, as shown by Fig. 3.

The models calibrated with chronic exposure data, except for coumarin, were not able to predict the short-term exposure data, especially at the intermediate concentrations of 2.53 mM for isoeugenol and 0.8 mM of benzophenone-2 (Supplementary data).

4. Discussion

We proposed here BK/TD models to describe the time course of the viability of cell populations exposed to cosmetic ingredients, thanks to impedance metric measurements. Although unspecific decrease and cellular metabolism could both influence the fate of the tested compounds, we assumed that one mechanism was predominant relative to the other. We would not be able to estimate the parameters if both phenomena were considered simultaneously in the model since it would require having kinetic data of the compounds (*i.e.*, free concentrations measurements over time or metabolism data on the tested HepaRG cells).

Depending on the selected fate model, the estimates of toxicity parameters (NEC and b) differed substantially (Table 2). It is therefore necessary to be able to select the most realistic model for a relevant application in performing a risk assessment. It was not possible to identify the most accurate models solely on the basis of the GOF of the impedance data (Table 2). Therefore, complementary experimental data are required, particularly in relation to metabolism. Fortunately, such information was available from other experiments (unpublished at the moment) performed at INRA Toxalim. Human primary hepatocytes were exposed to the three compounds we studied here in order to measure metabolism. Coumarin's maximum velocity (V_{max}) on human hepatocytes was 0.0065 nmol/min/10^5

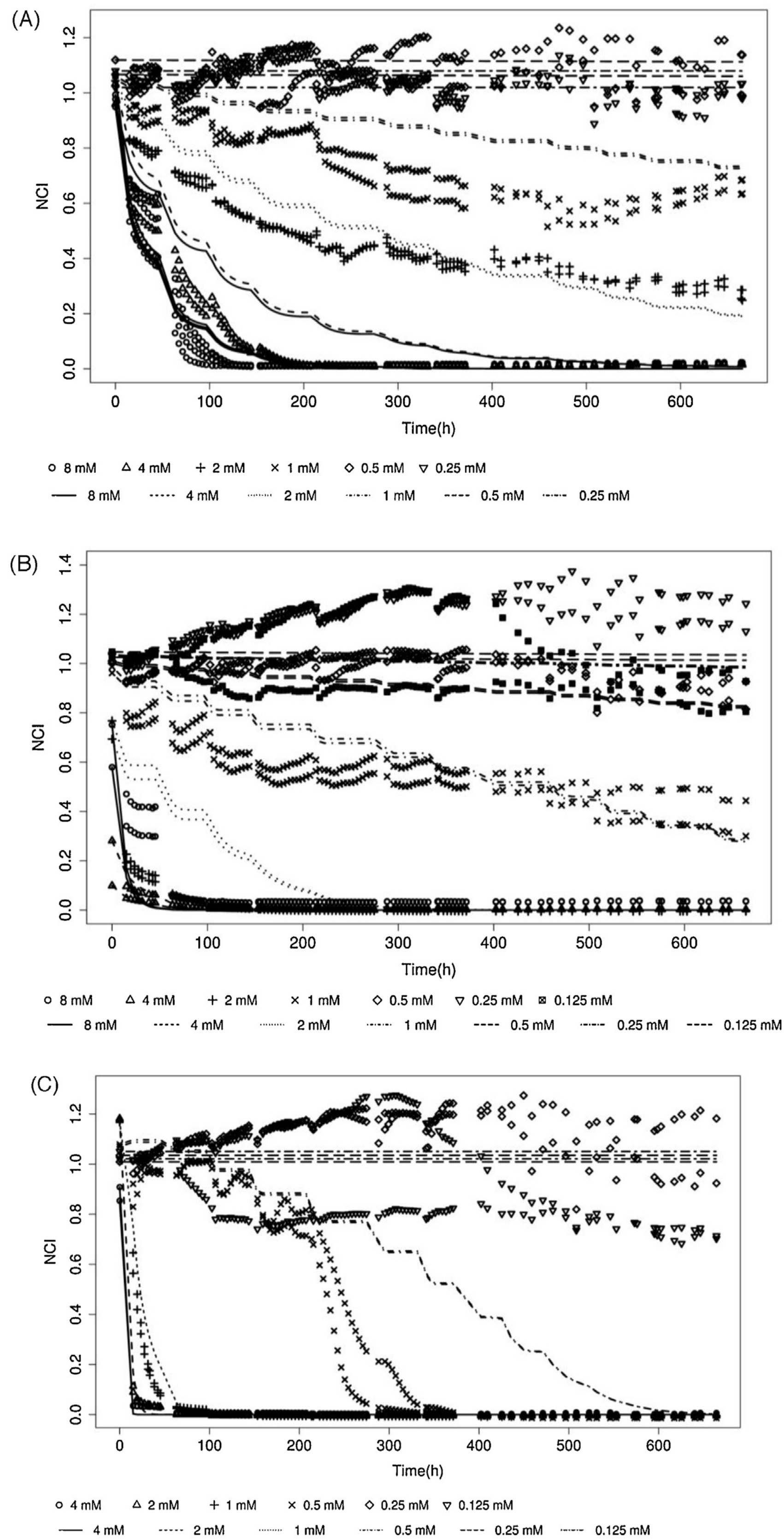


Fig. 3. Prediction of the Normalized Cell Index (NCI) of HepaRG cells after long-term exposure (every 2–3 days for 4 weeks) based on acute BK/TD models. Predictions were performed for coumarin (A) with a TK model due to unspecific phenomena (Eq. (3)), for isoeugenol (B) with a TK model due to cell metabolism (Eq. (4)), and for benzophenone-2 (C) with a TK model due to cell metabolism and a TD model accounting for the cell spreading phenomenon (Eq. (6)). Experimental data and predictions by the acute models are respectively represented by differently shaped points and lines. In order to improve the clarity of the figures, controls are not represented in the present figure but are shown in the Supplementary data.

cells in 100 μL of medium, which corresponds to a metabolism rate (V_{max}) equal to 0.0025 mmol/L/h/65.10^3 cells. This value is far below the value estimated by our BK/TD model with metabolism to account for the decrease of exposure concentration (0.283 mmol/L/h/65.10^{3}cells). Thus, the decrease of coumarin concentration is unlikely to be related to metabolism, and the model of a decrease of coumarin concentration due to an unspecific phenomenon (Eq. (3)) should be selected.

Metabolism experiments on human primary hepatocytes exposed to isoeugenol and benzophenone-2 showed cell metabolism for these compounds with V_{max} values of 0.295 nmol/min/10^5 cells and 0.324 nmol/min/10^5 cells, respectively. According to the metabolism rate ($V_{max,estim}$) estimated by our acute BK/TD model for isoeugenol and benzophenone-2 in HepaRG cells and their experimental Michaelis–Menten constant (K_m) ($V_{max,estim}$ of 0.113 mmol/L/h for 65.10^3 cells and 0.0343 mmol/L/h for 65.10^3 cells and K_m of 7.9 μM and 34.19 μM respectively), their respective maximum of velocity (V_{max}) after a single exposure would be 0.290 nmol/min/10^5 cells and 0.0879 nmol/min/10^5 cells. The metabolism rate of benzophenone-2 and isoeugenol we estimated with our BK/TD models were thus equivalent or only 4 times lower in HepaRG cells than in human primary hepatocytes (Table 1), which suggests that metabolism is a likely explanation for the decrease of exposure concentration of isoeugenol and THB. This also indicates that HepaRG cells metabolism for these two compounds is comparable to primary hepatocytes, even in the presence of DMSO to maintain the cells in the differentiated state. This is consistent with literature data, since benzophenone-2 and isoeugenol have been reported to be metabolized by phase II enzymes (Badger et al., 2002; Hong et al., 2013) *via* sulfo or glucurono conjugation. Some phase II enzymes, especially some sulfotransferases and UDP-glucuronyltransferases, are reported to be expressed at a lower level in HepaRG cells compared to human primary hepatocytes (Hart et al., 2010; Kanebratt and Andersson, 2008).

Regarding the toxicodynamics model, the cell viability of the three compounds was modelled using a simple equation based on a decrease in cell population (Eq. (5)). For one of the studied compounds, benzophenone-2, we had to add to the toxicodynamics model an equation (Eq. (6)) that accounted for a transitory spreading cells phenomenon due to an early-stage reversible toxic effect (Solly et al., 2004). This made it possible to adequately account for the toxicity phenomena leading to a decrease in cell population.

Our estimate of NEC from acute exposure to coumarin is consistent with the literature data on cytotoxicity for this compound. No cytotoxicity was observed below 0.2 mM of Coumarin exposure on human hepatocytes for 24 h or 72 h (Kienhuis et al., 2006; Ratanasavanh et al., 1996). We did not find cytotoxicity data on isoeugenol and benzophenone-2 or on coumarin after chronic exposure in the literature to compare with the threshold concentrations we estimated in our study.

Simulations of single exposure by the chronic BK/TD model showed that the chronic BK/TD models of isoeugenol and benzophenone-2 were not able to accurately predict the time course of cell viability after a single exposure at intermediate concentrations (2.53 mM for isoeugenol and 0.8 mM for benzophenone-2 (Supplementary data). Based on modelling, this is suggestive of different toxicological phenomena for acute and chronic exposure and means that acute and chronic data have to be analysed separately. Note, however, that in the case of benzophenone-2, in order to fully understand its toxicity, acute data were required to explain cells' spreading in addition to chronic toxicity data. Long-term cytotoxicity results from complex interactions with multiples pathways, than cannot be assessed only with impedance data. Further in-depth investigations on the cellular biological interactions, involving other measurements than impedance, would thus be needed.

The inability of the acute BK/TD models to predict chronic data might be due to a loss of some cellular mechanism of defence after chronic exposure. When exposed to a xenobiotic, the cellular response can either be a reversible adaptive response to a chemical-induced stress, which usually occurs at subtoxic exposures, or it can produce a disruption of homeostasis, leading to an irreversible decrease of cellular response which compromises cell viability (Martindale and Holbrook, 2002; Williams and Iatropoulos, 2002). Nevertheless, exposure which induces an adaptive response can produce toxicity with either longer or higher exposure (Williams and Iatropoulos, 2002). The drastic drop in NCI in HepaRG cells after the fourth exposure (Fig. 3) of benzophenone-2 and after the second exposure of isoeugenol at the respective subtoxic concentrations of 0.5 mM and 2 mM resulted from subtler changes that cannot be observed with impedance metrics. This decrease could be a consequence of an overwhelming phenomenon of the adaptive cellular response.

Another hypothesis for explaining the drastic drops of 2 mM for isoeugenol and 0.5 mM for benzophenone-2 would be a decrease of cell metabolic capacity after repeated exposures at these concentrations. This decrease in metabolism would indeed result in a higher exposure concentration over time.

The simulations with the coumarin acute BK/TD model for repeated exposure did well in predicting the experimental chronic (*i.e.*, long-term exposure) data (Fig. 3). Unlike isoeugenol and benzophenone-2, no additional or different mechanism for long-term exposure compared to short-term exposure of HepaRG cell to coumarin has been shown.

The models we proposed in the present study make it possible to monitor and, in fine, to predict real-time cell viability, but they are limited to cytotoxicity and do not address, for instance, cell specific function loss. Cytotoxicity assay has been validated as cell-based method to estimate starting doses for acute oral systemic toxicity tests (OECD, 2010; Prieto et al., 2014) and to identify negative oral toxicity for non-classified substances (Prieto et al., 2014). However, *in vivo* toxicity induced by exposure to chemicals involves several complex interactions at different scale *e.g.*, multi-pathways, multi-cellular, multi organ. Observed toxicity may results in modifications of physiological homeoastasis leading to functional alterations at different scales and more particularly at cellular scale. Quantifying and analyzing mechanisms of cellular function loss is still challenging.

In vivo-in vitro extrapolation (IVIVE) can be performed using our models to contribute to the prediction of *in vivo* hepatotoxicity under the hypothesis that effects on hepatic cell viability could be indicative of potential hepatotoxicity. Usually risk assessment in humans is based on data extrapolated from animals. As Prieto et al. (2006) pointed out, this approach is not based on scientific evidence, and additional data on the mechanism of animal and human toxic effects are needed. With the ban of animal testing in cosmetic ingredients, *in vitro* techniques and IVIVE methods are required. One of the hopes is that mechanistic information gained from *in vitro* techniques would partly compensate for not using full organisms.

In vitro-in vivo extrapolation is based on models accounting for toxicodynamics and biokinetics. The different phases of absorption – distribution – metabolism – excretion (ADME) can be predicted using physiology-based biokinetic (PBBK) models. These models represent the organism as a series of relevant compartments of organs or groups of similar organs linked by physiological blood flow. Coupling *in vitro* TD model with PBBK models (called PBBK/TD models) to relate hepatic concentration to the dynamic model can be used to extrapolate *in vitro* cytotoxicity on hepatic cells to *in vivo* hepatotoxicity. Using reverse dosimetry, PBBK/TD models would

provide a threshold dose for hepatoxicity under the assumptions that (i) *in vitro* toxicity data reflects the relevant toxicity parameters for *in vivo* situation, (ii) the appropriate parameters for building an adequate PBBK model are available (Blaauboer et al., 2012). Pery et al. (2013) have already showed that such approach is feasible by using IVIVE to estimate a human *in vivo* threshold dose of acetaminophen, which appeared to be closed to the usual range of human overdose, can be estimated. Such an estimated threshold could also account for the variability between different sub-populations, at least regarding kinetics, with different physiological characteristics such as age, renal insufficiency, polymorphisms and adapt risk assessment to targeted populations.

As ECVAM has highlighted in their report and recommendation of ECVAM Workshop 56, only a few attempts have been made to obtain toxicological data from long-term exposure of cells to a xenobiotic (Pfaller et al., 2001). Among these few attempts, the EU 7th Framework project, Predict-iv has been conducted to improve the predictivity of *in vitro* systems by developing mechanistic strategies. Thus, within this project, datasets have been generated in order to investigate long term repeated dose toxicity in three targeted organs (*i.e.*, the liver, the kidney and the central nervous system), and a mechanism-based model of cellular toxicity has been developed for renal epithelial cells (RPTEC/TERT1) exposed repeatedly for 14 days to the nephrotoxin cyclosporine A (Wilmes et al., 2013). In the present study, we proposed an approach providing long-term, real-time toxicological data using impedance metrics and also a methodology for analyzing these data. Due to the cost of long-term toxicity testing, we also addressed the issue of using acute toxicity data to extrapolate to chronic toxicity data. Through our study, we showed that acute-to-chronic extrapolation might lead to inaccurate predictions of chronic toxicity.

The proposed models to describe the cell viability decrease exposed to the different compounds, depending on the models, with three to five parameters, were able to reasonably describe the data. Nevertheless, additional metabolism data such as metabolite concentration measurements would be required to select the BK model that should be used for hazard assessment.

Conflict of interest

The authors declare that there are no conflicts of interests.

Transparency document

The Transparency document associated with this article can be found in the online version.

Acknowledgments

This work was supported by the European Community's Seventh Framework Program(FP7/2007-2013) and by Cosmetics Europe through the COSMOS project (grants agreement no. 266835).

The authors thank Frederic Bois, Cleo Tebby and Enrico Mombelli for their thorough review of this article. The authors thank Morningside Translation Inc., for the review of this article.

The authors thank the reviewers for their thorough review of this article which greatly helped to improve both the content and the clarity of the manuscript.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.toxlet.2015.03.011.

References

Adler, S., Basketter, D., Creton, S., Pelkonen, O., van Benthem, J., Zuang, V., Andersen, K.E., Angers-Loustau, A., Aptula, A., Bal-Price, A., Benfenati, E., Bernauer, U., Bessems, J., Bois, F.Y., Boobis, A., Brandon, E., Bremer, S., Broschard, T., Casati, S., Coecke, S., Corvi, R., Cronin, M., Daston, G., Dekant, W., Felter, S., Grignard, E., Gundert-Remy, U., Heinonen, T., Kimber, I., Kleinjans, J., Komulainen, H., Kreiling, R., Kreysa, J., Leite, S.B., Loizou, G., Maxwell, G., Mazzatorta, P., Munn, S., Pfuhler, S., Phrakonkham, P., Piersma, A., Poth, A., Prieto, P., Repetto, G., Rogiers, V., Schoeters, G., Schwarz, M., Serafimova, R., Tähti, H., Testai, E., van Delft, J., van Loveren, H., Vinken, M., Worth, A., Zaldivar, J.-M., 2011. Alternative (non-animal) methods for cosmetics testing: current status and future prospects-2010. Arch. Toxicol. 85 (5), 367–485. doi:http://dx.doi.org/10.1007/s00204-011-0693-2.

Aninat, C., Piton, A., Glaise, D., Le Charpentier, T., Langouët, S., Morel, F., Guguen-Guillouzo, C., Guillouzo, A., 2006. Expression of cytochromes P450, conjugating enzymes and nuclear receptors in human hepatoma HepaRG cells. Drug Metab. Dispos.: Biol. Fate of Chem. 34 (1), 75–83. doi:http://dx.doi.org/10.1124/dmd.105.006759.

Anthérieu, S., Chesné, C., Li, R., Camus, S., Lahoz, A., Picazo, L., Turpeinen, M., Tolonen, A., Uusitalo, J., Guguen-Guillouzo, C., Guillouzo, A., 2010. Stable expression, activity, and inducibility of cytochromes P450 in differentiated HepaRG cells. Drug Metab. Dispos. 38 (3), 516–525. doi:http://dx.doi.org/10.1124/dmd.109.030197.

Atienza, J.M., Zhu, J., Wang, X., Xu, X., Abassi, Y., 2005. Dynamic monitoring of cell adhesion and spreading on microelectronic sensor arrays. J. Biomol. Screening 10 (8), 795–805. doi:http://dx.doi.org/10.1177/1087057105279635.

Badger, D.A., Smith, R.L., Bao, J., Kuester, R.K., Sipes, I.G., 2002. Disposition and metabolism of isoeugenol in the male Fischer 344 rat. Food Chem. Toxicol. 40 (12), 1757–1765. doi:http://dx.doi.org/10.1016/S0278-6915(02) 183-7.

Bitsch, A., Jacobi, S., Melber, C., Wahnschaffe, U., Simetska, N., Mangelsdorf, I., 2006. REPDOSE: a database on repeated dose toxicity studies of commercial chemicals—a multifunctional tool. Regul. Toxicol. Pharmacol. 46 (3), 202–210. doi:http://dx.doi.org/10.1016/j.yrtph.2006.05.013.

Blaauboer, B.J., Boekelheide, K., Clewell, H.J., Daneshian, M., Dingemans, M.M.L., Goldberg, A.M., Heneweer, M., Jaworska, J., Kramer, N.I., Leist, M., Seibert, H., Testai, E., Vandebriel, R.J., Yager, J.D., Zurlo, J., 2012. The use of biomarkers of toxicity for integrating in vitro hazard estimates into risk assessment for humans. Altex-Altern. Anim. Exp. 29 (4), 411–425.

Ceriotti, L., Ponti, J., Colpo, P., Sabbioni, E., Rossi, F., 2007. Assessment of cytotoxicity by impedance spectroscopy. Biosens. Bioelectron. 22 (12), 3057–3063. doi:http://dx.doi.org/10.1016/j.bios.2007.01.004.

Gerets, H.H.J., Tilmant, K., Gerin, B., Chanteux, H., Depelchin, B.O., Dhalluin, S., Atienzar, F.A., 2012. Characterization of primary human hepatocytes, HepG2 cells, and HepaRG cells at the mRNA level and CYP activity in response to inducers and their predictivity for the detection of human hepatotoxins. Cell Biol. Toxicol. 28 (2), 69–87. doi:http://dx.doi.org/10.1007/s10565-011-9208-4.

Giaever, I., Keese, C.R., 1984. Monitoring fibroblast behavior in tissue culture with an applied electric field. Proc. Natl. Acad. Sci. 81 (12), 3761–3764.

Gripon, P., Rumin, S., Urban, S., Seyec, J.L., Glaise, D., Cannie, I., Guyomard, C., Lucas, J., Trepo, C., Guguen-Guillouzo, C., 2002. Infection of a human hepatoma cell line by hepatitis B virus. Proc. Natl. Acad. Sci. 99 (24), 15655–15660. doi:http://dx.doi.org/10.1073/pnas.232137699.

Guillouzo, A., 1998. Liver cell models in in vitro toxicology. Environ. Health Perspect. 106 (Suppl. 2), 511–532.

Guillouzo, A., Morel, F., Fardel, O., Meunier, B., 1993. Use of human hepatocyte cultures for drug metabolism studies. Toxicology 82 (1–3), 209–219. doi:http://dx.doi.org/10.1016/0300-483X(93) 90,065-Z 1993.

Hart, S.N., Li, Y., Nakamoto, K., Subileau, E., Steen, D., Zhong, X., 2010. A comparison of whole genome gene expression profiles of HepaRG cells and HepG2 cells to primary human hepatocytes and human liver tissues. Drug Metab. Dispos. 38 (6), 988–994. doi:http://dx.doi.org/10.1124/dmd.109.031831.

Hewitt, N.J., Lechón, M.J.G., Houston, J.B., Hallifax, D., Brown, H.S., Maurel, P., Kenna, J.G., Gustavsson, L., Lohmann, C., Skonberg, C., Guillouzo, A., Tuschl, G., Li, A.P., LeCluyse, E., Groothuis, G.M.M., Hengstler, J.G., 2007. Primary hepatocytes: current understanding of the regulation of metabolic enzymes and transporter proteins, and pharmaceutical practice for the use of hepatocytes in metabolism, enzyme induction, transporter, clearance, and hepatotoxicity studies. Drug Metab. Rev. 39 (1), 159–234. doi:http://dx.doi.org/10.1080/03602530601093489.

Hong, S.P., Fuciarelli, A.F., Johnson, J.D., Graves, S.W., Bates, D.J., Smith, C.S., Waidyanatha, S., 2013. Toxicokinetics of isoeugenol in F344 rats and $B6C3F_1$ mice. Xenobiotica 43 (11), 1010–1017. doi:http://dx.doi.org/10.3109/00498254.2013.790576.

Jossé, R., Aninat, C., Glaise, D., Dumont, J., Fessard, V., Morel, F., Poul, J.-M., Guguen-Guillouzo, C., Guillouzo, A., 2008. Long-term functional stability of human HepaRG hepatocytes and use for chronic toxicity and genotoxicity studies. Drug Metab. Dispos. 36 (6), 1111–1118. doi:http://dx.doi.org/10.1124/dmd.107.019901.

Kanebratt, K.P., Andersson, T.B., 2008. Evaluation of HepaRG cells as an in vitro model for human drug metabolism studies. Drug Metab. Dispos. 36 (7), 1444–1452. doi:http://dx.doi.org/10.1124/dmd.107.020016.

Ke, N., Wang, X., Xu, X., Abassi, Y.A., 2011. The xCELLigence system for real-time and label-free monitoring of cell viability. Methods Mol. Biol. (Clifton, N.J.) 740, 33–43. doi:http://dx.doi.org/10.1007/978-1-61779-108-6_6.

Kienhuis, A.S., Wortelboer, H.M., Hoflack, J.-C., Moonen, E.J., Kleinjans, J.C.S., van Ommen, B., van Delft, J.H.M., Stierum, R.H., 2006. Comparison of coumarin-

induced toxicity between sandwich-cultured primary rat hepatocytes and rats in vivo: a toxicogenomics approach. Drug Metab. Dispos. 34 (12), 2083–2090. doi:http://dx.doi.org/10.1124/dmd.106.011262.

Martindale, J.L., Holbrook, N.J., 2002. Cellular response to oxidative stress: signaling for suicide and survival*. J. Cell. Physiol. 192 (1), 1–15. doi:http://dx.doi.org/10.1002/jcp.10119.

Mebane, W.R., Sekhon, J.S., 2011. Genetic optimization using derivatives: the rgenoud package for R. J. Stat. Software 42 (11), 1–26.

Mitra, P., Keese, C.R., Giaever, I., 1991. Electric measurements can be used to monitor the attachment and spreading of cells in tissue culture. BioTechniques 11 (4), 504–510.

Nawaz, A., Razpotnik, A., Rouimi, P., Sousa, G., de Cravedi, J.P., Rahmani, R., 2014. Cellular impact of combinations of endosulfan, atrazine, and chlorpyrifos on human primary hepatocytes and HepaRG cells after short and chronic exposures. Cell Biol. Toxicol. 30 (1), 17–29. doi:http://dx.doi.org/10.1007/s10565-013-9266-x.

OECD, (2010). Guidance Document No 129 on using Cytotoxicity Tests to Estimate Starting Doses for Acute Oral Systemic Toxicity Tests.

Pery, A.R.R., Brochot, C., Zeman, F.A., Mombelli, E., Desmots, S., Pavan, M., Fioravanzo, E., Zaldivar, J.-M., 2013. Prediction of dose-hepatotoxic response in humans based on toxicokinetic/toxicodynamic modeling with or without in vivo data: a case study with acetaminophen. Toxicol. Lett. 220 (1), 26–34. doi:http://dx.doi.org/10.1016/j.toxlet.2013.03.032.

Pfaller, W., Balls, M., Clothier, R., Coecke, S., Dierickx, P., Ekwall, B., Hanley, B.A., Hartung, T., Prieto, P., Ryan, M.P., Schmuck, G., Sladowski, D., Vericat, J.A., Wendel, A., Wolf, A., Zimmer, J., 2001. Novel advanced in vitro methods for long-term toxicity testing: the report and recommendations of ECVAM workshop 45. European Centre for the Validation of Alternative Methods. Altern. Lab. Anim.: ATLA 29 (4), 393–426.

Prieto, P., Baird, A.W., Blaauboer, B.J., Castell Ripoll, J.V., Corvi, R., Dekant, W., Dietl, P., Gennari, A., Gribaldo, L., Griffin, J.L., Hartung, T., Heindel, J.J., Hoet, P., Jennings, P., Marocchio, L., Noraberg, J., Pazos, P., Westmoreland, C., Wolf, A., Wright, J., Pfaller, W., 2006. The assessment of repeated dose toxicity in vitro: a proposed approach. The report and recommendations of ECVAM workshop 56. Altern. Lab. Anim.: ATLA 34 (3), 315–341.

Prieto, P., Burton, J., Graepel, R., Price, A., Whelan, M.P., Worth, A., 2014. Eurl Ecvam Strategy to Replace, Reduce and Refine the Use of Animals in the Assessment of Acute Mammalian Systemic Toxicity. Publications Office of the European Union, Luxembourg.

Rajan, N., Habermehl, J., Coté, M.-F., Doillon, C.J., Mantovani, D., 2006. Preparation of ready-to-use, storable and reconstituted type I collagen from rat tail tendon for tissue engineering applications. Nat. Protoc. 1 (6), 2753–2758. doi:http://dx.doi.org/10.1038/nprot.2006.430.

Ratanasavanh, D., Lamiable, D., Biour, M., Guédès, Y., Gersberg, M., Leutenegger, E., Riché, C., 1996. Metabolism and toxicity of coumarin on cultured human, rat, mouse and rabbit hepatocytes. Fundam. Clin. Pharmacol. 10 (6), 504–510. doi:http://dx.doi.org/10.1111/j. 1472-8206.1996. tb00607. x.

Solly, K., Wang, X.B., Xu, X., Strulovici, B., Zheng, W., 2004. Application of real-time cell electronic sensing (RT-CES) technology to cell-based assays. Assay Drug Dev. Technol. 2 (4), 363–372. doi:http://dx.doi.org/10.1089/adt.2004.2.363.

R Development Core Team, 2013. R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna, Austria.

Williams, G.M., Iatropoulos, M.J., 2002. Alteration of liver cell function and proliferation: differentiation between adaptation and toxicity. Toxicol. Pathol. 30 (1), 41–53. doi:http://dx.doi.org/10.1080/01926230252824699.

Wilmes, A., Limonciel, A., Aschauer, L., Moenks, K., Bielow, C., Leonard, M.O., Hamon, J., Carpi, D., Ruzek, S., Handler, A., Schmal, O., Herrgen, K., Bellwon, P., Burek, C., Truisi, G.L., Hewitt, P., Di Consiglio, E., Testai, E., Blaauboer, B.J., Guillou, C., Huber, C.G., Lukas, A., Pfaller, W., Mueller, S.O., Bois, F.Y., Dekant, W., Jennings, P., 2013. Application of integrated transcriptomic, proteomic and metabolomic profiling for the delineation of mechanisms of drug induced cell stress. J. Proteomics 79, 180–194. doi:http://dx.doi.org/10.1016/j.jprot.2012.11.022.

Xing, J.Z., Zhu, L., Gabos, S., Xie, L., 2006. Microelectronic cell sensor assay for detection of cytotoxicity and prediction of acute toxicity. Toxicol. In Vitro 20 (6), 995–1004. doi:http://dx.doi.org/10.1016/j.tiv.2005.12.008.

Xing, J.Z., Zhu, L., Jackson, J.A., Gabos, S., Sun, X.-J., Wang, X.-B., Xu, X., 2005. Dynamic monitoring of cytotoxicity on microelectronic sensors. Chem. Res. Toxicol. 18 (2), 154–161. doi:http://dx.doi.org/10.1021/tx049721s.