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Bringing *in vitro* analysis closer to *in vivo*: Studying doxorubicin toxicity and associated mechanisms in 3D human microtissues with PBPK-based dose modelling

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ABSTRACT

Doxorubicin (DOX) is a chemotherapeutic agent of which the medical use is limited due to cardiotoxicity. While acute cardiotoxicity is reversible, chronic cardiotoxicity is persistent or progressive, dose-dependent and irreversible. While DOX mechanisms of action are not fully understood yet, 3 toxicity processes are known to occur *in vivo*: cardiomyocyte dysfunction, mitochondrial dysfunction and cell death. We present an *in vitro* experimental design aimed at detecting DOX-induced cardiotoxicity by obtaining a global view of the induced molecular mechanisms through RNA-sequencing. To better reflect the *in vivo* situation, human 3D cardiac microtissues were exposed to physiologically-based pharmacokinetic (PBPK) relevant doses of DOX for 2 weeks. We analysed a therapeutic and a toxic dosing profile. Transcriptomics analysis revealed significant gene expression changes in pathways related to "striated muscle contraction" and "respiratory electron transport", thus suggesting mitochondrial dysfunction as an underlying mechanism for cardiotoxicity. Furthermore, expression changes in mitochondrial processes differed significantly between the doses. Therapeutic dose reflects processes resembling the phenotype of delayed chronic cardiotoxicity, while toxic doses resembled acute cardiotoxicity. Overall, these results demonstrate the capability of our innovative *in vitro* approach to detect the three known mechanisms of DOX leading to toxicity, thus suggesting its potential relevance for reflecting the patient situation. Our study also demonstrated the importance of applying physiologically relevant doses during toxicological research, since mechanisms of acute and chronic toxicity differ.

1. Introduction

Doxorubicin (DOX, also known by the brand name Adriamycin) is a chemotherapeutic agent belonging to the class of anthracyclines, which are cytostatic antibiotics. DOX was isolated from *Streptomyces peucetius var. caesius* in 1967 (Arcamone et al., 1967) and it is still one of the most frequently used anti-cancer agents for treating a variety of cancers (*e.g.* hematological cancers, soft-tissue tumors, and solid tumors), even though it causes severe side effects. Next to nausea, vomiting, alopecia, myelosuppression, stomatitis, and gastrointestinal disturbances, DOX is known to induce cardiotoxicity. Cardiotoxicity is classified as acute or chronic. Acute cardiotoxicity may already occur during treatment with a single high dose or within 2–3 days after multiple DOX treatments (Zhang et al., 2009; Chatterjee et al., 2010). Though its prevalence is higher than for chronic cardiotoxicity, these side effects are reversible and clinically manageable. A chronic cardiotoxic phenotype may emerge between one month and decades after DOX treatment. This persistent or progressive cardiotoxicity is dose-dependent and irreversible. In cases where congestive heart failure has developed, the prognosis for the patient is poor, with a mortality rate of 50% within one year (Chatterjee et al., 2010; Chen et al., 2007; Carvalho et al., 2014; Kumar et al., 2012; Takemura and Fujiwara, 2007). Given this dose-

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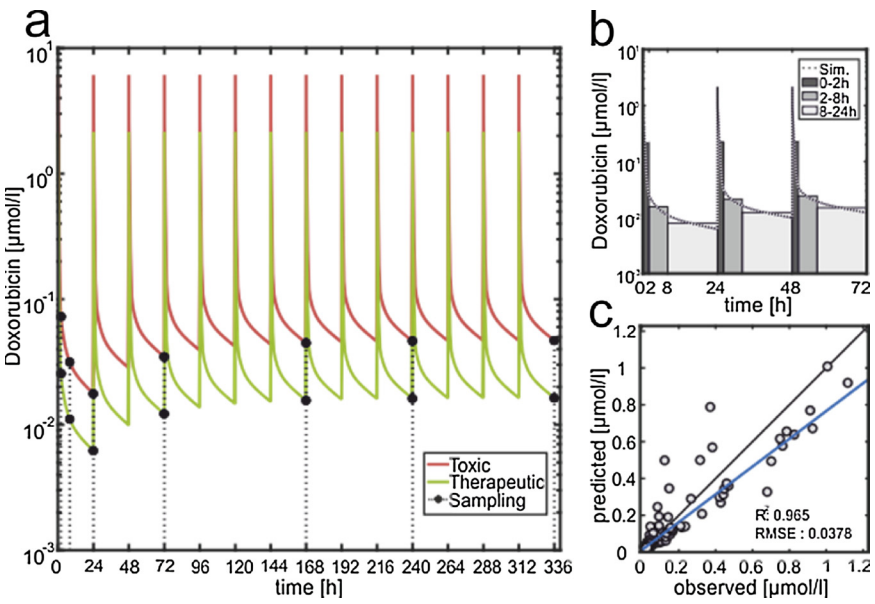

Fig. 1. Doxorubicin PBPK model simulations. a) Simulations of DOX interstitial heart concentration profiles following a therapeutic (green) and toxic (red) once daily dosing regimen with experimental sampling points (black circles). b) Exemplary translation of PBPK simulations into discrete *in vitro* assay concentration with equal exposure, applied to cardiac microtissues for the first three days. c) Validation of PBPK model predicted DOX blood plasma concentrations with literature data (Eksborg et al., 1985) (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

dependency, a maximum cumulative dose of 450–600 mg/m^2 is recommended for DOX (Varga et al., 2015; Edwardson et al., 2015).

DOX mechanisms of action are not fully understood yet. Nevertheless, it is generally accepted that the main mode of action is related to killing dividing cells. These effects are more severe for tumor cells since these cells divide more rapidly than non-cancer cells. However, DOX is not tumor cell-specific and can accumulate in the nucleus and mitochondria of heart, liver and blood cells, thereby contributing to toxic side effects, in particular chronic cardiotoxicity, even at therapeutic doses (Carvalho et al., 2014). DOX has been found to 1) intercalate into DNA, 2) target DNA topoisomerases, and 3) generate reactive oxygen species (ROS) (Damiani et al., 2016; Sorensen et al., 2016; Burridge et al., 2016). The first two processes inhibit unwinding of DNA, DNA replication, RNA transcription and protein biosynthesis. As a result, proliferation of dividing cells is also inhibited (Edwardson et al., 2015; Yang et al., 2014). This is thought to be the efficacy of the anti-cancer effects of DOX, while ROS generation is mainly ascribed to toxic effects (Berthiaume and Wallace, 2007). ROS can be generated as a result of mitochondrial dysfunction, but also by the oxidative semiquinone formed at complex I of the electron transport chain (ETC) during DOX metabolism. The induced oxidative stress may damage cells and cause cell death (Varga et al., 2015; Sorensen et al., 2016). Other detrimental actions of DOX can be related to death of non-cancer cells or to decreased cardiomyocyte functioning, which may partly result from mitochondrial dysfunction causing an imbalance in cellular energetics. Therefore, any effect of DOX on mitochondria structure or function can cause cardiomyocyte dysfunction and thereby cardiotoxicity (Tokarska-Schlattner et al., 2006). The detrimental actions of DOX, known to occur *in vivo*, can thus be summarized into 3 toxicity processes: cardiomyocyte dysfunction, mitochondrial dysfunction and cell death. However, it should be noted that the split between efficacy and detrimental actions is not fully justified because of overlapping processes, such as oxidative stress and cell death.

To predict molecular mechanisms underlying long term toxicity, toxicological risk assessment traditionally relies on animal models (Verheijen et al., 2015). However, notably due to increasing ethical pressure, the field has to reduce the amount of animal experiments. In this article, we present an innovative *in vitro* experimental design aimed to detect cardiotoxicity by obtaining a complete view of the induced mechanisms of a compound. By better reflecting the human *in vivo* conditions, we aim to find an *in vitro* model capable of reliably replacing animal models used within the field of toxicology and drug safety testing. First, instead of the regularly used monolayer cell cultures, a Human 3D cardiac microtissue (InSphero, SWL) model was used, which better resembles the *in vivo* heart (Elliott and Yuan, 2011; Achilli et al., 2012; Zuppinger, 2016). This *in vitro* spheroid cell model contained approximately 4000 iPSC-derived human cardiomyocytes and 1000 cardiac fibroblasts. Second, in contrast to using a relatively high dose to treat the cells, physiologically based pharmacokinetic (PBPK) modelling (Kuepfer et al., 2017) was included within the study design to better reflect the *in vivo* exposure. PBPK simulates the absorption, distribution, metabolism and excretion of a specific dose of DOX within the human body, therefore enabling us to predict the concentration to which a specific organ is exposed over time. The PBPK model was used to design a two weeks repetitive dosing profile in which microtissues were exposed to a decreasing dose over each day by means of 3 medium changes. The microtissues were exposed to either a therapeutic or a toxic dose of DOX using this PBPK-based repetitive dosing profile, and functional parameters for mitochondrial function and programmed cell death were assessed. Finally, by applying next-generation total RNA-sequencing to ribo-depleted RNA samples, we were able to analyze global changes in gene expression. By using this innovative experimental *in vitro* experimental design, we were able to successfully detect biological changes in the three main identified toxicity processes of DOX (cardiomyocyte dysfunction, mitochondrial dysfunction and cell death).

2. Methods

2.1. Samples

The human 3D cardiac microtissue (InSphero, SWL) model was used, containing approximately 4000 iPSC-derived human cardiomyocytes (female Caucasian donor) and 1000 cardiac fibroblasts (male Caucasian donor) per microtissue. The microtissues were cultured in 3D Insight™ Human Cardiac Microtissue Maintenance Medium (InSphero, Cat #CS-07-010-01).

2.2. PBPK model establishment

A PBPK model for DOX was established with the PBPK modelling software PK-Sim following a previously described workflow (Kuepfer et al., 2016). In particular, the model was used to quantify interstitial heart concentrations following administration of therapeutic and toxic drug doses. These concentration profiles were subsequently translated into discrete daily exposure profiles (Fig. 1a, b). During model