UNIVERZITA KARLOVA

Farmaceutická fakulta v Hradci Králové

Katedra farmakologie a toxikologie

HODNOCENÍ VLIVU VYBRANÝCH NOVÝCH ANTIRETROVIRÁLNÍCH LÉČIV NA TRANSPORT KARNITINU V PLACENTĚ

Diploma thesis

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Hradec Králové 2019 Eliška Marková

Prohlašuji, že tato práce je mým původním autorským dílem. Veškerá literatura a další zdroje, z nichž jsem při zpracování čerpala, jsou uvedeny v seznamu použité literatury a v práci řádně citovány. Tato práce nebyla použita k získání jiného či stejného titulu.

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ABSTRAKT

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Antiretrovirální léčba HIV-pozitivních těhotných žen představuje v současnosti standardní přístup pro omezení přenosu HIV z matky na plod. Přes nezbytnost této terapie je nicméně důležité, znát její bezpečnost a rizika. Pro správný vývoj plodu a funkci placenty je mj. nezbytný optimální přísun L-karnitinu, klíčového faktoru pro oxidaci mastných kyselin, z krve matky do placenty a krevního oběhu plodu. Publikované studie naznačují častější výskyt onemocnění kardiovaskulárního systému a kardiomyopatií u dětí narozených matkám, jímž byla v průběhu těhotenství podávána antiretrovirální terapie. Optimální transport karnitinu do buněk placenty je zajištěn díky přítomnosti funkčního transportního proteinu OCTN2 v apikální mikrovilózní membráně trofoblastu. Cílem této práce bylo vyhodnotit, zda antiretrovirální léčiva ze skupin nenuklesidových inhibitorů reverzní transkriptázy (rilpivirin a efavirenz), inhibitorů proteáz (ritonavir, sachinavir, tipranavir, lopinavir a atazanavir) a inhibitorů integrázy (dolutegravir a elvitegravir) nejsou schopna inhibovat funkci OCTN2 transportéru, a tím omezovat aktivní přenos karnitinu do buněk. Pro experimenty byl využit *in vitro* model buněčné linie BeWo odvozené od choriokarcinomu placenty a *ex vivo* model membránových vezikul z izolované mikrovilózní membrány lidských placent odebraných po porodu.

Naše výsledky prokázali výrazný inhibiční efekt proteázových inhibitorů ritonaviru a sachinaviru na uptake L-karnitinu u obou použitých modelů. Inhibice OCTN2 elvitegravirem a rilpivirinem se projevila pouze u buněk a nebyla pak potvrzena na izolovaných mikrovilózních membránách. Tato práce tak naznačuje, že pro terapii využívající proteázové inhibitory (ritonavir a sachinavir) by bylo vhodné vzít v potaz

možný deficit karnitinu, zatímco u ostatních testovaných léčiv se jeví jejich používání v těhotenství z pohledu dostupnosti L-karnitinu v placentě jako bezpečné.

ABSTRACT

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Nowadays, the antiretroviral treatment of HIV-positive pregnant women is the standard approach for restriction of transmission of HIV infection from mother to the fetus. In spite of necessity of this pharmacotherapy, it is important to know its safety and risks. For the correct fetal development and function of placenta it is (besides others) essential to ensure the optimal supply of L-carnitine, the key factor for oxidation of fatty acids from mother´s blood to the placenta and fetal blood circulation.

The deficiency of L-carnitine generally leads to significant metabolic changes in the cells and in it usually demonstrated with cardiomyopathies and myopaties. Published studies indicate higher incidence of cardiovascular diseases and cardiomyopathies in children born to mothers treated with antiretroviral therapy during pregnancy. Optimal transport of carnitine into the placental cells, is ensured due to the presence of functional transport protein OCTN2 in the apical membrane of trophoblast. The aim of this study was to evaluate, if antiretrovirals from groups of non-nucleoside reverse transcriptase inhibitors (rilpivirne and efavirenz), protease inhibitors (ritonavir, saquinavir, tipranavir, lopinavir and atazanavir) and integrase inhibitors (dolutegravir and elvitegravir) are able to inhibit OCTN2 transporter and thereby restrict the active transfer of carnitine into the cells. *In vitro* model of BeWo cell line derived from choriocarcinoma of the placenta and *ex vivo* model of microvillous plasma membrane vesicles isolated from human placenta obtained after the delivery were employed.

Our results demonstrate significant inhibitory effect of protease inhibitors ritonavir and saquinavir on the uptake of L-carnitine in both used models. The inhibitory effect of elvitegravir and rilpivirine on the OCTN2 was demonstrated only in BeWo cells but was not confirmed on isolated microvillous membranes. Our study indicates that

possible carnitine defficit should be considered therapeutic regimens involving protease inhibitors (mainly ritonavir and saquinavir). On the other hand, the tested antiretroviral drugs seem to be safe from the perspective of L-carnitine availability for placenta.

LIST OF CONTENT

1. LIST OF ABBREVIATIONS

ABC – ATP-binding cassette

ANOVA – one-way analysis of variance

ATA – atazanavir

BCA – bicinchoninic acid

BCRP – breast cancer resistant protein

CAR – carnitine

CNTs – concentrative nucleoside transporters

DOL – dolutegravir

EFA – efavirenz

ELV – elviregravir

ENTs – equilibrative nucleoside transporters

FAO – fatty acid oxidation

HEPES – 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

HLA – human leucocytes antigen

LOP – lopinavir

MATE – multidrug and toxin extrusion proteins

MRP – multidrug resistance-associated proteins

MVM – microvillous plasma membrane

NRTIs – nucleosidic reverse transcriptase inhibitors

OATPs – organic anion transporting polypeptides

OATs – organic anion transporters

OCTs – organic cation transporters

OCTNs – organic cation/carnitine transporters

P-gp – P-glycoprotein

PBS – phosphate buffered saline

RIL – rilpivirine

RIT – ritonavir

SAQ – saquinavir

SDS – sodium dodecyl sulfate

SLC – solute carrier

TIP – tipranavir

Tris – 2-amino-2-hydroxymethyl-propane-1,3-diol

VER – verapamil

WHO – World Health Organization

2. INTRODUCTION

The general belief is, that pharmacotherapy during pregnancy should be minimized. But there are some conditions, that require this therapy even in pregnant women – this therapy can target the mother, the fetus or both of them. Example of the last state is the HIV antiretroviral therapy. This therapy serves as a maintenance therapy for the mother and also works as the prophylaxis of the vertical transmission. In the absence of this therapy, the risk of the transmission is nearly fifty percent. Antiretroviral therapy during pregnancy is nowadays a gold standard in developed countries (Roustit et al., 2008).

Placenta as a temporary organ has many functions – among others is the transfer of the nutrition to the fetus and protection of the fetus from the harmful compounds. To do so, there are many transporters in placenta, comprising primary active ABC transporters as well as solute carriers of SLC transporter family (Staud et al., 2012). Because of them, the pharmacokinetics and the interactions of the drugs administered during the pregnancy must be studied very carefully. Administration of drugs can lead to their interaction on transporter(s) and/lead to the affected administration of nutrients to the fetus and in extreme situation, to fetal undernutrition and birth defects.

One of the important compounds delivered to the fetus from maternal blood circulation via transplacental transmission is the carnitine. It plays a major role in the β-oxidation of fatty acids and also in the developing fetus. Carnitine deficiency is connected primarily to cardiac defects and muscle weakness (Frreira and McKenna, 2017; Grube et al., 2005; Oey et al., 2006).

Several cases of cardiomyopathies were observed in newborns of mothers, who were treated with antiretroviral therapy during pregnancy (Blanche et al., 2006). We hypothesized that these states could be linked to the lack of carnitine resulting from interference of antiretroviral drugs with transport of carnitine from maternal circulation to placenta and fetus. In this study, we therefore aimed to address this issue using *in vitro* and *ex vivo* experimental approaches.

3. THEORETICAL PART

3.1. PLACENTA – STRUCTURE AND FUNCTIONS

Although the placenta is a temporary organ, its functions are essential for the growth and development of the fetus. Specifically it performs exchange of the nutrients from the mother to the fetus, waste elimination, as well as endocrine, metabolic and immune functions (Verma and Verma, 2013).

Fetal-derived chorionic villi carrying fetoplacental vessels represent the main functional unit of the placenta. Spiral arteries are carried by the decidua bringing maternal blood to intervillous space. It washes chorionic villi, thereby supplying placenta and further fetus by oxygen and nutrients. The outer surface of the villi is formed with a thin epithelial layer of multinucleated syncytiotrophoblasts and underlaying cytotrophoblasts and represents important barrier in transfer of compounds like xenobiotics and drugs. This barrier separates the maternal and fetal blood, so they are not becoming close enough, albeit still apart. (Vahakangas and Myllynen, 2009).

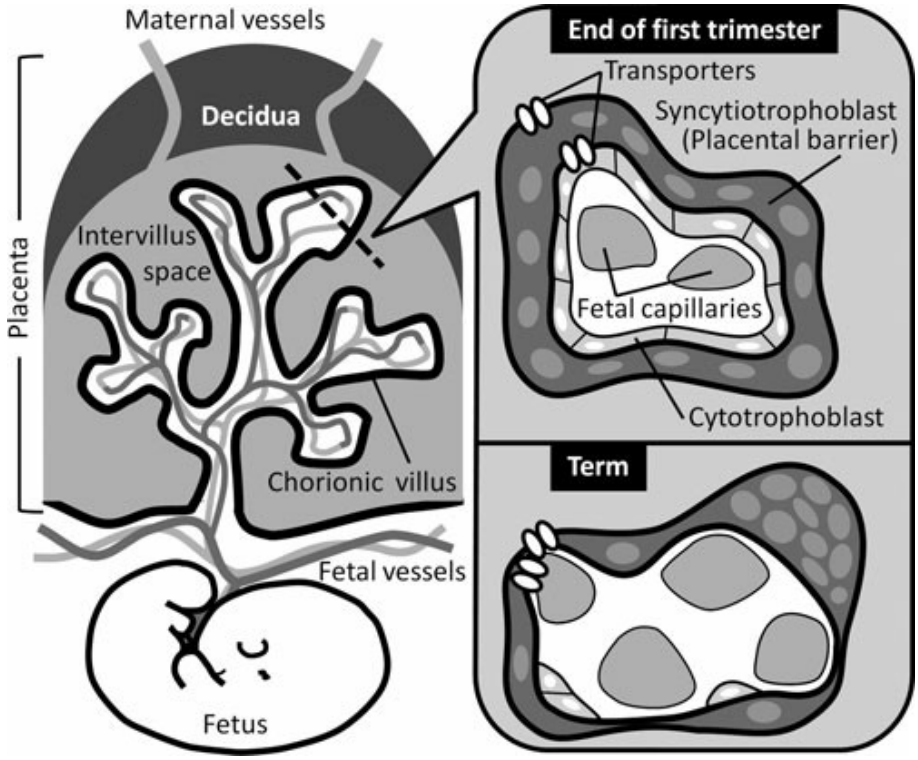

Figure 1. Schematic diagram of the maternal and fetoplacental circulations in the human placenta, together with the cross-section through the chorionic villus at the end of the first trimester and term (adopted from Gude et al., 2004).

Endocrine function of placenta is crucial for the succes of gestation – hormones produced by placenta play important role in both, pregnancy maintainance and fetal growth. Placenta produces the wide variety of hormones, these can be divided into two groups – peptide (e.g. human chorionic gonadotropin, human placental lactogen, placental growth factor) and steroid hormones - including estrogen, progesterone and the glucocorticoids (Verma and Verma, 2013; Costa, 2016).

Another very important function of the placenta is providing nutrients to the fetus and removing its waste metabolites. Transport between the fetus and the mother is based both on concentration difference and unidirectional carrier-mediated transport. Delivery and removal from the exchange area, as well as the speed of exchange, is regulated by the blood flow. Substances transported to the fetus are oxygen (and the carbon dioxide is removed), glucose, amino acids, fatty acids, vitamins, immunoglobulins, drugs and other essential compounds necessary for fetal development (Goplerud and Delivoria-Papadopoulos, 1985; Sibley et al., 1998; Verma and Verma, 2013).

Besides the transport of nutrients, placenta also fulfills metabolic function. It expressed enzymes involved in glycolysis, gluconeogenesis, oxidation, protein synthesis, amino acid interconversion, triglyceride synthesis and metabolism of fatty acids (Dube et al., 2012; Holzman et al., 1979; Verma and Verma, 2013).

Least, but not the last, placenta plays also important immunological role. The embryo exhibits both maternal and parental HLA proteins that could activate the maternal immune system. However, due to placental derived HLA-G antigens an immunosuppressive role is achieved (Szekeres-Bartho et al., 1997; Verma and Verma, 2013). Additionaly the maternal antibodies are transferred across the placenta providing protection to the fetus.

3.2. MEMBRANE TRANSPORTERS IN PLACENTA

In general, two major superfamilies of membrane transporters are distinguished. While the ATP-binding cassette (ABC) transporters are believed to be mainly responsible for efflux of potentially toxic compounds from the syncytiotrophoblast (Roth et al., 2012), the solute carrier (SLC) transporters, mediate uptake of nutrients and many essencial compounds into the placenta.

3.2.1. THE ATP-BINDING CASSETTE SUPERFAMILY

The ABC superfamily consists of 48 members, which are divided into seven subfamilies of membrane proteins with a broad substrate range (Dean and Annilo, 2005). Considering the transport of drugs, there are three most important members of distinct subfamilies: P-glycoprotein (P-gp, *ABCB1*) and breast cancer resistance protein (BCRP, *ABCG2*) (Staud et al., 2012).

P-glycoprotein and BCRP are expressed in the maternal blood-facing membrane of the placental syncytiotrophoblast layer, they provide protection for the fetus against maternal-derived xenobiotics via pumping them back into the maternal circulation (Ceckova-Novotna et al., 2006; Hahnova-Cygalova et al., 2011). P-gp substrates are usually neutral or cationic hydrophobic molecules including anticancer drugs, antiviral drugs, antibiotics, antiepileptics, opioids, antiemetics and many others (Ceckova-Novotna et al., 2006; Staud et al., 2010; Staud et al., 2012). BCRP substrates are mainly endogenous conjugates (e.g. porphyrines) and exogenous substances such as anticancer drugs, nucleoside analogues and oral hypoglycemic drugs (Ceckova et al., 2006; Evseenko et al., 2006; Jonker et al., 2002).

3.2.2. SOLUTE CARRIER FAMILY

SLC is so far the largest family of transporters, consisting of more than three hundred members expressed in many different tissues of the body. These transporters are both substrate-specific (transporting endogenous compounds – sugars, amino acids, nucleotides) as well as substrate-unspecific (recognizing endogenous as well as exogenous compounds). SLC-mediated transport across the placenta is in most cases facilitated energy-independent uptake of hydrophilic or charged molecules by the trophoblast cells (Koepsell and Endou, 2004, Staud et al., 2012).

The main SLC transporters family members include organic cation transporters (OCTs), organic cation/ carnitine transporters (OCTNs), organic anion transporters (OATs), organic anion transporting polypeptides (OATPs), multidrug and toxin extrusion proteins (MATE), concentrative nukleoside transporters (CNTs) and equilibrative nukleoside transporters (ENTs) (Staud et al., 2012).

First transporter family of SLC superfamily are OATs, which operate as anion exchangers for wide variety of substrates (Burckhardt and Burckhardt, 2003; Rizwan and Burckhardt, 2007). In human placenta there is located OAT4 (basolateral membrane of syncytiotrophoblasts in terminal and intermediate villi). This transporter is specific to human and its physiological function is to transport sulfoconjugated estrogens and C19-steroid precursors for placenta metabolism (Cha et al., 2000; Ugele et al., 2003; Ugele et al., 2008).

OATPs are mainly localized in hepatocytes, but they are also expressed in many other tissues including placenta (Staud et al., 2012). One of the mostly expressed OATP members in the placenta is OATP2B1 located to the basal membrane of the syncytiotrophoblast, where it cooperates with OAT4 in the uptake of steroid sulfates (Ugele et al., 2003). OATP4A1 is located in the apical membrane of syncytiotrophoblast and its suggested role is the transport of thyroid hormones and other endogenous compounds (Sato et al., 2003).

Slightly different in function are MATE transporters working as efflux transporters using the H^+ gradient as a driving force. They mediate the efflux across the apical membrane and usually cooperate with members of organic cation transporter family (OCTs) when transferring their substrates across the polarized cells, mainly in kidney and liver (Giacomini et al., 2010; Terada and Inui, 2008; Tsuda et al., 2007). This collaboration has a wide range of substrates (both endogenous molecules and drugs used in clinical practice (Nies et al., 2011; Motoshashi and Inui, 2013). Such cooperation, between OCT3 and MATEs has been reported also in rat placenta (Ahmadimoghaddam 2012), nevertheless its analogy in humans is rather questionable, since both, MATE1 and MATE2 show only negligible expression in human placenta (Ceckova et al., 2016; Staud et al., 2013).

Main substrates of OCT transporters are small monovalent organic cations, which are transferred by mechanism of facilitated diffusion (OCTs are in general believed to be uniports), acting mainly in electrogenic, sodium-independent manner (Koepsell and Endou, 2004; Staud et al., 2012). OCTs play role in many physiological and pathophysiological processes, such as disposition of endogenous substrates and elimination of drugs. They allow the first step in transport across the membrane from

basolateral side to cell, while other transporters mediate the second step out of the cells across the apical membrane (DeGorter and Kim, 2011; Staud et al., 2012). In the placenta, OCT3, which is located on the fetal circulation-facing basal membrane, represents the mostly expressed member of this subfamily. Its physiological role is not completely discovered, it is supposed that OCT3 plays a role in the clearance of catecholamines from fetal circulation and in co-operation with other transporters it mediates the cellular release of acetylcholine from the placenta (Ganapathy and Prasad, 2005; Wessler et al., 2001).

Another group from SLC superfamily are CNTs and ENTs, which role is to transfer nucleosides since they are usually hydrophilic molecules and require specific transport proteins (Molina-Arcas et al., 2009). Human placenta expresses CNT1, ENT1 and ENT2 in the apical (maternal side facing) membrane of syncytiotrophoblast. ENT2 and CNT1 are also functional in the basal membranes and CNT2 and CNT3 are expressed in human term placentas. In general, both ENTs and CNTs are responsible for controling maternal-to-fetal transfer of nucleosides and also some nucleoside-derived drugs (Cerveny et al., 2018; Ma et al., 2017).

OCTNs can transport cation molecules and H^+ in an antiport manner, but are mainly responsible for influx of carnitine to the cells. Since this study focuses on the OCTNs-mediated uptake of carnitine, this family of transporters is discussed in more detail here.

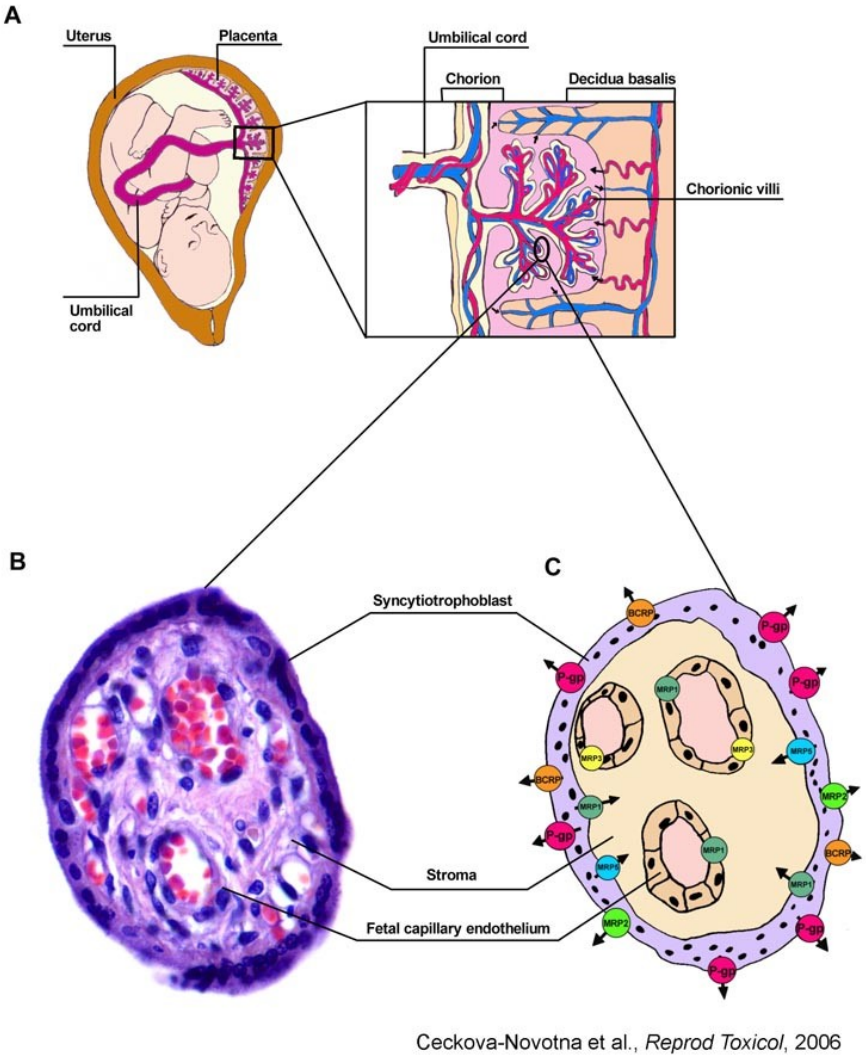

Figure 2. Placental structure depicted schematically. A) Cross section of uterus and a closeup of placental functional unit, chorionic villi. B) Paraffin sections of hematoxylin-eosin stained sections of villus from human third trimester placenta. C) Schematic illustration of main placental transporters located in apical and basolateral membrane of syncytiotrophoblast as well as fetal capillary endothelium (adopted from Ceckova-Novotna et al., 2006).

3.2.2.1 OCTN TRANSPORTERS

Two of three members of this SLC family are known to be present in humans, namely OCTN1 (*SLC22A4*) and OCTN2 (*SLC22A5*). They play a key role in maintainance of appropriate concentrations of carnitine in body and its tissues, when transporting it across the membranes during intestinal absorption, tissue distribution and renal reabsorption (Tamai, 2013). The expression of a carnitine transporting proteins in human placenta is believed to be essencial for placental fatty acid oxidation (FAO)

carnitine supply of the growing fetus (Grube, 2004). While OCTN2 shows high affinity for L-carnitine uptake, only low affinity transport was observed in OCTN1.

OCTN1 *(SLC22A4)*

While it is well confirmed that OCTN2 is located in the apical membrane facing the maternal circulation, OCTN1 localization is believed to be present in the apical microvillous membrane of the syncytiotrophoblast (Ganapathy and Prasad, 2005; Grube et al., 2005; Lahjouji et al., 2004). Transport of carnitine by OCTN1 is Na^+-independant, whereas OCTN2 requires Na^+ in order to shuttle L-carnitine to the trophoblast cells (Tamai et al.,1998).

OCTN2 *(SLC22A5)*

According to Lahjouji (2004), OCTN2 is responsible for most materno-fetal transport of carnitine and it is possible that even all of the placental carnitine transport is mediated by this specifc transporter (Lahjouji et al., 2004). Besides L-carnitine, many xenobiotic drugs are recognized as substrates of OCTN1 as well, e.g. pyrilamine, quinine, quinidine or verapamil. Other drug substrates are recognized by the OCTN2, such as cephaloridine, quinidine, spironolactone, cimetidine, clonidine, procainamide, valproic acid and verapamil (Klaassen and Aleksunes, 2010; Ohashi et al., 1999). In addition, some of these drugs may inhibit the transporter and hence the supply of carnitine to trophoblast and fetus. From confirmed inhibitors the most potent include desipramine and verapamil (Lahjouji et al., 2004).

Besides the pharmacological role, the mutation of the *SLC22A5* gene encoding Octn2 in mice was revealed to play a role in the pathophysiology of primary systematic carnitine deficiency. The defect of function in OCTN2 results in urinary loss of carnitine, rapid decrease of plasma concentration of carnitine and low levels of carnitine in tissues such as heart and muscles (Gallant et al., 2017; Tang et al., 1999; Wu et al., 1999). Experiments on OCTN2 null mice revealed impaired OCTN2-mediated transport and less than 20% of placental and fetal carnitine content compared to the OCTN2 wild-type mice, which was lethal for the developing embryos (Shekhawat et al., 2004. More recent studies than present this mortality as a definite result of non-functional OCTN2 transporter (Shekhawat et al., 2018).

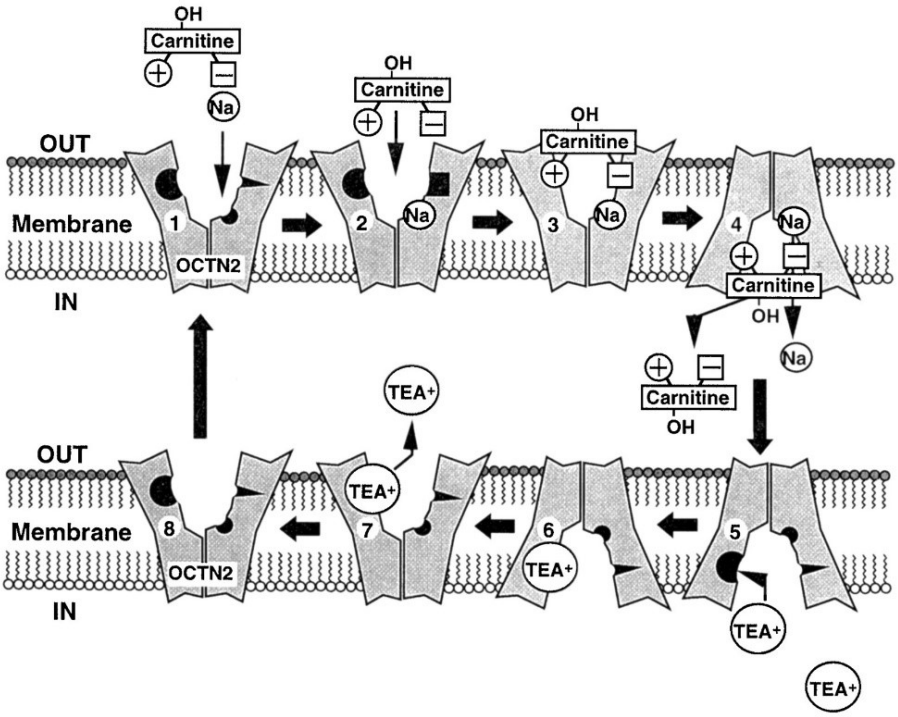

Figure 3. Transport of carnitine mediated by the OCTN2 transporter. OCTN2 – carnitine transporter 2 (adopted from Ohashi et al., 2001).

3.3. CARNITINE AND ITS PHYSIOLOGICAL ROLE

Carnitine (3-hydroxy-4-N-trimethylaminobutyric acid) is an essential compound for the human body. It has the irreplaceable role in beta-oxidation of fatty acids (transportation of activated long-chained fatty acids across the membrane of mitochondria is facilitated by carnitine). In addition, it is also involved in transfer of peroxisomal beta-oxidation products to the mitochondria, storage of energy (in a form of acetylcarnitine) and modulates the toxic effects of acyl-CoA esters (accumulated after the treatment with some drugs – e.g. valproic acid or as a result of some genetic disorders in fatty acid oxidation) (Longo et al., 2016; Oey et al., 2006). During the neonatal period, carnitine is essential for the proper development of the fetus, since fatty acids represent (besides glucose utilization) an important source of energy (Oey et al., 2003).

Approximately three-quarters of carnitine in human body are obtained from food; major sources being meat, fish and dairy products. The rest of carnitine is supplied from *de novo* synthesis. Complete synthesis is possible only by the liver, kidney and brain (Rebouche et al., 1980; Tein et al., 1996). Other tissues are dependent on uptake of carnitine from the circulation (Vaz et al., 2002). Such is also the placental tissue and the

fetus, which are dependent on uptake of carnitine from maternal circulation due to limited synthesizing ability (Arenas et al.,1998; Girarnd et al., 1985; Grube et al., 2005). Although it is suggested that upon compromised transplacental transport, the placental and fetal structures may be able to fully synthesize carnitine themselves due to the confirmed mRNA expression of the enzymes required for carnitine synthesis (Oey et al., 2006). On the other hand, the observations obtained in OCTN2 null mice indicate rather deterious effect of OCTN2 absence for carnitine levels and fetal growth and development (Shekhawat et al., 2004, Shekhawat et al., 2018).

3.3.1. CARNITINE DEFICIENCY

With the low levels of carnitine in the human body are connected two main conditions – primary and secondary carnitine deficiency.

Primary carnitine deficiency is the autosomal recessive disorder that leads to defect in OCTN2 transporter and subsequently defective fatty acid oxidation. It is characterized with development of conditions targeting the tissues highly dependent on FAO such as heart and skeletal muscle; namely cardiomyopathy, cardiac arrhythmias and generalized muscle weakness are the ones mostly reported in infants (Rasmussen et al., 2013; Stanley, 2004; Tein, 2002).

Secondary carnitine deficiency may be caused by many reasons, such as inborn fatty acid oxidation disorders, but also by OCTN2 inhibition by xenobiotics (eg. valproate or cefditoren pivoxil), renal dysfunction (this leads to a massive loss of carnitine in urine) or malnutrition. This deficit leads to accumulation of metabolites from incomplete fatty acid oxidation and there are complications connected to central nervous system observed - such as convulsion, lethargy or coma (Ferreira and McKenna, 2017; Stanley, 2004).

3.4. ANTIRETROVIRAL THERAPY DURING PREGNANCY

According to the World Health Organization every year there are approximately 1.5 million pregnant women with HIV, in the absence of antiretroviral therapy, 15-45% of their babies are infected during pregnancy and delivery (WHO, 2019). So the antiretroviral therapy during the pregnancy is used as prophylaxis against the vertical transmission and of course as the treatment for the medical condition of the mother.

Although in the developed countries is antiretrovirals treatment of HIV positive woman a standard, only 62% of pregnant women were receiving this therapy in 2012 (UNAIDS, 2013).

According to the World Health Organisation´s guidelines most widely used antiretroviral used for prophylaxis is NRTI lamivudine (WHO, 2013). Newer recommendations are more in favor of the administration of a combination of antiretrovirals (WHO, 2016). The combination antiretroviral therapy is the most effective among all the options in prevention the vertical transmission. Most of the nowadays used combination has a basis in two nucleoside or nucleotide reverse transcriptase inhibitors in combination with the third antiretroviral with the different mechanism of action. The British Medical Journal´s practical guidelines from the same year are more specific and recommend the combination based on two NRTIs zidovudine and lamivudine (combined mostly with ritonavir-boosted lopinavir, ritonavir-boosted darunavir, ritonavir-boosted atazanavir, raltegravir, efavirenz, rilpivirine or abacavir). This combination has a slightly lower number of pregnancy difficulties. On the other hand, this combination is served two times a day, it can´t be co-formulated in one tablet with other antiretrovirals and it is not always available despite it being quite old and cheap therapy. The other often used combination is again two NRTIs tenofovir and emtricitabine (can be combined with ritonavir-boosted darunavir, ritonavir-boosted atazanavir, raltegravir, efavirenz or rilpivirine). This option may raise adverse pregnancy outcomes – early prematurity, stillbirth and neonatal death (compared to lamivudine plus zidovudine). Its benefits are dosing once a day and possibility to co-formulate in one tablet with other antiretrovirals (Siemieniuk et al., 2017).

Many antiretrovirals that are used in prevention of mother-to-child transmission (MTCT) of HIV have been shown to interact with placental transporters, either as inhibitors or transported substrates (table 1). Even though the prophylaxis of MTCT is really desired, its safety aspects must be studied very carefully. Interaction of two or more antiretrovirals on drug membrane transporters can lead to affected pharmacokinetics and inhibition of membrane proteins that are involved in transfer of essencial compounds can lead to the shortage of nutrients for the fetus (Staud et al., 2012).

Transporter name	Transporter family	Antiretroviral substrates/ inhibitors
P-gp	ABC	abacavir, tenofovir, efavirenz, nevirapine, ritonavir, delaviridine, atazanavir, lopinavir, indinavir, saquinavir, nelfinavir,
BCRP	ABC	zidovudine, lamivudine, abacavir, efavirenz, delaviridine, lopinavir, ritonavir, nelfinavir, saquinavir, atazanavir
MRP1	ABC	emtricitabine, abacavir, tenofovir, lamivudine, delaviridine, efavirenz, nevirapine, indinavir, ritonavir, lopinavir, atazanavir
MRP2	ABC	emtricitabine, abacavir, tenofovir, lamivudine, delaviridne, efavirenz, nevirapine, saquinavir, ritonavir, indinavir, atazanavir
OCT3	SLC	abacavir, emtricitabine, tenofovir, lamivudine, saquinavir, ritonavir, indinavir, nelfinavir
OAT4	SLC	zidovudine
OATP2B1	SLC	lopinavir, saquinavir, nelfinavir,
CNT1		zidovudine, ribavirin
ENT1		zidovudine, ribavirin
ENT2		zidovudine, ribavirin

Table 1. List of antiretrovirals as substrates of placental transporters (Staud et al., 2012).

According to published cohort studies there is a link between some drugs used for antiretroviral therapy in pregnancy and cardiac and musculoskeletal dysfunctions in newborns of mothers receiving this therapy. These symptoms are most likely due to dysfunction of mitochondria in exposed fetuses (Blanche et al., 2006, Van Dyke et al., 2016). Tha causative mechanism of these findings has not been described yet. In particular, the possible link between antiretroviral drugs and non-functional FAO as a

result of insufficient carnitine supply to the placenta and fetus have not been addressed yet.

4. AIMS OF STUDY

The primary aim of this thesis was to determine the inhibitory potential of the broad range of new antiretroviral drugs on the placental carnitine uptake. Following drugs have been involved in the study:

- non-nucleoside reverse transcriptase inhibitors rilpivirine and efavirenz
- integrase inhibitors dolutegravir and elvitegravir
- protease inhibitors saquinavir, tipranavir, lopinavir, ritonavir and atazanavir

Inhibitory activity of these drugs towards OCTN2 uptake transporter was evaluated using

1) *in vitro* approach (accumulation studies in BeWo cells) and
2) *ex vivo* studies (uptake by the microvillous membrane vesicles islolated from human term placenta).

Addressing the above aims, the general goal of this thesis was to enlarge the knowledge on safety of the antiretrovirals potentially administered to pregnant women in terms of carnitine supply to the placenta and fetus.

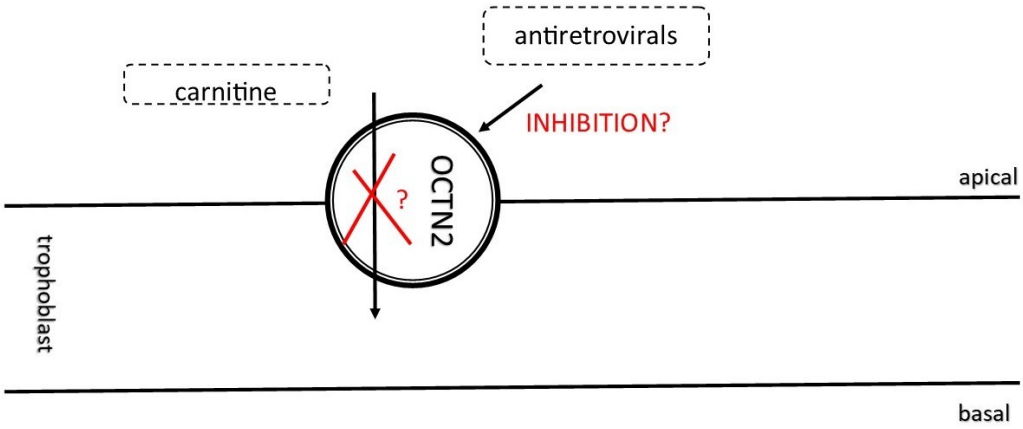

Figure 4. Schematic representation of the main hypothesis of this thesis. OCTN2 – carnitine transporter 2.

5. EXPERIMENTAL PART

5.1. MATERIAL AND CHEMICALS

Antiretrovirals used in this thesis were received from the National Institute of Health as a part of the National Institute of Health AIDS Reagent Program and comprise (atazanavir, efavirenz, lopinavir, rilpivirine, ritonavir, saquinavir and tipranavir) or obtained from MedChemExpress Europe (dolutegravir and elvitegravir). The radiolabeled carnitine (specifically [^{3}H]-L-carnitine 60 Ci/mmol) was purchased from M.G.P. spol. s.r.o. (Zlin, Czech Republic). Both unlabeled carnitine and verapamil were purchased from Sigma-Aldrich (St. Louis, Missouri, USA). Bicinchoninic acid assay kit was obtaineded from Thermo Scientific (Rockford, Illinois, USA). Tri Reagent solution was purchased from Molecular Research Centre (Cincinnati, Ohio, USA). All other used chemicals were of analytical grade.

5.2. ACCUMULATION OF CARNITINE IN BEWO CELLS

5.2.1. CELL CULTURE

The human choriocarcinoma-derived BeWo cell line (b30 clone) was kindly provided by Prof. Ch. Albrecht (University of Bern, Switzerland) with permission from Dr. A. Schwartz (Washington University, St. Louis, USA). Cells were cultured in Dulbecco's Modified Eagle Medium (high glucose) supplemented with 10% FBS, at 37 °C / 5% CO_2.

5.2.2. ACCUMULATION EXPERIMENTS

BeWo b30 cells ($2x10^5$) were seeded on 24-well culture plates (TPP, Trasadingen, Switzerland) and cultured for 2 days until confluent (the confluency was checked under the inverted optical microscope). At the beginning of experiment the medium was removed from all wells and the cells were rinsed twice with phosphate buffer saline (PBS). Then, the pre-incubation solutions alone (control) or containing tested antiretrovirals (at 10 μM concentration), verapamil (1 mM, control inhibitor) or carnitine (at saturating concentration of 1 mM) in OptiMEM were added and incubated for 15 minutes. After this time, the solutions were removed from wells and replaced with incubation solutions. They were of the same composition as the pre-incubation solutions, however, with addition of 0.25 μCi/ml [^{3}H]-L-carnitine. The accumulation took 15 minutes and was stopped by cooling the plate and washing the cells with ice-

cold PBS. The cells were checked under the microscope and 0.02% SDS was added for cell lysis. Accumulated concentrations of the radioisotope were determined by liquid scintillation counting and normalized to protein content (determined by BCA assay).

The same protocol was used for time-dependancy experiments, except from incubation times differing at designated time-points. In addition, Na^+-dependency was determined by comparing the uptake in Opti-MEM to that of Na^+-free buffer (140 mM N-methyl-D-glucamine, 5.4 mM potassium chloride, 1.8 mM calcium chloride, 0.8 mM magnesium sulfate, 5 mM glucose, 25 mM Tris and pH of this solution adjusted to pH 7.4).

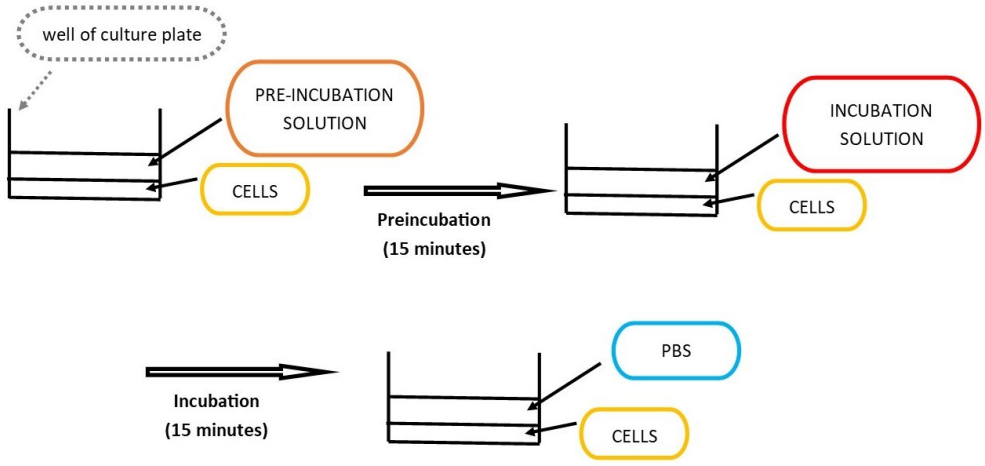

Figure 5. Schematic illustration of performed accumulation experiments on BeWo cells. PBS – phosphate buffered saline.

5.3. UPTAKE ASSAY ON HUMAN PLACENTAL MICROVILLOUS MEMBRANE VESICLES

5.3.1. ISOLATION OF MICROVILLOUS MEMBRANE VESICLES

Placentas were obtained from the hospital in Hradec Králové after informed consent from healthy mothers with uncomplicated pregnancies at term (38-40 weeks of gestation) and with the approval of the University Hospital Research Ethics Committee (201006S15P).

Firstly, the placenta was cut into small pieces (without the chorionic plate, blood clots or calcifications) and the tissue was homogenized in Brown´s buffer (300 mM mannitol, 10 mM HEPES, and 1 mM magnesium sulfate). The microvillous membrane vesicles

are negatively charged, therefore precipitation with magnesium chloride and differential centrifugation was used for isolation (principle described by Glazier et al., 1988). The gained final pellet was resuspended in intravesicular buffer (5 mM Tris, 5 mM HEPES and 290 mM sucrose and adjusted to pH 7.4 with hydrochloric acid). Shear force was applied to suspension (pellet was repeatedly passed through a 25G needle) to allow vesiculation. Isolated vesicles were stored at -80°C and equilibrated to room temperature before use.

5.3.2. ALKALINE PHOSPHATASE ACTIVITY

Samples of homogenate (dilution 1:10) and of vesicle pellets (dilution 1:300) was separately added to diethanolamine buffer (diethanolamine 1 M and magnesium chloride 1 M) in the microplate well and mixed. The reaction was initiated by added comix (1 tablet of p-nitrophenylphosphate dissolved in 1.3 ml of diethanolamine buffer) and the increase in absorbance was measured at time 0, 2, 4, and 6 minutes at or near 410 nm.

Confirmation of successful isolation was done by determination of alkaline phosphatase activity of MVM and its comparison to the whole homogenate (enrichment factor). The alkaline phosphatase enrichment factor in MVM used in this study was 23.39 ± 6.56 (mean ± SD, n = 11).

5.3.3. PROTEIN ANALYSIS

To determine the protein concentration the bicinchoninic (BCA) assay was performed. This assay was done using Microplate procedure from Thermo Scientific Pierce BCA Assay Kit. This kit includes Reagent A (sodium carbonate, sodium bicarbonate, bicinchoninic acid and sodium tartrate in 0.1 M sodium hydroxide), Reagent B (4% cupric sulfate) and albumin standard (bovine serum albumin in concentration 2mg/ml in 0.9% saline and 0.05% sodium azide). Protein concentration was calculated from the measurement of absorbance at 562 nm (after thirty minutes incubation).

5.3.4. [^{3}H] - L-CARNITINE UPTAKE ASSAY

For these experiments, the concentration of protein in MVM vesicles was adjusted to 20-30 mg/ml with intravesicular buffer (IVB; 5 mM Tris, 5 mM HEPES and 290 mM sucrose and adjusted to pH 7.4 with hydrochloric acid). The first step was the

preincubation of the vesicles with/without the selected antiretrovirals (1 or 10 µM concentration) or verapamil (1 mM) in extravesicular buffer (EVB; 5 mM Tris, 5 mM HEPES and 145 mM sodium chloride and adjusted to pH 7.4 with hydrochloric acid) for ten minutes. Incubation was initiated by addition of [^{3}H] - L-carnitine in EVB to the pre-incubated vesicles and after one minute the reaction was stopped by adding two millilitres of ice-cold stop solution (130 mM sodium chloride, 10 mM sodium phosphate dibasic, 4.2 mM potassium chloride, 1.2 mM magnesium sulfate and 0.75 mM calcium chloride). The samples were than filtered using rapid vakuum filtration (principle described by Glazier, 2006) through the Millipore filters (HAWP, pore size 0,45 µm). The filter was washed with stop solution and the radioactivity of filter was measured by liquid scintillation counting.

For time dependency experiments the pre-incubation time was the same as for the inhibitory studies, however, the accumulation (uptake) phase was performed at designated time-points. In addition, Na^+-dependency of [^{3}H]-L-carnitine uptake by the MVM vesicles and effect of control inhibitor verapamil (1 mM) over time was examined. For Na^+-free conditions, the sodium chloride was replaced with potassium chloride in the EVB.

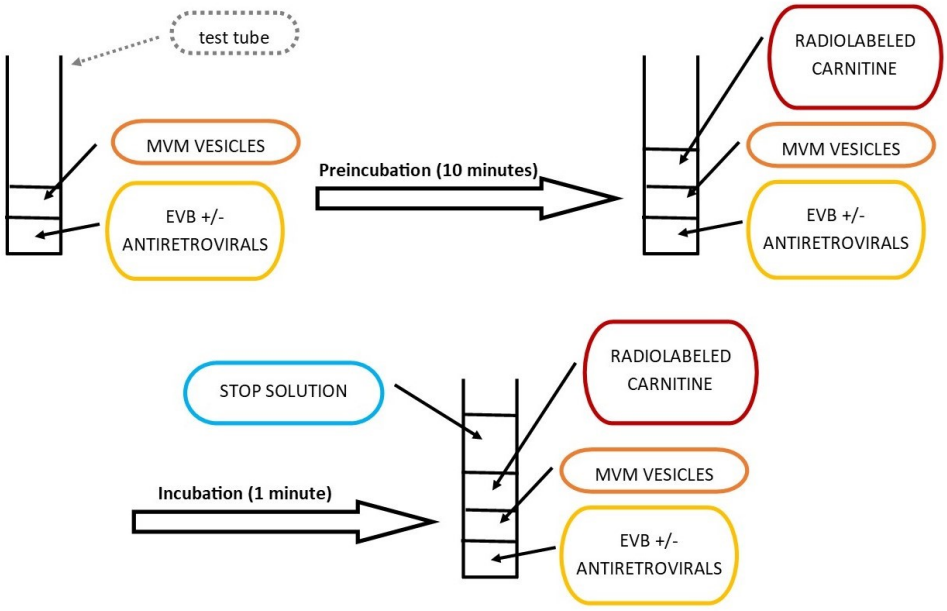

Figure 6. Schematic illustration of performed [^{3}H]-L-carnitine amino acid uptake assay. EVB – extravesicular buffer, MVM – microvillous plasma membrane.

5.4. STATISTICAL ANALYSIS

Statistical evaluation of the data and graph formation were done using Graph Pad Prism 7.0 software. Results from BeWo cells experiments were analysed with One-way ANOVA test, uptake assays on human placenta MVM vesicles were analyzed using multiple paired t-tests. * stands for $p \leq 0.05$, ** for $p \leq 0.01$ and *** for $p \leq 0.001$.

6. RESULTS

6.1. UPTAKE EXPERIMENTS IN BEWO CELLS

6.1.1 TIME DEPENDENCY

Time dependency experiments were performed to describe the course of the accumulation of carnitine in BeWo cells – regarding the time of the accumulation and the used buffer (Na^+-rich or Na^+-free). As showed in figure 7, Na^+ dependency of carnitine uptake was confirmed indicating OCTN2-mediated uptake. The uptake revealed time-dependency showing increase over the whole time of experiment (up to 2 hours). The time point 15 minutes was chosen for the following uptake experiments in order to ensure it is studied within the linear phase of uptake.

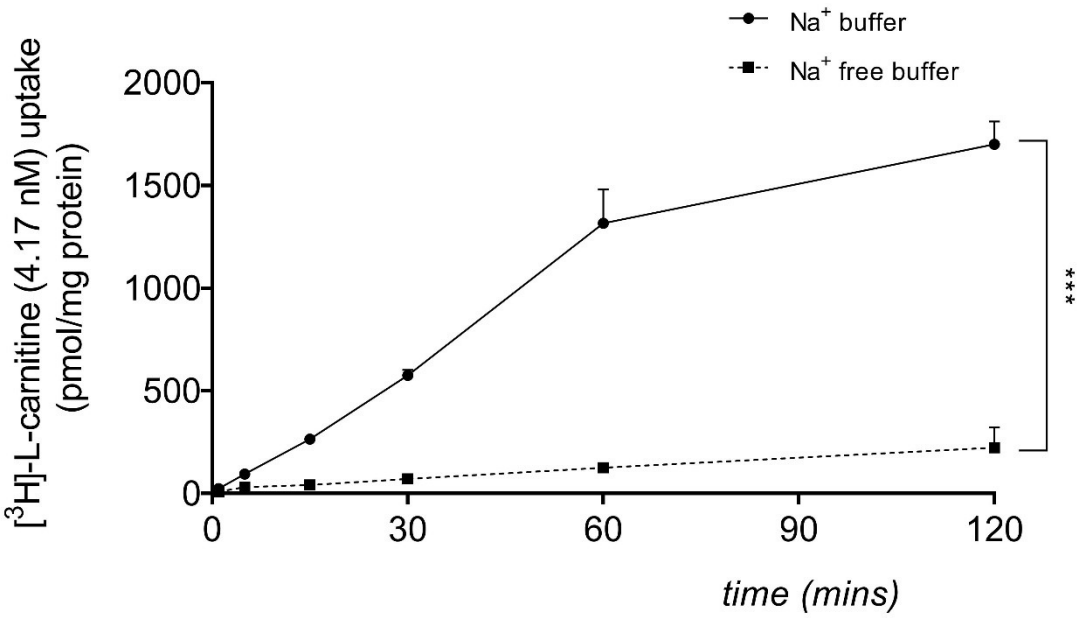

Figure 7. Time and sodium dependency of [^{3}H]-L-carnitine uptake in BeWo cells.

6.1.2. ACCUMULATION OF CARNITINE ON BEWO CELLS

Figure 8 presents the high inhibitory effect of some of the antiretrovirals – specifically protease inhibitor ritonavir and integrase inhibitor elvitegravir. There is also some potential inhibition by protease inhibitor saquinavir and non-nucleoside reverse transcriptase inhibitor rilpivirine. In this figure, there is also shown the inhibitory effect of verapamil, which inhibitory effect was already confirmed as positive control and carnitine to certify saturation of Na^+-dependent carnitine uptake.

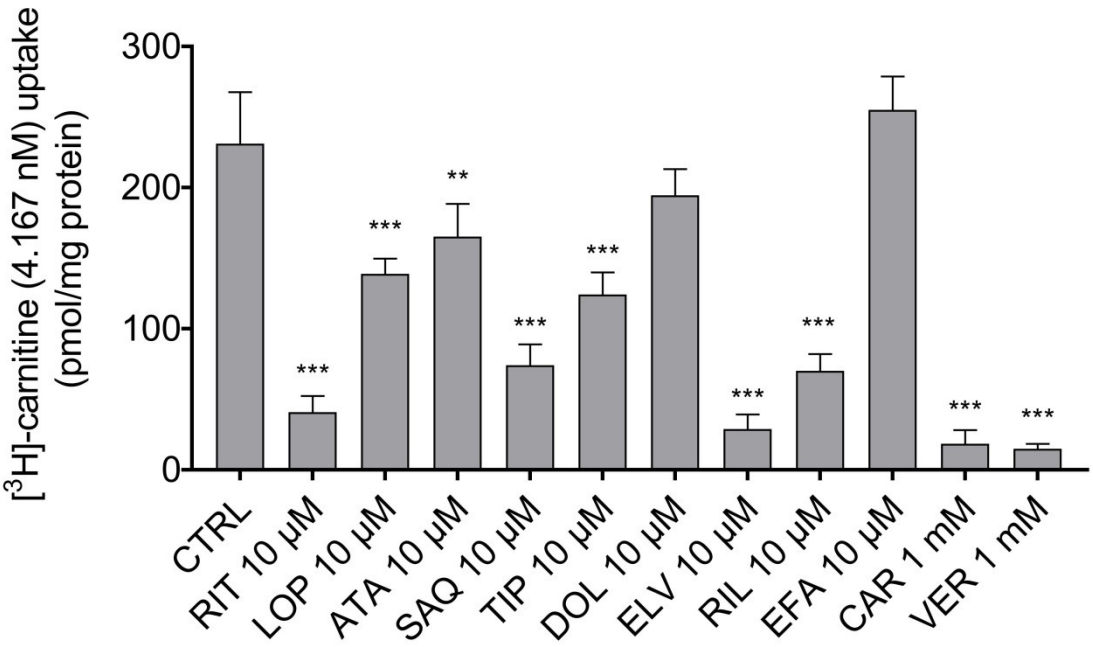

Figure 8. Uptake of [^{3}H] - L-carnitine and the inhibitory effect of tested antiretrovirals (all applied at 10 µM concentration). RIT – ritonavir, LOP – lopinavir, ATA – atazanavir, SAQ – saquinavir, TIP – tipranavir, DOL – dolutegravir, ELV – elvitegravir, RIL – rilpivirine, EFA – efavirenz, VER - control inhibitor verapamil (1 mM), CAR - carnitine (1 mM) used to address a saturation of the Na^+-dependent carnitine uptake; One-way ANOVA ** $p<0.01$, *** $p<0.001$.

6.2. L-CARNITINE UPTAKE ASSAYS IN MVM VESICLES

6.2.1 TIME DEPENDENCY

Characterization of carnitine uptake in human placental membrane vesicles is presented in figure 9. This figure clearly shows the Na^+ dependency of L-carnitine uptake. The time dependency was also observed and based on these data one-minute uptake was selected for further experiments.

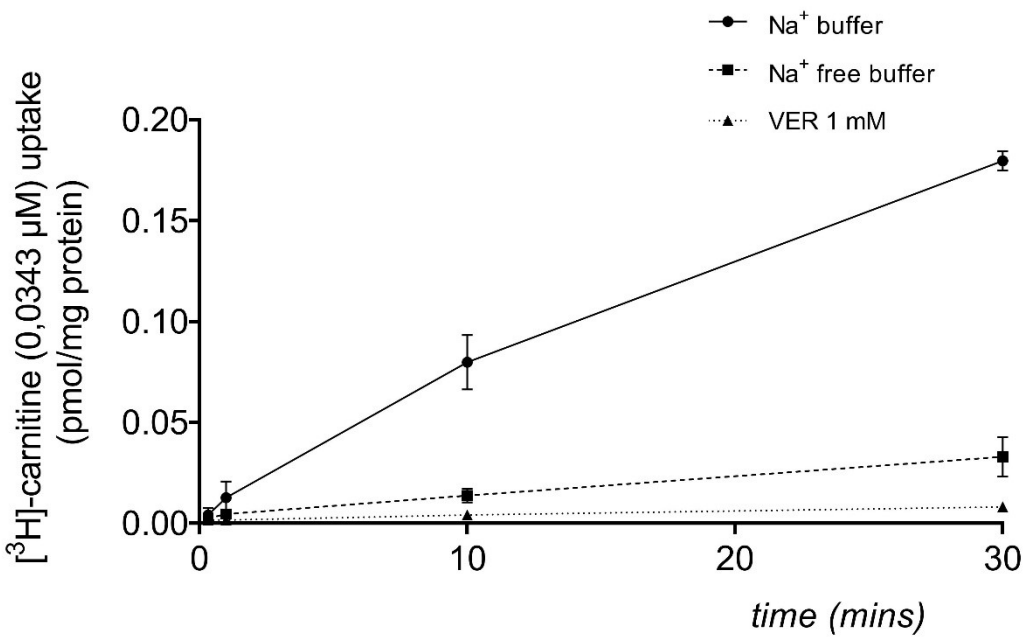

Figure 9. Time and sodium dependency of [^{3}H]-L-carnitine uptake in human placental microvillous membrane vesicles. VER – control inhibitor verapamil.

6.2.2. INHIBITORY EFFECT OF ANTIRETROVIRALS TO UPTAKE IN HUMAN PLACENTAL MVM VESICLES

Figures 10 and 11 show the inhibitory effect of antiretrovirals, when human placental microvillous membrane vesicles model is used. The highest inhibitory effect was observed with protease inhibitor ritonavir when applied in both 1 and 10 μM concentrations. An inhibitory effect was also observed with 1 and 10 μM protease inhibitor saquinavir and with integrase inhibitor elvitegravir. The experimental model was verified by verapamil as control inhibitor (1 mM) and high concentration of carnitine indicating saturation of the transport mechanism.

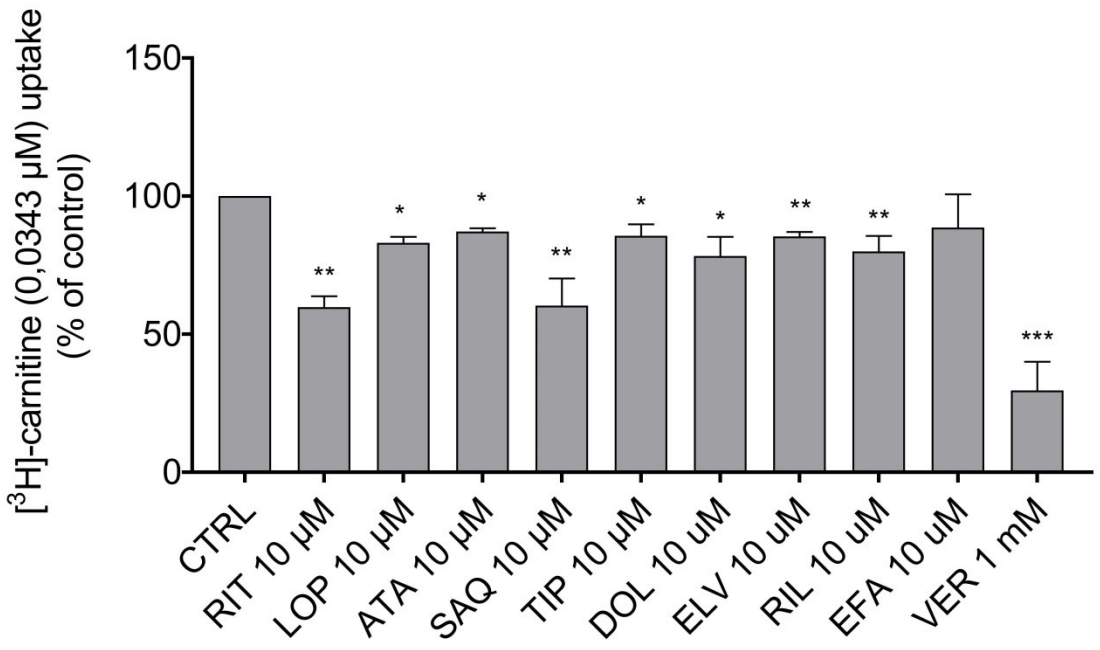

Figure 10. Inhibitory effect of studied antiretrovirals (10 μM) on the one-minute uptake of [^{3}H] - L-carnitine in MVM vesicles. RIT – ritonavir, LOP – lopinavir, ATA – atazanavir, SAQ – saquinavir, TIP – tipranavir, DOL – dolutegravir, ELV – elvitegravir, RIL – rilpivirine, EFA – efavirenz, VER - control inhibitor verapamil (1 mM), CAR - carnitine (1 mM) used to address a saturation of the Na^+-dependent carnitine uptake. Multiple t-test, * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

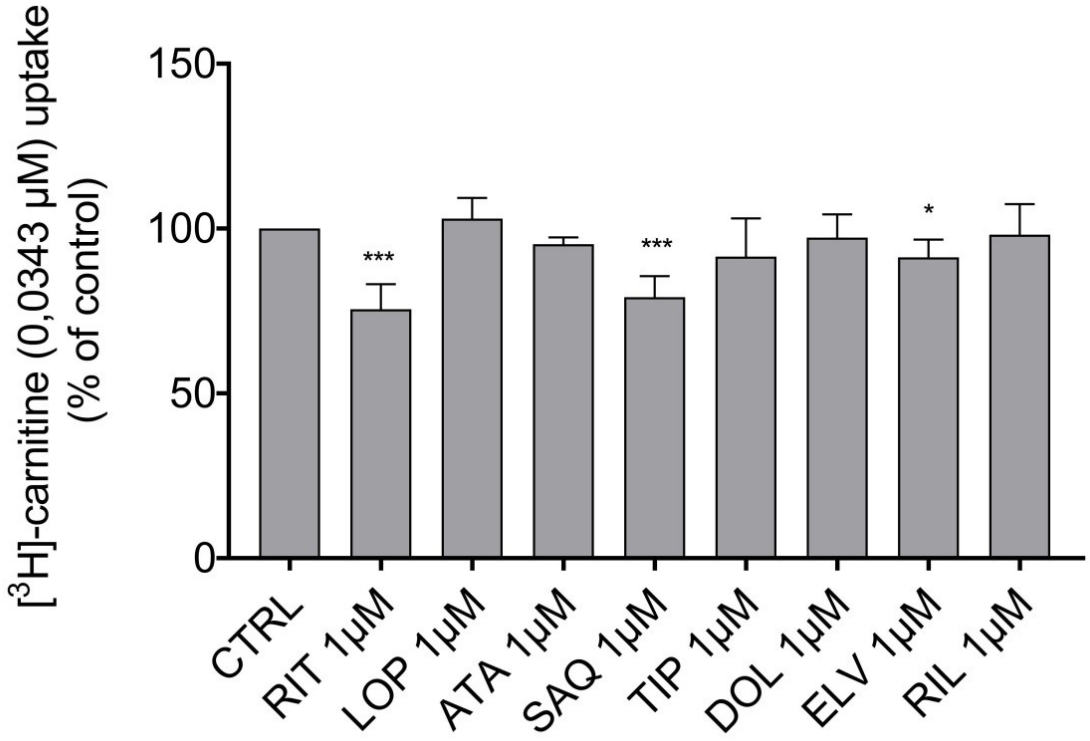

Figure 11. Inhibitory effect of studied antiretrovirals (1 µM) on the one-minute uptake of [^{3}H] - L-carnitine in MVM vesicles. RIT – ritonavir, LOP – lopinavir, ATA – atazanavir, SAQ – saquinavir, TIP – tipranavir, DOL – dolutegravir, ELV – elvitegravir, RIL – rilpivirine, EFA – efavirenz, VER - control inhibitor verapamil (1 mM), CAR - carnitine (1 mM) used to address a saturation of the Na^+-dependent carnitine uptake; Multiple t-test, * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

7. DISCUSSION

L-carnitine is an essential compound for β-oxidation of fatty acids, which is an important supply of energy for the human body. In pregnancy, this metabolic process is crucial also for correct placental function and fetal development. Transport of carnitine into the cells is mediated by OCTN2 in a sodium-dependent manner. Contribution of OCTN1 to L-carnitine supply is considered to be rather minor (Grube et al., 2005; Oey et al., 2006). With the shortage of carnitine in humans, there are two conditions connected – primary and secondary deficiency. Primary carnitine deficiency is hereditary defect and it is caused by mutation in *SLC22A5* gene coding the transporters for carnitine. This condition is connected primarily with heart dysfunction and muscle weakness. This genetic abnormality is nowadays one of the mutations addressed in newborns screening in developed countries (Gallant et al., 2017; Rasmussen et al., 2013). Carnitine deficiency can also develop as secondary, inquired by many conditions, comprising also drug-induced inhibition of OCTN2 resulting into shortage of carnitine uptake (Ferreira and McKenna, 2017; Stanley, 2004).

Since mother-to-fetus transmission is the main cause of HIV infection in children, antiretroviral therapy during pregnancy is nowadays common prophylaxis in the modern world. This highly active retroviral therapy helped significantly decrease HIV infection of newborns (from 50% to less than 1%). Unfortunately, some of the studies showed the connection between some antiretrovirals used in pregnancy and birth defects. A very strong connection was found between nucleoside reverse transcriptase inhibitor zidovudine and inborn cardiomyopathies. Since cardiomyopathy is one of the main symptoms of carnitine deficiency and the fact, that according to Ferraresi et al. (2006) acetyl-L-carnitine can mitigate this negative impact of therapy with NRTIs, it is highly possible, that this heart damage is caused by the lack of carnitine in newborn. This is the main idea of this thesis – that cardiomyophathies in babies born to mothers with antiretroviral therapy are caused by a lack of carnitine. Furthermore, we suspect, that this carnitine deficit is caused by the inhibition of its placental transporter OCTN2 (Ferraresi et al., 2006; Roustit et al., 2008; Sibiude et al., 2014).

To examine carnitine transmission via OCTN2 inhibition we used two models: (i) *in vitro* accumulation experiments in BeWo cells and (ii) uptake assay on *ex vivo* isolated

human placental microvillous membrane vesicles. In both of these models, we confirm the presence of sodium-dependent uptake of L-carnitine corresponding to the OCTN2 transporter, which shows high placental expression. Nine antiretroviral drugs have been tested in total, including atazanavir, dolutegravir, efavirenz, elvitegravir, lopinavir, rilpivirine, ritonavir, saquinavir and tipranavir. All the drug were initially evaluated for their potential interference with L-carnitine uptake whe applied at concentration of 10 μM. For drugs that show significant inhibitory effect the experiments were repeated at 1 μM. This concentration range of 1-10 μM covered and for most drugs exceeded the clinically achived plasma levels reported in patients during the therapy. The only exceptions were tipranavir and lopinavir, which can reach even higher maximum plasma concentrations in clinical settings than 10 μM. We aimed to take into consideration clinical plasma concentration reported for pregnant women, in drugs for which these data were not available, levels reported for non-pregnant patients were used (Best et al., 2010; Cressey et al., 2012; Gilbert et al., 2015; King and Acosta, 2006; Mulligan et al., 2018; Ripamonti et al., 2007; Roustit et al., 2008; Tran et al., 2016).

None of our tested drugs is a risk in pregnancy according to The Antiretroviral Pregnancy Registry. Contrary, the cohort study created by Van Dyke (2016) suggest, that there is the decrease in myocardial contractility connected with exposure to ritonavir-boosted lopinavir and the elevation of left ventricular wall thickness with the administration of atazanavir, which are subclinical findings that may indicate the future cardiac risk. Interestingly, our experiments demonstrate, that ritonavir is an inhibitor of carnitine transport in BeWo cells as well as MVM vesicles, so the contractility decline might be connected to the therapy outcomes. This finding needs further verification using other experimental models and ideally, *in vivo* clinical settings, since it might be calling for amendments in antiretroviral therapy stratagy, in which the ritonavir is widely used as pharmacoenhancer. On the other hand, we register no significant inhibition caused by atazanavir, so the cardiac defects might be inflicted by another mechanism. Moreover, our study reveals the inhibition of OCTN2 by rilpivirine, saquinavir and elvitegravir, where no evidence of harmful effect is described. But this may be caused by the fact, that for all of those drugs there are not enough data to evaluate their safety (Van Dyke et al., 2016).

The possible limitation of this study is that not all of the available models for studying transporters in placenta were used and none of the used models copy all of the conditions of the placental transport in the human body. Both of the methods themselves have some issues. MVM does not include the placental regulatory factors and since the BeWo cells are malignant trophoblast cells they do not fully correspondent to the structure and function of physiological trophoblast (Staud and Ceckova, 2015).

8. CONCLUSION

This thesis showed the inhibitory effect of some of antiretroviral drugs, which are already used in the treatment of the pregnant women infected with HIV and in the prophylaxis of vertical transmission in those women.

According to our data, protease inhibitor ritonavir is able to cause a significant inhibition of the OCTN2 transporter by. This inhibitory effect was demonstrated in both of our models – microvillous plasma membrane vesicles carnitine uptakes and BeWo cells accumulation experiments. In both models also protease inhibitor saquinavir demonstrated some inhibitory effect. In BeWo cells positive inhibitory effects of non-nucleoside reverse transcriptase inhibitor elvitegravir and non-nucleoside reverse transcriptase inhibitor rilpivirine were observed, but those results have not been confirmed by the MVM vesicles experiments.

This study should serve as a primary source for follow up series of experiments comprising other complementary experimental models of placenta to L-carnitine uptake studies in BeWo cells and MVM vesicles. Also, there should be thorough surveillance of the cardiac defects in babies born to mothers, who received the antiretrovirals that revealed some inhibitory effect towards OCTN2 in our study.

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