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**The cytotoxic effect of unconjugated bilirubin in human neuroblastoma  
SH-SY5Y cells is modulated by the expression level of MRP1  
but not MDR1**

**Lucia Corich\***, **Alejandro Aranda\***, **Laura Carrassa†**, **Cristina Bellarosa\***,  
**J. Donald Ostrow‡** and **Claudio Tiribelli\***

\* Centro Studi Fegato, Bld Q, AREA Science Park, Basovizza Campus, and Department of Life Sciences, University of Trieste, 34012 Trieste, Italy

† Laboratory of Molecular Pharmacology, Department of Oncology, Istituto di Ricerche Farmacologiche Mario Negri, Milan, Italy.

‡ GI/Hepatology Division, Department of Medicine, University of Washington, Seattle, WA, 98195, USA.

**Short Title: Role of MRP1 and MDR1 in bilirubin neurotoxicity**

Footnote: *In Memoriam:* This paper is dedicated to the late Prof. Rudi Schmid for his outstanding contributions in the bilirubin field.

Corresponding author:

Claudio Tiribelli, MD PhD  
Centro Studi Fegato,  
AREA Science Park, Basovizza Campus  
Ss 12 km 163.5  
34012 Trieste – Italy  
Tel +39-040-375 7840  
Fax +39-040-375 7832  
e-mail [ctliver@csf.units.it](mailto:ctliver@csf.units.it)

## Synopsis

*In vitro* and *in vivo* studies have demonstrated that unconjugated bilirubin (UCB) is neurotoxic. Although previous studies suggested that both MRP1 and MDR1 proteins may protect cells against accumulation of UCB, direct comparison of their role in UCB transport was never performed. To this end, we used an inducible siRNA expression system to silence the expression of MRP1 and MDR1 in human neuroblastoma SH-SY5Y cells. The effects of *in vitro* exposure to clinically-relevant levels of unbound UCB were compared between unsilenced (control) cells and cells with similar reductions in the expression of MRP1 or MDR1, documented by RT-PCR (mRNA), immunoblotting (protein), and for MDR1, the enhanced net uptake of a specific fluorescent substrate. Cytotoxicity was assessed by the MTT test. MRP1-deficient cells accumulated significantly more UCB and suffered greater cytotoxicity than controls. By contrast, MDR1-deficient cells exhibited UCB uptake and cytotoxicity comparable to controls. At intermediate levels of silencing, the increased susceptibility to UCB toxicity closely correlated with the decrease of the expression of MRP1, but not of MDR1. These data support the concept that limitation of cellular UCB accumulation, due to UCB export mediated by MRP1, but not MDR1, plays an important role in preventing bilirubin encephalopathy in the newborn.

Word Count: 201

Key Words: neurotoxicity, bilirubin, MRP1, MDR1, kernicterus

## INTRODUCTION

In humans and rodents, unconjugated bilirubin (UCB) may be neurotoxic. In some neonates with severe hyperbilirubinemia, UCB accumulates in neurons and astroglial cells in specific brain regions, causing encephalopathy and kernicterus [1]. Initial *in vitro* studies of the effects of UCB on mouse neuroblastoma cell lines revealed a higher sensitivity to UCB toxicity of mitotically active N2AB-1 cells than of “mature” neurons [2], and a progressive and irreversible toxicity in N-115 cells exposed to UCB for one or more hours [3].

Toxic effects of UCB are greatly decreased by tight binding to plasma albumin, since only the unbound diacid species of UCB, which is dominant at physiological pH [4;5], can diffuse rapidly across cell membranes [6]. *In vitro* exposure of neurons or astrocytes to UCB has shown neuroprotection at low, physiological concentrations of unbound UCB ( $B_f$ ), but neurotoxicity at modestly higher  $B_f$  values [5;7]. Others reported that UCB activated the mitochondrial pathway of apoptosis in rat brain neurons, inducing mitochondrial depolarization and Bax translocation *via* physical interaction with membranes [8]. Studies performed on human NT2-N neurons showed that UCB induces rapid necrosis at high and moderate concentrations and delayed apoptosis at low and moderate concentrations [9]. In human neuroblastoma SH-SY5Y cells, clinically relevant UCB concentrations cause early disruption of the mitochondrial membrane potential and subsequently induce apoptosis [10].

Collectively these findings indicate that intracellular accumulation of UCB may cause serious damage to nerve cells. This prompted us to explore the mechanisms that may limit toxic intracellular accumulation of UCB. The multidrug resistance-associated protein, MRP1 (ABCC1), and the multidrug resistance protein, MDR1 (P-Glycoprotein, ABCB1), belong to the superfamily of the ATP-binding cassette (ABC) transporters [11]. MRP1 transports amphipathic anionic, glucuronide and sulphate conjugates of steroid hormones and bile salts. In the presence of physiological concentrations of reduced glutathione, MRP1 also transports unconjugated xenobiotics and uncharged drugs [11-13]. MDR1 transports hydrophobic, either uncharged or weakly positively charged compounds in their unmodified forms and, in particular conditions, even highly charged, anionic compounds [11]. Both MRP1 and MDR1 protect nerve cells from toxic substances by promoting their active export from the cells [14-18].

Studies using various experimental models demonstrated that MRP1 is directly involved in the transport of UCB [19-21]. In plasma-membrane vesicles from MDCKII cells (Madin-Darby canine kidney II cells), the stable expression of human MRP1 increased significantly the export of UCB with high affinity,  $K_m=10\pm 3$  nM ( $B_f$ ) [22]. In cultured mouse astrocytes and

primary cultures of rat neurons and astrocytes, functional inhibition of Mrp1 with MK571 engendered an increase in UCB-induced toxicity [15], even at a normally nontoxic  $B_f$  of 40 nM [23]. Collectively, these findings show that MRP1 exports UCB at relatively low, physiologically-relevant  $B_f$ , and thus may protect the cell from accumulation of toxic levels of UCB.

Other studies have suggested that Mdr1/MDR1 might also transport UCB. UCB may be a weak substrate for Mdr1a [24] and competitively inhibits the labelling of brain capillary MDR1 with a photoaffinity substrate [25]. A greater proportion of UCB administered intravenously is taken up by the brains of *mdr1a* (-/-) knockout mice compared with their *mdr1a* (+/+) controls [26]. Although these studies suggest that MDR1 may be involved in the transport of UCB, they were all performed with high doses of UCB that yielded bilirubin concentrations vastly higher than clinically-relevant levels [27;28]. However, drugs known to inhibit MDR1 function may increase the risk of bilirubin encephalopathy in jaundiced neonates [29].

The aim of the present work was to compare directly the role of MRP1 and MDR1 in the prevention of UCB related cytotoxicity in the human neuroblastoma SH-SY5Y cell line, which expresses both proteins [30;31] and is highly susceptible to UCB toxicity [10]. The expression of either MRP1 or MDR1 in these cells was modulated by using small interference RNAs (siRNAs) produced by an inducible vector, and net accumulation and cytotoxicity of UCB were assessed. Our experiments are the first to directly compare the protective role of MRP1 and MDR1 against UCB toxicity in a single cell line under identical experimental conditions. We demonstrate that knockdown of MRP1, but not MDR1: a) decreases the cellular export of UCB and increases the cytotoxicity of UCB over a range of clinically-relevant  $B_f$  levels (40-140 nM); b) renders the cells sensitive to UCB toxicity at low  $B_f$  levels that are non-toxic to control cells.

## EXPERIMENTAL

### Chemicals

Dulbecco's Modified Eagle's Medium (DMEM), L-Glutamine, Penicillin Streptomycin and Trypsin 0.05%/EDTA 0.02% in PBS, without Phenol Red,  $Ca^{2+}/Mg^{2+}$  were purchased from Euroclone (Pero, Milano, Italy). Fetal Bovine Serum Tetracycline-free (Tet System Approved FBS, US-Sourced) was purchased from Clontech (Mountain View, CA, USA). MEM Non Essential Amino Acids, MEM Vitamin Solution, Opti-MEM I Reduced Serum Medium and Lipofectamine 2000 were purchased from Invitrogen (Carlsbad, CA, USA). Blasticidin S hydrochloride, puromycin dihydrochloride, doxycycline hyclate, PBS (Dulbecco's Phosphate

Buffered Saline) without  $\text{Ca}^{2+}/\text{Mg}^{2+}$ , TRI REAGENT<sup>TM</sup>, bicinchoninic acid protein assay kit, Ponceau S-solution (0.1% w/v) in 5% acetic acid (v/v), MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide], dimethyl sulfoxide (DMSO) and Hoechst 33342 were purchased from Sigma (St. Louis, MO, USA). Chlorophenol red- $\beta$ -D-galactopyranoside (CPRG) was purchased from Boehringer Mannheim, (Indianapolis, Ind., USA). iScript<sup>TM</sup> cDNA Synthesis Kit and iQ<sup>TM</sup> SYBR Green Supermix were purchased from Bio-Rad Laboratories (Hercules, CA, USA). Cell Lysis Buffer was purchased from Cell Signaling (Beverly, MA, USA). ECL-Plus Western Blotting detection system solutions were purchased from Amersham-Pharmacia Biotech (Buckinghamshire, UK). UCB was purchased from Sigma (St. Louis, MO, USA) and purified as described by McDonagh and Assisi [32]. [<sup>3</sup>H]-bilirubin (29.25 mCi/mmol) was biosynthetically labelled *in vivo* and then highly purified from the bilirubin conjugates in bile as described [33]. Liquid scintillation cocktail for radioassay (Filter counter N° 6013149) was purchased from Packard Bioscience (Groningen, The Netherlands). MRP1-A23 rabbit antibody was prepared at our laboratory [34], anti-Mouse IgG (Fc specific)-Peroxidase antibody produced in goat affinity isolated antibody, rabbit anti-actin affinity isolated antibody and peroxidase conjugated-goat anti-rabbit IgG-whole molecule affinity isolated antigen specific antibody were purchased from Sigma (St. Louis, MO, USA). Anti-C219 (MDR1) Monoclonal Antibody was purchased from Signet Laboratories (Dedham, MA, USA).

### **Construction of pSUPERIOR-MRP1 and pSUPERIOR-MDR1 clones**

We used pSUPERIOR.puro vector (Oligoengine, Seattle, WA, USA), a tetracycline (or doxycycline)-regulated vector for inducible siRNA expression. The siRNA sequence against *MDR1* was obtained by Wu *et al.* [35] while that against *MRP1* was the Silencer Validated *MRP1*-siRNA (#51321-1651 Ambion, Austin TX, USA). The pSUPERIOR.puro vector was digested by BglIII and HindIII and the annealed oligonucleotides ligated and cloned into the vector. The constructs were confirmed by sequencing. SH-SY5Y cells were transfected (Lipofectamine 2000) with the pcDNA6/TR vector (Invitrogen, Carlsbad, CA, USA) expressing high levels of the Tetracycline repressor (TetR) gene and cells grown in medium containing 5  $\mu\text{g}/\text{mL}$  blasticidin. After transient transfection of the pcDNA4/TO/*lacZ* vector (Invitrogen, Carlsbad, CA, USA), single colonies were isolated and expanded. They were screened for expression of the highest levels of Tet repressor from pcDNA6/TR, by assaying  $\beta$ -galactosidase expression upon induction with 3.9  $\mu\text{M}$  doxycycline, used as an alternative inducing agent to tetracycline (half-life: 48 vs. 24 hours, respectively) according to the pSUPERIOR manufacturer's suggestions. The clone showing the highest ratio of  $\beta$ -galactosidase activity

between induced and non-induced samples was selected for transfecting the pSUPERIOR vector containing the siRNA-expressing sequence targeting *MRP1* or *MDR1*. The empty pSUPERIOR.puro vector was also transfected and used as control. Puromycin (0.8  $\mu\text{g}/\text{mL}$ )-resistant colonies were expanded for two additional months. The clones showing the highest knockdown of *MRP1* (pSUPERIOR-*MRP1*) or *MDR1* (pSUPERIOR-*MDR1*) upon induction with 3.9  $\mu\text{M}$  doxycycline were selected by gene, protein and functional analysis.

### Cell culture

SH-SY5Y cells were kindly supplied from Pharmacological Research Institute “Mario Negri” (Laboratory of Molecular Pharmacology) in Milan. Stable SH-SY5Y clones expressed constitutively the Tet repressor from pcDNA6/TR vector and inducibly the siRNAs from pSUPERIOR vector containing the siRNA-expressing sequence targeting *MRP1* (pSUPERIOR-*MRP1* clone) or *MDR1* (pSUPERIOR-*MDR1* clone). The cells were grown in DMEM medium supplemented with 2 mM L-Glutamine, 10% (v/v) fetal bovine serum Tetracycline-free, 1% (v/v) MEM Non Essential Amino Acids, 1% (v/v) MEM Vitamin Solution and 1% (v/v) Penicillin/Streptomycin (100 units/ml Penicillin and 100  $\mu\text{g}/\text{ml}$  Streptomycin). Blasticidin (5  $\mu\text{g}/\text{mL}$ ) and puromycin (0.8  $\mu\text{g}/\text{mL}$ ) were added as selection antibiotics for pcDNA6/TR and pSUPERIOR.puro, respectively. Clones were maintained at 37° C in humidified air with 5% CO<sub>2</sub>. On the day prior to the experiment, confluent cells were trypsinized and plated in: 1) 35 mm plates at a density of  $2.5 \times 10^6$  cells/well for RT-Real Time PCR and Western Blot analyses; 2) in 12-well plates at a density of  $1 \times 10^6$  cells/well for the [<sup>3</sup>H]UCB-uptake assay; and 3) in 96-well plates at a density of  $0.2 \times 10^6$  cells/well for MTT assay and for Hoechst 33342 Dye Accumulation assay. Doxycycline (3.9  $\mu\text{M}$ ) was used to induce the siRNA expression from pSUPERIOR.puro vector. Before the harvest or treatments, clones were incubated with and without doxycycline (induced and non-induced samples, respectively) at 37°C for various time intervals.

### RNA isolation, reverse transcription and RT-Real Time PCR

pSUPERIOR-*MRP1* clone was incubated with doxycycline (3.9  $\mu\text{M}$ ) for 24, 48 and 72 hours or for 48 hours followed by additional 48 hours without doxycycline (induced samples). pSUPERIOR-*MDR1* was incubated with doxycycline (3.9  $\mu\text{M}$ ) for 24 and 48 hours or for 24 hours followed by additional 24 hours without doxycycline (induced samples). The control clone, stably transfected with pcDNA6/TR vector and with an empty pSUPERIOR.puro vector, was treated with doxycycline (3.9  $\mu\text{M}$ ) as described for pSUPERIOR-*MRP1* and pSUPERIOR-

*MDR1* clones. For each induced sample, a non-induced sample was tested. Total RNA was isolated from each clone and from its control using TRI REAGENT™, according to the manufacturer's recommendations. One µg of total RNA from cell culture was reverse transcribed using the iScript cDNA Synthesis Kit, according to manufacturer's instructions, on a thermal cycler (Gene Amp PCR System 2400, Perkin-Elmer, Boston, MA, USA). The relative levels of *MRP1* mRNA with sense (GCCAAGAAGGAGGAGACC) and anti-sense (AGGAAGATGCTGAGGAAGG) primers and the *MDR1* mRNA with sense (TGCTCAGACAGGATGTGAGTTG) and anti-sense (AATTACAGCAAGCCTGGAACC) primers were assayed by RT-Real Time PCR using iQ SYBR Green Supermix, as described previously [15]. Amplification, data acquisition and data analysis were carried out using the iCycler IQ (Bio-Rad Laboratories, Hercules, CA). Each sample was analyzed in triplicate. For each amplificate, a blank (nuclease free water) was added in place of cDNA. The thermal cycler conditions were: 95°C for 3 min; 40 cycles at 95°C for 20 minutes, 60°C for 20 minutes and 72°C for 30 minutes. In each reaction, a standard curve was generated by serial dilution of isolated and quantified cDNA (chosen among the cDNA samples) and a melting curve was generated to verify the specificity of the amplification. The results were normalized to each housekeeping gene and the initial amount of the template of each sample was determined as relative expression versus the non-induced control sample chosen as reference. *β-ACTIN* with sense (CGCCGCCAGCTCACCATG) and anti-sense (CACGATGGAGGGGAAGACGG) primers and *GAPDH* with sense (CCCATGTTTCGTCATGGGTGT) and anti-sense (TGGTCATGAGTCCTTCCACGATA) primers were used as endogenous controls to normalize the expression level of target genes.

### **Western Blotting**

pSUPERIOR-MRP1 clone was incubated with doxycycline (3.9 µM) for 24, 48 and 72 hours or for 72 hours followed by 72 hours without doxycycline (induced samples). pSUPERIOR-MDR1 clone was incubated with doxycycline (3.9 µM) for 24 and 48 hours or for 48 hours followed by 48 hours without doxycycline (induced samples). The control clone, stably transfected with pcDNA6/TR vector and with an empty pSUPERIOR.puro vector, was treated with doxycycline as described for pSUPERIOR-MRP1 and pSUPERIOR-MDR1 clones. For each induced sample, a non-induced sample was tested. Proteins of each sample were subjected to SDS-PAGE in 10% acrylamide and the resolved protein bands transferred onto a nitrocellulose membrane (0.2 lm Protran BA 83, Schleicher and Schuell, Dassel, Germany). Membranes were blocked for 1 hour at room temperature in 4% milk-TTBS (0.2% Tween 20,



20mM Tris and 500mM NaCl; pH 7.5) and then incubated overnight at 4°C with a 1:600 dilution of the MRP1-A23 rabbit antibody or 1:50 dilution of the anti-C219 (MDR1) mouse antibody or 1:1500 dilution of rabbit anti-actin antibody. After washing three times with 4% milk-TTBS, the membranes were incubated in blocking solution for 1 hour at room temperature with secondary antibodies: peroxidase conjugated-goat anti-rabbit (1:6000) or anti-Mouse IgG (Fc specific)–Peroxidase (1:2000). Protein bands were detected by ECL-Plus, transferred to Kodak film and visualized by Kodak EDAS 260 (Kodak Instruments, New Haven, CT, USA) using Kodak 1D image software. The intensities of protein bands were quantified by Scion Image (Frederick, MD, USA). To quantify the relative expression of the proteins (MRP1/ACTIN or MDR1/ACTIN), serial dilutions of a reference sample were loaded on each gel, optical density recorded and a standard curve was generated from the serial dilutions by non linear regression analysis (Curver Expert 1.3 software, Starkville, MS, USA). The correlation coefficient of the calibration curve was never lower than 0.995.

### **[<sup>3</sup>H]-UCB uptake in cultured cells**

pSUPERIOR-MRP1 clone was treated with UCB 72 hours after induction, by doxycycline (3.9 μM), of the siRNA-expressing sequence transcription. pSUPERIOR-MDR1 clone was treated with UCB 48 hours after removing the doxycycline from cells previously incubated with doxycycline for 48 hours. These incubation conditions were predetermined to be the best for observing the silencing of MRP1 and MDR1, respectively (see *Results*). [<sup>3</sup>H]-UCB (29.25 mCi/mmol) was dissolved in 0.3% (v/v) DMSO and diluted in culture medium supplemented with 15% (v/v) Fetal Bovine Serum containing 54 μM albumin. Cells were incubated for 30 minutes in the presence of [<sup>3</sup>H]-UCB (15 μM) at B<sub>f</sub>=40 nM, determined as described previously [36]. The cells were then carefully washed with ice-cold PBS to arrest UCB uptake and remove loosely-bound [<sup>3</sup>H]-UCB. The quantity of [<sup>3</sup>H]-UCB retained by cells, normalized for protein concentration, was measured as previously described [19].

### **MTT assay and Hoechst 33342 Dye Accumulation Assay**

MTT assay is used for measuring the activity of enzymes that reduce MTT to formazan. Yellow MTT is reduced to purple formazan in the mitochondria of living cells by active mitochondrial reductase enzymes; therefore conversion is used as a measure of viable cells.

pSUPERIOR-MRP1 and pSUPERIOR-MDR1 clones were incubated with doxycycline (3.9 μM) for the same durations selected to analyze protein content (see above). For each induced sample, a non-induced sample was included, and each treatment was performed in

quadruplicate. After the appropriate doxycycline incubation time, cells were treated for 4 hours with three different concentrations of UCB (15  $\mu$ M or 20  $\mu$ M or 30  $\mu$ M). UCB was dissolved in 0.3%, 0.4% or 0.6% v/v of DMSO respectively, and diluted in 100  $\mu$ l of complete medium supplemented with 15% Fetal Bovine Serum containing 54  $\mu$ M albumin. B<sub>f</sub> concentrations were 40 nM, 70 nM and 140 nM respectively, measured as described previously [36]. For each induced and non-induced sample treated with UCB, an induced and a non-induced sample treated with the same volume of DMSO, but not UCB, were included as controls (CTRL). Induced and non-induced samples, not treated with either UCB or DMSO, were tested also. MTT assay was performed as described previously [37], with absorbance measured at 562 nm, using a microtiter plate reader (Beckman Coulter). MTT assay was also performed on pSUPERIOR-MDR1 clone exposed to ceftriaxone, a cephalosporin antibiotic transported by MDR1 but not MRP1 [29;38;39]. Cell viability, assessed by MTT reduction ability, of induced and non-induced samples treated with UCB+DMSO was expressed as a percentage of the respective samples treated with DMSO alone.

To assess MDR1 export function, accumulation of the Hoechst 33342 dye, a substrate for MDR1 [40], was measured in a microtiter plate fluorescence reader (Beckman Coulter) at 350 nm (excitation)/460 nm (emission), using  $0.2 \times 10^6$  cells/well, in 96-well black plates. After the appropriate incubation time with doxycycline, cells were washed twice with Hank's solution and exposed for 6 minutes to three different concentrations of Hoechst 33342 (1.0, 2.5 and 5.0  $\mu$ M), dissolved in Hank's solution. Cellular dye uptake was assessed by the increase in fluorescence. At the end of each experiment cell viability was assessed by the MTT test, as described above.

### **Statistical analysis**

Data are given as mean  $\pm$  SD of at least three separate experiments. Statistical analysis was performed by Student "t" test and a *p* value less than 0.05 was considered statistically significant.

## **RESULTS**

### ***Gene and protein expression changes according to the incubation time with doxycycline***

MRP1/MRP1 expression of pSUPERIOR-MRP1 clone. In the samples induced with doxycycline for 72 hours, *MRP1* mRNA expression (Fig. 1 A) decreased by about 45% as compared to the non-induced samples (*p*<0.02); no decrease was observed with a shorter incubation time (48 hours). A 40% decrease (*p*<0.03) was also observed in samples incubated with doxycycline for 48 hours and then without doxycycline for 48 additional hours.

Protein analysis confirmed the variations observed in gene expression. As shown in Fig. 1 B, no change in the MRP1 level was observed in both induced and non-induced samples after 48 hours of incubation with doxycycline. On the contrary, a 60% reduction ( $p < 0.03$ ) was found after 72 hours of incubation. A similar decrease in MRP1 content (about 50%) occurred in samples induced by doxycycline for 72 hours and then incubated without doxycycline for 72 additional hours ( $p < 0.04$ ). The control clone, stably transfected with pcDNA6/TR vector and with an empty pSUPERIOR.puro vector, showed a 10% up-regulation of *MRP1*/MRP1 after 72 hours of incubation with doxycycline (data not shown).

*MDR1*/MDR1 expression of pSUPERIOR-MDR1 clone. After a 24 hour incubation with doxycycline, the expression of *MDR1* mRNA (Fig. 1 C) decreased by 30% ( $p < 0.05$ ), but partially recovered by 48 hours. The reduction in gene expression was greater than 50% ( $p < 0.02$ ) when the clone was incubated for 24 hours in presence of doxycycline followed by additional 24 hours in its absence. The expression of MDR1 protein (Fig. 1 D) did not show any decrease after 24 or 48 hours of incubation with doxycycline, but was reduced by about 60% ( $p < 0.02$ ) 48 hours after the removal of doxycycline from cells previously incubated with doxycycline for 48 hours. Taking into account the limited specificity of the anti-C219 antibody, these results are compatible with the observation that the silencing by siRNA was hidden by the induction of *MDR1*/MDR1 expression caused by doxycycline, as previously reported [41]. Doxycycline-related induction of 25% was observed also in the control clone stably transfected with pcDNA6/TR vector and with an empty pSUPERIOR.puro vector, after a 24 (for gene) or 48 hour (for protein) incubation in presence of the antibiotic followed by a 24 (for gene) or 48 hour (for protein) incubation in its absence (data not shown). This finding explains why the silencing effect could be observed mainly only after removing the doxycycline. The small reduction in the gene but not in the protein expression observed after a 24 hour incubation with doxycycline (Fig. 1 C,D) as well as the different incubation times needed to achieve maximum silencing in gene or protein expression is related to the shorter half life of mRNA than the protein [42].

These findings defined the optimal incubation times to achieve comparable decreases in the expression levels of MRP1 or MDR1 to correlate with UCB accumulation and cytotoxicity.

#### ***UCB accumulates in MRP1-deficient cells, not in MDR1-deficient cells***

Fig. 2 shows the effects of decreased expression of MRP1 or MDR1 on the intracellular accumulation of UCB after incubation for 30 minutes with [ $^3$ H]UCB ( $B_f = 40$  nM). Accumulation of [ $^3$ H]-UCB was markedly higher (2.6-fold) in the doxycycline-induced samples of the pSUPERIOR-MRP1 than in the non-induced ones ( $p < 0.0006$ ). By contrast, the intracellular accumulation of [ $^3$ H]-UCB was similar in the induced and non-induced pSUPERIOR-MDR1.

Since the decreased expression of both proteins was comparable in the two clones (see Fig. 1 B,D), these data confirm that MRP1, but not MDR1, can regulate the cellular content of UCB, at the low  $B_f$  of 40 nM.

#### ***Impairment of transport function and viability in MDR1-deficient cells***

As shown in Figure 3, compared to non-induced cells, induced pSUPERIOR-MDR1 samples showed a dramatic increase in the cellular accumulation of three different concentrations of the fluorescent dye Hoechst 33324. These results demonstrate that the knockdown of MDR1 expression resulted in functional impairment of the export of the dye, a known substrate for MDR1 [40].

When the induced clone with its MDR1 level decreased by 60% was exposed for 24 hours to 15 mM ceftriaxone, cell viability was decreased by two-thirds compared to the non-induced clone ( $35 \pm 6\%$  vs.  $100 \pm 4\%$ ,  $p < 0.002$ ) (data not shown). This confirmed that the decreased export function of MDR1 in the induced cells engendered greater susceptibility to cytotoxicity from a known MDR1 substrate.

#### ***UCB-induced cytotoxicity occurs in cells deficient in MRP1 but not MDR1***

Figure 4 shows the cytotoxic effects of 4 hours of exposure to UCB at  $B_f = 40, 70$  and 140 nM, on pSUPERIOR-MRP1 and pSUPERIOR-MDR1 clones with comparable silencing of MRP1 and MDR1, respectively. Cell viability was assessed by MTT assay. All pSUPERIOR-MRP1 samples, whether or not induced with doxycycline for 72 hours, showed progressive reduction in cell viability by increasing  $B_f$ . However, the decrease in viability was significantly greater in the induced cells with a 60% reduction of MRP1 (see Fig. 1B), at each  $B_f$  level (Fig. 4A), and this effect was UCB dose-dependent (Fig. 4C).

In contrast to the pSUPERIOR-MRP1, no difference in cytotoxicity was found between induced and non-induced samples of the pSUPERIOR-MDR1 (Fig. 4 B,C), even at the highest  $B_f$  (140 nM) although the induced clone reached a 60% reduction of MDR1 expression by incubation with doxycycline for 48 hours followed by additional 48 hours in its absence (see Fig. 1 D). In spite of the substantial reduction in MDR1 expression, the susceptibility of the induced clone to UCB was comparable to the one of the non-induced clone, in keeping with the lack of difference in UCB accumulation (Fig. 2). This is in sharp contrast to the clear increase, in induced pSUPERIOR-MDR1 cells, of accumulation of Hoechst 33342 (Figure 3), and cytotoxicity of ceftriaxone, two known MDR1 substrates, which confirmed the decreased export function of MDR1 in the induced cells.

In summary, greater accumulation of UCB markedly affected the cell viability of MRP1-deficient cells and this effect was dose-dependent. On the contrary, the MDR1-deficient cells showed UCB accumulation and cell viability identical to their non-induced control, in spite of a demonstrated impairment in transport activity of the protein for a known substrate (Fig. 3). Collectively these data strongly point to a role of MRP1 but not of MDR1 in the protection from UCB-induced cytotoxicity. This conclusion is further supported by the strong linear correlation between the relative expression of MRP1 and the cytotoxic effect of UCB at all  $B_f$  tested (Fig. 5).

## DISCUSSION

Previous studies have indicated that nerve cells are particularly sensitive to UCB toxicity [2;3;7-10;27]. MRP1 and MDR1 have each been proposed to protect cells against UCB toxicity, by limiting the intracellular accumulation of the pigment. We had previously demonstrated that MRP1 transports UCB with high affinity [22] and protects MEF cells from UCB toxicity by exporting the pigment from the cells [19]. Both Gennuso *et al.*, using differentiated mouse astrocytes [23] and, more recently Falcão *et al.* using mature rat astrocytes and neurons [15], have shown dramatic increases in UCB-induced cytotoxicity (impaired MTT reduction) following blockade of Mrp1 by MK571, an inhibitor of Mrp1 function [43]. Gennuso *et al.* [23] showed further that MK571 increased vulnerability of astrocytes to UCB-induced membrane, mitochondrial and nuclear damage, even at a normally non toxic  $B_f$  of 40 nM. Others have suggested that UCB is also a substrate for MDR1 [26] and that this transporter may protect neural cells from toxicity by limiting accumulation of UCB [25;26;29]. However, those studies were performed at vastly high and clinically irrelevant concentrations of UCB.

The present study is the first to directly compare the functional roles of MRP1 and MDR1 in protecting against UCB toxicity in identical cells exposed to identical, increasing concentrations of UCB at clinically-relevant  $B_f$  levels. The comparable reduction we obtained in the expression levels of MRP1 and MDR1, utilizing RNA interference technology, also allowed a direct comparison of their relative role in the transport of UCB and in the protection against UCB neurotoxicity in cells differing only in the reduced expression of either transporter.

Our experiments demonstrated that, at clinically-relevant  $B_f$  levels, protection from UCB cytotoxicity was correlated with the level of expression of MRP1 but not MDR1. No significant UCB-induced impairment in cell viability was observed even when maximal MDR1 silencing was achieved and its impaired transport function confirmed by the significantly greater cellular accumulation, in the cells with induced siRNA expression, of Hoechst 33342 dye, a substrate for MDR1 [40]. In contrast, the cytotoxic effect caused by ceftriaxone, another known substrate for

MDR1 [29;38;39], was significantly greater in the induced cells than in non-induced cells. These findings clearly indicate that decreased expression and function of MDR1 is not associated with greater UCB cytotoxicity at low  $B_f$  values.

The [ $^3$ H]-UCB uptake data confirm further the involvement of MRP1 in UCB export, previously demonstrated by using MEF cells isolated from Mrp1 knockout mice [19], and exclude participation of MDR1 in the transport of UCB at low  $B_f$  levels. They indicate also that the protective effects of MRP1 against UCB cytotoxicity are largely related to the ability of this transporter to export UCB from the cell, thus limiting intracellular accumulation of the pigment [19;20;22]. This is in agreement with the chemical characteristics of the substrates of the two proteins; MDR1 “prefers” uncharged or slightly positively charged compounds, while MRP1 primarily transports amphipathic, anionic conjugates, as well as unconjugated xenobiotics and uncharged drugs [11-13]. While our results show no role for MDR1 in the transport of UCB at  $B_f = 40$  nM, they do not exclude the possibility, suggested by the work of others [24-26;29], that MDR1 transports UCB at much higher  $B_f$  levels. Many compounds are substrates for both high affinity (low  $K_m$ ) transporters that operate at low concentrations, and low affinity (high  $K_m$ ) transporters that are operative at much higher concentrations of substrate [15;44;45]. In the case of UCB, MRP1 clearly is the high-affinity transporter, whereas MDR1 may become operative only at  $B_f$  levels that are far above clinically-relevant concentrations.

Our MRP1-knockdown model validly mimics the increases in mRNA and protein expression of Mrp1 [15;46], and the decreased susceptibility to UCB toxicity [47], that occur during maturation of rodent neurons and astrocytes. Similar changes in the expression of MRP1 might explain the higher vulnerability of premature than term infants to UCB-induced neurological dysfunction during moderate to severe neonatal jaundice [48-50].

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## FIGURE LEGENDS

**Figure 1. Time course of the relative gene and protein expression of *MRP1*/*MRP1* and *MDR1*/*MDR1* in SH-SY5Y clones.** **Panel A,** Relative *MRP1* mRNA expression in pSUPERIOR-*MRP1* clone at 48, 72 hours after induction by doxycycline (DOX, 3.9  $\mu$ M) and 48 hours after the DOX removal from cells previously incubated with DOX for 48 hours (48/48 h). RT-Real Time PCR was performed normalizing *MRP1* expression values to housekeeping genes *GAPDH* and  $\beta$ -*ACTIN*. Each bar represents the mean  $\pm$  SD of three separate experiments. \* $p$ <0.02 and  $^{\S}p$ <0.03 for Induced vs. Non-induced samples. **Panel B,** Western Blot analysis of *MRP1* and *ACTIN* proteins in the same clones under the same treatments as in Panel A except DOX was removed for 72 hours from cells previously incubated with DOX for 72 hours (72/72 h). Bands were visualized by Kodak 1D image software and quantified by Scion Image software. The relative expression values were obtained by the Curver Expert software as described in Methods. Each bar represents the mean  $\pm$  SD of three separate experiments. # $p$ <0.03 and \* $p$ <0.04 for Induced vs. Non-induced samples. **Panel C,** Relative *MDR1* mRNA expression in pSUPERIOR-*MDR1* clone at 24, 48 hours after induction by doxycycline (DOX, 3.9  $\mu$ M) and 24 hours after DOX removal from cells previously incubated with DOX for 24 hours (24/24h). RT-Real Time PCR was performed normalizing *MDR1* expression values to housekeeping genes *GAPDH* and  $\beta$ -*ACTIN*. Each bar represents the mean  $\pm$  SD of three separate experiments. \* $p$ <0.05 and  $^{\S}p$ <0.02 for Induced vs. Non-induced samples. **Panel D,** Western Blot analysis of *MDR1* and *ACTIN* proteins in the same clones under the same treatments as in Panel C, except DOX removed for 48 hours from cells previously incubated with DOX for 48 hours (48/48 h). Band visualization and quantification as in *Panel B*. Each bar represents the mean  $\pm$  SD of three separate experiments.  $^{\S}p$ <0.02 for Induced vs. Non-induced samples.

**Figure 2.  $^3$ [H]-UCB accumulation by SH-SY5Y clones *MRP1*- or *MDR1*-deficient.** Before exposure to  $^3$ [H]-UCB, the induced samples of pSUPERIOR-*MRP1* clone were incubated with DOX (3.9  $\mu$ M) for 72 hours while the induced samples of pSUPERIOR-*MDR1* clone were incubated with DOX (3.9  $\mu$ M) for 48 hours followed by additional 48 hours in medium without DOX (48/48 h). At these incubation times, the levels of *MRP1* and *MDR1* proteins were comparably decreased (see Fig.1 B and D). Both induced and non-induced samples were incubated with  $^3$ [H]-UCB at  $B_f = 40$  nM for 30 minutes. The  $^3$ [H]-UCB contents of the induced and non-induced samples were measured and normalized for the respective cell protein content. Data are reported as the mean  $\pm$  SD of experiments performed in triplicate. Induced samples of pSUPERIOR-*MRP1* clone accumulated 2.6 times as much  $^3$ [H]-UCB (# $p$ <0.0006) as non-

induced samples, whereas no difference was observed between induced and non-induced samples of pSUPERIOR-MDR1 clone.

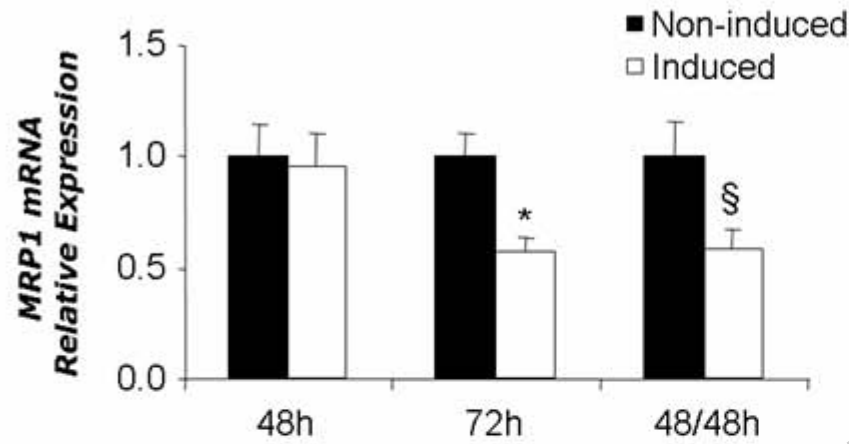
**Figure 3. Cellular accumulation of a fluorescent MDR1 substrate by SH-SY5Y clone MDR1-deficient.** Before exposure to the fluorescent dye Hoechst 33342, the induced samples of pSUPERIOR-MDR1 clone were treated with DOX as described in Fig. 2. Cell content of Hoechst 33342 was measured in 96-well, black microtiter plates by a fluorescence reader at 350 nm (excitation)/ 460 nm (emission), using  $0.2 \times 10^6$  cells/well. **Panel A:** Time dependent increase in cell fluorescence due to the uptake of 5  $\mu$ M Hoechst in pSUPERIOR-MDR1 clone induced (-□-) or non-induced (-▲-). **Panel B:** Cell fluorescence determined after 5 minutes of incubation with three different concentrations of Hoechst dye (1, 2.5 and 5  $\mu$ M). Data are expressed as the mean  $\pm$  SD of three independent measurements. # $p < 0.001$  for Induced vs. Non-induced samples. Dramatic increases in dye accumulation by induced cells are observed at all dye concentrations.

**Figure 4. Cell viability of SH-SY5Y MRP1- or MDR1-deficient clones after exposure to UCB.** Before exposure to UCB, the induced samples of pSUPERIOR-MRP1 clone (**Panel A and C**) and of pSUPERIOR-MDR1 clone (**Panel B and C**) were treated with DOX as described in Fig. 2. Clones were then exposed for 4 hours to increasing concentrations of unbound UCB ( $B_f = 40, 70, \text{ and } 140 \text{ nM}$ ), and viability then assessed by their ability to reduce MTT. Both induced and non-induced samples were incubated in the presence of DMSO 0.3, 0.4 or 0.6% v/v (used to dissolve the UCB) and 15% Fetal Bovine Serum (albumin concentration 54  $\mu$ M). Cell viability of induced and non-induced clones is expressed as a percentage of the corresponding control (CTRL) treated with DMSO only. Each bar represents the mean  $\pm$  SD of three separate experiments. # $p < 0.001$  and \* $p < 0.0002$  for Induced vs. Non-induced samples. § $p < 0.02$  for  $B_f = 40 \text{ nM}$  vs.  $B_f = 70 \text{ nM}$  and ~ $p < 0.01$  for  $B_f = 70 \text{ nM}$  vs.  $B_f = 140 \text{ nM}$ .

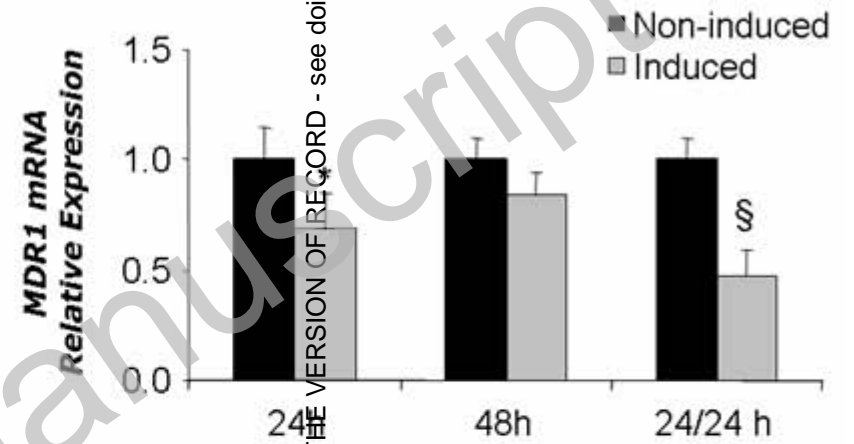
**Figure 5. Correlation between UCB-induced cytotoxicity and MRP1 protein expression in SH-SY5Y clone.** pSUPERIOR-MRP1 clone was incubated with DOX as reported in Figure 1B and UCB treatment performed as described in Figure 4. At each free bilirubin concentration ( $B_f$ ), there is a significant linear correlation between the MRP1 protein content (normalized for actin) of induced cells relative to non-induced (control) cells, and UCB cytotoxicity, expressed as cell viability assessed by MTT assay. The slope of the correlation increases with increasing  $B_f$ . Symbols: ■ =  $B_f 40 \text{ nM}$ ; ▲ =  $B_f 70 \text{ nM}$ ; ● =  $B_f 140 \text{ nM}$ .

**Figure 1**

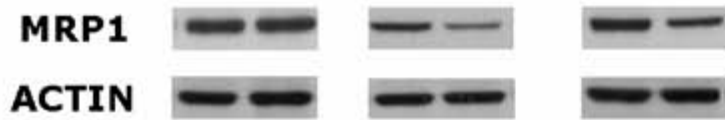
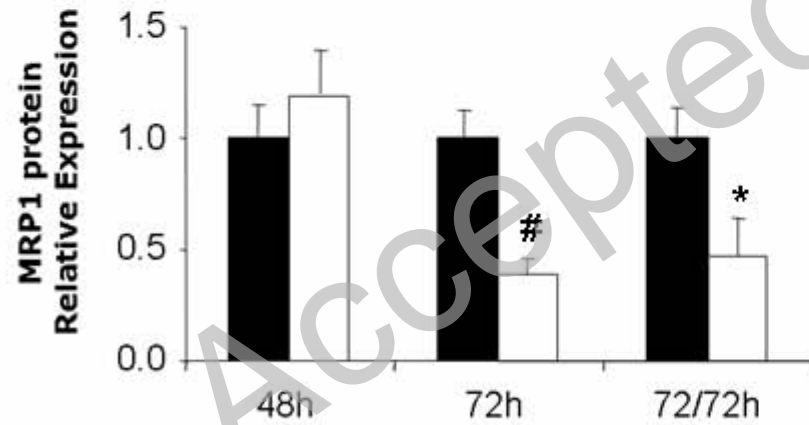
**A.**



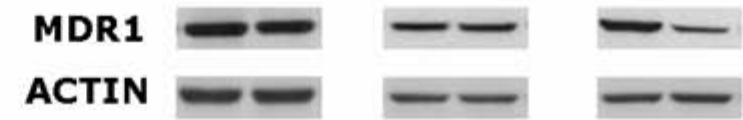
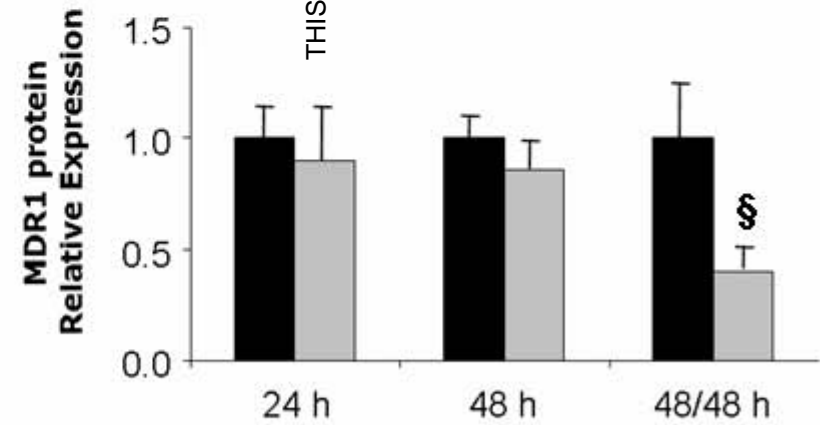
**C.**



**B.**

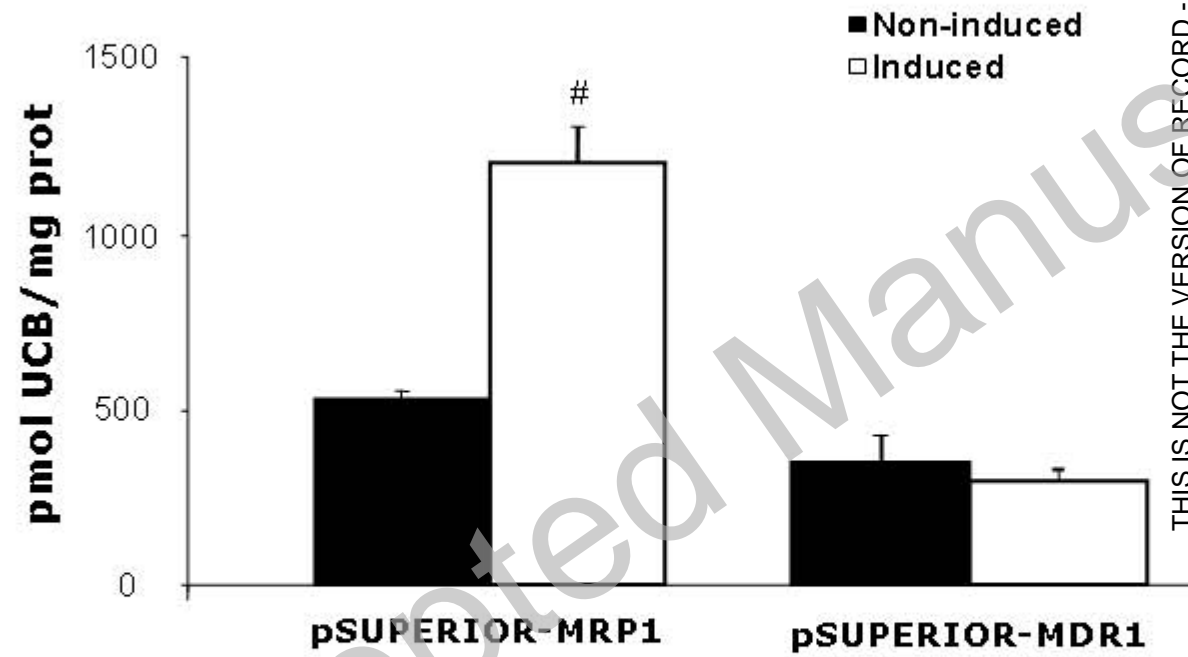


**D.**



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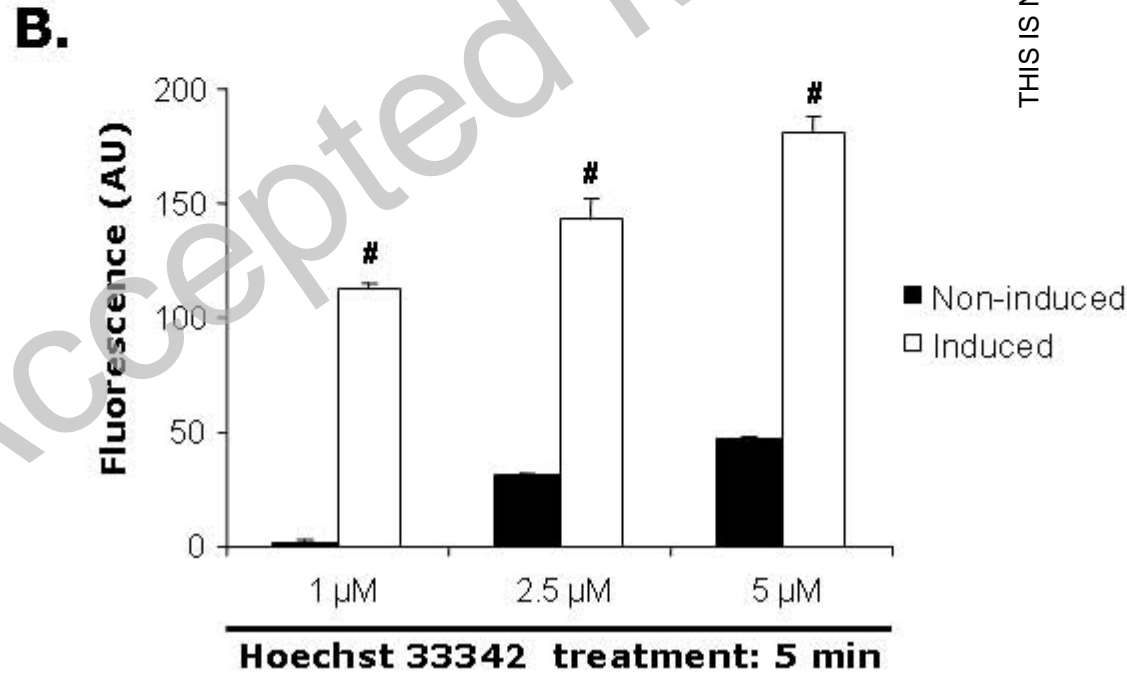
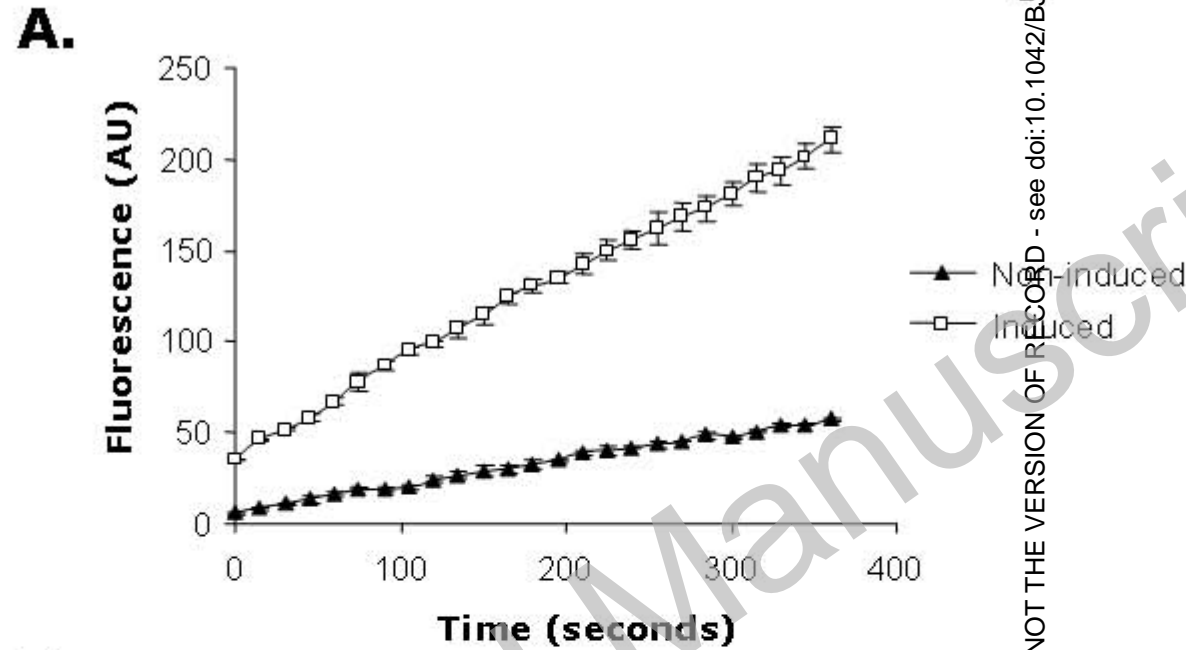
**Figure 2**



<b>Non-Induced</b>	534 ± 25	361 ± 81
<b>Induced</b>	1205 ± 110 (#)	306 ± 31

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**Figure 3**



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