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Human placental cell and tissue uptake of doxorubicin and its liposomal formulations

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HIGHLIGHTS

- Human placental cell and tissue uptake of doxorubicin was significant.
- Doxorubicin crossed placenta at low levels within 4-h human placental perfusion.
- Pegylated liposomal doxorubicin was weakly taken up by human placental cells.
- Pegylated liposomal doxorubicin did not cross human placenta in 4-h perfusion.

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ABSTRACT

The anticancer drug doxorubicin and its liposomal formulations are in clinical use, doxorubicin also during pregnancy. However, little is known about how doxorubicin and its liposomal formulations are taken up by placental cells and whether they can cross human placenta. We therefore investigated quantitative cellular uptake and toxicity of doxorubicin and its two liposomal formulations, pH-sensitive liposomal doxorubicin (L-DOX) and commercially available pegylated liposomal doxorubicin (PL-DOX), in human placental choriocarcinoma (BeWo) cells. PL-DOX showed significantly lower cellular uptake and toxicity compared with doxorubicin and L-DOX. In preliminary studies with human placental perfusion, PL-DOX did not cross the placenta at all in 4 h, whereas doxorubicin and L-DOX crossed the placenta at low levels (max 12% of the dose). Furthermore, PL-DOX did not accumulate in placental tissue while doxorubicin did (up to 70% of the dose). Surface pegylation probably explains the low placental cell and tissue uptake of PL-DOX. Formulation of doxorubicin thus seems to enable a decrease of fetal exposure.

1. Introduction

Doxorubicin is an amphiphilic small molecular weight drug (544 g/mol) and belongs to a group of anthracyclines (Minotti et al., 2004). It is widely used for the treatment of cancers such as breast cancer, childhood solid tumors, soft tissue sarcomas, and aggressive lymphomas (Minotti et al., 2004). Some of these cancer types are also diagnosed during pregnancy, although relatively rarely (Van Calsteren et al., 2010; Van Calsteren and Amant, 2014), and often treated with doxorubicin (Amant et al., 2012). However, only limited and discrepant *in vivo* data on human placental transfer of doxorubicin is available. In four case reports doxorubicin was administered to pregnant patients before elective (Roboz et al., 1979) or therapeutic abortion (d'Incalci et al., 1983), or normal delivery (Barni et al., 1992; Karp et al., 1983). High amounts of doxorubicin were found in fetal tissues such as liver, kidney and lung (*e.g.,* in liver 10 times higher concentration than in maternal plasma) (d'Incalci et al., 1983), while doxorubicin was not found in amniotic fluid (Barni et al., 1992; Roboz et al., 1979) or in cord plasma (Karp et al., 1983).

Despite of high effectiveness, doxorubicin causes severe side-effects including chronic cardiomyopathy and congestive heart failure (Minotti et al., 2004). By its encapsulation inside liposomes the tissue distribution of doxorubicin is, however, changed and the

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side-effects can be drastically diminished (Barenholz, 2012). The clinically approved liposomal formulations of doxorubicin are non-pegylated liposomal doxorubicin and pegylated liposomal doxorubicin. Because of pegylation, the circulation time of liposomal doxorubicin is extended (half-life with pegylation 30–90 h) (Gabizon et al., 2003). Thereafter, the passive accumulation into a solid tumor is possible *via* enhanced permeability and retention effect (Barenholz, 2012; Gabizon et al., 2003). Pegylated liposomal doxorubicin is used for the treatment of recurrent ovarian cancer, metastatic breast cancer (except in the USA), AIDS-related Kaposi's sarcoma and multiple myeloma (Barenholz, 2012; Coukell and Spencer, 1997; Gordon et al., 2001). However, according to the U.S. Food and Drug Administration the use of pegylated liposomal doxorubicin is not recommended during pregnancy (www.fda.gov). Pegylated liposomal doxorubicin has not been studied systematically in pregnant patients, and to our knowledge no studies on human placental transfer exist.

Human placental transfer of nanoparticles have only been described in a few studies in the literature (Bajoria et al., 2013; Grafmueller et al., 2015; Menjoge et al., 2011; Myllynen et al., 2008; Sonnegaard Poulsen et al., 2013; Wick et al., 2010). Myllynen et al. (2008) showed in their study that pegylated gold nanoparticles sized 10–30 nm were not able to cross human placenta during 6-h perfusion. On the other hand, other perfusion studies showed that fluorescently labeled polystyrene beads, silica nanoparticles and Alexa conjugated dendrimers sized 5–300 nm crossed placenta at variable extents (Grafmueller et al., 2015; Menjoge et al., 2011; Sonnegaard Poulsen et al., 2013; Wick et al., 2010). In addition, after 5.5-h perfusion warfarin encapsulated inside cationic liposomes crossed placenta (Bajoria et al., 2013). However, it was suggested that the liposomes may release the encapsulated warfarin intracellularly in tissue before the penetration of drug through placenta (Bajoria et al., 2013). Cellular transfer of nanoparticles is dependent on the physical and chemical properties of the formulation (*e.g.,* surface chemistry, size and charge), and more systematic studies are needed on cellular and transplacental transfer of nanoparticles.

In this study, quantitative cellular uptake and toxicity of doxorubicin, pegylated liposomal doxorubicin (PL-DOX) and less pegylated pH-sensitive liposomal doxorubicin (L-DOX) were evaluated in human placental choriocarcinoma (BeWo) cells. In addition, preliminary studies on human placental uptake and transfer of doxorubicin and the liposomal formulations were carried out in recirculating 4-h human placental perfusion. Currently, to the best of our knowledge, there is only one human placental perfusion study with doxorubicin (Grohard et al., 1989) but no publications with liposomal doxorubicin.

2. Methods

2.1. Drugs and liposomal formulations

Doxorubicin hydrochloride was purchased from Sigma–Aldrich® (St. Louis, MO, USA) and commercially available pegylated liposomal doxorubicin (DOXIL® in USA and CAELYX® in Europe), abbreviated as PL-DOX in this work, was purchased from Janssen-Cilag International NV (Beerse, Belgium). pH-sensitive liposomal doxorubicin (L-DOX) was prepared and characterized as described earlier (Soininen et al., 2012).

2.2. Cell culture

Human choriocarcinoma cell line (BeWo; American Type Culture Collection, Manassas, VA, USA) was cultured in BioWhittaker® RPMI 1640 without phenol-red (Lonza, Verviers, Belgium) supplemented with 100 U/ml penicillin-streptomycin (Lonza), 0.3 g/l L-glutamine (Sigma–Aldrich®), 1 mM sodium pyruvate (Lonza), 0.1 mM non-essential amino acids (Lonza), and 9% heat-inactivated foetal bovine serum (Lonza) at 37 °C in an incubator with a humidified atmosphere containing 95% air and 5% CO_2.

2.3. Cell viability assay

Cell viability was determined by the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay (Mosmann, 1983) as described before (Soininen et al., 2012) with minor modifications. Briefly, cells were plated on 96-well plates (10,000 cells/well) and a day after (cell culture 80% confluent) were exposed to doxorubicin (0–8 μM), L-DOX (0–8 μM) or PL-DOX (0–200 μM) in non-supplemented serum-free growth medium for 4 h. After exposure, the cells were washed and further incubated for 20 h. Three independent experiments with three replicates in each experiment were performed. The half maximal inhibitory concentrations (IC_{50}) were calculated by an equation of $Y = \text{Bottom} + (\text{Top - Bottom})/(1 + 10\text{^}(X - \text{Log } IC_{50}))$ where Top is the maximal response, Bottom the maximally inhibited response, Y the response with the used concentration and X the Log concentration of drug with GraphPad Prism version 5.03 (GraphPad Software, San Diego, SA, USA).

2.4. Cellular uptake studies

Cells were plated on 24-well plates (60,000 cells/well) and next day (cell culture 80% confluent) exposed to 0.5 or 5 μM doxorubicin, L-DOX or PL-DOX in non-supplemented serum-free growth medium for 1, 2 or 4 h at 37 °C. Then, the cells were washed twice with Dulbecco's phosphate buffered saline (Gibco®, Life Technologies™, Grand Island, NY, USA) and detached with 0.05% Trypsin-EDTA (Gibco®). Number of cells was counted from each sample by Countess® Automated Cell Counter according to the manufacturer's instructions (Invitrogen™, Life Technologies™, Eugene, OR, USA). After this, the samples were centrifuged (300 g, 2 min, 4 °C) and the cell pellets were stored at −20 °C until the analysis of doxorubicin. Three independent experiments with three replicates in each experiment were performed.

2.5. Human placental perfusions

A dual re-circulating perfusion of a placental cotyledon with separate maternal and fetal circulations was applied as described in detail earlier (Karttunen et al., 2015). This study protocol has been approved by the official Research Ethics committee of the University Hospital District of Kuopio (2005 and 2007). Placentas from healthy non-smoking mothers with a written informed consent were donated anonymously.

The mothers gave birth at the Kuopio University Hospital, Finland. Within 10 min after a normal delivery or a caesarean section, the term placentas were injected with a self-made Krebs Ringer phosphate buffer containing 25 IU/ml heparin (Leo Pharma, Malmo, Sweden) and 2 g/l human serum albumin (Finnish Red Cross, Finland). After 30 min pre-perfusion, either 5 μM doxorubicin, L-DOX or PL-DOX together with the reference compound antipyrine (final concentration 130 or 530 μM; Sigma–Aldrich® Chemie GmbH, Steinheim, Germany) were added in the maternal circulation. Samples of perfusion medium were collected from the maternal and the fetal circulations at several time-points (0.5, 1, 1.5, 2, 3 and 4 h) and stored at −20 °C until the analysis of doxorubicin and antipyrine. The maximum loss of volume from the fetal to the maternal circulation was 4 ml/h. Altogether 16 perfusions were started, of which two perfusions with doxorubicin and one with L-DOX and PL-DOX each filled the criteria of a successful perfusion (Karttunen et al., 2015).