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In vitro biokinetics of chlorpromazine and the influence of different dose metrics on effect concentrations for cytotoxicity in Balb/c 3T3, Caco-2 and HepaRG cell cultures

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ABSTRACT

The extrapolation of *in vitro* to *in vivo* toxicity data is a challenge. Differences in sensitivity between cell systems may be due to intrinsic properties of the cell but also because of differences in exposure. In this study, the cytotoxicity and biokinetics of the antipsychotic chlorpromazine (CPZ) were studied in *in vitro* assays using different cell types and exposure conditions. Different dose metrics were assessed to express the sensitivity to CPZ.

The biokinetics of CPZ were measured in cell cultures of Balb/c 3T3, Caco-2 and HepaRG cells. Cytotoxicity was measured by Alamar Blue and expressed using different dose metrics, including the nominal, measured total and measured free CPZ medium concentrations.

CPZ was taken up by the cells; the highest amounts in the cell compartments were found in the Caco-2 and HepaRG cells. CPZ was highly protein-bound in the Caco-2 cell medium containing 10% fetal bovine serum, resulting in lower bioavailable exposure concentrations. Moreover, also uptake into the cells strongly influenced the concentration in the medium. The Balb/c 3T3 cells were the most sensitive to the toxic effect of CPZ. The use of different dose metrics influenced the cytotoxicity results found in the three cell types. The data show that in comparing the sensitivity of the tested cell systems, the freely dissolved concentration is a more appropriate dose metric than total concentration in the medium. The ranking in sensitivity of the three cell types for CPZ was dependent on the dose metric used.

1. Introduction

Within Europe the REACH legislation (Registration, Evaluation and Authorization and restriction of CHemicals), installed in 2007, requires additional tests for 'existing' and 'new' chemicals that are produced in high volumes (EC, 2006). The number of animal tests needed to provide full information on the chemicals is estimated to be 2.6–3.9 million depending on the implementation of alternative testing methods (Van der Jagt et al., 2004). Besides the REACH legislation, animal experiments are also needed in the pre-clinical part of the development phase for new chemical entities. Both for REACH as in the development of pharmaceuticals, information should be provided on different toxicological endpoints, including acute and chronic toxicity, eye and skin irritation, kinetics, reproduction toxicity and genotoxicity (Lilienblum et al., 2008). For several of these studies *in vitro* assays are in the testing guidelines of the OECD for regulatory use, e.g. 3T3 NRU phototoxicity assay, embryonic stem cell test and whole embryo culture, the Ames test and cell transformation assays (Lilienblum et al., 2008; Punt et al., 2011). Alternatives for all toxicity end points are not available yet, but improving the already existing ones might reduce the use of laboratory animals (Spielmann et al., 1999; ICCVAM, 2001).

For acute toxicity studies, *in vitro* tests can be performed aimed at measuring the cytotoxicity (Eisenbrand et al., 2002). Results from these *in vitro* tests may assist in selecting the test concentrations for the *in vivo* studies (Spielmann et al., 1999; ICCVAM, 2001; Gülden and Seibert, 2003b). Different cell lines can be used in the *in vitro* experiments aimed at studying multiple toxicity parameters. However, discrepancies are often seen in the results obtained with the different cell types (Gülden et al., 2005). Possible reasons for these differences are specific cell properties, the use of different end points and the set-up of the cell assay, including differences in

Abbreviations: BSA, bovine serum albumin; CPZ, chlorpromazine; DMEM, Dulbecco's Modified Eagle Medium; DMSO, dimethyl sulfoxide; FBS, fetal bovine serum; HBSS, Hank's Balanced Salt Solution; NCS, newborn calf serum; nd-SPME, negligible depletion-solid phase microextraction; NEAA, non-essential amino acids; PBS, phosphate buffered saline.

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exposure conditions and spiking procedures (Gülden et al., 2001, 2010; Schirmer, 2006; Tanneberger et al., 2010; Kramer et al., 2012). The differences complicate the interpretation and extrapolation of the results found.

The end point most often used in cytotoxicity assays is basal cytotoxicity often expressed by a 50% effect concentration (EC_{50}-value). In most cases, EC_{50}s are based on nominal concentrations that are calculated from the amount added to the cell system. This nominal concentration might not reflect the concentration of test compound to which the cells are actually exposed (Gülden et al., 2002; Heringa et al., 2004). The compound can bind to serum proteins in the medium, can bind to the well plastic and can evaporate (Heringa et al., 2004).

Recent studies into the factors influencing the bioavailable amount of compound in *in vitro* assays have shown that the amount of proteins in the medium and the amount of cells present in the *in vitro* system play an important role (Seibert et al., 2002; Gülden et al., 2010; Kramer et al., 2012). These factors are not routinely taken into account (Gülden et al., 2003a). In a number of studies, however, actual and also freely dissolved concentrations are measured (Heringa et al., 2004; Tanneberger et al., 2010; Kramer et al., 2012). Besides that, also new exposure systems have been developed and applied where the concentration in the test system is kept constant (Kramer et al., 2010; Smith et al., 2010). In addition to these developments in the experimental work itself, exposure modeling, in combination with quantitative structure-activity relationships, represents a powerful tool to predict the actual exposure in *in vitro* tests (Vaes et al., 1998; DeBruyn and Gobas, 2007; Endo and Goss, 2011).

For the determination of EC_{50}s, researchers have often focused on nominal concentrations as the dose metric. Not only the topic of nominal concentrations versus measured concentrations is an issue in the interpretation of the outcome of an *in vitro* test, but also the selection of a suitable dose metric is an important aspect in analyzing dose–effect relationships. For example, in a test where the concentration decreases over time, one may select the concentration of the test compound at the beginning of the exposure, at the end of the exposure, or the mean of the concentrations at the beginning and the end (Knöbel et al., 2012). Other examples of dose metrics are peak concentrations or "areas under the curve" (AUC). The selection of a certain dose metric is also related to the mode or mechanism of action (Reinert et al., 2002; Gülden et al., 2010; Louisse et al., 2010).

In this study, the cytotoxicity and biokinetics of the model compound chlorpromazine hydrochloride (CPZ) were studied in cytotoxicity assays with Balb/c 3T3 cells, Caco-2 cells and HepaRG cells. CPZ was chosen because it is a lipophilic compound (apparent $LogPC_{(n\text{-octanol/water})}$ is 3.16 at pH = 7.4) that is ionized at a physiological pH (p*K*a = 9.3) (Sawada et al., 1994). It is highly protein bound *in vivo* (Sawada et al., 1994; Aungst et al., 2000) and *in vitro* (Sawada et al., 1994; Broeders et al., 2012) and for these reasons it is an interesting compound to compare the three different cell types with each other.

The Balb/c 3T3 fibroblasts are often used to determine basal cytotoxicity (ICCVAM, 2001), but these are murine cells and the use of human cell lines (for extrapolation to the human situation) is encouraged. Therefore, two human cell lines (Caco-2 cells and HepaRG cells) were included as well. The Caco-2 cells represent intestinal cells and the HepaRG cells are a human liver cell line. In addition to different cell properties, the medium composition (presence of serum) used in the different cell lines played an important role in selecting these cells.

Differences in apparent sensitivity to CPZ within the three cell lines tested can be caused by (i) differences in exposure (kinetics), (ii) differences in biotransformation and (iii) differences in intrinsic sensitivity (presence of a specific receptor). Differences in biotransformation can influence the sensitivity to the test compound in two ways: inactivation of the compound decreases the toxic effect; activation into toxic metabolites will increase the sensitivity of the cells to the compound. In this study we focus on the potential influence of differences in biokinetics and try to identify to what extent differences in the actual exposure and the change in exposure over time may explain differences in the observed effect concentrations (EC_{50}) in the three cell assays. For that reason, besides measuring the cytotoxicity, the exposure in the three cell systems was measured in detail by measuring the total and freely dissolved concentrations in the medium as well as the amounts accumulated in the cells. Freely dissolved concentrations were measured by negligible depletion-solid phase microextraction (Broeders et al., 2011). Furthermore, the amount of cells was determined to correct the results for the different cell assays. The results show that medium composition, the number of cells and the dose parameter used influence the cytotoxicity results.

2. Materials and methods

2.1. Chemicals and solutions

Dulbecco's Minimum Essential Medium (DMEM), William's E medium, fetal bovine serum (FBS), newborn calf serum (NCS), penicillin, streptomycin, sodium pyruvate (100 mM), L-glutamine (200 mM), MEM non-essential amino acids (NEAA, 100×), phosphate buffered saline (PBS, 10×), trypsin-EDTA (0.05% – 0.2 g/L), Hank's Balanced Salt Solution (HBSS), HEPES buffer and Alamar Blue were from Invitrogen (Breda, The Netherlands). Chlorpromazine hydrochloride, DMSO, hydrocortisone 21-hemisuccinate (10 mM in distilled water), bovine serum albumin (BSA, lyophilized powder ⩾96%), fluorescamine (⩾98% TLC), insulin (1 mM in acidified distilled water), formaldehyde solution 37% wt% in H_2O and calcium chloride ($CaCl_2$, ⩾96%) were purchased from Sigma–Aldrich (Zwijndrecht, The Netherlands). Methanol HPLC grade 99.9% purity was from Labscan (Dublin, Ireland), acetone was from Interchema (Zwolle, The Netherlands) and orthophosphoric acid 85% HPLC-grade was from VWR Prolabo (Amsterdam, The Netherlands). Deionized water was prepared with a Millipore water purification system with an organic free kit (Millipore Water, Amsterdam, The Netherlands).

2.2. Cell culture

The murine fibroblast cell line Balb/c 3T3 was purchased from American Type Culture Collection (ATCC, CCL-163, Manassas, VA). These cells were cultured in DMEM supplemented with 10% NCS, 110 mg/L sodium pyruvate, 100 U/mL penicillin and 100 μg/mL streptomycin. For the cytotoxicity assays, the cells were plated on 12-well plates (Greiner Bio-One, Alphen aan den Rijn, The Netherlands) at a seeding density of 1.5×10^4 cells/well. The cells were cultured on the plate for 24 h in the culture medium containing 10% NCS (ICCVAM, 2006).

The human intestinal cell line Caco-2 was from ATCC (HTB-37) and was cultured in DMEM supplemented with 10% FBS, 2 mM L-glutamine, 1× NEAA, 1 mM sodium pyruvate, 100 U/mL penicillin and 100 μg/mL streptomycin. For the cytotoxicity assays, the Caco-2 cells were plated on 12-well plates at a seeding density of 2.6×10^4 cells/well. The cells were cultured on the plate for 1 week; culture medium was refreshed twice (Konsoula and Barile, 2005).

The human liver cell line HepaRG was purchased from Biopredic International (Rennes, France, HPR101002). The HepaRG cells were routinely cultured in William's E medium supplemented with 10% FBS, 50 μM hydrocortisone 21-hemisuccinate, 5 μg/mL insulin,