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Amiodarone biokinetics, the formation of its major oxidative metabolite and neurotoxicity after acute and repeated exposure of brain cell cultures

Giuliana Pomponio [a,d,1], Marie-Gabrielle Zurich [b,1], Luise Schultz [c,1], Dieter G. Weiss [c], Luca Romanelli [d], Alexandra Gramowski-Voss [c], Emma Di Consiglio [a,*], Emanuela Testai [a]

[a] Mechanism of Toxicity Unit, Environment and Primary Prevention Department, Istituto Superiore di Sanità, Rome, Italy
[b] Department of Physiology, University of Lausanne, Lausanne, Switzerland
[c] Department of Animal Physiology, Institute of Biological Sciences, University of Rostock, Rostock, Germany
[d] Department of Physiology and Pharmacology "V. Erspamer", Università Sapienza, Rome, Italy

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ABSTRACT

The difficulty in mimicking nervous system complexity and cell–cell interactions as well as the lack of kinetics information has limited the use of *in vitro* neurotoxicity data.

Here, we assessed the biokinetic profile as well as the neurotoxicity of Amiodarone after acute and repeated exposure in two advanced rodent brain cell culture models, consisting of both neurons and glial cells organized in 2 or 3 dimensions to mimic the brain histiotypic structure and function. A strategy was applied to evidence the abiotic processes possibly affecting Amiodarone *in vitro* bioavailability, showing its ability to adsorb to the plastic devices. At clinically relevant Amiodarone concentrations, known to induce neurotoxicity in some patients during therapeutic treatment, a complete uptake was observed in both models in 24 h, after single exposure. After repeated treatments, bioaccumulation was observed, especially in the 3D cell model, together with a greater alteration of neurotoxicity markers. After 14 days, Amiodarone major oxidative metabolite (mono-N-desethylamiodarone) was detected at limited levels, indicating the presence of active drug metabolism enzymes (i.e. cytochrome P450) in both models.

The assessment of biokinetics provides useful information on the relevance of *in vitro* toxicity data and should be considered in the design of an Integrated Testing Strategy aimed to identify specific neurotoxic alerts, and to improve the neurotoxicity assay predictivity for human acute and repeated exposure.

1. Introduction

The nervous system is one of the most complex organ systems in terms of both structure and function; in addition, the lack of regeneration after severe damage renders the nervous system particularly vulnerable to toxic insult (Gramowski et al., 2004). Neurotoxicity is indeed one of the toxicity endpoints generally assessed in the safety evaluation of many chemicals as requested by EU Regulations, such as EC Regulations 1907/2006 (REACH); 1107/2009 (pesticides) and 528/2012 (biocides). Last but not least, the increasing onset of neuronal disorders and neurodegenerative diseases, linked to the aging of the population, represents a clear demand for drugs active on the nervous system for which safety has to be assessed in the early phase of development.

The currently accepted neurotoxicity studies include *in vivo* tests, usually on rodents (Bal-Price et al., 2010a); in this kind of studies direct adverse effects on the nervous system are often difficult to distinguish from indirect effects, linked to hormonal and immunological stimuli. This makes the interpretation of the observed functional changes quite difficult and leads to the conclusion that *in vivo* toxicity tests are not always ideal for the detection of neurotoxic effects (Harry and Tiffany-Castiglioni, 2005).

Abbreviations: AMI, Amiodarone; MDEA, mono-N-desethylamiodarone; DDEA, di-N-desethylamiodarone; 2D mouse model, murine neuronal network culture in two dimensional structure; 3D rat model, re-aggregating rat brain cell cultures in three dimensional structure; DIV, days *in vitro*; LDH, lactate dehydrogenase; CYP, cytochrome P450; DMSO, Dimethylsulfoxide; DMEM, Dulbecco's Modified Eagle's medium; PBS, phosphate buffered saline; PNGM, Primary Neuron Growth Medium; PNBM, Primary Neuron Basal Medium; d0, day 0; d13, day 13; TP, time point; LOD, limit of detection; LOQ, limit of quantification; BBB, blood–brain barrier.

* Corresponding author at: Istituto Superiore di Sanità, Viale Regina Elena 299, 00161 Rome, Italy. Tel.: +39 (0)6 49902959.
E-mail address: emma.diconsiglio@iss.it (E. Di Consiglio).

[1] These authors equally contributed to the work.

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There is nowadays a large consensus that the animal testing needs to be replaced by a combination of *in silico* and *in vitro* approaches, as evidenced by ethical and economic arguments, besides scientific ones (Bal-Price et al., 2010a). However, so far *in vitro* studies have been proposed to be used as complementary to animal testing (Harry et al., 1998; Bal-Price et al., 2010b), essentially to support the development of biomarkers as early indicators of adverse effects (e.g. initial biochemical alterations), or the identification of mechanism of action. Indeed, none of the *in vitro* available tests could be considered as a standing alone method, relying on for the evaluation of the neurotoxic hazard of a chemical. This is mainly related to the complex anatomical structure of the nervous system, its physiology, as well as cell–cell interaction among different cell types (neuronal and glial) (Weiss, 2011). However, promising models to be used as alternatives to *in vivo* neurotoxicity testing have been proposed (Bal-Price et al., 2010b), addressing the issues of complexity and cell–cell interactions, such as (i) the primary cortical (glia-containing) murine neuronal network culture, established on the surface of micro-electrode arrays (neurochips) in a two dimensional structure (2D mouse model) (Weiss, 2011) and (ii) the tri-dimensional re-aggregating rat brain cell cultures (3D rat model), containing different brain cell types (Honegger and Zurich, 2011). The 2D mouse model allows to directly monitor changes of 200 features of the electrical firing patterns by deriving quantitative parameters belonging to five general categories: burst structure, oscillatory aspects, synchronicity, connectivity and general activity (Gramowski et al., 2010; Johnstone et al., 2010; Weiss, 2011). The 3D rat model is composed of neurons, astrocytes, oligodendrocytes and microglial cells, allowing multiple cell–cell interactions, and the development of histotypic structures such as extracellular matrix, synapses and myelinated axons (Zurich et al., 2004). It has been extensively used for neurotoxicological investigations, and several structural and functional endpoints have proved to be useful specific markers of neurotoxicity, such as the activity of cell type-specific enzymes, the expression of selected genes, as well as astroglial and microglial reactivities (Monnet-Tschudi et al., 1995; Zurich et al., 2004, 2013).

The long-term stability of both models makes them suitable for both acute and repeated exposure to chemicals or drugs, giving in addition the possibility to study different functional endpoints, allowing to distinguish between general cytotoxicity and possible specific chemical-induced neurotoxicity.

The combination of the 2D and 3D brain cell models with a blood brain barrier (BBB) *in vitro* model (Culot et al., 2013) was evaluated as an improved *in vitro* neurotoxicity testing strategy (Schultz et al., 2015), aimed to overcome the lack of the *in vivo* filtering processes associated with the BBB or lipid-aqueous partitioning, contributing to the artificial nature of the culture exposure (Hallier-Vanuxeem et al., 2009). However, another important step forward to increase the predictability of *in vitro* testing is the measurement of *in vitro* kinetics, including all the abiotic and biological processes such as *in situ* metabolic capability, determining the actual exposure of cells, which can greatly influence the toxicological outcome, especially after repeated exposure (Coecke et al., 2013). Despite its relevance, this aspect is most of the times neglected, and the nominal concentration is used to refer to concentration-effect relationship.

Here we describe the *in vitro* biokinetics of Amiodarone (2-n-butyl-3-[3,5-diiodo-4-diethylaminoethoxybenzoyl]-benzofuran; AMI), the formation of its major oxidative metabolite mono-N-desethylamiodarone (MDEA), and some neurotoxicity end-points induced after acute and 14 day-repeated treatments in the 2D and 3D brain models. AMI, a Class III antiarrhythmic medication, is a non-competitive inhibitor of alpha- and beta-adrenergic receptors, commonly used to treat patients with refractory ventricular tachycardia and paroxysmal atrial fibrillation. In some patients under therapeutic treatment, during which the drug reaches plasma concentrations in the range of 1.3–2 μM (Lafuente-Lafuente et al., 2009), AMI is known to induce a variety of side effects including neurotoxicity (Brief et al., 1987), characterised by a set of symptoms such as headache, dizziness, fatigue, tremor, peripheral sensorimotor neuropathy, proximal muscle weakness, ataxia, with few cases of parkinsonism (Ishida et al., 2010).

2. Materials and methods

2.1. Chemicals and reagents

AMI was purchased from Sigma–Aldrich (Switzerland, Germany and Italy, catalogue No A8423-1g, lot #109K1307), MDEA (purity: 98.9%) was synthesized and kindly provided by Sanofi-Aventis (Germany, catalogue No RDS 8262). Dimethylsulfoxide (DMSO) (purity ⩾99.9%; Sigma–Aldrich catalogue No 472301). 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. Stability, solubility and cross-contamination among wells of the test compound

Preliminary assays were carried out to check AMI chemical stability: AMI solutions in distilled water, culture media and DMSO (the vehicle used to prepare stock solutions), were kept at 37 °C and under controlled pH, for different incubation times in glass and plastic tubes and analysed by HPLC.

To quantify cross-contamination among different wells during 2D mouse model incubations, DMSO was added in wells next to the ones with cells treated with AMI. At the end of the experiment, well content was stored at −80 °C and then analysed by HPLC.

2.2.2. Adsorption to the plastic devices

In order to measure the unspecific binding to the plastic devices used in the 2D mouse model, once the content of each well was removed, the empty cell culture plate was washed twice with PBS before adding 3 ml MeOH per well. The plate was sealed with Parafilm and incubated at room temperature under gentle shaking for 2 h before transferring the complete volume into glass vials for storage at −80 °C. This estimation was not carried out for the 3D rat model since glass labware was used.

2.3. Cell culture

2.3.1. 2D mouse model

NMRI mice (Charles River Laboratories, Sulzfeld, Germany) were sacrificed at embryonic day 16 by cervical dislocation in accordance with the regulations of the German Animal Protection Act §4. The frontal cortex tissue was dissected out, dissociated and cultured according to Gramowski et al. (2010) with minor modifications, which include the use of DNase I (8000 units/ml, Roche, Mannheim, Germany, catalogue No 10104159001) and papain (10 U/ml, Roche, Mannheim, Germany, catalogue No 10108014001) for the enzymatic dissociation of the tissue (Huettner and Baughman, 1986), which was then further mechanically dissociated with a transfer pipette. The cells were resuspended in Primary Neuron Basal Medium (PNBM), supplemented with Primary Neuron Growth Medium PNGM™-A SingleQuots™ Kit containing the necessary volumes of L-glutamine, antibiotic, and NSF-1 (a supplement supporting neuronal growth and survival) to complement the basal medium (both from Lonza Sales AG, Verviers, Belgium, catalogue No CC-3256 and CC-4462). The