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Effects of drugs in subtoxic concentrations on the metabolic fluxes in human hepatoma cell line Hep G2

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ABSTRACT

Commonly used cytotoxicity assays assess the toxicity of a compound by measuring certain parameters which directly or indirectly correlate to the viability of the cells. However, the effects of a given compound at concentrations considerably below EC_{50} values are usually not evaluated. These subtoxic effects are difficult to identify but may eventually cause severe and costly long term problems such as idiosyncratic hepatotoxicity. We determined the toxicity of three hepatotoxic compounds, namely amiodarone, diclofenac and tacrine on the human hepatoma cell line Hep G2 using an online kinetic respiration assay and analysed the effects of subtoxic concentrations of these drugs on the cellular metabolism by using metabolic flux analysis. Several changes in the metabolism could be detected upon exposure to subtoxic concentrations of the test compounds. Upon exposure to diclofenac and tacrine an increase in the TCA-cycle activity was observed which could be a signature of an uncoupling of the oxidative phosphorylation. The results indicate that metabolic flux analysis could serve as an invaluable novel tool for the investigation of the effects of drugs. The described methodology enables tracking the toxicity of compounds dynamically using the respiration assay in a range of concentrations and the metabolic flux analysis permits interesting insights into the changes in the central metabolism of the cell upon exposure to drugs.

Introduction

The development of drugs is a very expensive and time-consuming process. The average clinical success rate in all therapeutic areas is approximately 11%. This means that just one in nine compounds makes it through the clinical development and reaches the market. The costs for the development of a drug are about $900 million and most of these incur later in the pipeline where major attrition occurs (Kola and Landis, 2004). One of the leading causes of attrition at all stages of drug development is toxicity (Kramer et al., 2007). In particular, hepato- and cardiovascular toxicities were accounted for two out of three post-market withdrawals of drugs (Schuster et al., 2005). Drug induced liver impairment is one of the major causes of acute liver failure in western countries and the mortality rate is about 80% in patients with acute liver failure (Ostapowicz and Lee, 2000). There is, therefore, an urgent need to identify better models to predict human hepatotoxicity (Sahu, 2007). While animal based toxicity assays could predict 70% of the toxicity in human beings in a retrospective analysis, hepatotoxicity has a very bad concordance between human beings and animals. Only half of the new compounds that caused clinical hepatotoxicity had a concordance with animal hepatotoxicity (Olson et al., 2000; O'Brien et al., 2006).

Primary hepatocytes represent the gold-standard for the analysis of the hepatotoxic potential of new drug entities but other cellular models can also be used for similar predictions (Davila et al., 1998; Castell et al., 2006). Several cell lines are available for the analysis of toxicity mechanisms. The human hepatoma cell line Hep G2, established in 1979, is the best-characterized and most frequently used cell line with respect to hepatotoxic endpoints and has been used to examine various mechanisms of hepatotoxicity (Aden et al., 1979; Brandon et al., 2003; Wilkening et al., 2003; Hewitt and Hewitt, 2004; Vermeir et al., 2005; Noor et al., 2009). The evaluation of multiple endpoints on Hep G2 cells allows the prediction of human hepatotoxicity with more than 80% sensitivity and 90% specificity (O'Brien et al., 2006). Therefore, the idea that Hep G2 cells can predict overall hepatotoxicity using hepatospecific endpoints is becoming more and more accepted (Sahu, 2007).

Various mechanisms can be involved in the onset and progression of hepatotoxicity. There are different cellular targets of drug-related toxicity. Moreover, liver injury can be initiated by mechanisms specific to the toxicant (Chang and Schiano, 2007; Sahu, 2007).

Biological systems are quite robust concerning their ability to adapt to environmental changes. For example alternative pathways can be activated, isoenzymes can undertake the task of an enzyme or alternative substrates can be used. The metabolic system may adapt without visible changes in the phenotype or even before the manifestation of toxicity. These metabolic changes could, therefore, be indications for the hepatotoxic potential of a test compound.

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It is highly probable that the effects of subtoxic concentrations contribute to the overall toxicity of the compound which may later lead to the failure of the drug. These effects may be difficult to identify but could later cause severe long term problems. To our knowledge very little is known about the effects of drugs in subtoxic concentrations on the cellular metabolism. In the emerging field of systems biology, the analysis of metabolic flux data is highly relevant since intracellular reaction rates represent the functional endpoints of gene, protein and metabolic interactions (Sauer, 2006). Metabolic flux analysis aims at the quantitative analysis of *in-vivo* carbon fluxes across metabolic networks and has revealed fascinating insights into various biological systems (Wittmann, 2007). Such quantitative analysis has been applied to analyse and engineer the metabolism of microorganisms (Wittmann and Heinzle, 2002; Kiefer et al., 2004; Becker et al., 2005; Antoniewicz et al., 2007; Kim et al., 2008), and to study mammalian cells (Bonarius et al., 1996; Balcarcel and Clark, 2003; Vo and Palsson, 2006; Hofmann et al., 2008; Maier et al., 2008; Yoo et al., 2008; Deshpande et al., 2009) and plants (Heinzle et al., 2007; Libourel and Shachar-Hill, 2008). Assessment of the effects of drugs on mammalian metabolism requires methods allowing high throughput analysis. Relatively few studies focused on developing and applying such methods (Balcarcel and Clark, 2003; Sauer, 2004; Wittmann et al., 2004; Velagapudi et al., 2007). We used the Hep G2 cell line as a model system to determine whether metabolic flux analysis in a high throughput setup can be used to analyse changes in the metabolism of cells upon exposure to subtoxic concentrations of drugs. A number of compounds namely amiodarone, diclofenac and tacrine as well as the commonly used solvent dimethylsulphoxide (Busby et al., 1999; Easterbrook et al., 2001) were tested. For the determination of the EC_{50} and subtoxic concentrations of the test drugs, we used a time-resolved respiration assay which is reported as an invaluable non-invasive tool for the assessment of toxicity (Deshpande et al., 2005; Noor et al., 2009). The anabolic demand of the Hep G2 cells was determined, the exometabolome was analysed and the metabolic fluxes were estimated using metabolite balancing. In the presented work, the following questions were addressed: (i) can differences in metabolic fluxes of the central metabolism of HepG2 cells be observed upon exposure to subtoxic concentrations of the tested drugs? (ii) Which changes occur and iii) is it possible to reconciliate these changes with literature data according to functions of the test drugs and mechanisms of toxicity of these drugs?

Materials and methods

Cell line and culture conditions. The human hepatoma cell line HepG2 (DSMZ, Braunschweig, Germany) was maintained in Williams Medium E (WME, PAN Biotech, Aidenbach, Germany) supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin (penicillin/streptomycin solution from C.C. Products, Oberdorla, Germany) and 3% fetal calf serum (FCS Gold, PAA Laboratories, Pasching, Austria). The cells were kept in culture flasks (Nunc, Wiesbaden, Germany) at 37 °C in a humidified 5% CO_2 cell culture incubator and were subcultivated at 90% confluency. Cell number was determined using a hemocytometer and viability was assessed using the trypan blue exclusion method.

Test compounds. Test drugs and DMSO were purchased from Sigma-Aldrich (Steinheim, Germany). Stock solutions of 50 mM were prepared in DMSO for all test compounds. The stock solutions were serially diluted with Dulbecco's phosphate buffered saline (PBS, PAA Laboratories, Pasching, Austria). An appropriate volume of the diluted stock solution was added in the medium to yield the desired final concentration of the test drug ($n=4$). The highest final concentration of the solvent in toxicity assay did not exceed 2% (v/v). The DMSO concentration in subtoxic concentrations was always less than 0.1% (v/v).

Evaluation of proliferation of Hep G2. For the determination of the proliferation of Hep G2 cells under serum free conditions, 5×10^4 cells/well were seeded in WME containing 3% FCS in 4-well plates (Nunc, Langenselbold, Germany). One well was always filled with medium as a control. After 24 h, the medium was aspirated, the cells were washed once with serum free WME and finally serum free WME was added. The cultivation volume was 500 µl. The proliferation was monitored using the Sulforhodamine B-assay (SRB-assay, Sigma-Aldrich). The SRB-assay is a rapid method for the quantification of the cellular protein content and is linear with the number of cells (Skehan et al., 1990). The assay was carried out according to the kit instructions. The absorption was measured at 540 nm against a reference wavelength at 660 nm. At each time point, one 4-well plate was used and the SRB-assay was performed.

Growth rates (μ) were calculated using

$$\mu = \frac{\ln N - \ln N_0}{t - t_0} \quad (1)$$

where N represents the absorbance measured in the SRB-assay and t is the time. Doubling time (T_D) was calculated using

$$T_D = \frac{\ln 2}{\mu}.$$

Determination of the biomass composition. Hep G2 cells were maintained in 175 cm^2 culture flasks under standard culture conditions in a humidified 5% CO_2 incubator at 37 °C. For the calculation of the anabolic demand of the cells, the biomass composition of Hep G2 cells was determined as follows:

Sample preparation. Cells were harvested in the late exponential phase by trypsinisation, collected in a 50 ml centrifuge tube (Falcon) and spun down in a centrifuge (1500 U/min, 5 min, 25 °C, Function Line, Heraeus Instruments, Hanau, Germany). The cell pellet was washed twice with PBS. The total cell number was then determined using a hemocytometer. An appropriate amount of cells was used for each biomass-related analysis.

Dry cell weight. Samples containing $5–15\times10^6$ cells were taken in preweighed 1.5 ml tubes (Eppendorf) and centrifuged (3000 U/min, 5 min, 25 °C, Biofuge Pico, Heraeus Instruments, Hanau, Germany). The supernatant was carefully discarded and the pellets were frozen, lyophilized and finally weighed.

Protein content. Samples containing $2–10\times10^6$ cells were taken in 1.5 ml Eppendorf tubes and centrifuged (3000 U/min, 5 min, 25 °C, Biofuge Pico, Heraeus Instruments, Hanau, Germany). The supernatant was carefully aspirated off and the cell pellets were resuspended in 200 µl CelLytic™ M solution (Sigma-Aldrich) and agitated for 15 min. After centrifugation (13,000 U/min, 10 min, 4 °C, Biofuge fresco, Heraeus Instruments, Hanau, Germany) the supernatant was transferred into fresh tubes. The cellular protein content was determined using the Bio-Rad Protein Assay which is based on the method described by Bradford (1976). The calibration was performed using different concentrations of bovine serum albumin (Sigma-Aldrich) and the absorbance was measured at 595 nm (iEMS Reader MF, Labsystems, Helsinki, Finland).

Amino acid composition of cellular protein. To determine the amino acid composition of the total cellular protein, $2–5\times10^6$ cells were taken in 1.5 ml Eppendorf tubes. After centrifugation (3000 U/min, 5 min, 25 °C, Biofuge Pico, Heraeus Instruments) the supernatant was discarded and the pellets were resuspended in 450 µl PBS. For hydrolysis 50 µl of 6 M HCl was added and the tubes were incubated for 24 h at 110 °C. The solution was then neutralized using an appropriate volume of 6 M NaOH and the amino acid composition was determined by high performance