Univerzita Karlova

1. lékařská fakulta

Studijní program: Biochemie a patobiochemie

UNIVERZITA KARLOVA
1. lékařská fakulta

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Studium bilirubinu a jeho oxidačních produktů

Study of bilirubin and its oxidation products

Disertační práce

Vedoucí závěrečné práce/Školitel: RNDr. Aleš Dvořák, PhD.

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V Praze, 30.3.2023 Nikola Capková

ABSTRAKT

Fototerapie (PT) modrozeleným světlem (420-490 nm) se řadí mezi standardní léčbu těžké novorozenecké žloutenky, která brání toxickému působení bilirubinu (BR) u kojenců. Vystavením se modrozelenému světlu je BR přeměněn na polárnější fotoizomer (PI) lumirubin (LR) a další oxidační produkty (mono-, di-, tripyrroly), které lze snadněji vyloučit z těla močí a/nebo žlučí. Ačkoli je PT považována za bezpečnou, je doprovázena zvýšeným rizikem různých patofyziologických stavů (zánětlivých procesů, alergií, cukrovky i některých typů rakoviny), zejména u novorozenců s extrémně nízkou porodní hmotností. Účelem této práce bylo pochopení mechanizmu vylučování BR v různých tkáních i buněčných liniích a zkoumání bioaktivních vlastností BR i jeho hlavního fotooxidačního produktu LR.

Nejprve jsme se zaměřili na detekci BR v žluči a stolici hyperbilirubinemických potkanů Gunn. Současně jsme testovali antioxidační a prooxidační účinky nekonjugovaného BR u lidských hepatoblastomových (HepG2), proximálních tubulárních (HK2), neuroblastomových (SH-SY5Y) a myších endotelových (H5V) buněk, jejich vystavením postupně se zvyšujícím koncentracím BR. Pro porovnání účinků BR a LR na markery metabolismu a oxidačního stresu byly biologické aktivity zkoumány *in vitro* na buňkách lidského hepatoblastomu (HepG2), fibroblastu (MRC5) a myších makrofázích (RAW 264.7). Zaměřili jsme se také na proliferaci, morfologii, expresi specifických genů i proteinů a diferenciaci neurálních kmenových buněk (NSC).

Naše experimenty potvrdily, že souvislost mezi regulací transintestinálního vylučování cholesterolu a plazmatickými koncentracemi nekonjugovaného BR u potkanů Gunn není přítomna. U všech studovaných buněčných linií jsme zjistili, že nízké koncentrace BR vedly k antioxidačním účinkům, zatímco vyšší koncentrace k prooxidačním nebo cytotoxickým účinkům, čím se potvrzuje, že každý typ buněk má jiný práh pro BR. Při porovnání s LR, jsme sledovali výrazně nižší toxicitu a zachování antioxidační kapacity v séru. LR také potlačil aktivitu vedoucí k produkci mitochondriálního superoxidu, avšak byl méně účinný v prevenci lipoperoxidace. Naše data také potvrdily vliv BR a LR na časnou fázi diferenciace NCS a schopnost LR ovlivňovat polaritu a identitu NSC během časného vývoje lidského neuronu, což může mít klinický význam, protože buněčná polarita hraje významnou roli během vývoje CNS.

Klíčová slova: Metabolismus hemu, bilirubin, fotooxidační produkty, novorozenecká žloutenka

ABSTRACT

Phototherapy (PT) with blue-green light (420-490 nm) is the standard treatment for severe neonatal jaundice to prevent infants from toxic bilirubin (BR). Upon blue-green light exposure, BR is converted to more polar photoisomer (PI) lumirubin (LR) and the other oxidation products (mono-, di-, tripyrrols) which can be more easily disposed of the body via urine and/or bile. Although generally considered to be safe, PT is accompanied by an increased risk of various pathophysiological conditions (inflammatory processes, allergies, diabetes, and some types of cancer), in extremely low-birth-weight newborns. Thus, to account for these consequences, our study aimed to understand the mechanism of BR secretion in different tissues and cell lines and investigate the bioactive properties of BR and its main photooxidation product LR.

At first, we focused on the detection of BR in the bile and feces of hyperbilirubinemic Gunn rats. Simultaneously, we tested the antioxidant and pro-oxidant effects of unconjugated BR in human hepatoblastoma (HepG2), proximal tubular (HK2), neuroblastoma (SH-SY5Y), and murine endothelial (H5V) cells by exposing them to progressively increasing concentrations of BR. To compare the BR and LR effects on metabolic and oxidative stress markers, the biological activities were investigated in vitro on human hepatoblastoma (HepG2), fibroblast (MRC5), and murine macrophage (RAW 264.7) cells. We also focused on proliferation, morphology, expression of specific genes and proteins, and differentiation of neural stem cells (NSC).

Our experiments confirmed no link between the regulation of transintestinal cholesterol excretion and plasma concentrations of unconjugated BR in Gunn rats. We observed in all studied cell lines, that low concentrations of BR exhibit antioxidant effects, whereas higher concentrations exhibit a prooxidant or cytotoxic effect, confirming that each cell type has a different threshold for BR. When compared to LR, significantly lower toxicity and maintenance of antioxidant capacity in serum were observed. LR also suppressed the activity leading to mitochondrial superoxide production but was less effective in preventing lipoperoxidation. Our data also confirmed the effect of BR and LR on the early phase of NCS differentiation and the ability of LR to influence the polarity and identity of NSCs during early human neuronal development, which may have clinical relevance since cell polarity has an important role during CNS development.

Key words: Haem metabolism, bilirubin, photo-oxidation products, neonatal jaundice

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1 INTRODUCTION

1.1 Heme catabolism

Heme is an important iron-containing cyclic tetrapyrrolic molecule expressed ubiquitously in organisms which serves as a prosthetic group for a variety of hemoproteins including hemoglobin, myoglobin, cytochrome P-450, catalase, peroxidase, tryptophan pyrrolase, cytochrome b5, and mitochondrial cytochromes which are implicated in multiple cellular functions including oxygen transport, energy generation, defence against increased oxidative stress or cell signalling (Vitek & Ostrow, 2009) (Jayanti et al., 2020) (B. Wu et al., 2019). Due to 65-75 % of the iron pool in the human body being derived from heme, this molecule also acts as a major storage of bioavailable iron (Korolnek & Hamza, 2014) (Schultz et al., 2010). After its release from red blood cells, heme is bound to hemopexin or haptoglobin and recycled or transported back to the splenic sinusoids in reticuloendothelial system where its degradation process occurs (Schultz et al., 2010).

The mammalian heme degradation pathway ***(Fig. 1)*** consists of two enzymatic steps, which are mediated by heme oxygenase (HMOX) and biliverdin reductase (BLVR) (Maines, 2005). HMOX is the enzyme classified as oxidoreductase crucial for the first step of the heme degradation. HMOX system consists of HMOX and NADPH–cytochrome P450 reductase with the protective effect of cells against heme-induced oxidative stimuli (B. Wu et al., 2019). The process of heme degradation is initiated when HMOX catalyses the opening of the heme ring at α -carbon bridge to yield equimolar quantities of non-toxic polar biliverdin (BV) with tetrapyrrole structure, carbon monoxide (CO) and free iron with the consumption of three molecules of oxygen (O_2) and the reducing equivalent of NADPH (Otterbein & Choi, 2000). After this cleavage, BV is directly converted by reduction of the middle -CH= bridge into non-polar unconjugated BR mainly by the cytosolic enzyme BVR with a unique feature which increases the production of bilirubin and thus enhancing defence against oxidative stress (Tenhunen et al., 1972) (Salim et al., 2001).

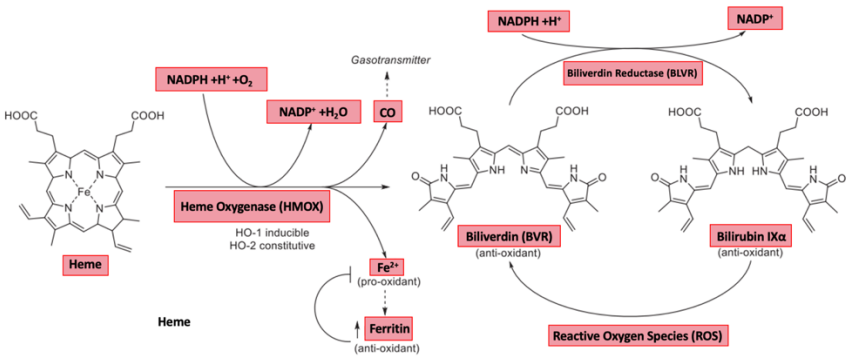

Fig.1. Heme degradation pathway. *Conversion of heme molecule generates an equimolar amount of CO, ferrous ion (Fe^{2+}), and biliverdin which is subsequently reduced by biliverdin reductase to non-polar unconjugated bilirubin IXα – 4Z, 15Z.* Modified from (Nocentini et al., 2022).

The presence of unconjugated BR produced entirely from the degradation of heme and heme proteins is 4.4 ± 0.7 mg/kg (Berk et al., 1974) accounting for an average of 300 – 375 mg of daily *de novo* unconjugated BR production (Salehi et al., 2014). Of this amount, 75% to 80% of unconjugated BR derives from hemoglobin released during destruction of senescent red blood cells in the reticuloendothelial system (Salehi et al., 2014). The remaining 25% of unconjugated BR synthesis is derived from non-hemoglobin heme proteins in the liver, from accelerated destruction in the spleen of immature or defectively formed red cells, and the bone marrow from heme formed in excess of globin (Vitek & Ostrow, 2009).

1.2 Bilirubin metabolism

Unconjugated BR was discovered in 1847 by Dr. Virchow. Its chemical tetrapyrrolic structure was defined by Fischer and Orth in 1937 (Tschesche, 1938) and in 1942 its successful synthesis was reported (Fisher H. & Plieninger H., 1942).

1.2.1 Bilirubin structure

The natural bilirubin in humans is the unconjugated BR IXα 4Z,15Z molecule and its other isoforms include IIIα and XIIIα isomers formed by a nonenzymatic process so called molecular scrambling, in which unconjugated BR IXα is split into two halves which then randomly re-assemble ***(Fig. 2)*** (Vitek & Ostrow, 2009).

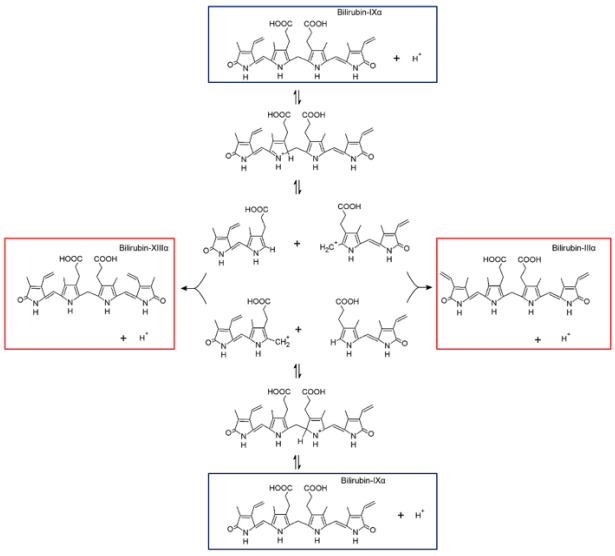

Fig.2. Formation of bilirubin IXα, the dominant bilirubin molecule in the circulation, and its constitutional isomers IIIα and XIIIα formed by dipyrrole exchange reaction. Modified from (Itoh et al., 2017).

The structure of unconjugated BR IXα 4Z,15Z molecule is nearly symmetrical and composed of two planar dipyrrole units (rings A-B and C-D) joined to each other by a central methylene group (-CH2-). In each dipyrrolic half, the two monopyrroles are linked by an unsaturated (double-bonded) methene group (-CH=) and lie in the same plane. Each outer pyrrole rings (A & D) have a polar lactam (-CO-NH-) group, while each central pyrrole ring (B & C) carries a carboxyethyl sidechain (-CH2-CH2-COOH), which can ionize by loss of the terminal proton. The remaining sites on the pyrrole rings are occupied by ethyl (-CH3) and vinyl (-CH=CH2) substituents; these are asymmetrically arranged in the A and D rings, giving to unconjugated BR optical activity ***(Fig. 3)*** (Vitek & Ostrow, 2009).

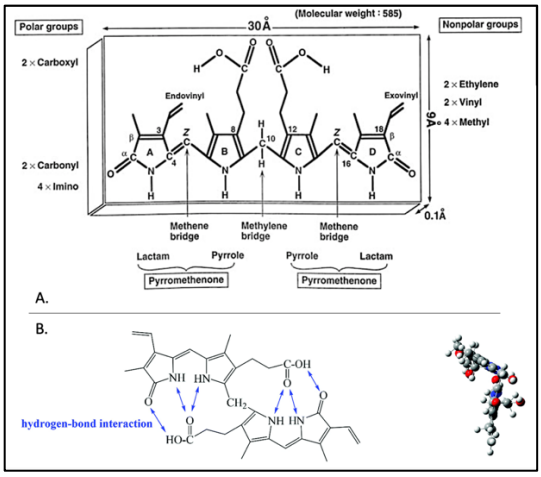

Fig. 3. Bilirubin-IX α molecule and its nomenclature (A) with visualization of an internal hydrogen bonds in its molecular configuration (B). Modified from (Itoh et al., 2017) (Kou & Wang, 2022).

Due to an internal hydrogen bonding of its polar groups hidden from interaction with water molecules ***(Fig. 3B)***, unconjugated BR appears to have very low solubility in aqueous media - ranging from 7 to 100 nM (the solubility threshold in plasma is 70 nM) at a pH of 7.4 and temperature of 37°C (Gazzin et al., 2017) (Levitt & Levitt, 2014). Transport of non-polar unconjugated BR in the plasma is provided mainly (90%) by bounding to human serum albumin (HSA) and secondary (10%) to the apolipoprotein D found primarily in the high density (HDL) (Jacobsen, 1969) (Suzuki et al., 1988) (Goessling & Zucker, 2000) or by high-affinity bilirubin transporter α1-fetoprotein in the fetus and early neonates (Aoyagi et al., 1979). Only < 0.1% of the concentration of unconjugated BR in plasma is not bound to any carrier molecule and is termed as „free unconjugated bilirubin“ (Bf) which represent a fraction with the ability to diffuse into tissues leading to cytotoxicity (Calligaris et al., 2007).

1.2.2 Transport and excretion of bilirubin

HSA with the primary high-affinity site binds one mol of unconjugated BR dianion through ionic bonds of its $-COO^-$ groups with the terminal $-NH^+$ groups of two lysine residues in an otherwise hydrophobic pocket (Gazzin et al., 2017). Binding with albumin keeps BR dissolved in the circulation and prevents excessive amounts of Bf from

passing through membranes when accumulating in cells with the exertion of cytotoxic effects (Lauff et al., 1983). Unbound unconjugated BR and its albumin enter the hepatocyte complex predominantly via passive diffusion across the porous sinusoidal endothelium to reach the basolateral membrane of the hepatocytes (Cui et al., 2001) (Briz et al., 2006). The conjugation of unconjugated BR in hepatocytes occurs when one or both -COOH groups are modified by covalent attachment of 1- 2 molecules of glucuronic acid by the action of the UDP- glucuronosyl transferase 1A1 isoform (UGT1A1), resulting information of conjugated bilirubin ***(Fig. 4)*** (Gazzin et al., 2017). Substantial fraction of bilirubin conjugates might be transported by the multidrug resistance-associated protein MRP3 at the sinusoidal membrane into the blood, from where is subsequently reuptaken by sinusoidal membrane-bound organic anion transporting polypeptides (OATP) 1B1 and 1B3 of downstream hepatocyte to prevent oversaturation of canalicular excretion mechanism in periportal hepatocytes (Sticova, 2013a). The activity of UGT1A1 is under hormonal control enhancing progesterone and inhibiting testosterone activity (Muraca & Fevery, 1984). In the following step, the excretion of conjugated BR into bile through the bile canaliculi is mediated by an ATP-dependent transporter identified as the multidrug resistance-associated protein MRP2/cMOAT and, to a lesser extent, also by ATP-binding cassette (ABC) efflux transporter ABCG2 (Sticova, 2013a). The absence of functionally active MRP2 prevents the secretion of conjugated BR into the bile and redirects this conjugate into sinusoidal blood (Gartung & Matern, 1997). Due to differences in the expression of the transporters OATP1B1/3 at the sinusoidal face which increases from the portal to the centrilobular space intralobular transport system (the sinusoidal liver- to- blood loop) is formed with the possible protecting function of the periportal hepatocytes from the excessive bilirubin and xenobiotics accumulation (Gazzin et al., 2017) (Sticova, 2013b).

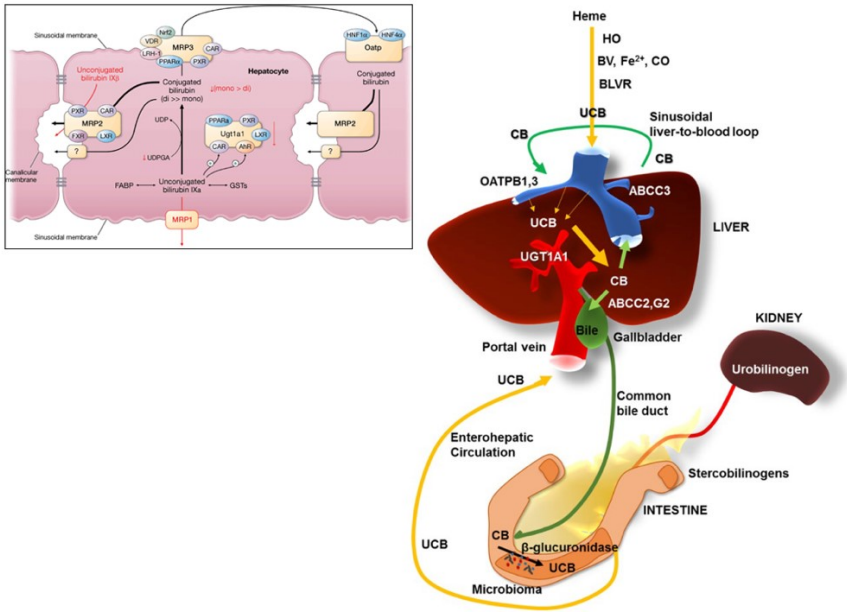

Fig. 4. Intracellular and extracellular metabolism of bilirubin. Modified from (Gazzin et al., 2017) and (Stevenson DK et al., 2012).

The vast majority of conjugated BR absorbed in the small intestine is deconjugated to unconjugated BR by the action of β-glucuronidases and reduced by the coliform bacteria to urobilinogen and stercobilinogen ***(Fig. 5)***. However, a part is excreted as unconjugated BR (Vítek et al., 2000) (Morelli, 2008) (Gritz & Bhandari, 2015). Most of the urobilinogen undergoes oxidation and feces excretion. Only a tiny fraction is filtered by the kidney and excreted in the urine due to enterohepatic / enterosystemic circulation. Under certain conditions, a small amount of unconjugated BR can also undergo these reabsorption processes. However, the reabsorption of a small amount of unconjugated BR occurs in the colon when delivered by portal circulation back to the liver (Vítek et al., 2000) (Tiribelli & Ostrow, 2005).

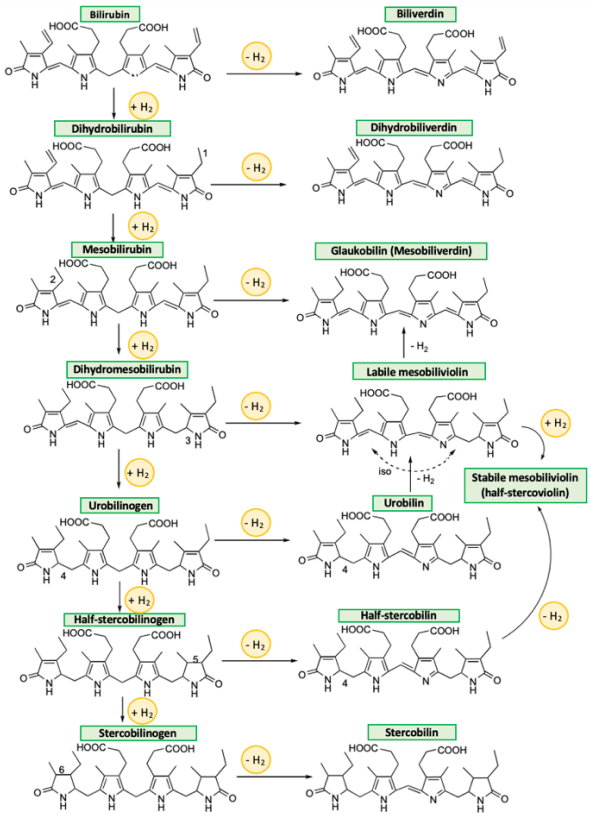

Fig. 5. Reduction of UCB by intestinal microflora showing the chemical structure of its formed products. Modified from (Vitek & Ostrow, 2009).

1. 3 Biological properties of bilirubin

Our understanding of bilirubin, originally considered only as a waste substance associated with liver disease, has rapidly changed during the recent fifty years of active in vivo and in vitro research. BR is now regarded as a molecule with many intricate biological functions from cell signalling (behaving almost like a “real” hormonal

substance), and modulation of metabolism, to immune regulation, affecting biological activities with apparent clinical and even therapeutic consequences. These may indicate the lower incidence of civilization diseases such as diabetes, obesity, cardiovascular diseases, arterial hypertension, metabolic syndrome, certain cancers, and autoimmune or neurodegenerative diseases observed in individuals with chronic mild unconjugated hyperbilirubinemia. Meanwhile, high concentrations of bilirubin are associated with the opposite effect when becomes toxic (Vítek & Tiribelli, 2021).

1.3.1 Positive effects of bilirubin

As early as the 1950s, research shows the protection effects of BR against lipid oxidation such as vitamin A or linoleic acid (Pranty et al., 2022) but intensive research of its antioxidant effects began later on in 1987 with a ground-breaking study of Roland Stocker found the ability of BR to inhibit fatty acid oxidation (Stocker, Yamamoto, et al., 1987a). In the research of Wu *et al*. was confirmed and demonstrated 20 times more effective antioxidant effect of BR than a vitamin E analogue Trolox (T.-W. Wu et al., 1994). These antioxidant effects are mainly due to the presence of the tetrapyrrole C-10 methylene group which provides an electron to reactive oxygen species (ROS) and serves as a free radical scavenger. However, when compared with the other antioxidants, BR physiological concentrations in the human body are relatively low and not sufficient to provide such intensive protection that has been observed in many clinical and experimental studies in relation to BR (Jansen & Daiber, 2012), (Gazzin et al., 2016), (DiNicolantonio et al., 2018).

A possible explanation is the existence of the so-called called biliverdin-bilirubin redox cycle ***(Fig. 1.)*** when BR is oxidized by ROS (hydrogen peroxide, lipid peroxide, or peroxyl radicals) back to BV and later on enzymatically regenerated back to BR (Greenberg, 2002), (Sedlak et al., 2009). In addition, BR enhances its antioxidant effect by inhibition of the common isoforms of the NADPH-oxidase enzyme which represent the superoxide-releasing complexes in the cells (Lanone et al., 2005), prevent peroxidation of proteins (Stocker & Ames, 1987), phospholipids (Sedlak et al., 2009), or LDL protein (T.-W. Wu et al., 1994) and reduce protein carbonylation. BR also reduces oxidative liver damage induced by the accumulation of bile acids during cholestasis (Muchova et al., 2011) and counteracts the harmful effects of pro-oxidants including bile acids *in vitro* and *in vivo* (Zelenka et al., 2012). The association between BR concentration and total serum antioxidant capacity is affecting the development of coronary heart disease (Schwertner et al., 1994) specifically, in middle-aged individuals with Gilbert's syndrome, when the incidence of coronary heart disease was 2% compared to the general population in the same age group, where this risk was up to 12% (Sedlak & Snyder, 2004). Another function of BR as an antioxidant is present in human vascular endothelial cells (Ziberna et al., 2016), and at low concentrations protects neuronal cells from oxidative stress (Doré et al., 1999).

Moderately elevated concentrations of BR are considered to be protective and directly associated with reduced atherosclerotic plaque formation in carotid arteries, which decreases the risk of stroke (Ishizaka et al., 2001) and reduction of developing atherosclerosis in the general population (Novotný & Vítek, 2003). On contrary, the

concentration of BR below 7 μmol/L increased risk of developing systemic diseases associated with higher oxidative stress (Wagner et al., 2015), including systemic lupus erythematosus (Vítek et al., 2010), multiple sclerosis (Peng et al., 2011), asthma (Horsfall et al., 2014), diabetes (Abbasi et al., 2015), hypertension (L. Wang & Bautista, 2015), and obesity (DiNicolantonio et al., 2018) or certain forms of cancer such as colon cancer (Temme et al., 2001).

Other important data were brought in experimental and clinical studies focused on the anti-inflammatory effects of BR with the first consistent evidence seen almost 80 years ago in patients with rheumatoid arthritis, who experienced a surprising alleviation of symptoms as a result of the development of liver disease due to increased BR concentrations (Sidel & Abrams, 1934) (Hench, 1938). Later on, in 2010, a large epidemiological study demonstrated a direct association between a reduced risk of developing rheumatoid arthritis and higher total serum BR concentrations (Fischman, 2010). When comparing the patients with ulcerative colitis or primary sclerosing cholangitis and hyperbilirubinemia when a higher concentration of BR was observed milder colitis occurred (Papatheodoridis et al., 1998). Likewise with Gilbert's syndrome when BR is naturally increased, individuals have a reduced predisposition for the development of inflammatory bowel disease (Crohn's disease) (Leníček et al., 2014) (Jangi et al., 2013).

1.3.2 Toxicity of bilirubin

Excessively elevated concentrations of BR are toxic due to the binding capacity of albumin being exceeded and Bf permeates the plasma membrane into the intracellular space which has the ability to interfere with the respiratory chain by inhibiting mitochondrial enzymes (Diamond, 1970) (Mancuso, 2017) resulting in the release and accumulation of cytochrome c into the cytosol, decrease in mitochondrial membrane potential with disruption of lipid or protein membrane structure and finally to the induction of apoptosis (Rodrigues, Solá, & Brites, 2002a). Bf has many other negative effects on the cell including inhibition of protein kinases like cAMP, cGMP, or Ca^{2+} dependent kinase affecting cellular phosphorylation (Hansen et al., 1996), inhibition of DNA synthesis (Yamada et al., 1977), or neuronal proteins (Gurba & Zand, 1974). High concentrations of BR lead to the inhibition of ion exchange and water transport in renal cells (Dennery et al., 2001). Due to its affinity for membrane phospholipids, BR inhibits also tyrosine uptake (Amato et al., 1994) and in the auditory nerve may disrupt neuroexcitation signals (Dennery et al., 2001). When focused on *in vivo* experiments, the toxicity of BR in the central nervous system (CNS) was observed in the brainstem, cerebral cortex, hippocampus, basal ganglia, and Purkinje cells (Ahdab-Barmada & Moossy, 1984) (Ahlfors & Shapiro, 2001) (Watchko, 2006) (Ye et al., 2019). Observations of high concentrations of BR *in vitro* reveal toxicity in astrocytes (Deliktaş et al., 2019), neurons (Grojean et al., 2000), and organotypic brain sections (Dani et al., 2019).

1.3.3 Hyperbilirubinemia

Physiological total serum bilirubin concentration varies within the range of 0.2- 1 mg/dL (3.4-17.1 μmol/L) (Gazzin et al., 2017). Elevated concentrations above 17 μmol/L so called hyperbilirubinemia are related to impaired

BR metabolism (Strassburg, 2010). Once bilirubin levels in the circulation rise above its physiological concentrations, icteric discoloration of sclera, mucosal surfaces and skin is observed. Much more severe hyperbilirubinemias (usually above 340 μmol/L) could be accompanied with deleterious bilirubin effects, among them bilirubin-induced neurological dysfunction and kernicterus being the worst and most dangerous complications (Watchko & Tiribelli, 2013).

Hyperbilirubinemias can be classified into conjugated (postmicrosomal), unconjugated (premicrosomal), and mixed hyperbilirubinemias (van Dijk et al., 2015). **Conjugated** hyperbilirubinemias are caused mainly by extrahepatic cholestasis (biliary obstruction), intrahepatic cholestasis (viral and alcoholic hepatitis, steatohepatitis, intrahepatic cholestasis of pregnancy, posttransplant conditions, etc.), when compared with **mixed** hyperbilirubinemias caused by hepatocellular damage (toxic, infectious, immunological, systemic damage, neoplasms, etc.) (Krige, 2001) (Stratta et al., 1989) (Brumbaugh & Mack, 2012) (Mendenhall et al., 1984). **Unconjugated** hyperbilirubinemias are caused mainly by following pathophysiological mechanisms: BR overproduction (intra/extravascular hemolysis, erythrocyte phagocytosis during extravasation, defective haemoglobin synthesis, impaired uptake of BR by the hepatocyte (during the administration of drugs such as certain antibiotics, etc.) and by impaired bilirubin conjugation due to UGT1A1 activity such as in Gilbert syndrome, Crigler-Najjar syndrome type I and II or due to its inhibition by specific drugs such as antibiotics, antivirotic atazanavir or irinotecan (Robinson et al., 1962) (Kenwright & Levi, 1974) (Bosma, 2003) (Muchová et al., 2004) (Dhawan et al., 2005) (Strassburg, 2010).

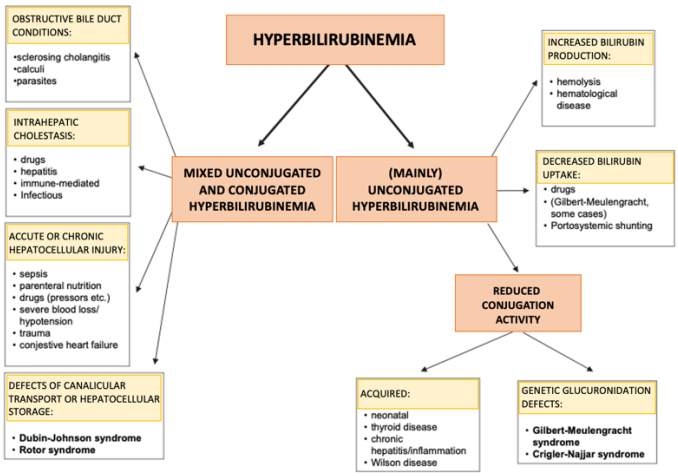

Fig. 6. Differential diagnostic approach to hyperbilirubinemia. Modified from (Strassburg, 2010).

1.3.4 Neonatal jaundice

Neonatal jaundice, one of the most common clinical conditions in the newborn period, is defined as the elevation of a total serum bilirubin concentration above 85 μmol/L (Stevenson DK et al., 2012). Nearly 60% of term

and 80% of preterm infants develop jaundice in the first week of life, and 10% of breastfed infants remain jaundiced until 1 month of age (Olusanya et al., 2014) (Battersby et al., 2017). Neonatal jaundice has multifactorial pathogenesis due to an imbalance between the production and excretion of bilirubin after birth (Hakan et al., 2015). The most important factors involved in its manifestation include immaturity of the blood-brain barrier, which is, therefore, more permeable to Bf, and immaturity of hepatic transporters and glucuronosylation mechanisms, where the body is unable to rapidly adapt to BR overproduction shortly after birth (Shapiro et al., 2006).

Mild to moderate neonatal jaundice is associated with protection from the development of various oxidative stress-mediated diseases and resolves spontaneously within a few days after birth. However, other risk factors such as low birth weight, ABO and rhesus blood group incompatibility, glucose-6-phosphate dehydrogenase (G6PD) deficiency, neonatal immaturity, sepsis, or breastfeeding are all prerequisites for the manifestation of severe neonatal hyperbilirubinemia with bilirubin concentrations above 342 μmol/L which could lead to accumulation of bilirubin in the basal ganglia and brainstem nuclei, resulting in acute or chronic bilirubin neurotoxicity and the risk of subsequent kernicterus which may result in acute bilirubin encephalopathy with clinical significance (MacDonald, 1995) (Dennery et al., 2001) (Watchko, 2003) (Ostrow et al., 2004) (Shapiro et al., 2006).

Many therapeutic approaches have been attempted in the past for the treatment of severe neonatal jaundice. To reduce the toxic effects of BR is still commonly used as the golden standard phototherapy (PT) with blue-green light (450-510 nm) in which BR is converted into more polar photoisomers that can be easily disposed of the body (McDonagh et al., 2009).

1.5 Phototherapy

During the neonatal period and 5–10% of them require treatment by phototherapy with visible light for which the range of wavelengths between 450 and 510 nm is the most effective (Bhutani & Johnson-Hamerman, 2015).

PT for unconjugated hyperbilirubinemia was discovered in 1958 by the team of Cremer et al. (Cremer et al., 1958) and later on in 1968 the first studies focused on the evaluation of the efficacy and safety were performed by the Lucey and co-workers (Lucey et al., 1968). The principle of this therapy has been based on the photoconversion of bilirubin to its more polar structural photo-isomers and photo-oxidative products easily excreted from the body by urine and/or bile ***(Fig. 7.)*** (McDonagh, 2001). Considering the fact that bilirubin concentrations in neonates can change within hours, it is quite difficult to determine the appropriate phototherapeutic treatment and its recommendations are constantly being updated (Porter & Dennis, 2002) (Hansen et al., 2020). The guidelines for clinics that use the definitions for quality of evidence and balance of benefits and harms established by the AAP Steering Committee on Quality Improvement Management when PT used for infants at age 25 - 48 hours with bilirubin levels above 256 μmol/L, infants at age 49 - 72 hours with levels 308 μmol/L, and infants older than 72 hours with bilirubin levels above 342 μmol/L (Porter & Dennis, 2002) (Kemper et al., 2022).

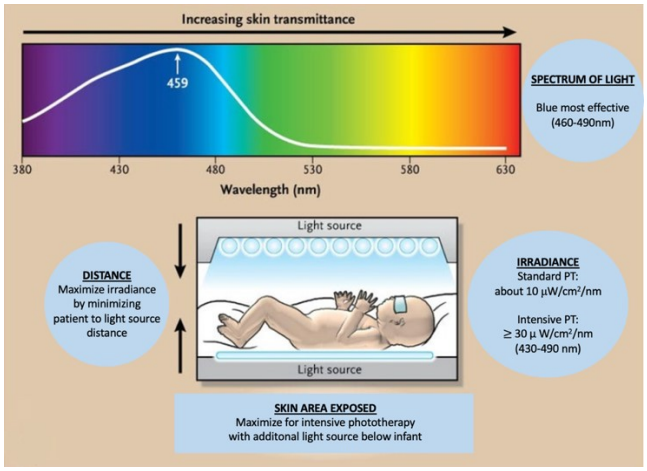

Fig. 7. The principle of phototherapeutic treatment. Modified from (Maisels & McDonagh, 2008).

Even though PT is worldwide used as the golden standard, is accompanied with side effects such as development of a bronze baby syndrome, water loss, impairment of thermoregulation, damage to unprotected eyes, and/or hypocalcaemia (Stevenson DK et al., 2012) (Khan et al., 2016). Surprisingly, recent studies suggest that PT might also be associated with an increased risk of ileus (Raghavan et al., 2005), allergic diseases (Wei et al., 2015), type 1 diabetes (McNamee et al., 2012), cancer (Wickremasinghe et al., 2016) (Cnattingius et al., 1995), and even mortality (Arnold et al., 2014; Morris et al., 2008) especially in extremely low birthweight (ELBW) neonates. The principle of the whole process is to reduce serum bilirubin concentrations and thus the toxic effects by transformation of unconjugated BR into easily excreted photoproducts (McDonagh et al., 2009).

1.5.1 Bilirubin photoisomers

During the therapeutic approach, BR is transformed by the action of blue or blue-green light (within the wavelength range 450 - 510 nm, close to the absorption maximum of BR) into its structural BR photoisomers (PI) (Maisels & McDonagh, 2008). Exact structures of PI were established by McDonagh et al. and Onishi et al. (McDonagh et al.1982) (McDonagh & Assisi, 1972) (Onishi et al., 1984) (McDonagh et al., 2009b). Configurational isomerization of BR leads to the fast and reversible formation of ZE- and EZ-bilirubin when compared to structural isomerization that leads to an irreversible change of BR into the most important PI E- and Z-lumirubin ***(Fig. 8.)*** (Onishi et al., 1984).

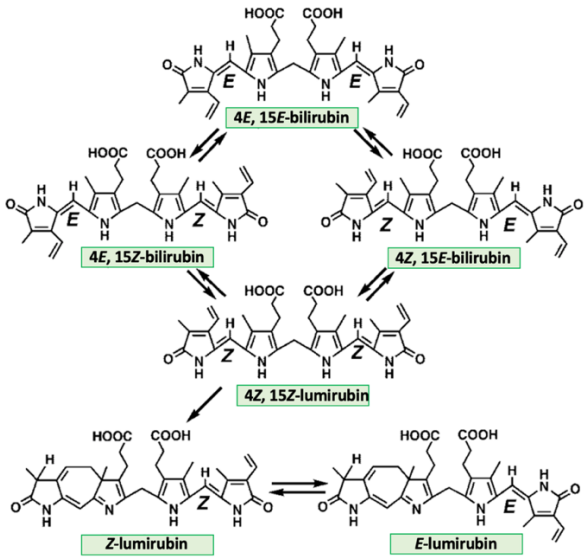

Fig. 8. Linear 2-dimensional representations of the chemical structures of bilirubin IX and its major PI lumirubin with photochemical interconversion pathways. Modified from (McDonagh et al., 2009).

Bilirubin PI could be detected by HPLC and LCMS/MS in bile, serum, and urine but none of these methods has been used in clinical practice. In 1982, McDonagh and co-workers focused on the LR determination, but this method has limited resolution of the separated PI (McDonagh, Palma, Trull, et al., 1982). Later on, another method based on the correction of the HPLC chromatogram peak areas according to the different relative molar absorption coefficients of bilirubin PI was established, but this method was not tested on the clinical samples (Itoh et al., 1999). Latest research from Jašprová and co-workers established a sensitive LC-MS/MS method for simultaneous determination of LR in HSA spiked with BR when exposed to continuous PT and in the serum of neonates that undergoes PT to understand the kinetics of bilirubin PI. Surprisingly, very low concentrations of LR 6.4 ± 2.9 μmol/L were observed in the serum of neonates, despite a dramatic decrease in unconjugated BR concentrations. When compared to the spiked HSA, LR was produced at a 24% yield from unconjugated BR, giving LR concentrations of 75 μmol/L and the sum of the LR with unconjugated BR molar concentrations accounted only for 43% of the initial unconjugated BR concentration. After 6h of irradiation LR concentrations decreased to only 3 μmol/L and the mass balance changed dramatically (Jašprová et al., 2020). The possible main factors accounting for the low concentration of LR in clinical samples are certainly the increased excretion of LR and bilirubin photoproducts via the urine and bile (McDonagh, 1985) and the efficient degradation to the secondary photoproducts (most likely tri-, di-, and monopyrroles) (Jašprová et al., 2020). However, no quantitative data focused on the efficiency of LR photoproduction, distribution among different biological compartments, or even its transfer across the blood-brain barrier exist. Difficulties in establishing such an analytical methods are most importantly related to the low stability of bile pigments and their photodegradation products, resulting in both preanalytical as well as analytical problems.

Another studies from Jašprová and co-workers (Jasprova et al., 2016) (Jašprová et al., 2018) focused on *in vitro* effects of bilirubin photo-oxidative products on cell viability using three CNC models (SH-SY5Y a human neuroblastoma line, U-87 a human glioblastoma line, and HMC3 a human microglial line) did not result to any negative effect on the cell viability even when a high concentration of LR (25 μmol/L) was used. A similar observation was explored when performed *in vivo* studies using organotypic rat hippocampal slices, which is more representative of the complex physiologic multicellular environment. Moreover, these findings are consistent with the early studies of Silberberg and co-workers who did not detect any toxic effects of photo-irradiated bilirubin on myelinating cerebellum cultures (Silberberg et al., 1970). Surprisingly, when Jašprová and co-workers focused on the effect of LR on the expression of pro-inflammatory genes (TNF-α, IL-1β, IL-6, and cyclooxygenase-2 (COX-2)), increasing expression of all studied pro-inflammatory genes was observed. LR, although not affecting the viability of neuronal cells (Falcão et al., 2006), can produce pro-inflammatory cytokines (Jašprová et al., 2018a). Collectively, all viability studies demonstrated that short-term exposure to LR did not lead to cell damage or apoptosis.

1.5.2 Bilirubin oxidation products

Photochemical reactions of bilirubin occurring during light exposure lead to the formation of more polar bilirubin oxidative metabolites (Jašprová et al., 2018). Although these photodegradation products are generally regarded as being benign (Stevenson DK et al., 2012), potential biological effects have never been properly investigated. These metabolites are divided into tripyrrolic, dipyrrolic, and monopyrrolic degradation products.

The first group of BR oxidation products are tripyrrolic biopyrrins firstly discovered and studied by Yamaguchi and co-workers in 1994 as diazo-negative pigments. They identified by mass spectroscopy (MS) and nuclear magnetic resonance (NMR) the structure of two metabolites 1,14,15,17-tetrahydro-2,7,13-trimethyl-1,14-dioxo-3vinyl-16H-tripyrrin-8,12-dipropionic acid (biopyrrin a) and1,14,15,17-tetrahydro-3,7,13-trimethyl-1,14-dioxo-2-vinyl-16H-tripyrrin-8,12-dipropionic acid (biopyrrin b) ***(Fig. 9.)*** from the urine of healthy people using anti-bilirubin monoclonal antibody 24G7 (Yamaguchi et al., 1994).

Biopyrrins were observed in higher concentrations in the urine of the patients who underwent laparotomy (Yamaguchi et al., 1994), after acute myocardial infarction (KUNII et al., 2009) and in the urine of mice exposed to social stress (Miyashita et al., 2006). The higher levels of biopyrrins were also found in patients with schizophrenia (Yasukawa et al., 2007) and during pregnancy were related to the smoking of mothers (Matsuzaki et al., 2014). Biopyrrins levels were studied by Vítek and co-workers in subjects with Gilbert syndrome (GS) who demonstrated that mild hyperbilirubinemia protecting from oxidative stress is associated with decreased urinary biopyrrin excretion (Vítek et al., 2007). Moreover, tripyrrols were also confirmed as markers of increased oxidative stress in rats subjected to endotoxin treatment (Yamaguchi et al., 1995) (Yamaguchi et al., 1997) or fenofibrate treatment (Kobayashi et al., 2003), as well as in the hepatic ischemia-reperfusion model in the rat (Yamaguchi et al., 1996).

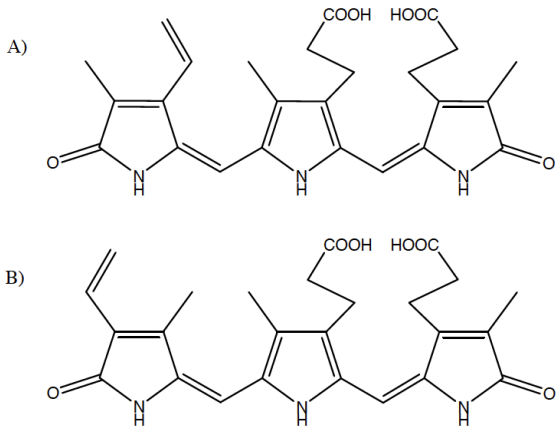

Fig. 9. Structure of biopyrrin a (A) and biopyrrin b (B). Modified from (Jašprová et al., 2018).

The second group of BR oxidation products are dipyrrolic propentdyopents, products of oxidative degradation of BR ***(Fig. 10.)*** firstly discovered by Stokvis and co-workers in 1870 by alkalization of icteral urine when its red coloration was observed and in 1934 by Bengold and co-workers (Dolphin, 1978). In 1957 was described a chromatographic and electrophoretic method to characterize propentdyopents by Heikel and co-workers (Heikel, 1958). Propentdyopents can be possibly determined by Stokvis reaction spectrometrically at 525 nm (Ostrow et al., 1961), and the first discovery of their *in vitro* formation was reported in 1972 (Lightner & Quistad, 1972). Lightner and co-workers observed the production of these dipyrrols in the urine of newborn undergoing PT (Lightner et al., 1984) and the same results were demonstrated by Kunikata and co-workers when also oxidation of BR to propentdyopents during PT in neonates was observed (Kunikata et al., 2000). The latest research from Joerk and co-workers performed experiments when propentdyopents have been considered potential additional effectors in the development of arterial vasoconstriction and are present in the cerebrospinal fluid of patients with subarachnoid hemorrhage (SAH) (Joerk et al., 2019).

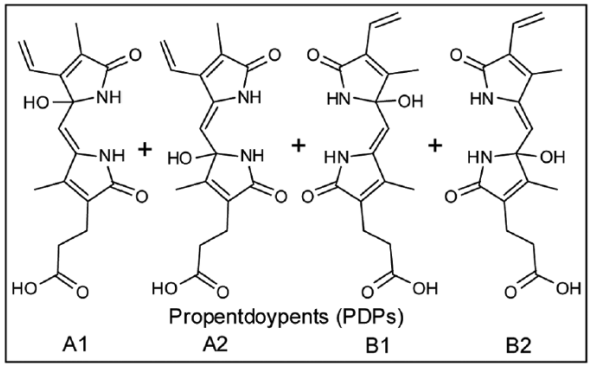

Fig. 10. Structure of propentdyopents. Modified from (Ritter et al., 2016).

The third group of BR oxidation products are mono-pyrrolic BOXes A-D ***(Fig. 11.)***. BOX A, 2-(4-methyl-5-oxo-3-vinyl-1,5-dihydro-2H-pyrrol-2-ylidene)acetamide and BOX B, 2-(3-methyl-5-oxo-4-vinyl-1,5-dihydro-2H-pyrrol-2-ylidene)acetamide were identified as first in the cerebrospinal fluid of patients after SAH with developed cerebral vasospasm (Kranc et al., 2000). Later in 2008 a significant production of BOXes, malondialdehyde, and superoxide dismutase, indicating a potent oxidizing environment was observed by Clark and co-workers in hematomas from the porcine model of intracerebral hemorrhage (ICH). To confirm the formation of bioactive molecules such as BOX-es by oxidation of UCB, Clark and co-workers synthesized in vitro BOX-es by oxidation of UCB at room temperature with a large excess of hydrogen peroxide. These results suggest potent oxidation processes in hematoma when the conversion of bilirubin to BOX-es is associated with a biochemical state that may cause or contribute to pathological sequelae after ICH (Clark et al., 2008).

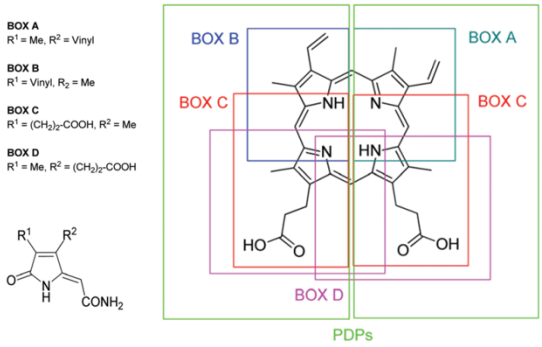

Fig. 11. The structure of bilirubin oxidation end products (BOX A-D) via intermediately formed di-pyrrolic propentdyopents (PDP) depicted in frames. Modified from (Schulze et al., 2019).

Total synthesis and characterization of BOX A and BOX B were performed by Seidel and co-workers *via* a five-step *de novo* synthesis (Seidel et al., 2014), and determination of these compounds in HSA was performed by LC-MS/MS one year later (Seidel et al., 2015). The total synthesis with NMR characterization of the BR oxidation end product BOX C (Z)-3-(5-(2-amino-2-oxoethylidene)-4-methyl-2-oxo-2,5-dihydro-1H-pyrrol-3-yl)propanoic acid and its isomeric form BOX D (Z)-3-(2-(2-amino-2-oxoethylidene)-4-methyl-5-oxo-2,5-dihydro-1H-pyrrol-3-yl)propanoic acid which might not be a direct product of oxidative degradation of BR but could derive from heme were performed by Schulze and co-workers (Schulze et al., 2019). Jašprová and co-workers demonstrated that BOX A and B are toxic *in vitro* only in very high, non-physiological concentrations contrasting to data published in SAH patients (Jašprová et al., 2018).

2 AIMS

The aim of this thesis was to study the biological properties of BR and its most common photoisomer LR. Specifically, our aims were:

- Isolation of LR for *in vitro* studies in cell lines.
- Determination of the BR excretion in different tissues and cell populations.
- Establishment of the intracellular unconjugated BR concentrations thresholds differentiating between anti- and pro-oxidant effects in different cell populations.
- Determination of the BR and LR stability and its effects on metabolic and oxidative stress markers in different cell populations.
- Determination of the effect of BR and LR on the proliferation, morphology, specific gene and protein expression, and differentiation of self-renewing neural stem cells (NSC) derived from human pluripotent stem cells (hPSC)
- Visualization of predicted morphological and genomic changes of NSC by 3D super-resolution microscopy.

3 METHODS

Following list represents the methods used in the submitted dissertation thesis by author. Detail description and other information about particular methods are listed in publications related to this thesis in section “Materials and Methods”.

- Cultivation of immortalized cell lines (HEPG2, SH-SY5Y, MRC5, RAW 264.7)
- Cultivation and differentiation of neurons (CoMo-NSC derived from ESI-017)
- Preparation and purification of BR and LR
- Stability and detection of BR and LR (LC-MS/MS)
- Detection of LR fragments (LC-MS/MS)
- Quantification of TNFα and FGF21 proteins (ELISA)
- Analysis of intracellular metabolites of the TCA Cycle (GC-MS)
- Viability/cytotoxicity measurement (MTT Assay)
- Determination if the glycolytic reserve (Oxygen Consumption Rate - OCR Seahorse)
- Cell cycle analyses (Flow cytometry)
- RNA isolation and Real-Time qPCR
- Western blot analyses
- DNA damage analyses (Comet Assay)
- Immunofluorescence and 3D Cell Imaging (fluorescent microscopy)
- Statistical analyses

4 RESULTS

The results of this thesis are presented in the form of four original manuscripts focused on BR and LR metabolism. Each publication is separately discussed in the context with current literature.

1 BLANKESTIJN, Maike, Ivo P. VAN DE PEPPEL, Aleš DVOŘÁK, **Nikola CAPKOVÁ**, Libor VÍTEK, Johan W. JONKER & Henkjan J. VERKADE.
Induction of fecal cholesterol excretion is not effective for the treatment of hyperbilirubinemia in Gunn rats.2021, Pediatric Research 89, 510–517.

2 BIANCO, Annalisa, Aleš DVOŘÁK, **Nikola CAPKOVÁ**, Camille GIRONDE, Claudio TIRIBELLI, Christophe FURGER, Libor VITEK, and Cristina BELLAROSA.
The extent of intracellular accumulation of bilirubin determines its anti- or pro-oxidant effect. 2021, International Journal of Molecular Sciences 21, no. 21: 8101.

3 DVOŘÁK, Aleš, Kateřina POSPÍŠILOVÁ, Kateřina ŽÍŽALOVÁ, **Nikola CAPKOVÁ**, Lucie MUCHOVÁ, Marek VECKA, Nikola VRZÁČKOVÁ, Jana KŘÍŽOVÁ, Jaroslav ZELENKA, Libor VÍTEK.
The effects of bilirubin and lumirubin on metabolic and oxidative stress markers. 2021, Frontiers Pharmacology 12, 567001.

4 **CAPKOVÁ, Nikola,** Veronika POSPÍŠILOVÁ, Veronika FEDOROVÁ, Jan RAŠKA, Kateřina POSPÍŠILOVÁ, Matteo DAL BEN, Aleš DVOŘÁK, Jitka VIKTOROVÁ, Dáša BOHAČIAKOVÁ, and Libor VÍTEK.
The effects of bilirubin and lumirubin on the differentiation of human pluripotent cell-derived neural stem cells. 2021, Antioxidants 10, no. 10: 1532.

5 DISCUSSION

The research of a presented thesis was focused on the understanding of the fecal cholesterol excretion related to the treatment of the hyperbilirubinemia in Gunn rats and to the biological properties of BR and its photo-oxidation products, which might have clinical relevance in phototherapy-treated hyperbilirubinemic neonates.

During the last decades, BR was determined as an important bioactive molecule, with substantial toxic effects when accumulated in high concentrations within the human body (Watchko & Tiribelli, 2013). However, mildly elevated systemic BR concentrations (such as in Gilbert syndrome) may protect against various oxidative stress-mediated and metabolic diseases including type 2 diabetes, cardiovascular diseases, or metabolic syndrome (Bosma et al., 1995) (Vítek, 2012). To understand the mechanism of BR excretion in different tissues and cells that can give us important data and information for possible therapeutic lowering of hyperbilirubinemia we tested the hypothesis that stimulation of fecal neutral sterol (FNS) excretion lowers total plasma bilirubin (TB) in hyperbilirubinemic Gunn rats *in vivo* in our paper "**Induction of fecal cholesterol excretion is not effective for the treatment of hyperbilirubinemia in Gunn rats**". Gunn rats are a mutant strain of Wistar rats that due to the deficiency of UGT1A1 activity exhibit lifelong nonhemolytic unconjugated hyperbilirubinemia inherited as an autosomal recessive trait. These rats are used as only natural mutant model for the studies that could provide important information on BR toxicity and have helped in developing new therapeutic modalities for hyperbilirubinemia, including cell transplantation and gene therapies (Roy-Chowdhury et al., 2020). In Gunn rats, around 2 - 15% of intestinal unconjugated BR originates from biliary disposal, while 85 - 98% is derived from transintestinal unconjugated BR excretion, which is stimulated by enhancement of the fecal fatty acid excretion, which makes transintestinal bilirubin excretion the major route of unconjugated BR disposal in Gunn rats (Kotal et al., 1997) (Nishioka et al., 2003) (Cuperus et al., 2009). However, the underlying mechanism of these processes is not fully understood.

Since transintestinal excretion route is present for both cholesterol (TICE) and unconjugated BR (Kotal et al., 1997) (de Boer et al., 2018) (Hafkamp et al., 2006) we assumed that TICE - stimulated treatment could affect unconjugated BR excretion. Earlier studies showed that plasma unconjugated BR decreased by administration of a high-fat diet (HFD) and/or the lipase inhibitor orlistat in Gunn rats (Nishioka et al., 2003) (Cuperus et al., 2011) and the increase in fecal fat excretion was correlated to the decrease in plasma unconjugated BR levels (Hafkamp et al., 2005) (Hafkamp et al., 2006). By use of radiolabelled BR, the decrease of unconjugated BR upon orlistat was observed due to an increase in transintestinal excretion (Hafkamp et al., 2006) and feeding HFD to mice enhanced TICE, resulting in increased fecal neural sterol (FNS) excretion (van der Velde et al., 2008). Therefore, a possible increase in fecal unconjugated BR and subsequent decrease in plasma unconjugated BR levels upon higher intestinal fat concentrations could be the results of unconjugated BR "capturing" by fatty acids, meaning that the reabsorption of unconjugated BR is decreased upon its association with non-absorbed fat in the intestinal lumen (Nishioka et al., 2003) (Bulmer et al., 2013). However, our results showed that the transintestinal excretion pathways for cholesterol and for unconjugated BR are not quantitatively linked.

Since liver X receptor (LXR) and farnesoid X receptor (FXR) are involved in the regulation of the hepatic and intestinal cholesterol metabolism we inhibited intestinal cholesterol absorption by its inhibitor ezetimibe (EZE) and stimulated TICE via LXR and FXR. However, our observation resulted in the conclusion that neither stimulation of FNS excretion nor LXR or FXR stimulation exerts hypobilirubinemic effects in Gunn rats, however, fecal unconjugated BR excretion was increased. The fecal unconjugated BR excretion only accounts for an estimated ~ 50% of TB turnover (van der Veere et al., 1996) and we cannot definitively determine whether the increase was caused by increased transintestinal unconjugated BR secretion, decreased transintestinal unconjugated BR reabsorption, or decreased intraluminal (microbial) unconjugated BR degradation due to not proper quantitative estimation of unconjugated BR turnover. When compared to the Gunn rats treated with a FXR agonist obeticholic acid (OCA) with or without EZE, the lower biliary bile acid concentrations and a more hydrophilic profile since hydrophilic muricholic bile acids inhibit intestinal cholesterol absorption, and promote FNS excretion. These observations support another previous study when the underlying mechanism by which OCA increases FNS excretion in mice has been suggested to be mediated by a smaller and more hydrophilic bile acid pool (de Boer et al., 2017). Moreover, simultaneous treatment with OCA and EZE slightly increased net intestinal cholesterol excretion further than either treatment alone. Therefore, most of the OCA effects are mediated through decreased cholesterol absorption in Gunn rats, a small part of the effects could be due to direct stimulation of TICE.

Interesting data were observed while Gunn rats treated with the liver X receptor agonist T0901317 (T09), resulting to increased bilirubin and severely increased triglycerides (TG) levels in plasma followed by optical yellow - coloured and turbid appearance. The possible effect should be explained by the data from another study when has been demonstrated that plasma TG levels > 12 mmol/L increase hemolysis, possibly due to increased membrane instability of erythrocytes (Dimeski et al., 2005) since BR is a degradation product of heme metabolism and the presence of increased hemolysis due to hypertriglyceridemia upon T09 treatment is supported by decreased plasma-free haptoglobin concentrations which is a marker for intravascular hemolysis (Shih et al., 2014). However, we did not have proper samples to perform another red blood cell analyses to determine whether hemolysis had been induced. Collectively, our data suggest that FNS excretion, LXR, or FXR activation do not result in a hypobilirubinemic effect in Gunn rats and the link between the regulation of transintestinal excretion of cholesterol and plasma unconjugated BR concentrations is not present.

In our second paper "**The extent of intracellular accumulation of bilirubin determines its anti- or pro-oxidant effect**" we aimed to establish the intracellular unconjugated BR concentrations thresholds differentiating between anti- and pro-oxidant effects in vitro on the cells derived from the normal human kidney (HK2), murine endothelium (H5V), human hepatoblastoma (HepG2) and human neuroblastoma (SH-SY5Y) since intracellular unconjugated BR concentration was found to be cell-specific due to several factors including the extent of uptake, excretion, and metabolic transformation, with each of these steps differing in various organs. Our results showed that HepG2 cells have the lowest concentrations, while the SH-SY5Y are the most sensitive. As expected, the HepG2 cell

line was less sensitive to unconjugated BR toxicity, even at the highest concentration. Conversely, the neuronal cells appeared the most sensitive since cytotoxicity started at the lowest unconjugated BR concentration while HK2 and H5V showed an intermediate behaviour.

Since cells developed multiple systems (such as enzymes with antioxidant actions including catalase and superoxide dismutase (SOD) which together convert superoxide to water) to protect against ROS, the principal endogenous intracellular antioxidant cytoprotective molecule is regarded as Glutathione (GSH). However, BR has been demonstrated to be a powerful antioxidant substance in *in vitro* studies (Gopinathan et al., 1994) (Farrera et al., 1994) (Marilena, 1997), suppressing oxidation more strongly than many other antioxidants, (Stocker, Yamamoto, et al., 1987b) (Stocker, Glazer, et al., 1987) (T.-W. Wu et al., 1991). *In vitro* studies focused on cells while depletion of GSH or bilirubin indicate that bilirubin is of comparable importance to GSH in cytoprotection (Barañano et al., 2002) (Gopinathan et al., 1994). Since bilirubin has the most potent superoxide and peroxide radical scavenger activities (Farrera et al., 1994) its potent physiological antioxidant action is further amplified by its oxidation to biliverdin and then recyclate by BVR back to BR (Barañano et al., 2002). Doré and Snyder with co-workers reported the maximal neuroprotective effects of BR in hippocampal cultures when reached at nanomolar concentrations (10–50 nM), while at higher concentrations the prooxidant effects of BR were observed (Dore & Snyder, 1999) and almost similar effect was reported by Liu and co-workers in the primary cultures of oligodendrocytes (Liu et al., 2003). Latest research by Zelenka and co-workers present data focused on long-term, mildly elevated BR concentrations resulting to protection of mitochondria and the respiratory chain, with a concomitant decrease of ROS and pro-inflammatory cytokine production (Zelenka et al., 2016). These observations are consistent with another *in vitro* and *in vivo* study, demonstrating the anti-inflammatory effects of BR (Valaskova et al., 2019). However, the exact concentration thresholds between pro- and anti-oxidant effects of BR remain still unclear (Gazzin et al., 2012).

To test the pro-oxidant ability of unconjugated BR, we measured intracellular ROS induction by H2O2 when unconjugated BR did not result in any significant increase in intracellular ROS production in HepG2 cells even at higher concentration. On the contrary, in SH-SY5Y cells resulted in a threefold increase in intracellular ROS production and in H5V cells and HK2 cells doubled the intracellular ROS concentration. These data clearly indicates that each cell type has a different BR threshold switching between beneficial or toxic effects and expand the previous studies by Dore and co-workers with Liu and co-workers (Dore & Snyder, 1999) (Liu et al., 2003). Another experiment focused on the anti-oxidant effect of UCB revealed direct antioxidant activity of lower concentrations of unconjugated BR in all four live cell lines. For HepG2 cells, the dose effect was measured at higher concentrations while for SH-SY5Y cells at the lowest concentrations, whereas in H5V and HK2 cells the antioxidant effect occurred at intermediate lower unconjugated BR concentration. No cytotoxic effect was observed on HepG2 and HK2 cells while it was present at low concentrations on SH-SY5Y and at very high concentrations on H5V cells. Since the cells use multiple systems to protect against ROS overproduction, we measured the total reduced GSH concentrations and SOD activity. While previously observed by Giraudi and co-workers that unconjugated BR modulated the GSH concentration in neuroblastoma cells through the induction of the System Xc-increasing cysteine uptake and intracellular GSH content

(Giraudi et al., 2011) our results confirmed no effect of unconjugated BR on GSH concentration except in SH-SY5Y cells where the concentration increased upon a lower UCB treatment. In addition, the induction of SOD activity was observed in the H5V, HK2, and SH-SY5Y cells at its unconjugated BR pro-oxidant/cytotoxic concentrations while in HepG2 cell line was not affected.

While a mild elevation of BR concentration is associated with anti-oxidant effects, severe hyperbilirubinemia can cause a permanent neurological damage in neonates. Although, PT is worldwide used as the golden standard for the treatment of neonatal jaundice, the biological properties of BR photoisomers and their oxidation products have not properly been investigated. However, the still scarce data obtained until now suggests some biological activity of these products (Jašprová et al., 2018), there is still a lack of complex data and research focused on these molecules. The main reason should be the stability of these photo-sensitive molecules and the difficulty in the preparation processes of bilirubin PI in their pure forms.

In our paper "**The effects of bilirubin and lumirubin on metabolic and oxidative stress markers**" we aimed to compare the effects of BR and LR, the major BR photo-oxidation product, on metabolic and oxidative stress markers. The biological activities of these pigments were investigated on human neuroblastoma SH-SY5Y cells, human hepatoblastoma HepG2 cells, fibroblast-like MRC5 cells from human lung tissue, and murine macrophage-like RAW 264.7 cells with a focus on mitochondrial respiration, substrate metabolism, ROS production, and the overall effects on cell viability. The stability of LR and BR in HSA and standard medium were tested before any experiments with the biological samples. The results showed relatively fast degradation of LR in both relevant biological matrices and the remaining LR level after the experiment in medium and in HSA. In contrast, BR was stable during the entire experiment. Due to the remaining concentrations of LR from previous experiment, we focused on its stability under different oxygen conditions (normoxia 21% O_2 and hypoxia 1% O_2) when the presence of oxygen contributed to LR degradation beginning only early after the start of incubation. The rate of degradation during this time was much faster under normoxic conditions and these results suggest that the degradation of LR can be triggered by higher oxygen concentration.

When focused on the effect of LR and BR on cell viability, LR was found to be much less toxic and had no effect on the viability in all four cell lines even in the highest concentrations, while all the BR concentrations were negatively correlated with cell viability. Cytotoxicity of BR was compromised by the cellular glycolytic reserve, which indicates the capability of a cell to respond to an energetic demand as well as how close the glycolytic function is to the cell's theoretical maximum. The results showed that the most affected cell line was HepG2 (***Annex 3 – Fig. 6F***). Production of mitochondrial superoxide was measured for both lower and higher concentrations of BR and LR since the highest concentration was too apoptotic and cell debris interfered with the determination of mitochondrial superoxide. Only the higher concentrations of LR or BR caused a significant drop in superoxide production in HepG2 and SH-SY5Y cells while in MRC5 cells, even the lower concentrations were significantly efficient. Therefore, both LR and BR were almost equally capable of scavenging mitochondrial superoxide.

Since previous studies had demonstrated an inhibitory role of BR on mitochondrial respiration (Mustafa et al., 1969) (Noir et al., 1972), (Almeida & Rezende, 1981) (Rodrigues, Solá, & Brites, 2002) we focused on the effects of LR in comparison to BR on mitochondrial respiration in our cell lines incubated overnight with BR and LR. We observed no changes in respiration except for the higher concentrations of BR which decreased both basal as well as maximal respiration and indicated an overall depression of mitochondrial respiration. The ratio of maximal to basal respiration, corresponding to the respiratory capacity (Brand & Nicholls, 2011), differed for each cell line with no significant changes between the controls and treated cells. Under the serum – free conditions in HepG2 and SH-SY5Y cell lines no effect on the basal and maximal respiration was observed.

An important finding was observed for the LR effect on oxidative stress since BR is known to be one of the most potent endogenous antioxidants (Stocker, Yamamoto, et al., 1987). The anti-oxidant capacity (AOX) was tested in the different biological matrices in the following experiments. First, BR and LR capability to scavenge peroxyl radicals in HSA with increasing concentrations was tested. Interestingly, LR had the same AOX as BR despite its degradation compared to vitamin E analog – Trolox in the same concentration. Since LR instability, the AOX of LR solutions with its spontaneous degradation was also tested and resulting in the decrease of AOX after 24 h approximately to 50% of the initial value. Moreover, substantial antioxidant effect of LR was observed in our cell models despite its marked degradation, suggesting a marked ROS - scavenging activity of LR degradation products. Nevertheless, in preventing lipoperoxidation, LR was much less efficient most likely due to its lower lipophilicity.

Since BR has impact on mitochondrial metabolism, we focused also on the possible effects of LR and BR on the production of intracellular metabolites of the TCA cycle well known for their ability to affect energy balance and to modulate multiple cellular functions as well being linked to oxidative phosphorylation (Martínez-Reyes & Chandel, 2020). Both BR/LR did not have any marked effect at lower concentrations in MRC5 and HepG2 cells; while in SH-SY5Y cells the concentrations significantly decreased in the presence of both compounds. A different response was observed with BR at higher concentration when most of the metabolites were significantly reduced in all cell lines; whereas virtually no effect was observed in cells exposed to LR (***Annex 3 – Fig. 10***.). These data are consistent with the previous reports of the impact of BR on the mitochondrial metabolism and morphology demonstrated mostly in brain cells (Mustafa et al., 1969) (Almeida & Rezende, 1981) (Rodrigues, Solá, & Brites, 2002b) while no harmful effect present for LR. However, these effects on mitochondrial metabolism were beneficial in the other tissues such hearth and liver (Mustafa et al., 1967) (Stumpf et al., 1985), same for the effect of BR on the mitochondrial function in adipocytes (Gordon et al., 2020). These data suggest the complex cell-specific and concentration-dependent effects of BR and its derivates in specific conditions.

Additionally, we observed that BR behave as a pro-inflammatory molecule in the macrophage-like RAW264.7 cells, while only a mild and insignificant effect was observed for LR. This contrasts with a study performed by Jašprová and co-workers on different cell models of CNS origin indicating substantial cell viability of BR/LR-induced pro-inflammatory effects (Jašprová et al., 2018). The possible explanation for this observation may be linked to the BR-induced TCA cycle dysregulation known to affect the inflammatory status, NO production, as well as post-

translational acetylation (Williams & O'Neill, 2018), since both treatments lead to a decrease in NO availability. Since BR is known for its ability to scavenge NO by forming N-nitroso derivatives (Barone et al., 2009), the same might also be possible for LR. Moreover, BR inhibits inducible NO synthase to prevent cells from its production of large amount of NO (Zucker et al., 2015), while LR may also act in a similar manner.

Based on the recent observations by Jašprová and co-workers who demonstrated the striking upregulation of proinflammatory cytokines in organotypic rat hippocampal slices after short-term exposure to LR (Jašprová et al., 2018), we hypothesized that photoproducts of BR could possibly affect early human neurodevelopment. In our paper "**The effects of bilirubin and lumirubin on the differentiation of human pluripotent cell-derived neural stem cells**" we aimed to compare the effects of BR and LR on the proliferation, differentiation, morphology, and specific gene and protein expressions of self-renewing neural stem cells (NSC) derived from human pluripotent stem cells (hPSC) which has the ability to self-renew and terminally differentiate into neurons and glia, and thus they represent a biologically and developmentally relevant surrogate human model to study the influence of the potentially biologically active compounds on these processes. In the initial phase of our studies, we focused to assess the possible cytotoxic effects of both BR and its major photoisomer LR. We observed significant decrease in the viability/metabolic activity of the cells exposed to BR within the whole range of tested concentrations. Compared to LR, the effect was much lower, and only visible at the highest concentration indicating the much higher toxicity of BR on NSC, thus its cytotoxic effect on the CNS when severe neonatal hyperbilirubinemia occurs in neonates. Previous studies by Genc and co-workers shown that exposure to increasing concentrations of unconjugated BR is cytotoxic to rat oligodendrocytes and increase its apoptosis *in vitro* (Genc et al., 2003). Several additional studies have shown the cytotoxic and pro-apoptotic effects of BR on neuronal cultures (Rodrigues, Solá, & Brites, 2002b) (Silva et al., 2002) (Rodrigues, Solá, Silva, et al., 2002) (Kumral et al., 2005). Although under our study conditions no significant changes in DNA damage were observed, while only a negligible modulation of the cell cycle of treated NSC exposed to BR was present.

To explore the possible effects on the protein expressions of the apoptotic or DNA damage-related markers we analysed NSC treated with both pigments. While BR exposure induced apoptotic and DNA damage markers, LR exposure in clinically relevant concentrations exerted protective effects against these changes. During the testing of toxicity, we noticed a significantly changed undifferentiated arrangement and acquired a different phenotype with increasing concentrations of LR. The neuroepithelium forming the neural tube represents the first polarized single-cell layer with a central lumen and cells displaying apicobasal polarity during the onset of neural differentiation (Wilson & Stice, 2006). These processes are mimicked under *in vitro* conditions by the radially organized neuroepithelial cells differentiated from hPSC so-called neural rosettes, a flower like structures, that represent the niche from which NSC are isolated (Wilson & Stice, 2006) (Banda et al., 2015) (Grabiec et al., 2016) (Fedorova et al., 2019). Such polarity ensures a different distribution of signalling molecules as well as of junction proteins (Miyamoto et al., 2015) (Banda et al., 2015) (Grabiec et al., 2016). When focused to Western blot analysis of transcription factors including those expressed upon differentiation of hPSC towards neuroectoderm and signalling

pathway important for neural cell differentiation from NSC we observed interesting data in expressions of NSC-specific markers upon exposure to LR (***Annex 4 – Fig. 3***.). These data strongly suggested that LR-treated self-renewing NSC acquire a significantly different morphology reminiscent of immature rosettes, with apically localized cell polarity proteins. Surprisingly, our study demonstrated for the first time that LR induces NSC to repolarize and that this induction is dose-dependent. Moreover, these repolarized NSC cultures, expressed higher amounts of phosphorylated ERK, important for the process of neurogenesis (possibly as a positive feedback mechanism), as well as showing an altered expression of NSC-specific. These data suggest that the potential to affect the identity and polarity of NCS during early human neural development by LR resulting to possible clinical relevance while aggressive PT used on preterm neonates is often accompanied by serious adverse effects (J. Wang et al., 2021) and the processes of neurogenesis and neurodevelopment are impaired in these neonates (Rice & Barone, 2000), which may even be exacerbated by BR photo-oxidation products generated during PT.

Lastly, we assessed the capacity of BR or LR to affect the terminal differentiation of NSC since previous studies by Brites and co-workers have shown that moderate to severe hyperbilirubinemia could induce neurological dysfunction and potentially impair brain myelination with long-term sequelae, particularly in preterm infants (Brites & Fernandes, 2015) and no possible effects of LR or other BR photo-oxidation products have been reported yet. We focused on the gene expression of selected differentiation-associated markers. The expression of NSC had gradually increased for glial markers as well as neuronal markers while there were no changes in the expression of the neural stem cells (***Annex 4 – Figure 4.***). However, we did not observe any significant changes in the expression after the treatment with BR and LR which is a notable observation since just a short-term exposure led to significant changes in these markers.

6 SUMMARY

Bilirubin has been for a long time considered only as a toxic waste product. But recent findings well documented this bioactive molecule as a powerful endogenous antioxidant with immunomodulatory, anti-inflammatory, antiproliferative and cell-signalling properties.

In the presented thesis, we investigated the kinetics and biological properties of BR compared to its photo-oxidation products, which might have clinical relevance in hyperbilirubinemic neonates treated by intensive phototherapy with blue-green light.

In Gunn rats, which represent the natural *in vivo* model for severe unconjugated hyperbilirubinemia, it is well known that only a tiny fraction of intestinal unconjugated BR originates from biliary disposal. At the same time, the biggest part is derived from transintestinal unconjugated BR excretion, which is stimulated by enhancing fecal fatty acid excretion, which makes transintestinal bilirubin excretion the major route of unconjugated BR disposal. Since transintestinal excretion also occurs for cholesterol, we hypothesized that increasing fecal cholesterol excretion and/or transintestinal excretion could also enhance fecal unconjugated BR disposal and subsequently lower plasma unconjugated BR concentrations. However, our data do not support the regulation of transintestinal excretion of cholesterol and bilirubin. Moreover, the FNS excretion, liver X receptor or farnesoid X receptor activation do not results in a hypobilirubinemic effect and has no potential for the therapy of unconjugated hyperbilirubinemia.

Unconjugated BR has the ability to diffuse into any cell while in mildly elevated concentrations is protective from various oxidative stress-mediated diseases. Hence, each cell has to maintain its intracellular concentration of unconjugated BR below the toxic threshold which is regulated by its intracellular metabolism. To understand these processes we performed an *in vitro* study using different human and murine cell lines exposed to increasing unconjugated BR concentration to find the thresholds differentiating between pro-oxidant and anti-oxidant effects while finding that a low concentration of unconjugated BR resulted in anti-oxidant effect while higher concentrations resulted to the pro-oxidant or cytotoxic effects in all studied cell lines. Our results expand and better substantiate that each cell type has a different bilirubin threshold switching between the beneficial and toxic effects of bilirubin. Total unconjugated BR concentration treatment is an uncertain predictor of its biological effects because intracellular levels of unconjugated BR are modulated by its oxidation, conjugation, and export from the cells by membrane ABC transporters. The ability to measure real unconjugated BR concentration in the cells helps to better understand cytotoxicity induced by unconjugated BR as well as its protective effects.

While a mild elevation of BR concentration is associated with anti-oxidant effects, severe hyperbilirubinemia can cause permanent neurological damage in neonates. Although, the golden standard of the treatment of severe unconjugated hyperbilirubinemia, the biological properties of BR photo-oxidation products remain still unknown. Since intracellular metabolic impact of BR photoisomers has never been properly investigated, although our previous

data suggest their biological importance we compared BR and its major photo-oxidation product LR on the metabolic and oxidative stress markers resulting in the data when LR was found to be much less toxic while still maintaining a similar anti-oxidant capacity in the serum as well as suppressing activity leading to the mitochondrial superoxide production. However, LR was less efficient in preventing lipoperoxidation due to its lower lipophilicity. Additionally, BR was found to behave as a pro-inflammatory molecule while only a mild and insignificant effect was observed for LR. Nevertheless, our data point to the biological effects of BR and its photo-oxidation products, which seem to have clinical relevance in phototherapy-treated hyperbilirubinemic neonates and adult patients.

Since aggressive PT used on preterm neonates is often accompanied by serious adverse effects and the processes of neurogenesis and/or neurodevelopment are impaired in these neonates we aimed to understand the possible impact of BR and LR on these processes using an *in vitro* model of neural stem cells. When compared to BR, LR exerted lower cytotoxicity on self-renewing neuronal stem cells. This dose-dependent effect was accompanied by mildly elevated pro-apoptotic markers for BR. Another interesting dose-dependent effect was observed for the morphology inducing cells to form highly polarized structures with lower expressions of some NSC-specific markers when treated by LR. Our data clearly indicate that BR and LR play a role in the earlier phases of differentiation, an influence which, however, was later lost and despite visible changes in the morphology, at the level of the terminal differentiation, no major changes can be detected toward neuronal and glial cell types. However, LR has the potential to affect the polarity and identity of NSC during early human neural development. This observation may be of clinical importance since cellular polarity plays a significant role during the development of the CNS.

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8 LIST OF ABBREVIATIONS

ABC	ATP-binding cassete
ABCG5/8	ATP-binding cassette (ABC) transporters G5 (ABCG5) and G8 (ABCG8)
AOX	anti-oxidant capacity
Bf	free unconjugated bilirubin
BR	bilirubin
BV	biliverdin
BVR	biliverdin reductase
Ca^{2+}	calcium ions
cAMP	cyclic adenosine monophosphate
CNS	central nervous system
CO	carbon monoxide
DNA	deoxyribonucleic acid
EZE	ezetimibe
Fe^{2+}	ferrous ion
FNS	fecal neutral sterol
FXR	farnesoid X receptor
G6PD	glucose-6-phosphate dehydrogenase
GSH	glutathione
H5V	hearth endothelial cells
HDL	high density lipoprotein
HepG2	hepatic cells
HFD	high fat diet
HK2	kidney tubular cells
HO	heme oxygenase
HPLC	high-performance liquid chromatography
hPSC	human pluripotent stem cells
HSA	human serum albumin
ICH	intracerebral hemorrhage
IL-1β	interleukin 1 beta
IL-6	interleukin 6
iUCB	intracellular unconjugated bilirubin
LC-MS/MS	liquid chromartography – mass spectrometry
LDL	low density lipoprotein
LR	lumirubin
LXR	liver X receptor

MRC5	fibroblast-like cells
MRP	multidrug resistance-associated protein
MS	mass spectroscopy
NADPH	nicotinamide adenine dinucleotide phosphate
NMR	nuclear magnetic resonance
NO	nitric oxide
NPC1L1	Niemann-Pick C1-Like 1
NSC	neural stem cells
O	oxygen
OATP	organic anion transporting polypeptides
OCA	obeticholic acid
PI	photoisomer
PT	phototherapy
RAW 264.7	murine macrophage-like cells
ROS	reactive oxigen species
SAH	subarachnoid hemorrhage
SOD	superoxide dismutase
SY5Y	neuronal cells
T09	liver X receptor agonist T0901317 (T09)
TB	total bilirubin
TCA	tricarboxylic Acid Cycle
TG	triglycerides
TICE	Transintestinal cholesterol excretion
TNF- α	tumor necrosis factor alpha
UGT1A1	UDP- glucuronosyl transferase 1A1 isoform