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"Determinants of bilirubin neurotoxicity by an *in vitro* molecular approach"

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To my family, friends and all the people who supported me on this big challenge…

CONTENTS

ABBREVIATIONS

ABE	Acute bilirubin encephalopathy
ABR	Auditory brainstem response
APE1/Ref1	Apurinic/apyrimidinic endonuclease 1/redox effector factor
ATP	Adenosine triphosphate
BBB	Blood Brain Barrier
Bf	Free unconjugated bilirubin
BIND	Bilirubin induced neurological dysfunction
BSA	Bovine serum albumin
BSO	Buthionine sulfoximine
BT	Total unconjugated bilirubin
CNS	Central nervous system
CP	Choroid plexus
CSF	Cerebrospinal fluid
DEM	Diethyl maleate
DMSO	Dimethyl sulfoxide
DTNB	5',5'- dithiolbis-2-nitrobenzoic acid
ER	Endoplasmic reticulum
Erg-1	Early growth response 1
FACS	Fluorescence activated cell sorting
FCS	Fetal calf serum
HO-1	Heme Oxigenase 1
4F2hc	4F2 cell-surface antigen heavy chain
GCLC	Gamma Cysteine Ligase – Catalytic Subunit
GCLM	Gamma Cysteine Ligase – Modifier Subunit
GSH	Reduced glutathione
GSSG	Oxidized glutathione
HSA	Human serum albumin
H_2DCFDA	2',7'- dichlorodihydrofluorescein diacetate
MEF	Mouse embryo fibroblast
MRI	Magnetic resonance imaging
MRP1	Multidrug resistance - associated protein 1
MDR1	Multidrug resistance protein 1
MTT	methylthiazoletetrazolium
NADPH	Nicotinamide – adenine dinucleotide phosphate
NMDA	N- methyl – D – aspartate receptor

NO	Nitric Oxide
NQO1	NAD(P)H Quinone Oxidoreductase 1
PBS	Phosphate-buffered saline
PTEN	Phosphatase and tensin homolog
PMSF	Phenylmethylsulphonylfluoride
SLC7A11	Solute carrier family 7, (cationic amino acid transporter, y+ system) member 11
SLC3A2	Solute carrier family 3 (activators of dibasic and neutral amino acid transport
xCT	Cystine/glutamate transporter

ABSTRACT

Unconjugated Bilirubin (UCB) is the final product of the heme catabolism. The high serum UCB concentrations in the first days of life of the newborns, due to immature mechanisms for hepatic uptake, conjugation and biliary secretion, is called physiological neonatal jaundice. This common condition is generally a benign and transient phenomenon, but in some cases the hyperbilirubinemia can progress to bilirubin encephalopaties ranging from minimally neurological injury to severe and permanent neurodevelopmental dysfunction.

In the present thesis the SH-SY5Y neuroblastoma cell line was used to approach the molecular events associated to bilirubin neurotoxicity and highlight the biochemical and molecular events that are induces in the neurons when get contact with the UCB.

Depending on the bilirubin concentration and the time of exposure to UCB, we were able to define experimental setups for the study of bilirubin resistance and bilirubin toxicity.

Using the model to study bilirubin resistance, it was demonstrated that the resistance is not entirely achieved by limiting the entrance or increasing extrusion of the pigment from the cell, but rather by enhancing the cellular defensive mechanisms, in particular against the oxidative stress. This was achieved by increasing the intracellular glutathione content *via* the specific induction of the genes and activity of the System X_c^-. Furthermore, the cells exposed to bilirubin over-expressed several additional genes that encode for important antioxidant and detoxifying proteins like Heme Oxygenase-1 and NAD(P)H:quinone oxidoreductase 1.

As far as the mechanisms of bilirubin neurotoxicity, we showed that UCB exposure lead to the induction of the intracellular ROS accumulation. Moreover, the data presented report evidences that the bilirubin toxicity could be displayed by a mechanism of excitotoxicity carried out by the cellular release of glutamate.

Further studies will be necessary to elucidate the molecular mechanisms by which bilirubin produces neurotoxicity and to understand how the cells avoid the damage. The information presented here could contribute to the identification of targets to avoid the bilirubin damage.

Introduction

INTRODUCTION

1. Bilirubin physiology and metabolism

The bilirubin is the oxidative product of the protoporphyrin portion of the heme groups present in different proteins currently found in the human body. Several studies have indicated that the 80% of the bilirubin produced derives from the heme group present in the hemoglobin transported by the red blood cells, 15-20% from the turnover of the myoglobin, cytochroms and other hemoproteins, and less than the 3% from the elimination of immature red blood cells coming from the bone marrow (Ostrow et al., 1962). All these sources let to one healthy adult person produce every day between 250 mg and 400 mg of bilirubin (LONDON et al., 1950).

The heme degradation is an enzymatic process mainly achieved in the spleen and the liver Kupffer cells, the principal places for red blood cells breakdown. The degradation begin when the microsomal heme oxygenase-1 (HO-1) directs the stereospecific cleavage of the heme ring, releasing the iron ion and the tetrapyrrolic chain with the final formation of the Biliverdin and carbon monoxide (figure 1.1). This reaction requires a reducing agent, such as, nicotinamide-adenine dinucleotide phosphate (NADPH) and three molecules of oxygen (Tenhunen et al., 1968). Two different isoforms for HO have been described: the constitutively isoform HO-2, and a inducible isoform HO-1 (Foresti et al., 2004; Rublevskaya and Maines, 1994; McCoubrey, Jr. et al., 1997). Following its synthesis the Biliverdin is subsequently converted in Bilirubin by the cytosolic enzyme biliverdin reductase (BVR), in the presence of the NADPH (Foresti et al., 2004).

The bilirubin molecule has a low aqueous solubility, most probably due to the hydrophobic groups it contains and the internal hydrogen bonding of all its polar groups precluding their interaction with water (Kaplan and Navon, 1982). The analysis of the experimental solubility at neutral pH, determined by chloroform-to-water partition, have indicated that the maximum aqueous solubility of the bilirubin at 25°C and ionic strength 0.15, is about 70 nM (Hahm et al., 1992). Because of its poor aqueous affinity, once released in the blood the bilirubin is tightly, but reversibly, bound to the serum albumin (Weisiger et al., 2001). Carried by the albumin, the bilirubin is transported in the blood to different organs, in particular to the liver where the modification of the molecule continues. Despite of the high affinity of the bilirubin for the albumin, a small portion (less than 0.1%) remains unbound and is known as Free Bilirubin (Bf). The total bilirubin composed by the Free Bilirubin and the Bilirubin bound to albumin conform the Unconjugated Bilirubin (UCB) (Ahlfors, 2001).

When UCB reaches the hepatocyte, it is rapidly dissociated and internalized at the sinusoidal surface of the cell through mechanisms not fully elucidated (Zucker et al., 1999; Cui et al., 2001).

Once inside the hepatocyte the bilirubin is bound to a group of cytosolic Glutathione-S-tranferases (GSTs) that prevents the backflow of the molecule from the cell (Zucker et al., 1995). The bilirubin is then conjugated with one or two glucuronic acid moieties from UDP-glucuronic acid (UDPGA) in a reaction catalyzed by a specific form of the uridine diphosphoglucuronate glucuronosyltransferase (UGT1A1). The mono- and di- glucuronide portions confer to the bilirubin (Conjugated Bilirubin, CB) a high polarity, rendering the molecule water soluble and unable to diffuse across the membranes (Bosma et al., 1994). The CB is then excreted into the bile canaliculus by canalicular multispecific anion transporter (cMOAT), also known as multidrug resistance-related protein 2 (MRP2) (Kamisako et al., 1999). Finally, the CB excreted in bile passes through the small intestine without significant absorption. In the colon, it is both deconjugated, presumably by the bacterial β-glucuronidase, and degraded by other bacterial enzymes to a large family of reduction-oxidation products, collectively known as urobilinoids, which are mostly excreted by feces.
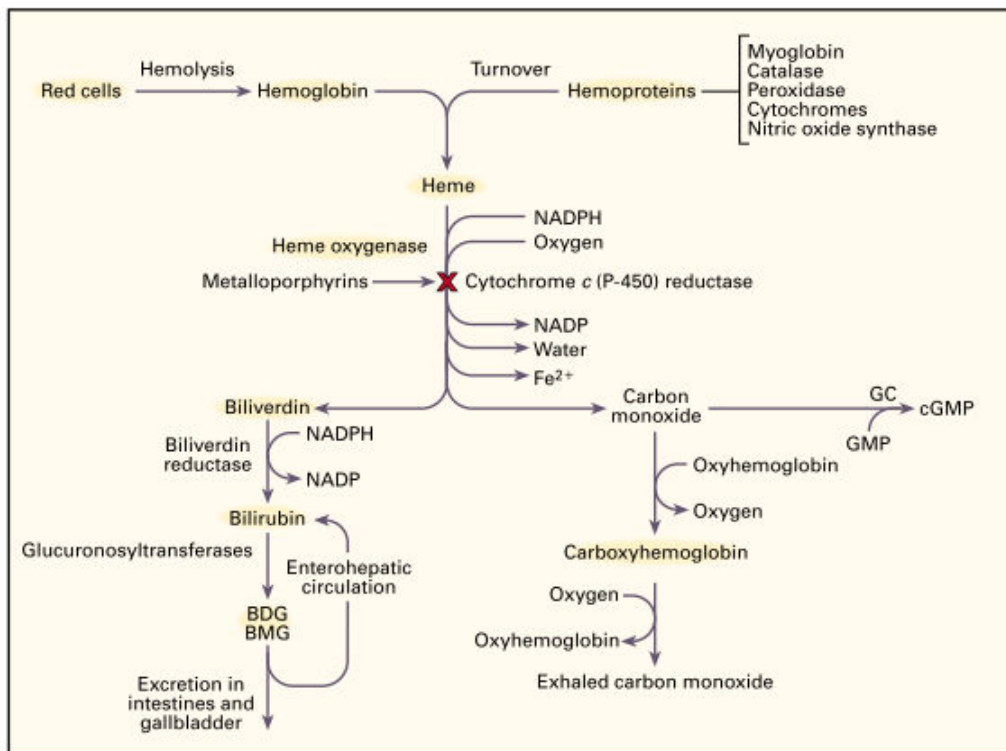
Figure 1.1 – Metabolic pathway of the degradation of the Heme and the bilirubin formation.
Reproduced from Dennery PA,- 2001 *N Engl J Med* 344:581-590.

2. Neonatal hyperbilirubinemia
2.1. The bilirubin in newborns

Neonatal hyperbilirubinemia results from an increased production of bilirubin in the newborn and their limited ability to excrete it. *In utero*, the very limited excretory function of the fetal liver is compensated by active transport of UCB across the placenta to the maternal circulation. At birth, the newborn is suddenly deprived of the placental protection just when a marked increase in catabolism of red-cell breakdown, and consequently, a high load of UCB to the liver is produced. Delayed maturation of hepatic transport processes results in significant retention of UCB even in healthy term neonates. In addition, the neonate lacks anaerobic ileo-colonic flora that convert UCB to urobilinogens, leaving more unmetabolized UCB available for absorption into the portal blood, thus increasing the entero-hepatic circulation of UCB. In normal infants after the age of 1 month, the processes of hepatic uptake, storage, conjugation and biliary secretion of bilirubin have matured to near adult levels (Ahlfors and Wennberg, 2004; Ostrow et al., 2003a). Together, these limitations lead to physiologic jaundice, characterized by a high serum bilirubin concentrations in the first days of life in full-term infants, followed by a decline during the next several weeks to values commonly found in adults. The average full-term newborn infant has a peak serum bilirubin concentration of 5 to 6 mg/dL (86 to 103 μmol/L) around the 5th day from birth. Exaggerated physiologic jaundice occurs at values above this threshold (7 to 17 mg/dL [104 to 291 μmol/L]). Serum bilirubin concentrations higher than 17 mg/dL in full-term infants are no longer considered physiologic, and a cause of pathologic jaundice can usually be identified in such infants (Dennery et al., 2001).

2.2. Bilirubin encephalophaties and Kernicterus

Usually, plasma bilirubin levels peak at less than 10 mg/dL at approximately 1 week of life and return to normal over the next 1–2 weeks. Neonatal physiological jaundice may be aggravated, however, by several abnormalities like the common increase that occur in all hyperhemolytic conditions and deficiency of uridine diphosphate glucuronosyltransferase (UGT1A1), the enzyme required for the conjugation of bilirubin (as in Gilbert syndrome and Crigler–Najjar syndrome) (Dennery et al., 2001; Reiser, 2004). The actual available treatments for this last condition, like phototherapy, exchange transfusion and liver transplantation seem to be efficient but implicate several risks to the patients and reduce a lot the quality of life of patients and their families (Ostrow et al., 2003b).

Bilirubin encephalopathy is usually reversible, particularly at the early stages, characterized by hypotonia, lethargy, poor suckling, and abnormal brainstem auditory evoked potentials (BAEPs). With more severe and/or prolonged jaundice, hypertonia, opisthotonus, a high-pitched cry,

impairment of upgaze or the 'setting sun sign', fever and worsening of the BAEPs may supervene. Later, irreversible neurological signs may develop, ranging from subtle deficits, including delay in motor development, impaired cognitive function, and auditory dysfunction (e.g. auditory neuropathy, hearing loss, and deafness), to more severe extrapyramidal motor, auditory, oculomotor, and cognitive disorders (Ostrow et al., 2003a).

Kernicterus is a devastating, chronic disabling neurological disorder whose central nervous system (CNS) sequelae reflect both a predilection of bilirubin toxicity for neurons (rather than glial cells) and the regional topography of bilirubin-induced neuronal injury that is characterized by yellow staining of the basal ganglia, hippocampus and in the several nuclear clusters of the brainstem and cerebellum observed in infants who died with severe jaundice (Watchko, 2006).

3. Bilirubin neurotoxicity

In the past years, interest in newborn's bilirubin encephalopathy has been reawakened by an increase in its prevalence. For a long time the serum or plasma total bilirubin concentration (TB) has been the standard clinical laboratory test for evaluating neonatal jaundice, associating it with acute bilirubin encephalopathy (ABE) and its sequelae including death, classical kernicterus, or bilirubin induced neurological dysfunction (BIND). In contrast, there is strong evidence suggesting that is the plasma non–protein-bound (unbound or free) bilirubin concentration (Bf), rather than TB, that is more closely associated with central nervous system bilirubin concentrations and therefore ABE and its sequelae (Ahlfors and Wennberg, 2004; Calligaris et al., 2007).

In vitro studies with neurons and astrocytes reveal that neurotoxic effects of UCB develops only near or above the aqueous saturation limit of 70 nM, a range in which only UCB monomers, soluble oligomers, and metastable small colloids are likely to be present (Ostrow et al., 2003b). By contrast, Bf levels well below 70 nM appear to protect CNS cells against oxidative damage and this protection is lost because of the countervailing toxic effects of UCB at higher Bf levels (Dore and Snyder, 1999; Baranano et al., 2002; Ostrow et al., 2003b; Ostrow and Tiribelli, 2003).

4. Molecular basis of neurotoxicity

The real mechanisms by which the bilirubin causes cellular toxicity are not yet completely understood. Because of the lipophilic characteristics of the bilirubin molecule, several works have suggested that the damage is originated at the level of membranes (plasma, mitochondrial, and endoplasmic reticulum (ER)) with resultant perturbations of membrane permeability and function (Rodrigues et al., 2002a; Rodrigues et al., 2002b; Watchko, 2006). These perturbations could contribute to the genesis of neuronal excitotoxicity (Grojean et al., 2001; McDonald et al., 1998),

increased intracellular Ca^{2+} concentration (Brito et al., 2004) and mithocondrial energy failure (DAY, 1954; Ernster and ZETTERSTROM, 1956; Vogt and Basford, 1968; Rodrigues et al., 2000; Oakes and Bend, 2005; Malik et al., 2010).

Excitotoxicity refers to an excessive activation of neuronal amino acid receptors. The specific type of excitotoxicity triggered by the amino acid glutamate is the key mechanism implicated in the mediation of neuronal death in many disorders. The discovery of excitotoxic injury is a major clue in the search for answers to fundamental questions as why neurons die in disease states and what is the precise or critical mechanism of neuronal death. The activation of the NMDA receptor is closely related with the intracellular accumulation of calcium. Excessive accumulation of intracellular calcium is the key observed process leading to neuronal death or injury, and the NMDA receptors activate channels that allow the influx of extracellular calcium (and sodium) (Mark et al., 2001).

Calcium influx *via* NMDA receptors elicits more potent toxicity than other modes of calcium entry. This type of toxicity involves nitric oxide (NO) since treatment with Nitric Oxide Sintetase (NOS) inhibitors, removal of L-arginine or reduced hemoglobin, which scavenges NO, blocks this form of toxicity. Understanding the neurotoxic mechanisms of NO requires uncovering how NO acts on numerous potential targets to which it can initiate neurotoxic cascades (Figure 1.3). NO probably mediates most of its toxic effects through interactions with O_2^- to form peroxynitrite. The best established candidate for the target of NO and peroxynitrite to achieve toxicity is poly(ADP-ribose) synthetase [PARS; also called poly(ADP-ribose) polymerase or NAD+:protein(ADP-ribosyl)transferase]. Another potentially important target of NO-mediated neurotoxicity is mitochondrial respiration. NO binds to mitochondrial complex I and II and cis-aconitase leading to inhibition of oxidative phosphorylation and glycolysis (Yun et al., 1997; Brito et al., 2010). It is also though that UCB interfere with mithocondrial membrane producing permeabilization that result in a perturbation of the cyclosporine A-sensitive large conductance channel. This processes consequently produce electron transport chain dysfunction, such as decreased ATP production and reactive oxygen species production, may lead to further mitochondrial damage, including oxidation of mitochondrial DNA, proteins, and lipids, and opening of the mitochondrial permeability transition pore (Rodrigues et al., 2000).
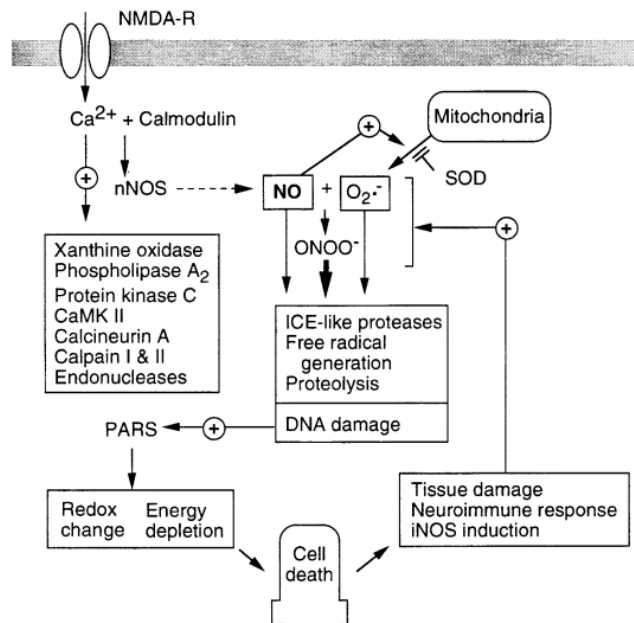
Collectively, these phenomena and downstream events trigger cell death by both apoptosis and necrosis. Process like DNA fragmentation, release of cytochrome c, activation of caspase-3 and cleavage of poly(ADP)ribose polymerase has been described in bilirubin cell death by apoptosis (Rodrigues et al., 2002b; Hanko et al., 2005; Hanko et al., 2006; Malik et al., 2010).

Conjugated bilirubin excreted in bile passes through the small intestine without significant absorption. In the colon, it is both deconjugated, presumably by the bacterial β-glucuronidase, and degraded by other bacterial enzymes to a large family of reduction-oxidation products, collectively known as urobilinoids, which are mostly excreted by feces.

Another possible explanations of the UCB neurotoxicity, at least in part, could be the oxidative damage that was observed on cellular components after UCB treatments at clinical concentrations (Brito et al., 2008). Despite of the cytoprotective properties of free bilirubin levels below aqueous saturation, above this value it enhances the formation of free radical that lead to protein oxidation, lipid peroxidation, increase the amount of intracellular ROS and decrease the glutathione levels. Signs of disturbance of the redox status indicate that oxidative stress is involved, at least in part, in UCB-induced neurotoxicity (Brito et al., 2004; Dore et al., 1999). In addition, recent evidences demonstrated that UCB-mediated apoptosis in Hepa 1c1c7 cells is associated with oxidative stress (Oakes and Bend, 2005) and in HeLa cells, the increase in intracellular reactive oxygen species, due to UCB, activate a signaling pathway involving APE1/Ref-1, Egr-1 and PTEN (Cesaratto et al., 2007).

5. Brain neuroprotective mechanisms

5.1. The blood brain barrier (BBB)

Except for the circumventricular region of the brain, two barriers limit penetration of drugs and other compounds into the CSF and brain parenchyma: the choroid plexus (CP, the blood–CSF barrier) and the brain capillary endothelium (the blood–brain barrier, BBB). The endothelial cells of the BBB have tight junctions and no fenestrae, so they severely restrict both paracellular and transcellular diffusion of many toxic compounds to the adjacent neurons and astrocytes, but might not restrict the free transmembrane diffusion of UCB (Ostrow et al., 2003a; Zucker et al., 1999) (Figure 1.2).
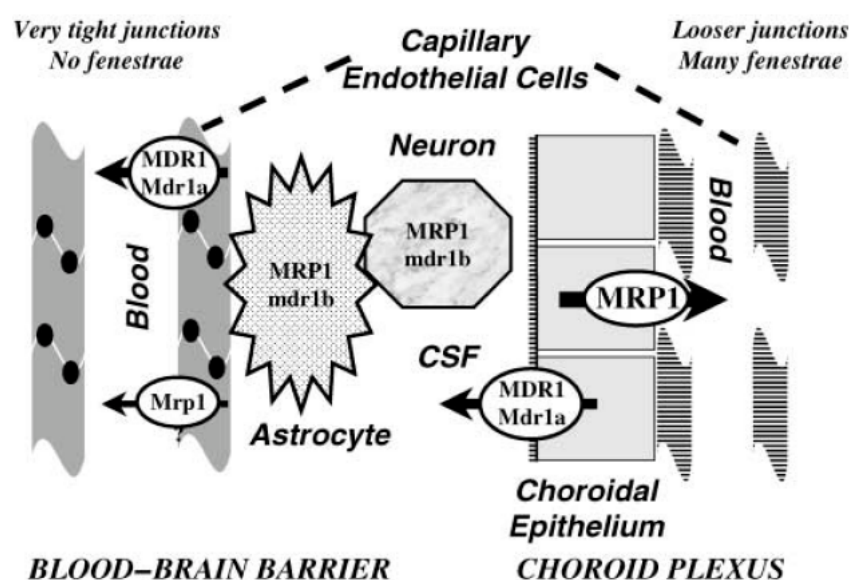
Figure 1.2 The Blood Brain Barrier: Schematic representation of the organization of the brain and its barriers, including the distribution and, where known, polarity (arrows) of the MDR1/Mdr1 and MRP1/Mrp1 transporters in the various cell types. Also indicated are the characteristics of the tight junctions and fenestrae that account for the lower permeability of the endothelium in the blood–brain barrier (left), as compared with the blood–CSF barrier (right). Reproduced from Ostrow, *J.D.-* 2003 *European Journal of Clinical Investigation*, 33, 988–997.

A maturational effect on bilirubin uptake by brain is better documented in experimental animals. In the rhesus monkey, the susceptibility of the brain-stem auditory evoked response to bilirubin is very dependent on gestational age. Ahlfors et al. found that both total and unbound bilirubin levels required to produce neurotoxicity (changes in the auditory evoked response) increased dramatically with increasing gestational age (Ahlfors et al., 1986). Blood–brain barrier permeability to bilirubin in the newborn piglet (2 days) is greater than in the 2-week-old piglet, with the highest concentration found in the subcortical (cerebellum and brainstem)regions at both ages (Lee et al., 1989; Lee et al., 1995).

The transport of bilirubin into brain in the presence of an intact blood–brain barrier presents unique problems since the dissociation rate of the albumin/bilirubin complex is slow and the time of exposure of the capillary endothelium to the circulating bilirubin–albumin is brief (Wennberg, 2000). Newborn serum albumin does not bind bilirubin as well as albumin in adults or older infants (Ahlfors and DiBiasio-Erwin, 1986; Weisiger et al., 2001; Kapitulnik et al., 1975) and may contain many weak competitors for binding, yet to be identified. Such competitive binding of the primary site would displace significant bilirubin to the weaker secondary sites. The dissociation rate of these sites has not been established, but the lower binding constant implies a higher dissociation rate of the secondary binding sites, which would greatly facilitate bilirubin transport into brain. This could explain why many babies develop bilirubin encephalopathy before the primary albumin binding sites are saturated (Wennberg, 2000).

5.2. Bilirubin transport

Several mechanisms have been postulated to explain how the bilirubin moves in and out from the cells in the different tissues. Uptake studies performed in isolated liver cells showed that UCB transport follows a bimodal behaviour being saturative at low concentration (carrier-mediated)while becoming concentration dependent (diffusion) when UCB reaches higher content (Mediavilla et al., 1999). On the contrary studies in hepatocytes performed by Zucker S. (Zucker et al., 1999; Zucker and Goessling, 2000) reported that bilirubin exhibit spontaneous diffusion through the membrane by a flip-flop mechanism.The penetration of more lipid-soluble molecules capable of diffusing across the cell membranes is limited by binding to plasma albumin and regulated by transport proteins present in both barriers. These transport systems belong to several subfamilies of the ATP Binding Cassette (ABC) efflux transporters and to solute carrier (SLCO) systems such as organic anion and cation transporters. Each displays broad substrate specificities, and they can work in concert at both cell membranes to achieve a high level of neuroprotection (Ghersi-Egea et al., 2009).

The OATP/SLCO superfamily consists of multi- and oligospecific membrane transport systems that mediate sodium-independent transmembrane solute transport. The multispecific transporters accept a broad range of amphi- pathic endo- and xenobiotics (Roma et al., 2008). Their multiple expression in the liver, kidney, small intestine, choroid plexus, blood-brain barrier and many other tissue barriers confer a strategic position for absorption, distribution and excretion of xenobiotic substances as shown Table 1.1 (Hagenbuch and Meier, 2004; Angeletti et al., 1997). As reported by Hagenbuch(Hagenbuch and Meier, 2004) different OATP transporters are able to transport bile salts and bilirubin like OATPC, OATPE, OATP1, OATP1a2 and OATP8. The role

for bilirubin transport of this transporters was studied by numerous works using different models like trophoblast cells from human an rat placenta (Briz et al., 2003b; Briz et al., 2003a), rat hepatocytes (Wang et al., 2003), HepG2 cells (Zucker and Goessling, 2000).

ATP-binding cassette (ABC) transporters are multi-domain integral membrane proteins that use the energy of ATP hydrolysis to translocate solutes across cellular membranes in all mammalian species. ABC transporters form one of the largest of all protein families and are central to many important biomedical phenomena, including resistance of cancers and pathogenic microbes to drugs (Jones and George, 2004).

Pgp, a member of the ABCB subfamily (MDR1), stands out among ABC transporters by conferring the strongest resistance to the widest variety of compounds. Pgp transports drugs that are central to most chemotherapeutic regimens. Is normally expressed in the transport epithelium of the liver, kidney and gastrointestinal tract, at pharmacological barrier sites, in adult stem cells and in assorted cells of the immune system. An additional member of the ABCB subfamily implicated in drug resistance are normally expressed in the liver, the ABCB4 (MDR3), a phosphatidylcholine flippase, has been shown to promote the transcellular transport of several Pgp substrates (Szakacs et al., 2006).

The ABCC subfamily members (the MRPs) also transport organic anions and Phase II metabolic products. ABCC1 (widely known as MRP1) is expressed in a wide range of tissues, clinical tumours and cancer cell lines. MRP1 confers resistance to several hydrophobic compounds that are also Pgp substrates. In addition, like other members of the ABCC subfamily, MRP1 can export glutathione (GSH), glucuronate or sulphate conjugates of organic anions. MRP1 homologues implicated in resistance to anticancer agents include ABCC2 (MRP2), ABCC3 (MRP3), ABCC6 (MRP6) and ABCC10 (MRP7). Despite the similarity of their sequences, MRP3 transports fewer compounds than MRP1 or MRP2. Interestingly, MRP3 has a preference for glucuronides over GSH conjugates. Substrates of MRP3 include anticancer drugs and some bile acid species, as well as several glucuronate, sulphate and GSH conjugates (Szakacs et al., 2006).

MDR1 and MRP1 are two major efflux transporters involved in neuroprotection by preventing access to, or increasing elimination, from brain of various endo- and xenobiotics. Both MDR1 at the blood-facing luminal membrane of the endothelium and MRP1 at the basolateral membrane of the choroidal epithelium (Gazzin et al., 2008; Strazielle and Ghersi-Egea, 2000) are ideally localized to prevent the brain entry of blood-borne substrates into the CNS, including UCB (Ghersi-Egea et al., 2009; Loscher and Potschka, 2005). UCB has been demonstrated to be a substrate for the MRP1 (Deeley et al., 2006). Net cellular uptake of UCB is decreased by specific inhibition of MRP1 expressed in polarized human BeWo trophoblastic cells (Pascolo et al., 2001).

Table 1 - Human and rodent members of the OATP/SLCO superfamily. Summary of new and old classification/nomenclature, predominant transport substrates, tissue distribution, chromosomal localization, accession number and known splice variants. Reproduced from Hagenbuch, B. – 2004 Eur J Physiol 447:653–665.

New gene symbol[a]	New protein name[a]	Old gene symbol	Old protein names	Predominant substrate	Tissue distribution and cellular/subcellular expression	Gene locus[b]	Sequence accession id	Splice variant
Slco1a1	Oatp1a1	Slc21a1	Oatp1, Oatp	Bile salts, organic anions, organic cations	Liver (basolateral membrane of hepatocytes), kidney (apical membrane of proximal tubule), choroid plexus (apical)	4q44 (r) 6A3-A5 (m)	NM_017111 NM_013797	
SLCO1A2	OATP1A2	SLC21A3	OATP-A, OATP	Bile salts, organic anions, organic cations	Brain (endothelial cells), kidney, liver, ciliary body	12p12 (h)	NM_021094	
Slco1a3	Oatp1a3_v1 Oatp1a3_v2	Slc21a4	OAT-K1 OAT-K2	Bile salts, organic anions	Kidney	4q44 (r)	NM_030837	AB012662
Slco1a4	Oatp1a4	Slc21a5	Oatp2	Digoxin, bile salts, organic anions, organic cations	Liver, blood-brain barrier, choroid plexus, ciliary body, retina	4 (r) 6G2 (m)	NM_131906 NM_030687	
Slco1a5	Oatp1a5	Slc21a7	Oatp3	Bile salts, organic anions	Jejunum, choroid plexus	4q44 (r) 6G2 (m)	NM_030838 NM_130861	
Slco1a6	Oatp1a6	Slc21a13	Oatp5			4q44 (r) 6G2 (m)	NM_130736 NM_023718	
SLCO1B1	OATP1B1	SLC21A6	OATP-C, LST-1, OATP2	Bile salts, organic anions	Liver	12p12 (h)	NM_006446	
Slco1b2	Oatp1b2	Slc21a10	Oatp4, Lst-1	Bile salts, organic anions	Liver, ciliary body	4q44 (r) 6G2 (m)	NM_031650 NM_020495	
SLCO1B3	OATP1B3	SLC21A8	OATP8	Bile salts, organic anions, digoxin	Liver, cancer cell lines	12p12(h)	NM_019844	
SLCO1C1 Slco1c1	OATP1C1 Oatp1c1 Oatp1c1	SLC21A14 Slc21a14 Slc21a14	OATP-F, OATP-RP5 Oatp14, BSAT1, Oatp2	T4, rT3, BSP	Brain, testis (Leydig cells)	12p12 (h) 4q44 (r)6G1 (m)	NM_017435 NM_053441 NM_021471	
SLCO2A1 Slco2a1	OATP2A1 Oatp2a1 Oatp2a1	SLC21A2 Slc21a2 Slc21a2	hPGT rPGT mPGT	Eicosanoids	Ubiquitous	3q21 (h) 8q32 (r)9F1 (m)	NM_005621 NM_022667 NM_033314	
SLCO2B1 Slco2b1	OATP2B1 Oatp2b1	SLC21A9 Slc21a9	OATP-B, OATP-RP2 Oatp9, moat1	E-3-S, DHEAS, BSP	Liver, placenta, ciliary body	11q13 (h)	NM_007256 NM_080786	
SLCO3A1 Slco3a1	OATP3A1 Oatp3a1 Oatp3a1	SLC21A11 Slc21a11 Slc21a11	OATP-D, OATP-RP3 Oatp11 MJAM	E-3-S, prostaglandin	Ubiquitous	15q26 (h) 7D1 (m)	NM_013272 AF239219 NM_023908	BC000585
SLCO4A1 Slco4a1	OATP4A1 Oatp4a1	SLC21A12 Slc21a12	OATP-E, OATP-RP1 Oatp12, oatpE	Taurocholate, T3, prostaglandin	Ubiquitous	20q13.1 (h) 2H4(m)	NM_016354 NM_133608NM_ 148933	
SLCO4C1	OATP4C1	SLC21A20	OATP-H		Kidney	5q21 (h)	AY273896	
SLCO5A1	OATP5A1	SLC21A15	OATP-J, OATP-RP4			8q13.1 (h)	NM_030958	
SLCO6A1	OATP6A1	SLC21A19	OATP-I, GST		Testis	5q21 (h)	NM_173488	
Slco6b1	Oatp6b1 Oatp6b1	Slc21a16	Oatp16, TST-1, GST-1	Taurocholate, T3, T4, DHEAS	Testis, epididymis, ovary, adrenal gland	9 (r)1 (m)	NM_133412 AK006249	
Slco6c1	Oatp6c1	Slc21a18	Oatp18, TST-2, GST-2	Taurocholate, T3, T4, DHEAS	Testis	9 (r)1 (m)	NM_173338 AK016647	
Slco6d1	Oatp6d1	Slc21a17	Oatp17			1 (m)	AK014872	

[a] All *capital symbols* denote human genes and gene products while *lower case symbols* stand for rodent genes and gene products (exception: OAT-K1 is a rat protein). To better discriminate between human, rat and mouse gene products sometimes a *lower case h, r* or *m* precedes the abbreviation. However, this is not part of the gene or protein symbol
[b] (h) human, (r) rat, (m) mouse

5.3. Bilirubin metabolism by CYPs

The cytochrome P450 (CYP) enzymes constitute a superfamily of haemoproteins that are involved in the oxidative activation or deactivation of both endogenous and exogenous compounds such as drugs, environmental toxins and dietary constituents. They play a critical role in the detoxification and activation of xenobiotics, and are expressed in a tissue-selective manner (Miksys and Tyndale, 2002).

Brain CYPs were originally reported to occur at only 1% of the levels found in liver. Many of the CYP subfamilies have been observed at the blood–brain interface and in circum ventricular organs (regions of the brain that are not protected by the blood–brain barrier) such as the choroid plexus and posterior pituitary (e.g., CYP1A, CYP2B and CYP2D). This may have evolved as a protection against harmful xenobiotics, but there is the caveat that these regions may also be exposed to toxic drug and steroid metabolites produced by local CYP activity (Miksys and Tyndale, 2002). CYP1A1 has been localized to the cortical regions, midbrain, basal ganglia and cerebellum. CYP1A2 has been found in most brain regions examined. A study in different strains of mouse, including Cyp1a2 null mutant mice, implicated both Cyp1a1 and Cyp1a2 in the bilirubin-degrading activity of the induced liver microsomes. CYP1A2 is believed to possess bilirubin-degrading activity intrinsically. CYP1A1 and CYP1A2, in particular, have been implicated in the inducibility of this pathway. However, their contribution to microsomal bilirubin oxidation appeared to be minor and therefore other CYP isoforms may contribute to the major part of the constitutive and inductive microsomal bilirubin oxidation. The induction of microsomal bilirubin degradation by CYP2a5 (CYP2A6 in human) were also reported in mice (Abu-Bakar et al., 2005).

5.4. Antioxidant players in neurons

5.4.1. The Glutathione protection against the oxidative environment

The glutathione (GSH) is the most abundant thiol present in mammalian cells. Conformed by three peptides (γ-l-glutamyl-l-cysteinyl-glycine), GSH is synthesized *in vivo* by the consecutive action of two enzymes (Figure 1.4). γ-Glutamylcysteine synthetase uses glutamate and cysteine as substrates to forms the dipeptide γ GluCys, which is combined with glycine in a reaction catalyzed by glutathione synthetase to finally generate GSH. Both reaction are supplied with the energy of the hydrolysis of ATP. The balance of cellular synthesis and consumption of GSH is regulated by feedback inhibition of the γ-Glutamylcysteine synthetase reaction by the end product GSH (Dringen et al., 2000).

GSH has important functions as an antioxidant, is a transport and storage form of cysteine, is a reaction partner for the detoxification of xenobiotica, and is a cofactor in isomerisation

reactions. In addition, GSH maintains the thiol redox potential in cells keeping sulfhydryl groups of cytosolic proteins in the reduced form (Dringen, 2000; Jia et al., 2008).
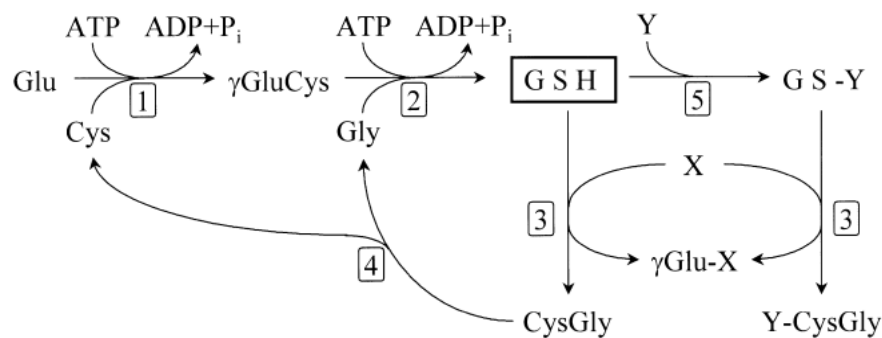
Figura 1.4 - Metabolism of glutathione: GSH is synthesized by the two consecutive ATP-consuming reactions of γ-glutamylcysteine synthetase (1) and glutathione synthetase (2). GSH is a substrate of the ectoenzyme γGT (3). X represents an acceptor of the glutamyl moiety transferred from GSH by γGT. The dipepetide CysGly is generated in equimolar concentrations to that of GSH used in the gGT reaction and is hydrolyzed by the reaction catalyzed by a dipeptidase (4). Intracellular GSH is conjugated by glutathione-S-transferase(s) (5) to xenobiotics or endogenous compounds (represented by Y). These conjugates are substrates of γGT. Reproduced from Dringen, R – 2000 Eur. J. Biochem. 267, 4912-4916.

The glutathione system is especially important for cellular defense against ROS. GSH reacts directly with radicals in nonenzymatic reactions and is the electron donor in the reduction of peroxides catalyzed by Glutathione peroxidise (GPx) (Figure 1.5). The product of the oxidation of GSH is glutathione disulfide (GSSG). GSH is regenerated from GSSG within cells in a reaction catalyzed by the flavoenzyme glutathione reductase (GR). This enzyme regenerates GSH by transferring reduction equivalent from NADPH to GSSG (Figure 1.5) (Dringen et al., 2000).
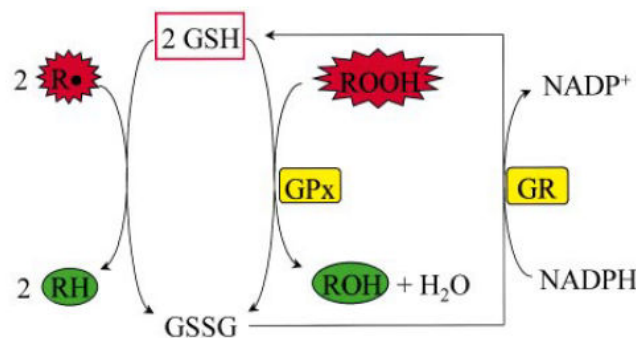
Figura 1.5 - Function of GSH as an antioxidant: GSH reacts nonenzymatically with radicals (R´) and is the electron donor for the reduction of peroxides (ROOH) in the reaction catalyzed by GPx. GSH is regenerated from GSSG by GR which uses NADPH as cofactor. Reproduced from Dringen, R – 2000 Eur. J. Biochem. 267, 4912-4916

5.4.2. The Cystine transporter System X_c^-

The System X_c^- is a highly regulated electroneutral system that exchanges cystine for glutamate and one of the main transporters responsible for central nervous system cystine transport (Burdo et al., 2006).

The antiporter is a heterodimer composed of: a heavy chain comprised of a cell surface antigen 4F2hc (non specific transporter subunit of the transporter, encoded by the *SLC3A2* gene) and a light chain known as xCT (encoded by the *SLC7A11* gene) (Markowitz et al., 2007). The xCT subunit is composed by 502 amino acids and 12 putative transmembrane domains (Figure 1.6) (La, V et al., 2007). The analysis in protein expression by Western blot of the System X_c^- performed by Shih, A. *et. al.* have demonstrated the presence of a 35 kDa band corresponded to the monomeric form of xCT. A 105 kDa band was detected using both xCT and 4F2hc antibodies under nonreducing conditions that represent the xCT plus 4F2hc disulfide-linked heterodimer. The monomeric 4F2hc was found to migrate at 80 kDa under reducing conditions. A fainter 55 kDa band (xCT-mod) was consistently detected in xCT and Nrf2-overexpressing astrocytes but was unlikely to be an xCT homodimer because it was too low in molecular weight and could not be disrupted by reducing conditions, suggesting that it is derived from the xCT transcript and may be an alternatively spliced or translationally modified form distinct from 35 kDa xCT. No heterodimerization was observed between 4F2hc and xCT-mod (Shih et al., 2006).

The System X_c^- works by exchanging the anionic form of cystine (which is transported into the cell) and glutamate (which is travelling outwards down its concentration gradient). The exchange is obligatory with a molar ratio of 1:1 and a in Cl^- dependent and Na^+-independent process. Cystine taken up by the cell via System X_c^- is then rapidly reduced to cysteine, which is the rate-limiting precursor for the synthesis of the potent intracellular reducing agent glutathione (GSH). Cysteine can in its turn be released back to the extracellular space, where the oxidizing conditions favour the formation of cystine. Thus, the System X_c^- contributes to driving the cystine/cysteine cycle and to maintaining the redox balance between the two amino acids in the medium (La, V et al., 2007; Liu et al., 2007). The System X_c^- activity can be quantified using either L-glutamate or L-cystine as a substrate, and in both instances, uptake is Cl^-dependent and Na^+-independent (Patel et al., 2004).
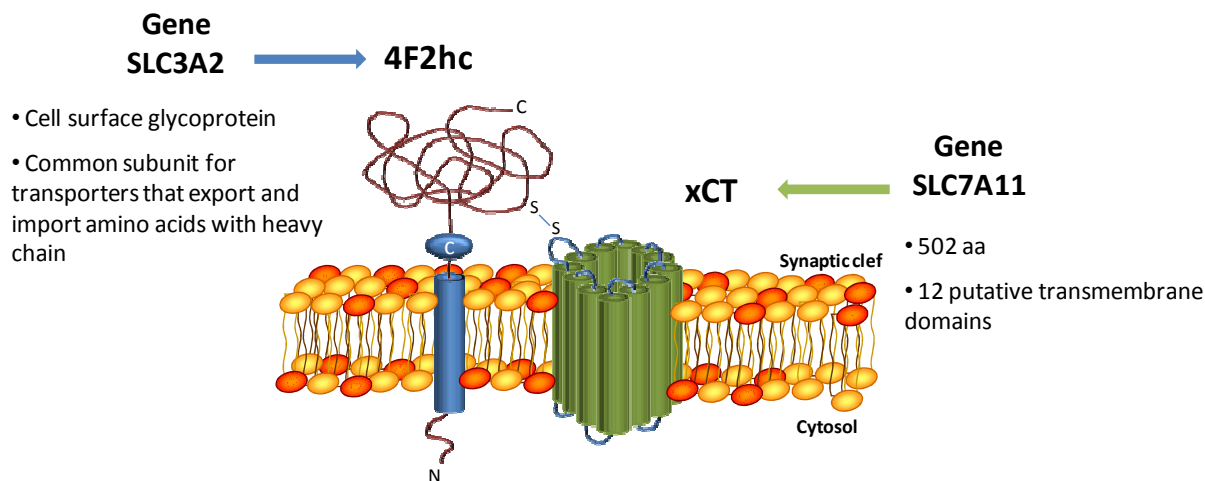
Figure 1.6 – Schematic representation of the System X_c^-: the system is an heterodimer composed by the heavy subunit 4F2hc (encode by the SLC3A2 gene) and the light subunit xCT (encode by the SLC7A11 gene).

Within the 5′-flanking region of the xCT gene are several sequences resembling the cis-acting electrophilic response element (EpRE), also known as the antioxidant response element (ARE) that is responsible for the induction of the adjacent genes. As such, xCT is transcriptionally regulated by electrophilic agents such as diethylmaleate (Bannai, 1984), oxygen (Bannai et al., 1989), bacterial lipopolysaccharide (Sato et al., 1995), cysteine and others amino acids (Sato et al., 2004) and by activation of the endogenous antioxidant response via the Nrf2 transcription factor (Sasaki et al., 2002). On the contrary, the cystine uptake carried out by the System X_c^- can be inhibit using the L-Quisqualate as demonstrated by Patel *et al.* and Knickelbein *et al.* (Patel et al., 2004; Knickelbein et al., 1997).

5.4.3. Cysteine providers for glutathione synthesis in neurons

Cysteine is one of the three amino-acid precursors of glutathione and it has been shown to be the rate-limiting substrate for glutathione synthesis in several different types of cells, presumably because the two other amino acids, glycine and glutamate, are normally present at much higher intracellular concentrations. Studies by Dringen and colleagues showed that the GSH released from astrocytes is used as substrate of the astroglial ectoenzyme γGT (encode by the GGT1 gene). The CysGly produced in this reaction is cleaved by the neuronal ectopeptidase ApN and the amino acids cysteine and glycine generated are subsequently taken up as precursors for neuronal glutathione synthesis by their respective transporters (Figure 1.7). The results presented here suggest that in these experimental models cysteine is generated from extracellular GSH by the consecutive reactions of γGT and ApN (Dringen, 2000; Dringen et al., 2001).
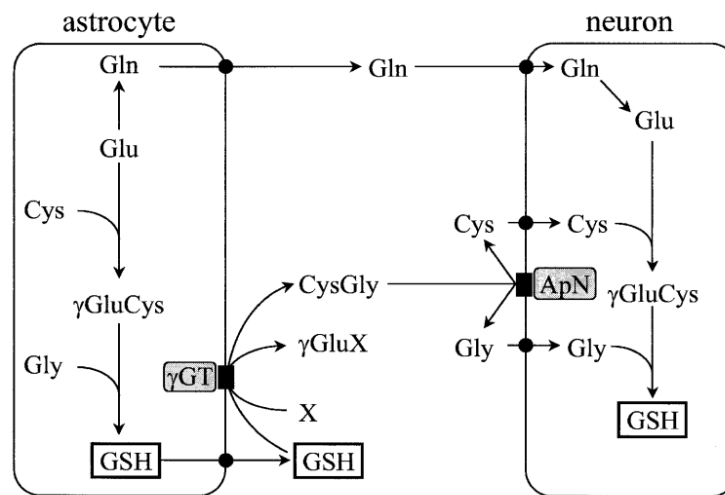
Figure 1.7 Scheme of the function of ApN in the proposed metabolic interaction between astrocytes and neurons in GSH metabolism: Astrocytes release GSH that serves as substrate for the astroglial ectoenzyme γGT. In addition, glutamine is released from astrocytes and used by neurons as precursor for the glutamate necessary for GSH synthesis. X represents an acceptor of the γ-glutamyl moiety transferred by γGT from GSH. The CysGly generated by the γGT reaction is hydrolyzed by the neuronal ectoenzyme ApN and the generated amino acids cysteine and glycine are taken up by neurons and serve as substrates for GSH synthesis. Reproduced from Dringen R – 2001 *J Neurosci Res* 66:1003-1008.

The mechanism by which free cysteine is then taken up by neurons has not been fully characterized yet. One potential route of cysteine uptake is *via* excitatory amino acid transporters (EAATs), which are members of the System X^-_{AG} family of electrogenic, Na^+ - dependent anionic amino acid transporters. Cysteine is known to be a substrate for EAAT3 (encode by the SLC1A1 gene) that is widely expressed by neurons in mature brain (Chen and Swanson, 2003).

5.4.4. Other antioxidant and detoxifying players

The antioxidant responsive element (ARE) is an enhancer element that initiates the transcription of a battery of genes encoding phase II detoxification enzymes and factors essential for neuronal survival. The ARE is activated through the binding of its transcription factor, Nrf2 (NF-E2-related factor 2) (Johnson et al., 2008). Nrf2 is sequestered in the cytosol by kelch-like ECH-associated protein 1 (Keap1). During oxidative challenge, the modification of Keap1 sulfhydryl groups results in the release and nuclear translocation of Nrf2. Nrf2 can transcriptionally activate several enzymes involved in cellular protection, including NAD(P)H:quinone oxidoreductase 1 (Nqo1), Heme oxygenase-1 (HO-1) and γ-glutamylcysteinyl synthetase (γ -GCS) (Maher et al., 2007).

NQO1 exemplifies a protein with multiple protective roles that include and extend beyond its catalytic function. It is a widely distributed FAD-dependent flavoprotein that catalyzes the reduction of quinones, quinoneimines, nitroaromatics, and azo dyes. The classical direct antioxidant

role of NQO1 is inherent in its catalytic mechanism: the obligatory two-electron reduction of a broad array of quinones to their corresponding hydroquinones by using either NADPH or NADH as the hydride donor. In doing so, NQO1 diverts quinone electrophiles from participating in reactions that could lead to either sulfhydryl depletion, or to one-electron reductions that can generate semiquinones and various reactive oxygen intermediates as a result of redox cycling. In addition, the hydroquinone products of the NQO1 reaction can be further metabolized to glucuronide and sulfate conjugates, thereby facilitating their excretion (Dinkova-Kostova and Talalay, 2010).

HO-1 is a ubiquitous and redox-sensitive inducible stress protein. In mammals, the crucial participation of HO-1 gene expression in alleviating organ dysfunction and counteracting metabolic disorders is supported by consistent reports showing a protective role for the products of the enzymatic activity of HO-1. The main evidences of the involvement of HO-1 come from experimental data like demonstrated by Poss, K. D. (Poss and Tonegawa, 1997) where HO-1 has been proposed to play an obligatory role in endogenous defense against oxidative stress because cells from HO-1$^{-/-}$ mice are highly susceptible to oxidative insults (Scapagnini et al., 2002).

Heme serves as a substrate for HO-1 in the formation of carbon monoxide, free ferrous iron, and biliverdin; the latter is rapidly converted to bilirubin by biliverdin reductase (BVR). The antioxidant actions of bilirubin are dramatically amplified by BVR in a biliverdin–bilirubin cycle. Thus, when bilirubin acts as an antioxidant, it is itself oxidized to biliverdin which is rapidly reduced by BVR to bilirubin. Depletion of BVR by RNA interference markedly diminishes the cytoprotective effects of exogenous bilirubin and leads to increased cellular levels of oxygen free radicals and cell death (Sedlak et al., 2009; Baranano et al., 2002).

The detoxification pathway via GSH-conjugation has been studied extensively. Glutathione S-transferases (GSTs) play an important role in the conjugation of xenobiotics with GSH, whose synthesis is rate determined by γ-GCS (Hayashi et al., 2003). The later is composed by a catalytic subunit (GCLC) and a modifier subunit (GCLM). Interestingly, the expression of both GCLC and GCLM is increased by Nrf2 activation (Johnson et al., 2008).

6. The SH-SY5Y cell line as a neuronal model

The SH-SY5Y cell line is the thrice cloned subline of SK-N-SH cells which were originally established from a bone marrow biopsy of a neuroblastoma patient with sympathetic adrenergic ganglial origin in the early 1970's (Biedler et al., 1973). The SK-N-SH cell line contains cells with three different phenotypes: neuronal (N type), Schwannian (S type), and intermediary (I type). The N-type cells appear to be immature neuroblasts characterise by small refractile cell bodies, a high nuclear-to-cytoplasmic ratio, and short, sometimes numerous, neuritis. They contain tyrosine

hydroxylase, dopamine-b-hydroxylase, the norepinephrine uptake transporter, and the low affinity nerve growth factor receptor–enzymes and cell surface receptors present in developing neuroblasts; vesicular granins chromogranin A and secretogranin II; and neurofilaments proteins like neurofilament 68. By contrast, S-type cells adhere tightly to the substrate, appearing as large flat cells with a prominent oval nucleus and abundant cytoplasm. The S cell phenotype is similar to that of an Schwannian/glial/melanoblastic precursor cell. In addition to the absence of neuronal marker proteins, these cells synthesize the intermediate filament vimentin, epidermal growth factor receptor, and fibronectin. Some S cells also express tissue-specific proteins such as the melanocyte enzyme tyrosinase, glial fibrillary acidic protein, desmin, or alpha-smooth muscle actin. Finally, the I-type cell has morphological features of both N and S cell types and are considered as a precursor that give origin to N and S cells (Ross and Spengler, 2007). These cells attach equally well to the substrate and to other cells, have a round, prominent nucleus, as do N cells, but with more cytoplasm, and may or may not have occasional neurites. No marker proteins specific to I cells have been identified yet (Ross et al., 2003; Xie et al., 2010).

Even if the SH-SY5Y cell line had origin as a homogeneous N type cell line, studies reported by Biagiotti, T. *et al* have demonstrated the presence of positive cells for S type markers. They have confirmed that actually the SH-SY5Y are an heterogeneous line composed by cells with characteristics of the both N and S types independently that can be inter-converted in culture through the I type (Biagiotti et al., 2006).

The SH-SY5Y cell line has been widely used in experimental neurological studies, including analysis of neuronal differentiation, metabolism, and function related to neurodegenerative and neuroadaptive processes, neurotoxicity, and neuroprotection.

7. Aims of the study

As indicated in the first part of this work, modifying the time of exposure and the concentration in which the SH-SY5Y cells are incubated with bilirubin, is possible to study both the bilirubin neurotoxicity and the mechanisms by which the neurons overcome the bilirubin damage. Based on these models we aimed to the identification of players that contribute to the bilirubin resistance induced by a first bilirubin treatment on neurons and molecular events that occur when UCB cause cell injury.

Materials and Methods

MATERIALS AND METHODS

1. Chemicals

Dulbecco's Phosphate Buffered saline (PBS), streptomycin and penicillin were purchased from Euroclone, Milan (Italy). Ham's Nutrient Mixture F12 (F12), Fetal calf serum (FCS), GlutaMAX^{TM} and TRIZOL reagent, obtained from Invitrogen (Carlsbad, CA) contained 24 g/L albumin. Ham's Nutrient Mixture F12 (F12), Eagle's Minimum Essential Medium (EMEM), nonessential amino acid solution (MEM), dimethy sulfoxide (DMSO), peroxide (H_2O_2, 30% wt/vol), 3(4,5-dimethiltiazolil-2)-2,5 diphenyl tetrazolium (MTT), L-buthionine-[S,R]-sulfoximine, NADPH, DEM, glutathione reductase, 2,2'-dinitro-5,5'-di-thiobenzoic acid (DTNB), Quisqualic Acid; Tri Reagent were purchased from Sigma Chemical Co.-Aldrich, Milan (Italy). Chloroform was obtained from Carlo Erba, Milan (Italy).

Unconjugated bilirubin (UCB)(Sigma Chemical Co, St. Louis MO), was purified as described by Ostrow & Murkerjee (Ostrow and Mukerjee, 2007). The 2,7-dichlorodihydrofluorescein diacetate (H_2DCFDA) was obtainded from Molecular Probes (Carlsbad, CA, USA) and iScript^{TM} cDNA Synthesis kit, iQ^{TM} SYBR Green Supermix were purchased from Bio-Rad Laboratories (Hercules, CA, USA).

2. Basic procedures and techniques

2.1. SH-SY5Y Cell Culture

Human neuroblastoma SH-SY5Y cells were cultured in a growth medium conformed by a mixture of EMEM/F12 (1:1 *v/v*) containing 15% (*v/v*) FCS, 1% (v/v) non essential amino acids, 1% (v/v) GlutaMAX^{TM}, penicillin (100 U/mL) and streptomycin (100 μg/mL) in 75 cm^2 tissue culture flasks at 37°C in a humidified atmosphere of 5% CO_2. The cells were fed every 2 days and sub-cultured once they reached 80-90% confluence. Cultures were stopped at the 20th passage, as recommended in the European Collection of Cell Cultures.

2.2. Bilirubin preparation

Among the experiments presented in this thesis different treatments were done exposing SH-SY5Y cells to bilirubin. Immediately before each incubation an aliquot of purified UCB was dissolved in DMSO (0.33 μL of DMSO per μg of UCB) obtaining a final concentration of 5 mM and then added to the culture with fetal calf serum. In the different UCB solutions, the concentration of free bilirubin (Bf) was measured with the peroxidase method (Calligaris et al., 2006). Because

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DMSO is used to dissolve the bilirubin, for each experiment cells exposed to growth medium with the same concentration of DMSO that carry the medium with bilirubin were used as controls.

2.3. Viability determination by MTT test

The stock of 3(4,5-dimethyltiazolyl-2)-2,5 diphenyl tetrazolium (MTT) was dissolved in PBS pH 7.4 at 5 mg/mL and finally diluted in Growth medium to a final concentration of 0.5mg/mL. The cells were incubated with the MTT solution during 1.5 hours at 37 °C. After incubation, to dissolve MTT formazan crystals, the medium was replaced discarded, 0.4 mL of DMSO were added and the sample gentle shook for 15 min. Absorbance values at 562 nm were determined in a LD 400C Luminescence Detector, Beckman Coulter, Milan, Italy. Results were expressed as percentage of control cells, not exposed to UCB, which was considered as 100% viability.

2.4. RNA extraction and quantification

Total RNA was isolated using Tri Reagent solution according to the manufacture's suggestions (T9424 Sigma, Missouri, USA). The total RNA concentration was quantified by spectrophotometric analysis in a Beckman DU640 Spectrophotometer. For each sample the A260/A280 ratio comprised between 1.8 and 2.0 was considered as good RNA quality criteria. Isolated RNA was re-uspended in RNAse free water and stored at -80°C until analysis.

2.5. cDNA Preparation

Single stranded cDNA was obtained from 1 µg of purified RNA using the iScripTMcDNA Synthesis Kit, according to the manufacture's suggestions. The reaction was run in a Thermal Cycler (Gene Amp PCR System 2400, Perkin-Elmer, Boston, MA, USA) in agreement with the reaction protocol proposed by the manufacturer.

2.6. Quantitative Real-Time PCR

Real Time quantitative PCR (qPCR) was performed with an iCycler (Bio-Rad Laboratories, Hercules, CA, USA) and a IQ5 Multicolor Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, USA). β-actin, HPRT and GAPDH were used as endogenous controls to normalize the expression level of the different gene analyzed (Primers sequence are shown in table 2.1). The primers were designed using Beacon Designer 4.02 software (PREMIER Biosoft International, Palo Alto, CA, USA). All primer pairs were synthesized by Sigma Genosys (Cambridgeshire, UK).

Table 2.1

Gene	Accession Number	Primer Forward	Primer Reverse	Product (bp)
B-actin	NM_001101.3	CGCCGCCAGCTCACCATG	CACGATGGAGGGGAAGACGG	120
HPRT	NM_000194	CTGGAAAGAATGTCTTGATTGTGG	TTTGGATTATACTGCCTGACCAAG	91
GAPDH	NM_002046	TCAGCCGCATCTTCTTTTG	GCAACAATATCCACTTTACCAG	146
OATPC	NM_006446.4	TCAATGGTTATACGAGCACTAGG	TGAAGACAAGCCCAAGTAGAC	165
OATPE	NM_016354.3	AGAAGGTGTACCGAGACTG	CGGACACATCGTAGAGTTG	187
OATP1	NM_001145946.1	ATTGAGCAGCAGTATGGACAG	ACAGGGAAAGAAATAGGAGGTAAC	190
OATP1A2	NM_021094.3	GCCCAACACACTTCCAAAG	GCATTGAACTGTATCACACTTAC	195
OATP8	NM_019844.2	TCCAGTCAATGGTTATAAGAACAC	AGCCCAAGTAGACCCTTCC	162
MRP1	NM_004996.3	TGATGGAGGCTGACAAGG	GCGGACACATGGTTACAC	127
MRP2	NM_000392.3	AGCACCGACTATCCAGCATCTC	GACGAAACCAAAGGCACTCCAG	117
MRP3	NM_003786.3	GCCATCTGTCTCCTGTATG	CCAGCATCACCAAGAACC	169
MDR1	NM_000927.	TGCTCAGACAGGATGTGAGTTG	AATTACAGCAAGCCTGGAACC	122
MDR3	NM_000443	TTTTTACTTTCTTCCTTCAGGGTTTC	TAAAAGCCATTGACCGCAGTCT	81
SLC7A11	NM_014331.3	GGTGGTGTGTTTGCTGTC	GCTGGTAGAGGAGTGTGC	107
GGT1	NM_001032364.1	TCTCTGACGACACCACTC	GACCTTGGAGCCAAAGTAG	152
SLC1A1	NM_004170.4	CCACTCTCATTGCTGTTATTC	CATCCACCGTACTGACTTC	120
HO-1	NM_002133.2	ATGCCCCAGGATTTGTCA	CCCTTCTGAAAGTTCCTCAT	95
HO-2	NM_001127204.1	TGAGTATAACATGCAGATATTCA	CCATCCTCCAAGGTCTCT	75
NQO1	NM_000903.3	CCTCTATGCCATGAACTT	TATAAGCCAGAACAGACTC	107
GCLC	NM_001498.3	AATGTCCGAGTTCAATAC	AATCTGGGAAATGAAGTTAT	111
CYP1A1	NM_000499.3	TATTGGTCTCCCTTCTCTA	GCTCAGGTAGTTGTTCTT	188
CYP1A2	NM_000761.3	TCCATCAACTGAAGAAGAC	TTGGCTAAAGCTGCTATT	119
CYP2A6	NM_000762.5	GCAGTTTAAGAAGAGTGA	GGTGAAGAAGAGAAAGAG	136
UGT1A1	NM_000463.2	CTGAATGTTCTGGAAATGACTT	CTTGTGAAGGCTGGAGAG	108
CACYBP	NM_001007214.1	ACAATCTCTTGAAACCCATCTCTG	TTCAGTGTCATAGGAGGGCTTC	164
VIM	NM_003380.3	AACTTCTCAGCATCACGATGAC	TTGTAGGAGTGTCGGTTGTTAAG	195
NFL	NM_006158.3	AAGAAGAAGGAGGTGAAGGTGAAG	TGGTTGGTTGGTTGGTGATGG	184
CHCA	NM_001275	GCATCGTTGAGGTCATCTC	AACCGCTGTGTTTCTTCTG	195
nNOS	NM_000620.3	CGTCTTGGAACTGACATAA	CTGGACAACTCTTACCTTC	84
iNOS	NM_000325.4	CCTCAAGTCTTATTTCCTCAAC	ATCAATCCAGGGTGCTAC	79
eNOS	NM_000603.4	ATTCCTCTTGCCTCTCTC	TAGTAGTTCTCCTAACATCTGG	114

Briefly, 25 ng of cDNA were amplified by PCR with 1x iQ SYBR Green Supermix (100 mM KCl, 40 mM Tris-HCl, pH 8.40; 0.4 mM each dNTP; 50 U/mL iTaq DNA polymerase; 6 mM MgCl2; SYBR Green I; 20 mM fluorescein; and stabilizers) (Bio-Rad Laboratories) and 250 nM gene specific sense and anti-sense primers, in a final volume of 25 µL for each well. The PCR was performed in 96-well plates, each sample was performed in triplicate and a no-template control was included for each amplificate. Standard curves using a "calibrator" cDNA (chosen among the cDNA samples) were prepared for each target and reference gene. In order to verify the specificity of the amplification, a melt-curve analysis was performed, immediately after the amplification protocol. Non-specific products of PCR were not found in any case. The expression analysis and relative quantification was made using the Bio-Rad IQ5 2.0 Standard Edition Optical System Software.

2.7. Protein extraction and Quantification

Total cells extracts were obtained by lising cells in ice-cold Cell Lysis Buffer (#9803, Cell Signaling Technology, Inc - Beverly, Massachusetts, USA) for 10 min, on ice, and using scrapper. The lysate was centrifugated at 14.000 g for 10 min, at 4°C, and the supernatans were collected and stored at -80°C. Protein concentration in the lysate was determined by the Bicinchoninic Acid Protein Assay (BCA) (Smith et al., 1985) following the instructions reported by the supplier (B-9643, Sigma, Missouri, USA).

2.8. Western Blot analysis

Equal amounts of protein were subjected to sodium dodecyl sulphate-poliacrilamide gel electrophoresis (SDS-PAGE). Molecular weight standards (10-250 kDa, #SM1811 Fermentas) were used as marker proteins. 2.5% β-mercapoethanol was added to the samples and finally they were subjected to denaturation protocol in a Thermal Cycler (Gene Amp PCR System 2400, Perkin-Elmer, Boston, MA, USA) for protein denaturation. Proteins were loaded on 10% polyacrylamide gel by electrophoresis in a Hoefer SE 250 System (Amersham Biosciences). After SDS-PAGE, gels were electrotransferred with a semi-dry blotting system at 100 V for 60 min to a immune-blot PVDF membranes (Bio-Rad Laboratories, Hercules, CA, USA).

After the protein transfer step, membranes were blocked in 4% BSA (fatty acid free, fraction V) in TTBS (0,2% Tween 20, 20 mM Tris-HCl (pH 7.5), 500 mM NaCl) and incubated at 4°C with the primary antibody. Then, membranes were washed three times with 5% BSA-TTBS and incubated for 60 min with a secondary antibody conjugated with peroxidase. For each particular test a normalization was performed by concomitant determination of β-actin using the polyclonal anti-

Actin antibody (A2066, Sigma Chemical, St. Louis, MO). Protein bands were detected by peroxide reaction using ECL-Plus Western Blot detection system solutions (ECL Plus Western Blot detection reagents, GE-Healthcare BioSciences, Italy) and visualized by autoradiography with Hyperfilm Sigma. The relative intensities of protein bands were analysed using the NIH Image software (Scion Corporation Frederick, MD, USA).

3. MODEL DEFINITION

3.1. Sensitivity of SH-SY5Y cells to free bilirubin (Bf)

SH-SY5Y cells were seeded in a 24 multiwell plates at a density of 60,000 cells/cm^2. Cell viability assays were conducted when 70% confluence was attained uniformly in all wells. After washing with pre-warmed PBS, cells were incubated with increasing doses of UCB dissolved in growth medium.

In the time course studies, cells were exposed to increasing concentrations Bf (10, 40, 70 and 140 nM) during different periods of time: 1h, 2h, 4h, 6h e 24h. At the end of the incubation, the medium containing UCB was discarded, the cells were washed 3 times with pre-warmed PBS and finally the viability was tested by the MTT test.

In order to standardize DMSO-related effects, cells were incubated with medium containing DMSO 0.6% (the quantity of DMSO necessary to dissolve the UCB and obtain the more concentrated solution Bf 140 nM) and the viability by MTT test was checked at the end of each incubation period.

3.2. Cellular growth curve of SH-SY5Y cells after bilirubin treatment

SH-SY5Y cells pre-treated with bilirubin (Bf final concentration 140 nM), DMSO 0.6% and growth medium during 24h were seeded at 10.000/cm^2 in a 24 multiwell plate. A growth curve was established counting the quantity of cells by cm^2 at 24h, 96h, 120h, 144h, 168h and 196h. At each time the cells were washes with PBS and detached incubating them with trypsin (Euroclone – Milan, Italy) during 2 minutes. After trypsin neutralization with growth medium cells were counted using a Burker chamber.

3.3. Response of SH cells to a second treatment with bilirubin during 4 hours

60.000 cells by cm^2 were seeded in two 6-multiwells plate and 100.000 cells by cm^2 in one 6-multiwells plate. The day after, the cells in the first two 6-multiwells plate were treated with growth medium and DMSO 0.6%, respectively, and the last 6-multiwells plate was treated with Bf 140 nM. All the treatments were performed for 24h. At the end of the first treatment cells in each 6-

multiwells plate were re-exposed to a second treatment with growth medium, DMSO 0.6% and Bf 140 nM during 4h. The cell viability was finally assessed by MTT test.

4. SH-SY5Y CELL MODEL TO STUDY BILIRUBIN RESISTANCE

4.1. The dynamic of the bilirubin

4.1.1. Bilirubin entrance analysis by 3H-UCB uptake analysis

The entrance of H^3-bilirubin was assessed in SH-SY5Y cells to measure the accumulation of the pigment in the cells. Briefly, cells were seeded in 35 mm dishes at 37 °C in atmosphere of 5% CO_2 and 95% humidity. H^3-bilirubin was dissolved first in DMSO and then in growth medium to obtain a final Bf concentration of 140 nM (controlled by absorbance at 486 nm) and DMSO 0.6%. 10 µL of this solution were used to measure the concentration. The cells were previously washed with the medium and then exposed to the labeled bilirubin for 0, 2, 5, 10, 30 and 60 min. After each time interval, the cells were washed three times with cold PBS and incubated with lysing solution (NaOH 0.2 N + SDS 2%) for 15 min. After homogenize with a scrapper 375 µL were mixed with 4 mL of scintillation cocktail (ULTIMA Gold^{TM}, Perkin Elmer Italia Life and Analytical Sciences) and the radioactivity was determined using a scintillation counter (Tri-Carb Liquid Scintillation Analyzer, Packard Instrument Company). 50 µL were taken for protein determination.

4.1.2. Bilirubin accumulation by HPLC analysis

The bilirubin accumulation inside the cells was also assessed by High Performance Liquid Chromatography (HPLC). Fourteen 75 cm2 flasks were seeded with 80.000 cells/cm2. Cells were exposed to bilirubin with a final Bf concentration of 140 nM for 0.25h, 0.5h, 1h, 1.5h, 2h, 2.5h, 3h, 3.5h, 4h, 6h, 18h and 24h.Cells exposed to DMSO 0.6% for 18h and 24h were used as controls. After the treatment, the cells were washed three times with PBS and collected in 500 µL of PBS detaching them using a scrapper. 50 µL were separated for protein quantification. Finally the total bilirubin quantity was determined using a HPLC with diode array (Agilent, Santa Clara, CA, USA) as described by Zelenka et al. (Zelenka et al., 2008).

4.1.3. Bilirubin entrance mediated by OATP transporters

The mRNA expression of membrane transporters considered to be involved in UCB entrance into the cells was tested by Real Time PCR, as described in point 2.6, in SH-SY5Y cells treated with Bf 140 nM, DMSO 0.6% and growth medium. For the cellular uptake the expression of the OATPC, OATPE, OATP1, OATP1a2 and OATP8 transporters were analyzed at 1h, 4h and 24h of treatment using the primers shown in Table 2.1.

4.1.4. Bilirubin Extrusion mediated by ABC transporters

Some ABC transporters are involved in the export of bilirubin from the cell. The mRNA expression of these membrane transporters was tested by RT-Real Time PCR (see point 2.6) in SH-SY5Y cells treated with Bf 140 nM, DMSO 0.6% and growth medium. The mRNA expression of the transporters MRP1, MRP2, MRP3, MDR1 and MRD3 was analyzed at 1h, 4h and 24h of treatment and 0h, 48h and 156h after a pre-treatment with BF 140 nM for 24h using the primers shown in table 2.1.

4.2. Metabolic changes inside the cells

4.2.1. The Cystine Transporter System X_c^-

4.2.1.1. Effect of bilirubin on mRNA expression levels of genes involved in cystine uptake.

The mRNA expression of the transporter able to internalize cystine into the neuron was analyzed by qPCR. SH-SY5Y cells were seeded at 80.000 cells/cm^2 in 75 cm2 the day before the experiment and then exposed to the following experimental conditions: growth medium, DMSO 0.6% medium or Bf 140 nM medium for 1h, 4h and 24h.

Total RNA was extracted, isolated and quantified as described in points 2.4-2.6.

4.2.1.2. L-[^{14}C]-Cystine uptake by bilirubin treated and untreated SH-SY5Y cells

SH-SY5Y cells were seeded in 6-multiwell plates at a density of 80.000 cell/cm^2. Cells were exposed to 24h treatment with Bf 140 nM or DMSO 0.6% and medium for controls the second day after the cells achieved a 70% confluence. Cells were then rinsed three times with 37 °C uptake buffer (140 mM NaCl, 25 mM HEPES, 5.4 mM KCl, 1.8 mM CaCl2, 0.8 mM MgSO4, 5 mM glucose (pH = 7.5).

Cystine uptake by SH-SY5Y cells was started by incubating the cells in uptake buffer containing 0.8 µM L-[14C] cystine (L- [U-14C]-Cystine, 250 mCi/mmol ; PerkinElmer Italia Life and Analytical Sciences) at 37°C during 0, 2, 5, 10 and 30 min. The uptake was stopped by rapidly rising the cells two times with ice-cold unlabelled uptake buffer. The cells were then lysed by adding 0.8 mL of 0.2 N NaOH containing 1% SDS. An aliquot of 50 µL was taken for protein determination. The remaining solution (750 µL) was mixed with 5 mL of scintillation cocktail (ULTIMA GoldTM, Perkin Elmer Italia Life and Analytical Sciences) and the radioactivity was determined using a scintillation counter (Tri-Carb ® Liquid Scintillation Analyzer, Packard Instrument Company).

4.2.1.3. Uptake contribution of neuronal L-Cystine transporters: System Xc⁻, X$_{AG}$⁻ and GGT

To analyze the individual contribution of each transporter to the high cystine uptake observed in SH-SY5Y cells after 24h of treatment with Bf 140 nM, the uptake was performed in presence or absence of sodium (For Na+ -free uptake, NaCl was replaced in the medium with an equal concentration of N-metyl-D-glucamine chloride) or In presence or absence of the specific inhibitor for the System Xc-, the quisqualic acid (Patel et al., 2004).

Briefly, cells were seeded in 6-multiwell plates at a density of 80.000 cell/cm^2. The cells were then rinsed three times and cystine uptake analyzed by incubating the cells in uptake buffer containing 0.8 µM L-[14C] cystine (L- [U-14C]-Cystine, 250 mCi/mmol ; PerkinElmer Italia Life and Analytical Sciences) at 37°C for 10 min. For the analysis of Na+-dependent and Na+-independent cystine transport and quisqualate inhbition, cells were incubated for10 min. The uptake was terminated by rapidly rising cells two times with ice-cold unlabelled uptake buffer. The cells were then lysed by adding 0.8 mL of 0.2 N NaOH containing 1% SDS. An aliquot of 50 µL was taken for protein determination. The remaining solution (750 µL) was mixed with 5 mL of scintillation cocktail (ULTIMA GoldTM, Perkin Elmer Italia Life and Analytical Sciences) and the radioactivity was determined using a scintillation counter (Tri-Carb ® Liquid Scintillation Analyzer, Packard Instrument Company).

4.2.1.4. Glutathione determinations after bilirubin treatment

Glutathione content was assessed at 1h, 4h and 24h of treatment with Bf 140 nM and 0h, 48h and 156h after a first treatment with Bf 140 nM for 24h. SH-SY5Y cells were seeded in 60 mm diameter dish at a density of 80.000 cell/cm2. After each treatment the cells were rinsed three times with PBS at 37°C and 500 µL ice-cold 5% perchloric acid was added. The cells were detached by scrapping, harvested, and the dishes rinsed twice with 500 µL ice-cold 5% perchloric acid. All the fractions were pooled together, homogenized and transferred to eppendorf tubes. The samples were centrifugated at 13.000 g and the acid-soluble fraction was separated from the pellet and both were stored at -80°C until analysis were performed. The proteins were quantified using the bicinchoninic acid assay from acid-precipitated pellet by treatment with 1 M NaOH.

Reduced glutathione (GSH) plus oxidized glutathione (GSSG) were measured as total glutathione content in the acid supernatants using an enzymatic method, after its neutralization with a 0.76 M KHCO3. Briefly, supernatant aliquots (100 µL) were assessed in 900 µL of the reaction mixture (0.1 M sodium phosphate buffer (pH 7.5) containing 1 mM EDTA, 0.3 mM DTNB, 0.4

mM NADPH). The rate of the enzymatic product formation (Tiobenzoic acid=TNB) was monitored after the addition of glutathione reductase (1 U/mL), in a termostated cuvette (30 °C), at 415 nm, for 3 min, with a Beckman DU 640 spectrophotometer. Glutathione concentrations were calculated using appropriate standards and normalized by mg of protein.

4.2.1.5. Cell viability of SH-SY5Y cells pre-treated with UCB after hydrogen peroxide stress

Cells were plated in 24-multiwell plates. Once they achieved a 70–80% confluence the growing medium was discarded, the cells were rinsed with PBS at 37°C and exposed to medium containing DMSO 0.6% or Bf 140 nM for 24h. Then, the cells were rinsed with PBS and maintained in optimal growth media until hydrogen peroxide treatment was performed. Cell response to hydrogen peroxide (0 – 700 µM) for 1 h was evaluated immediately after Bf or DMSO treatment and at 48h and 156h upon release in growth medium. After the incubation with H_2O_2, the medium was removed and MTT test was performed as described in point 2.3. The results were expressed as percentage of MTT reduction respect to cells not exposed to H_2O_2.

4.2.1.6. Small interference RNA-mediated System Xc⁻ (SLC7A11) gene silencing

30.000 SH-SY5Y cells/cm^2 (for DMSO treatment) or 60.000 SH-SY5Y cells/cm^2 (for Bf treatment) were plated either in 24-multiwell plates or in 6-multiwell plates for H_2O_2 treatment or Western blot analysis, respectively. Cells were untransfected (MOCK) or transfected with 50 nM siGENOME Non-Targeting siRNA #1 (Dharmacon, Lafayette, CO, USA) (NT, silencing control) or with 50 nM siGENOME SMARTpool siRNA against human SLC7A11 gene (Dharmacon, Lafayette, CO, USA) (anti-xCT). 1.25 µL/well (for 24-multiwell plates) or 5 µL/well (for 6-multiwell plates) of DharmaFECT 1 transfection reagent were used, according to manufacturer's instructions. Media were changed after 24h, and cells were growth for additional 24h. At this time, cells were rinsed once with PBS and exposed to medium containing DMSO 0.6% or UCB (Bf 140 nM) for 24h, as previously described. Cells seeded in 24-multiwell plates were exposed to H_2O_2 during 1h and the viability was assessed by MTT-assay. Cells seeded in 6-multiwell plates were lysed with 200 µL of cell lysis buffer 1X (Cell Signaling, Danvers, MA, USA) supplemented with 1 mM PMSF and the total cell extracts were harvested by scraping.. The protein content of xCT in the cell extract was determined by Western blot analysis using an anti-xCT rabbit polyclonal antibody (ab37185, Abcam, Cambridge, UK) and normalized by actin protein content using an anti-actin antibody (a2066, Sigma, Saint Louis, MO, USA). Bands were analyzed using Kodak 1D image software and quantified by Scion image software.

4.2.1.7.   xCT Protein expression and glutathione content after DEM treatment

The DEM is an electrolyte able to confer a high glutathione content to the cell by increasing the expression of System X_c^- and subsequently the cystine uptake (Sasaki et al., 2002). SH-SY5Y cells at a density of 60.000 cells/cm^2 were seeded two 75 cm^2 flasks and then treated with DEM 0.1 mM or medium for 24h, respectively. The xCT protein expression after the treatments was assessed by Western blot analysis as described in point 2.8, an anti-xCT rabbit polyclonal antibody (ab37185, Abcam, Cambridge, UK) and normalized by actin protein content using an anti-actin antibody (a2066, Sigma, Saint Louis, MO, USA). Bands were analyzed using Kodak 1D image software and quantified by Scion image software.

The intracellular glutathione content after the treatment with DEM 0.1 mM for 24h was tested using the same methodology described in point 4.2.1.4.

4.2.1.8.   SH-SY5Y cell viability after DEM and bilirubin treatment

SH-SY5Y cells at a density of 60.000 cells/cm^2 were seeded in two 24-multiwell plates. One was treated with medium and the other with DEM 0.1 mM. After 24h, the cells in both multiwells were exposed to DMSO 0.6%, Bf 40 nM, Bf 70 nM and Bf 140 nM during 4h. Finally, the viability after the last treatment was analyzed by MTT test as described in point 2.3.

4.2.1.9.   SH-SY5Y cell viability after DEM and hydrogen peroxide treatment

SH-SY5Y cells at a density of 60.000 cells/cm^2 were seeded in two 24 multiwell plates. One multiwell was treated with medium and the other with DEM 0.1 mM for 24h. Once finish the treatment, the cells were exposed for 1h to increasing concentration of hydrogen peroxide (50 µM, 100 µM, 200 µM, 300 µM, 400 µM and 500 µM) and medium as control. The viability at the end of the experiment was evaluated by MTT test as described in point 2.3.

4.2.2. Other possible targets involved in Bilirubin SH-SY5Y resistance
4.2.2.1.   HO-1, HO-2, NQO1 and GCLC mRNA expression

SH-SY5Y cells were exposed for 1h, 4h and 24h to Bf 140 nM and cells treated with DMSO 0.6% and Medium were used as controls. The mRNA that encode for the proteins HO-1, HO-2, NQO1 and GCLC, involved in detoxifying and antioxidant processes in the cells, was tested by qPCR as described in point 2.6 using the primers shown in table 2.1.

4.2.2.2. Bilirubin metabolism by CYP1A1, CYP1A2, CYP2A6 and UGT1A1

The mRNA expression of genes CYP1A1, CYP1A2, CYP2A6 and UGT1A1 was analyzed by qPCR in cells exposed to Bf 140 nM, DMSO 0.6% and medium during 1h. 4h and 24h. The procedure is described in point 2.6 and the primers used are shown on table 2.1.

4.2.3. SH-SY5Y Cell line population. Possible different susceptibilities to bilirubin treatment

4.2.3.1. Morphologic SH-SY5Y separation and bilirubin treatment

A morphologic analysis of the SH-SY5Y cell line was carried out by flow cytometry using a BD FACS Callibur (Becton Dickinson, Franklin Lakes, NJ, USA). The FSC-A and SSC-A scatter was used to distinguish the main populations regarding the cell morphology. Once determined the groups of cells morphologically different, they were sorted using a BD FACS Aria (Becton Dickinson, Franklin Lakes, NJ, USA). The various populations were incubated with growth medium for 48h and then treated for 24h with Bf 140 nM or DMSO 0.6% (Control). The viability at the end of the experiment was assessed by MTT test.

4.2.3.2. Specific markers mRNA expression for the "S" and "N" SH-SY5Y subpopulations

The mRNA expression of some specific markers for "S" and "N" SH-SY5Y subpopulation was analyzed by qPCR. After the treatment with Bf 140 nM for24h, the mRNA expression of the markers for the "S" subpopulation encoding for the proteins Calcyclin Binding Protein (CACYBP) and Vimentin (VIM), and markers for the "N" subpopulation encoding for the proteins Neurofilament 68 (NFL) and Chromograning A (CHGA) was assessed. The primers used for each gene are reported in table 2.1.

4.2.3.3. Vimentin expression in SH-SY5Y cells after bilirubin treatment

The expression of Vimentin protein, a specific marker of the "S" subpopulation, was studied in SH-SY5Y cells by Flow cytometry. Briefly, 60.000 cells/cm2 were seeded in three 25 cm^2 flasks and, the day after, treated with growth medium, DMSO 0.6% and Bf 140 nM during 24h. At the end of the treatment the cells were rinsed 3 times with PBS and detached incubating them with trypsin and finally neutralized with growth medium. After centrifugation at 200 g for 5 min and 4°C (centrifuge Beckman Coulter – Allegra 25R), the pellet was washed in Cold Washing Buffer (10% FBS - 1% azide in PBS) and fixed with 0.1% formaldehyde for 15 min. The cells were then

permeabilizided with 50% (V/V) methanol on ice for other 15 min, blocked in BSA 3% for 30 min and stained with and antibody anti-Vimentin conjugated with Phycoerythin (Vimentin antibody [VI-RE/1] (Phycoerythrin) ab49918, Abcam, Cambridge, UK) at concentration 5 mg/mL in BSA 3% for 1h. Finally the cells were washed with Cold Washing Buffer and the percentage of positive cells stained for Vimentin was assessed measuring the intensity of fluorescence with a BD FACS Callibur (Becton Dickinson, Franklin Lakes, NJ, USA) and analyzed with CellQuest Pro software.

5. SH-SY5Y MODEL TO STUDY BILIRUBIN NEUROTOXICITY

5.1. Oxidative stress generation by UCB

5.1.1. Intracellular ROS accumulation after bilirubin treatment

The Intracellular ROS accumulation after bilirubin treatment was determined using the 2'7'-dichlorofluorescein diacetate (DCFH-DA) compound. 60.000 cells/cm^2 were seeded in 6 multiwell plates and growth up to 70 % of confluence. Cells were pre-treated for 15 min with DCFH-DA 40nM diluted in serum free medium added with Hepes 25 mM, and finally exposed to a Bf 140 nM for15 min, 30 min, 1h, 1.5h, 2h and 4h. Cells treated with DMSO 0.6% (negative control) or H_2O_2 200 µM (positive control) were used as controls. At the end of the treatment, cells were washed with PBS, detached by trypsinization and re-suspended in PBS for FACS analysis. The intensity of fluorescence was measured with a BD FACS Callibur (Becton Dickinson, Franklin Lakes, NJ, USA) and analyzed with CellQuest Pro software.

5.2. Glutamate excitotoxicity

5.2.1. Glutamate release by SH-SY5Y cells exposed to UCB

60.000 cells/cm^2 were seeded in 24 multiwell plate and growth up to 70 % of confluence. Then they were treated Bf 140 nM (to dissolve the bilirubin DMEM High Glucose medium W/O phenol red was used to avoid interference in the spectrofotometric reading) for 1h, 2h, 3h and 4h, and DMSO 0.6% as controls. The glutamate released by the cells in the culture medium was measured using a EnzyChrom^{TM} Glutamate Assay Kit (EGLT-100) (BioAssay Systems, Hayward, CA, USA). The glutamate determination is based on glutamate dehydrogenase catalyzed oxidation of glutamate, in which the formed NADH reduces a formazan (MTT) reagent. The intensity of the product color, measured at 565 nm, is proportionate to the glutamate concentration in the sample. The glutamate levels in the medium were determined using a standard curve provided by the kit.

5.2.2. nNOS, iNOS and eNOS mRNA expression after bilirubin treatment

SH-SY5Y cells were exposed for 1h, 4h and 24h to Bf 140 nM for the analysis while cells treated with DMSO 0.6% and medium were used as controls. The mRNA encoding for the enzymes nNOS, iNOS and eNOS, all involved in nitric oxide generation in the cells, was tested by qPCR as described in point 2.6 using the primers shown in table 2.1.

6. Statistical analysis

All experiments were performed in triplicate and repeated in at least three different cell preparations. Results are expressed as means ± SD. One way ANOVA with Tukey–Kramer post-test was performed using GraphPad InStat version 3.00 for Windows 95 (GraphPad Software, San Diego, CA, USA). Probabilities <0.05 were considered statistically significant.

Results

RESULTS

1. MODEL DEFINITION

1.1. Sensitivity of SH-SY5Y cells to free bilirubin (Bf)

The sensitivity of the SH-SY5Y cells to bilirubin was tested exposing cells to Bf 10 nM, Bf 40 nM, Bf 70 nM and Bf 140 nM during different periods of time (1h, 2h, 4h, 6h and 24 h). In all the cases the exposition to DMSO 0.6% was used as control. After the time of exposure the viability was evaluated by MTT test (Figure 3.1).
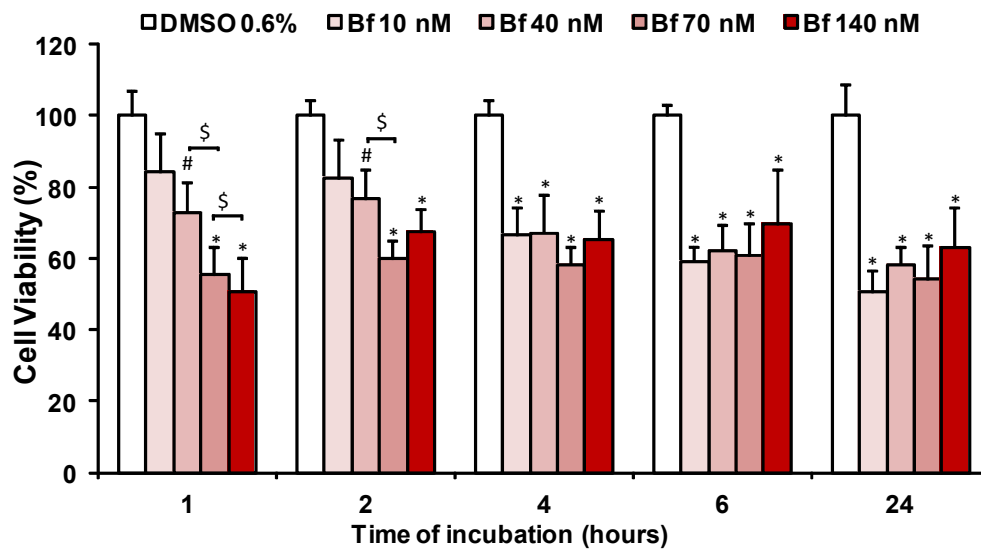
Figure 3.1 - Effect of UCB on cell viability: SH-SY5Y cells were incubated with different concentrations of free UCB (Bf) for different times, and the viability was evaluated by MTT test. * $p<0.001$ and # $p<0.01$ treated cells vs. control cells. $ $p<0.001$ between treatments.

The reduction on cell viability due to the incubation with different concentrations of bilirubin displayed a dose-dependent behavior at times shorter than 4h. At 4h, 6h and 24h, independently on the bilirubin concentration, the cell viability always arrived to a plateau at 60-70%, even at the highest bilirubin concentration (Bf 140 nM) and after a rather long time of exposure (24h).

1.2. Cellular growth curve of SH-SY5Y cells after bilirubin treatment

To assess the growth capability of cells that resist the bilirubin treatment, a growth curve with cells pre-treated with Bf 140 nM during 24h was performed. Pre-treated cells were incubated in medium and counted each 24h during 192h. Cells pre-treated with growth medium and DMSO 0.6% were used as controls (Figure 3.2).
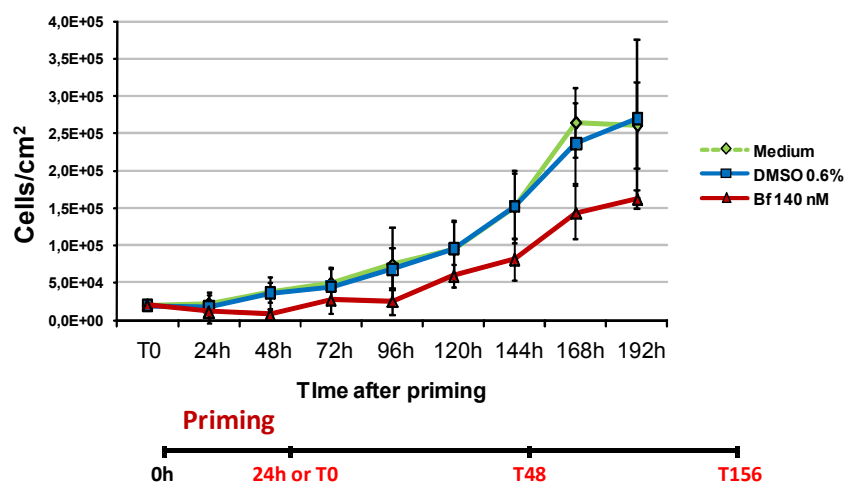
Figure 3.2 - Growth capability of SH-SY5Y cells that have resisted the bilirubin treatment: SH-SY5Y cells were pre-treated with Bf 140 nM during 24h and then released in growth medium to analyze the growth and duplication capability.

The cells pre-treated with bilirubin showed difficulty to grow, showing a lag phase during the first 96h after the treatment. After this time the cells started growing at the same rate as controls did. Based in this behavior three different times of study were defined for analysis in the next experiments: *T0*, immediately after bilirubin treatment (this time coincides with the 24h of bilirubin treatment); *T48*, 48h after the end of the incubation with bilirubin when the cells are in the lag phase; and *T156*, 156h after the end of the bilirubin exposure when they recover the growth ability (Figure 3.2).

1.3. Response of SH-SY5Y cells to a second treatment with bilirubin during 4 hours

In order to study the effect on the cell viability to a second bilirubin treatment, cells at *T0*, *T48* and *T156* were re-exposed to a second treatment with medium, DMSO 0.6% and Bf 140 nM for 4h. The cell viability was assessed after the 4h by the MTT test (figure 3.3).

Independently on the time of analysis, SH-SY5Y cells previously exposed to medium and DMSO 0.6% showed around a 70% of viability after the treatment with Bf 140 nM for 4h. On the contrary 100% viability was observed in cells that were previously treated with bilirubin during 24h, demonstrating the cells continue to be resistant to the pigment.
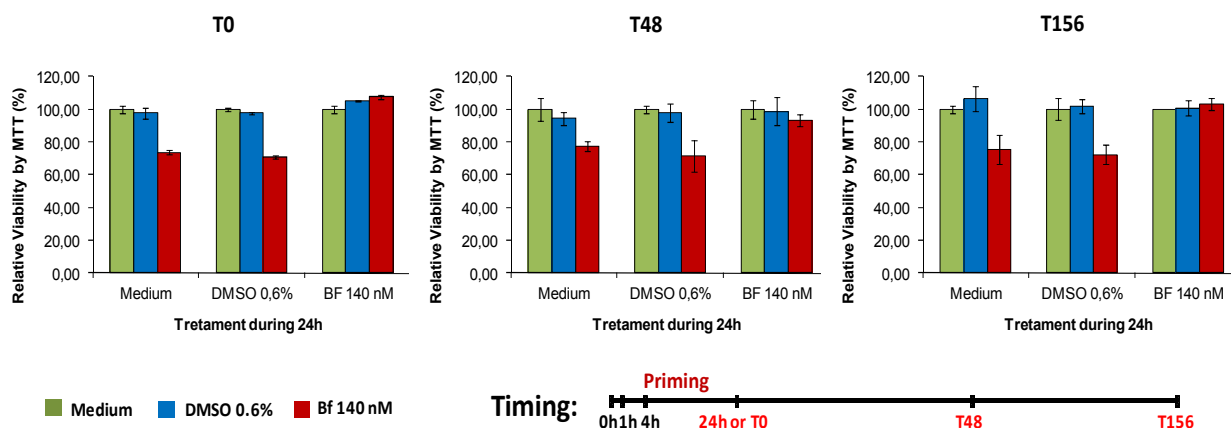
Figure 3.3 - Cell viability after a second bilirubin treatment: Cells at *T0*, *T48* and *T156* were re-exposed to medium, DMSO 0.6% and Bf 140 nM in order to study the effect of a second bilirubin exposure on the cells viability.

1.4. Model differentiation to study bilirubin resistance and bilirubin toxicity using SH-SY5Y cells

The previous results let us highlight two different behaviors achieved by SH-SY5Y cells when they are exposed to bilirubin. On one side, when the cells are treated with different bilirubin concentration for times shorter than 4h, the cell viability was reducedin a dose-dependent manner, thus showing the toxic effect of the bilirubin. These observation allowed us to define the conditions to establish a model to study the bilirubin toxicity where the mechanism by which the UCB cause the damage could be analyzed. On the other side, when the cells are treated during times larger than 4h till 24h, independently on the bilirubin concentration, the 60%-70% of the cells not only resist the expose to UCB, they are also able to overcome a second bilirubin treatment 156h after the first one. This indicate that the cells become resistant to UCB and let us define the conditions to have a model to study the mechanism by which the cells are able to resist the bilirubin damage.

Due to the double behavior of SH-SY 5Y observed in response to bilirubin treatment, the studies carried out in this thesis will be presented separately according to each model proposed for **bilirubin resistance** and **bilirubin neurotoxicity**.

2. SH-SY5Y MODEL TO STUDY BILIRUBIN RESISTANCE

2.1. The dynamic of the bilirubin

2.1.1. Bilirubin entrance analysis by ^{3}H-UCB uptake analysis

To understand the mechanism by which the bilirubin enter in the cells, SH-SY5Y cells were exposed to the bilirubin labeled with tritium (^{3}H-UCB) during 0, 2, 5, 10, 30 and 60 min, and the accumulation of bilirubin was determined measuring the radioactivity using a scintillation counter. In order to assess if the bilirubin entrance is achieved by an active transport the study was performed at 4°C and 37°C. Throughout the 60 min of uptake studied, the bilirubin cell content was 2 folds higher than the initial amount at both temperature, going from 120.82 ± 5.91 pmol/mg of protein to 183.56 ± 18.10 pmol/mg of protein at 4°C and from 106.14 ± 11.18 pmol/mg of protein to 178.89 ± 14.10 pmol/mg of protein at 37°C (Figure 3.4). The data obtained showed that the bilirubin uptake is not modified when the activity of the possible transporters involved is slowed reducing the work temperature. This behavior correlates better with a passive diffusion rather than an active transport carry out by a transporter.
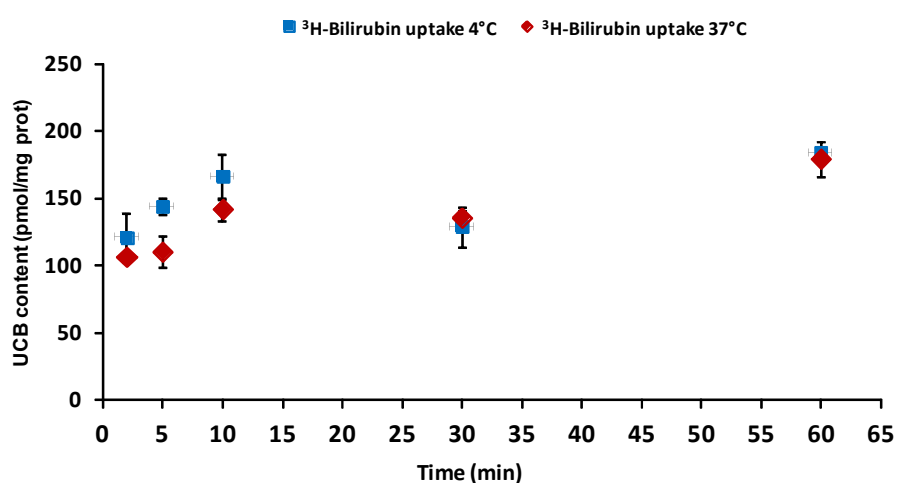
Figure 3.4 - ^{3}H-UCB uptake by SH-SY5Y cells: cells were incubated during 0, 2, 5, 10, 30 and 60 min with bilirubin labeled with tritium at a Bf concentration of 140 nM. The bilirubin accumulation after the uptake was measured using a scintillation counter and normalized by mg of protein.

2.1.2. Bilirubin accumulation by HPLC analysis

To confirm that the bilirubin is being accumulated inside the cell and not eliminated, the accumulation of the pigment was measured by High Performance Liquid Chromatography (HPLC). SH-SY5Y cells were exposed to Bf 140 nM during 0.25h, 0.5h, 1h, 1.5h, 2h, 2.5h, 3h, 3.5h, 4h, 6h, 18hand 24h. Control cells were exposed to DMSO for 18h and 24h. During the time frame

analyzed, bilirubin entered in the cell progressively with the time. The data plotted respect to the time adjust to a linear trend with a correlation coefficient $r^2=0.9234$ (Figure 3.5). Supporting the results obtained at short times by ^{3}H-UCB uptake in point 2.1.1, longer bilirubin incubations confirmed the entrance by passive diffusion.

HPLC

$r^2= 0.9234$
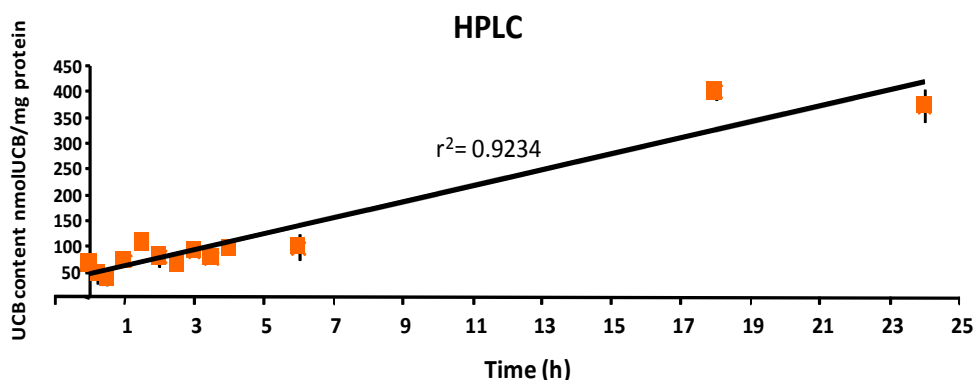
Figure 3.5 - Bilirubin accumulation inside SH-SY5Y cells: cells were incubated during 0.25h, 0.5h, 1h, 1.5h, 2h, 2.5h, 3h, 3.5h, 4h, 6h, 18h and 24h with Bf 140 nM and after each time the bilirubin accumulation inside the cells was measured by HPLC. Cells incubated with DMSO 0.6% for 18h and 24h were used as controls.
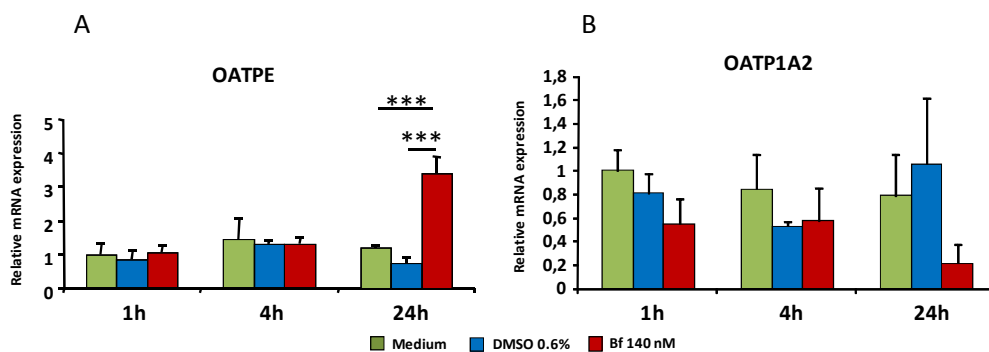
2.1.3. Bilirubin entrance mediated by OATP transporters

The hypothesis that the bilirubin resistance could be due to a partial inability of the pigment to enter into the cell was considered. Even if a specific bilirubin carrier was not identified yet, there are many indication regarding the role of Organic Anions Transporters Proteins (OATP transporters) in bilirubin active transport inside the cell (Hagenbuch and Meier, 2004). In order to study if the uptake of bilirubin on SH-SY5Y cells is carried out by an active transport, the expression of OATP1, OATPC, OATP8, OATPE and OATP1A2 mRNA was analyzed by qPCR on SH-SY5Y cells treated with medium, DMSO 0.6% and Bf 140 nM during 1h, 4h and 24h. The results showed that OATP1, OATPC and OATP8 are not expressed in SH-SY5Y cells. In the case of OATPE a low expression was observed in control cells treated with medium and DMSO 0.6 % at the three times analyzed. The cells exposed to Bf 140 nM had the same level of expression as controls at 1h and 4h while there was an increase of 3.4 folds in mRNA expression of OATPE at 24h ($p < 0.001$ - Figure 3.6A). When OATP1A2 was assessed, no significant changes were observed among the treatments and times (Figure 3.6B). This results indicate that the bilirubin is unable to modulate the expression of the OATP transporters analyzed to let the pigment enter in the cell. The increased expression of the OATPE at 24h could be attributed to a late response in the induction of the transporter by the bilirubin or to a secondary response not directly correlated with the bilirubin itself.

Figure 3.6 - mRNA relative expression of OATP transporters possibly involved in SH-SY5Y bilirubin entrance: mRNA expression for OATPE (A) and OATP1A2 (B) analyzed on cells exposed to medium, DMSO 0.6% and Bf 140 nM during 1h, 4h and 24h. *** $p < 0.001$.

2.1.4. Bilirubin Extrusion mediated by ABC transporters

ABC transporters family is involved in the extrusion of many toxic compounds from the cells. In particular 4 transporters belonging of that family, MRP1, MRP3, MDR1 and MDR3, are considered involved in UCB extrusion (Szakacs et al., 2006; Deeley et al., 2006). To address if the bilirubin resistance could be due to an increased extrusion of the compound from the cell, the mRNA expression of these ABC transporters was analyzed on cells exposed to medium, DMSO 0.6% and Bf 140 nM for1h, 4h and 24h by qPCR. The results showed that noneof the ABC transporters is over-expressed at the conditions studied (Figure 3.7).
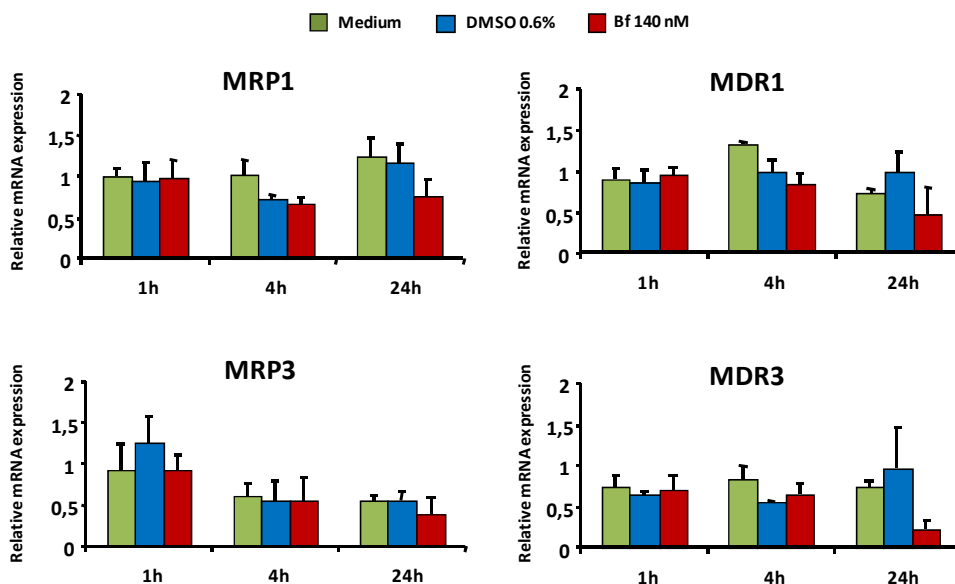
Figure 3.7 - mRNA relative expression of ABC transporters possibly involved in SH-SY5Y bilirubin extrusion from the cells: The mRNA expression for MRP1, MRP3, MDR1 and MDR3 was analyzed on cells exposed to medium, DMSO 0.6% and Bf 140 nM during 1h, 4h and 24h.

The expression of the same transporters was analyzed at *T0*, *T48* and *T156*, when previous results indicated that the cells become resistant to bilirubin. At this three times the results obtained were the same than 1h, 4h and 24h, with no significant changes on the mRNA expression for the MRP1, MRP3, MDR1 and MDR3 genes (Figura 3.8).

These results indicate that cell resistance to bilirubin treatment is not conferred by an increased extrusion at least as suggested by the gene expression of the main ABC trasporters related to UCB trasport. .
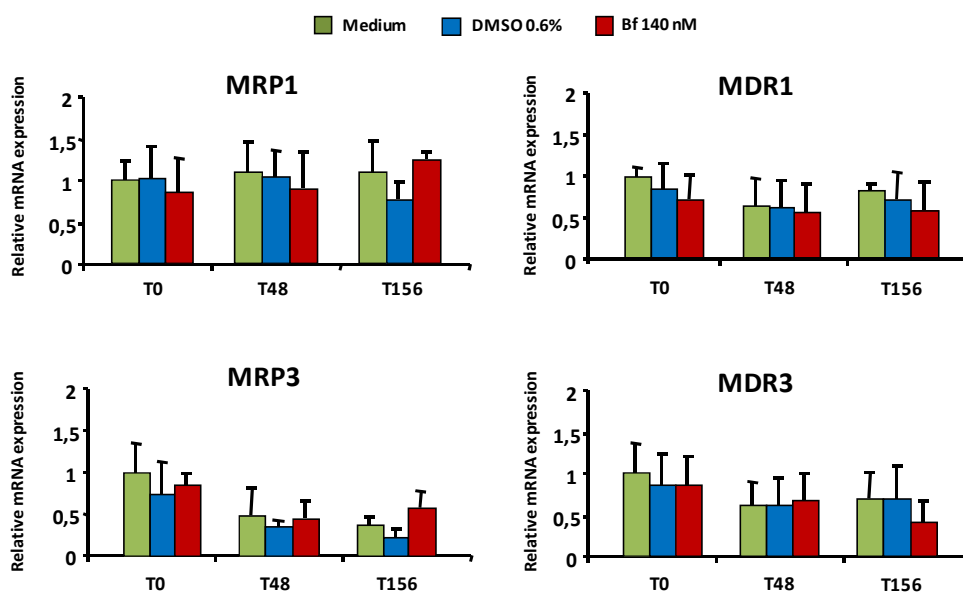
Figure 3.8 - mRNA relative expression of ABC transporters possibly involved in SH-SY5Y bilirubin extrusion from the cells: The mRNA expression for MRP1, MRP3, MDR1 and MDR3 was analyzed on cells exposed to medium, DMSO 0.6% and Bf 140 nM during *T0, T48* and *T156*.

2.2. Cell metabolic changes

2.2.1. The Cystine Transporter System X_c^-

2.2.1.1. Effect of bilirubin on mRNA expression levels of genes involved in cystine uptake

In order to confirm the previous observation by microarray RNA on SH-SY5Y treated with Bf 140nM for 24h (Calligaris et al., 2009) SH-SY5Y cells were treated with medium, DMSO 0.6% and Bf 140 nM for 1h, 4h and 24h, and the mRNA expression of the genes involved in cystine uptake (*SLC7A11*, *SLC1A1* and *GGT1*) analyzed by qPCR. As shown in Figure 3.9, after 24h of treatment a significant induction of *SLC7A11* was observed with mRNA level 5 folds higher (p<0.001) respect to the controls. No changes in the expression were observed after 1h or 4h of UCB treatment. On the contrary, the *SLC1A1* gene expression was decreased (p<0.01) 24h after UCB treatment while no change respect to controls (0.6% DMSO and untreated) was observed at 1h and

4h. The expression of GGT1 was not affected by UCB treatment at any time in the study (Figure 3.9).
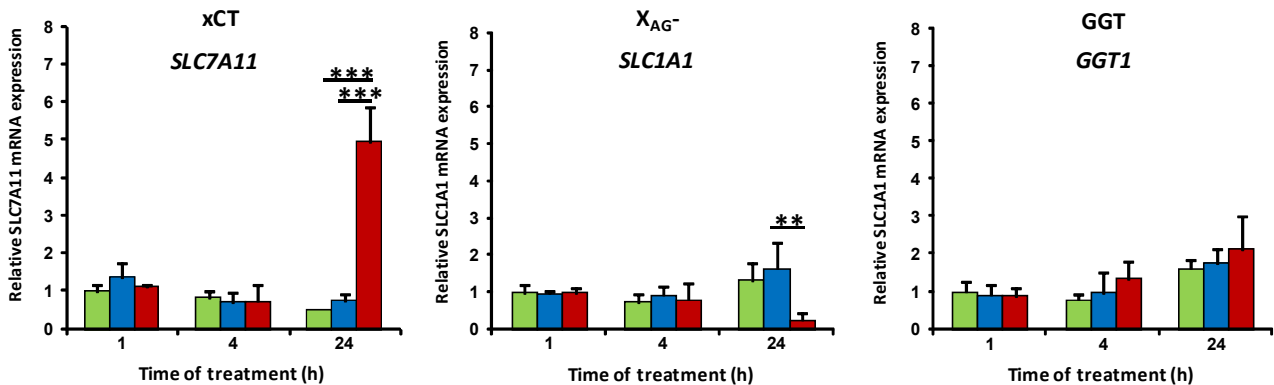
Figure 3.9 - Effect of bilirubin on mRNA expression levels of genes involved in cystine uptake: SH-SY5Y cells exposed to medium , DMSO 0.6% or Bf140 nM were collected after 1h, 4h and 24h of treatment . Specific mRNA expressions was analysed by qPCR, the results represent the mean ± SD of 3 experiments relative to 1 h medium control set at 1.0. ***p < 0.001 and **p < 0.01 versus DMSO or medium controls.

2.2.1.2.   L-[^{14}C]-Cystine uptake by bilirubin treated and no treated SH-SY5Y cells

Once shown that SLC7A11 mRNA expression is up-regulated after 24h of UCB exposure, we examined whether the cystine uptake was also increased. Untreated SH-SY5Y, exposed to medium, DMSO 0.6% or to Bf 140 nM cells for 24h were incubated for 0, 2, 5, 10, and 30 min with an uptake buffer containing 0.2 µCi/mL of L-[^{14}C] cystine (0.8 µM) and cystine uptake was measured thereafter. The results are reported in Figure 3.10.
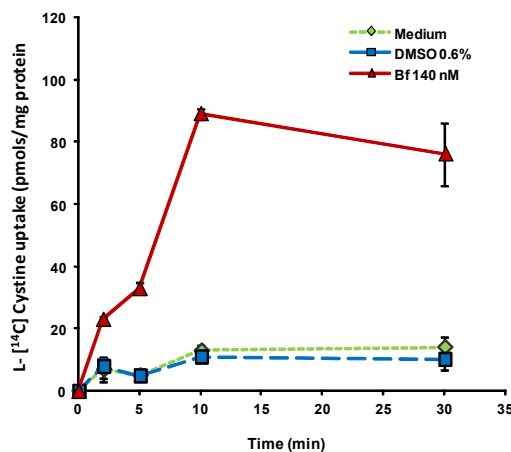
Figure 3.10 - Cellular cystine intracellular accumulation in SH-SY5Y cells treated with bilirubin: Cells were exposed for 24h to medium, DMSO 0.6% or Bf 140 nM for 24h. At the end of the treatment cells were incubated with L- [^{14}C] Cystine (0.8 µM) for 0, 2, 5, 10 and 30 min and the bilirubin accumulated inside the cells was determined and expressed as pmols/mg protein.

SH-SY5Y cells previously treated with UCB showed a cystine uptake significantly higher than controls (medium and DMSO 0.6% treated cells) over a 30 min period. The uptake in Bf

treated cells was about 8 times higher than controls at 10 min and remained high thereafter. No differences were observed between cells exposed to medium and DMSO 0.6%.

2.2.1.3. Uptake contribution of neuronal L-Cystine transporters: Systems X_c^-, X_{AG}^- and GGT

The individual contribution of Systems X_c^-, X_{AG}^- and GGT to the whole L-cystine transport was studied taking advantage that System X_{AG}^- is a sodium-dependent transporter and System X_c^- and GGT are sodium-independent transporters. We also used for the analysis on the contribution of System X_c^- the modulation of its activity with L-quisqualate, a specific inhibitor of this System (Patel et al., 2004; Knickelbein et al., 1997). The uptake of L-[^{14}C] cystine was measured at 10 min in cells previously exposed during 24h to UCB (Bf 140 nM); cells exposed to 0.6% DMSO were used as controls. The experiment was performed in presence or absence of sodium ions (140 mM) and with or without L-quisqualate (500 µM).

As shown in Figure 3.11 - panel A, when all the transporters are active (presence Na^+ and absence of L-quisqualate), cystine uptake was about 8 folds higher in Bf pre-treated cells than the control. In presence of sodium and after L-quisqualate addition (Figure 3.11 - panel B), L-cystine uptake in UCB treated cells showed values similar to control cells (from 74.7 ± 0.9 to 11.9 ± 0.2 pmols/mg protein respectively, p<0.001). This result suggests that System X_c^- plays a major role in accounting for the increased cystine uptake induced by UCB treatment.
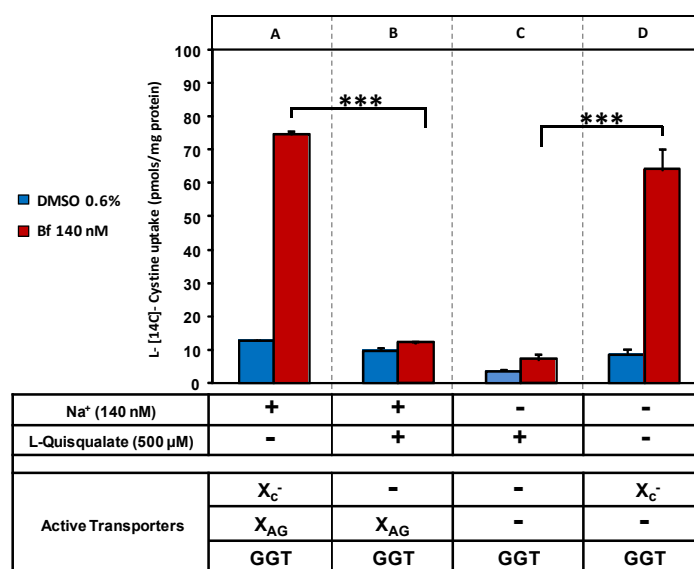
**Figure 3.11 - Neuronal cystine transporters contribution to cystine uptake in SH-SY5Y cells treated with bilirubin:** Transport activity was measured in control (DMSO 0.6%, 24h) and treated (Bf 140 nM, 24h) cells. L-[^{14}C] cystine (0.8 µM) uptake was measured after 10 min incubation at 37°C in the presence and absence of sodium ions. L-quisqualate was used as a specific inhibitor for System X_c^-. (*** p<0.001).

When sodium was removed from the medium (Figure 3.11, panel C) but in presence of L-quisqualate (System X_c^- blocked), the sodium dependent System X_{AG}^- was further inhibited leaving cystine transport accounted only by GGT activity. Under these conditions cystine uptake was not significantly decreased both in controls (from 9.7 ± 0.8 to 3.6 ± 0.5 pmols/mg protein) and treated cells (from 11.9 ± 0.4 to 7.0 ± 1.6 pmols/mg protein). These results indicate that the contribution of System X_{AG}^- is not modified by UCB treatment.

When the activity of System X_c^- is added to that of GGT (absence of both Na^+ and L-quisqualate), L-cystine uptake was again significantly higher in UCB treated than in control cells (64.0 ± 12.6 pmols/mg protein vs 7.0 ± 1.6 pmols/mg protein, respectively, $p<0.001$) (Figure 3.11, panel D), indicating that only System X_c^- activity and not System X_{AG}^- and GGT activity is induced by bilirubin treatment.

2.2.1.4. Glutathione determinations after bilirubin treatment

The reduced intracellular glutathione content (GSH) was determined at 1h, 4h and 24h after the exposure of cells to medium, DMSO 0.6% and Bf 140 nM. The time frame used was the same as in the study of the mRNA expression of the cystine transporters (see Figure 3.9). GSH content was also evaluated at *T0*, *T48* and *T156* when it has been demonstrated that the cells are resistant to a second bilirubin treatment.
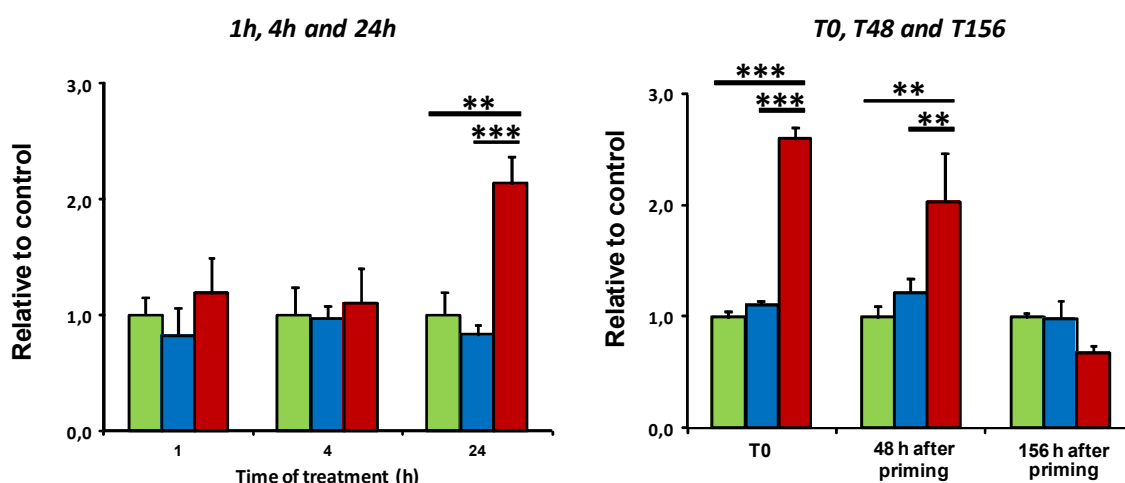
Figure 3.12 - Reduced intracellular glutathione levels in SH-SY5Y cells treated with bilirubin: SH-SY5Y cells were exposed to medium, DMSO0.6% or Bf 140 nM and reduced intracellular content of glutathione was determined after 1h, 4h and 24 h. **p<0.01 and ***p<0.001

As shown in Figure 3.12, 1h and 4h after UCB exposure no changes in GSH levels were observed in SH-SY5Y cells treated with UCB as compared to controls. On the contrary, after 24 h

the total GSH content was 2.5 folds higher in UCB treated cells than controls (25.1 ± 5.3 vs. 7.6 ± 0.6 nmols/mg protein for DMSO 0.6%, $p<0.001$; and vs. 9.2 ± 1.1 nmols/mg protein for medium, $p<0.001$). At *T0* the same behavior as 24h exposure was observed with 2 folds higher glutathione content in cells treated with bilirubin (18.5 ± 0.54 vs. 7.9 ± 0.41 nmols/mg protein for DMSO, $p<0.001$; and vs. 7.6 ± 0.64 nmols/mg protein for medium, $p<0.001$). At *T48* the levels of glutathione started to decrease (14.1 ± 3.13 nmols/mg protein) in cells treated with UCB to finally return to be normal at T156 (5.5 ± 0.90 nmols/mg protein).

2.2.1.5. Cell viability of SH-SY5Y cells pre-treated with UCB after hydrogen peroxide stress

Experiments were performed to examine if the reductive intracellular environment due to GSH increased content in UCB-treated cells may be protective against an oxidative stress insult induced by H_2O_2 treatment.

As shown in Figure 3.13, the exposure of SH-SY5Y cells previously treated with DMSO 0.6% for 24h (*T0*) to increasing concentrations of H_2O_2 during 60 min produced a dose-dependent reduction in cell viability. On the contrary, cells exposed for 24h (*T0*) to Bf 140 nM showed a significantly lower damage. The same analysis was performed at *T48*, where the results have shown that cells exposed to different concentration of H_2O_2 are not as resistant as *T0,* however continue to be resistant at high concentrations of hydrogen peroxide. When cells pre-treated with 140 nM Bf for 24h were grown for 156h in the absence of UCB, the sensitivity to the H_2O_2 was comparable to control cells. These results are in agreement with the GSH intracellular contents at each time of analysis (Figure 3.12).
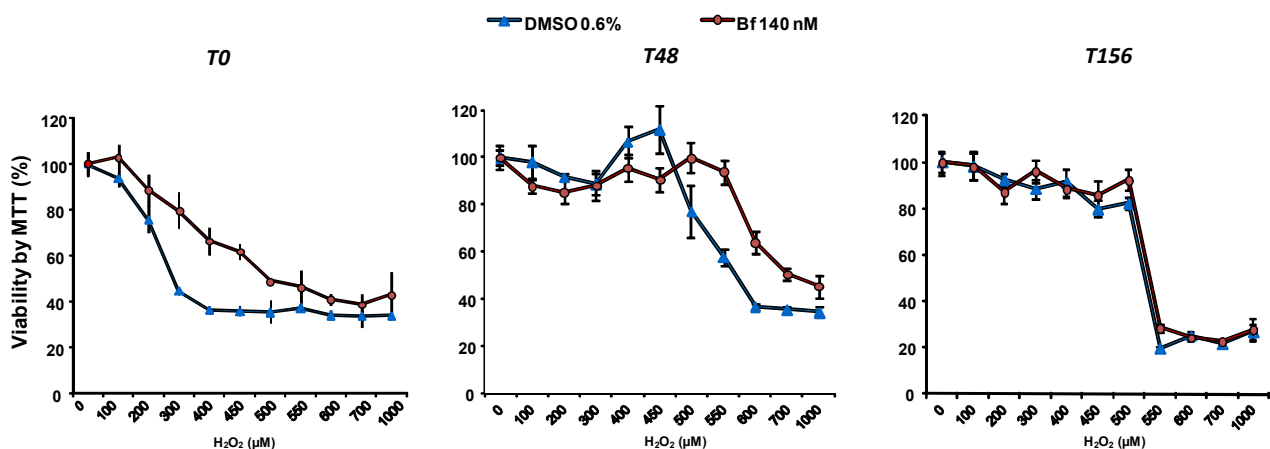

Figure 3.13 - Cell viability of SH-SY5Y cells previously treated with bilirubin exposed to increasing concentrations of H_2O_2: SH-SY5Y cells previously treated with DMSO 0.6% and Bf 140 nM at *T0, T48* and *T156,* were exposed to increasing concentrations of H_2O_2.

2.2.1.6. Response to H₂O₂ oxidative stress in xCT silenced SH-SY5Y cells after bilirubin treatment

To test the contribution of System X_c^- to the cytoprotective effects induced by UCB we performed experiments gene silencing of SLC7A11 using siRNA. The expression of xCT protein (55 kDa and 35 kDa) was studied in SH-SY5Y cells untransfected (MOCK), transfected with not targeting siRNA (NT) and transfected with siRNA against SLC7A11 gene (anti-xCT) after 24 h of Bf 140 nM or DMSO 0.6% treatment. The analysis of protein expression was performed by Western blot identifying two bands for xCT protein, one at 35 kDa and the other at 55 kDa. The former correspond to the monomeric form of xCT and the latter, even if specific only to xCT it function remain still unknown (Shih et al., 2006). As shown in Figure 3.14A, xCT protein expression was decreased only in siRNA anti-xCT treatments (lanes 3 and 6). The expression of the xCT band of 35 kDa was undetectable in DMSO treated cells (Figure 3.14B, lane 3) and reduced by 88% in Bf treated cells (Figure 3.14B, lane 6). Reduction of 55 kDa band was 89% in DMSO treated cells (Figure 3.14B, lane 3) and 66% in Bf treated cells (Figure 3.14B, lane 6).

Figure 3.14C shows the cytotoxic effects of 60 min exposure to increasing concentration of H_2O_2 in DMSO or Bf pre-treated cells, after SLC7A11 genesilencing. A dose-dependent reduction in cell viability was observed for MOCK and NT groups. Pre-exposure to Bf resulted in a significantly lower cytotoxicity than DMSO pre-treated cells ($p < 0.05$ at 300 μM H_2O_2 and $p < 0.01$ at 600 and 700 μM of H_2O_2. On the contrary, in anti-xCT group (Figure 3.14C), the sensitivity to the oxidative stress was identical between Bf pre-treated cells and controls, supporting a direct contribution of the System X_c^- to the protection against H_2O_2 oxidative stress.
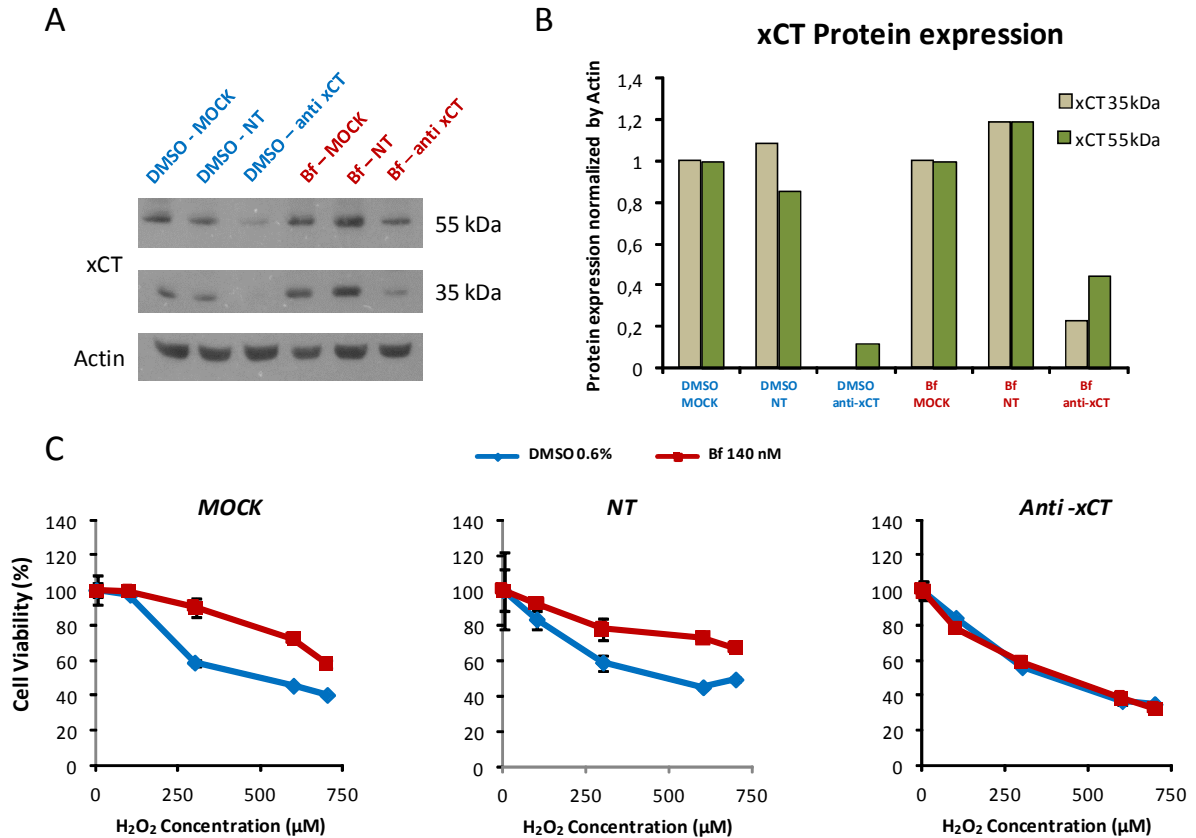
Figure 3.14 - Response to H_2O_2 oxidative stress in xCT silenced SH-SY5Y cells pre-treated with bilirubin: (A) Protein expression of xCT (55 kDa and 35 kDa) analyzed by Western blot after siRNA and Bf treatment. Lane 1: MOCK and 0.6% DMSO; lane 2: NT and 0.6% DMSO; lane 3: anti-xCT and DMSO 0.6%. Lane 4: MOCK and Bf 140nM; lane 5: NT and Bf 140nM ; lane 6: anti-xCT and Bf 140nM. (B) Quantification of bands shown in lanes from 1 to 6 of A. xCT 35 kDa and 55 kDa bands normalized by actin and expressed as relative to MOCK . (C) H_2O_2 dose-response by MTT test was evaluated in untransfected (MOCK – left), transfected with not targeting siRNA (NT – middle) and transfected with siRNA against SLC7A11 gene (anti-xCT - right) SH-SY5Y cells. After silencing, and before 1h H_2O_2 treatment, SH-SY5Y cells were exposed for 24h to 0.6% DMSO or Bf 140 nM as indicated in the legend of each picture.

2.2.1.7. xCT Protein expression and glutathione intracellular content after DEM treatment

The ability of the DEM to induce the expression of the xCT protein was analyzed by Western blot. SH-SY5Y cells treated with DEM 0.1 mM for 24h increased an 80% the expression of the xCT protein as demonstrated in Figure 3.15A-B.

The contribution of the high xCT expression to the GSH generation was determined. SH-SY5Y cells that were previously exposed to DEM 0.1 mM during 24h showed a glutathione intracellular content 4.5 folds higher (41.5 ± 5.8 nmols/mg protein) than control cells treated with medium (8.7 ± 0.8 nmols/mg protein) (Figure 3.15C).
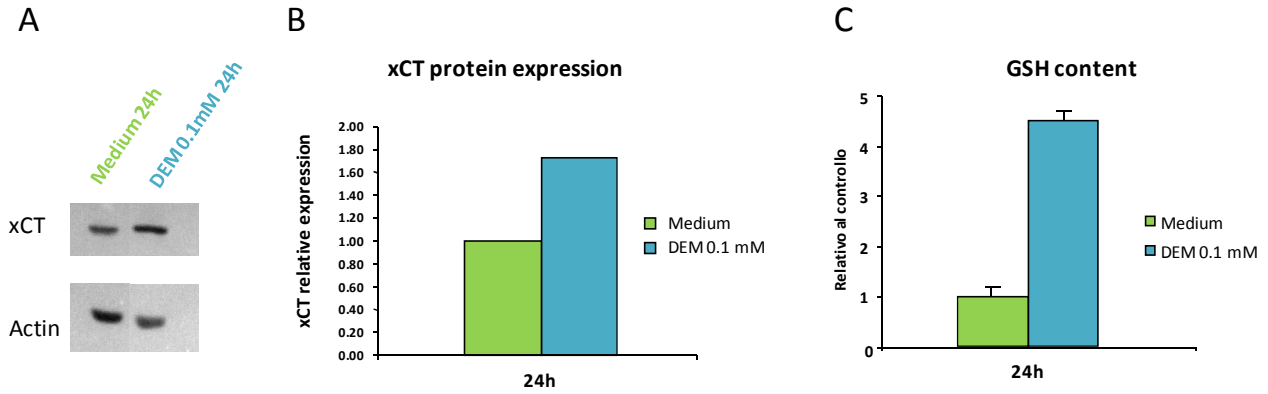
Figure 3.15 - xCT over-expression induction by DEM: the induction of xCT protein was analyzed by Western blot on SH-SY5Y cells treated with DEM 0.1 mM for 24h (A) and then quantified (B). The GSH content after the same treatment was determined (C).

2.2.1.8. Cell viability after bilirubin treatment on SH-SY5Y cells pre-treated with DEM

In order to identify if the over-expression of the System X_c^- and the high glutathione content are the main players on the bilirubin resistance, SH-SY5Y cells treated with DEM 0.1 mM or released in medium during 24h were then exposed to a second treatment with Bf 40, 70 and 140 nM or DMSO 0.6% for another 4h. If these players are directly involved in bilirubin resistance, cells pre-treated with DEM should have a 100% on viability after MTT test.

As shown in Figure 3.16, cells that were firstly treated with DEM, independently on the bilirubin concentration that were then exposed, arrived to 80 % on viability compare to DMSO 0.6. The same behavior was observed on cells that have never been treated with DEM. These results demonstrate that even if the System X_c^- is up-regulated and the cells contain high intracellular glutathione levels, these condition are not enough to confer the resistance against to the UCB injury.
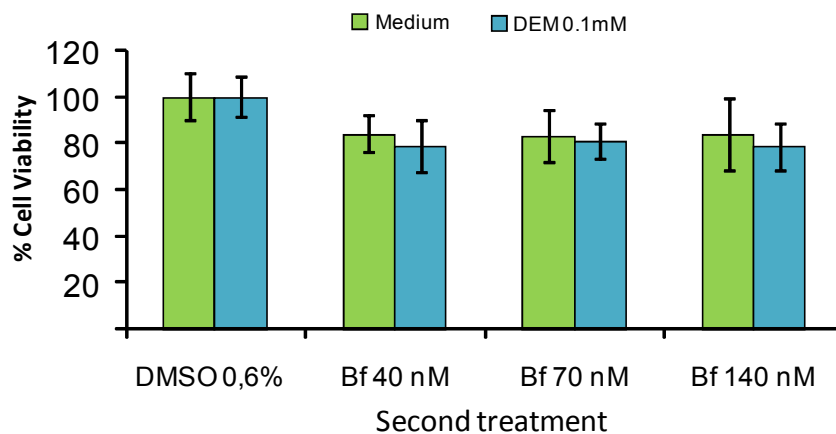
Figure 3.16 - System X_c^- and glutathione role in bilirubin resistance: SH-SY5Y cells were incubated for 24h with medium or DEM 0.1 mM and then exposed to DMSO 0.6% or Bf 40, 70 and 140 nM for another 4h to analyze the bilirubin resistance. The cell viability after the whole treatment was assessed by MTT.

2.2.1.9. Cell viability after hydrogen peroxide treatment on SH-SY5Y cells pre-treated with DEM

To corroborate the implication of the System X_c^- and the glutathione on the resistance of SH-SY5Y cells to oxidative environment, the over-expression of the system and the increase on glutathione intracellular content was induce incubating SH-SY5Y cells with DEM 0.1 mM for 24h (control cells were exposed to medium). Then the cells were exposed to increasing concentrations of hydrogen peroxide (medium for control, 50, 100, 200, 300, 400 and 500 μM H_2O_2) and the viability after the whole treatment was assessed by MTT (Figure 3.17).
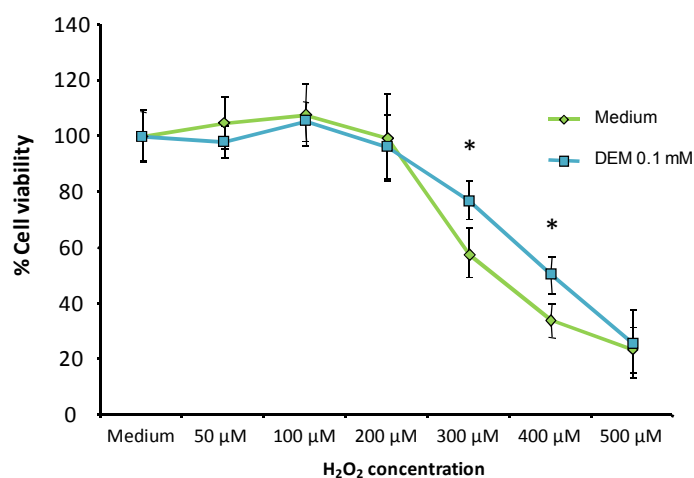
Figure 3.17 - System Xc- and glutathione role in hydrogen peroxide resistance: SH-SY5Y cells were incubated for 24h with medium or DEM 0.1 mM and then exposed to medium or 50, 100, 200, 300, 400 and 500 μM of H2O2. The cell viability after the whole treatment was assessed by MTT. *p<0.05.

The results have demonstrated that cells previously treated with DEM, when are exposed to H_2O_2 300 and 400 μM, are significantly more resistant to the oxidative environment respect control cells pre-treated with medium (76.97% ± 7.8% vs. 57.64% ± 8.3% and 50.41% ± 9.1% vs. 34.01% ± 5.2%, respectively. *p*<0.05).

2.2.2. Other possible targets involved in Bilirubin SH-SY5Y resistance

2.2.2.1. HO-1, HO-2, NQO1 and GCLC mRNA expression on SH-SY5Y cells treated with bilirubin

Other targets that are usually involved in detoxifying and antioxidant processes in the cell where considered as possible players in the mechanisms of bilirubin resistance. To approach this targets, the mRNA expression of the genes Heme Oxigenase 1 (HO-1), Heme Oxigenase 2 (HO-2), NADPH Quinone Oxidoreductase 1 (NQO1) and GCLC, the catalytic subunit of the enzyme Gama

Glutathione-Cysteine Sintetase was analyzed by qPCR on cells treated with medium, DMSO 0.6% and Bf 140 nM for 1h, 4h and 24h (Figure 3.18).
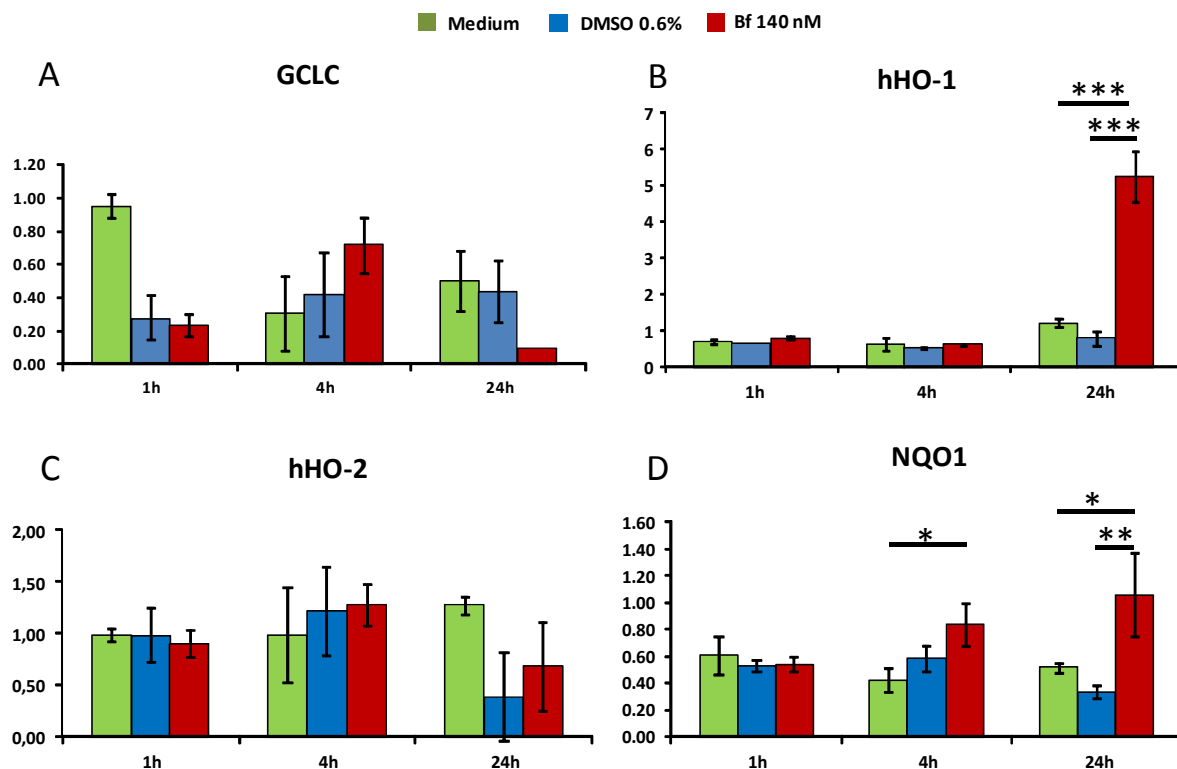
Figure 3.18 - mRNA expression of detoxifying and antioxidant genes: the mRNA expression of the genes GCLC (A), HO-1 (B), HO-2 (C) and NQO1 (D) was analyzed by qPCR after the incubation of SH-SY5Y cells with medium, DMSO 0.6% and Bf 140 nM during 1h, 4h and 24h. $*p<0.05$, $**p<0.01$ and $***p<0.001$.

In Figure 3.18A is shown the mRNA expression of GCLC. It was observed a very low expression of this gene on SH-SY5Y cells under the conditions above described.

Interesting results were obtained on mRNA expression analysis of HO-1 (Figure 3.18B). It was observed that cells treated with Bf 140 nM for 24h over-expressed (5 folds) the mRNA respect to the controls at 24h (Medium and DMSO 0.6%, $p<0.001$) and the treatments at 1h and 4h. Contrary to the over-expression in HO-1, the expression of the HO-2 was low and without significant changes among the treatments (Figure 3.18C). The mRNA expression of the NQO1 was significantly increased in cells treated with Bf 140 nM for both 4h and 24h as compared to their controls and 1h (Figure 3.18D).

2.2.2.2.   Bilirubin metabolism by CYP1A1, CYP1A2, CYP2A6 and UGT1A1

The hypothesis that the bilirubin resistance could be due to the metabolism of the pigment by an increase in the expression of the enzymes Cytochrome P450, family 1, member A1 (CYP1A1); Cytochrome P450, family 1, member A2 (CYP1A2); Cytochrome P450, family 2, member A6 (CYP2A6) and UDP glucuronosyltransferase 1 family, polypeptide A1 (UGT1A1) was

evaluated. For this reason mRNA extracted from SH-SY5Y cells treated with Medium, DMSO 0.6**%** and Bf 140 nM for 1h, 4h and 24h was used to analyze the expression of this two genes by qPCR.

The results indicate that SH-SY5Y cells do not express the genes CYP1A2, CYP2A6 and UGT1A1. Only the gene CYP1A1 was found to be expressed in SH-SY5Y cells, but no change was observed on the mRNA expression under the conditions analyzed (Figure 3.19).
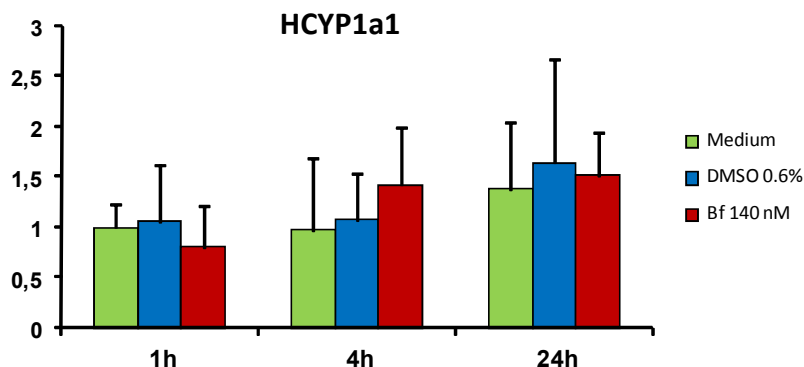
Figure 2. CYP1A1 mRNA expression on SH-SY5Y cells treated with bilirubin: the mRNA expression of the gene CYP1A1 that for the enzyme Cytochrome P450, family 1, member A1 able to metabolize the bilirubin was analyzed by qPCR on SH-SY5Y cells exposed to medium, DMSO 0.6% and Bf 140 nM for 1h, 4h and 24h.

2.2.3. SH-SY5Y Cell line populations - Possible different susceptibilities to bilirubin treatment

2.2.3.1.   Morphologic SH-SY5Y separation and bilirubin treatment

Considering that SH-SY5Y cell line is an heterogeneous cell line (Biagiotti et al., 2006), cells were initially subjected to a morphologic study. To analyze if the resistance to bilirubin observed on SH-SY5Y cells could be due to the presence of different susceptible and resistant subpopulation, SH-SY5Y cells were studied by flow cytometry. Based on the FSC-A and SSC-A parameters, two main morphologic subpopulation (P1 and P2) were identified (Figure 3.20A). The subpopulations P1 and P2 were then sorted and treated separately with DMSO 0.6% and Bf 140 nM for 24h to analyze the capability to resist the bilirubin exposure.
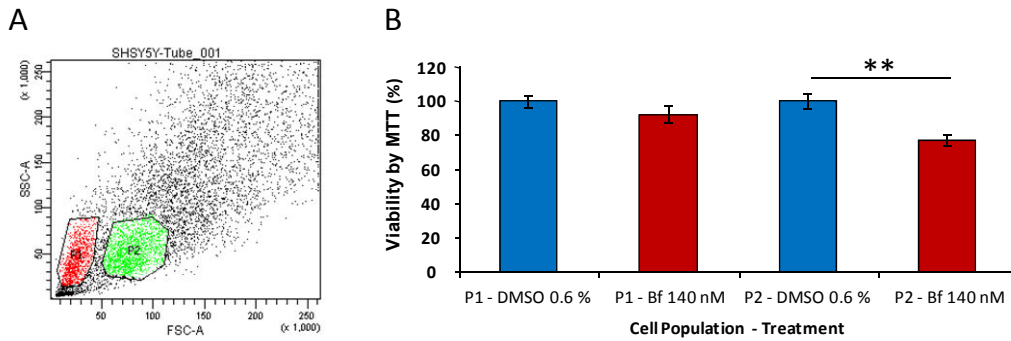
Figure 3.20 - Morphologic SH-SY5Y subpopulation exposed to bilirubin: P1 and P2 SH-SY5Y cells morphologic subpopulation were identified by flow cytometry based on the FSC-A and SSC-A analysis (A). The susceptibility to bilirubin of both subpopulations was assessed exposing them to Bf 140 nm and DMSO 0.6% (control) for 24h (B).

The viability of P1 subpopulation cells treated with bilirubin did not differ significantly than their controls treated with DMSO 0.6% (100% ± 3.54% vs. 92% ± 4.62%). Instead of P1, P2 have shown to be significantly less resistant to the bilirubin treatment (100% ± 4.86% vs. 77.21% ± 3.35%, $p < 0.01$) (Figure 3.20B). This result indicates that the morphologic separation is not enough to identify a subpopulation on the SH-SY5Y cell line that cannot overcome the bilirubin treatment.

2.2.3.2. Specific markers mRNA expression for the "S" and "N" SH-SY5Y subpopulations

In order to identify the subpopulations reported to be present in the SH-SY5Y cell line and their susceptibilities to bilirubin, the mRNA expression for the specific markers of the "S" (Calcyclin [CACYBP] and Vimentin [VIM]) and "N" (Neurofilament 68 [NFL] and Chromogranin A [CHGA]) populations was analyzed after bilirubin treatment. mRNA obtained from SH-SY5Y cells treated with medium, DMSO 0.6% and Bf 24h were used in the study.

The results shown in Figure 3.21 demonstrated that the four markers analyzed are expressed after the bilirubin treatment. These findings indicate that the cell population able to resist the bilirubin exposure is composed by N and S cells.
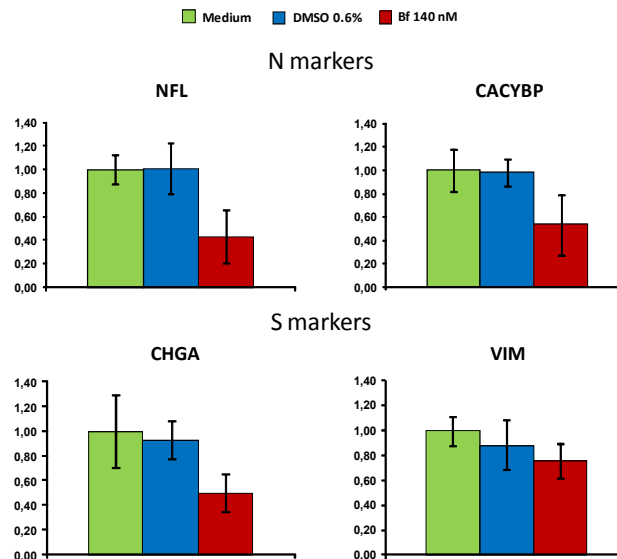
Figure 3.21 - mRNA expression of SH-SY5Y N and S cell subpopulation markers after bilirubin treatment: the mRNA expression of the specific markers for the N and S SH-SY5Y subpopulation was analyzed on cells after the treatment with medium, DMSO 0,6% and Bf 140 nM for 24h.

2.2.3.3. Vimentin expression in SH-SY5Y cells after bilirubin treatment

With the objective of identify if only one of the two main population present in the SH-SY5Y cell line (N or S) remain after the bilirubin treatment, the Vimentin protein expression (specific marker for the S population) was study by flow cytometry. Cells treated with medium, DMSO 0.6% and Bf 140 nM for 24h expressed the same percentage of Vimentin protein without significant differences (68.41% ± 11.37% vs 69.33% ± 6.74% vs. 65.92% ± 6.11%, respectively) (Figure 3.22). The fact that the percentage of positive cells for vimentin is the same despite of the bilirubin treatment means that the bilirubin is not able to select one of the two populations.
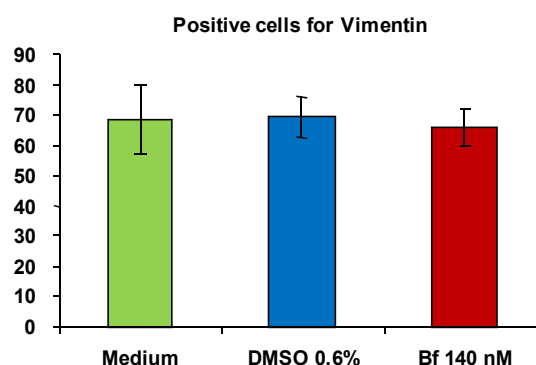
Figure 3.22 - Vimentin protein expression after bilirubin treatment: The Vimentin protein expression, a specific markers for the S SH-SY5Y subpopulation, was analyzed after the cell treatment with medium, DMSO 0,6% and Bf 140 nM for 24h.

3. SH-SY5Y MODEL TO STUDY BILIRUBIN NEUROTOXICITY

3.1. Oxidative stress generation by UCB

3.1.1. Intracellular Reactive Oxygen Species accumulation induced by bilirubin

The intracellular ROS accumulation induced by bilirubin was evaluated using 2'7'-dichlorofluorescein diacetate (DCFH-DA). SH-SY5Y cells were exposed to Bf 140 nM for 15 min, 30 min, 1h, 1.5h, 2h and 4h and the ROS generated, reported by the DCF fluorescence, was determined by Flow Cytometry. Cells treated with DMSO 0.6% for 4h were used as negative control and cells treated with H_2O_2 200 µM for 1h were used as positive control.
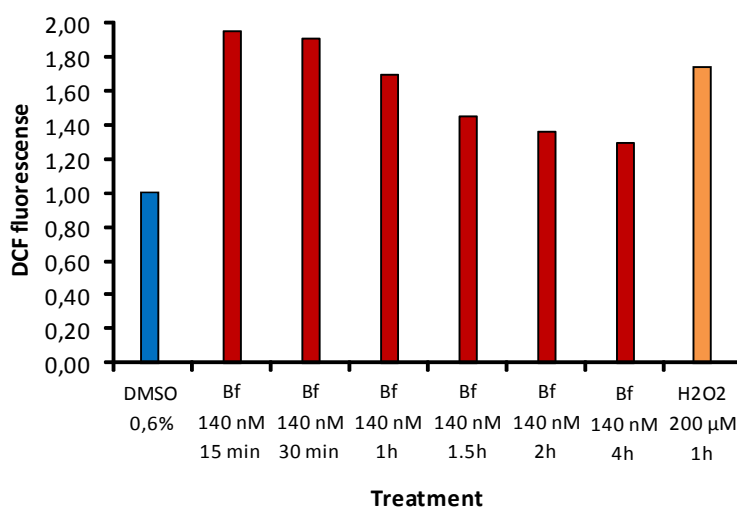
Figure 3.23 - ROS induction by bilirubin treatment: SH-SY5Y cells were treated with Bf 140 nM for 15 min, 30 min, 1h, 1.5h, 2h and 4h and the ROS generation after the treatment was analyzed measuring the DCF fluorescence intensity. For controls cells exposed to DMSO 0.6% (Negative) and H_2O_2 200 µM were used.

The results demonstrated that when the SH-SY5Y cells enter in contact with the bilirubin high levels of ROS are rapidly induced but then decrease within the 4h (Figure 3.23).

3.2. Glutamate excitotoxicity

3.2.1. Glutamate release in the medium by SH-SY5Y cells exposed to UCB

The mechanism of excitotoxicity was considered as a possible cause of cell death produce by bilirubin. Because this pathway start with the activation of the N-methyl-D-aspartate receptor by glutamate, the glutamate release in the medium by SH-SY5Y cells exposed to bilirubin was determined (figure 3.24).

The glutamate determinations on the medium obtained from cells exposed to Bf 140 nM during 1h, 2h, 3h and 4h have shown that the concentration of the glutamate increases accordingly with the time of exposure. The medium from cells treated with DMSO 0.6 % for 4h was used as control.
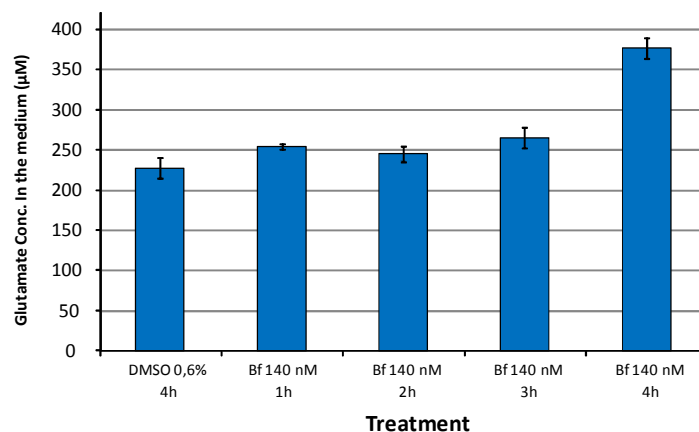
Figure 3.24 - Glutamate release in the medium after bilirubin treatment: The glutamate concentration in the medium was determined after the treatment of cells with Bf 140 nM for 1h, 2h, 3h and 4h using a EnzyChrom^{TM} Glutamate Assay Kit . Medium obtained from cells treated with DMSO 0.6% were used as controls.

3.2.2. nNOS, iNOS and eNOS mRNA expression after bilirubin treatment

To evidence a possible activation of the excitotoxicity pathway the expression of the mRNA that encode for the enzyme nNOS, iNOS and eNOS, involved in nitric oxide generation in the cells, was tested by qPCR. mRNA obtained from SH-SY5Y cells treated with medium, DMSO 0.6% and Bf 140 nM for 1h, 4h and 24h was analyzed. None of the genes was found over-expressed respect to the controls after the bilirubin treatment indicating that the excitotoxicity pathway is apparently not activated (Figure 3.25).
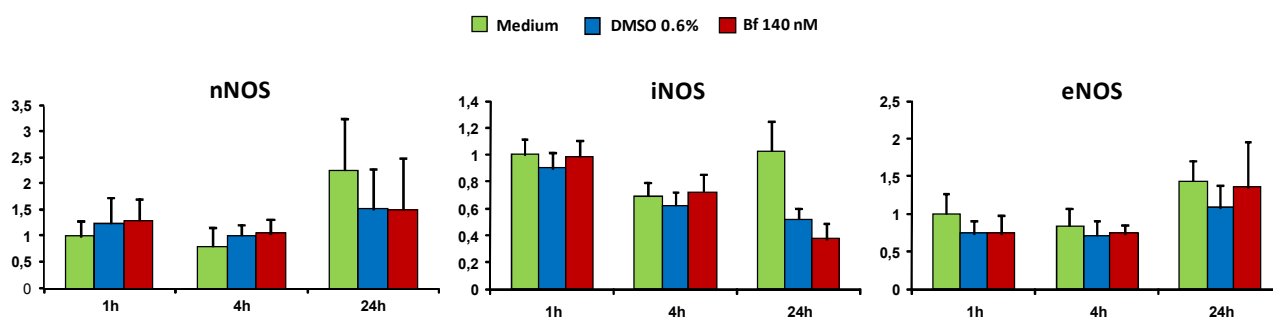
Figure 3.25 - mRNA expression of Nitric Oxide Sintetase enzymes on cells treated with bilirubin: The mRNA obtained from cells treated with medium , DMSO 0.6% and Bf 140 nM for 1h, 4h and 24h was used to analyzed the expression of the enzymes nNOS, iNOS and eNOS after each treatment.

Discussion and Conclusion

DISCUSSION

For a long time the bilirubin has been consider as a simple end product of the heme catabolism (Foresti et al., 2004; Dennery et al., 2001). Nowadays this overall picture has been changed extremely because of the high quantity of data involving the bilirubin as a main player in pathologies (Ostrow et al., 2003a; Dennery et al., 2001; CRIGLER, Jr. and NAJJAR, 1952; Reiser, 2004). In the present study the SH-SY5Y neuroblastoma cell line was used to approach the molecular events associated to bilirubin neurotoxicity and highlight the biochemical and molecular events that are induces in the neurons when get contact with the yellow pigment.

A dose-dependent decrease in cell viability was observed on cells exposed for times lower than 4h to Bf concentrations of 10 nM, 40 nM, 70 nM and 140 nM, arriving to a 60-70% of viability at the highest concentration. Of notice the observation that when the cells were incubated for 4h, 6h or 24h, the viability was always around 60-70%, independently on the Bf concentration tested. Several mechanisms could be hypothesized to explain this behavior. One possibility would be that two different populations of cells are present. Upon exposure to bilirubin, only a portion of the cells will activate cellular mechanisms to prevent accumulation/toxicity of the pigment at the cellular level. The other hypothesis, not excluding the former, is the presence of 2 cell subpopulations with different susceptibility. According to this, the first "hit" will remove the sensitive subpopulation leaving the resistant growing. It is know that the SH-SY5Y cell line is mainly composed by two subpopulation (Biagiotti et al., 2006; Ciccarone et al., 1989; Ross and Spengler, 2007) and it has been demonstrated that different cell types can react differentially to the bilirubin damage (Ngai et al., 2000). The idea that one of the two populations is more sensitive to bilirubin could explain the observations. This two hypothesis were considered along this thesis.

An interesting observation was done when the cells that survived to the treatment with Bf 140 nM for 24h were released in growth medium. These cells had difficulties to grow during the first 96h, showing a lag phase. After this time the cells re-start the duplication with the same rate as controls. The influence of the bilirubin on the cell cycle has been previously reported by Ollinger *et al.*(Ollinger et al., 2005; Ollinger et al., 2007a; Ollinger et al., 2007b) who showed that UCB acts as a natural inhibitor of the proliferation in vascular smooth muscle cells and HRT-18 colon cancer cells. Other evidence on the effect of the bilirubin causing arrest on the cell cycle derives from the work carried out by Rao *et al*. (Rao et al., 2006) where it was demonstrated the anti-cancer activity of bilirubin acting as a pro-oxidant, inhibited the growth of human carcinoma cell lines. These particular behaviors on our studies led us to define 3 different times respect to the growing state (*T0*, when the Bf 140 nM treatment for 24h is just finish; *T48*, when the cells are in the lag phase

not growing; and *T156* when the cells have recovered the growth capacity) for further analysis on the experiments.

When cells able to resist a the first bilirubin treatment were re-exposed to second bilirubin exposition at *T0, T48* and *T156*, all the cells continue to be resistant. Despite of that the cells have not been in contact to bilirubin for almost 7 days, the capacity to resist the bilirubin treatment persisted.

The analysis of the previous results let us define two different model to explain the dual behavior observed when SH-SY5Y cells are exposed to bilirubin. From one side, working at 24h of bilirubin treatment when the cells become resistant to the pigment, we have defined a ***model to study the bilirubin resistance***, where the mechanism involved in the bilirubin resistance could be addressed. On the other side, working at times shorter than 4h, when the cells exposed to bilirubin die, we have defined a ***model to study the bilirubin toxicity*** where the mechanism by which the UCB cause the damage could be analyzed. The discussion of the results obtained in each model will be addressed separately.

SH-SY5Y MODEL TO STUDY BILIRUBIN RESISTANCE

The first approach considered to analyze was why the cells became resistant to the UCB treatments. This study addressed mainly the bilirubin movements, such as the mechanism by which the bilirubin enter and exit in the cell. In addition, we also addressed if the lack of toxic effects may be related to intracellular events leading to detoxification of the pigment.

The possibility that the UCB resistance is achieved by reducing/preventing the entrance of the bilirubin was first considered. The mechanisms by which the bilirubin cross the cell membranes has not been completely understood. Studies in hepatocytes performed by Zucker S. (Zucker et al., 1999; Zucker and Goessling, 2000) has proposed that bilirubin exhibit spontaneous diffusion through the membrane by a flip-flop mechanism. On the contrary, other studies have shown the presence of distinct transporters in the two domains of human placental trophoblast that couldcooperate to transfer UCB from the fetus to the maternal circulation(Serrano et al., 2002). Similar results were observed in freshly isolated hepatocytes where at low, physiological UCB concentrations, UCB uptake showed saturative kinetics with an apparent K(m) of 41 nM, indicating carrier-mediated transport. With aqueous supersaturation, UCB entered hepatocytes mainly by passive diffusion (Mediavilla et al., 1999). Our results on SH-SY5Y neuroblastoma cells did not show differences in the uptake rate of bilirubin measured at 4°C and 37°C, suggesting a passive diffusion process rather than an active transport.

In order to confirm the passive diffusion we studied the mRNA expression of the OATP transporters reported to be involved in UCB transport (Hagenbuch and Meier, 2004; Briz et al., 2003b). We revealed that the OATP1, OATPC and OATP8 were not expressed in SH-SY5Y cells. Neither the expression of the OATP1A2 nor OATPE was found down-regulated. On the contrary, the OATPE was up-regulated at 24h. OATPE mRNA is expressed ubiquitously with strongest expression in liver, heart, placenta and pancreas. At the protein level it has been localized to the apical surface of the syncytiotrophoblast and has so far been only minimally characterize (Sato et al., 2003). In transiently transfected HEK293 cells this transporter has been implicated in the uptake of steroid conjugates, PGE2 and benzylpenicillin (Fujiwara et al., 2001; Tamai et al., 2000). The fact that the up-regulation is accounted at 24h when most of the cells have already died, and not at 1h and 4h, suggest us that the up-regulation may be regarded as a secondary reaction not directly related with the bilirubin resistance.

Furthermore, the accumulation of the bilirubin analyzed by HPLC have shown that the bilirubin entrance follow a linear trend supporting the passive diffusion hypothesis. This results showing a constant bilirubin accumulation into the cells plus the studies carried out on the ABC transporter mRNA expressions, where none of the transporters analyzed was up-regulated, strongly suggest that UCB resistance is not achieved by pumping out the bilirubin from the cell.

The exclusion of the theory where the UCB resistance is conferred avoiding the permanence of the bilirubin inside the cells led us to concentrate the attention in the metabolic changes induced by bilirubin in the cells. Several genes have been demonstrated by microarray analysis to be induced when SH-SY5Y cells are exposed to Bf 140 nM during 24h, most of them related with the ER stress (Calligaris et al., 2009). Among the up-regulated genes where those of the SLC7A11 and the SLC3A2 encoding respectively for the xCT and 4F2hc subunits of the System X_c^-. This system together with the System X_{AG}^- (SLC1A1) and the γ-GT (GGT1), is the main suppliers of cystine/cysteine for the Glutathione synthesis (La, V et al., 2007; Liu et al., 2007; Dringen, 2000; Dringen et al., 2001; Chen and Swanson, 2003; Shanker and Aschner, 2001). In the present study we have confirmed that bilirubin induces only the System X_c^- and not the other transporters,. Even more, the mRNA data was correlated with an specific functional induction of this system. Cells treated with bilirubin for 24h showed a 8 folds higher uptake of cystine, accounted entirely by the System X_c^-. As for bilirubin, other compounds have been reported to induce the expression of this transporter. Sasaki *et al.*(Sasaki et al., 2002) demonstrated in BHK21 cells that the activity of System X_c^- was significantly induced by various electrophilic agents like diethyl maleate, arsenite, $CdCl_2$, hydroquinone. The induction of SLC7A11 gene (the specific subunit of the System X_c^-) was mediated by Keap1/Nrf2 pathway. In 1984, Bannai S. (Bannai, 1984) described that electrophilic

compounds (diethyl maleate, sulfobromophthalein, ethacrynate) at relatively low concentrations, caused an increase in cellular glutathione due to the enhanced uptake of cystine via Na^+ independent transport.

Additional observations regarding the role of the intracellular glutathione content were provided in this thesis. Cells exposed to bilirubin for 24h showed 2.5 fold higher GSH level respect to the controls, and 2 folds higher 48h after UCB removal and growth on medium. Interestingly was the observation that at 156h after the deprivation of bilirubin the levels of glutathione returned to normal values. The effect of this levels of GSH to overcome an oxidative stress insult was assessed in the studies where the cells pre-treated with bilirubin at *T0*, *T48* and *T156* were exposed to different concentrations of H_2O_2. This experimental scheme demonstrated that, as expected, the higher the intracellular glutathione content, the higher the resistance to the oxidative environment. The contribution of the glutathione on the cell resistance could not only derive from the antioxidant properties of the molecule. GSH is also recognized as a general regulatory molecule with several functions like the modification of proteins as part of normal cell physiology and signaling (Rigacci et al., 1997; Gomez et al., 2004; Nakamura et al., 1997; Shackelford et al., 2005). Recent studies have shown that intracellular reductive environment is the key requirement for allowing DNA synthesis to occur(Chen et al., 2007).

By analyzing the resistance to H_2O_2 we observed that the cell exposed to the oxidative environment had a higher content of intracellular bilirubin, as shown on the HPLC accumulation studies at 24h. This higher bilirubin content could contribute to the cell resistance since previous evidence showed that bilirubin has antioxidant capacity at low concentration as described by Doré S. *et al.* in 1999 by studying the effect of nM concentrations of UCB in the cytoprotection of primary hippocampal cultures to H_2O_2. Furthermore, Baranano *et al.*(Baranano et al., 2002) propone, a biosynthetic cycle wherein oxidize bilirubin is generated from biliverdin by biliverdin reductace in a redox cycle. Recently Sedlak *et al.*(Sedlak et al., 2009) demonstrated that bilirubin protects against lipid peroxidation while GSH primarily prevents the oxidation of water soluble proteins. In this regard a possible overlapping between the two systems could contibute to confer the resistance.

The direct implication of the System X_c^- on the H_2O_2 resistance was proven by the silencing the xCT protein using a specific anti-xCT siRNA. Removal of the expression of System X_c^- was followed by loss of resistance. Moreover, the increased expression of this system by the exposition of the cells to DEM also conferred resistance to hydrogen peroxide. Despite of this results, the involvement of this transporter could not be directly associated with bilirubin resistance since the over-expression of the System X_c^- did not prevent the damage when the cells were exposed to different bilirubin concentrations.

Other possible targets that could be contributing to the resistance were analyzed. The γ-GCS, which forms γ-glutamylcysteine by ligatingglutamate and cysteine using ATP, is known to mediate the first rate-limiting step in the glutathione synthesis. γ-GCS is a heterodimericenzyme composed of a catalytic subunit encoded by GCLC anda modifier subunit encoded by GCLM(Johnson et al., 2008). The importance of this enzyme was demonstrated by several *in vivo* studies with genetically modified mice (Fujii et al., 2011). Shi, *ZZ et al.* showed that GCLC null cells isolated from embryos die, but can survive by supplementation with glutathione or N-acetylcysteine (NAC) (Shi et al., 2000). Because the gene GCLC, as the System X_c^-, is transcriptional regulated by Nrf2 (Wild et al., 1999; Wild et al., 1998; Sasaki et al., 2002), we analyzed the mRNA expression. No change on the mRNA expression was observed in cells treated with bilirubin in spite of an higher intracellular glutathione. γ-GCS activity was demonstrated to be rapidly regulated by post-translational modification of pre-existing GCLC and/or GCLM protein. In this regard, oxidative stress has been shown to stimulate γ-GCS activity prior to, or in the absence of, an increase in γ-GCS subunits protein expression. Sub-toxicconcentrations of hydrogen peroxide, menadione, phorone, or other oxidativeagents, lead to the transient stimulation of γ-GCS activity without detectable increases in γ-GCSsubunits protein levels (Franklin et al., 2009; Ochi, 1995; Ochi, 1996; Toroser et al., 2006).

Another important proteins with antioxidant and detoxifying properties analyzed were the HO-1, HO-2 and NQO1 (Baranano et al., 2002; Dinkova-Kostova and Talalay, 2010). Interestingly, except for HO-2, the expression of these genes was found up-regulated which could be indicating the participation of these enzymes in the protection processes. To finally corroborate their involvement, the enzymatic activity of these two proteins should be analyzed.

The cytochrome P450 (CYP) enzymes play a critical role in the detoxification and activation of xenobiotics(Miksys and Tyndale, 2002). In our studies it was demonstrated that only CYP1A1 is expressed in the SH-SY5Y cells, but its expression is not modified by the bilirubin incubation, discarding this enzyme as a possible player in the bilirubin resistance.

The differential susceptibility to bilirubin of the two main subpopulation that composed the SH-SY5Y cell line was considered as a possible explanation for the resistance observed after the UCB incubation. As described previously, the SH-Y5Y cell line is composed by two main, morphologic and phenotypic different cells (N and S type cells) (Biagiotti et al., 2006; Ciccarone et al., 1989; Lautrette et al., 2003; Acosta et al., 2009). A morphologic separation was done obtaining two main subpopulations, P1 and P2. Even if the exposition of them to Bf 140 nM for 24h demonstrated that P2 is more sensitive to the bilirubin treatment, both population remained alive. A second approach was considered analysing the Vimentin expression, a specific marker for the S-

type cells, after the bilirubin treatment. The same percentage of Vimentin expression (around 70%) was observed among bilirubin treated cells and the controls suggesting that the bilirubin is not able to select one of the subpopulation. Therefore we cannot conclude that the behaviour of SH-SY5Y is due to a different susceptibility of UCB toxicity of the 2 subpopulations of this cell line.

SH-SY5Y MODEL TO STUDY BILIRUBIN TOXICITY

The exposition of SH-SY5Y cells to different Bf concentration for periods of time no longer than 4h demonstrated that the cells die in a dose-dependent manner. This findings allowed us to define the conditions to analyze the bilirubin toxicity in the SH-SY5Y model.

Firstly we examined the bilirubin capacity to generate Reactive Oxygen Species (ROS) accumulation that could damage the cells. Our results demonstrate that the UCB induces the accumulation of ROS with the highest levels at shorter times of exposure. The effect of UCB in ROS generation was also reported on primary culture of rat neurons where the protein oxidation and lipid peroxidation is observed after bilirubin treatment (Brito et al., 2008). Additional studies, based on spin-labeling electron paramagnetic resonance spectroscopy analysis, indicated that UCB disrupts the redox status of isolated mitochondria (Rodrigues et al., 2002a) and intact nerve cells (Rodrigues et al., 2002b). Moreover, studies performed by Brito *et al* showed that injury to neocortical synaptosomes was linked to oxidative stress (Brito et al., 2004).

Neurons submitted to oxidative stress are prone to excitotoxicity and therefore control of extracellular glutamate levels is most important to prevent cell death (Yun et al., 1997). In our study extracellular glutamate was found increased according with the time of exposure to bilirubin. These findings could indicate a possible mechanism of excitotoxicity that enhance the oxidative stress damage through the NMDA receptor. This activation leads to the generation of NO and the consecutive increase of the Reactive Nitrogen Species (RNS). Excessive amount of these molecules leads to oxidative modification and, therefore, dysfunction of proteins, nucleic acids, and lipids(Wang and Michaelis, 2010). Even if the mRNA expression of the nNOS, eNOS and iNOS was not modified in bilirubin treated cells, the enzymatic activity and NO production should be tested to finally confirm this hypothesis.

CONCLUSION

In the present study the SH-SY5Y neuroblastoma cell line was used as a model to approach the molecular events that take place when the neuron are exposed to bilirubin. The results obtained in this thesis let us identify different players in the bilirubin damage and the neuron response to the pigment.

The incubation of cells with pathological concentrations of free bilirubin showed that initially the cells are sensitive to the damage generated by the UCB and die in a dose-dependent manner. Data supporting the role of the intracellular ROS accumulation and the extracellular glutamate release after bilirubin exposure was provided. This findings contributes to the hypothesis that the cellular death is achieved by an excitotoxic mechanism.

After 4h of incubation with bilirubin, the cells develop the ability to resist the UCB injury. The presented data do not support the hypothesis that resistance is accounted by preventing accumulation of the pigment inside the cells but rather suggest the conclusion that the resistance is produced by changes at a metabolic level.

Even if the exacts mechanisms by which the cells resist to bilirubin damage could not be identified, important players that certainly are contributing to the cell defense were recognized. For the first time, our work have demonstrated that the bilirubin is able to induce the System X_c^- with a consequent increased cysteine uptake and glutathione synthesis. The final product of this pathway, the GSH, could contribute to restore the equilibrium of the cellular redox status. Furthermore, we showed induction of other antioxidant and detoxifying players like the HO-1 and the NQO1, which may contribute further to UCB resistance.

Much work remains to be performed to finally unravel the mechanism(s) by which the bilirubin produce neurotoxicity. The data presented in this thesis not only could help to get closer to this goal but may also contribute to the identification of targets that could be used to prevent bilirubin damage.

References

REFERENCE LIST

Abu-Bakar,A., Moore,M.R., and Lang,M.A. (2005). Evidence for induced microsomal bilirubin degradation by cytochrome P450 2A5. Biochem. Pharmacol. *70*, 1527-1535.

Acosta,S., Lavarino,C., Paris,R., Garcia,I., de,T.C., Rodriguez,E., Beleta,H., and Mora,J. (2009). Comprehensive characterization of neuroblastoma cell line subtypes reveals bilineage potential similar to neural crest stem cells. BMC. Dev. Biol. *9*, 12.

Ahlfors,C.E. (2001). Bilirubin-albumin binding and free bilirubin. J. Perinatol. *21 Suppl 1*, S40-S42.

Ahlfors,C.E., Bennett,S.H., Shoemaker,C.T., Ellis,W.G., Davis,S.L., Wennberg,R.P., and Goetzman,B.W. (1986). Changes in the auditory brainstem response associated with intravenous infusion of unconjugated bilirubin into infant rhesus monkeys. Pediatr. Res. *20*, 511-515.

Ahlfors,C.E. and DiBiasio-Erwin,D. (1986). Rate constants for dissociation of bilirubin from its binding sites in neonatal (cord) and adult sera. J. Pediatr. *108*, 295-298.

Ahlfors,C.E. and Wennberg,R.P. (2004). Bilirubin-albumin binding and neonatal jaundice. Semin. Perinatol. *28*, 334-339.

Angeletti,R.H., Novikoff,P.M., Juvvadi,S.R., Fritschy,J.M., Meier,P.J., and Wolkoff,A.W. (1997). The choroid plexus epithelium is the site of the organic anion transport protein in the brain. Proc. Natl. Acad. Sci. U. S. A *94*, 283-286.

Bannai,S. (1984). Induction of cystine and glutamate transport activity in human fibroblasts by diethyl maleate and other electrophilic agents. J Biol. Chem. *259*, 2435-2440.

Bannai,S., Sato,H., Ishii,T., and Sugita,Y. (1989). Induction of cystine transport activity in human fibroblasts by oxygen. J Biol. Chem. *264*, 18480-18484.

Baranano,D.E., Rao,M., Ferris,C.D., and Snyder,S.H. (2002). Biliverdin reductase: a major physiologic cytoprotectant. Proc. Natl. Acad. Sci. U. S. A *99*, 16093-16098.

Biagiotti,T., D'Amico,M., Marzi,I., Di,G.P., Arcangeli,A., Wanke,E., and Olivotto,M. (2006). Cell renewing in neuroblastoma: electrophysiological and immunocytochemical characterization of stem cells and derivatives. Stem Cells *24*, 443-453.

Biedler,J.L., Helson,L., and Spengler,B.A. (1973). Morphology and growth, tumorigenicity, and cytogenetics of human neuroblastoma cells in continuous culture. Cancer Res. *33*, 2643-2652.

Bosma,P.J., Seppen,J., Goldhoorn,B., Bakker,C., Oude Elferink,R.P., Chowdhury,J.R., Chowdhury,N.R., and Jansen,P.L. (1994). Bilirubin UDP-glucuronosyltransferase 1 is the only relevant bilirubin glucuronidating isoform in man. J. Biol. Chem. *269*, 17960-17964.

Brito,M.A., Brites,D., and Butterfield,D.A. (2004). A link between hyperbilirubinemia, oxidative stress and injury to neocortical synaptosomes. Brain Res. *1026*, 33-43.

Brito,M.A., Lima,S., Fernandes,A., Falcao,A.S., Silva,R.F., Butterfield,D.A., and Brites,D. (2008). Bilirubin injury to neurons: contribution of oxidative stress and rescue by glycoursodeoxycholic acid. Neurotoxicology *29*, 259-269.

Brito,M.A., Vaz,A.R., Silva,S.L., Falcao,A.S., Fernandes,A., Silva,R.F., and Brites,D. (2010). N-methyl-aspartate receptor and neuronal nitric oxide synthase activation mediate bilirubin-induced neurotoxicity. Mol. Med. *16*, 372-380.

Briz,O., MacIas,R.I., Serrano,M.A., Gonzalez-Gallego,J., Bayon,J.E., and Marin,J.J. (2003a). Excretion of foetal bilirubin by the rat placenta-maternal liver tandem. Placenta *24*, 462-472.

Briz,O., Serrano,M.A., MacIas,R.I., Gonzalez-Gallego,J., and Marin,J.J. (2003b). Role of organic anion-transporting polypeptides, OATP-A, OATP-C and OATP-8, in the human placenta-maternal liver tandem excretory pathway for foetal bilirubin. Biochem. J. *371*, 897-905.

Burdo,J., Dargusch,R., and Schubert,D. (2006). Distribution of the cystine/glutamate antiporter system xc- in the brain, kidney, and duodenum. J. Histochem. Cytochem. *54*, 549-557.

Calligaris,R., Bellarosa,C., Foti,R., Roncaglia,P., Giraudi,P., Krmac,H., Tiribelli,C., and Gustincich,S. (2009). A transcriptome analysis identifies molecular effectors of unconjugated bilirubin in human neuroblastoma SH-SY5Y cells. BMC. Genomics *10*, 543.

Calligaris,S., Cekic,D., Roca-Burgos,L., Gerin,F., Mazzone,G., Ostrow,J.D., and Tiribelli,C. (2006). Multidrug resistance associated protein 1 protects against bilirubin-induced cytotoxicity. FEBS Lett. *580*, 1355-1359.

Calligaris,S.D., Bellarosa,C., Giraudi,P., Wennberg,R.P., Ostrow,J.D., and Tiribelli,C. (2007). Cytotoxicity is predicted by unbound and not total bilirubin concentration. Pediatr. Res. *62*, 576-580.

Cesaratto,L., Calligaris,S.D., Vascotto,C., Deganuto,M., Bellarosa,C., Quadrifoglio,F., Ostrow,J.D., Tiribelli,C., and Tell,G. (2007). Bilirubin-induced cell toxicity involves PTEN activation through an APE1/Ref-1-dependent pathway. J. Mol. Med. *85*, 1099-1112.

Chen,Y. and Swanson,R.A. (2003). The glutamate transporters EAAT2 and EAAT3 mediate cysteine uptake in cortical neuron cultures. J. Neurochem. *84*, 1332-1339.

Chen,Z., Odstrcil,E.A., Tu,B.P., and McKnight,S.L. (2007). Restriction of DNA replication to the reductive phase of the metabolic cycle protects genome integrity. Science *316*, 1916-1919.

Ciccarone,V., Spengler,B.A., Meyers,M.B., Biedler,J.L., and Ross,R.A. (1989). Phenotypic diversification in human neuroblastoma cells: expression of distinct neural crest lineages. Cancer Res. *49*, 219-225.

CRIGLER,J.F., Jr. and NAJJAR,V.A. (1952). Congenital familial nonhemolytic jaundice with kernicterus. Pediatrics *10*, 169-180.

Cui,Y., Konig,J., Leier,I., Buchholz,U., and Keppler,D. (2001). Hepatic uptake of bilirubin and its conjugates by the human organic anion transporter SLC21A6. J. Biol. Chem. *276*, 9626-9630.

DAY,R.L. (1954). Inhibition of brain respiration in vitro by bilirubin; reversal of inhibition by various means. Proc. Soc. Exp. Biol. Med. *85*, 261-264.

Deeley,R.G., Westlake,C., and Cole,S.P. (2006). Transmembrane transport of endo- and xenobiotics by mammalian ATP-binding cassette multidrug resistance proteins. Physiol Rev. *86*, 849-899.

Dennery,P.A., Seidman,D.S., and Stevenson,D.K. (2001). Neonatal hyperbilirubinemia. N. Engl. J. Med. *344*, 581-590.

Dinkova-Kostova,A.T. and Talalay,P. (2010). NAD(P)H:quinone acceptor oxidoreductase 1 (NQO1), a multifunctional antioxidant enzyme and exceptionally versatile cytoprotector. Arch. Biochem. Biophys. *501*, 116-123.

Dore,S. and Snyder,S.H. (1999). Neuroprotective action of bilirubin against oxidative stress in primary hippocampal cultures. Ann. N. Y. Acad. Sci. *890*, 167-172.

Dore,S., Takahashi,M., Ferris,C.D., Zakhary,R., Hester,L.D., Guastella,D., and Snyder,S.H. (1999). Bilirubin, formed by activation of heme oxygenase-2, protects neurons against oxidative stress injury. Proc. Natl. Acad. Sci. U. S. A *96*, 2445-2450.

Dringen,R. (2000). Metabolism and functions of glutathione in brain. Prog. Neurobiol. *62*, 649-671.

Dringen,R., Gutterer,J.M., Gros,C., and Hirrlinger,J. (2001). Aminopeptidase N mediates the utilization of the GSH precursor CysGly by cultured neurons. J. Neurosci. Res. *66*, 1003-1008.

Dringen,R., Gutterer,J.M., and Hirrlinger,J. (2000). Glutathione metabolism in brain metabolic interaction between astrocytes and neurons in the defense against reactive oxygen species. Eur. J. Biochem. *267*, 4912-4916.

Ernster,L. and ZETTERSTROM,R. (1956). Bilirubin, an uncoupler of oxidative phosphorylation in isolated mitochondria. Nature *178*, 1335-1337.

Foresti,R., Green,C.J., and Motterlini,R. (2004). Generation of bile pigments by haem oxygenase: a refined cellular strategy in response to stressful insults. Biochem. Soc. Symp. 177-192.

Franklin,C.C., Backos,D.S., Mohar,I., White,C.C., Forman,H.J., and Kavanagh,T.J. (2009). Structure, function, and post-translational regulation of the catalytic and modifier subunits of glutamate cysteine ligase. Mol. Aspects Med. *30*, 86-98.

Fujii,J., Ito,J.I., Zhang,X., and Kurahashi,T. (2011). Unveiling the roles of the glutathione redox system in vivo by analyzing genetically modified mice. J. Clin. Biochem. Nutr. *49*, 70-78.

Fujiwara,K., Adachi,H., Nishio,T., Unno,M., Tokui,T., Okabe,M., Onogawa,T., Suzuki,T., Asano,N., Tanemoto,M., Seki,M., Shiiba,K., Suzuki,M., Kondo,Y., Nunoki,K., Shimosegawa,T., Iinuma,K., Ito,S., Matsuno,S., and Abe,T. (2001). Identification of thyroid hormone transporters in humans: different molecules are involved in a tissue-specific manner. Endocrinology *142*, 2005-2012.

Gazzin,S., Strazielle,N., Schmitt,C., Fevre-Montange,M., Ostrow,J.D., Tiribelli,C., and Ghersi-Egea,J.F. (2008). Differential expression of the multidrug resistance-related proteins ABCb1 and ABCc1 between blood-brain interfaces. J. Comp Neurol. *510*, 497-507.

Ghersi-Egea,J.F., Gazzin,S., and Strazielle,N. (2009). Blood-brain interfaces and bilirubin-induced neurological diseases. Curr. Pharm. Des *15*, 2893-2907.

Gomez,L.D., Noctor,G., Knight,M.R., and Foyer,C.H. (2004). Regulation of calcium signalling and gene expression by glutathione. J Exp. Bot. *55*, 1851-1859.

Grojean,S., Lievre,V., Koziel,V., Vert,P., and Daval,J.L. (2001). Bilirubin exerts additional toxic effects in hypoxic cultured neurons from the developing rat brain by the recruitment of glutamate neurotoxicity. Pediatr. Res. *49*, 507-513.

Hagenbuch,B. and Meier,P.J. (2004). Organic anion transporting polypeptides of the OATP/ SLC21 family: phylogenetic classification as OATP/ SLCO superfamily, new nomenclature and molecular/functional properties. Pflugers Arch. *447*, 653-665.

Hahm,J.S., Ostrow,J.D., Mukerjee,P., and Celic,L. (1992). Ionization and self-association of unconjugated bilirubin, determined by rapid solvent partition from chloroform, with further studies of bilirubin solubility. J. Lipid Res. *33*, 1123-1137.

Hanko,E., Hansen,T.W., Almaas,R., Lindstad,J., and Rootwelt,T. (2005). Bilirubin induces apoptosis and necrosis in human NT2-N neurons. Pediatr. Res. *57*, 179-184.

Hanko,E., Hansen,T.W., Almaas,R., Paulsen,R., and Rootwelt,T. (2006). Synergistic protection of a general caspase inhibitor and MK-801 in bilirubin-induced cell death in human NT2-N neurons. Pediatr. Res. *59*, 72-77.

Hayashi,A., Suzuki,H., Itoh,K., Yamamoto,M., and Sugiyama,Y. (2003). Transcription factor Nrf2 is required for the constitutive and inducible expression of multidrug resistance-associated protein 1 in mouse embryo fibroblasts. Biochem. Biophys. Res. Commun. *310*, 824-829.

Jia,Z., Hallur,S., Zhu,H., Li,Y., and Misra,H.P. (2008). Potent upregulation of glutathione and NAD(P)H:quinone oxidoreductase 1 by alpha-lipoic acid in human neuroblastoma SH-SY5Y cells: protection against neurotoxicant-elicited cytotoxicity. Neurochem. Res. *33*, 790-800.

Johnson,J.A., Johnson,D.A., Kraft,A.D., Calkins,M.J., Jakel,R.J., Vargas,M.R., and Chen,P.C. (2008). The Nrf2-ARE pathway: an indicator and modulator of oxidative stress in neurodegeneration. Ann. N. Y. Acad. Sci. *1147*, 61-69.

Jones,P.M. and George,A.M. (2004). The ABC transporter structure and mechanism: perspectives on recent research. Cell Mol. Life Sci. *61*, 682-699.

Kamisako,T., Leier,I., Cui,Y., Konig,J., Buchholz,U., Hummel-Eisenbeiss,J., and Keppler,D. (1999). Transport of monoglucuronosyl and bisglucuronosyl bilirubin by recombinant human and rat multidrug resistance protein 2. Hepatology *30*, 485-490.

Kapitulnik,J., Horner-Mibashan,R., Blondheim,S.H., Kaufmann,N.A., and Russell,A. (1975). Increase in bilirubin-binding affinity of serum with age of infant. J. Pediatr. *86*, 442-445.

Kaplan,D. and Navon,G. (1982). Studies of the conformation of bilirubin and its dimethyl ester in dimethyl sulphoxide solutions by nuclear magnetic resonance. Biochem. J. *201*, 605-613.

Knickelbein,R.G., Seres,T., Lam,G., Johnston,R.B., Jr., and Warshaw,J.B. (1997). Characterization of multiple cysteine and cystine transporters in rat alveolar type II cells. Am. J. Physiol *273*, L1147-L1155.

La,B., V, Valentino,F., Piccoli,T., and Piccoli,F. (2007). Expression and developmental regulation of the cystine/glutamate exchanger (xc-) in the rat. Neurochem. Res. *32*, 1081-1090.

Lautrette,C., Cardot,P.J., Vermot-Desroches,C., Wijdenes,J., Jauberteau,M.O., and Battu,S. (2003). Sedimentation field flow fractionation purification of immature neural cells from a human tumor neuroblastoma cell line. J. Chromatogr. B Analyt. Technol. Biomed. Life Sci. *791*, 149-160.

Lee,C., Oh,W., Stonestreet,B.S., and Cashore,W.J. (1989). Permeability of the blood brain barrier for 125I-albumin-bound bilirubin in newborn piglets. Pediatr. Res. *25*, 452-456.

Lee,C., Stonestreet,B.S., Oh,W., Outerbridge,E.W., and Cashore,W.J. (1995). Postnatal maturation of the blood-brain barrier for unbound bilirubin in newborn piglets. Brain Res. *689*, 233-238.

Liu,R.R., Brown,C.E., and Murphy,T.H. (2007). Differential regulation of cell proliferation in neurogenic zones in mice lacking cystine transport by xCT. Biochem. Biophys. Res Commun. *364*, 528-533.

LONDON,I.M., WEST,R., SHEMIN,D., and RITTENBERG,D. (1950). On the origin of bile pigment in normal man. J. Biol. Chem. *184*, 351-358.

Loscher,W. and Potschka,H. (2005). Blood-brain barrier active efflux transporters: ATP-binding cassette gene family. NeuroRx. *2*, 86-98.

Maher,J.M., Dieter,M.Z., Aleksunes,L.M., Slitt,A.L., Guo,G., Tanaka,Y., Scheffer,G.L., Chan,J.Y., Manautou,J.E., Chen,Y., Dalton,T.P., Yamamoto,M., and Klaassen,C.D. (2007). Oxidative and electrophilic stress induces multidrug resistance-associated protein transporters via the nuclear factor-E2-related factor-2 transcriptional pathway. Hepatology *46*, 1597-1610.

Malik,S.G., Irwanto,K.A., Ostrow,J.D., and Tiribelli,C. (2010). Effect of bilirubin on cytochrome c oxidase activity of mitochondria from mouse brain and liver. BMC. Res. Notes *3*, 162.

Mark,L.P., Prost,R.W., Ulmer,J.L., Smith,M.M., Daniels,D.L., Strottmann,J.M., Brown,W.D., and Hacein-Bey,L. (2001). Pictorial review of glutamate excitotoxicity: fundamental concepts for neuroimaging. AJNR Am. J. Neuroradiol. *22*, 1813-1824.

Markowitz,A.J., White,M.G., Kolson,D.L., and Jordan-Sciutto,K.L. (2007). Cellular interplay between neurons and glia: toward a comprehensive mechanism for excitotoxic neuronal loss in neurodegeneration. Cellscience. *4*, 111-146.

McCoubrey,W.K., Jr., Huang,T.J., and Maines,M.D. (1997). Isolation and characterization of a cDNA from the rat brain that encodes hemoprotein heme oxygenase-3. Eur. J. Biochem. *247*, 725-732.

McDonald,J.W., Shapiro,S.M., Silverstein,F.S., and Johnston,M.V. (1998). Role of glutamate receptor-mediated excitotoxicity in bilirubin-induced brain injury in the Gunn rat model. Exp. Neurol. *150*, 21-29.

Mediavilla,M.G., Pascolo,L., Rodriguez,J.V., Guibert,E.E., Ostrow,J.D., and Tiribelli,C. (1999). Uptake of [(3)H]bilirubin in freshly isolated rat hepatocytes: role of free bilirubin concentration. FEBS Lett. *463*, 143-145.

Miksys,S.L. and Tyndale,R.F. (2002). Drug-metabolizing cytochrome P450s in the brain. J. Psychiatry Neurosci. *27*, 406-415.

Nakamura,H., Nakamura,K., and Yodoi,J. (1997). Redox regulation of cellular activation. Annu. Rev. Immunol. *15*, 351-369.

Ngai,K.C., Yeung,C.Y., and Leung,C.S. (2000). Difference in susceptibilities of different cell lines to bilirubin damage. J. Paediatr. Child Health *36*, 51-55.

Oakes,G.H. and Bend,J.R. (2005). Early steps in bilirubin-mediated apoptosis in murine hepatoma (Hepa 1c1c7) cells are characterized by aryl hydrocarbon receptor-independent oxidative stress and activation of the mitochondrial pathway. J Biochem. Mol. Toxicol. *19*, 244-255.

Ochi,T. (1995). Hydrogen peroxide increases the activity of gamma-glutamylcysteine synthetase in cultured Chinese hamster V79 cells. Arch. Toxicol. *70*, 96-103.

Ochi,T. (1996). Menadione causes increases in the level of glutathione and in the activity of gamma-glutamylcysteine synthetase in cultured Chinese hamster V79 cells. Toxicology *112*, 45-55.

Ollinger,R., Bilban,M., Erat,A., Froio,A., McDaid,J., Tyagi,S., Csizmadia,E., Graca-Souza,A.V., Liloia,A., Soares,M.P., Otterbein,L.E., Usheva,A., Yamashita,K., and Bach,F.H. (2005). Bilirubin: a natural inhibitor of vascular smooth muscle cell proliferation. Circulation *112*, 1030-1039.

Ollinger,R., Kogler,P., Troppmair,J., Hermann,M., Wurm,M., Drasche,A., Konigsrainer,I., Amberger,A., Weiss,H., Ofner,D., Bach,F.H., and Margreiter,R. (2007a). Bilirubin inhibits tumor cell growth via activation of ERK. Cell Cycle *6*, 3078-3085.

Ollinger,R., Yamashita,K., Bilban,M., Erat,A., Kogler,P., Thomas,M., Csizmadia,E., Usheva,A., Margreiter,R., and Bach,F.H. (2007b). Bilirubin and biliverdin treatment of atherosclerotic diseases. Cell Cycle *6*, 39-43.

Ostrow,J.D., JANDL,J.H., and Schmid,R. (1962). The formation of bilirubin from hemoglobin in vivo. J. Clin. Invest *41*, 1628-1637.

Ostrow,J.D. and Mukerjee,P. (2007). Solvent partition of 14C-unconjugated bilirubin to remove labeled polar contaminants. Transl. Res. *149*, 37-45.

Ostrow,J.D., Pascolo,L., Shapiro,S.M., and Tiribelli,C. (2003a). New concepts in bilirubin encephalopathy. Eur. J. Clin. Invest *33*, 988-997.

Ostrow,J.D., Pascolo,L., and Tiribelli,C. (2003b). Reassessment of the unbound concentrations of unconjugated bilirubin in relation to neurotoxicity in vitro. Pediatr. Res. *54*, 926.

Ostrow,J.D. and Tiribelli,C. (2003). Bilirubin, a curse and a boon. Gut *52*, 1668-1670.

Pascolo,L., Fernetti,C., Garcia-Mediavilla,M.V., Ostrow,J.D., and Tiribelli,C. (2001). Mechanisms for the transport of unconjugated bilirubin in human trophoblastic BeWo cells. FEBS Lett. *495*, 94-99.

Patel,S.A., Warren,B.A., Rhoderick,J.F., and Bridges,R.J. (2004). Differentiation of substrate and non-substrate inhibitors of transport system xc(-): an obligate exchanger of L-glutamate and L-cystine. Neuropharmacology *46*, 273-284.

Poss,K.D. and Tonegawa,S. (1997). Reduced stress defense in heme oxygenase 1-deficient cells. Proc. Natl. Acad. Sci. U. S. A *94*, 10925-10930.

Rao,P., Suzuki,R., Mizobuchi,S., Yamaguchi,T., and Sasaguri,S. (2006). Bilirubin exhibits a novel anti-cancer effect on human adenocarcinoma. Biochem. Biophys. Res. Commun. *342*, 1279-1283.

Reiser,D.J. (2004). Neonatal jaundice: physiologic variation or pathologic process. Crit Care Nurs. Clin. North Am. *16*, 257-269.

Rigacci,S., Iantomasi,T., Marraccini,P., Berti,A., Vincenzini,M.T., and Ramponi,G. (1997). Evidence for glutathione involvement in platelet-derived growth-factor-mediated signal transduction. Biochem. J *324 (Pt 3)*, 791-796.

Rodrigues,C.M., Sola,S., Brito,M.A., Brites,D., and Moura,J.J. (2002a). Bilirubin directly disrupts membrane lipid polarity and fluidity, protein order, and redox status in rat mitochondria. J. Hepatol. *36*, 335-341.

Rodrigues,C.M., Sola,S., Castro,R.E., Laires,P.A., Brites,D., and Moura,J.J. (2002b). Perturbation of membrane dynamics in nerve cells as an early event during bilirubin-induced apoptosis. J Lipid Res. *43*, 885-894.

Rodrigues,C.M., Sola,S., Silva,R., and Brites,D. (2000). Bilirubin and amyloid-beta peptide induce cytochrome c release through mitochondrial membrane permeabilization. Mol. Med. *6*, 936-946.

Roma,M.G., Crocenzi,F.A., and Mottino,A.D. (2008). Dynamic localization of hepatocellular transporters in health and disease. World J. Gastroenterol. *14*, 6786-6801.

Ross,R.A., Biedler,J.L., and Spengler,B.A. (2003). A role for distinct cell types in determining malignancy in human neuroblastoma cell lines and tumors. Cancer Lett. *197*, 35-39.

Ross,R.A. and Spengler,B.A. (2007). Human neuroblastoma stem cells. Semin. Cancer Biol. *17*, 241-247.

Rublevskaya,I. and Maines,M.D. (1994). Interaction of Fe-protoporphyrin IX and heme analogues with purified recombinant heme oxygenase-2, the constitutive isozyme of the brain and testes. J. Biol. Chem. *269*, 26390-26395.

Sasaki,H., Sato,H., Kuriyama-Matsumura,K., Sato,K., Maebara,K., Wang,H., Tamba,M., Itoh,K., Yamamoto,M., and Bannai,S. (2002). Electrophile response element-mediated induction of the cystine/glutamate exchange transporter gene expression. J Biol. Chem. *277*, 44765-44771.

Sato,H., Fujiwara,K., Sagara,J., and Bannai,S. (1995). Induction of cystine transport activity in mouse peritoneal macrophages by bacterial lipopolysaccharide. Biochem. J *310 (Pt 2)*, 547-551.

Sato,H., Nomura,S., Maebara,K., Sato,K., Tamba,M., and Bannai,S. (2004). Transcriptional control of cystine/glutamate transporter gene by amino acid deprivation. Biochem. Biophys. Res. Commun. *325*, 109-116.

Sato,K., Sugawara,J., Sato,T., Mizutamari,H., Suzuki,T., Ito,A., Mikkaichi,T., Onogawa,T., Tanemoto,M., Unno,M., Abe,T., and Okamura,K. (2003). Expression of organic anion transporting polypeptide E (OATP-E) in human placenta. Placenta *24*, 144-148.

Scapagnini,G., Foresti,R., Calabrese,V., Giuffrida Stella,A.M., Green,C.J., and Motterlini,R. (2002). Caffeic acid phenethyl ester and curcumin: a novel class of heme oxygenase-1 inducers. Mol. Pharmacol. *61*, 554-561.

Sedlak,T.W., Saleh,M., Higginson,D.S., Paul,B.D., Juluri,K.R., and Snyder,S.H. (2009). Bilirubin and glutathione have complementary antioxidant and cytoprotective roles. Proc. Natl. Acad. Sci. U. S. A *106*, 5171-5176.

Serrano,M.A., Bayon,J.E., Pascolo,L., Tiribelli,C., Ostrow,J.D., Gonzalez-Gallego,J., and Marin,J.J. (2002). Evidence for carrier-mediated transport of unconjugated bilirubin across plasma membrane vesicles from human placental trophoblast. Placenta *23*, 527-535.

Shackelford,R.E., Heinloth,A.N., Heard,S.C., and Paules,R.S. (2005). Cellular and molecular targets of protein S-glutathiolation. Antioxid. Redox. Signal. *7*, 940-950.

Shanker,G. and Aschner,M. (2001). Identification and characterization of uptake systems for cystine and cysteine in cultured astrocytes and neurons: evidence for methylmercury-targeted disruption of astrocyte transport. J. Neurosci. Res. *66*, 998-1002.

Shi,Z.Z., Osei-Frimpong,J., Kala,G., Kala,S.V., Barrios,R.J., Habib,G.M., Lukin,D.J., Danney,C.M., Matzuk,M.M., and Lieberman,M.W. (2000). Glutathione synthesis is essential for mouse development but not for cell growth in culture. Proc. Natl. Acad. Sci. U. S. A *97*, 5101-5106.

Shih,A.Y., Erb,H., Sun,X., Toda,S., Kalivas,P.W., and Murphy,T.H. (2006). Cystine/glutamate exchange modulates glutathione supply for neuroprotection from oxidative stress and cell proliferation. J. Neurosci. *26*, 10514-10523.

Smith,P.K., Krohn,R.I., Hermanson,G.T., Mallia,A.K., Gartner,F.H., Provenzano,M.D., Fujimoto,E.K., Goeke,N.M., Olson,B.J., and Klenk,D.C. (1985). Measurement of protein using bicinchoninic acid. Anal. Biochem. *150*, 76-85.

Strazielle,N. and Ghersi-Egea,J.F. (2000). Choroid plexus in the central nervous system: biology and physiopathology. J. Neuropathol. Exp. Neurol. *59*, 561-574.

Szakacs,G., Paterson,J.K., Ludwig,J.A., Booth-Genthe,C., and Gottesman,M.M. (2006). Targeting multidrug resistance in cancer. Nat. Rev. Drug Discov. *5*, 219-234.

Tamai,I., Nezu,J., Uchino,H., Sai,Y., Oku,A., Shimane,M., and Tsuji,A. (2000). Molecular identification and characterization of novel members of the human organic anion transporter (OATP) family. Biochem. Biophys. Res. Commun. *273*, 251-260.

Tenhunen,R., Marver,H.S., and Schmid,R. (1968). The enzymatic conversion of heme to bilirubin by microsomal heme oxygenase. Proc. Natl. Acad. Sci. U. S. A *61*, 748-755.

Toroser,D., Yarian,C.S., Orr,W.C., and Sohal,R.S. (2006). Mechanisms of gamma-glutamylcysteine ligase regulation. Biochim. Biophys. Acta *1760*, 233-244.

Vogt,M.T. and Basford,R.E. (1968). The effect of bilirubin on the energy metabolism of brain mitochondria. J Neurochem. *15*, 1313-1320.

Wang,P., Kim,R.B., Chowdhury,J.R., and Wolkoff,A.W. (2003). The human organic anion transport protein SLC21A6 is not sufficient for bilirubin transport. J. Biol. Chem. *278*, 20695-20699.

Wang,X. and Michaelis,E.K. (2010). Selective neuronal vulnerability to oxidative stress in the brain. Front Aging Neurosci. *2*, 12.

Watchko,J.F. (2006). Kernicterus and the molecular mechanisms of bilirubin-induced CNS injury in newborns. Neuromolecular. Med. *8*, 513-529.

Weisiger,R.A., Ostrow,J.D., Koehler,R.K., Webster,C.C., Mukerjee,P., Pascolo,L., and Tiribelli,C. (2001). Affinity of human serum albumin for bilirubin varies with albumin concentration and buffer composition: results of a novel ultrafiltration method. J. Biol. Chem. *276*, 29953-29960.

Wennberg,R.P. (2000). The blood-brain barrier and bilirubin encephalopathy. Cell Mol. Neurobiol. *20*, 97-109.

Wild,A.C., Gipp,J.J., and Mulcahy,T. (1998). Overlapping antioxidant response element and PMA response element sequences mediate basal and beta-naphthoflavone-induced expression of the human gamma-glutamylcysteine synthetase catalytic subunit gene. Biochem. J. *332 (Pt 2)*, 373-381.

Wild,A.C., Moinova,H.R., and Mulcahy,R.T. (1999). Regulation of gamma-glutamylcysteine synthetase subunit gene expression by the transcription factor Nrf2. J. Biol. Chem. *274*, 33627-33636.

Xie,H.R., Hu,L.S., and Li,G.Y. (2010). SH-SY5Y human neuroblastoma cell line: in vitro cell model of dopaminergic neurons in Parkinson's disease. Chin Med. J. (Engl.) *123*, 1086-1092.

Yun,H.Y., Dawson,V.L., and Dawson,T.M. (1997). Nitric oxide in health and disease of the nervous system. Mol. Psychiatry *2*, 300-310.

Zelenka,J., Lenicek,M., Muchova,L., Jirsa,M., Kudla,M., Balaz,P., Zadinova,M., Ostrow,J.D., Wong,R.J., and Vitek,L. (2008). Highly sensitive method for quantitative determination of bilirubin in biological fluids and tissues. J. Chromatogr. B Analyt. Technol. Biomed. Life Sci. *867*, 37-42.

Zucker,S.D. and Goessling,W. (2000). Mechanism of hepatocellular uptake of albumin-bound bilirubin. Biochim. Biophys. Acta *1463*, 197-208.

Zucker,S.D., Goessling,W., and Hoppin,A.G. (1999). Unconjugated bilirubin exhibits spontaneous diffusion through model lipid bilayers and native hepatocyte membranes. J. Biol. Chem. *274*, 10852-10862.

Zucker,S.D., Goessling,W., Ransil,B.J., and Gollan,J.L. (1995). Influence of glutathione S-transferase B (ligandin) on the intermembrane transfer of bilirubin. Implications for the intracellular transport of nonsubstrate ligands in hepatocytes. J. Clin. Invest *96*, 1927-1935.

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LIST OF PUBLICATIONS

"Functional induction of the cystine-glutamate exchanger system Xc- activity in SH-SY5Y cells by unconjugated bilirubin". Pablo Giraudi, Cristina Bellarosa, **Carlos D. Coda-Zabetta** and Claudio Tiribelli. PlosOne December 2011, Vol 6. 12 – e29078.

"Postnatal changes of bilirubin accumulation in selective brain regions in hyperbilirubinemic Gunn rat puppies". Gazzin S. & Zelenka J., Zdrahalova L., Konickova R., **Coda Zabetta C.D.**, Giraudi J.P., Berengeno A.L.; Raseni A., Robert M.C, Vitek L. and Tiribelli C. *In press* on Pediatric Research.

Congress presentations

Workshop " Yellow Retreat", June 6th – 7th 2011, Trieste, Italy.
"Functional Induction of the Cystine-Glutamate Exchanger System X_c^- Activity in SH-SY5Y Cells by Unconjugated Bilirubin" Authors: **Carlos Coda Zabetta**, Pablo Giraudi, Cristina Bellarosa and Claudio Tiribelli. *Oral presentation*

7th seminar SIBBM – Frontier in Molecular Biology. May 26th – 28th 2011, Trieste, Italy
"Functional induction of the cystine-glutamate exchanger system X_c^- activity in SH-SY5Y cells by unconjugated bilirubin". Authors: **Carlos Coda-Zabetta**, Pablo Giraudi, Cristina Bellarosa and Claudio Tiribelli. *Poster presentation.*

Pediatric Academic Socities & Asian Society for Pediatric Research. 30th – May 3rd 2010, Denver, Colorado, USA. April.
"A New Point of Care System to Measure Plasma Bilirubin Concentration". Authors: Richard Wennberg, Pablo Giraudi, **Carlos Coda-Zabetta**, Cristina Bellarosa, Chiara Greco and Claudio Tiribelli. *Poster presentation*.

Workshop " Yellow Retreat" March 8th 2010, Trieste, Italy.
"Bilirubin content and Bilirubin transporter expression in SH-SY5Y cells treated with UCB" Authors: **Carlos Coda Zabetta**, Pablo Giraudi, Cristina Bellarosa and Claudio Tiribelli. *Oral presentation*

3rd Congress of the of Pediatric Societies – EAPS. October 23rd – 26th 2010, Copenhagen, Denmark.

"Bilirubin upregulates gene expression of SLC7A11 and increases cystine uptake mediated by System X_c^- in SH-SY5Y neuroblastoma cells". <u>Authors:</u> Pablo Giraudi, Cristina Bellarosa, **Carlos Coda-Zabetta** and Claudio Tiribelli. *Poster presentation*.

3rd FEBS Special Meeting "ATP-Binding Cassette Proteins: From Multidrug Resistance to Genetic Disease". February 27th – March 5th 2010, Innsbruck, Austria.
"mRNA expression of some ABC proteins in SH-SY5Y neuroblastoma cells treated with bilirubin". <u>Authors:</u> Pablo Giraudi, Cristina Bellarosa, **Carlos Coda-Zabetta** and Claudio Tiribelli. *Poster presentation*.