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In vitro kinetics of amiodarone and its major metabolite in two human liver cell models after acute and repeated treatments

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ABSTRACT

The limited value of *in vitro* toxicity data for the *in vivo* extrapolation has been often attributed to the lack of kinetic data. Here the *in vitro* kinetics of amiodarone (AMI) and its mono-N-desethyl (MDEA) metabolite was determined and modelled in primary human hepatocytes (PHH) and HepaRG cells, after single and repeated administration of clinically relevant concentrations. AMI bioavailability was influenced by adsorption to the plastic and the presence of protein in the medium (e.g. 10% serum protein reduced the uptake by half in HepaRG cells). The cell uptake was quick (within 3 h), AMI metabolism was efficient and a dynamic equilibrium was reached in about a week after multiple dosing. In HepaRG cells the metabolic clearance was higher than in PHH and increased over time, as well as CYP3A4. The interindividual variability in MDEA production in PHHs was not proportional to the differences in CYP3A4 activities, suggesting the involvement of other CYPs and/or AMI-related CYP inhibition. After repeated treatment AMI showed a slight potential for bioaccumulation, whereas much higher intracellular MDEA levels accumulated over time, especially in the HepaRG cells, associated with occurrence of phospholipidosis.

The knowledge of *in vitro* biokinetics is important to transform an actual *in vitro* concentration–effect into an *in vivo* dose–effect relationship by using appropriate modelling, thus improving the *in vitro*-to-*in vivo* extrapolation.

1. Introduction

The use of large numbers of laboratory animals and the distress that toxicity testing inevitably evokes on the animals have resulted in extensive discussions on the ethical, scientific and economical feasibility of the current process of toxicity testing. Apart from the ethical objections against the use of animals, the scientific motivation for criticism relies in the qualitative/quantitative differences between animals and humans, very often related to kinetic parameters, but the exploration of pharmacodynamic differences is just starting and will probably reveal further discrepancies. Actually, if the focus is on human cells *in vitro*, bypassing animal testing, inter-individual differences will be of major relevance (Zeise et al., 2013).

Over the last decades an increasing number of *in vitro* test systems for evaluating the possible toxicological hazard of chemical compounds have been developed (Hartung, 2011). These *in vitro* systems have been useful in studying the mechanism/mode of action, in prioritizing chemicals mainly as screening tools, especially by the pharmaceutical industry; so far their use in risk assessment has been very limited. This is due to the needs to develop a series of *in vitro* tests resembling the steps leading to the occurrence of toxic effects in humans (Liebsch et al., 2011),

Abbreviations: AMI, amiodarone; MDEA, mono-N-desethylamiodarone; DDEA, di-N-desethylamiodarone; PHH, primary human hepatocytes; CYP, cytochrome P450; DMSO, dimetylsulfoxide; MeOH, methanol; HMM, Hepatocyte Maintenance Medium; FCS, fetal calf serum; SW, sandwich; d0, day 0; d13, day 13; TP, time point; LOD, limit of detection; ADR, adverse drug reactions; PBPK, Physiologically Based Pharmacokinetic models; PBBK, physiologically based biokinetic models.

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considering that a single system cannot replace the complexity of the organism. The obtained data should then be integrated, by using appropriate modelling such as Physiologically Based Pharmacokinetic (PBPK) models (Coecke et al., 2013).

The use of *in vitro* toxicity data for the risk assessment of a chemical highly depends on the relevance of the *in vitro*-derived data and the possibility for the *in vitro–in vivo* extrapolation (IVIVE) (Kramer et al., 2012). A prerequisite for the extrapolations is the identification of the actual toxic concentration in the *in vitro* system and its relevance in the *in vivo* context. This requires knowledge of the biokinetic behaviour of the chemical (Blaauboer, 2002), since nominal concentrations can change over time, as a consequence of binding to the medium proteins, adsorption to the plastic devices, chemical instability or metabolism by the cells (Kramer et al., 2012).

The most commonly *in vitro* systems used to study the influence of drugs in development on hepatocellular functions and viability and to analyse the *in vitro* metabolism of xenobiotics are primary hepatocyte cultures from rat and human (Ullrich et al., 2009). A considerable species difference exists (Tuschl et al., 2009), particularly in the kinetic of compounds and in CYPs involved in metabolic reactions. Therefore human liver cell systems, i.e. primary human hepatocytes (PHH) and the HepaRG cell line, offer a definite advantage.

Freshly isolated PHH are considered the gold standard model for xenobiotic metabolism, drug–drug interactions and drug toxicity (Hewitt et al., 2007). These contain metabolically active enzymes at their physiological levels immediately after isolation, then most liver-specific gene expressions associated with metabolic capacities and consequently their activities decrease during the initial stages of cultivation (Guillouzo, 1998; Richert et al., 2003). However, the metabolic capabilities of PHH can thereafter be maintained at acceptable levels over time when cultured in a sandwich (SW) configuration and with appropriated medium (Moeller et al., 2012). Freshly isolated PHH have on the other hand a restricted accessibility, which can be overcome by using cryopreserved PHH allowing to study interindividual differences, particularly useful during preclinical studies of drugs (Hrach et al., 2011) on a sufficiently high number of samples.

Another possibility to overcome restricted accessibility is the use of human hepatic immortalized cells. Unfortunately, for most cell lines, the metabolic enzyme expression levels are very low and they are therefore inappropriate for metabolite identification studies (Hewitt et al., 2007). Recently, the HepaRG cells derived from a human cholangio-hepatocarcinoma were shown to differentiate into hepatocyte-like cells expressing drug metabolizing enzymes to an extent comparable to primary hepatocytes (Guillouzo et al., 2007). HepaRG cells particularly express high levels of the major cytochrome P450 (CYP) such as CYP1A2, CYP2B6, CYP2C9, and CYP3A4 (Aninat et al., 2006; Lübberstedt et al., 2011) and phase II enzymes and transporters (Anthérieu et al., 2010). Thus, HepaRG cells are used to study the *in vitro* metabolism of xenobiotics and to determine if the parent compound and its metabolites have hepatotoxic effects.

Differences in cytotoxicity profiles between PHH and HepaRG cells have been reported and explained by differences in expression of certain liver-specific functions (Gerets et al., 2012) as well as differences in culture conditions such as sandwich culture or medium composition (i.e. presence or absence of serum).

Both PHH and HepaRG cells cultured in appropriate conditions maintain the morphological and functional properties over time, and were included in the established cell culture models within the European Seventh Framework Programme PREDICT-IV (FP7/2007–2013) aimed to develop an *in vitro* strategy to identify adverse drug reactions after repeated exposure in the early phases of the drug development process. The rationale was to combine biological effects (toxicodynamics) with toxicokinetics and PB-Biokinetic (PBBK) modelling ensuring the generation of data in which the real exposure to cells was linked to early pharmacological and toxicological effects. This would allow a better quantitative IVIVE. Since hepatotoxicity is among the major cause for failure or withdrawal of drugs from the market, liver models were used with hepatotoxic pharmaceuticals in repeated treatment to mimic long term human exposure.

Here we describe results obtained with amiodarone (2-n-butyl-3-[3,5-diiodo-4-diethylaminoethoxybenzoyl]-benzofuran; AMI), a class III antiarrhythmic drug with additional classes I and II properties used in the treatment of a wide spectrum of cardiac arrhythmias (Zahno et al., 2011). AMI's therapeutic use is limited because of its numerous side effects that include thyroidal, pulmonary, ocular and liver toxicity (Waldhauser et al., 2006). At therapeutic dosages, AMI reaches plasma concentrations in the range of 2 μM (Lafuente-Lafuente et al., 2009). In the liver, AMI concentrations are 10–20 times higher than in plasma (Weiss, 1999). AMI is metabolized to mono-N-desethyl-amiodarone (MDEA) and di-N-desethyl-amiodarone (DDEA) by cytochrome P450 mainly CYP3A4, but also 2C8, 1A2 and 2D6 (Elsherbiny et al., 2008). Recent *in vitro* investigations suggested that both parent compound and metabolites cause AMI's hepatotoxicity (Zahno et al., 2011). Both AMI and MDEA have also been shown to inhibit CYP activities (Ohyama et al., 2000; Ricaurte et al., 2006), highlighting the importance of knowing the intracellular concentrations of these compounds in repeat treatment regimen.

Thus, the aim of the present study was to compare the *in vitro* biokinetics of AMI and MDEA in fresh PHH and HepaRG cells, after acute and repeated dose experiment.

2. Materials and methods

2.1. Chemicals and reagents

AMI was purchased from Sigma–Aldrich (Gallarate, Milan, Italy or St. Quentin-Fallavier, France), MDEA (purity: 98.9%) was synthesized and kindly provided by Sanofi-Aventis (Germany). For the sandwich culture of freshly isolated primary human hepatocytes, BD™ collagen I, rat tail (BD Biosciences, France) and Geltrex™ from Gibco® (Thermo Fisher Scientific, Illkirch, France) were used. The culture medium, HMM (Hepatocyte Maintenance Medium) for freshly isolated PHH was purchased from Lonza (Verviers, Belgium). FCS was from HyClone® Gibco or Perbio (Thermo Fisher Scientific, Illkirch, France) and hydrocortisone hemisuccinate from Upjohn Pharmacia (Guyancourt, France). Further reagents were from Sigma–Aldrich (St. Quentin-Fallavier, France) included Dexamethasone, and DMSO or Gibco (Thermo Fisher Scientific, Illkirch, France) for ITS-G Liquid Media Supplement, Williams' E medium, Penicillin/Streptomycin, Insulin, Glutamine.

Cell culture plastics were purchased by Becton Dickinson (Le Pont-De-Claix, France). For AMI and MDEA quantification HPLC grade chemicals were obtained from commercially available sources. The Milli-Q water purification system (Millipore, Italy) was used to obtain deionised water.

2.2. Abiotic processes

2.2.1. AMI stability, solubility and cross-contamination among wells

Preliminary assays were carried out in the actual experimental conditions, in order to check the AMI stability in both aqueous solutions and in MeOH or DMSO, the vehicle used to prepare stock solutions. Thus solutions of AMI dissolved in distilled water, culture media or vehicle were kept at 37 °C and controlled pH, for dif-

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