UNIVERSITÀ DEGLI STUDI DI TRIESTE

DIPARTIMENTO DI BIOCHIMICA, BIOFISICA E CHIMICA DELLE MACROMOLECOLE

DOTTORATO DI RICERCA IN SCIENZE BIOMOLECOLARI
XIX CICLO
Settore scientifico-disciplinare: Biochimica (Bio/10)

USE OF A TETRACYCLINE-INDUCIBLE SILENCING SYSTEM TO INVESTIGATE THE ROLE OF MRP1 AND MDR1 IN THE TRANSPORT OF ORGANIC ANIONS IN NEURONAL CELLS

Dottoranda
Lucia Corich

Coordinatore del Collegio Docenti:
Prof. Franco Vittur
Università degli Studi di Trieste

Tutore:
Prof. Claudio Tiribelli
Università degli Studi di Trieste

CONTENTS

CHAPTER 1

GENERAL INTRODUCTION

1.1 ATP-BINDING CASSETTE PROTEINS

The ATP-binding cassette transporter genes (ABC-transporter genes) are a family of genes which encode the ABC-transporter proteins. It is one of the largest and most ancient family with representatives in all phyla from prokaryotes to humans [1]. ABC transporters utilize the energy of ATP hydrolysis to transport various substrates (including metabolic products, lipids and sterols, and drugs) across extra- and intracellular membranes, often against a concentration gradient.

The name ABC transporters was introduced in 1992 by Chris Higgins [2]. The designation ABC was based on the highly conserved ATP-binding cassette, the most characteristic feature of the superfamily [3]. Traffic ATPases is another name used for this family.

ABC transporters share a common structural organisation (Fig. 1.1):

- **two transmembrane domains (TMD)**, each of which consists of α-helices that cross the phospholipid bilayer multiple times. These transmembrane domains provide the specificity for the substrate and prevent unwanted molecules from using the transporter. Between the TMDs there is a ligand binding-domain. It is on the extracellular side of proteins involved in the import of substrates (importers) and on the cytoplasmic side of proteins involved in the export (exporters).

- **two ATP-binding domains** (called ATP-binding cassette, **ABC** or also called nucleotide binding domains, **NBD**), that are located on the cytoplasm side of the membrane. These domains are divided into parts of motifs, called Walker A and Walker B, which are separated by approximately 90-120 amino acids. In addition, there is a third short and highly conserved motif (called signature motif) located after the Walker motif B. Unlike the Walker A and Walker B motifs, which are found in other proteins which hydrolyze ATP, the signature motif is unique to ABC transporters. These domains, called also folds, form the "cassettes" which the protein family is named after.

ABC transporters may be divided into half transporters or full transporters. Full transporters consist of typical two TMDs and NBDs. Half transporters consist of only one TMD and one NBD and must combine with another half transporter to gain function ability. Half transporters can thus form homodimers if two identical ABC transporters join, and heterodimers if two unlike ABC transporters join.

ABC proteins are mostly unidirectional. In bacteria, they are predominantly involved in the import of essential compounds that cannot be achieved by diffusion (e.g., sugars, vitamins, metal ions, etc.) into the cell. In eukaryotes, most ABC genes move compounds from the cytoplasm to the outside of the cell or into an extracellular compartment (endoplasmic reticulum, mithocondria, peroxisome)[4]. More recently, ABC-transporters have been shown to exist in the placenta, indicating they could play a protective role for the developing fetus against xenobiotics [5-9].

Most of the known functions of eukaryotic ABC transporters involve the shuttling of hydrophobic compounds either within the cell as part of a metabolic process or outside the cell for transport to other organs, or secretion from the body [4].

Physiologically, mammalian ABC proteins transport lipids, bile salts, toxic compounds and peptides for antigen presentation or other purposes [3]. Each protein is specific for a substrate or a group of related substrates.

There are 49 (+1?) known ABC transporters genes present in humans. The ABC proteins are grouped into seven subfamilies, ranging from ABCA to ABCG [updated information is on line available at this website: http://nutrigene.4t.com/humanabc.htm] based on genomic organization, order of domains and sequence homology.

In general, the ABC transporters are important in human physiology, toxicology, pharmacology and disease.
It has been found that:

1. ABC proteins transport drugs (xenotoxins) and drug conjugates.
2. Mammalian secretory epithelia use ABC transporters to excrete a large number of substances, sometimes against a steep concentration gradient.

Several inborn errors in liver metabolism are due to mutations in one of the genes for these pumps. A rapidly increasing number of ABC transporters plays a role in lipid transporter. In addition, there are evidence that a subfamily of human ABC proteins is an excellent transporter of hydrophobic peptides [10] [11]; a heterodimeric ABC protein transports peptides for antigen presentation [12] and an ABC transporter related to it exports peptides from mitochondria [13]. Defects in each of these transporters are involved in human inborn or acquired diseases [3].

The main characteristics and the role in the organic anion transport of 2 human ABC proteins, **MRP1 and MDR1 (P-gp)**, will be explained in this work.

1.2 MDR1 AND MRP1 DIFFERENCES AND ANALOGIES

1.2.1 SUBSTRATE SPECIFICITY

MDR1 transports large **hydrophobic**, either uncharged or slightly positively charged compounds in their **unmodified** forms, while **MRP1** primarily transports hydrophobic **anionic conjugates**, but also **unconjugated xenobiotics** and uncharged drugs. The MRP1-related uncharged drug transport is quite an enigma, and is somehow linked to the transport or allosteric effect of cellular free reduced gluthatione [14] .

1.2.2 CELLULAR AND TISSUE DISTRIBUTION

MRP1 is almost ubiquitously expressed, while the expression of **MDR1** is more restricted to tissues involved in absorption and secretion [15] [16]. High level MDR1 expression has also been shown in certain pharmacological barriers of the body, such as the blood-brain barrier and the choroid plexus [17] [18]. Multi-drug transporters are localized predominantly in the plasma membrane. In polarized cells, MDR1 is localized in the apical (luminal) membrane surface (e.g. in the epithelial cells of the intestine and the proximal tubules of kidney, or in the biliary canalicular membrane of hepatocytes) [19] [20]. In contrast, MRP1 expression in polarized cells is restricted to the basolateral membrane.

1.2.3 MOLECULAR MECHANISM

There are strong indications that the hydrophobic substrates of MDR1 are recognized within the membrane bilayer or in its vicinity, and this type of recognition makes the MDR1 protein a highly effective pump, preventing the cellular entry of toxic compounds [21]. In the case of MRP1 a similar picture has emerged.

On the basis of the three-dimensional structures of bacterial NBD-units, it has been shown that the close interaction of the two NBD units results in the formation of a fully competent catalytic site. The regions connecting the NBD units to the transmembrane domains have a key role in the transfer of conformational information within the protein, and the signature region may have a special function in this regard [22].

The ATP-hydrolytic cycle of both MDR1 and MRP1 have been investigated in detail. Interaction with the transported drugs enhances the catalytic ATPase activity of MDR1. In all ABC transporters, ATP binding and hydrolysis occurs at the

sites localized in the NBD domains. Thus, an allosteric control of the drugs on the ATPase activity requires intramolecular interaction between the drug binding and the catalytic regions of the protein, but the mechanism by which the transported drugs accelerate ATP-hydrolysis is presently unknown. It has been documented in detail that the interaction of the two NBD units is an essential requirement for the catalytic reaction [23] [24]. Several lines of evidence indicate that both NBDs can bind ATP, and both catalytic sites are active and, in the case of MDR1 but not of MRP1 [3], the two ABC domains enter alternately into the catalytic cycle [25-27].

1.2.4 MRP1

The human multidrug resistance-associated protein MRP1, also called **ABCC1**, is one of the 13 members of the ABCC subfamily. It is a 190 kDa ATP-dependent membrane-bound transporter. Regarding the membrane topology, the MRP1 structure is different compared to the basic structure of ABC proteins because it contains an additional N-terminal segment of about 280 amino acids. A major part of this region is membrane-embedded with five transmembrane helices (TMD_0), while a small cytoplasmic loop of about 80 amino acids (L_0) connects this area to the core region [28-31]. Recent studies revealed that the TMD_0 domain of MRP1 does not play a crucial role in either the transport activity or the proper routing of the protein. However, the presence of the membrane-associated cytoplasmic L_0 region (together with the core region) is necessary for both the transport activity and the proper intracellular routing of the protein. These studies indicate that the L_0 region forms a distinct structural and functional domain, which interacts with the membrane and the core region of the MRP1 transporter [32] (Fig. 1.2).

Physiological function of MRP1

Former transport studies on intact cells and isolated membrane vesicles suggested that MRP1 transports **conjugated xenobiotics, particularly organic anions** and dyes, preferentially glutathione *S*-conjugates (GS *S*-conjugates), but also glucuronides and sulphate conjugates. Reduced glutathione (GSH) is ubiquitously present in all cells of the human body and plays an important role in detoxification of ROS, electrophiles and oxyanions either by reduction or conjugation [33]. GS S-conjugates are substrates with high affinity for MRP1 and the affinity increases with the length of the alkyl chain [34] [35]. Based on its ubiquitous expression [36] and its substrate specificity, it was proposed that extrusion of endogenously formed GSH-dependent detoxification products is the physiological function of MRP1 [37]. Indeed, **endogenous organic anions** known

to be natural occurring substrates of MRP1 include the cysteinyl leukotriene LTC_4 [35] [38], the glutathione conjugate of prostaglandin A2 ($GS\text{-}PGA_2$) [39] [40], oxidized gluthatione (GSSG) [41] and GS-4HNE [(4-hydroxynonenal, that is a α,β-unsaturated aldehyde (the most prevalent toxic lipid peroxidation product formed during oxidative stress)] [42]. LTC_4 and PGA_2 are involved in the inflammation process and cell cycle arrest [43] [44], while GSSG and GS-4HNE are detoxification products generated under conditions of a changed redox state [33] [45]. A role of MRP1 in immune responses was recently proposed based on the induction of its murine orthologue upon activation of T-helper 1 cells [46]. MRP1 mediated transport of GSSG and GS-4HNE suggests that MRP1 functions as part of the cellular defence system against oxidative stress [41] [42]. Thus, extrusion of the metabolites by MRP1 may be required to retain the response against inflammatory stimuli and to prevent cellular damage.

The generation of Mrp1 -/- knockout mice has significantly contributed to the understanding of the physiological role of MRP1. Mice lacking Mrp1 show a poor response to inflammation induced by arachidonic acid, probably due to impaired export of LTC_4 from LTC_4-secreting cells [47]. In addition, Mrp1 plays a role in the protection of testicular tubules, tongue, cheek, and the urinary collecting duct against etoposide-induced damage [48]. Recently, the presence of Mrp1 in mouse and rat choroid plexus (CP) was shown [17] [49] [50]. It is located on the basolateral site of CP epithelial cells [17]. Mrp1 expression in rat CP was higher than in the lung, a tissue with a relative high basal Mrp1 expression [49]. Functionality of Mrp1 was strongly suggested by a rapid elimination of the conjugated organic anion Mrp1 substrate, estradiol 17-ß-D-glucuronide (E_2 17ßG), from the CP [49] and by the MK571 (MRP1 inhibitor)-mediated inhibition of translocation of ^{99m}Tc-sestamibi (^{99m}Tc-labeled methoxyisobutil isonitrile, a myocardial perfusion agent) through CP epithelial cells [17]. Comparison of double (Mdr1a/Mdr1b) and triple (Mdr1a/Mdr1b/Mrp1) knockout mice clearly demonstrated that Mrp1 eliminates the anticancer drug etoposide from the CP [50]. Thus Mrp1 appears to function also as part of the blood-cerebrospinal fluid barrier by preventing drug entry into the brain. Some studies showed that Mrp1 was highly expressed in rat brain parenchyma [51]. More recently, Mrp1 was found in rat astrocytes cultures where a high functional activity was detected. In addition, it was observed a higher expression of Mrp1 in primary astrocytes, compared with primary brain endhotelial cells [52]; finally, Mrp1 was found in rat primary microglia [53] and very recently its expression was demonstrated also in rat neurons [54].

Experiments with membrane vesicles from MRP1-overexpressing cells demonstrated that MRP1 is a transporter for the **unconjugated xenobiotics** such as aflatoxin B1, vincristine and daunorubicin, but only in the presence of physiological amounts of GSH [35] [55]. In addition, it transports also **neutral/basic amphipathic drugs and even oxyanions**, cotransported by GSH [3]. These results extend the earlier observations that GSH is a critical factor in MRP1-mediated drug resistance [56] [57] (Fig. 1.3b).

Conclusions: MRP1 acts as a multispecific conjugated organic anion transporter, with (oxidized) glutathione, cysteinyl leukotrienes and activated aflatoxin B1 as substrates. This protein also transports glucuronides and sulphate conjugates of steroid hormones and bile salts. In addition, it transports unconjugated drugs and other hydrophobic compounds in presence of glutathione [see:http://nutrigene.4t.com/humanabc.htm]. In fact it confers resistance to doxorubicin, daunorubicin, vincristine, colchicines, and several other xenobiotic compounds [58]. MRP1 is expressed in many tissues and its expression is restricted to the basolateral membranes.

1.2.5 MDR1

The human multidrug resistance P-glycoprotein MDR1, also called **P-gp (P-glycoprotein)** or **ABCB1**, is one of the 11 members of the ABCB subfamily. It is a 170 kDa transmembrane glycoprotein which includes 10-15 kDa of N-terminal glycosylation. The N-term half of the molecule contains 6 transmembrane domains, followed by a large cytoplasmic domain with an ATP binding site, and then a second section with 6 transmembrane domains and an ATP binding site which shows over 65% of amino acid similarity with the first half of the polypeptide (Fig.1.2).

The gene coding for MDR1 was the first identified human ABC transporter gene [59].

MDR1 is a key player in the defense of the body against amphipatic xenotoxins. It transports a vast range of drugs in their **unmodified form**. The preference of MDR1 is for large amphipathic molecules that are neutral or weakly basic, but if pushed, MDR1 can also inefficiently handle an anionic highly charged compound, such as methotrexate (MTX). It is therefore difficult to define the elements common to all MDR1 substrates [3].

Juliano and Ling [60] were the first to describe MDR1 in drug-resistant cells with a defined pattern of "multidrug resistance" including anthracyclines, anthracendiones, *vinca*-alkaloids, taxanes, and epipodophyllotoxins. MDR1 confers

drug resistance by lowering the intracellular drug concentrations to sub-lethal levels. Since the initial drug export model [59], several modified models have been proposed. Roepe [61] suggested that MDR1 channels ions alters pH values and drugs follow the pH gradient out of the cells. This model, however, is not generally accepted [62] [63]. There is considerable evidence that MDR1 extracts its substrates directly out of the plasma membrane before they get into the cell. Concerning this, Raviv *et al.* [64] suggested a "vacuum cleaner" model. Intra-membranous molecules, which do not belong to the membrane, are recognized by MDR1, enter MDR1 from the membranous site, and then leave the cell (Fig. 1.3 a). On the contrary, Higgins and Gottesman [21] suggested a flippase hypothesis: P-glycoprotein acts as a drug exporter by flipping drugs from the inner leaflet of the plasma membrane to the outer leaflet against a concentration gradient. The flippase model for MDR1 was substantiated by other human ABC transporters (MDR2, MRP1) as well as LmrA, an ABC drug transporter from *Lactococcus lactus* [65-68].

The broad spectrum specificity of MDR1 for that many chemically and functionally different compounds have been ascribed to two features [69]:

1. MDR1 has at least two different drug binding sites with different preferences for allocrites. Both sides reveal cooperative interaction. Though a compound binds to one binding site, stimulation of the other binding site is required for translocation.
2. Lipophilic compounds concentrate in the membrane bilayer. High local concentrations facilitate translocation by MDR1 without the need for high affinity binding sites.

The *MDR1* gene is expressed at high levels in various normal organs such as brain vessels, adrenal gland, kidney, liver, and gastrointesinal tract [70][71]. In placental trophoblasts, testis and bone marrow it provides protection of vital body parts; in the gut mucosa it prevents entry of toxins into the body; in the blood brain barrier transports a number of lipophilic molecules that enter the endothelial cells back to the blood [72]; in the gut, liver and kidney, MDR1 helps to eliminate toxins from the body [3]. In addition, MDR1 translocates hormones, and detoxifies xenobiotics taken up along with nutrients. Studies on knock-out mice demonstrated that P-glycoprotein is not a housekeeping gene. The animals are vital, fertile, and do not show phenotypic abnormalities. They are hypersensitive to cytotoxic agents, especially in the brain [73] [74] [52]. In fact, recent studies demonstrated that Mdr1 in the rat brain was localized predominantly in microvessel endothelial cells (as also observed in humans) [75] [76] and weak

expression was seen in brain parenchyma [51]. In brain parenchyma, Mdr1 was identified in astrocytes [51][77][78]. More recently, it was found that Mdr1 is also expressed and functional in brain microglia [53].

The exploitation of the clinical relevance of MDR1 has been a matter of intense research and has been evaluated in meta-analyses [79] [80]. Although clinical drug resistance is frequently multifaceted [81] [82], P-glycoprotein's role for drug resistance is evident.

Apart from multidrug resistance in cancer cells, MDR1 also may contribute to resistance of AIDS patients towards protease inhibitors like indinavir, nelfinavir, or saquinavir [83] [84]. MDR1 expression in the normal gastrointestinal tract prevents drug absorption after oral administration. Likewise, MDR1 expression in the brain prevents penetration of antiviral drugs across the blood-brain barrier.

Conclusions: MDR1 is an ATP-dependent drug efflux pump for xenobiotic compounds with broad substrate specificity. It is responsible for decreased drug accumulation in multidrug-resistant cells and often mediates the development of resistance to anticancer drugs.

MDR1 is expressed in many tissues (especially those with barrier functions such as liver, BBB, kidney, intestine, placenta) and its expression is restricted to the apical membranes [see:http://nutrigene.4t.com/humanabc.htm].

1.3 CELL LINE ANALYSED

In order to investigate the role of MRP1 and MDR1 in the organic anion transport, we performed our studies in **SH-SY5Y cells, a human neuroblastoma cell line**. Neuroblastoma is thought to arise from the anomalous arrest of multipotential embryonal cells of neuronal crest during differentiation. It is this disordered differentiation that contributes to the pathogenesis of the disease [85]. Both MRP1 and MDR1 are expressed in this cell line, as reported in literature [86][87].

The choice of a neuronal cell line was based on the aim to investigate the MRP1 and MDR1 involvement in the transport of an unconjugated organic anion that could cause encephalopathy in infants.

1.4 ORGANIC COMPOUNDS EXAMINED

Two different categories of **organic anions** were considered: **xenobiotic drugs** and **endogenous metabolites**.

Xenobiotic drugs, substrates of MRP1 or MDR1, were used in order to assay the knockdown (triggered by small interference RNA, siRNA) level of MRP1 or MDR1 in SH-SY5Y neuroblastoma clones, compared to their respective control.
If we should establish the **MRP1** transport activity, we used:

- **Indomethacin**, a non-steroidal anti-inflammatory drug, transported by MRP1, but not by MDR1 [88-91].

If we should establish the **MDR1** transport activity, we used:

- **Ceftriaxone**, a cephalosporin antibiotic, transported by MDR1, but not MRP1. [92-98].

Moreover, if we should establish the **MRP1** and **MDR1** transport activity, we used:

- **Doxycycline**, member of the tetracycline antibiotics group, transported by both MRP1 and MDR1 [99][100].

As regards the endogenous metabolites, we focused our attention on the **unconjugated bilirubin (UCB)**, to investigate the involvement of MRP1 and MDR1 in its transport out of the cells.

1.5 BILIRUBIN

1.5.1 OVERVIEW

The chemistry, metabolism and disposal of bilirubin have been studied systematically during the last two centuries as a model for hepatic disposal of biologically important organic anions of limited aqueous solubility.

The discovery of several inherited disorders of bilirubin metabolism and excretion during the twentieth century has led to renewed interest in inherited diseases associated with jaundice, some of which continue to pose a therapeutic challenge, providing stimulus for further research.

Several studies are mainly concerned with the toxic effect of bilirubin and its importance as a liver function test, however the antioxidant property of bilirubin may provide a physiological defence against oxidative injury [101].

1.5.2 FORMATION OF BILIRUBIN

Sources of bilirubin

Bilirubin is the breakdown product of the haem moiety of haemoglobin, other haemoproteins, such as cytochromes, catalase, peroxidase and tryptophan pyrrolase, and a small pool of free heme.

In humans, 250–400 mg of bilirubin is produced daily, of which approximately 20% is produced from non-haemoglobin sources [102].

Enzymatic mechanism of bilirubin formation

The microsomal heme oxygenase (HO) enzymes catalyse the oxidation of heme (Fig. 1.4). Three molecules of O2 are consumed in this reaction and a reducing agent, such as nicotinamide adenine dinucleotide phosphate hydrogenase (NADPH), is needed. The α-methene bridge carbon is eliminated as CO and the iron molecule is released [103]. Of the three forms of HO, HO-1 is ubiquitous and inducible by heme [104] and stress [105]; HO-2 is a constitutive protein, expressed mainly in the brain and the testis. The catalytic activity of HO-3 is low, and this protein may function mainly as a heme binding protein. CO produced by HO activity has a vasodilatory effect and regulates the vascular tone in the liver, heart and other organs during stress. Similarly, biliverdin and its product bilirubin are potent antioxidants, which may protect tissues under oxidative stress [105] [106].

Biliverdin is reduced to bilirubin by the action of cytosolic biliverdin reductases, which require NADH or NADPH for activity [107]. As discussed later, bilirubin requires energy-consuming metabolic steps for excretion in bile. Thus, the physiological advantage of its formation is not clear. The strong antioxidant activity of bilirubin may be particularly important during the neonatal period, when other antioxidants are scarce in body fluids.

1.5.3 CHEMICAL CHARACTERISTICS OF BILIRUBIN

The tetrapyrrole structure of bilirubin IXa (1,8-dioxo-1,3,6,7tetramethyl-2,8-divinylbiladiene-a,c-dipropionic acid [108]) was solved by Fischer and Plieninger [109]. X-ray crystallography has revealed that the propionic acid side-chains of bilirubin form hydrogen bonds with the pyrrolic and lactam sites on the opposite half of the molecule, giving rise to a distorted 'ridge tile' structure [110] (Fig. 1.5). Engagement of all polar groups (two propionic acid carboxyls, four NH groups and two lactam oxygens) of bilirubin by the hydrogen bonds makes the molecule insoluble in water, necessitating chemical modification of excretion in

bile. Disruption of the hydrogen bonds is accomplished *in vivo* by enzyme-catalysed esterification of the propionic acid carboxyl groups with a glycosyl moiety, mainly glucuronic acid.

The hydrogen bonds 'bury' the central methane bridge, so that the unconjugated bilirubin (see further) reacts very slowly with diazo reagents, whereas bilirubin glucuronides, which lack hydrogen bonds, react rapidly ('direct' van den Bergh reaction). The addition of 'accelerators' such as methanol, ethanol, 6 M urea or dimethyl sulphoxide to plasma disrupts the hydrogen bonds of bilirubin, so that both conjugated and unconjugated bilirubin (see further) react rapidly with diazo reagents ('total' van den Bergh reaction).

In cases of prolonged accumulation of conjugated bilirubin in plasma, as in cases of cholestasis or Dubin–Johnson syndrome, the pigment may become covalently bound to albumin [111]. This irreversibly protein-bound form, often termed delta-bilirubin, is included in the 'direct' fraction of bilirubin and is not eliminated in the bile or urine, which results in delayed clearance even after biliary obstruction or cholestasis is resolved.

1.5.4 BILIRUBIN TOXICITY

Unconjugated bilirubin is toxic to many cell types, intracellular organelles and physiological processes. Bilirubin inhibits DNA synthesis [112] and ATPase activity of brain mitochondria [113], and uncouples oxidative phosphorylation. It has been reported to inhibit Ca2+-activated, phospholipid-dependent protein kinase C activity and cAMP-dependent protein kinase activity [114]. Which of these toxic effects is the predominant cause of bilirubin encephalopathy remains unclear at this time.

Clinically, toxic effects of bilirubin, particularly to the brain, are seen in neonates and patients with severe inherited deficiency of bilirubin conjugation. Yellow discoloration of the hippocampus, basal ganglia and nuclei of the cerebellum and brain stem, found in infants with acute bilirubin encephalopathy, is termed **kernicterus**. Such discoloration is not found in patients with chronic encephalopathy, in whom focal necrosis of neurons and glia is seen [115].

As all toxic effects of bilirubin are abrogated by tight binding to albumin, cerebral toxicity is usually seen when there is a molar excess of bilirubin in plasma over albumin. At serum unconjugated bilirubin concentrations over 20 mg/dL, newborn babies are at risk of kernicterus. However, kernicterus can occur at lower concentrations in the presence of substances, such as sulphonamides, radiographic contrast dyes and coumarin, that inhibit albumin–bilirubin binding by competitive

or allosteric displacement [116]. Although immaturity of the blood–brain barrier in neonates has been implicated in the increased susceptibility of neonates to kernicterus, evidence to support the concept is insufficient. Normally, bilirubin entering the brain is cleared rapidly, but the pigment may bind to damaged and oedematous brain inhibiting its clearance, thereby increasing the susceptibility to bilirubin encephalopathy [117].

1.5.5 POTENTIAL BENEFICIAL EFFECTS OF PRODUCTS OF HEME BREAKDOWN

Although clinicians are mainly concerned with the importance of bilirubin levels as a marker of liver disease and with the toxic effects of the pigment, biliverdin and bilirubin may exert some beneficial effects by virtue of their strong antioxidant property.

This may be relevant during the newborn period, when the level of other natural antioxidants is low. Bilirubin, which is toxic to neuronal cells at high concentrations, has been reported to have cytoprotective activity at lower concentrations [118]. An inverse relationship between serum bilirubin levels and risk of ischaemic coronary artery disease has been observed [119], although whether such a protective effect extends to subjects with Gilbert syndrome is questionable [120]. Study of a large number of subjects in the United States has shown that the odds ratio for colorectal cancer is reduced to 0.295 in men and 0.186 in women per 1 mg/dL increment in serum bilirubin levels [121].

Similarly, a previous large study showed an inverse relationship between serum bilirubin levels and cancer mortality in a Belgian population [122]. However, such associations do not conclusively prove a causative role for bilirubin, because possible confounding variables may exist.

1.5.6 DISPOSITION OF BILIRUBIN

Disposition of bilirubin by hepatocytes comprises several specific steps, including transport of bilirubin to hepatocytes from sites of production, uptake by and storage within hepatocytes, enzyme-catalysed conjugation with glucuronic acid, active transport into the bile canaliculus and degradation in the intestinal tract.

Transport in plasma

Unconjugated bilirubin circulates in plasma bound tightly but reversibly to albumin, which prevents its excretion in urine, except during albuminuria. Albumin

binding keeps bilirubin in solution and abrogates its toxic effects. Conjugated bilirubin is bound less tightly to albumin, and the unbound fraction is excreted in the urine. As mentioned above, during prolonged conjugated hyperbilirubinaemia, a fraction of conjugated bilirubin becomes irreversibly bound to albumin. This fraction, termed delta-bilirubin, is not excreted in the bile or urine and disappears slowly, reflecting the long half-life of albumin [111].

A small unbound fraction of unconjugated bilirubin is thought to be responsible for its toxicity [123]. Albumin has one high-affinity primary binding site for bilirubin. Additional sites are occupied when bilirubin is in molar excess. Normal plasma concentration of albumin (500–700 μmol/L) exceeds that of bilirubin (3–17 μmol/L). However, during exaggerated neonatal jaundice and in patients with Crigler–Najjar syndrome, the molar concentration of unconjugated bilirubin may exceed that of albumin. Hypoalbuminaemia resulting from inflammatory states, chronic malnutrition or liver disease may precipitate bilirubin toxicity. Sulphonamides, anti-inflammatory drugs, cholecystographic contrast media, fusidic acid, azapropazone, sodium caprylate and N-acetyl tryptophan displace bilirubin from albumin and increase the risk of kernicterus in jaundiced infants [124]. Binding of short-chain fatty acids to albumin causes conformational changes, decreasing bilirubin binding.

Uptake by hepatocytes

At the sinusoidal surface of the hepatocyte (Fig. 1.6), bilirubin dissociates from albumin and is taken up by the hepatocyte by facilitated diffusion that requires inorganic anions, such as Cl^-.

Storage within the liver cell

After entering the hepatocyte, bilirubin binds to the major cytosolic proteins, glutathione-S-transferases (GSTs, formerly designated ligandin or Y-protein). Bilirubin is a ligand for GSTs, but not a substrate for glutathione transfer. Binding to GSTs reduces the efflux of bilirubin from hepatocytes, thereby increasing its net uptake (Fig. 1.6). GST binding inhibits non-specific diffusion of bilirubin into various subcellular compartments, thereby preventing specific organellar toxicity, such as inhibition of mitochondrial respiration by bilirubin in vitro [127].

Conjugation of bilirubin

Conversion of unconjugated bilirubin to bilirubin diglucuronide or monoglucuronide by esterification of both or one of the propionic acid carboxyl groups is critical for efficient biliary excretion of bilirubin (Fig. 1.6).

1.5.7 Bilirubin-uridine diphosphoglucuronate glucuronosyltransferase

Bilirubin is one of the many endogenous and exogenous substrates, whose conjugation with glucuronic acid is mediated by one or more isoform of uridine diphosphoglucuronate glucuronosyltransferase (UGTs). UGTs are enzymes concentrated in the endoplasmic reticulum and nuclear envelope of many cell types [128]. They catalyse the transfer of the glucuronic acid moiety of UDP-glucuronic acid to the aglycone substrates, forming polar and usually less bioreactive products. Bilirubin glucuronidation is catalysed predominantly by a single UGT isoform, UGT1A1 [129]. The UGT superfamily of genes comprises two major families, UGT1 and UGT2. Four consecutive exons (exons 2–5) located at the 3' end of the UGT1A locus are used in nine different mRNAs. These encode the identical carboxy-terminal domains of these UGT isoforms, which contain the UDP-glucuronic acid binding site. Upstream of these four common region exons is a series of unique exons, each preceded by a separate promoter. Only one of these exons is utilized in a specific UGT mRNA. The presence of a separate promoter upstream from each unique region exon permits differential regulation of individual UGT isoforms during development and in response to inducing agents. UGT1A1 develops after birth [130] and is induced by phenobarbital and clofibrate [131]. Delayed development of UGT1A1 is a major cause of neonatal hyperbilirubinaemia in primates. Treatment of rats with triiodothyronine markedly reduces UGT activity towards bilirubin, whereas the activity towards 4-nitrophenol is increased [132].

In humans, the expression of UGT1A1 is limited to hepato-cytes and, to a lesser extent, in the proximal small intestine. UGTs are integral to endoplasmic reticulum (ER) membranes.

Canalicular excretion of conjugated bilirubin

Conjugated bilirubin undergoes unidirectional transport into the bile against a concentration gradient, so that bilirubin concentration in the bile can be as high as 150-fold that in the hepatocyte. The electrochemical gradient of −35 mV, generated by the sodium pump, may help in the canalicular transport but, by itself, is too small to account for this large concentration gradient. The energy for the uphill transport of bilirubin and many other non-bile salt organic anions is derived from adenosine triphosphate (ATP) hydrolysis by the canalicular ATP-

binding cassette protein, ABCC2 [also termed the MDR-related protein 2 (MRP2) or the multispecific organic anion transporter, MOAT]. ABCC2 pumps glutathione-, glucuronic acid- or sulphate-conjugated compounds across the canalicular membrane [133] [134]. Canalicular transport of organic anions is unidirectional from the cytoplasm of the hepatocyte into the bile.

Canalicular transport may be assisted by the membrane potential, but the contribution of membrane potential in organic anion transport has not been quantified. The ATP-dependent canalicular organic anion transport is mediated by a canalicular membrane protein, termed canalicular multispecific organic anion transporter (cMOAT) or MRP2 [135].

1.5.8 FATE OF BILIRUBIN IN THE GASTROINTESTINAL TRACT

Although conjugated bilirubin is not substantially absorbed from the intestines, a fraction of the small amount of unconjugated bilirubin that is excreted in bile is absorbed and undergoes enterohepatic circulation.

Degradation of bilirubin by intestinal bacteria generates urobilinogen and related products [136]. A major portion of the urobilinogen reabsorbed from the intestine is excreted in bile, but a small fraction is excreted in urine. Urobilinogen is colourless; its oxidation product, urobilin, contributes to the colour of normal urine and stool.

1.5.9 NEUROTOXICITY OF BILIRUBIN *IN VITRO*.

Neurotoxicity is determined mostly by the $\mathbf{B_f}$, the concentration of the unbound (free) fraction of UCB in plasma [137]. In physiological condition, over 99,9% of UCB is tightly bound to albumin, then the B_f is 0,1% of total UCB, only. The major unbound UCB species is an electrically neutral diacid [138], which, because of extensive internal hydrogen bonding [139], can diffuse passively across any cell membrane [140]. *In vitro* exposure of neurons and astrocytes to UCB has revealed neuroprotection at Bf below aqueous saturation with UCB (70 nM) [141], but neurotoxicity at B_f modestly above aqueous saturation with UCB [141] [142]. Accumulation of UCB might be limited by its conjugation and oxidation, binding to cytosolic glutathione-S-transferases and export by membrane transporters that are known to extrude other compounds from the CNS [138].

1.6 SH-SY5Y CELLS and UNCONJUGATED BILIRUBIN

Some studies have been performed in order to investigate different aspects of bilirubin toxicity in SH-SY5Y cells and in other sublines of neuroblastoma cell line. They shown that:

- After UCB exposure of 1 hour or longer, the neuroblastoma cell line N-115 develops evidence of toxicity which is progressive and irreversibile [143].
- In human SH-SY5Y cells, clinically relevant UCB concentration caused early disruption of the mitochondrial membrane potential (MMP) and following induced apoptosis [144].

1.7 MRP1 and UNCONJUGATED BILIRUBIN

In 1997, it was demonstrated that:

- in membrane vesicles from *MRP1*-transfected HeLa (human cervical cancer) cells, ATP-dependent transport of both monoglucuronosylbilirubin and bisglucuronosyl bilirubin is mediated by the multidrug resistance protein (MRP1) [145].

Further studies conducted in our laboratory, demonstrated that MRP1 is involved also in the transport of **UNCONJUGATED BILIRUBIN (UCB)** and supported its role in protecting cells from bilirubin toxicity.

It was found that:

- UCB is a substrate for both *YCF1* and *YLL015* gene products in *Saccaromhyces cerevisiae*. Of the six multi drug resistance genes expressed, *YCF1* and *YLL015* show a high homology for human *MRP1* [146].
- In BeWo (human trophoblastic) cells, grown in polarized manner, concomitantly increased the export of UCB and the expression of MRP1; morever, the efflux of UCB was almost abolished by MK571 [147], a general inhibitor of MRPs [148].
- In cultured astrocytes exposed to clinically relevant concentrations of UCB *in vitro*, MK571 increased apoptosis and impairment of mitochondrial function [(3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) test] and decreased Trypan Blue exclusion. In addition, it was shown that UCB upregulated the expression of MRP1 and engendered its translocation from the Golgi to the plasma membrane [149].
- In rats, the upregulation of Mrp1 in hemolysis is mediated by UCB and/or other products of heme oxygenase [150].
- Plasma-membrane vesicles from MDCKII cells (Madin-Darby canine kidney II cells) transfected stably with human MRP1 showed a transport activity of UCB

three and five times higher respectively compared with the same vesicles transfected stably with MRP2 or wild type vesicles. In addition, UCB inhibited the transport of LTC4 [151].

- In mouse embryo fibroblasts (MEF), isolated from Mrp1 knockout (-/-) mice, the UCB accumulation was twice higher compared to wild type cells. This was associated with greater, dose-related cytotoxicity, assessed by the MTT test, lactate dehydrogenase release and cellular ATP content [152].
- In primary cultures of rat neurons and astrocytes, the inhibition of Mrp1 with MK571 was associated with an increase in UCB-induced toxicity, demonstrated by the higher levels of cell death, cell dysfunction, cytokines secretion and glutamate release [54].

These findings show that MRP1 is involved in the transport of unconjugated bilirubin and that the affinity of this transporter for UCB is high (Km=10 nM) [151].

1.8 MDR1 and UNCONJUGATED BILIRUBIN

In 1991 it was reported that:

- UCB may be a weak substrate for Mdr1a [153].

Further studies showed that:

- UCB competitively inhibits the labelling of brain capillary MDR1 with a photoaffinity substrate [154].
- A greater proportion of UCB is taken up by the brains of *Mdr1a*(-/-) knockout mice compared with their *Mdr1a*(+/+) controls [155].
- Drugs known to inhibit MDR1 function may increase the risk of bilirubin encephalopathy in the hyperbilirubinemic infant [99].
- Inhibition of human MDR1 is associated with increased level of UCB-induced cell apoptosis *in vitro* [156].

From these findings, it seems that **MDR1** may be somehow iinvolved in the transport of UCB. However, these studies were performed with doses of UCB that yielded B_f much higher than clinically relevant concentrations [138] [142].

As mentioned above, we have attempted to reduce the MRP1 and MDR1 expression by small interfering RNAs (siRNAs). Their introduction in the cell causes the **RNA interference (RNAi)** phenomenon, a biological response to double-stranded RNA, that has proved to be an efficient means to manipulate the gene expression experimentally and to probe rapidly the gene function on a whole-genome scale.

1.9 RNA INTERFERENCE

1.9.1 OVERVIEW

RNA interference (also called "RNA-mediated interference", but abbreviated RNAi) is a cellular mechanism for the targeted destruction of RNA molecules. RNAi involves double-stranded ribonucleic acid (dsRNA) that can specifically interfere with the expression of genes with sequences that are complementary to the dsRNA. RNAi is a form of post-transcriptional gene silencing (PTGS) in which an antisense RNA strand targets a complementary gene transcript such as a messenger RNA for cleavage by a ribonuclease. RNAi has been shown to be a common cellular process in many eukaryotes.

The ability of RNAi to selectively reduce the expression of an individual protein in a cell makes RNAi a valuable laboratory research tool, both in cell culture and in vivo in living organisms. Synthetic dsRNA can be added to cells in order to artificially induce RNAi. RNAi can be used for large-scale screens that systematically shut down each protein in the cell in an attempt to identifying the necessary components for a particular cellular process or event such as cell survival or replication. RNAi also holds promise as a therapeutic technique in human disease. RNAi has been particularly well-studied in certain organisms such as the fruit fly *Drosophila melanogaster*, in plants where the effect can spread from cell to cell within the organism and in the nematode worm *Caenorhabditis elegans*, in which the gene silencing phenotype is heritable. In addition, in C. elegans the delivery of the dsRNA is exceptionally easy. Via a mechanism whose details are poorly understood, bacteria such as *Escherichia coli* that carry the desired dsRNA can be fed to the worms and will transfer their RNA payload to the worm via the intestinal tract. This "delivery by feeding" yields essentially the same magnitude of gene silencing as do more costly and time-consuming traditional delivery methods, such as soaking the worms in dsRNA solution and injecting dsRNA into the gonads [157].

Before RNA interference was well characterized, the phenomenon was known by other names, including post transcriptional gene silencing, transgene silencing, and quelling. Only after these were also characterized at the molecular level did it become clear that they described the RNAi phenomenon. Before RNAi was discovered, RNA was used to reduce gene expression in plant genetics. Single-stranded antisense RNA was introduced into plant cells and hybridized to the homologous single-stranded "sense" messenger RNA. It is now clear that the resulting dsRNA was responsible for reducing gene expression.

1.9.2 HISTORY

The revolutionary finding of RNAi was proceeded by reports of unexpected outcomes in experiments performed by plant scientists in the USA and The Netherlands [158]. The goal was to produce petunia plants with improved flower colors. To achieve this goal, they introduced additional copies of a gene encoding a key enzyme for flower pigmentation into petunia plants. Surprisingly, many of the petunia plants carrying additional copies of this gene did not show the expected deep purple or deep red flowers but carried fully white or partially white flowers. When the scientists had a closer look they discovered that both types of genes, the endogenous and the newly introduced transgenes, had been turned off. Evidence was obtained for posttranscriptional inhibition of gene expression that involved an increased rate of mRNA degradation [159]. This phenomenon was called "co-suppression of gene expression", but the molecular mechanism remained unknown.

A few years later plant virologists made a similar observation. In their research they aimed towards improvement of resistance of plants against plant viruses. At that time it was known that plants expressing virus-specific proteins showed enhanced tolerance or even resistance against virus infection. However, they also made the surprising observation that plants carrying only short regions of viral RNA sequences not coding for any viral protein showed the same effect. They concluded that viral RNA produced by transgenes can also attack incoming viruses and stop them from multiplying and spreading throughout the plant [160]. They did the reverse experiment and put short pieces of plant gene sequences into plant viruses. Indeed, after infection of plants with these modified viruses the expression of the targeted plant gene was suppressed. They called this phenomenon "virus-induced gene silencing" or simply "VIGS". These phenomena are collectively called post transcriptional gene silencing [161].

After these initial observations in plants many laboratories around the world searched for the occurrence of this phenomenon in other organisms. Mello and Fire's 1998 Nature paper based on research conducted with their colleagues (SiQun Xu, Mary Montgomery, Stephen Kostas, Sam Driver) at the Carnegie Institution of Washington and the University of Massachusetts reported a potent gene silencing effect after injecting double stranded RNA into *C. elegans* [162]. In investigating the regulation of muscle protein production, they observed that neither mRNA and antisense RNA injections had an effect on protein production, but double-stranded RNA successfully silenced the targeted gene. As a result of this work, they coined the term RNAi. The discovery of RNAi in *C. elegans* is particularly notable, as it represented the first identification of the causative agent

(double stranded RNA) of this not yet explained phenomenon. Fire and Mello were awarded the Nobel Prize in Physiology or Medicine in 2006 for their work [163].

1.9.2 CELLULAR MECHANISM

RNAi is an RNA-dependent gene silencing process that is mediated by the same cellular machinery that processes microRNA, known as the RNA-induced silencing complex (RISC). The process is initiated by the ribonuclease protein Dicer [164], which binds and cleaves exogenous double-stranded RNA molecules to produce double-stranded fragments of 20-25 base pairs with a few unpaired overhang bases on each end [165] (Fig. 1.7a). The short double-stranded fragments produced by Dicer, called small interfering RNAs (siRNAs), are separated and integrated into the active RISC complex. Although it was first believed that an ATP-dependent helicase separated the two strands [166], it has since been shown that the process is ATP-independent and effected directly by the protein components of RISC [167][168].

The catalytically active components of the RISC complex are known in animals as argonaute proteins, endonucleases which mediate the siRNA-induced cleavage of the target mRNA strand. Because the fragments produced by Dicer are double-stranded, they could each in theory produce a functional siRNA; however, only one of the two strands - known as the guide strand - binds the argonaute protein and leads to gene silencing (Fig. 1.7b). The other anti-guide strand or passenger strand is degraded as a RISC substrate during the process of RISC activation [169]. The strand selected as the guide tends to be the strand whose 5' end is more stable, but strand selection is not dependent on the direction in which Dicer cleaves the dsRNA before RISC incorporation [170].

It is not yet well understood how the activated RISC complex locates complementary mRNA molecules within the cell. Although the cleavage process has been proposed to be linked to translation, it has been shown that translation of the mRNA target is not a prerequisite for RNAi-mediated degradation [171]. In fact, one study found an increase in RNAi activity against mRNA targets that were not translated [172]. Argonaute proteins, the catalytic components of RISC, have been identified as localized to specific regions in the cytoplasm called cytoplasmic bodies, which are also local regions of high mRNA decay rates [173].

The native cellular purpose of the RNA interference machinery is not well characterized, but it is known to be involved in microRNA (miRNAs) processing and the resulting translational repression. MicroRNAs, which are encoded in the genome and have a role in gene regulation, typically have incomplete base pairing and only inhibit the translation of the target mRNA; by contrast, RNA interference as used in the laboratory typically involves perfectly base-paired dsRNA molecules

that induce mRNA cleavage [174]. After integration into the RISC, siRNAs base pair to their target mRNA and induce the RISC component protein argonaute to cleave the mRNA, thereby preventing it from being used as a translation template (Fig. 1.8).

Organisms vary in their cell ability to take up foreign dsRNA and use it in the RNAi pathway. The effects of RNA interference are both systemic and heritable in plants and in *C. elegans*, although not in *Drosophila* or mammals due to the absence of RNA replicase in these organisms. In plants, RNAi is thought to propagate through cells via the transfer of siRNAs through plasmodesmata [166].

1.9.4 BIOLOGICAL SIGNIFICANCE OF RNAi DISCOVERY

The far-reaching consequences of the discovery can be summed up as follows (Fig. 1.9):

1. RNAi protects against viral infections: what Fire and Mello found in the 1998 [162] that is cells can process injected dsRNA and eliminate homologous single-stranded RNA suggested that RNAi could constitute a defence mechanism against viral attacks. It had earlier been shown that plant cells have an efficient defence against viruses based on the PTGS phenomenon [160] [161]. When it became apparent that PTGS is the plant equivalent to RNAi, this early work in plants supported the proposition that RNAi is involved in protecting cells from viral attacks. Today, we know that this anti-viral mechanism is at work in plants, worms and flies, whereas it is still unclear how relevant it is for vertebrates, including man.

2. RNAi secures genome stability by keeping mobile elements silent: it was proposed early on that RNAi/PTGS in *C. elegans* and plants could block the action of transposons (mobile elements in the genome). Subsequently, it could be shown that when components of the RNAi machinery are mutated in *C. elegans,* transposons are activated and the mobile elements cause disturbances in the function of the genome [175] [176]. It has been proposed that in transposon-containing regions of the genome both DNA strands are transcribed, dsRNA is formed, and the RNAi process eliminates these undesirable products. As short dsRNAs can also operate directly on chromatin and suppress transcription, this would be another mode to keep transposons inactive (see n.4). Even if the mechanisms are not yet fully revealed, it is clear that if the RNAi machinery is not efficient, the transposons are not kept under control and can start to jump and cause deleterious effects in the genome.

It has been argued that RNA silencing could represent an "immune defence" of the genome [177]. Close to 50% of our genome consists of viral and transposon elements that have invaded the genome in the course of evolution. The RNAi machinery can recognize invading double-stranded viral RNA (or the double-stranded replicative form of the viral RNA) and suppress the infection by degradation of the RNA. The RNAi system thus shares important features with the vertebrate immune system: it recognizes the invading parasite (dsRNA), raises an initial response and subsequently amplifies the response to eliminate the foreign element.

3. RNAi-like mechanisms repress protein synthesis and regulate the development of organisms: soon after the discovery that short RNA is the effector of RNAi, it was shown that there is a class of endogenous RNA molecules of the same size in worms, flies, mice and humans; this small RNA was called microRNA (miRNA) [178-180]. Plants also contain this class of endogenous RNA [181] . The small miRNAs are processed from larger hairpin-like precursors by an RNAi-like machinery [182] [183]. The miRNAs can regulate gene expression by base-pairing to mRNA, which results in either degradation of the mRNA or suppression of translation. Consequently, the RNAi machinery is important to regulate endogenous gene activity. This effect was first described for the worm *Caenorhabditis elegans* in 1993 by R. C. Lee et al. of Harvard University [184]. In plants, this mechanism was first shown in the "JAW microRNA" of *Arabidopsis thaliana*; it is involved in the regulation of several genes that control the plant shape [185]. Genes have been found in bacteria that are similar in the sense that they control mRNA abundance or translation by binding an mRNA by base pairing, however they are not generally considered to be miRNAs because the Dicer enzyme is not involved [186]. It has been suggested that CRISPR systems in prokaryotes are analogous to eukaryotic RNA interference systems, although none of the protein components is orthologous [187]. Today, it is estimated that there are about 500 miRNAs in mammalian cells, and that about 30% of all genes are regulated by miRNAs. It is known that miRNAs play an important role during development in plants, *C. elegans* and mammals. Thus, the miRNA-dependent control of gene expression represents a new major principle of gene regulation. However, the full significance of small regulatory RNAs is probably still not apparent.

4. RNAi-like mechanisms keep chromatin condensed and suppress transcription: it was known from work in plants that gene silencing could take place at the transcriptional level (TGS). After the discovery of RNAi, it was soon shown that TGS in plants operates via RNAi-like mechanisms [188] [189]. In the fission yeast *Schizosaccharomyces pombe* [190] [191], and later on in *Drosophila* and vertebrates, it was found that similar processes keep heterochromatic regions

condensed and transcriptionally suppressed. In addition, the RNAi-like machinery regulates the activity of genes in the immediate vicinity of the condensed blocks of chromatin. The phenomenon is still not understood at the molecular level although histone modifications, binding of specific chromatin condensing proteins (HP1), and DNA methylation all play important roles [192]. It is, however, evident that this action on chromatin is most important for proper functioning of the genome and for maintenance of genome integrity.

1.9.5 GENE KNOCKDOWN

RNAi has recently been applied as an experimental technique to study the function of genes in several organisms. Studying the effects of the decrease in production of the protein of interest caused by the introduction of dsRNA targeting that specific gene, insights into the protein role and function can be found. Since RNAi may not totally abolish expression of the gene, this technique is sometimes referred as a "knockdown", to distinguish it from "knockout" procedures in which expression of a gene is entirely eliminated by removing or destroying its DNA sequence.

1.9.6 CROSSTALK BETWEEN RNA EDITING AND RNA INTERFERENCE

The type of RNA editing that is most prevalent in higher eukaryotes converts adenosine (A) residues into inosine (I) in double-stranded (ds)RNAs through the action of ADAR (adenosine deaminase acting on RNA) enzymes [193].

The idea that the RNAi and A→I RNA editing pathways might compete for a common substrate dsRNA was originally proposed in 2000 [194]. Recent studies showed that precursor RNAs of certain miRNAs indeed undergo A→I RNA editing [195] and editing seems to regulate the processing and expression of mature miRNAs [196]. Furthermore, one of the mammalian ADAR-family members sequesters siRNAs, thereby reducing RNAi efficacy [197]. Last, analysis of ADAR-null *C. elegans* strains indicates that A→I RNA editing might counteract RNAi silencing of endogenous genes and transgenes [198].

1.9.7 RNAi IN MAMMALIAN CELLS

1.9.7.1 Non-specific Gene Silencing by Long dsRNAs

While the natural presence of RNAi had been observed in a variety of organisms (plants, protozoa, insects, and nematodes), evidence for the existence of RNAi in mammalian cells took longer to establish. Transfection of long dsRNA molecules (>30 nt) into most mammalian cells causes nonspecific suppression of gene expression, as opposed to the gene-specific suppression seen in other organisms. This suppression has been attributed to an antiviral response, which takes place through one of two pathways.
In one pathway, long dsRNAs activate a protein kinase, PKR. Activated PKR, in turn phoshorylates and inactivates the translation initiation factor, eIF2a, leading to repression of translation [199]. In the other pathway, long dsRNAs activate RNase L, which leads to nonspecific RNA degradation [200].
A number of groups have shown that the dsRNA-induced antiviral response is absent from mouse embryonic stem (ES) cells and at least one cell line of embryonic origin [201] [202]. It is therefore possible to use long dsRNAs to silence specific genes in these specific mammalian cells. However, the antiviral response precludes the use of long dsRNAs to induce RNAi in most other mammalian cell types.

1.9.7.2 Antiviral Response Bypass by siRNAs

Interestingly, dsRNAs less than 30 nt in length do not activate the PKR kinase pathway. This observation, as well as knowledge that long dsRNAs are cleaved to form siRNAs in worms and flies and that siRNAs can induce RNAi in *Drosophila* embryo lysates, prompted researchers to test whether introduction of siRNAs could induce gene-specific silencing in mammalian cells [203]. Indeed, siRNAs introduced by transient transfection were found to effectively induce RNAi in mammalian cultured cells in a sequence-specific manner. The effectiveness of siRNAs varies — the most potent siRNAs result in >90% reduction in target RNA and protein levels [204-206]. The most effective siRNAs turn out to be 21 nt dsRNAs with 2 nt 3' overhangs. Sequence specificity of siRNA is very stringent, as single base pair mismatches between the siRNA and its target mRNA dramatically reduce silencing [204] [207]. Unfortunately, not all siRNAs with these characteristics are effective. The reasons for this are unclear but may be a result of positional effects [206] [208] [209].

1.9.7.3 RNAi as a Tool for Functional Genomics

With the knowledge that RNAi can be induced in mammalian cells by the transfection of siRNAs, many more researchers are beginning to use RNAi as a tool in human, mouse and other mammalian cell culture systems. In early experiments with mammalian cells, the siRNAs were synthesized chemically and transfected transiently into cells. Shortly afterwards, companies introduced kits to produce siRNAs by in vitro transcription, which is a less expensive alternative to chemical synthesis, particularly when multiple different siRNAs need to be synthesized.

Recently, a number of groups have developed expression vectors to continually express siRNAs in transiently and stably transfected mammalian cells [210-216]. Some of these vectors have been engineered to express small hairpin RNAs (shRNAs), which get processed in vivo into siRNAs-like molecules capable of carrying out gene-specific silencing [210] [213] [214] [216]. The vectors contain the shRNA sequence between a polymerase III (pol III) promoter and a 4-5 thymidine transcription termination site. The transcript is terminated at position 2 of the termination site (pol III transcripts naturally lack poly(A) tails) and then folds into a stem-loop structure with 3' UU-overhangs. The ends of the shRNAs are processed in vivo, converting the shRNAs into ~21 nt siRNA-like molecules, which in turn initiate RNAi [210]. This latter finding correlates with experiments in *C. elegans*, *Drosophila*, plants and Trypanosomes, where RNAi has been induced by an RNA molecule that folds into a stem-loop structure [217].

Another siRNA expression vector developed by a different research group encodes the sense and antisense siRNA strands under control of separate pol III promoters [212]. The siRNA strands from this vector, like the shRNAs of the other vectors, have 5 thymidine termination signals. Comparing to chemically synthesized siRNA, DNA vector-based siRNA technology has several advantages. Some of them are listed here below:

- unlike synthetic siRNA, vector based siRNA is the same as DNA, it is very stable and can be easily transfected into cell using routine DNA transfection reagents, such as Lipofectamine;
- stable cell line can be established and observe long-term effects of RNAi;
- inducible system can be estabilished by using a vector with an inducible promoter;
- once a DNA construct is made, an unlimited supply of siRNA is available.

1.9.7.4 Role in medicine

It may be possible to exploit the RNA interference process for therapeutic purposes. Although it is difficult to introduce long dsRNA strands into mammalian cells due to the interferon response, the use of short interfering RNA mimics has been more successful [202]. The first applications to reach clinical trials are in the treatment of macular degeneration and respiratory syncytial virus [218]. RNAi has also been shown effective in the complete reversal of induced liver failure in mouse models [219].

Other proposed clinical uses explored in cell culture center on antiviral therapies, including the inhibition of viral gene expression in cancerous cells [220], the silencing of hepatitis A [221] and hepatitis B [222] genes, silencing of influenza gene expression [223], and inhibition of measles viral replication [224]. Potential treatments for neurodegenerative diseases have also been proposed, with particular attention to the polyglutamine diseases such as Huntington's disease [225].

Despite the proliferation of promising cell culture studies for RNAi-based drugs, some concern has been raised regarding the safety of RNA interference, especially the potential for "off-target" effects in which a gene with a coincidentally similar sequence to the targeted gene is also repressed [226]. A computational genomics study estimated that the error rate of off-target interactions is about 10% [227]. One major study of liver disease in mice led to high death rates in the experimental animals, suggested by researchers to be the result of "oversaturation" of the dsRNA pathway [228].

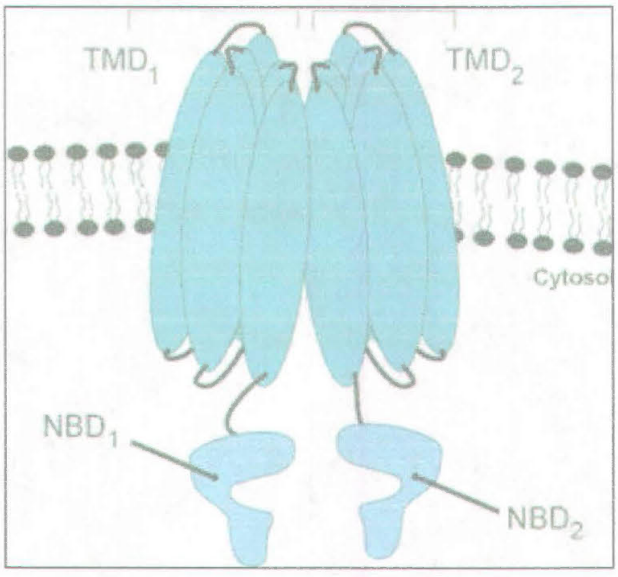


Fig. 1.1 Functional unit of an ABC transporter.

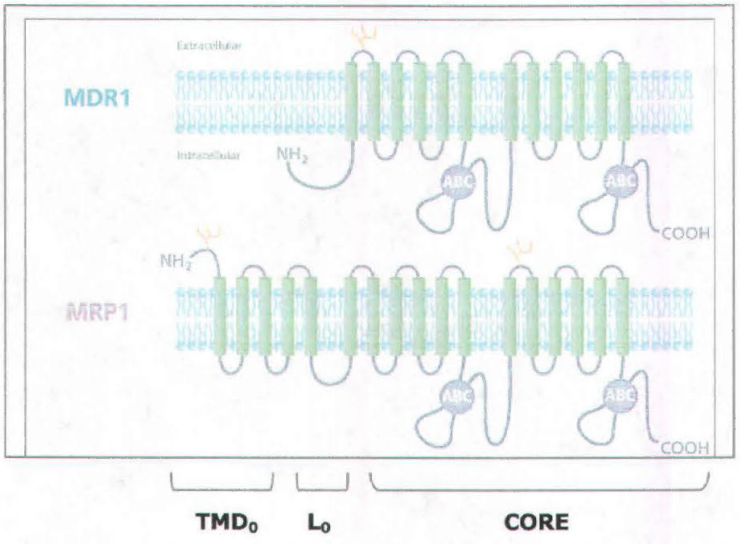


Fig. 1.2 Membrane topology models for MDR1 and MRP1 proteins.

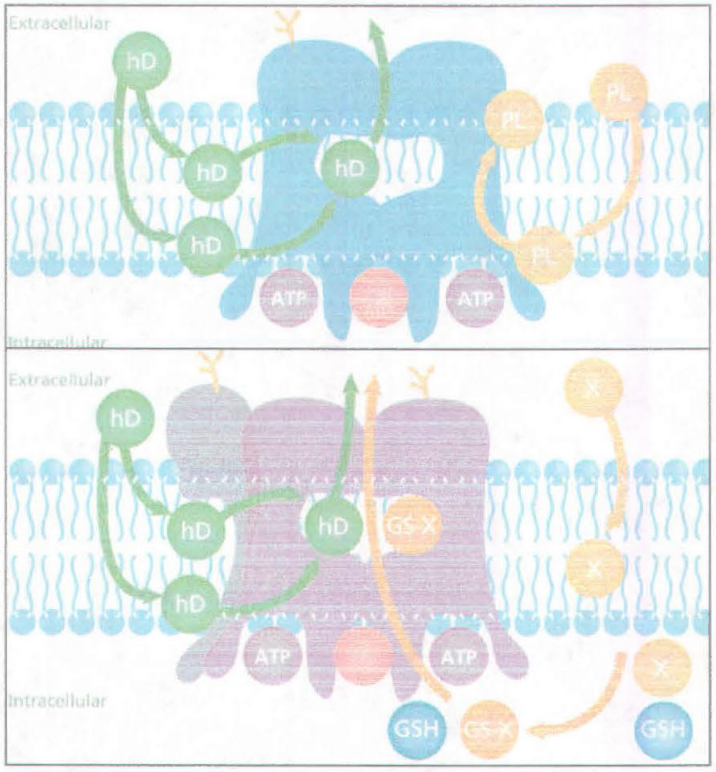


Fig. 1.3 a MDR1-P- glycoprotein.
Substrates are ricognized in, or near to the membrane lipid phase.
hD, Hydrophobic drugs
PL,phospholipid.

Fig. 1.3 b MRP1.
Both hydrophobic drugs and anionic conjugates, such as glutathione conjugates, are transported. The transport of some hydrophobic drugs may be coupled to reduced glutathione (GSH) as GS-X molecules.

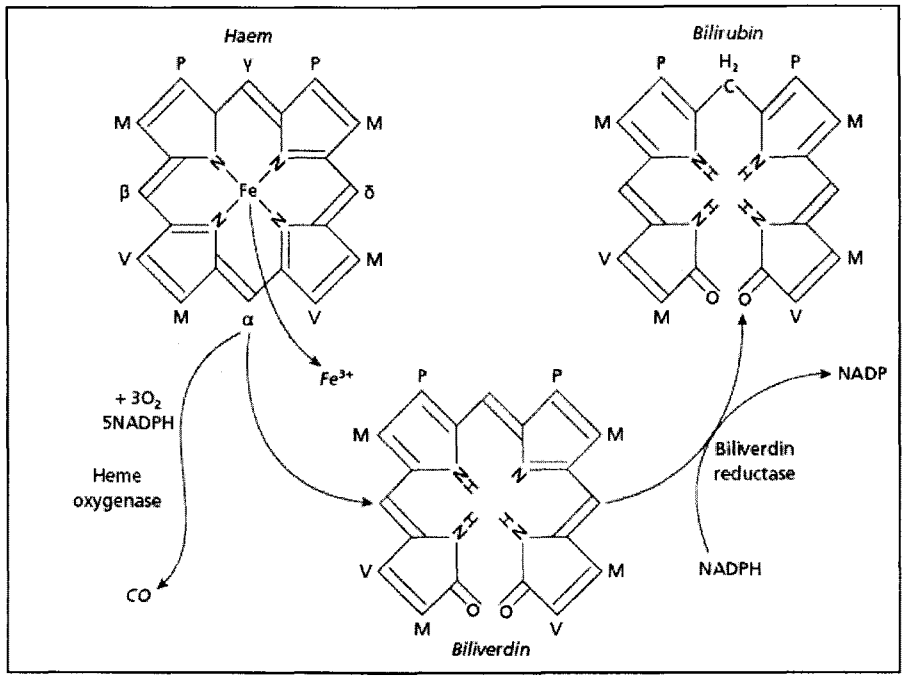


Fig. 1.4 Enzyme-catalysed degradation of haem. Haem degradation begins by haem oxygenase-catalysed oxidation of the a-bridge carbon of haem, which is converted to CO, NADPH leading to opening of the tetrapyrrole ring and release of the iron molecule. The resulting biliverdin molecule is subsequently reduced to bilirubin by cytosolic biliverdin reductase.

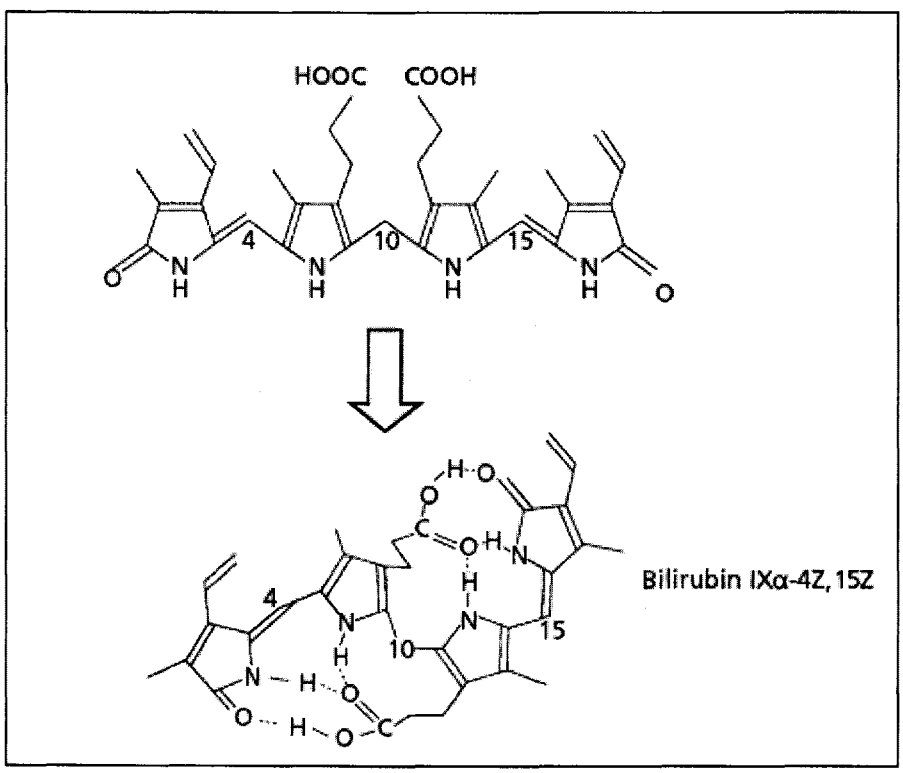


Fig. 1.5 Internal hydrogen bonding. The carboxylic acid moiety of the propionic acid side-chains of bilirubin form internal hydrogen bonds with contralateral NH groups and the lactam oxygen, thereby engaging all polar groups of the molecule and making it insoluble in water.

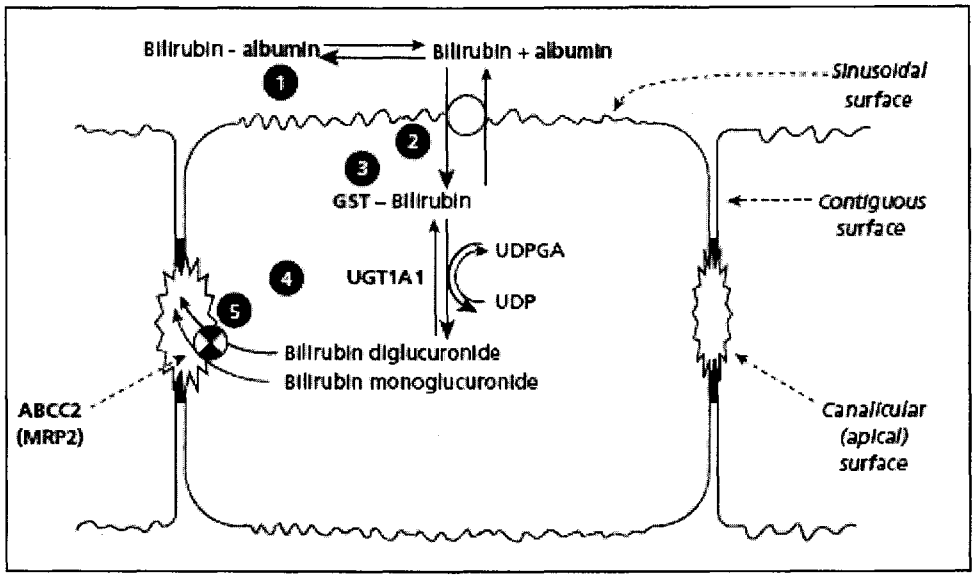
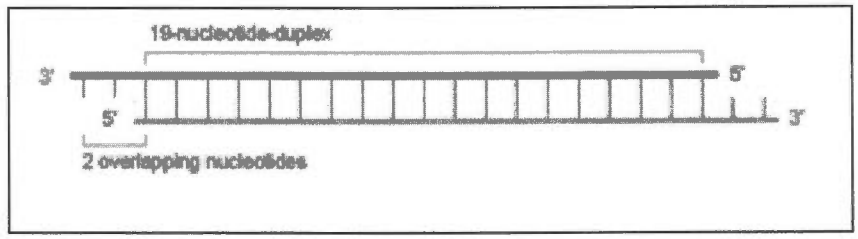
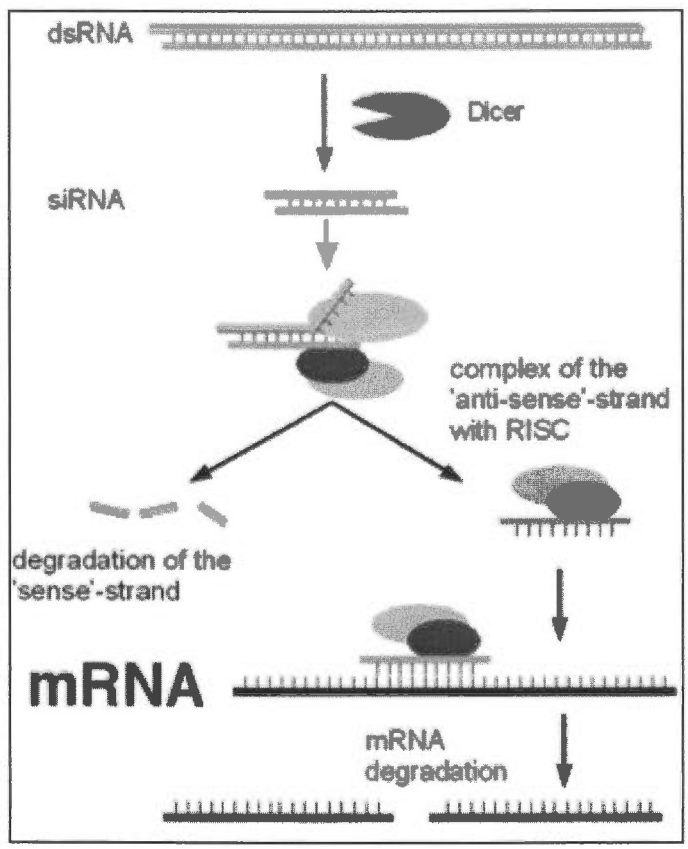


Fig. 1.6 Bilirubin throughput by hepatocytes. Bilirubin is transported from sites of production to hepatic sinusoids bound to albumin. At the sinusoidal surface of hepatocytes, bilirubin dissociates from albumin and enters hepatocytes by facilitated diffusion. Binding to cytosolic glutathione-S-transferases (GSTs) increases net uptake of bilirubin by inhibiting its efflux. Bilirubin is converted to mono- and diglucuronide by the action of UGT1A1, which catalyses the transfer of the glucuronic acid moiety from UDP-glucuronic acid to bilirubin.
Bilirubin glucuronides are actively transported into bile against a concentration gradient by the ATP-utilizing pump ABCC2 (also termed MRP2).



A



B

Fig. 1.7 A. The RNAi process is initiated by the ribonuclease protein Dicer, which binds and cleaves exogenous double-stranded RNA molecules to produce double-stranded fragments of 20-25 base pairs with a few unpaired overhang bases on each end.

B. Cells can use Dicer to trim double stranded RNA and form small inhibitory RNA (siRNA). An siRNA can be processed by to the single strand anti-sense RNA and used to target mRNAs for destruction. Several proteins (ovals) are required for efficient RNA interference. The protein-containing complex was named RNA-induced silencing complex (RISC).

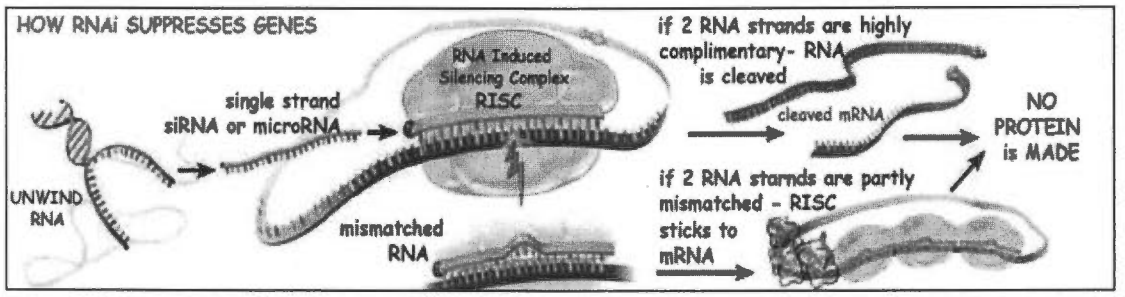


Fig. 1.8 The short siRNA duplexes are unwinded into single strand RNAs and integrated into the RNA-Induced Silencing Complex (RISC). The RISC then captures a native mRNA molecule that complements the short siRNA sequence. If the pairing is essentially perfect, the native mRNA is cut into useless RNA fragments that aren't translated. If however, the pairing is less than perfect then the RISC complex binds to the mRNA and blocks ribosome movement along the native mRNA also halting translation. The net effect is NO PROTEIN IS MADE.

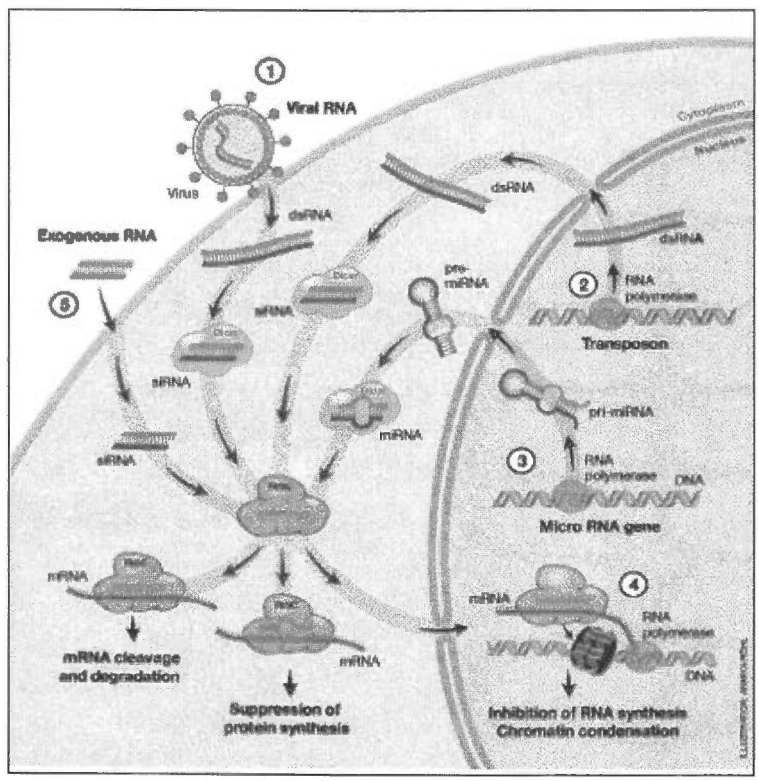


Fig. 1.9 Cellular processes dependent on the RNAi machinery. The Dicer and RISC complexes play a central role in the destruction of invading viral RNA (1), the elimination of transcripts from mobile elements (transposons) and repetitive DNA (2), the block of protein synthesis brought about by small RNAs generated within the cell (3), and the RNAi-mediated suppression of transcription (4). The machinery is also utilized when siRNA is introduced into the cell experimentally to inhibit the activity of specific genes (5). The figure is schematic, and the Dicer and RISC complexes can vary dependent on cellular process.

1.10 REFERENCES

1 Jones PM, George AM (2004) The ABC transporter structure and mechanism: perspectives on recent research. Cellular and Molecular Life Sciences 61, 682-699.

2 Higgins CF (1992) ABC transporters: from microorganisms to man. Annu Rev Cell Biol 8, 67-113.

3 Borst P, Oude Elferink R (2002) Mammalian ABC transporters in health and disease. Annu Rev Biochem 71, 537-92.

4 Dean M, Rzhetsky A, Allikmets R (2001) The Human ATP-Binding Cassette (ABC) Transporter Superfamily. Genome Research 11, 1156-1166.

5 Lankas GR, Wise LD, Cartwright ME, Pipper T, Umbenhauer DR (1998) Placental P-glycoprotein deficiency enhances susceptibility to chemically induced birth defects in mice. Reprod Toxicol 12, 457-63.

6 Smit JW, Huisman T, Van Tellingen O, Wiltshire HR, Schinkel AH (1999) Absence or pharmacological blocking of placental P-glycoprotein profoundly increases fetal drug exposure. J Clin Invest 104, 1441-47.

7 Pascolo L, Fernetti C, Pirulli D, Crovella S, Amoroso A, Tiribelli C (2003) Effects of maturation on RNA transcription and protein expression of four MRP genes in human placenta and in BeWo cells Biochem Biophys Res 303, 259-265.

8 St-Pierre MV, Stallmach T, Freimoser Grundshober A, Dufour JF, Serrano MA, Marin JJG, Sugiyama Y, Meier PJ (2004) Am J Physiol Integr Comp Physiol 287, 1505-1516.

9 Meyer zu Schwabedissen HE, Jedlitschky G, Gratz M, Haenish S, Linnemann K, Fush C, Cascorbi I, Kroemer HK (2005) Variable expression of MRP2 (ABCC2) in human placenta: influence of gestational age and cellular differentiation. Drug Metabol Dispos 33, 896-904.

10 Sarkadi B, Müller M, Homoloya L, Holló Z, Sepródi J, Germann UA, Gottesman MM, Price EM, Boucher RC (1994) Interaction of bioactive hydrophobic peptides with the human multidrug transporter. FASEB J 8(10), 766-70.

11 Borgniaq MJ, Eytan GD, Assaraf YG (1996) Competition of hydrophobic peptides, cytotoxic drugs, and chemosensitizers on a common P-glycoprotein pharmacophore as revealed by its ATPase activity. J Biol Chem 271(6), 3163-71.

12 Schmitt L, Tampe R (2000) Affinity, specificity, diversity: a challenge for the ABC transporter TAP in cellular immunity. Chembiochem 1(1):16-35.

13 Young L, Leonhard K, Tatsuta T, Trowsdale L, Langer T (2001) Role of the ABC transporter Mdl1 in peptide export from mitochondria. Science 291, 2135-38.

14 Borst P, Evers R, Kool M, Wijnholds J (2000) A family of drug transporters: the multidrug resistance-associated proteins. J Natl. Cancer Inst 92(16), 1295-1302.

15 Endicott JA, Ling V (1989) The biochemistry of P-glycoprotein-mediated multidrug resistance. Annu Rev Biochem 58, 137-171.

16 Gottesman MM, Fojo T, Bates SE (2002) Multidrug resistance in cancer: role of ATP-dependent transporters. Nat Rev Cancer 2(1), 48-58.

17 Rao VV, Dahlheimer JL, Bardgett ME, Snyder AZ, Finch RA, Sartorelli AC, Piwnica-Worms (1999) Choroid plexus epithelial expression of *MDR1* P glycoprotein and multidrug resistance-associated protein contribute to the blood–cerebrospinal-fluid drug-permeability barrier Proc Natl Acad Sci USA 96(7), 3900–3905.

18 Bart J, Groen HJ, Hendrikse NH, van der Graaf WT, Vaalburg W, de Vries EG (2000) The blood-brain barrier and oncology: new insights into function and modulation. Cancer Treat Rev 26(6) 449-462.

19 Thiebaut F, Tsuruo T, Hamada H, Gottesman MM, Pastan I, Willingham MC (1987) Cellular localization of the multidrug-resistance gene product P-glycoprotein in normal human tissues. Proc Natl Acad Sci USA 84(21), 7735-7738.

20 Kipp H, Arias IM (2000) Intracellular trafficking and regulation of canalicular ATP-binding cassette transporters. Semin Liver Dis 20(3), 339-351.

21 Higgins CE, Gottesman MM (1992) Is the multidrug transporter a flippase? Trends Biochem Sci 17(1), 18-21.

22 Szakacs G, C Ozvegy, E Bakos , B Sarkadi, and A Váradi (2001) Role of glycine-534 and glycine-1179 of human multidrug resistance protein (MDR1) in drug-mediated control of ATP hydrolysis. Biochem J 356(Pt 1), 71–75.

23 Szabo K, Welker E, Bakos E, Muller M, Roninson I, Varadi A, Sarkadi B (1998) Drug-stimulated Nucleotide Trapping in the Human Multidrug Transporter MDR1. J Biol Chem 273, 10132-10138.

24 Hrycyna CA, Airan LE, Germann UA, Pastan I, Gottesman MM (1998) Structural flexibility of the linker region of human P-glycoprotein permits ATP hydrolysis and drug transport. Biochem 37, 13660-13673.

25 Hrycyna CA, Ambudkar SV, Ramachandra M, Ko, YH, Pedersen PL, Pastan I, Gottesman MM (1998) Mechanism of action of human P-glycoprotein ATPase activity. Photochemical cleavage during a catalytic transition state using orthovanadate reveals cross-talk between the two ATP sites. J Biol Chem 273, 16631-16634.

26 Sauna ZE, Ambudkar SV (2001) Characterization of the Catalytic Cycle of ATP Hydrolysis by Human P-glycoprotein. J Biol Chem 276(15), 11653-11661.

27 Senior AE, Al-Shawi MK, Urbatsch IL (1995) The catalytic cycle of P-glycoprotein. FEBS Lett 377, 285-89.

28 Gao M, Loe DW, Grant CE, Cole SPC, Deeley RG (1996) Reconstitution of ATP-dependent leukotriene C_4 transport by co-expression of both half-molecules of human multidrug resistance protein in insect cells. J Biol Chem 271(44), 27782-27787.

29 Bakos E, Hegedus T, Hollo Z, Welker E, Tusnady GE, Zaman GJ, Flens MJ, Varadi A, Sarkadi B (1996) Membrane topology and glycosylation of the human multidrug resistance-associated protein. J Biol Chem 271, 12322-12326.

30 Gao M, Yamazaki M, Loe DW, Westlake CJ, Grant CE, Cole SPC, Deeley RG (1998) Multidrug resistance protein. Identification of regions required for active transport of leukotriene C_4. J Biol Chem 273(17), 10733-10740.

31 Bakos E, Evers R, Szacks G, Tusnady GE, Welker E, Szabo K, de Haas M, van Deemter L, Borst P, Varadi A, Sarkadi B (1998) Functional multidrug resistance (MRP1) lacking the N-terminal transmembrane domain. Biol Chem 273(48), 32167-32175.

32 Bakos E, Evers R, Calenda G, Tusnady GE, Szakacs G, Varadi A, Sarkadi B (2000) Characterization of the amino-terminal regions in the human multidrug resistance protein (MRP1). J Cell Sci 113(24), 4451-61.

33 Wang W, Ballatori N (1998) Endogenous glutathione conjugates: occurrence and biological functions. Pharmacol Rev 50, 335-356.

34 König J, Nies AT, Cui Y, Leier I, Keppler D (1999) Conjugate export pumps of the multidrug resistance protein (MRP) family: localization, substrate specificity, and MRP2-mediated drug resistance. Biochim Biophys Acta 1461, 377-394.

35 Loe DW, Almquist KC, Deeley RG, Cole SPC (1996) Multidrug resistance protein (MRP) mediated transport of leukotriene C4 and chemotherapeutic agents in membrane vesicles. J Biol Chem 271, 9675-9682.

36 Flens MJ, Zaman GJR, van der Valk P, Izquierdo MA, Schroeijers AB, Scheffer GL, van der Groep P, de Haas M, Meijer CJLM, Scheper RJ (1996) Tissue distribution of the multidrug resistance protein. Am J Pathol 148, 1237-1247.

37 Müller M, Roelofsen H, Jansen PLM (1996) Secretion of organic anions by hepatocytes: involvement of homologues of the multidrug resistance protein. Semin Liver Dis 16, 211-220.

38 Müller M, Meijer C, Zaman GJR, Borst P, Scheper RJ, Mulder NH, de Vries EGE, Jansen PLM (1994) Overexpression of the gene encoding the multidrug resistance-associated protein results in increased ATP-dependent glutathione S-conjugate transport. Proc Natl Acad Sci USA 91, 13033-13037.

39 Evers R, Cnubben NHP, Wijnholds J, van Deemter L, van Bladeren P, Borst P (1997) Transport of glutathione prostaglandin A conjugates by the multidrug resistance protein 1. FEBS Lett 419, 112-116.

40 Ishikawa T, Akimaru K, Nakanishi M, Tomokiyo K, Furuta K, Suzuki M, Noyori R (1998) Anticancer-prostaglandin-induced cell-cycle arrest and its modulation by an inhibitor of the ATP-dependent gluathione S-conjugate export pump (GS-X pump). Biochem J 336, 569-576.

41 Leier I, Jedlitschky G, Buchholz U, Center M, Cole SPC, Deeley RG, Keppler D (1996) ATP-dependent glutathione disulphide transport mediated by the MRP gene-encoded conjugate export pump. Biochem J 314, 433-437.

42 Renes J, de Vries EGE, Hooiveld GJEJ, Krikken I, Jansen PLM, Müller M (2000) The multidrug resistance protein MRP1 protects against the toxicity of the major lipid peroxidation product 4hydroxynonenal. Biochem J 350, 555-561.

43 Hay DW, Torphy TJ, Undem BJ (1995) Cysteinyl leukotrienes in asthma: old mediators up to new tricks. Trends Pharmacol Sci 16, 304-309.

44 Parker J (1995) Prostaglandin A2 protein interactions and inhibition of cellular proliferation. Prostaglandins 50, 359-375.

45 Esterbauer H, Schaur RJ, Zollner H (1991) Chemistry and biochemistry of 4-hydroxynonenal, malondialdehyde and related aldehydes. Free Rad Biol Med 11, 81-128.

46 Prechtl S, Roellinghoff M, Scheper RJ, Cole SPC, Deeley RG, Lohoff M (2000) The multidrug resistance protein 1: a functionally important activation marker for murine Th1 cells. J Immunol 164, 754-761.

47 Wijnholds J, Evers R, van Leusden MR, Mol CA, Zaman GJ, Mayer U, Beijnen JH, van der Valk M, Krimpenfort P, Borst P (1997) Increased sensitivity to anticancer drugs and decreased inflammatory response in mice lacking the multidrug resistance-associated protein. Nat Med 3, 1275-1279.

48 Wijnholds J, Scheffer GL, van der Valk M, van der Valk P, Beijnen JH, Scheper RJ, Borst P (1998) Multidrug resistance protein 1 protects the oropharyngeal mucosal layer and the testicular tubules against drug-induced damage. J Exp Med 188, 797-808.

49 Nishino J, Suzuki H, Sugiyama D, Kitazawa T, Ito K, Hanano M, Sugiyama Y (1999) Transepithelial transport of organic anions across the choroid plexus: possible involvement of organic anion transporter and multidrug resistance-associated protein. J Pharmacol Exp Ther 290, 289-294.

50 Wijnholds J, de Lange EC, Scheffer GL, van Den Berg DJ, Mol CA, van der Valk M, Schinkel AH, Scheper RJ, Breimer DD, Borst P (2000) Multidrug resistance protein 1 protects the choroid plexus epithelium and contributes to the blood-cerebrospinal fluid barrier. J Clin Invest 105, 279-285.

51 Regina A, Koman A, Piciotti M et al (1998) Mrp1 multidrug resistance-associated protein and P-glycoprotein expression in rat brain microvessel endothelial cells. J Neurochem 71, 705-715.

52 Declèves X, Regina A, Laplanche JL, Roux F, Boval B, Launay JM, Scherrmann JM (2000) Functional expression of P-Glycoprotein and Multidrug Resistance-Associated Protein (Mrp1) in primary cultures of rat astrocytes. J Neurosci Res 60, 594-601.

53 Lee G, Schlichter L, Bendayan M, Bendayan R (2001) Functional expression of P-glycoprotein in rat brain microglia. J Pharmacol Exp Ther 299, 204-212.

54 Falcão AS, Bellarosa C, Fernandes A, Brito MA, Silva RFM, Tiribelli C, Brites D (2007) Role of multidrug resistance-associated protein 1 expression in the *in vitro* susceptibility of rat nerve cell to unconjugated bilirubin. Neurosci 144, 878-888.

55 Renes J, de Vries EGE, Nienhuis EF, Jansen PLM, Müller M (1999) ATP-and glutathione-dependent transport of chemotherapeutic drugs by the multidrug resistance protein MRP1. Br J Pharmacol 126, 681-688.

56 Versantvoort CHM, Broxterman HJ, Bagrij T, Scheper RJ, Twentyman PR (1995) Regulation by glutathione of drug transport in multidrug-resistant human lung tumour cell lines overexpressing multidrug resistance-associated protein. Br J Cancer 72, 82-89.

57 Zaman GJR, Lankelma J, van Tellingen O, Beijnen J, Dekker H, Paulusma CC, Oude Elferink RPJ, Baas F, Borst P (1995) Role of glutathione in the export of compunds from cells by the multidrug-associated protein. Proc Natl Acad Sci USA 92, 7690-7694.

58 Kuwano M, Toh S, Uchiumi T, Takano H, Kohno K, Wada M (1999) Multidrug resistance-associated protein subfamily transporters and drug resistance. Anticancer Drug Des. 14, 123-31.

59 Chen CJ, Chin JE, Ueda K, Clark DP, Pastan I, Gottesman MM, Roninson IB (1986) Internal duplication and homology with bacterial transport proteins in the mdr1 (P-glycoprotein) gene from multidrug-resistant human cells. Cell 47, 381-389.

60 Juliano RL, Ling V (1976) A surface glycoprotein modulating drug permeability in Chinese hamster ovary cell mutants. Biochim Biophys Acta, 455, 152- 162.

61 Roepe PD (1995) The role of the MDR protein in altered drug translocation across tumor cell membranes. Biochim Biophys Acta 1241(3), 385-405.

62 Ruetz S, Gros P (1994) A mechanism for P-glycoprotein action in multidrug resistance: are we there yet? Trends Pharmacol Sci 15(7), 260-263.

63 Germann UA (1996) P-glycoprotein: a mediator of multidrug resistance in tumour cells. Eur J Cancer, 32A(6), 927-944.

64 Raviv Y, Pollard HB, Brüggemann EP, Pastan I, Gottesman MM (1990) Photosensitized labeling of a functional multidrug transporter in living drug-resistant tumor cells. J Biol Chem 265(7), 3975-3980.

65 Smit JJ, Schinkel AH, Oude Elferink RPJ, Grown AK, Wagenaar E and 9 others (1993) Homozygous disruption of the murine mdr2 P-glycoprotein gene leads to a complete absence of phospholipid from bile and to liver disease. Cell 75, 451-462.

66 van Helvoort A, Smith AJ, Sprong H, Fritzsche I, Schinkel AH, Borst P, van Meer G (1996) MDR1 P-glycoprotein is a lipid translocase of broad specificity, while MDR3 P-glycoprotein specifically translocates phosphatidylcholine. Cell, 87(3), 507-517.

67 Kamp D, Haest CW (1998) Evidence for a role of the multidrug resistance protein (MRP) in the outward translocation of NBD-phospholipids in the erythrocyte membrane. Biochim Biophys Acta, 1372(1), 91-101.

68 Bolhuis H, van Veen HW, Molenaar D, Poolman B, Driessen AJ, Konings WN (1996) Multidrug resistance in Lactococcus lactis: evidence for ATP-dependent drug extrusion from the inner leaflet of the cytoplasmic membrane. EMBO J 15(16), 4239-4245.

69 Shapiro AB, Ling V (1998) The mechanism of ATP-dependent multidrug transport by P-glycoprotein. Acta Physiol Scand 163 Suppl. 643, 227-234.

70 Fojo AT, Uda K, Slamon DJ, Poplack DG, Gottesman MM, Pastan I (1987) Expression of a multidrug-resistance gene in human tumors and tissues. Proc Natl Acad Sci USA 84(1), 265-269.

71 Cordon-Cardo C, O'Brien JP, Casals D, Rittman-Grauer L, Biedler JL, Melamed MR, Bertino JR (1989) Multidrug-resistance gene (P-glycoprotein) is expressed by endothelial cells at blood-brain barrier sites. Proc Natl Acad Sci USA 86(2), 695-698.

72 Ramakrishnan P (2003) The role of P-glycoprotein in the blood-brain barrier. J Biol Med 19, 160-165.

73 Schinkel AH, Smith JJ, van Tellingen O, Beijnen JH, Wagenaar E and 7 others (1994) Disruption of the mouse mdr1a P-glycoprotein gene leads to a deficiency in the blood-brain barrier and to increased sensitivity to drugs. Cell 77(4), 491-502.

74 Schinkel AH, Wagenaar E, Mol CA, van Deemter L (1996) P-glycoprotein in the blood-brain barrier of mice influences the brain penetration and pharmacological activity of many drugs. J Clin Invest 97(11), 2517-2524.

75 Silverman JA (1999) Multidrug-resistance transporters. Pharm Biotechnol 12, 353-86.

76 Fromm MF (2000) P-glycoprotein: a defense mechanism limiting oral bioavailability and CNS accumulation of drugs. Int J Clin Pharmacol Ther 38, 69-74.

77 Pardrige WM, Golden PL, Kang YS et al (1997) Brain microvascular and astrocyte localization of P-glycoprotein. J Neurochem 68, 1278-85.

78 Zhang L, Ong WY, Lee T (1999) Induction of P-glycoprotein expression in astrocytes following intracerebroventricular kainite injections. Exp Brain Res 126, 509-516.

79 Efferth T, Osieka R (1993) Clinical relevance of the MDR-1 gene and its gene-product, P-glycoprotein, for cancer chemotherapy: A meta-analysis. Tumor Diagn Ther 14, 238-243.

80 Trock BJ, Leonessa F, Clarke R (1997) Multidrug resistance in breast cancer: a meta-analysis of MDR1/gp170 expression and its possible functional significance. J Natl Cancer Inst 89(139, 917-931.

81 Efferth T, Mattern J and Volm M (1992) Immunohistochemical detection of P glycoprotein, glutathione S transferase and DNA topoisomerase II in human tumors. Oncology 49(5), 368- 375.

82 Volm M, Kästel M, Mattern J, Efferth T (1993) Expression of resistance factors (P-glycoprotein, glutathione S-transferase-pi, and topoisomerase II) and their interrelationship to proto-oncogene products in renal cell carcinomas. Cancer 71(12), 3981-3987.

83 Kim RB, Fromm MF, Wandel C, Leake B, Wood AJ, Roden DM, Wilkinson GR (1998) The drug transporter P-glycoprotein limits oral absorption and brain entry of HIV-1 protease inhibitors. J Clin Invest 101(2), 289-294.

84 Lee CG, Gottesman MM, Cardarelli CO, Ramachandra M, Jeang KT, Ambudkar SV, Pastan I, Dey S (1998) HIV-1 protease inhibitors are substrates for the MDR1 multidrug transporter. Biochemistry, 37(11), 3594-3601.

85 Castleberry RP (1999) Predicting outcome in neuroblastoma. N Engl J Med 340, 1992-1993.

86 Bates SE, Mickley LA, Chen YN, Richert N, Rudick J, Biedler JL, Fojo AT (1989) Expression of a drug resistance gene in human neuroblastoma cell lines: modulation by retinoic acid-induced differentiation. Mol Cell Biol 9(10), 4337-4344.

87 Bordow SB, Haber M, Madafiglio J, Cheung B, Marshall GM, Norris MD (1994) Expression of the multidrug resistance-associated protein (*MRP*) gene correlates with amplification and overexpression of the N-*myc* oncogene in childhood neuroblastoma. Cancer Res 54, 5036-5040.

88 Draper MP, Martell RL, Levy SB (1997) Indomethacin-mediated reversal of multidrug resistance and drug efflux in human and murine cell lines overexpressing MRP, but not P-glycoprotein. Br J Cancer 75(6), 810-815.

89 Dekkers DWC, Comfurius P, van Gool RGJ, Bevers EM, Zwaal RFA (2000) Multidrug resistance protein 1 regulates lipid asymmetry in erythrocyte membranes. Biochem J 350, 531-535.

90 Raggers RJ, Vogels I, van Meer G (2001) Multidrug-resistance P-glycoprotein (MDR1) secretes platelet-activating factor. Biochem J 357, 859-865.

91 Kamm W, Hauptmann J, Behrens I, Stürzebecher J, Dullweber F, Gohlke H, Stubbs M, Klebe G, Kissel T (2001) Transport of peptidomimetic thrombin inhibitors with a 3-amidino-phenylalanine structure: permeability and efflux mechanism in monolayers of a human intestinal line (Caco-2). Pharmac Res 18(8), 1110-1118.

92 Gosland MP, Lum BL, Sikic BI (1989) Reversal of cefoperazone of resistance to etopside, doxorubicin, and vinblastine in multidrug resistant human sarcoma cells. Cancer Res 49, 6901-6905.

93 Cavalier A, Leveque D, Peter JD, Salmon J, Elkhaili H, Salmon Y, Nobelis P, Geisert J, Monteil H, Jehl F (1997) Pharmacokinetic interaction between itraconazole and ceftriaxone in yucatan miniature pigs. Antimicrob Agents Chemother 41(9), 2029-2032.

94 Rodriguez I, Abernethy DR, Woosly RL (1999) P-Glycoprotein in clinical cardiology. Circulation 99, 472-474.

95 Hendrikse NH, Bart J, de Vries EG, Groen HJ, van der Graaf WT, Vaalburg W (2001) P-glycoprotein at the bllod-brain barrier and analysis of drug transport with positron-emission tomography. J Clin Pharmacol 41S-54S.

96 Basic S, Hajnsek S, Poljakovic Z, Basic M (2006) Lack association between the C3435T Polymorphism in the human multidrug rsistance (MDR1) gene and response to antiepileptic drug treatment. Epilepsia 47(2), 449-452.

97 Hanko E, Tommarello S, Watchko JF, Hansen TW (2003) Administration of drugs known to inhibit P-glycoprotein increases brain bilirubin and alters the regional distribution of bilirubin in rat brain. Pediatr Res 54(4), 439-40

98 Watchko JF, Daood MJ, Mahmood B, Vats K, Hart C, Adhab-Barmada M (2001) P-Glycoprotein and bilirubin disposition, J Perinatol 21, S43-S47.

99 Mealey KL, Borhoumi R, Burghardt RC, Safe S, Kochevar DT (2002) Doxycycline induces expression of P Glycoprotein in MCF-7 breast carcinoma cells. Antimicrob Agents Chemother 46(3) 755-761.

100 de Jong S, Holtrop M, de Vries H, de Vries EG, Mulder NH (1992) Increased sensitivity of an adriamycin-resistant human small cell lung carcinoma cell line to mitochondrial inhibitors. Biochem Biopys res Commun 182, 877-885.

101 Chen TS, Chen PS (1984) Understanding the Liver. A History. CT: Greenwood Press, p. 99.

102 London IM, West R, Shemin D et al. (1950) On the origin of bile pigment in normal man. J Biol Chem 184, 351–358.

103 Tenhunen R, Marver HS, Schmid R (1969) Microsomal heme oxygenase: characterization of the enzyme. J Biol Chem 244, 6388–6394.

104 Ishizawa S, Yoshida T, Kikuchi G (1983) Induction of heme oxygenase in rat liver. J Biol Chem 258, 4220–4225.

105 Elbirt KK, Bonkovsky HL (1999) Heme oxygenase: recent advances in understanding its regulation and role. Proc Assoc Am Phys 111, 438–447.

106 Hayashi S, Takamiya R, Yamaguchi T et al. (1999) Induction of heme oxygenase-1 suppresses venular leukocyte adhesion elicited by oxidative stress: role of bilirubin generated by the enzyme. Circ Res 85, 663–671.

107 Tenhunen R, Ross ME, Marver HS et al. (1970) Reduced nicotinamide-adenine dinucleotide phosphate dependent biliverdin reductase: partial purification and characterization. Biochemistry 9, 298–303.

108 Grandchamp B, Bissel DM, Licko V et al. (1981) Formation and disposition of newly synthesized heme in adult rat hepatocytes in primary cultures. J Biol Chem 256, 11677–11683.

109 Fischer H, Plieninger H (1942) Synthese des biliverdins (uteroverdins) und bilirubins der biliverdine XIII, und III, sowie der Vinulneoxanthosaure. Hoppe Seyler Z Physiol Chem 274, 231.

110 Bonnett R, Davis E, Hursthouse MB (1976) Structure of bilirubin. Nature 262(5566), 327–328.

111 Lauff JJ, Kasper ME, Ambros RT (1983) Quantitative liquid chromatographic estimation of bilirubin species in pathological serum. Clin Chem 29, 800–805.

112 Schiff D, Chan G, Poznasky MJ (1985) Bilirubin toxicity in neural cell lines N115 and NBR10A. Pediatr Res 19(9), 908–911.

113 Mustafa MG, Cowger ML, King TE (1969) Effects of bilirubin on mitochondrial reactions. J Biol Chem 244(23), 6403–6414.

114 Sano K, Nakamura H, Tamotsu M (1985) Mode of inhibitory action of bilirubin on protein kinase C. Pediatr Res 19(6), 587– 590.

115 Vaughan VC, Allen FC, Diamond LK (1950) Erythroblastosis fetalis. IV. Further observations on kernicterus. Pediatrics 6, 706.

116 Gourley GR (1997) Bilirubin metabolism and kernicterus. Adv Pediatr 44, 173–229.

117 Lee KS, Gartner LM (1983) Management of unconjugated hyperbilirubinemia in the newborn. Semin Liver Dis 3(1), 52–64.

118 Rigato I, Ostrow JD, Tiribelli C (2005) Bilirubin and the risk of common non-hepatic diseases. Trends Mol Med 11(6), 277-283.

119 Breimer LH, Wannamethee G, Ebrahim S et al. (1995) Serum bilirubin and risk of ischemic heart disease in middle-aged British men. Clin Chem 41, 1504-1508.

120 Bosma PJ, van dM, I, Bakker CT et al. (2003) UGT1A1*28 allele 17 and coronary heart disease: the Rotterdam Study. Clin Chem 49, 1180–1181.

121 Zucker SD, Horn PS, Serman KE (2004) Serum bilirubin levels in the U.S. population: gender effect and inverse correlation with colorectal cancer. Hepatology 40, 827–835.

122 Temme EHM, Zhang J, Schouten EG et al. (2001) Serum bilirubin and 10-year mortality risk in a Belgian population. Cancer Causes Control 12, 887–894.

123 Bowen WR, Porter E, Waters WF (1959) The protective action of albumin in bilirubin toxicity in new born puppies. Am J Dis Child 98, 568–573.

124 Brodersen R (1986) Aqueous solubility, albumin binding and tissue distribution of bilirubin. In: Ostrow JD (ed.) Bile Pigments and Jaundice. New York: Marcel Dekker, pp. 157–181.

125 Cui Y, Konig J, Leier I et al. (2000) Hepatic uptake of bilirubin and its conjugates by the human organic anion-transporting polypeptide 2 (symbol SLC21A6). J Biol Chem 276, 9626–9630.

126 Wang P, Kim RB, Roy-Chowdhury J et al. (2003) organic anion transport protein SLC21A6 (OATP2) is not sufficient for bilirubin transport. J Biol Chem 278(23), 20695–20696.

127 Kamisaka K, Gatmaitan Z, Moore CL et al. (1975) Ligandin reverses bilirubin inhibition of liver mitochondrial respiration in vitro. Pediatr Res 9(12), 903–905.

128 Roy Chowdhury J, Novikoff PM, Roy Chowdhury N et al. (1985) Distribution of uridinediphosphoglucuronate glucuronosyl transferase in rat tissues. Proc Natl Acad Sci USA 82, 2990–2994.

129 Bosma PJ, Seppen J, Goldhoorn B et al. (1994) Bilirubin UDPglucuronosyltransferase 1 is the only relevant bilirubin glucuronidating isoform in man. J Biol Chem 269(27), 17960–17964.

130 Wishart GJ (1978) Functional heterogeneity of UDP-glucuronosyl transferase as indicated by its differential development and inducibility by glucocorticoids. Biochem J 174(2), 485–489.

131 Roy Chowdhury J, Roy Chowdhury N, Moscioni AD et al. (1983) Differential regulation by triiodothyronine of substrate-specific uridinediphosphoglucuronate glucuronyl transferases in rat liver. Biochim Biophys Acta 761(1), 58–65.

132 Lilienblum W, Walli AK, Bock KW (1982) Differential induction of rat liver microsomal UDP-glucuronosyltransferase activities by various inducing agents. Biochem Pharmacol 31(6), 907–913.

133 Ishikaowa T, Muller M, Klunemann C et al. (1990) ATP-dependent primary active transport of cysteinyl leukotrienes transport system for glutathione S-conjugates. J Biol Chem 265(31), 19279–19286.

134 Nishida T, Gatmaitan Z, Roy-Chowdhury J et al. (1992) Two distinct mechanisms for bilirubin glucuronide transport by rat bile canalicular membrane vesicles. J Clin Invest 90(5), 2130–2135.

135 Gatmaitan ZC, Nies AT, Arias IM (1997) Regulation and translocation of ATP-dependent apical membrane proteins in rat liver. Am J Physiol 272, G1041–G1049.

136 Watson CJ (1977) The urobilinoids: milestones in their history and some recent developments. In: Berk PD, Berlin NI (eds) Bile Pigments: Chemistry and Physiology. Washington, DC: US Government Printing Office, pp. 469–482.

137 Ahlfors CE (2001) Bilirubin-albumin binding and free bilirubin. J Perinatol 21(1), S40-S42.

138 Ostrow JD et al (2003) New concepts in bilirubin encephalopathy. Eur J Clin Invest 33, 988-997.

139 Ostrow JD et al (1994) Structure and binding of unconjugated bilirubin: relevance for physiological and pathophysiological function. J Lipid Res 35, 1715-1737.

140 Zucker SD et al (1999) Unconjugated bilirubin exhibits spontaneous diffusion through model lipid bilayers and notive hepatocyte membranes. J Biol Chem 274, 10582-10862.

141 Dore S, Snyder SH (1999) Neuroprotective action of bilirubin against oxidative stress un primary hippocampal cultures. Ann N Y Acad Sci 890, 167-172.

142 Ostrow JD et al (2003) Reassessment of the unbound concentrations of inconjugated bilirubin in relation to neurotoxicity in vitro. Pediatr Res 54, 98-104.

143 Amit Y, Poznansky MJ, Schiff D (1989) Bilirubin toxicity in a neuroblastoma cell line N-115. Delayed effects and recovery. Pediatr Res 25(4), 369-372.

144 Han Z, Hu P, Ni D (2002) Bilirubin induced apoptosis of human neuroblastoma cell line SH-SY5Y and affected the mitochondrial membrane potential. Zhonghua Er Bi Yan Hou Ke Za Zhi 37(4), 243-246.

145 Jedlitschky G, Leier I, Buchholz U, Hummel-Eisenbeiss J, Burchell B (1997) ATP-dependent transport of bilirubin glucuronides by the multidrug resistance protein MRP1 and its hepatocyte canalicluar isoform MRP2. Biochem J 327, 305-310.

146 Petrovic S, Pascolo L, Gallo R, Cupelli F, Ostrow JD, Goffeau A, Tiribelli C, Bruschi CV (2000) The products of YCF1 and YLL015w (BPT1) cooperate for the ATP-dependent vacuolar transport of unconjugated bilirubin in *Saccaromhyces cerevisiae.* Yeast 16, 561-571.

147 Pascolo L, Fernetti C, Garcia-Mediavilla MV, Ostrow JD, Tiribelli C (2001) Mechanism for the transport of unconjugated bilirubin in human trophoblastic BeWo cells. FEBS Lett 495, 94-99.

148 Gekeler V, Ise W, Sanders KH, Ulrich WR, Beck J (1995) The leukotriene LTD4 receptor antagonist MK571 specifically modulates MRP associated multidrug resistance. Biochem Biophys Res Commun 208, 345-352.

149 Gennuso F, Fernetti C, Tirolo C, Testa N, L'Episcopo F, Caniglia S, Morale MC, Ostrow JD, Pascolo L, Tiribelli C et al (2004) Bilirubin protects astrocytes frm its own toxicity by inducine up-regulation and translocation of multidrug resistance-associated protein 1 (Mrp1). Proc Natl Acad Sci USA 101, 2470-2475.

150 Cekic D, Bellarosa C, Garcia-Mediavilla MV, Rigato I, Pascolo L, Ostrow JD, Tiribelli C (2003) Upregulation in the expression of multidrug resistance protein *Mrp1* mRNA and protein by increased production in rat. Biochem Biophys Res Comm 311, 891-896.

151 Rigato I, Pascolo L, Fernetti C, Ostrow JD, Tiribelli C (2004) The human multidrug-resistance-associated protein MRP1 mediates ATP-dependent transport of unconjugated bilirubin. Biochem J 383, 335-341.

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

153 Gosland MP, Brophy NA, Duran GE, et al. (1991) Bilirubin: a physiological substrate for the multidrug transporter. Proc Am Assn Cancer Res 32, 426.

154 Jettè L, et al. (1995) Interaction of drugs with P-glycoprotein in brain capillaries. Biochem Pharmacol 50, 1701-1709.

155 Watchko JF, Daood MJ, Hansen TW (1998) Brain bilirubin content is increased in P-glycoprotein-deficient transgenic null mutant mice. Pediatr Res 44(5), 763-766.

156 Watchko JF, Ziegler J, Daood MJ, Balkundi D (2003) Inhibition of human MDR1 P-glycoprotein increases bilirubin-induced cell apoptosis *in vitro*. Pediatr Res 53, 400A.

157 Fortunato A, Fraser AG (2005) Uncover genetic interactions in *Caenorhabditis elegans* by RNA interference. Biosci Rep 25 (5-6), 299-307.

158 Napoli C, Lemieux C, Jorgensen R (1990) Introduction of a chalcone synthase gene into Petunia results in reversible co-suppression of homologous genes in trans. Plant Cell 2, 279-289.

159 van Blokland K, van der Geest N, Mol J, Kooter J (1994) Transgene-mediated suppression of chalcone synthase expression in Petunia hybrida results from an increase in RNA turnover. Plant J 6, 861-877.

160 Covey S, Al-Kaff N, Lángara A, Turner D (1997) Plants combat infection by gene silencing. Nature 385, 781-782.

161 Ratcliff F, Harrison B, Baulcombe D (1997) A similarity between viral defense and gene silencing in plants. Science 276, 1558-1560.

162 Fire A, Xu S, Montgomery MK, Kostas SA, Driver SE, Mello CC (1998) Potent and specific genetic interference by double-stranded RNA in Caenorhabditis elegans. Nature 391, 806-811.

163 Review for the 2006 Nobel Prize in Physiology or Medicine, Advanced Information: RNA interference by Bertil Daneholt.

164 Bernstein E, Caudy AA, Hammond SM, Hannon GJ (2001) Role for a bidentate ribonuclease in the initiation step of RNA interference. Nature 409(6818), 363-366.

165 Vermeulen A, Behlen L, Reynolds A, Wolfson A, Marshall WS, Karpilow J, Khvorova A (2005) The contributions of dsRNA structure to Dicer specificity and efficiency. RNA 11(5), 674-682.

166 Lodish H, Berk A, Matsudaira P, Kaiser CA, Krieger M, Scott MP, Zipurksy SL, Darnell J (2004) Molecular Cell Biology 5th ed. WH Freeman, New York, NY.

167 Matranga C, Tomari Y, Shin C, Bartel DP, Zamore PD (2005) Passenger-strand cleavage facilitates assembly of siRNA into Ago2-containing RNAi enzyme complexes. Cell 123(4), 607-620.

168 Leuschner PJ, Ameres SL, Kueng S, Martinez J (2006) Cleavage of the siRNA passenger strand during RISC assembly in human cells. EMBO Reports 7(3), 314-320.

169 Gregory RI, Chendrimada TP, Cooch N, Shiekhattar R (2005) Human RISC couples microRNA biogenesis and posttranscriptional gene silencing. Cell 123(4), 631-640.

170 Preall JB, He Z, Gorra JM, Sontheimer EJ (2006) Short interfering RNA strand selection is independent of dsRNA processing polarity during RNAi in Drosophila. Curr Biol 16(5), 530-535.

171 Sen GL, Wehrman TS, Blau HM (2005) mRNA translation is not a prerequisite for small interfering RNA-mediated mRNA cleavage. Differentiation 73(6), 287-293.

172 Gu S, Rossi JJ (2005) Uncoupling of RNAi from active translation in mammalian cells. RNA 11(1), 38-44.

173 Sen GL, Blau HM (2005) Argonaute 2/RISC resides in sites of mammalian mRNA decay known as cytoplasmic bodies. Nat Cell Biol 7(6), 633-636.

174 Schramke V, Allshire R (2003) Hairpin RNAs and retrotransposon LTRs effect RNAi and chromatin-based gene silencing. Science 301(5636), 1069-74.

175 Ketting RF, Haverkamp TH, van Luenen HG, Plasterk RH (1999) Mut-7 of *C. elegans,* required for transposon silencing and RNA interference, is a homolog of Werner syndrome helicase and RNase D. Cell 99, 133-141.

176 Tabara H, Sarkissian M, Kelly WG, Fleenor J, Grishok A, Timmons L, Fire A, Mello CC (1999) The rde-1 gene, RNA interference, and transposon silencing in *C. elegans*. Cell 99, 123-132.

177 Plasterk RHA (2002) RNA Silencing: The Genome's Immune System. Science 296, 1263-1265.

178 Lee RC, Ambros V (2001) An extensive class of small RNAs in *Caenorhabditis elegans*. Science 294, 862-864.

179 Lau NC, Lim LP, Weinstein EG, Bartel DP (2001) An abundant class of tiny RNAs with probable regulatory roles in *Caenorhabditis elegans*. Science 294, 858-862.

180 Lagos-Quintanta M, Rauhut R, Lendeckel W, Tushl T (2001) Identification of novel genes coding for small expressed RNAs. Science 294, 853-858.

181 Reinhart BJ, Weinstein EG, Rhodes MW, Bartel B, Bartel DP (2002) MicroRNAs in plants. Genes Dev 16, 1616-1626.

182 Murchison EP, Hannon GJ (2004) miRNAs on the move: miRNA biogenesis and the RNAi machinery. Curr Opin Cell Biol 16, 223-229.

183 Zamore PD, Haley B (2005) Ribo-gnome: The Big World of Small RNAs. Science 309, 1519-1524.

184 Lee RC, Feinbaum RL, Ambros V (1993) The *C. elegans* heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin-14. Cell 75(5), 843-854.

185 Palatnik JF, Allen E, Wu X, Schommer C, Schwab R, Carrington JC, Weigel D (2003) Control of leaf morphogenesis by microRNAs. Nature 425(6955), 257-263.

186 Morita T, Mochizuki Y, Aiba H (2006) Translational repression is sufficient for gene silencing by bacterial small noncoding RNAs in the absence of mRNA destruction. Proc Natl Acad Sci 103(13), 4858-63.

187 Makarova KS, Grishin NV, Shabalina SA, Wolf YI, Koonin EV (2006) A putative RNA-interference-based immune system in prokaryotes: computational analysis of the predicted enzymatic machinery, functional analogies with eukaryotic RNAi, and hypothetical mechanisms of action. Biol Direct 16,1-7.

188 Mette MF, Aufsatz W, van der Winden J, Matzke M, Matzke A (2000) Transcriptional gene silencing and promoter methylation triggered by double stranded RNA. EMBO J 19, 5194-5201.

189 Sijen T, Vijn I, Rebocho A, van Blokland R, Roelofs D et al. (2001) Transcriptional and posttranscriptional gene silencing are mechanistically related. Curr Biol 11, 436-440.

190 Hall IM, Shankaranarayana GD, Noma K, Ayoub N, Cohen A, Grewal SI (2002) Establishment and maintenance of a heterochromatic domain. Science 297(5590), 2232-2237.

191 Volpe TA, Kidner C, Hall IM, Teng G, Grewal SI, Martienssen RA (2002) Regulation of heterochromatic silencing and histone H3 lysine-9 methylation by RNAi. Science 297, 1833-1837.

192 Sharp PA (2006) The Biology of Short RNAs. In "RNA World", Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

193 Bass BL (2002) RNA editing by adenosine deaminases that act on RNA. Annu Rev Biochem 71, 817–846.

194 Bass BL (2000) Double-stranded RNA as a template for gene silencing. Cell 101, 235–238.

195 Luciano DJ, Mirsky H, Vendetti NJ, Maas S (2004) RNA editing of a miRNA precursor. RNA 10, 1174–1177.

196 Yang W et al. (2006) Modulation of microRNA processing and expression through RNA editing by ADAR deaminases. Nature Struct Mol Biol 13, 13–21.

197 Yang W et al. (2005) ADAR1 RNA deaminase limits short interfering RNA efficacy in mammalian cells. J Biol Chem 280, 3946–3953.

198 Kazuko Nishikura (2006) Nature Reviews Molecular Cell Biology 7, 919-931.

199 Manche L, Green SR, Schmedt C, Mathews MB (1992) Interactions between double-stranded RNA regulators and the protein kinase DAI. Mol Cell Biol 12, 5238-5248.

200 Minks MA, West DK, Benvin S, Baglioni C (1979) Structural requirements of double-stranded RNA for the activation of 2'-5'-oligo(A) polymerase and protein kinase of interferon-treated HeLa cells. J Biol Chem 254, 10180-10183.

201 Yang S, Tutton S, Pierce E, Yoon K (2001) Specific double-stranded RNA interference in undifferentiated mouse embryonic stem cells. Mol Cell Biol 21(22), 7807-7816.

202 Paddison PJ, Caudy A, Hannon GJ (2002) Stable suppression of gene expression by RNAi in mammalian cells. Proc Natl Acad Sci USA 99(3), 1443-1448.

203 Elbashir SM, Lendeckel W, Tuschl T (2001) RNA interference is mediated by 21- and 22-nucleotide RNAs Genes Dev 15(2), 188-200.

204 Elbashir SM, Harborth J, Lendeckel W, Yalcin A, Weber K, Tuschl T (2001) Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. Nature 411, 494-498.

205 Caplen NJ, Parrish S, Imani F, Fire A, Morgan RA (2001) Specific inhibition of gene expression by small double-stranded RNAs in invertebrates and vertebrate systems. Proc Natl Acad Sci USA 98, 9746-9747.

206 Holen T, Amarzguioui M, Wiiger M, Babaie E, Prydz H (2002) Positional effects of short interferning RNAs targeting the human coagulation trigger Tissue Factor. Nucleic Acids Research 30(8), 1757-1766.

207 Elbashir SM, Martinez J, Patkaniowska A, Lendeckel W, Tuschl T (2001) Functional anatomy of siRNA for mediating efficient RNAi in *Drosophila melanogaster* embryo lysate. EMBO J 20, 6877-6888.

208 Jarvis RA, Ford LP (2001) The siRNA Target Site Is an Important Parameter for Inducing RNAi in Human Cells. TechNotes 8(5), 3-5.

209 Brown D, Jarvis R, Pallotta V, Byrom M, Ford L (2002) RNA Interference in Mammalian Cell Culture: Design, Execution and Analysis of the siRNA Effect. TechNotes 9(1), 3-5.

210 Brummelkamp TR, Bernards R, Agami R (2002) A system for stable expression of short interfering RNAs in mammalian cells. Science 296, 550-553.

211 Lee NS, Dohjima T, Bauer G, Li H, Li M-J, Ehsani A, Salvaterra P, Rossi J (2002) Expression of small interfering RNAs targeted against HIV-1 rev transcripts in human cells. Nature Biotechnol 20, 500-505.

212 Miyagishi M, Taira K (2002) U6-promoter-driven siRNAs with four uridine 3' overhangs efficiently suppress targeted gene expression in mammalian cells. Nature Biotechnol 20, 497-500.

213 Paddison PJ, Caudy AA, Bernstein E, Hannon GJ, Conklin DS (2002) Short hairpin RNAs (shRNAs) induce sequence-specific silencing in mammalian cells. Genes and Dev 16, 948-958.

214 Paul CP, Good PD, Winer I, Engelke DR (2002) Effective expression of small interfering RNA in human cells. Nature Biotechnol 20, 505-508.

215 Sui G, Soohoo C, Affar EB, Gay F, Shi Y, Forrester WC, Shi Y (2002) A DNA vector-based RNAi technology to suppress gene expression in mammalian cells. Proc Natl Acad Sci USA 99(6), 5515-5520.

216 Yu J-Y, DeRuiter SL, Turner DL (2002) RNA interference by expression of short-interfering RNAs and hairpin RNAs in mammalian cells. Proc Natl Acad Sci USA 99(9), 6047-6052.

217 Hammond SM, Caudy AA, Hannon GJ (2001) Post-transcriptional Gene Silencing by Double-stranded RNA. Nature Rev Gen 2, 110-119.

218 Sah DW (2006) Therapeutic potential of RNA interference for neurological disorders. Life Sci 79(19),1773-80.

219 Zender L, Hutker S, Liedtke C, Tillmann HL, Zender S, Mundt B, Waltemathe M, Gosling T, Flemming P, Malek NP, Trautwein C, Manns MP, Kuhnel F, Kubicka S (2003) Caspase 8 small interfering RNA prevents acute liver failure in mice. Proc Natl Acad Sci USA 100(13), 7797-7802.

220 Jiang M, Milner J (2002) Selective silencing of viral gene expression in HPV-positive human cervical carcinoma cells treated with siRNA, a primer of RNA interference. Oncogene 21(39), 6041-6048.

221 Kusov Y, Kanda T, Palmenberg A, Sgro JY, Gauss-Muller V (2006) Silencing of hepatitis A virus infection by small interfering RNAs. J Virol 80(11), 5599-610.

222 Jia F, Zhang YZ, Liu CM (2006) A retrovirus-based system to stably silence hepatitis B virus genes by RNA interference. Biotechnol Lett 28(20), 1679-85.

223 Li YC, Kong LH, Cheng BZ, Li KS (2005) Construction of influenza virus siRNA expression vectors and their inhibitory effects on multiplication of influenza virus. Avian Dis 49(4), 562-73.

224 Hu L, Wang Z, Hu C, Liu X, Yao L, Li W, Qi Y (2005) Inhibition of Measles virus multiplication in cell culture by RNA interference. Acta Virol 49(4), 227-34.

225 Raoul C, Barker SD, Aebischer P (2006) Viral-based modelling and correction of neurodegenerative diseases by RNA interference. Gene Ther 13(6), 487-95.

226 Tong AW, Zhang YA, Nemunaitis J (2005) Small interfering RNA for experimental cancer therapy. Curr Opin Mol Ther 7(2), 114-24.

227 Qiu S, Adema CM, Lane T (2005) A computational study of off-target effects of RNA interference. Nucleic Acids Res 33(6), 1834-47.

228 Grimm D, Streetz KL, Jopling CL, Storm TA, Pandey K, Davis CR, Marion P, Salazar F, Kay MA (2006) Fatality in mice due to oversaturation of cellular microRNA/short hairpin RNA pathways. Nature 441(7092), 537-41.

CHAPTER 2

MATERIALS AND METHODS

2.1 siRNA SYNTHESIS BY *IN VITRO* TRANSCRIPTION

In order to produce 21-nt siRNAs (small interfering RNAs, that are 21-nt double strand RNAs) to transfect them transiently into the cells, the *Silencer* siRNA Construction kit (Ambion, 1620) was used.

2.1.1 *Silencer* siRNA CONSTRUCTION KIT PROCEDURE

The Silencer siRNA Construction Kit (US patent pending) overcomes the sequence requirements of traditional in vitro transcription strategies by using siRNA template oligonucleotides containing a **"leader" sequence** that is complementary to the **T7 Promoter Primer** included in the kit. Inclusion of **leader sequences** provides two benefits:

- the 8 nt leader sequence is optimized for maximal RNA yield;
- after transcription and hybridization of the sense and antisense strands of the siRNA, the leader sequences are efficiently removed from the dsRNA preparation, eliminating the need to select target mRNA sequences that are compatible with T7 transcription.

The steps described here below, are shown in Fig. 2.1.

a. Two 29-mer DNA oligonucleotides (template oligonucleotides) with 21 nt encoding the siRNA and 8 nt complementary to the T7 Promoter Primer are synthesized and desalted.

b. In separate reactions, the 2 template oligonucleotides are hybridized to a T7 Promoter Primer (an oligonucleotide provided with the kit that contains a T7 promoter sequence and 8 nt complementary to the template oligonucleotides).

c. The 3' ends of the hybridized DNA oligonucleotides are extended by the Klenow fragment of DNA polymerase to create double-stranded siRNA transcription templates.

d. The sense and antisense siRNA templates are transcribed by T7 RNA polymerase and the resulting RNA transcripts are hybridized to create dsRNA. The dsRNA consists of 5' terminal single-stranded leader sequences, a 19 nt target specific dsRNA, and 3' terminal UUs.

e. The leader sequences are removed by digesting the dsRNA with a single strand specific ribonuclease. Overhanging UU dinucleotides will remain on the siRNA because the RNase does not cleave U residues. The DNA template is removed at the same time by a deoxyribonuclease.

f. The resulting siRNA is purified by glass fiber filter binding and elution which removes excess nucleotides, short oligomers, proteins, and salts in the reaction.

The end product is a double-stranded 21-mer siRNA with 3' terminal uridine dimers that can effectively reduce the expression of target mRNA when transfected into mammalian cells.

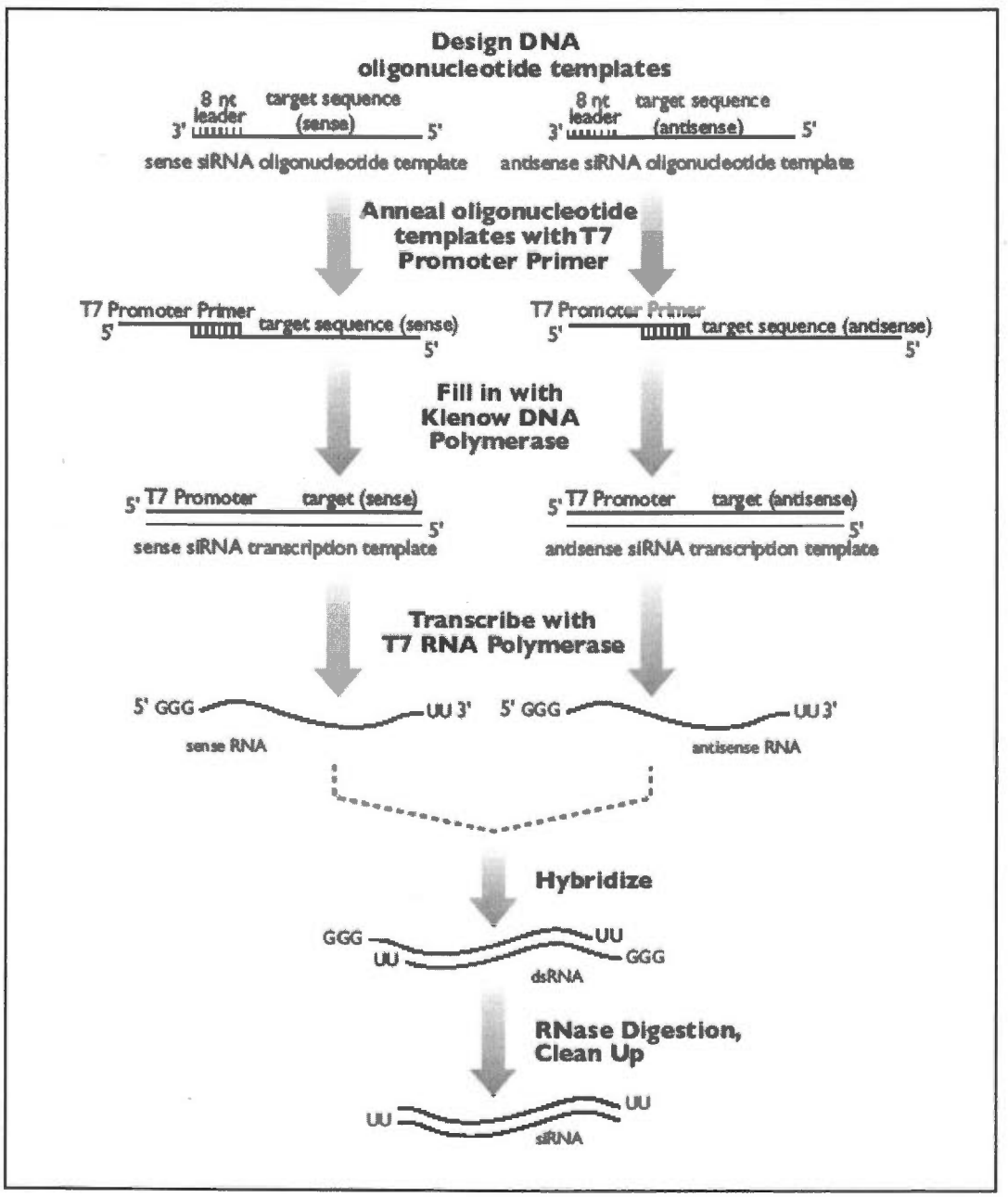


Fig. 2.1 Silencer siRNA Construction Kit procedure.

2.1.2 *Silencer* siRNA CONSTRUCTION KIT: INSTRUCTIONS

A. siRNA Design

For siRNA design, instructions based on both the current literature, and on empirical observations by scientists at Ambion (www.ambion.com/techlib/misc/siRNA_design.html), were followed.

1. Finding of 21 nt sequences in the target mRNA that begin with an AA dinucleotide.

Beginning with the AUG start codon, the transcript was scanned for AA dinucleotide sequences. Each AA and the 3' adjacent 19 nucleotides were recorded as potential siRNA target sites.

This strategy for choosing siRNA target sites is based on the observation by Elbashir et al. [1] that siRNA with 3' overhanging UU dinucleotides are the most effective. Since then, however, siRNA with other 3' terminal dinucleotide overhangs have been transfected into cells and shown to induce RNAi, but it is essential to avoid G residues in the overhang because the siRNA is cleaved by RNase at single-stranded G residues.

2. Selecting of 2–4 target sequences

Among the sequences identified in step 1, target sites were chosen, based on the following guidelines:

- since some regions of mRNA may be either highly structured or bound by regulatory proteins, generally siRNA target sites at different positions along the length of the gene sequence were selected.

Generally there aren't any correlation between the position of target sites on the mRNA and siRNA potency. However, the potential target sites were compared to the appropriate genome database (NM_004996 and NM_000927) and any target sequences with more than 16–17 contiguous base pairs of homology to other coding sequences have been eliminated from consideration. For finding out these sequences, BLAST (Basic Local Alignment Search Tool) (www.ncbi.nlm.nih.gov/BLAST,www.ambion.com/techlib/misc/siRNAdesign.html) was used.

siRNAs with 30–50% GC content were preferred to those with a higher G/C content, because considered more active.

3. Design of template oligonucleotides (DNA)

According to the guidelines, the **antisense** template oligonucleotide should have 21 nt at the 5' end that is the DNA counterpart of the target mRNA

sequence chosen, i.e. the same sequence as the target RNA except that U residues are replaced with T's.

The **sense** template oligonucleotide should start with an AA dinucleotide at the 5' end followed by 19 nt that are complementary to the target sequence identified in step 2.

The 8 nt at the 3' end of both oligonucleotides should be the following sequence: **5'-CCTGTCTC-3'**.

This 8 nt sequence is complementary to the T7 Promoter Primer provided with the Silencer siRNA Construction Kit.

Hybridization of the template oligonucleotides to the T7 Promoter Primer adds the T7 promoter sequence to the 5' ends of the template oligonucleotide so that after the fill-in reaction, they can be efficiently transcribed.

In order to attempt the silencing of ***MRP1***, 5 target sequences were identified by scanning the gene sequence (NM_004996), following the instructions listed above.

• The first siRNA molecule targeting *MRP1* corresponded to the coding region 299–319 (5'-AACACGGTCCTCGTGTGGGTG-3') relative to the start codon.

The **sense template oligonucleotide (DNA)** for the first siRNA was the following : 5′-AACACCCACACGAGGACCGTG[illegible]-3′

The **antisense template oligonucleotide (DNA)** for the first siRNA was the following : 5′-AACACGGTCCTCGTGTGGGTG[illegible]-3′

• The second siRNA molecule targeting *MRP1* corresponded to the coding region 1469–1489 (5'-AACCTCATGTCTGTGGACGCT-3') relative to the start codon.

The **sense template oligonucleotide (DNA)** for the second siRNA was the following : 5′-AAAGCGTCCACAGACATGAGG[illegible]-3′

The **antisense template oligonucleotide (DNA)** for the second siRNA was the following: 5′-AACCTCATGTCTGTGGACGT[illegible]-3′

• The third siRNA molecule targeting *MRP1* corresponded to the coding region 1682–1702 (5'-AAGAGCAAAGACAATCGGATC-3') relative to the start codon.

The **sense template oligonucleotide (DNA)** for the third siRNA was the following : 5′-AAGATCCGATTGTCTTTGCTC[illegible]-3′

The **antisense template oligonucleotide (DNA)** for the third siRNA was the following : 5′-AAGAGCAAAGACAATCGGATC[illegible]-3′

• The fourth siRNA molecule targeting *MRP1* corresponded to the coding region 2867–2887 (5'-AAGGAAGCAAAGCAAATGGAG-3') relative to the start codon.
The **sense template oligonucleotide (DNA)** for the fourth siRNA was the following : 5′-AACTCCATTTGCTTTGCTTCC[redacted]-3′
The **antisense template oligonucleotide (DNA)** for the fourth siRNA was the following : 5′-AAGGAAGCAAAGCAAATGGAG[redacted]-3′

• The fifth siRNA molecule targeting *MRP1* corresponded to the coding region 4535–4555 (5'-AAGACGAAGATCCTTGTGTTG-3') relative to the start codon.
The **sense template oligonucleotide (DNA)** for the fifth siRNA was the following : 5′-AACAACACAAGGATCTTCGTC[redacted]-3′
The **antisense template oligonucleotide (DNA)** for the fifth siRNA was the following : 5′-AAGACGAAGATCCTTGTGTTG[redacted]-3′

The sequence chosen to attempt the silencing of ***MDR1*** was that published by Wu et al. [2].
• The siRNA molecule targeting *MDR1* corresponded to the coding region 79-99 (5'-AAGGAAAGAAACCAACTGTC-3') relative to the start codon of the gene sequence (NM_000927).
The **sense template oligonucleotide (DNA)** for the siRNA was the following : 5′-AAGACAGTTGGTTTCTTTTCC[redacted]-3′
The **antisense template oligonucleotide (DNA)** for the siRNA was the following : 5′-AAGGAAAAGAAACCAACTGTC[redacted]

Both oligonucleotides (DNA) for each siRNA were synthesised by Sigma-Genosis. The smallest scale synthesis (40 nmol or less) was chosen, because it is sufficient for hundreds of transcription reactions. Desalting was selected as purification.

B. Transcription Template Preparation

The siRNA duplexes were synthesised by using *Silencer* siRNA Construction Kit (Ambion, Inc.)

The transcription of the antisense oligonucleotide generates RNA that is complementary to the target mRNA.

The transcription of the sense template generates a 3' terminal UU that is not complementary to the antisense strand of the siRNA. This UU sequence does not need to be part of the mRNA sequence because the sense strand of the siRNA appears to have no function in targeting mRNAs for degradation.

To make an efficient transcription template, the sense and antisense template oligonucleotides (DNA) for each siRNA must be converted to dsDNA with a T7 promoter at the 5' end. This is accomplished by hybridizing the 2 oligonucleotides to the T7 Promoter Primer provided with the Silencer siRNA Construction Kit and extending the T7 Promoter Primer and template oligonucleotides using a DNA polymerization reaction.

1. Resuspending of the template Oligonucleotides to 200 µM in nuclease-free water.

Oligonucleotides were supplied dry; so the tubes containing the oligonucleotides were tapped on the bench to force the powder to the bottom of the tubes. The sense and antisense template oligonucleotides were dissolved in nuclease-free water to approximately 200 µM.

2. Determination of the template oligonucleotide concentration by A260.

A small sample of the sense and antisense template oligonucleotides was diluted 1:250 into TE (10 mM Tris-HCl pH 8, 1 mM EDTA) and read the absorbance at 260 nm in the spectrophotometer (UV/Vis Scanning Spectrophotometer (DU Series 700, BECKMAN COULTER). The spectrophotometer was blanked with the same TE that was used for sample dilution.

The absorbance was multiplied by 5000 to determine the concentration of the oligonucleotides in µg/ml. (See the explanation below.)

5000 = 250-fold dilution X 20 µg oligo/ml per absorbance unit*

* 20 µg/ml is used to compensate for the non-full length oligonucleotide that is typically present in chemically synthesized oligonucleotide preps.

The molar concentration of the oligonucleotides in µM was determined by dividing the µg/ml concentration by 9.7. (See the explanation below.)

- There are 9.7 µg of DNA in 1 nmole of an average 29-mer:
  29 nt X 0.333 µg/nmol for each nt = 9.7 µg/nmol

- Dividing the µg/ml concentration by 9.7 yields the µM concentration as shown below:

$$\frac{\frac{X\ \mu g}{ml}}{\frac{9.7\ \mu g}{nmol}} = \frac{X\ \mu g}{ml} \quad \frac{nmol}{9.7\ \mu g} = \frac{X\ nmol}{ml\ (9.7)} = \frac{X\ \mu mol}{L\ (9.7)} = \frac{X\ \mu M}{9.7}$$

Therefore µM = X ÷ 9.7

3. Preparation of a 100 µM solution of each oligonucleotide

An aliquot of each template oligonucleotide was diluted to 100 µM using nuclease-free water or TE (10 mM Tris-HCl pH 8, 1 mM EDTA). ~20 µl of 100 µM oligonucleotide solutions were prepared.

4. Thawing the frozen template preparation reagents

The following kit components were thawed at room temperature, then briefly vortexed each before use.

- T7 Promoter Primer
- 10X Klenow Reaction Buffer
- 10X dNTP Mix
- Nuclease-free Water

The tube of Exo– Klenow was kept at –20°C and not vortexed.

5. Hybridizing of each template to the T7 Promoter Primer

In separate tubes were mixed the following:

2 µl T7 Promoter Primer

6 µl DNA Hyb Buffer

2 µl either sense or antisense template oligonucleotide

The mixture was heated to 70°C for 5 min, then left at room temp for 5 min.

6. Filling in with Klenow DNA polymerase

The following components were added to the hybridized oligonucleotides:

2 µl 10X Klenow Reaction Buffer

2 µl 10X dNTP Mix

4 µl Nuclease-free Water

2 µl Exo– Klenow

The components were gently mixed by pipetting or slow vortexing and then centrifuged briefly to collect the mixture at the bottom of the tube.
The mixtures were transfered into 37°C incubator and incubated for 30 min.
After this step, the siRNA templates can be used directly in a transcription reaction or stored at −20°C until they are needed for transcription.

C. dsRNA Synthesis

The sense and antisense siRNA templates were transcribed for 2 hours in separate reactions. The reactions were then mixed, and the combined reaction was incubated overnight.
Transcribing the templates separately eliminates potential competition between templates for transcription reagents that might limit the synthesis of 1 of the 2 strands of the siRNA duplex. Mixing the transcription reactions facilitates hybridization of the 2 siRNA strands and enables continued RNA synthesis to maximize the dsRNA yield.

1. Thawing the 2X NTP Mix and 10XT7 Reaction Buffer

The 2X NTP Mix and 10X T7 Reaction Buffer were thawed at room temperature. After that, each tube was vortexed. The 10XT7 Reaction Buffer was checked to see if a precipitate was visible, and if so, the tube was vortexed until the solution was completely resuspended. Briefly both tubes were span prior to using to ensure that no solution was lost when the tubes were opened.
The tube of T7 Enzyme Mix were kept at −20°C and do not vortexed.

2. Assembling **of the transcription reactions**

For each siRNA, 2 transcription reactions were assembled at room temperature to synthesize the sense and antisense RNA strands of the siRNA. For each transcription reaction, the following components were mixed in the order shown:
2 µl sense or antisense siRNA template (from step 6. section B)
4 µl Nuclease-free Water
10 µl 2X NTP Mix
2 µl 10X T7 Reaction Buffer
2 µl T7 Enzyme Mix

The mix contents were gently thoroughly by flicking or brief vortexing and then microfuged briefly to collect the reaction mixture at the bottom of the tube.

3. Incubation of reactions

The transcription reactions were incubated for 2 hr at 37°C, preferably in a cabinet incubator. (This prevents condensation, which may occur if the tube is incubated in a heat block.)

4. Combination of the sense and antisense transcription reactions and incubation

The sense and antisense transcription reactions were combinated into a single tube and continue incubation at 37°C overnight.

The overnight incubation maximizes the yield of RNA and facilitates hybridization of the sense and antisense strands of the siRNA.

D. siRNA Preparation/Purification

The dsRNA made by in vitro transcription has 5' overhanging leader sequences that must be removed prior to transfection. The leader sequence is digested by a single-strand specific ribonuclease. In the same digestion reaction, the DNA template is eliminated by DNase digestion.

The resulting siRNA is recovered from the mixture of nucleotides, enzymes, short oligomers, and salts in the reaction by column purification. The purified siRNA is eluted from the column into Nuclease-free Water, providing siRNA that is ready for transfection.

1. Digestion of the siRNA with RNase and DNase

The Digestion Buffer was thawed at room temperature and the tube was vortexed to mix the contents thoroughly.

To the tube of dsRNA (step C.4), the following reagents were added in the indicated order:

6 µl Digestion Buffer

48.5 µl Nuclease-free Water

3 µl RNase

2.5 µl DNase

The reagents were mixed gently, and incubated for 2 hr at 37°C.

Before using the siRNA Binding and Wash Buffers for the first time, 100% ethanol was added as shown here below:

5.3 ml of 100% ethanol to siRNA Binding Buffer

11 ml of 100% ethanol to siRNA Wash Buffer

400 µl of siRNA Binding Buffer were added to the nuclease digestion reaction and incubated 2–5 min at room temperature.

For each siRNA preparation, a Filter Cartridge was placed in a 2 ml Tube (provided with the kit).

100 µl of siRNA Wash Buffer was applied to the filter of the Filter Cartridge.

The siRNA was added in the siRNA Binding Buffer to a prewet Filter Cartridge and spinned at ~10,000 rpm in a microcentrifuge for 1 min.

The flow-through was discarded from the Collection Tube, and replaced the Filter Cartridge in the 2 ml Tube.

500 µl of siRNA Wash Buffer was applied to the filter of the Filter Cartridge and span at 10,000 rpm for 1 min. The flow-through was discarded from the Collection Tube, and the Filter Cartridge was replaced in the 2 ml Tube.

The wash was repeated with a second 500 µl of siRNA Wash Buffer.

The Filter Cartridge was transfered to a new 2 ml Tube.

Nuclease-free Water was heated to 75°C.
100 µl of the preheated Nuclease-free Water were added to the filter of the Filter Cartridge and incubate at room temperature for 2 min.

The Filter Cartridge was spinned at 12,000 rpm for 2 min. The purified siRNA was in the eluate (in the 2 ml Tube).
siRNAs were stored at or –80°C until they are prepared for transfection.

E. siRNA Quantification

The siRNA concentration used for transfection is critical to the success of gene silencing experiments. Transfecting too much siRNA causes nonspecific reductions in gene expression and toxicity to the transfected cells. Transfecting too little siRNA does not change the expression of
the target gene. Assuming that the UV spectrophotometer is accurate, measuring the absorbance of the siRNA sample at 260 nm is the simplest method to assess the concentration of the siRNA preparation.

1. Measurement of the A260 of the siRNA

A small sample of the siRNA was diluted 1:25 into TE (10 mM Tris-HCl dilution of the siRNA pH 8.1 mM EDTA) and read the absorbance at 260 nm in the spectrophotometer (DU Series 700, BECKMAN COULTER). The spectrophotometer was blanked with the same TE that was used for sample dilution.

2. Determination of siRNA concentration in µg/ml

The absorbance was multiplied by 1,000 to determine the concentration of the purified siRNA in µg/ml (explanation below).

1,000 = 25-fold dilution x 40 µg siRNA/ml per absorbance unit

3. Determination of the siRNA molar concentration

The molar concentration of the siRNA in µM was determined by dividing the µg/ml concentration of the siRNA by 14 (explanation below).

- There are 14 µg of RNA in 1 nmole of an average 21-mer dsRNA:

21 nt x 2 strands = 42 nt x 0.333 µg/nmol for each nt = 14 µg/nmol

- Dividing the µg/ml concentration by 14 yields the µM concentration as shown below:

$$\frac{\frac{X\ \mu g}{ml}}{\frac{14\ \mu g}{nmol}} = \frac{X\ \mu g}{ml} * \frac{nmol}{14\ \mu g} = \frac{X\ nmol}{ml\ (14)} = \frac{X\ \mu mol}{L\ (14)} = \frac{X\ \mu M}{14}$$

Therefore µM = X ÷ 14

All the steps described above were followed also to generate an siRNA specific to GAPDH siRNA from the Sense and Antisense Control DNA templates supplied with the siRNA Construction Kit. This GAPDH siRNA can be used both as a control to confirm that the kit is working properly and as a positive control for many siRNA experiments.

The outcome of this reaction was analyzed by measuring the A260 of the purified siRNA, and determining the yield in µg as described in step E 2. The size of the siRNA was checked by running 10 µl of the purified siRNA on a 2% agarose gel.

G. Gel Analysis of siRNA

siRNA was assessed by gel electrophoreisis on 2% agarose in TBE, following these instructions:

1. 10 µl of siRNA sample was mixed with 2 µl of a native gel loading buffer.
2. The sample was loaded on a 2% agarose gel and electrophoresed at about 5–10 mAmps/cm.
3. The electrophoresis was stopped when the bromophenol blue dye front had migrated two-thirds of the way down the gel.
4. The gel was stained for ~10 min in a 1 µg/ml solution of ethidium bromide.
5. The siRNA was visualized by Kodak EDAS 260 (Kodak Instruments, New Haven, CT, USA) using Kodak 1D image software.

2.2 CELL CULTURE

The cells were grown at 37°C in a 5% CO_2 incubator.

Media:

Growth medium of HeLa cells (human cervical cancer cell line) and HepG2 cells (human hepatocellular liver carcinoma cell line): DMEM (Dulbecco's Modified Eagle's Medium) with high glucose (Euroclone), supplemented with 1% L-Glutamine 100X (Euroclone), 10% Fetal Bovine Serum (FBS) (SIGMA), 1% Penicillin Streptomycin 100X (Euroclone).

Growth medium of SH-SY5Y cells (human neuroblastoma cell line): DMEM (Dulbecco's Modified Eagle's Medium) with high glucose (Euroclone), supplemented with 1% L-Glutamine 100X (Euroclone), 10% FBS Tetracycline-free (Tet System Approved FBS, US-Sourced)* (BD Biosciences Clontech), 1% MEM Non Essential Amino Acids (100X) (GIBCO), 1% MEM Vitamin Solution (100X) (GIBCO), 1% Penicillin Streptomycin 100X (Euroclone).

* **SH-SY5Y cells** have been used in pSUPERIOR RNA interference system. As it is a vector system for inducible expression of short interfering RNA, that uses tetracycline as inducing agent, FBS Tetracycline-free has been used. That's why many lots of FBS contain tetracycline, as FBS is generally isolated from cows that have been fed a diet containing tetracycline. If the growth medium contains FBS that is not reduced in tetracycline, it's possible to observe low basal expression of the gene of interest even if tetracycline has not been added into the medium.

Cell passage

Reagents used:

- Media– pre-warmed to 37°C
- PBS (Dulbecco's Phosphate Buffered Saline) without Ca_{2+}/Mg_{2+} (SIGMA-ALDRICH)
- Trypsin 0.05%/EDTA 0.02% in PBS, without Phenol Red, Ca_{2+}/Mg_{2+} (EuroClone)

Main equipment:

- 5% CO_2 Incubator (Thermo Forma)
- Waterbath (Stuart Scientific) set to 37°C

- Class II Microbiological safety cabinet (Steril-VBH)
- Centrifuge SIGMA 2-5 (Laborzentrifugen GmbH)
- Bürker camera (Precicolor HBG)
- Pre-labeled flasks
- Sterile pipettes
- Inverted microscope (Nikon)

Procedure

1. Before passaging the cells, the culture was viewed using an inverted microscope to assess the degree of confluency and confirm the absence of bacterial and fungal contaminants.
2. The medium was removed.
3. The cell monolayer was washed with pre-warmed PBS without Ca_{2+}/Mg_{2+} using a volume equivalent to half volume of culture medium.
4. Pre-warmed Trypsin/EDTA was added onto the washed cell monolayer using 1 ml per 25 cm^2 of surface area. The flask was rotated to cover the monolayer with trypsin.
5. The flask was returned to the incubator and left for about 2 minutes.
6. The cells were examined using an inverted microscope to ensure that all the cells were detached and floating. The side of the flask was gently tapped to release any remaining attached cells.
7. The cells were resuspended in a small volume of pre-warmed fresh serum-containing medium to inactivate the trypsin and transferred in a 15 ml tube.
8. Cells were centrifuged at 1000 rpm for 5 minutes.
9. The supernatant was removed and the pellet was gently tapped for dissociate the cells and then immediately resuspended in the appropriate amount of pre-warmed medium.
10. If necessary, 100-200 ul of cell culture were removed in order to perform a cell count using a Bürker camera.
11. The required number of cells was transferred to a new labeled flask containing pre-warmed medium according to the required seeding density.
12. The cells were incubated at 37°C in a 5% CO_2 incubator.
13. This process was repeated as demanded by the growth characteristics of the cell line.

2.3 siRNA TRANSFECTING INTO MAMMALIAN CELLS

The transient transfection of siRNA (small interference RNAs) targeting *MRP1* and *MDR1* was performed by Lipofectamine2000 (Invitrogen,11668-027). The siRNA sequence targeting *MDR1* was synthesized by *in vitro* transcription using *Silencer* siRNA Construction kit (see paragraph 2.1). The sequence chosen to attempt the silencing of *MDR1* had already been published, as reported in the mentioned paragraph. The 5 siRNA sequences targeting *MRP1*, designed by ourselves, were also synthesised by using *Silencer* siRNA Construction kit, while the *Silencer* Validated siRNA was designed and chemically synthesized by Ambion.

Procedure

1. One day before transfection, cells were plated in wells of 6-well plate containing 2 ml of growth medium without antibiotics so that they will be 60-70% confluent at the time of transfection.
2. For each transfection sample, siRNA:Lipofectamine2000 complexes were prepared as follows:
   a. 100 nM siRNA (for siRNA targeting *MRP1*) or 200 nM siRNA (for siRNA targeting *MDR1*), chosen on the basis of dose-response studies, were diluted in 250 µl of Opti-MEM I Reduced Serum Medium (Invitrogen, 31985-062) without serum and mixed gently.
   b. The appropriate amount of Lipofectamine 2000 (Invitrogen, 1168-027) was diluted in 250 µl of Opti-MEM I Medium (according to the manufacturer's protocol). The dilution was mixed gently and incubated for 5 minutes at room temperature.
   c. After the 5 minute incubation, the diluted siRNA was combined with the diluted Lipofectamine 2000 (total volume was 500 µl), mixed gently and incubated for 20 minutes at room temperature to allow the siRNA:Lipofectamine 2000 complexes to form.
3. 500 µl of siRNA:Lipofectamine 2000 complexes were added to each well and mixed gently by rocking the plate back and forth.
4. The cells were incubated at 37°C in a CO_2 incubator for 24-72 hours until they were ready to assay for gene knockdown.

In order to investigate the gene knockdown, the incubation of cells transfected by siRNA targeting *MRP1*, was 2 days long, while the incubation of cells transfected by siRNA targeting *MDR1*, was 1 day long. 1 and 2 days were suggested as the best assay time for observing the silencing of MRP1 and MDR1, in a transient silencing experiments respectively (see *Results*).

2.4 RNA ISOLATION

The RNA was isolated by TRI REAGENT (SIGMA, T9424). This product, a mixture of guanidine thiocyanate and phenol in a mono-phase solution, dissolves DNA, RNA and protein on homogenization or lysis of tissue or cell samples. After adding chloroform and centrifuging, the mixture separates into 3 phases: an aqueous phase containing the RNA, the interphase containing DNA and an organic phase containing proteins. Each component can then be isolated.

Sample Preparation

Monolayer cells, grown in 6-well plate, were lysed directly on the culture dish.

1. After washing cells by PBS without Ca^{2+}/Mg^{2+}, 0,5 ml of TRI REAGENT were added in each well. The cell lysate was passed several times through a pipette to form a homogenous lysate and transferred in a microtube.
2. To ensure complete dissociation of nucleoprotein complexes, the samples should stand for 5 minutes at room temperature. 0,1 ml of chloroform was added and the sample was covered tightly, shacken vigorously for 15 seconds and let stand for 2-15 minutes at room temperature. The resulting mixture was centrifuged at 12,000 x g for 15 minutes at 4 °C.

   Centrifugation separates the mixture into 3 phases: a red organic phase (containing protein), an interphase (containing DNA), and a colorless upper aqueous phase (containing RNA).

RNA Isolation

1. The aqueous phase was transferred to a fresh tube and 0.25 ml of isopropanol were added and mixed. The sample stood for 5-10 minutes at room temperature and then centrifuged at 12,000 g for 10 minutes at 4 °C. The RNA precipitate formed a pellet on the side and bottom of the tube.
2. The supernatant was removed and the RNA pellet was washed by adding 0,5 ml (minimum) of 75% ethanol. The sample was vortexed and then centrifuged at 12,000 x g for 5 minutes at 4 °C.
3. The RNA pellet was briefly dried for 5-10 minutes by air drying, making sure the RNA pellet didn't dry completely, because this decreases greatly its solubility. About 30 µl of sterile water were added to the RNA pellet and the sample was mixed by repeated pipetting.
4. Final preparation of RNA was stored at – 80°C.

The RNA absorbances at 230 nm, 260 nm and 280 nm, including the calculation of absorbance ratios (260/280) and concentrations, were assessed by UV/Vis Scanning Spectrophotometer (DU Series 700, BECKMAN COULTER).

2.5 RNA REVERSE TRANSCRIPTION AND REAL TIME QUANTITATIVE PCR

RNA integrity was checked on agarose–formaldehyde gel. The ratio OD_{260nm}/OD_{280nm} was always between 1.6 and 1.8.

Single-strand cDNA was obtained from 1 µg of total RNA from cell culture, using the iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, CA, USA) according to manufacturer's instructions. RT was performed in a thermal cycler (Gene Amp PCR System 2400, Perkin–Elmer, Boston, MA, USA) at 25°C for 5 min, 42°C for 30 min, 85°C for 5 min, and 4°C for 5 min. cDNA was stored at - 20°C.

Real Time quantitative PCR was performed with an iCycler IQ (Bio-Rad Laboratories, Hercules, CA, USA); *β-ACTIN* and/or *GAPDH* were used as an endogenous control to normalize the expression level of target genes.
Primers used were:

MRP1— GCCAAGAAGGAGGAGACC (sense)
MRP1— AGGAAGATGCTGAGGAAGG (anti-sense)
MDR1—TGCTCAGACAGGATGTGAGTTG (sense)
MDR1—AATTACAGCAAGCCTGGAACC (anti-sense)
β-ACTIN — CGCCGCCAGCTCACCATG (sense)
β-ACTIN — CACGATGGAGGGGAAGACGG (anti-sense)
GAPDH— CCCATGTTCGTCATGGGTGT (sense)
GAPDH— TGGTCATGAGTCCTTCCACGATA (anti-sense)

The PCR was performed in 96-well plates. In each well, a final volume of 25 µl was loaded. It contained: 1X iQ SYBR Green Supermix [100mM KCl; 40mM Tris–HCl, pH 8.4; 0.4mM each dNTP; 50 U/ml iTaq DNA polymerase; 6mM MgCl2; SYBR Green I; 20nM fluorescein; and stabilizers], 250 nM gene specific sense and anti-sense primers and 25 ng of cDNA. Primers for gene of interest and for housekeeping gene/s were added in separate wells.

Each sample was performed in triplicate. For each amplificate, a blank (nuclease free water was added in place of cDNA).

The thermal cycler conditions were: 3 min at 95°C; 40 cycles at 95°C for 20 minutes, 60°C for 20 minutes and 72°C for 30 minutes.

In order to verify the specificity of the amplification, a melt-curve analysis was performed, immediately after the amplification protocol, under the following conditions: 1 min denaturation at 95 °C, 1 min annealing at 55°C, and 80 cycles of

0.5°C increments (10 seconds each) beginning at 55°C. Non-specific products of PCR were not found in any case.

A standard curve was generated using a "calibrator" cDNA (chosen among the cDNA samples), which was serially diluted. In a relative quantification, a standard curve allows to calculate the correlation coefficient and determine the efficiency of the Real Time reaction for each gene.

The iCycle iQ Real Time PCR Detection System Software generated the equation describing the plots of the log10 of the starting quantity (micromoles) of 5 dilutions (200, 100, 50, 25, and 12.5 ng) of the calibrator cDNA versus the corresponding threshold cycle (Ct). Only the reactions that shown a correlation coefficient of the ≥ 0.99, were accepted.

The results were normalized to each housekeeping gene/s and the initial amount of the template of each sample was determined as relative expression versus one of the samples chosen as reference (in this case the control sample) which is considered the 1. The relative expression of each sample was calculated by the formula $2^{-\Delta\Delta C}{}_t$. ΔC_t is a value obtained, for each sample, by the difference between the mean C_t value of the interested gene and the mean C_t value of the housekeeping gene/s. $\Delta\Delta C_t$ of one sample is the difference between its ΔC_t value and C_t value of the sample chosen as reference (User Bulletin 2 of the ABI Prism 7700 Sequence Detection System).

2.6 CELL LYSIS AND PROTEIN EXTRACTION

The following protocol was used to prepare total protein extracts from cells. This protocol precedes the analysis of protein levels by Western blot.

In order to lyse cells under nondenaturing conditions, **Cell Lysis Buffer** (Cell Signaling, 9803) was used.

All reagents and lysates were kept on ice.

Procedure

1. Cells were plated in 12-well or 6-well plates.
2. Medium was removed from the cell plate.
3. Cells were washed with cold Dulbecco's phosphate buffered saline without calcium and magnesium, 1X (1X PBS).
4. The wash buffer was removed.
5. 30 µl of Lysis Buffer (1X) were added into each well of the 12-well plate or 60 µl into each well of the 6-well plate.
6. The plate was incubated on ice for 5 minutes.
7. After incubation, cells were scraped, lysed thoroughly and dissociated from plate by repetitive pipetting.
6. Cell lysate was plated into a sterile eppendorf tube and centrifuged at 14000 rpm for 10 minutes at 4°C in a Microfuge 18 Centrifuge (BECKMAN COULTER).
8. Supernatant was transferred to a new sterile eppendorf tube and kept on ice. The required amount of protein extracts was used for assessing the protein concentration, performed by bicinchininc acid protein assay kit (SIGMA,BCA-1). The remaining amount was stored at -80°C.

2.7 PROTEIN DETERMINATION

The protein determination was performed by bicinchininc acid protein assay kit (SIGMA, BCA-1).

The principle of the bicinchoninic acid (BCA) assay is based on the formation of a Cu^{2+} - protein complex under alkaline conditions, followed by reduction of the Cu^{2+} to $Cu1^{+}$. The amount of reduction is proportional to the protein present. It has been shown that cysteine, cystine, tryptophan, tyrosine, and the peptide bond are able to reduce Cu^{2+} to $Cu1^{+}$. BCA forms a purple-blue complex with $Cu1^{+}$ in alkaline environments, thus providing a basis to monitor the reduction of alkaline Cu^{2+} by proteins.

The BCA assay has a linear concentration range between 200-1000 µg/ml of protein.

Reagents required and provided by kit

Bicinchoninic Acid Solution (B9643): contains bicinchoninic acid, sodium carbonate, sodium tartrate, and sodium bicarbonate in 0.1 N NaOH (final pH 11.25).

Copper(II) Sulfate Pentahydrate 4% Solution (C2284): contains 4% (w/v) copper(II) sulfate pentahydrate.

Protein Standard (Bovine Serum Albumin - BSA) Solution (P0914): contains 1.0 mg/ml bovine serum albumin in 0.15 M NaCl with 0.05% sodium azide as a preservative.

Procedure

1. Sample preparation:
   - Standards were prepared dissolving different volumes of BSA protein standards (1 mg/ml) in deionized water:
     - 2.5 µl of BSA protein standard in 47.5 µl of deionized water.
     - 5 µl of BSA protein standard in 45 µl of deionized water.
     - 10 µl of BSA protein standard in 40 µl of deionized water.
     - 20 µl of BSA protein standard in 30 µl of deionized water.
   - Each unknown protein sample was prepared diluting 5 µl of cell lysate in 45 µl of deionized water.
   - Blank (consisting of 50 µl of deionized water without protein) was included.

Blank, BSA standard protein and unkown samples as well were triple assayed.

- The BCA Working Reagent was prepared by mixing 50 parts of Bicinchoninic Acid Solution with 1 part of Copper(II) Sulfate Pentahydrate 4% Solution. The BCA Working Reagent was mixed until it was light green in color.

2. 50 µl of standard, blank and unknown sample were loaded in a 96-well plate.
3. 100 µl of BCA Working Reagent were added into each well.
4. The 96-well plate was sealed with film and covered with a lid.
5. Samples were incubated at 37°C for 30 minutes.
6. After incubation, the absorbance of the samples was measured at 562 nm by a microtiter plate reader (BECKMAN COULTER).
7. The protein concentration was determined by comparison of the absorbance of the unknown samples to the standard curve prepared using the BSA protein standards.
   The absorbance of each sample was calculated subtracting the absorbance of the blank.
   The **total amount of protein in the unknown sample (µg)** was calculated dividing the average absorbance, measured in the 3 wells in which the same unknown sample was loaded, by the angular coefficient of the standard curve.
   The **concentration of the unknown protein sample (µg/µl)** was calculated dividing the total amount of protein of the sample by the volume of the sample loaded in the well.

2.8 WESTERN BLOTTING

Proteins, extracted from cells by lysis buffer and assessed by bicinchininic acid reaction, were separated by SDS–PAGE in 7% acrylamide and blotted onto a nitrocellulose membrane (0.2 Im Protran BA 83, Schleicher and Schuell, Dassel, Germany) with a semi-dry blotting system (Sigma, St. Louis, MO, USA). Molecular weight standards (212–53 kDa, Amersham–Pharmacia, Buckinghamshire, UK) were used as marker proteins. The blots were stained with Ponceau S-solution (0.1% wt/vol) in 5% acetic acid (vol/vol) (Sigma Chemical, St. Louis, MO), and after blocking for 60 min with 4% milk- TTBS (0.2% Tween 20; 20mM Tris; and 500mM NaCl; pH 7.5), the membrane was incubated overnight with a appropriate dilution of the specific antibody (primary antibody) against both the gene of interest and the housekeeping gene. After washing three times with 4% milk-TTBS, the membrane was incubated for 60 minutes with a antibody conjugated with peroxidase (secondary antibody). The peroxidase reaction was obtained by exposure of the membrane in the ECL-Plus Western Blotting detection system solutions (Amersham–Pharmacia Biotech, Buckinghamshire, UK). After transfer to Kodak film, the bands were visualized by Kodak EDAS 260 (Kodak Instruments, New Haven, CT, USA) using Kodak 1D image software. Protein expression was quantified by both Scion Image and Curver Expert 1.3 softwares.

GENES OF INTEREST:

Primary antibody against MRP1: MRP1-A23 rabbit antibody [3]. Dilution 1:600.

Secondary antibody against A23: peroxidase conjugate-goat anti-rabbit IgG-whole molecule affinity isolated antigen specific antibody (SIGMA, A6154). Dilution 1:6000.

Primary antibody against MDR1: anti-C219 (MDR1) Monoclonal Antibody (Signet, 8710). Dilution 1:50.

Secondary antibody against C219: anti-Mouse IgG (Fc specific)–Peroxidase antibody produced in goat affinity isolated antibody (SIGMA, A2554). Dilution 1:2000.

HOUSEKEEPING GENE:

Primary antibody against ACTIN: rabbit anti-actin affinity isolated antibody (SIGMA, A2066). Dilution 1:1500.

Secondary antibody against ACTIN: peroxidase conjugate-goat anti-rabbit IgG-whole molecule affinity isolated antigen specific antibody (SIGMA, A6154). Dilution 1:8000.

2.9 A VECTOR SYSTEM FOR INDUCIBLE EXPRESSION OF siRNA

A vector system had been used in order to obtain an inducible expression of the siRNAs targeting the interested gene. I start describing one of these vectors: the pSUPERIOR.puro vector (OligoEngine, VEC-IND-0006).

2.9.1 pSUPERIOR VECTOR: OVERVIEW

The pSUPERIOR vectors are inducible versions of the widely-used pSUPER suite of vectors for siRNA expression in mammalian cells. pSUPERIOR vectors are tetracycline-regulated expression vectors that utilize regulatory elements from the E. Coli Tn10-encoded tetracycline (Tet) resistance operon [4][5]. Tetracycline regulation in pSUPERIOR vectors is based on the binding of tetracycline to the Tet repressor and derepression of the promoter controlling expression of the gene of interest [6].

2.9.2 REQUIRED COMPONENTS FOR REGULATION OF TRANSCRIPTION

In addition to the pSUPERIOR vector, the two critical items required for controlled regulation of transcription are:

1) a TetR expressing vector (or a cell line that stably expresses the TetR protein);
2) tetracycline.

Doxycycline may be used as an alternative inducing agent with pSUPERIOR; Doxycycline is similar to Tetracycline in its mechanism of action, and exhibits similar dose response and induction characteristics as tetracycline when used with pSUPER. Doxycycline has been shown to have a longer half-life than tetracycline (48 hours vs. 24 hours, respectively).

2.9.3 THE pSUPER RNAi SYSTEM

The pSUPER RNAi system provides a mammalian expression vector that directs intracellular synthesis of siRNA-like transcripts. The vector uses the polymerase-III H1-RNA gene promoter, as it produces a small RNA transcript lacking a polyadenosine tail and has a well-defined start of transcription and a termination signal consisting of five thymidines in a row (T5). Most important, the

cleavage of the transcript at the termination site is after the second uridine, yielding a transcript resembling the ends of synthetic siRNAs, which also contain two 3′ overhanging T or U nucleotides (nt).

2.9.3.1 pSUPERIOR INDUCIBLE SYSTEM

With pSUPERIOR vectors, expression of siRNA is repressed in the absence of tetracycline and induced in the presence of tetracycline. When induced, transcription of the siRNA-precursor RNA hairpin occurs in the same manner as in the "standard" pSUPER vectors.
In fact, the siRNA expression cassettes in all pSUPER and pSUPERIOR vectors are completely identical, except for one key feature: a sequence modification of the H1 promoter between the TATA box and the RNA hairpin transcription start site.

The following two sequences illustrate the difference:

• H1 promoter (from 35nt upstream of BglII / HindIII cloning site AGATCTaagctt):
5′...GAATCT**TATA**AGTTCTGTATGAGACCACAGATCTaagctt...3′

• Inducible H1 promoter (from 35nt upstream of BglII / HindIII cloning site AGATCTaagctt):
5′...GAATCT**TATA**AGTTCCCTATCAGTGATAGAGATCTaagctt...3′

The underlined 19-nt region of the second sequence indicates the modification, which corresponds to the tetracycline operator 2 (TetO2) site. The TetO2 sequence serves as the binding site for 2 molecules of the Tet repressor, and the change of the H1 promoter sequence in this manner does not in itself affect the transcription activity of the vector.

Unlike other tetracycline-regulated systems that use hybrid regulatory molecules and viral transactivation domains [7], pSUPERIOR vectors use only regulatory elements from the native Tet operon [6]. This method more closely resembles the regulation of the native bacterial tet operon [4][5] and – importantly for RNAi research – avoids the potentially toxic effects of viral transactivation domains observed in some mammalian cell lines.
The map of pSUPERIOR.puro vector is shown here below (Fig. 2.2).

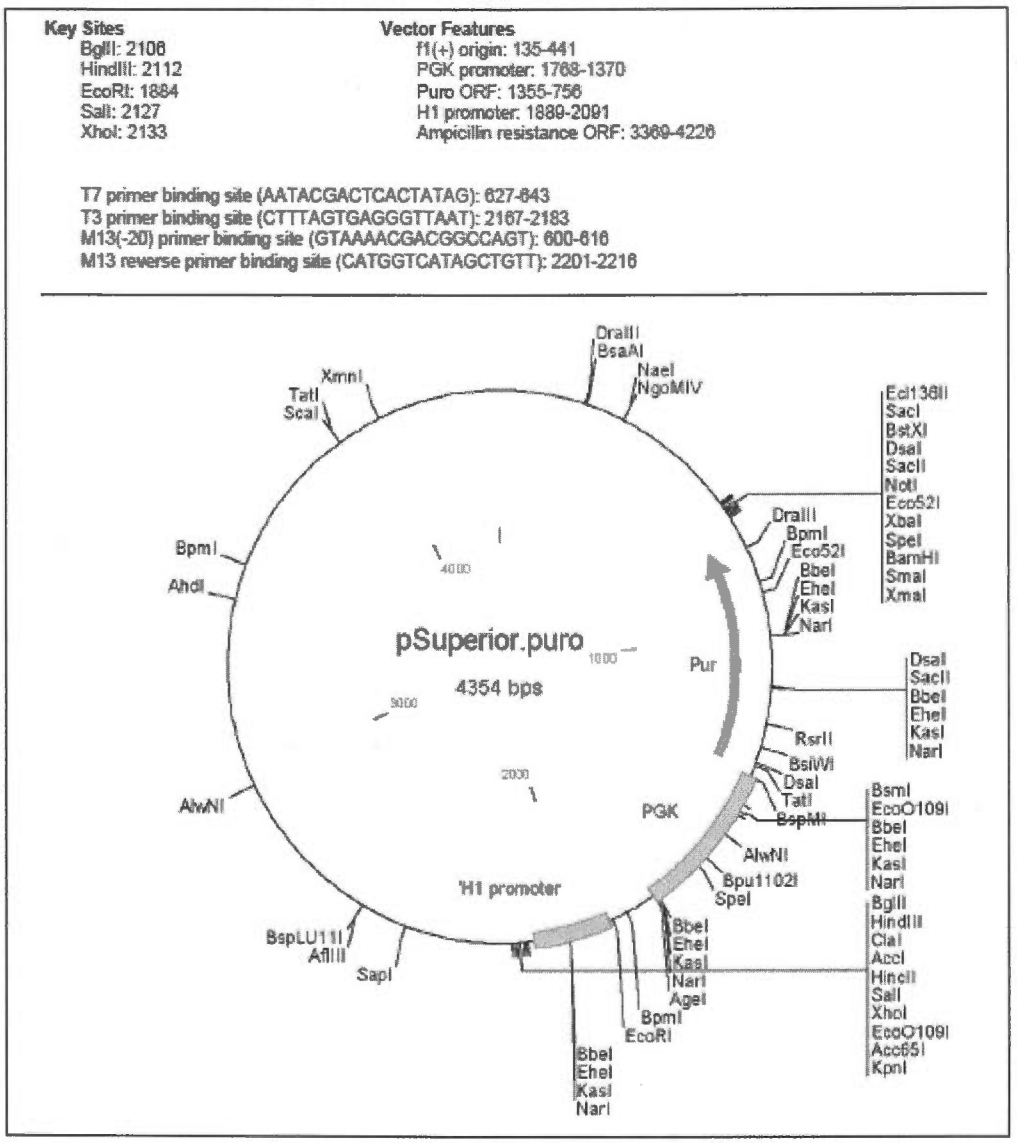


Fig. 2.2 Map of pSUPERIOR.puro

2.9.4 OLIGO INSERT DESIGN

To effect the silencing of a specific gene, the pSUPERIOR vector is used in concert with a pair of oligonucleotides that contain, among other features, a unique 19-nt sequence derived from the mRNA transcript of the gene targeted for suppression (the "N-19 target sequence").

The N-19 target sequence corresponds to the sense strand of the pSUPER-generated siRNA, which in turn corresponds to a 19-nt sequence within the mRNA. In the mechanism of RNAi, the antisense strand of the siRNA duplex hybridizes to this region of the mRNA to mediate cleavage of the molecule.

These forward and reverse oligos are annealed and cloned into the vector, between the unique BglII and HindIII enzyme sites. This positions the forward oligo at the correct position downstream from the H1 promoter's TATA box to generate the desired siRNA duplex.

The sequence of this forward oligo includes the unique N-19 target in both sense and antisense orientation, separated by a 9-nt spacer sequence. The 5′ end corresponds to the BglII site, while the 3′ end contains the T5 sequence and any HindIII- corresponding nucleotides. While the 5′ overhang of the oligo corresponds to the 3′ BglII overhang of the plasmid, the overhang sequence of the oligo actually corresponds to the BamH1, and thus destroys the BglII site upon ligation to enable more efficient screening of positive clones.

The resulting transcript of the recombinant vector is predicted to fold back on itself to form a 19–base pair stem-loop structure.

Analysis indicates that the stem-loop precursor transcript is quickly cleaved in the cell to produce a functional siRNA. Fig.2.3 provides an overview of the insert design, and how the oligos are transcribed and processed to functional siRNA.

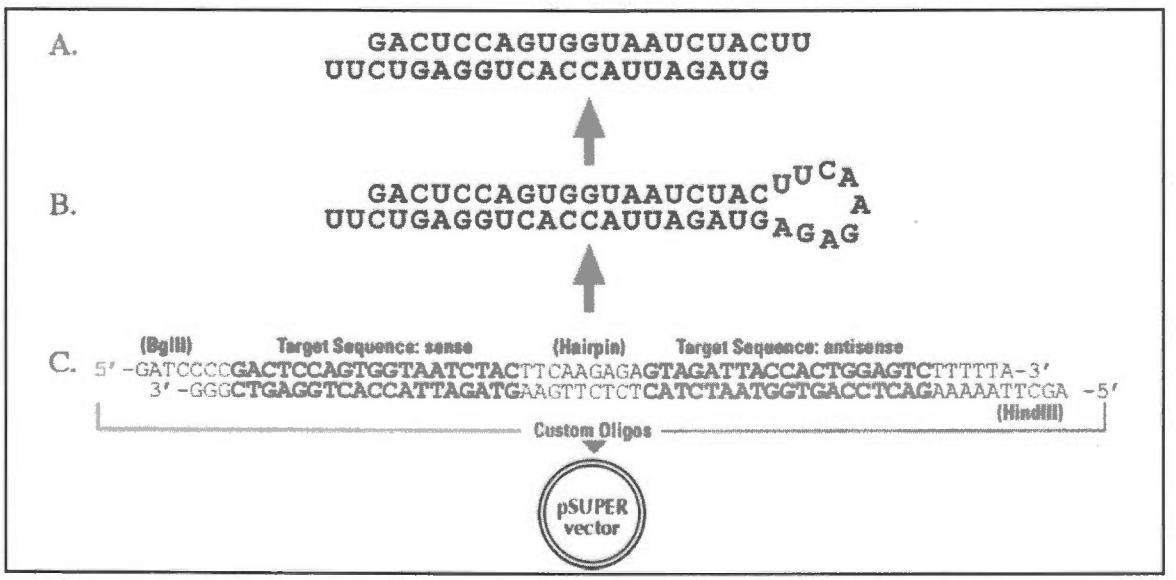


Fig. 2.3 Transcription of 60-nt oligo to hairpin RNA, processed to functional siRNA.

As mentioned in paragraph 2.1.2, the sequence chosen to attempt the silencing of ***MDR1*** was published by Wu et al. [2].

The siRNA molecule targeting ***MDR1*** corresponded to the coding region 79-99 (**5'-AAGGAAAGAAACCAACTGTC-3'**) relative to the start codon of the gene sequence (NM_000927).

The **sense template oligonucleotide (DNA)** for the siRNA was the following: **5'-AAGACAGTTGGTTTCTTTTCC-3'**
The **antisense template oligonucleotide (DNA)** for the siRNA was the following: **5'-AAGGAAAAGAAACCAACTGTC**

The target sequences to insert in the pSUPERIOR vector had 19 nt, as described by the vector manufacturer and they were the following:
Sense sequence: (5' → 3') GACAGTTGGTTTCTTTTCC
Antisense sequence: (5' → 3') GGAAAAGAAACCAACTGTC

The whole insert of 60 nt, containing the *MDR1* target sequence, reads as follows:

Forward Primer
(BglII) Sense Anti-sense
5'-GATCCCC**AAAGGTCTTGTATAACACC**ttcaagaga**GGTGTTATACAAGACCTTT**TTTTTA**-3'**

Reverse Primer
(HindIII) Sense Anti-sense
5'-AGCTTAAAAA**GACAGTTGGTTTCTTTTCC**tctcttgaa**GGAAAAGAAACCAACTGTC**GGG**-3'**

The sequence chosen to attempt the silencing of ***MRP1*** was bought from Ambion, Inc. **(*Silencer* Validated *MRP1*-siRNA, Ambion 51321)**. As the composition of the sequence was also required to the manufacturer, the oligo insert could be designed following the same rules described above.
Both oligonucleotides (DNA) were synthesised by Sigma-Genosis.

2.9.5 PROCEDURE

The following steps have been carried out by me at the **Pharmacological Research Institute "Mario Negri" (Laboratory of Molecular Pharmacology) in Milan.**
Here are the general steps performed for the experiment utilizing the pSUPERIOR vector:

1. The forward and reverse strands of the oligos containing the siRNA-expressing sequence targeting the gene of interest (*MRP1*-siRNA and *MDR1*-siRNA, respectively), were annealed.

2. The circular pSUPERIOR.puro vector was linearized with BglII and HindIII.
3. The annealed oligos were cloned into the vectors.
4. The vectors were transformed in bacteria.
5. pcDNA6/TR vector (Invitrogen, V1025-20) was transfected into SH-SY5Y cells and a stable cell line that constitutively expressed the Tet repressor was established by blasticidin (SIGMA, 15205) and the cell line which expressed the highest Tet repressor level was determined by pcDNA4/TO/*LacZ* vector (Invitrogen, V1020-20) transfection and β-galactosydase assay.
6. pSUPERIOR vectors were tranfected into SH-SY5Y cells TetR expressing vector, selected in step 5.
7. A selection with puromycin (SIGMA, P8833) was carried out to find a stable cell line for siRNA expression.
8. The cells were treated with doxycycline (SIGMA, D9891) to induce transcription of the siRNA.
8. The effects on protein expression and/or mRNA levels were assayed.

2.9.5.1 Step One: Oligo Annealing

The oligos targeting *MRP1* and *MDR1* were dissolved in sterile, nuclease-free water to a concentration of 3 mg/ml.

Each of the two annealing reactions was assembled by mixing 1 µl of each oligo (forward + reverse) with 48 µl annealing buffer (100 mM NaCl and 50 mM HEPES pH 7,4).

The mixture was incubated at 90°C for 4 min, and then at 70°C for 10 minutes. The annealed oligos were slowly cooled down to 37°C for 20 minutes, next at room temperature for some hours and then down to 4°C. For longer storage, the annealed oligos were kept at -20°C.

2.9.5.2 Step Two: Vector Linearization

In order to linearize the pSUPERIOR.puro vector (OligoEngine, VEC-IND-0006) supplied by "Mario Negri" Lab, the restriction enzymes used were HindIII and BglII.

Three different digestion reactions have been performed:

1. pSUPERIOR with HindIII and BglII;
2. pSUPERIOR with HindIII;
3. pSUPERIOR with BglII.

The digestion reaction of pSUPERIOR with HindIII and BglII was prepared mixing the components in the following order: 15 µl of water, 2 µl of Buffer (10X), 1 µl of pSUPERIOR vector (800 ng/µl), 1 µl of HindIII, 1 µl of BglII and (final volume 20 µl).

The digestion reaction of pSUPERIOR with HindIII was prepared mixing the components in the following order: 16 µl of water, 2 µl of Buffer (10X), 1 µl of pSUPERIOR vector (800 ng/µl) and 1 µl of HindIII (final volume 20 µl).

The digestion reaction of pSUPERIOR with BglII was prepared mixing the components in the following order: 16 µl of water, 2 µl of Buffer (10X), 1 µl of pSuperior vector (800 ng/µl) and 1 µl of BglII (final volume 20 µl).

The 3 digestion reactions were kept at 37°C for about 1 hour.

In order to assay the digestion reaction results, the samples were loaded on a 1% agarose gel. This step was also helpful to perform the gel purification of the linearized vector to remove the fragment between BglII and HindIII, to help separate the prep from any undigested circular plasmid and to decrease the background in ligation and transformation.

1% agarose gel

0.5 g of agarose were weighed out into a 250mL conical flask and 50 mL of 1X TAE (Tris Acetate EDTA) were added.

The mix was swirled and then heated in a microwave for about 1 minute to dissolve the agarose.

The agarose was left to cool on the bench down to about 60°C for 5 minutes.

4 µL of ethidium bromide (2.5 mg/mL) were added, then the mix was swirled.

The gel was slowly poured into the tank and the comb was inserted.

The gel was left to set for 1 hour.

The 1X TAE buffer was poured into the gel tank to submerge the gel to 2–5 mm depth.

Sample Preparation

The samples obtained by digestion reactions and the undigested circular pSuperior vector were prepared to be loaded into the gel:

4 µl of loading buffer (6X) (25 mg bromophenol blue, 4 g sucrose and distilled water to 10 ml) were added into 20 µl of each samples.

The samples were loaded into the gel

1 kb marker and 100 pb marker were loaded into the gel too.

The gel was let run at 70 V.

The gel was let stop when the bromophenol blue had run 3/4 the length of the gel.

The gel was looked at on the UV light-box to view the DNA bands and a photo was taken.

The DNA band of the pSuperior vector digested by both HindIII and BglII was cut out of the gel, and then was dissolved to extract and purify the DNA by MinElute Gel Extraction Kit (QIAGEN, 28604).

DNA extraction and purification by MinElute Gel Extraction Kit (QIAGEN, 28604)

1. The gel slice was weighed in a micro tube.
2. Three volumes of Buffer QG (provided by the kit) were added to 1 volume of gel (as the band weighed 110 mg, 330 µl of Buffer QG were added).
3. The gel was incubated at 50°C for about 10 min, until the gel slice has completely dissolved. To help dissolve the gel, the tube was vortexed every 2–3 min during the incubation.
4. 1 gel volume of isopropanol (110 µl) was added to the sample and mixed by inverting the tube several times.
5. A MinElute column was placed in a provided 2 ml collection tube in a suitable rack.
6. To bind DNA, the sample was applied to the MinElute column, and centrifuged at 12000 rpm for 1 min.

7. The flow-through was discarded and the MinElute column was placed back in the same collection tube.
8. 500 µl of Buffer QG were added to the spin column and centrifuged at 12000 rpm for 1 min.
9. The flow-through was discarded and the MinElute column was placed back in the same collection tube.
10. To wash, 750 µl of Buffer PE (provided by the kit, ethanol (96%) were added to it, before using) were added to the MinElute column and centrifuged for 1 min at 12000 rpm.
11. The flow-through was discarded and the MinElute column was centrifuged for an additional 1 min at 12000 rpm.
12. The MinElute column was placed into a clean 1.5 ml microcentrifuge tube.
13. To elute DNA, 10 µl of sterile water were added to the center of the membrane, let the column stand still for 1 min, and then centrifuged for 1 min at 12000 rpm.
14. The elution contained the purified DNA and it was stored at – 20°C.

2.9.5.3 Step Three: Ligation into pSUPERIOR.puro Vector

Two distinct reactions were assembled for ligation of annealed oligos targeting *MRP1* and *MDR1*.

Each of the two cloning reaction was assembled by adding 5 µl of T4 DNA ligase buffer, 2 µl of the annealed oligos, 1 µl pSUPERIOR.puro vector (800 ng/µl), 1 µl nuclease-free water, and 1 µl T4 DNA ligase.

A negative control cloning reaction was performed with the linearized vector alone and no insert. This reaction was assembled by adding 5 µl of T4 DNA ligase buffer, 1 µl pSUPERIOR.puro vector, 3 µl nuclease-free water, and 1 µl T4 DNA ligase.

The mix was incubated at room temperature for about 4 hours.

2.9.5.4 Step Four: Transformation of Bacteria

The purpose of this technique is to introduce a foreign plasmid into a bacteria and to use that bacteria to amplify the plasmid in order to make large quantities of it.

Since DNA is a very hydrophilic molecule, it won't normally pass through a bacterial cell membrane. In order to make bacteria take the plasmid, they must first be made "competent" to take up DNA. This is done by creating small holes in

the bacterial cells by suspending them in a solution with a high concentration of calcium. DNA can then be forced into the cells by incubating the cells and the DNA together on ice, placing them briefly at 42°C (heat shock), and then putting them back on ice. This causes the bacteria to take in the DNA. The cells are then plated out on antibiotic containing media.

Making competent cells

To carry out every experiment with bacteria, *E.coli XL1* (supplied by "M. Negri" Lab) was used.

Procedure:

1. A single colony was picked from a freshly grown plate of *E. coli XL1* by sterile loop and it was inoculated in 10 ml of sterile LB broth in a 50 ml sterile tube.
2. The culture was incubated at 37°C with vigorous shaking overnight.
3. The day after, 1 ml of bacterial culture was taken from the 50 ml tube and it was added to 100 ml of LB broth in a 500 ml sterile flask.
4. The culture was incubated at 37°C with vigorous shaking for approximately 2 hours.
5. Cell density was monitored by determining optical density (OD) at 600 nm (OD_{600}) until it was between 0,2 and 0,3.
6. 50 ml of this culture were transferred to a 50 ml conical tube and placed on ice for about 10 minutes.
7. The culture was centrifuged at 3000 rpm for 10 minutes.
8. The supernatant was gently removed by pipette. The pellet was resuspended in 50 ml (1/2 of initial culture volume) of ice cold 50 mM $CaCl_2$ (it was diluted in Ultrapure water) and placed on ice for 1 hour.
9. After the ice incubation, the culture was centrifuged at 3000 rpm for 5 minutes at 4°C.
10. The supernatant was gently removed by pipette. The pellet was resuspended in 10 ml (1/10 of initial culture volume) of a solution consisting of ice cold 50 mM $CaCl_2$ and sterile glycerol (20%).
11. The suspension was shared into eppendorf tubes (0,4 ml-0,5 ml each tube) and placed into an ice bucket in the fridge (4°C) overnight.
12. The day after, the competent bacteria were freezed in dry ice and ethanol and then stored at - 80°C.

The **TRANSFORMATION** was performed by the following protocol:

1. 300 µl of competent bacteria (previously prepared and stored at – 80°C) were removed from – 80°C and placed directly into a ice bucket. They were let thaw on ice for about 15 minutes.
2. After thawing, three tubes were placed on ice and 100 µl of competent bacteria were put into each tube.
3. 50 µl of 2X TCM (10 mM Tris-Cl, pH 7,5; 10 mM $CaCl_2$ and 10 mM $MgCl_2$) and 40 µl of nuclease-free water were added to 10 µl of each of three ligation reactions previously prepared (Step Three).
4. The three previously reactions (100 µl each) were added to the each of the three tubes containing 100 µl of competent bacteria.
5. The tubes were placed on ice for 45 minutes.
6. After 45 minutes, the tubes were put at 42°C for 2 minutes and then returned to ice for 2 minutes.
7. 1 ml of sterile LB (Luria-Bertani Broth) were added to three tubes and bacteria were let grow with shaking for 1 hour at 37°C.
8. The tubes were centrifuged for 10 minutes at 2000 rpm.
9. The most of supernatant was removed.
10. The cell pellet of each tube was resuspended in the remaining supernatant and then plated onto a ampicilin-agarose plate and spread around using a sterilized, bent glass rod spreader.
11. The three plates were placed upside down in the 37°C incubator overnight.
12. The day after, the number of well-isolated colonies on the plates was counted.
13. The colonies were picked using a sterile loop, inoculated in 3 ml of LB broth containing 3 µl of Ampicillin (1000X) and grown with shacking overnight at 37°C.
14. The day after, plasmid extraction was performed by FastPlasmid Mini Kit (eppendorf,0032 007 653).

Plasmid extraction by FastPlasmid Mini Kit (eppendorf, 0032 007 653)

The following steps were carried out for each of the 14 bacterial cultures chosen:

a. 1,5 ml of bacterial culture were centrifuged at 12000 rpm for 1 minute in the provided 2 ml culture tube (the remaining bacterial culture of each colony was stored at 4°C).
b. The medium was removed by decanting, taking care not to disturb bacterial pellet.
c. 400 µl of ICE-COLD Complete Lysis Solution were added in the tube .
d. The tube was mixed thoroughly by constant vortexing at the highest setting for a full 30 seconds.
e. The lysate was incubated at room temperature for 3 minutes.
f. The lysate was transferred to a Spin Column Assembly by decanting or pipetting.
g. The Spin Column Assembly was centrifuged for 30–60 seconds at maximum speed.
h. 400 µl of diluted Wash Buffer were added to the Spin Column Assembly.
i. The Spin Column Assembly was centrifuged for 30–60 seconds at maximum speed.
l. The Spin Column was removed from the centrifuge and the filtrate was decanted from the Waste Tube. The Spin Column was placed back into the Waste Tube and returned to the centrifuge.
m. The Spin Column was centrifuged at maximum speed for 1 minute to dry it.
n. The Spin Column was transferred to a Collection Tube.
o. 50 µl of nuclease-free water were added directly to the center of the Spin Column membrane and the Collection Tube was capped over the Spin Column.
p. The Spin Column was centrifuged at maximum speed for 30–60 seconds.
q. The Spin Column was removed and discarded.
r. The Collection Tube contained the eluted DNA. Some was used to check the presence of positive clones (i.e. containing vèctor with oligo insert) and some was stored at – 20°C.

Checking the positive clones

The colonies were checked for the presence of positive clones (i.e., containing vector with oligo insert) by digesting the vector with EcoRI and XhoI (the digestion by EcoRI and HindIII was also possible):
15 reactions were assembled to digest the 14 vectors extracted from the bacterial colonies transformed by pSUPERIOR ligated to the oligo insert

targeting *MRP1* and *MDR1* respectively, and 1 reaction for digesting pSUPERIOR without insert (negative control).

a. Each of the 14 digestion reactions of vector ligated to the oligo insert was assembled by adding 1 µl of EcoRI, 1 µl of XhoI, 2 µl of BSA (10X), 2 µl of Buffer EcoRI (this Buffer provides reaction conditions that are amenable to both restriction enzymes) and 14 µl of plasmid (extracted by FastPlasmid Mini Kit, eppendorf).
In the digestion reaction of pSUPERIOR without insert (extracted by Plasmid Midiprep Kit, GENOMED), 1 µl of plasmid only was added to 13 µl of nuclease-free water. This was why the concentration of pSUPERIOR without insert after extraction by Plasmid Midiprep Kit (800 ng/ µl) was much higher than the concentration that we could have obtained from extraction of the 14 plasmids by FastPlasmid Mini Kit. In fact, generally the maximum plasmid DNA yields obtained from FastPlasmid Mini Kit are much lower compared to the ones obtained from Plasmid Midiprep Kit.
b. The 15 digestion reactions were kept at 37°C for about 1 hour.
In order to assay the digestion reaction results, the samples were loaded on a 1% agarose gel (see Step Two for 1% agarose gel preparation).

After digestion and loading of the samples on a 1% agarose gel, the results were determined as follows:

	Cut with Eco RI & XhoI
Positive clone: vector with insert	281 pb
Negative clone*: no insert	248 pb

* e.g. supercoil that was nicked and not fully linearized with BglII and HindIII, has a fragment of 248 pb.

Among the positive clones, 2 bacterial colonies containing vector with oligo insert targeting *MRP1* and 2 bacterial colonies containing vector with oligo insert targeting *MDR* were chosen.

c. From each of 4 colonies chosen, 20 µl were taken, put into 3 ml of LB broth containing 3 µl of Ampicillin (1000X) and grown with shacking overnight at 37°C (the remaining bacterial culture of each colony was stored at 4°C).
d. The day after, plasmid extraction was performed by QIAprep Miniprep (QIAGEN,27104).

Plasmid extraction by QIAprep Miniprep (QIAGEN,27104)

The following steps were performed for each of the 4 bacterial cultures chosen:

a. The pelleted bacterial cells were resuspended in 250 µl Buffer P1 (containing RNase A) and transfered to a micro-centrifuge tube.
b. 250 µl Buffer P2 were added and the tube was gently inverted 4–6 times to mix. The lysis reaction had not to proceed for more than 5 minutes.
c. 350 µl Buffer N3 were added and the tube was inverted immediately but gently 4–6 times, to avoid localized precipitation.
d. The mix was centrifuged for 10 minutes at maximum speed in a microcentrifuge. A compact white pellet was formed.
e. The supernatant from step d. was applied to the QIAprep spin column by pipetting.
f. The QIAprep spin column was centrifuged for 30–60 seconds and the flow-through was discarded.
g. The QIAprep spin column was washed by adding 0.75 ml Buffer PE and centrifuging for 30–60 seconds.
h. The flow-through was discarded, and centrifuged for an additional 1 minute to remove residual wash buffer.
i. The QIAprep column was placed in a clean 1.5 ml microcentrifuge tube. To elute DNA, 50 µl nuclease-free water were added to the center of each QIAprep column, let stand for 1 min, and centrifuged for 1 min.
j. The microcentrifuge tube contained the eluted DNA. Some was used to check the presence of positive clones (i.e. containing vector with oligo insert) and some was stored at – 20°C.

Checking the positive clones

The colonies were checked for the presence of positive clones (i.e., containing vector with oligo insert) by digesting the vector with EcoRI and XhoI (the digestion by EcoRI and HindIII was also possible).
3 reactions were assembled for digestion:

- 1 vector extracted from the bacterial colonies transformed by pSUPERIOR ligated to the oligo insert targeting *MRP1*.
- 1 vector extracted from the bacterial colonies transformed by - pSUPERIOR ligated to the oligo insert targeting *MDR1*.

- 1 reaction for digesting pSUPERIOR without insert (negative control).

The digestion was performed as described at Step Four.

In order to assay the digestion reaction results, the samples were loaded on a 2% agarose gel (see Step Two for agarose gel).

The presence of the correct insert within the recombinant pSUPERIOR vector was confirmed by sequencing prior to transfection in SH-SY5Y cells.

Vector Sequencing

For sequencing primer options, the sequence file, which is available for download from the pSUPER RNAi System section of the OligoEngine Web site (www.oligoengine.com) was consulted. It suggested to use primers T7 and T3 [T7 primer binding site (AATACGACTCACTATAG): 627-643; T3 primer binding site (CTTTAGTGAGGGTTAAT): 2167-2183].
As the laboratory "M. Negri" had designed 2 primers that had demonstrated to work even better, these primers were used.
The DNA Sequencing was performed at the Europe and WorldWide Primm Lab, Milan and then tested by BLAST (Basic Local Alignment Search Tool).

In the meantime, the 4 bacterial cultures chosen were frozen.

Preservation of bacteria by freezing

a. The 4 bacterial cultures chosen were let grow to high density but still in the logarithmic phase.

b. 0.25 ml of sterile glycerol and 0.75 ml of bacterial culture [final glycerol concentration 25%] was added into each cryovial, mixed fast and freezed immediately in dry ice and ethanol).

c. The bacterial frozen stocks were stored at - 80°C.

After making sure by sequencing that the oligos targeting *MRP1* and *MDR1* were inserted correctly in the two respective recombinant pSUPERIOR vectors, high copy of those plasmid DNAs from *E.coli Xl1* were extracted and purified by JETSTAR Plasmid Midiprep kit (GENOMED, 210025).

Plasmid extraction by JETSTAR Plasmid Midiprep kit (GENOMED, 210025)

The following steps were performed for both bacterial cultures where recombinant pSUPERIOR vector containing the siRNA-expressing sequence targeting MRP1 and MDR1 respectively, had been cloned.

1. Columns (provided from kit) were equilibrated by applying 10 ml of solution E4 (600 mM NaCl, 100 mM Sodium Acetate, 0,15% Triton X-100, acetic acid ad pH 5.0).
2. The bacterial culture, growth overnight in 100 ml of LB Broth with Ampicillin, was shared in two 50 ml tubes.
3. Each of the 50 ml bacterial culture was centrifuged at 3000 x g for 20 minutes.
4. The supernatant was removed carefully.
5. 4 ml of solution E1 (50 mM Tris, 10 mM EDTA, HCl ad pH 8.0) were added to the pellet and the cells were resuspended until the suspension was homogeneous.
6. 4 ml of solution E2 [200 mM NaOH, 1.0% SDS (w/v)] were added into the suspension and mixed gently, by inverting until the lysate appeared to be homogeneous.
7. 4 ml of solution E3 (3.1 M potassium acectate, acetic acid ad pH 5.5) were added and mixed immediately by multiple inverting until a homogeneous suspension was obtained.
8. The mixture was centrifuged at room temperature at 3000 x g for 20 minutes.
9. The supernatant was applied to the equilibrated column, a sterile gauze had been applied to before. The lysate was let drop by gravity flow.
10. The gauze was removed from the column.
11. The column was washed with 10 ml of solution E5 (800 mM NaCl, 100 mM sodium acetate, acetic acid ad pH 5.0) twice. The column was let empty after each wash by gravity flow.
12. The column was placed on a 15 ml tube.
13. The DNA was let elute with 5 ml of solution E6 (1250 mM NaCl, 100 mM Tris, HCl ad pH 8.5).
14. The DNA was let precipitate with 3,5 ml of isopropanol.
15. The solution was centrifuged at 13000 rpm for 30 minutes at 4°C.

16. The supernatant was removed and the plasmid DNA was washed with about 1,5 ml of 70% ethanol and recentrifuged at 13000 rpm for 5 minutes at 4°C.
17. The supernatant was removed and the pellet was let dry for about 15 minutes by air drying.
18. The DNA was dissolved in about 1,5 ml of sterile water.
19. The DNA absorbances at 230 nm, 260 nm and 280 nm were read by UV/Vis Scanning Spectrophotometer (BECKMAN COULTER)

2.9.5.5 Step Five: Transfection of SH-SY5Y Cells

Before transfecting the pSUPERIOR.puro plasmid into the target cells (SH-SY5Y), a stable line that constitutively expresses only the Tet repressor from pcDNA6/TR vector (Invitrogen, V1025-20) had to be created. Then that cell line had to be used to create a second cell line that expresses the siRNA from the pSUPERIOR.puro vector. In this way, a stable cell line that constitutively expresses the Tet repressor and inducibly expresses the siRNA was established. (Alternatively, transfection with both plasmids (pcDNA6/TR and pSUPERIOR.puro) and dual-select with to isolate a single stable cell line expressing both the Tet repressor and the gene of interest could be possible).

THE T-Rex System: OVERVIEW

The T-REx™ System is a tetracycline-regulated mammalian expression system that uses regulatory elements from the E. coli Tn10-encoded tetracycline (Tet) resistance operon [4][5]. Tetracycline regulation in the T-REx™ System is based on the binding of tetracycline to the Tet repressor and derepression of the promoter controlling expression of the gene of interest [6].

The major components of the T-REx™ System include:

- An inducible expression plasmid for expression of the gene of interest under the control of the strong human cytomegalovirus immediate-early (CMV) promoter and two tetracycline operator 2 (TetO2) sites.
- A regulatory plasmid, pcDNA6/TR, which encodes the Tet repressor (TetR) under the control of the human CMV promoter.
- Tetracycline for inducing expression.
- A control expression plasmid containing the lacZ gene, which when cotransfected with pcDNA6/TR, expresses ß-galactosidase upon induction with tetracycline.

THE-TRex SYSTEM: DESCRIPTION

In the T-REx System, expression of the gene of interest is repressed in the absence of tetracycline and induced in the presence of tetracycline. The T-REx System uses only regulatory elements from the native Tet operon [6]. Tetracycline-regulated gene expression in the T-REx System more closely resembles the regulation of the native bacterial tet operon [4][5] and avoids the potentially toxic effects of viral transactivation domains observed in some mammalian cell lines.

Expression of the gene of interest from the inducible expression vector is controlled by the strong CMV promoter [8-10] into which 2 copies of the tet operator 2 (TetO2) sequence have been inserted in tandem. The TetO2 sequences consist of 2 copies of the 19 nucleotide sequence, 5′-TCCCTATCAGTGATAGAGA-3′ separated by a 2 base pair spacer [4][5]. Each 19 nucleotide TetO2 sequence serves as the binding site for 2 molecules of the Tet repressor.

The second major component of the Vector System used in this work is the **pcDNA6/TR** regulatory vector which expresses high levels of the TetR gene [11] under the control of the human CMV promoter Both T-REx vectors can be introduced into mammalian host cells by standard transfection methods.

THE TRex SYSTEM: MECHANISM OF REPRESSION

In the absence of tetracycline, the Tet repressor forms a homodimer that binds with extremely high affinity to each $TetO_2$ sequence in the promoter of the inducible expression vector [4]. The 2 $TetO_2$ sites in the promoter of the inducible expression vector serve as binding sites for 4 molecules (or 2 homodimers) of the Tet repressor. The affinity of the Tet repressor for the tet operator is KB = 2 x 1011 M-1 (as measured under physiological conditions), where KB is the binding constant [4]. Binding of the Tet repressor homodimers to the $TetO_2$ sequences represses transcription of the gene of interest. Upon addition, tetracycline binds with high affinity to each Tet repressor homodimer in a 1:1 stoichiometry and causes a conformational change in the repressor that renders it unable to bind to the Tet operator. The association constant, KA, of tetracycline for

the Tet repressor is 3 x 109 M-1 [4]. The Tet repressor:tetracycline complex then dissociates from the Tet operator and allows induction of transcription from the gene of interest (Fig 2.4).

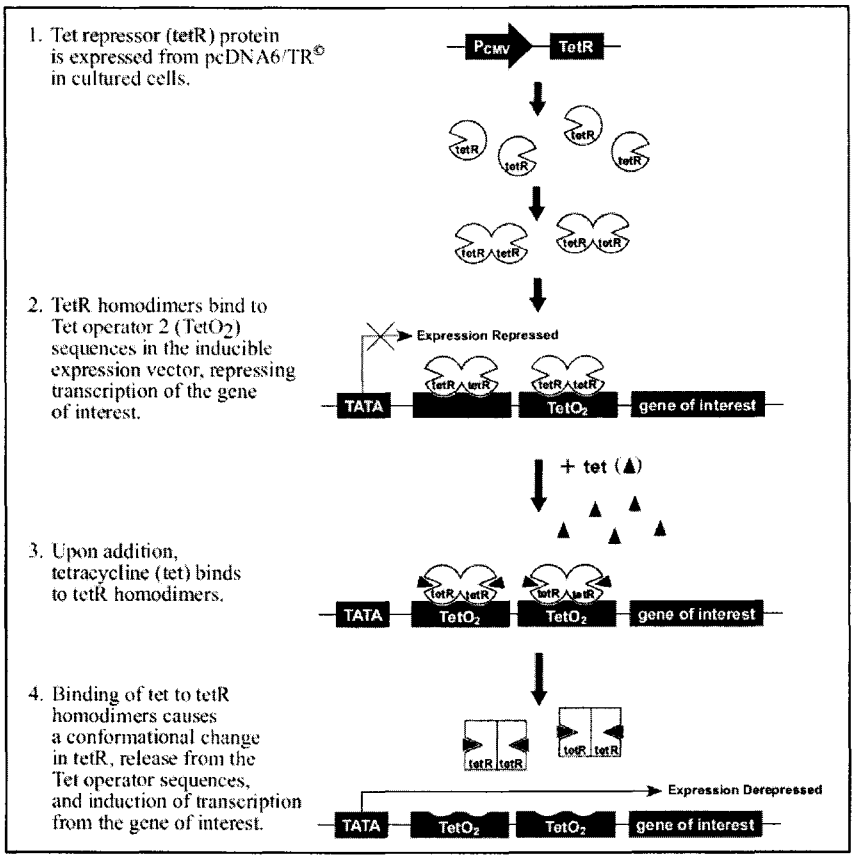


Fig. 2.4 The components of T-Rex System

THE T-Rex SYSTEM: EXPERIMENTAL OUTLINE

The gene of interest is cloned into the multiple cloning site of the inducible expression vector, and the resulting construct cotransfected with the regulatory plasmid, pcDNA6/TR into mammalian cells. After transfection, cells are treated with tetracycline to derepress the hybrid CMV/ $TetO_2$ promoter in the inducible expression vector and induce transcription of the gene of interest.

The positive control vector containing the lacZ gene can be transiently cotransfected into mammalian cells with pcDNA6/TR to demonstrate that the system is working properly in the cell line. Stable cell lines expressing Tet repressor from pcDNA6/TR can be established to serve as hosts for inducible expression vector-based constructs (Fig. 2.5).

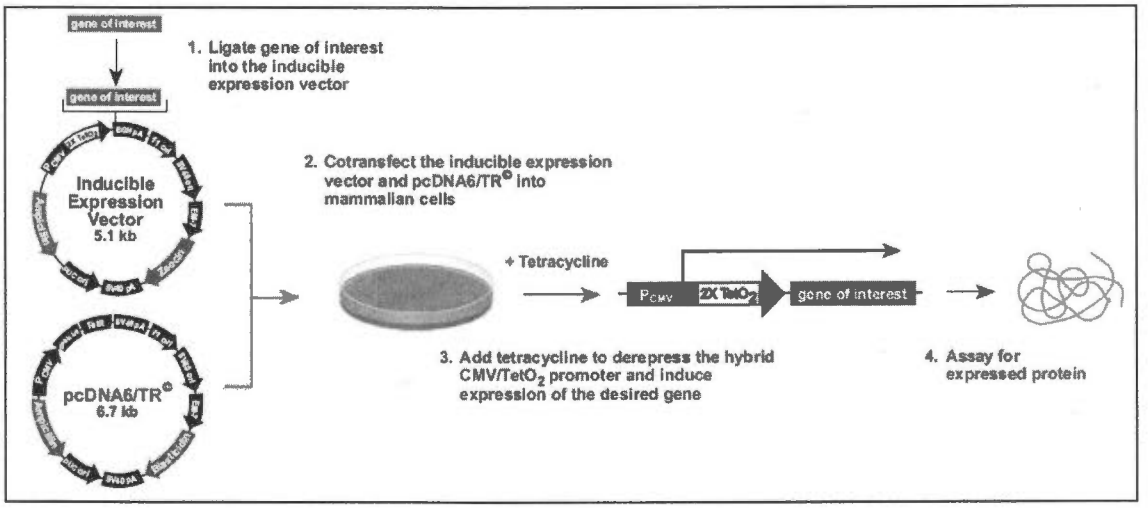


Figure 2.5 Experimental outline in the T-Rex System

pcDNA6/TR vector (Invitrogen, V1025-20)

The pcDNA6/TR vector was gently supplied by M. Negri Laboratory, Milan.

pcDNA6/TR is a 6.7 kb vector designed to be used with the T-REx System. The vector expresses high levels of the tetracycline (Tet) repressor under the control of the human cytomegalovirus immediate-early (CMV) promoter. High-level stable and transient expression of the Tet repressor can be carried out in most mammalian cells. Tetracycline-regulated expression of a gene of interest may then be tested by transfecting the inducible expression plasmid into host cells expressing the Tet repressor.

The TetR gene used in pcDNA6/TR was originally isolated from the Tn10 transposon which confers resistance to tetracycline in E. coli and other enteric bacteria [11].

The TetR gene from Tn10 encodes a class B Tet repressor and is often referred to as TetR(B) in the literature [4] .

The TetR gene encodes a repressor protein of 207 amino acids with a calculated molecular weight of 23 kDa.

The pcDNA6/TR vector contains the ampicillin resistance gene and the blasticidin resistance gene, either of which allows selection of the plasmid in E. coli.

The map of pcDNA6/TR vector is shown here below (Fig. 2.6).

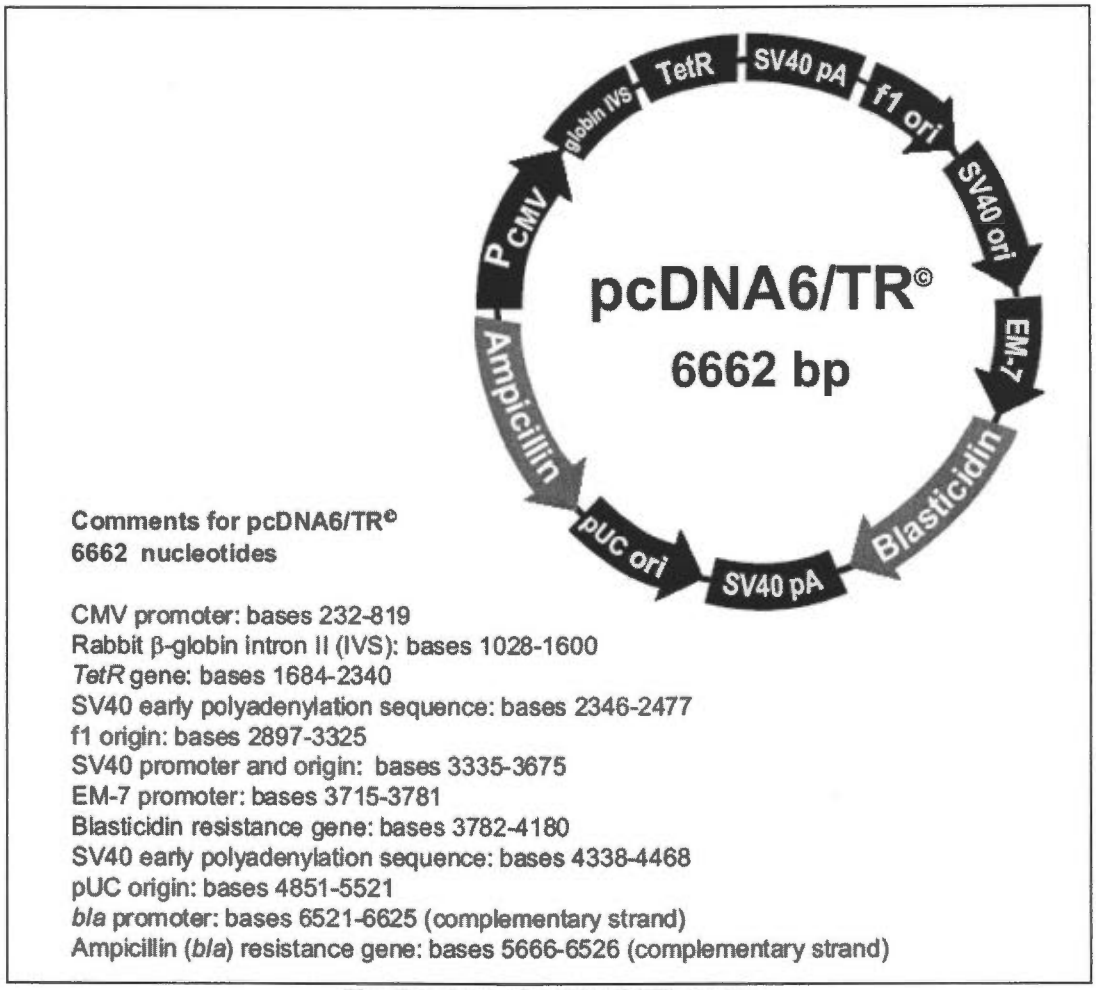


Fig. 2.6 Map of pcDNA6/TR vector

The relevant features of pcDNA6/TR are described in table 2.1 .

Feature	Benefit
Human cytomegalovirus (CMV) immediate early promoter	Permits high-level expression of the *TetR* gene [8-10]
Rabbit ß-globin intron II (IVS)	Enhances expression of the *TetR* gene [12]
TetR gene	Encodes the Tet repressor that binds to tet operator sequences to repress transcription of the gene of interest in the absence of tetracycline [6] [11]
SV40 early polyadenylation signal	Permits efficient transcription termination and polyadenylation of mRNA
f1 origin	Allows rescue of single-stranded DNA
SV40 early promoter and origin	Allows efficient, high-level expression of the blasticidin resistance gene in mammalian cells and episomal replication in cells expressing SV40 large T antigen

EM-7 promoter	Synthetic prokaryotic promoter for expression of the blasticidin resistance gene in *E. coli*
Blasticidin (*bsd*) resistance gene	Allows selection of stable transfectants in mammalian cells [13] and transformants in *E. coli*
SV40 early polyadenylation signal	Allows efficient transcription termination and polyadenylation of mRNA
pUC origin	Permits high-copy number replication and growth in *E. coli*
bla promoter	Allows expression of the ampicillin (*bla*) resistance gene
Ampicillin (*bla*) resistance gene (ß-lactamase)	Allows selection of transformants in *E. coli*

Table 2.1 Features of pcDNA6/TR

To generate a cell line that stably expresses the Tet repressor, pcDNA6/TR has to be transfected into the mammalian host cell line and cells have to be selected with blasticidin. Cells expressing suitably high levels of the Tet repressor may then be used as hosts to stably or transiently express the gene of interest from the inducible expression vector.
Before transfection, the sensitivity of SH-SY5Y cells to blasticidin (as the pcDNA6/TR vector contains the blasticidin resistance gene) was tested.

Determination of blasticidin sensitivity

The determination of blasticidin sensitivity was performed estabilishing a kill curve for blasticidin to determine its optimal effective dose. It is the minimum concentration of blasticidin required to kill the untransfected host cell line. According to the blasticidin manufacturer's instructions, concentrations between 2 and 10 µg/ml blasticidin are sufficient to kill the untransfected host cell line.
A range of concentrations was tested as suggested by blasticidin manufacturer.
As plating density can have a strong impact on antibiotic selection because cells at higher densities are less effectively killed off than cells at lower concentration, cells were plated at different density.

Procedure

- One day before adding blasticidin, SH-SY5Y cells were plated in the following plates:
  - 24-well plate in growth medium (without blasticidin), so that cells were 35% confluent at the time of blasticidin addition;
  - 24-well plate in growth medium (without blasticidin), so that cells were 70% confluent at the time blasticidin addition;
  - 10 cm plate in growth medium (without blasticidin), at a concentration of 3000 cells/ml;
  - 10 cm plate in growth medium (without blasticidin), at a concentration of 1500 cells/ml;

- The next day, the culture medium was substituted with medium containing these concentrations of blasticidin S hydrochloride (SIGMA, 15205): 0, 1, 3, 5, 7.5, and 10 µg/ml.

- The selective medium was replenished every 3-4 days.
  Cells sensitive to blasticidin appeared rounded up and detached from the plate.

- The number of viable cells was counted at regular intervals to determine the appropriate concentration of blasticidin that prevents growth within 1-2 weeks after addition of the antibiotic.

Once the appropriate blasticidin concentration to use for selection was determined, a stable cell line expressing pcDNA6/TR vector could be generated.

Transfection of pcDNA6/TR vector (Invitrogen, V1025-20)

The transfection of pcDNA6/TR vector (Invitrogen) was performed using Lipofectamine 2000 (Invitrogen) and according to the manufacturer's instructions.

Procedure

1. One day before transfection, SH-SY5Y cells were plated in a T25 flask in presence of 5 ml of growth medium without antibiotics so that cells were 90-95% confluent at the time of transfection.
2. The DNA-Lipofectamine 2000 complexes were prepared as follows:

a. 8 µg of **pcDNA6/TR vector** were diluted in 500 µl of Opti-MEM I Reduced Serum Medium (GIBCO). The dilution was mixed gently.

b. 20 µl of Lipofectamine 2000 were diluted in 500 µl of Opti-MEM I Medium. The dilution was mixed gently and incubated for 5 minutes at room temperature.

c. After the 5 minute incubation, the diluted DNA was combined with the diluted Lipofectamine 2000. The solution was mixed gently and incubated for 20 minutes at room temperature to allow the DNA-Lipofectamine 2000 complexes to form.

3. The complexes were added to the T25 flask containing cells and medium. The medium was mixed gently by rocking the flask back and forth.
4. The cells were incubated at 37°C in a CO_2 incubator for 48 hours prior to testing for transgene expression.

Selection of stable cell lines

1. After a 48 hour incubation, cells were dissociated from the culture flask by trypsin (EuroClone) and plated in several 10 cm plates at a concentration of 3000 cells/ml.
2. The following day, **blasticidin** (5 µg/ml) was added into the medium.
3. The selective medium was replenished every 3-4 days until blasticidin-resistant colonies were detected.

2 untransfected control cultures were included:

- 1 untransfected control culture was subjected to blasticidin selection to check the cells that spontaneously became or were already antibiotic resistant; it was helpful to determine the effectiveness of the transfection and selection. Cells were plated at two different concentrations: 3000 cells/ml (as the transfected cells) and 15000 cells/ml. This was to assay the blasticidin selection at different cell confluences.
- 1 untransfected control culture was grown without blasticidin selection as a positive control for cell viability. Cells were plated at two different concentrations: 3000 cells/ml (as the transfected cells) and 15000 cells/ml.

2 transfected control culture was included:

- 1 transfected control culture was grown without blasticidin selection to check a possible toxicity of the transfection. Cells were plated at a concentration of 3000 cells/ml.

- 1 transfected control culture was grown with blasticidin selection. Cells were plated at a concentration of 15000 cells/ml. It was to check a possible toxicity of the blasticidin.

4. The positive colonies were picked and expanded.
In order to isolate single colonies, a sterile glass ring was put on the culture plate to surround each clone, before scraping the adherent cells. The ring could stick on the plate because sterile vaseline was put downside. The cells were dissociated from the culture plate by trypsin and every isolated clone was plated in a 2 cm^2 well (24-wells plate). The clones were expanded in a growth medium that always contained blasticidin, even if all of the cells in the untransfected control culture were killed.

Among the clones that were blasticidin resistant and therefore should have stably expressed the Tet repressor, the ones that expressed the highest levels of Tet repressor to use as hosts for the inducible expression construct, had to be selected. These clones, as synthesised the highest levels of Tet repressor, should exhibit the most complete repression of basal transcription of the gene of interest.

5. To screen the clones for those expressing the highest levels of Tet repressor from pcDNA6/TR, the **pcDNA4/TO-based expression vector containing the *lacZ* gene (Invitrogen, V1020-20)** was transfected transiently into the cells and assayed for **ß-galactosidase expression** after induction with tetracycline. Those clones exhibiting the lowest basal levels and highest inducible levels of ß-galactosidase expression, had to be selected.

pcDNA4/TO vector (Invitrogen, V1020-20)

pcDNA4/TO is a 5.1 kb expression vector designed to use with the T-REx. System. The vector allows tetracycline-regulated expression of the gene of interest in mammalian host cells cotransfected with the pcDNA6/TR vector.
The vector contains:
• Hybrid promoter consisting of the human cytomegalovirus immediate-early (CMV) promoter and tetracycline operator 2 ($TetO_2$) sites for high-level tetracycline-regulated expression in a wide range of mammalian cells.

The pcDNA4/TO vector contains two tetracycline operator 2 ($TetO_2$) sites within the human cytomegalovirus immediate-early (CMV) promoter for tetracycline-regulated expression of the gene of interest. The $TetO_2$ sequences serve as binding sites for 4 Tet repressor molecules (comprising two Tet repressor homodimers) and confer tetracycline-responsiveness to the gene of interest.

Yao et al. [6] have recently demonstrated that the location of tet operator sequences in relation to the TATA box of a heterologous promoter is critical to the function of the tet operator. Regulation by tetracycline is only conferred upon a heterologous promoter by proper spacing of the $TetO_2$ sequences from the TATA box. For this reason, the first nucleotide of the $TetO_2$ operator sequence has been placed 10 nucleotides after the last nucleotide of the TATA element in the CMV promoter in pcDNA4/TO.

In the absence of tetracycline, expression of the gene of interest is repressed by the binding of Tet repressor homodimers to the $TetO_2$ sequences. Addition of tetracycline to the cells derepresses the hybrid CMV/$TetO_2$ promoter in pcDNA4/TO and allows expression of the gene of interest.

The relevant features of pcDNA4/TO are described in table 2.9.2.

Feature	**Benefit**
Human cytomegalovirus (CMV) immediate early promoter	Permits high-level expression of your gene of interest [8-10]
CMV Forward priming site	Allows sequencing in the sense orientation
Tetracycline operator (O2) sequences	Two tandem 19 nucleotide repeats which serve as binding sites for Tet repressor homodimers [4]
Multiple cloning site	Allows insertion of your gene of interest
BGH Reverse priming site	Permits sequencing of the non-coding strand
Bovine growth hormone (BGH) polyadenylation signal	Permits efficient transcription termination and polyadenylation of mRNA [14]
f1 origin	Allows rescue of single-stranded DNA

SV40 early promoter and origin	Allows efficient, high-level expression of the Zeocin. resistance gene in mammalian cells and episomal replication in cells expressing SV40 large T antigen
EM-7 promoter gene in *E. coli*	Synthetic prokaryotic promoter for expression of the Zeocin. resistance
Zeocin resistance (Sh ble) gene (expressed from the SV40 early promoter or the EM-7 promoter)	Permits selection of stable transfectants in mammalian cells [15] [16] and transformants in *E. coli*
SV40 early polyadenylation signal	Allows efficient transcription termination and polyadenylation of mRNA
pUC origin	Allows high-copy number replication and growth in *E. coli*
bla promoter	Allows expression of the ampicillin (bla) resistance gene
Ampicillin (bla) resistance gene (ß-lactamase)	Permits selection of transformants in *E. coli*

Table 2.2 Features of pcDNA4/TO

The **pcDNA4/TO/*lacZ*** vector, that was transfected transiently into the SH-SY5Y cells, in order to assay the clones expressing stably the Tet repressor from pcDNA6/TR, is a 8224 bp control vector containing the gene for ß-galactosidase.

This vector was constructed by ligating a 3.1 kb Hind III-EcoR I fragment containing the *lacZ* gene from pcDNA.3/His/*lacZ* into the Hind III-EcoR I site of pcDNA4/TO.

The *lacZ* gene is fused to an N-terminal peptide containing an ATG initiation codon, a polyhistidine (6xHis) tag, and the Xpress. epitope. The size of the ß-galactosidase fusion protein is approximately 120 kDa in size.

The map of **pcDNA4/TO/*lacZ*** vector is shown here below (Fig. 2.7).

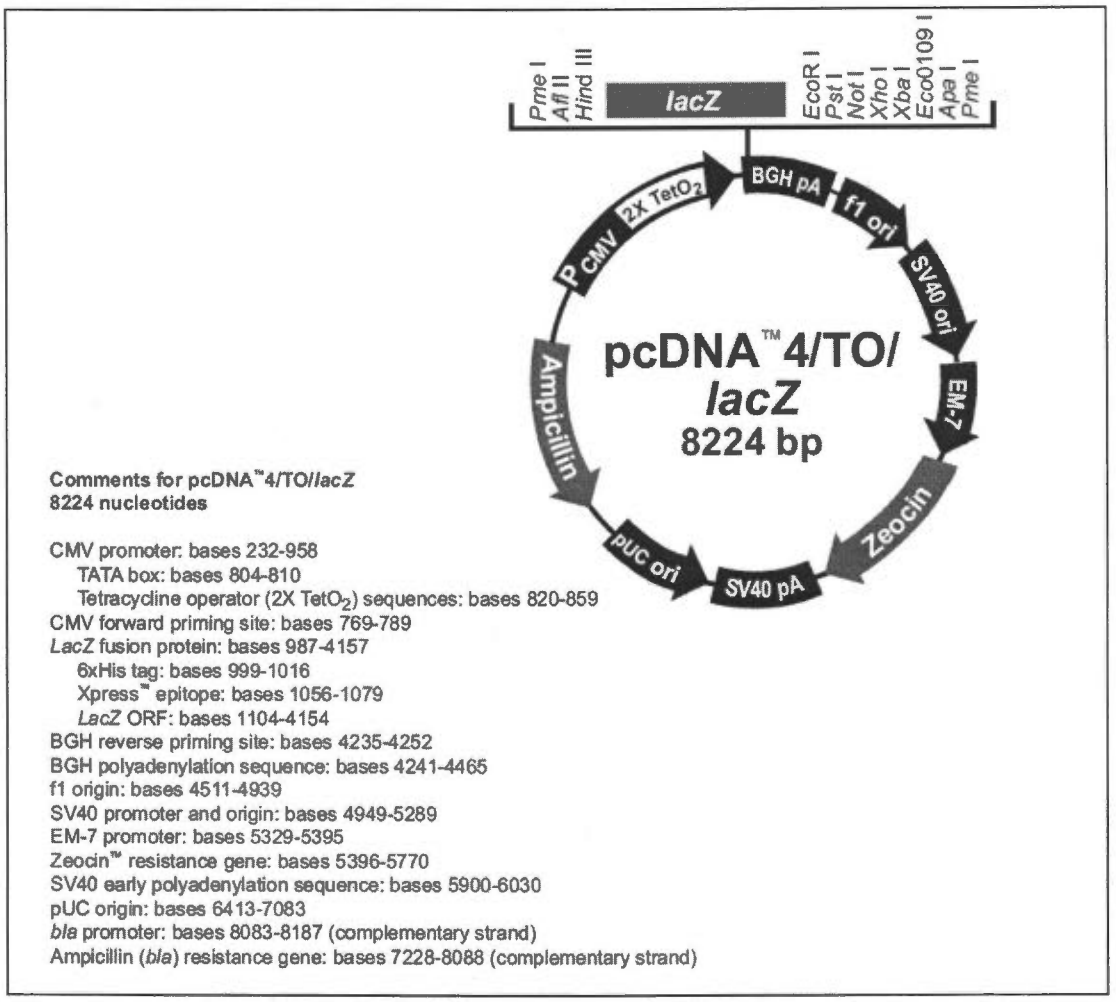


Fig. 2.7 Map of pcDNA4/TO/*lacZ* vector

The transfection of **pcDNA4/TO/*lacZ*** vector in clones that stably express the Tet repressor from pcDNA6/TR, results in the induction of **ß-galactosidase expression** upon addition of tetracycline.

The pcDNA4/TO/*lacZ* vector was gently supplied by M. Negri Laboratory, Milan.

Transfection of pcDNA4/TO/*lacZ* vector

Each SH-SY5Y clone, selected by blasticidin after a transfection of pcDNA6/TR vector, was made grow and then plated in three 4-cm^2 wells (12-well plate).

The cells plated in 1 of the 3 wells were used to expand the clone.

The cells plated in the remaining 2 wells were transfected by **pcDNA4/TO/*lacZ* vector** using Lipofectamine 2000 Reagent (Invitrogen).

Procedure

The following steps were performed for each selected clone.

1. One day before transfection, SH-SY5Y cells were plated in two 4 cm^2 wells (12-well plate) in presence of 1 ml of growth medium without antibiotics so that cells were 90-95% confluent at the time of transfection.
2. The DNA-Lipofectamine 2000 complexes were prepared as follows:
   a. 1 µg of **pcDNA4/TO/*lacZ* vector** were diluted in 100 µl of Opti-MEM I Reduced Serum Medium (GIBCO). The dilution was mixed gently.
   b. 2,8 µl of Lipofectamine 2000 were diluted in 100 µl of Opti-MEM I Medium. The dilution was mixed gently and incubated for 5 minutes at room temperature.
   c. After the 5 minute incubation, the diluted DNA was combined with the diluted Lipofectamine 2000. The solution was mixed gently and incubated for 20 minutes at room temperature to allow the DNA-Lipofectamine 2000 complexes to form.
3. The complexes were added to each of the two 4 cm^2 wells containing cells and medium. The medium was mixed gently by rocking the flask back and forth.
4. The cells were incubated at 37°C in a CO_2 incubator for 24 hours.
5. After a 24 hour incubation, **doxycycline hyclate** (2 µg/ml or 3.9 µM) (SIGMA, D9891) was added into 1 of the 2 wells and cells were incubated at 37°C in a CO_2 incubator for 24 hours more.

Note: Instead of tetracycline, doxycycline has been used as inducing agent in all of gene expression experiments. Doxycycline is similar to tetracycline in its mechanism of action, as suggested from pSUPER manufacturer.

6. The following day, the ß-galactosidase assay was performed.

ß-galactosidase assay

ß-galactosidase, encoded by *LacZ* gene of *E.coli* (contained in the pcDNA4/TO/*lacZ* vector), is an enzyme that catalyzes the hydrolysis of ß-galactosides, including lactose and the galatoside analog chlorophenol red-ß-D-galactopyranoside (CPRG). The ß-galactosidase gene functions well as a reporter gene because the protein product is extremely stable, resistant to proteolytic degradation in cellular lysates, and easily assayed. When

cleaved by ß-galactosidase, catalytic hydrolysis of the colorless CPRG substrate, yields a dark red water-soluble product (Fig. 2.8). The levels of active ß-galactosidase can then be measured by a microtiter plate reader or spectrophotometer (absorbance at λ= 560-595 nm).

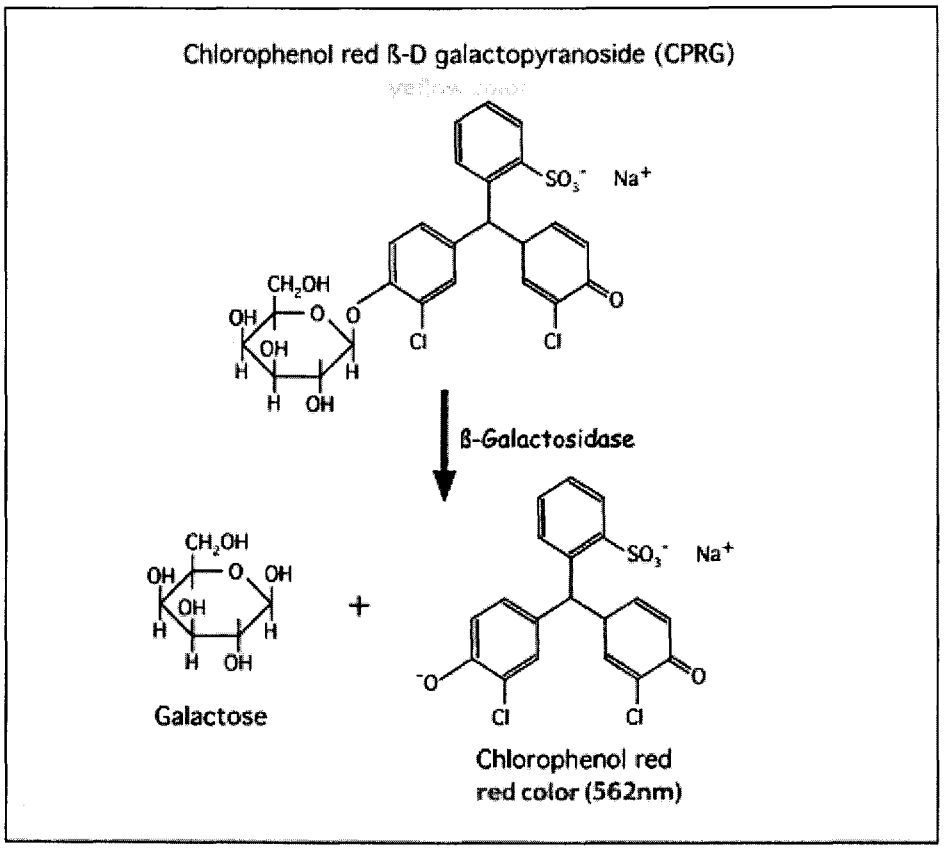


Fig. 2.8: cleavage of clorophenol red ß-D-galactopyranoside (CPRG) by ß-galactosidase: a dark red water soluble product is yielded.

Procedure:

The following steps were performed for the cells contained in each of two 4-cm^2 wells (both induced and uninduced cells by doxycycline), in which every clone was plated.

1. Tranfected cells were washed with 300 µl of 1× PBS.
2. The PBS was aspirate from the dish.
3. 500 µl of PBS was added into the well.
4. Cells were gently scraped down and then put in a eppendorf tube.
5. The tube was centrifuged at 12000 rpm for 1 minute.
6. The supernatant was removed and the pellet was resuspended with PBS (the PBS volume depends on the amount of the pellet).

7. The tube (containing pellet with PBS) was incubated on dry ice for 5 minutes and then directly in a water bath at 37°C for 5 minutes. This step was repeated 3 consecutive times.
8. The tube was centrifuged at 12000 rpm for 20 minutes at 4°C.
9. The supernatant was collected and 5 µl were used for protein concentration determination by Bicinconinc Acid Method (see paragraph 2.8). The remaining cell lysate was used in the **ß-galactosidase assay**.

Reagents required by ß-galactosidase assay:

The Reaction Buffer was prepared by mixing the following components:

- 80 mM NaH_2PO_4/ Na_2HPO_4, pH=7.3
- 9 mM $MgCl_2$
- 104 mM ß-Mercaptoethanol

The chlorophenol red-ß-D-galactopyranoside (CPRG) (Boehringer Mannheim/ROCHE, 10884308001) was used at a concentration of 80 mM.

10. Blank and samples were loaded in a 96-well plate:
    - the blank consisted of 100 µl of Reaction Buffer and 80 µl of PBS;
    - the samples were consisted of 100 µl of Reaction Buffer, 60 µl of PBS and 20 µl of cell lysate.

11. 20 µl of CPRG (80 mM) were added into blank and sample wells.
12. The reaction mixture was let incubate at room temperature until the color changed from yellow to red.
13. The blank and sample absorbance was read by a microtiter plate reader (BECKMAN COULTER) at 562 nm, every 5 minutes, starting from 0 minutes to 30 minutes after the beginning of the reaction.
14. The absorbance values showed the ß-galactosidase activity of each sample. They were normalized for the absorbance at 562 nm relative to the amount of total protein of the sample (obtained by bicinconinic acid method).
15. The ratio between induced and uninduced sample was calculated. This value met the ß-galactosidase activity increase of induced clone compared to uninduced one.
16. The clone which shown the highest ratio between induced and uninduced sample, was chosen. In fact, this clone should express the highest levels of Tet repressor in absence of doxycycline.

17. The clone chosen was used for transfecting the pSUPERIOR vector containing the siRNA-expressing sequence targeting *MRP1* and the pSUPERIOR vector containing the siRNA-expressing sequence targeting *MDR1*, in order to obtain clones showing a high level of *MRP1*/MRP1 suppression and clones showing a high level of *MDR1*/MDR1suppression.

Before transfection, the sensitivity of SH-SY5Y cells to puromycin (as the pSUPERIOR vector contains the puromycin resistance gene) was tested.

Determination of puromycin sensitivity

As for the blasticidin, the determination of puromycin sensitivity was performed estabilishing a kill curve for puromycin to identify its optimal effective dose. It is the lowest level of antibiotic that kills untransfected cells.

Procedure:

- One day before adding puromycin, SH-SY5Y cells were plated in the following plates:
  - 24-well plate in growth medium (without puromycin), so that cells were 35% confluent at the time of puromycin addition;
  - 24-well plate in growth medium (without puromycin), so that cells were 70% confluent at the time puromycin addition;
  - 10 cm plate in growth medium (without puromycin), at a concentration of 3000 cells/ml;
  - 10 cm plate in growth medium (without puromycin), at a concentration of 1500 cells/ml;

- The next day, the culture medium was substituted with medium containing the following concentrations of puromycin dihydrochloride (SIGMA, P8833): 0, 0.1, 0.2, 0.4, 0.8, 1, 2, 4, 6, 8 and 10 µg/ml.

- The selective medium was replenished every 3-4 days.

Cells sensitive to puromycin appeared rounded up and detached from the plate.

- 5 days after the addition of puromycin:
  - the medium was removed;
  - the cells were washed by PBS;

- 250 µl of methylene blue hydrate (SIGMA, MB1) dissolved in methanol (C. Erba, 414816) (w/v = 0.5%) were added into the wells;
- cells were incubated for 20 minutes at room temperature;
- the methylene blue was removed;
- cells were washed by PBS;
- the number of viable cells was counted by Bürker camera under an inverted microscope (Nikon) to determine the appropriate concentration of puromycin that prevents growth within 5 days after addition of the antibiotic.

Once the appropriate puromycin concentration to use for selection was determined, a stable cell line expressing costitutively the Tet repressor from pcDNA6/TR vector and inducibly the siRNA from pSUPERIOR vector, could be generated.

2.9.6 Step Six: Transfection of recombinant pSUPERIOR vector

The transfections of pSUPERIOR vector containing the siRNA-expressing sequence targeting *MRP1* and pSUPERIOR vector containing the siRNA-expressing sequence targeting *MDR1* [both vectors were sequenced and then extracted from *E.coli XL1* by JETSTAR Plasmid Midiprep kit (GENOMED)] were performed utilizing Lipofectamine 2000 (Invitrogen) and according to the manufacturer's instructions.

Procedure

The following steps were carried out for the transfection of both pSUPERIOR vector containing the siRNA-expressing sequence targeting *MRP1* and pSUPERIOR vector containing the siRNA-expressing sequence targeting, transfected in cells deriving from the same clone.

1. One day before transfection, the clone of SH-SY5Y cells, transfected by pcDNA6/TR vector and selected to express costitutively the highest Tet repressor level, was plated in a T25 flask in presence of 5 ml of growth medium without antibiotics so that cells were 90-95% confluent at the time of transfection.
2. The DNA-Lipofectamine 2000 complexes were prepared as follows:
   a. 8 µg of recombinant pSUPERIOR vector were diluted in 500 µl of Opti-MEM I Reduced Serum Medium (GIBCO). The dilution was mixed gently.
   b. 20 µl of Lipofectamine 2000 were diluted in 500 µl of Opti-MEM I Medium. The dilution was mixed gently and incubated for 5 minutes at room temperature.

c. After a 5 minute incubation, the diluted DNA was combined with the diluted Lipofectamine 2000. The solution was mixed gently and incubated for 20 minutes at room temperature to allow the DNA-Lipofectamine 2000 complexes to form.

3. The complexes were added to the T25 flask containing cells and medium. The medium was mixed gently by rocking the flask back and forth.
4. The cells were incubated at 37°C in a CO_2 incubator for 48 hours prior to testing for transgene expression.

2.9.5.7 Step Seven: Selection of stable transfectants

1. After a 48 hour incubation, cells were dissociated from the culture flask by trypsin and plated in several 10 cm plates at a concentration of 3000 cells/ml.
2. The following day, **puromycin dihydrochloride** (SIGMA) (0,8 µg/ml) was added into the medium.
3. The selective medium was replenished every 3-4 days until puromycin-resistant colonies were detected.

   2 untransfected control cultures were included:

   - 1 untransfected control culture was subjected to puromycin selection to check for cells that spontaneously became or were already antibiotic resistant; it was helpful to determine the effectiveness of the transfection and selection. Cells were plated at two different concentrations: 3000 cells/ml (as the transfected cells) and 15000 cells/ml. This was useful to assay the puromycin selection at different cell confluences.
   - 1 untransfected control culture was grown without puromycin selection as a positive control for cell viability. Cells were plated at two different concentrations: 3000 cells/ml (as the transfected cells) and 15000 cells/ml.

   2 transfected control culture was included:

   - 1 transfected control culture was grown without puromycin selection to check a possible toxicity of the transfection. Cells were plated at a concentration of 3000 cells/ml.
   - 1 transfected control culture was grown with puromycin selection. Cells were plated at a concentration of 15000 cells/ml. It was to check a possible toxicity of the puromycin.

4. The positive colonies were picked and expanded.

   In order to isolate single colonies, a sterile glass ring was put on the culture plate to surround each clone, before scraping the adherent cells. The ring could stick on the plate because sterile vaseline was put downside. The cells were

dissociated from the culture plate by trypsin (EuroClone) and every isolated clone was plated in a 2 cm^2 well (24-wells plate). The clones were expanded in a growth medium that always contained blasticidin and puromycin, even if all of the cells in the untransfected control culture were killed.

pSUPERIOR-transfected cells that survive antibiotic selection may not have a significant reduction in expression of the target gene. Instead, they may have found a way to mitigate the effects of a reduction in the target gene expression by compensating in another fashion or by shutting down expression of the siRNA. Therefore, isolated clones can be screened to identify the cells that cause the desired reduction in target gene expression.

In order to induce the derepression mechanism that causes a conformational change in the Tet repressor and renders it unable to bind to the Tet operator in the promoter of the inducible expression vector, the inducing agent **doxycycline** was added into the growth medium of the SH-SY5Y clones. This allowed the transcription of siRNA-expressing sequence.

2.9.5.8 Step Eight: Induction with doxycycline

1. Cells were plated in growth medium without antibiotics.
2. Before induction, the medium was removed and fresh medium containing doxycycline (2 µg/ml) was added to the cells. A sample of cells, grown in a medium without doxycycline, was included in order to compare the differences between induced and uninduced samples.
3. Cells were incubated at 37°C in a CO_2 incubator for at least 24 hours.
   As doxycycline has a 48 hour half-life, a second addition of the same doxycycline amount was carried out, if the incubation time was longer than 2 days.
4. Both induced and uninduced cells were harvested to assay for expression of the gene and protein or used for specific treatment.

Measurement of siRNA-induced silencing

The level of suppression of the target gene was measured by using different techniques:

• To determine the amount of protein expressed by the gene, a **Western Blot** analysis was performed.

- For a measurement of the mRNA transcript of the target gene, **quantitative RT-PCR** was used.
- To investigate the efficiency of the target proteins in the transport of different compounds, MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay was performed.

2.10 MTT ASSAY

MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay is based on the ability of a mitochondrial dehydrogenase enzyme from viable cells to cleave the tetrazolium rings of the pale yellow MTT and form a dark blue formazan crystals which is largely impermeable to cell membranes, thus resulting in its accumulation within healthy cells (Fig. 2.9). Solubilisation of the cells by the addition of a detergent results in the liberation of the crystals which are solubilized. The number of surviving cells is directly proportional to the level of the formazan product created. The color can then be quantified using a colorimetric assay based on the ability of the viable cells to reduce a soluble yellow tetrazolium salt to blue formazan crystals. The results can be read on a multiwell scanning spectrophotometer [17-20].

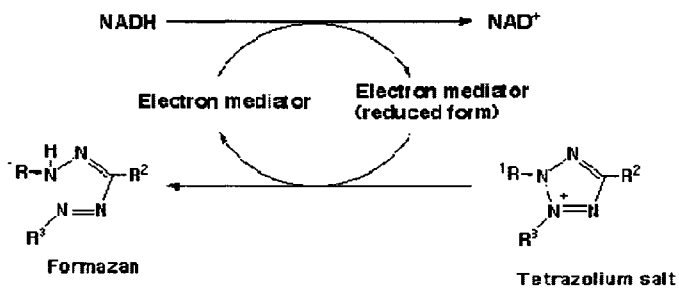


Fig 2.9 MTT reduction

The MTT assay was performed on the stable SH-SY5Y cells expressing costitutively the Tet repressor from pcDNA6/TR vector and inducibly the siRNA from pSUPERIOR vector containing the siRNA-expressing sequence targeting *MRP1* and pSUPERIOR vector containing the siRNA-expressing sequence targeting *MDR1.* The 2 recombinant pSUPERIOR vectors were transfected in different cells of the same clone (see paragraph 2.9)

In order to investigate the drug citotoxicity in SH-SY5Y cells siRNA-expressing sequence targeting *MRP1*, drug treatment was performed 3 days after induction (by doxycycline) of siRNA-expressing sequence transcription.

In order to investigate the drug citotoxicity in SH-SY5Y cells siRNA-expressing sequence targeting *MDR1*, drug treatment was performed 2 days after induction (by doxycycline) of siRNA-expressing sequence transcription.

3 days and 2 days were suggested as the best assay time for observing the silencing of MRP1 and MDR1, respectively (see *Results*).

Procedure:

- SH-SY5Y cells were seeded onto 96-well plates in 100 µl of complete medium in presence of the antibiotics causing selection: 5 µg/ml Blasticidine S hydrochloride (SIGMA, 15205) and 0,8 µg/ml Puromycin dihydrochloride (SIGMA, P8833).
- 24 hours after the sowing, doxycycline hyclate (SIGMA, D9891) was added into the wells of the induced samples. For each induced sample, an uninduced sample was loaded. Each treatment was performed on four samples.
- 2 days (for SH-SY5Y cells siRNA-expressing sequence targeting *MDR1*) or 3 days (SH-SY5Y cells siRNA-expressing sequence targeting *MRP1*) after induction by doxycycline, cells were treated by adding 100 µl of complete medium containing the test compound at the appropriate concentrations*.
  4 well of induced samples and 4 wells of uninduced samples that weren't treated with drug, were included as control.
- After 24 hrs of treatment, the compound was removed and the MTT dye, 20 µl per 200 µl of medium of a 5 mg/ml solution, was added and plates were incubated for 2 hrs at 37 °C in the dark.
- Absorbance was measured, after removing MTT solution and dissolving the blue formazan crystals with DMSO (200 µM) (Sigma D5879), at 562 nm using a microtiter plate reader (BECKMAN COULTER).

* The cells were treated with the following drugs:
  - Indomethacin (Liomtacen, Promedica)
  - Doxycycline hyclate (SIGMA, D9891)
  - Ceftriaxone (FIDATO, Fidia)

These compounds were chosen among the substrates of MRP1 and/or MDR1. Particularly, Indometacin is substrate of MRP1, but not of MDR1, Cefriaxone is substrate of MDR1, but not of MRP1 and Doxycycline is substrate of both MRP1 and MDR1 (see *Results*).

The starting solution of the compound **Indomethacin** was prepared dissolving 50 mg of lyophilised powder into 1.4 ml of sterile physiological solution, obtaining a 100 mM concentration. Final tested concentrations were: 0.5 mM; 1 mM; 2 mM and 3 mM.

The starting solution of the compound **Ceftriaxone** was prepared dissolving 100 mg of lyophilised powder into 5 ml of sterile physiological solution, obtaining a 20 mg/ml concentration. Final tested concentrations were: 0.1 mg/ml; 1 mg/ml; 5 mg/ml; 8 mg/ml and 15 mg/ml.

The starting solution of the compound **Doxycycline** was prepared dissolving 12 mg of lyophilised powder into 6 ml of sterile physiological solution, obtaining a 100 mM solution. Final tested concentrations were: 0.8 mg/ml; 1.2 mg/ml; 1.6 mg/ml.

The final tested concentrations were chosen after assaying a large range of drug concentrations and they were the more significance ones to observe the decreasing of the cell viability due to the increasing of the drug amount.

The drug citotoxicity effect was correlated to the functional activity of MRP1 and MDR1 transporters to extrude the drugs out of the cells.

Data are expressed as mean ± SD. Statistical analysis was performed by Student "t" test and a p value less than 0.05 was considered statistically significant.

2.11 [^{3}H] BILIRUBIN UPTAKE IN CULTURED CELLS

[^{3}H] Bilirubin uptake was performed on the stable SH-SY5Y cells expressing costitutively the Tet repressor from pcDNA6/TR vector and inducibly the siRNA from pSUPERIOR vector containing the siRNA-expressing sequence targeting *MRP1* and pSUPERIOR vector containing the siRNA-expressing sequence targeting *MDR1*.

The 2 recombinant pSUPERIOR vectors were transfected in different cells of the same clone. (see paragraph 2.9)

In order to investigate the [^{3}H]Bilirubin uptake in SH-SY5Y cells siRNA-expressing sequence targeting *MRP1*, the uptake experiment was performed 3 days after induction (by doxycycline) of siRNA-expressing sequence transcription.

In order to investigate the [^{3}H]Bilirubin uptake in SH-SY5Y cells siRNA-expressing sequence targeting *MDR1*, an experiment was performed after the following treatment: after a 48 hour induction (by 3.9 μM doxycycline,) of siRNA-expressing sequence transcription, the medium containing doxycycline was removed, washed by PBS, and incubated in fresh medium without doxycycline, for 48 hours more (see *Results*).

Loading and Washing out Procedure:

1. One day before performing the uptake experiment, SH-SY5Y cells were plated at a suitable concentration to obtain 70% surface confluence in a 4 cm^2 well (12-well plate). Cells were grown in complete medium together with antibiotics causing: 5 μg/ml Blasticidine S hydrochloride (SIGMA, 15205) and 0,8 μg/ml Puromycin dihydrochloride (SIGMA, P8833), under 5% CO_2. Each sample was performed in quadruplicate—3 for radiolabel counts, 1 for protein content (without radiolabel).
2. 125 μg of [^{3}H]-UCB [previously dissolved in 40 μL of Dimethyl sulfoxide (DMSO, Sigma D2438)] were diluted in culture medium supplemented with 15% Fetal Bovine Serum (FBS) (SIGMA, F7524) containing an albumin concentration of 54 μM. The unbound "free" bilirubin concentration (B_f) was 40 nM and it was determined as previously described by Roca et al. [21].
3. A sample of 10 μL was taken from culture medium containing [^{3}H]UCB-Albumin to measure the final bilirubin concentration.

$$(Dpm/\mu L)*10^6 / (MW_{UCB} \times \text{Specific Act.}) = \mu M \text{ UCB}$$

d.p.m.: disintegrations per minute

Specific Activity units: Dpm/μg

4. After removing medium, cells were washed with PBS.
5. **Loading**: A medium containing [^{3}H]-bilirubin 10 µM/BSA 10 µM (BSA concentration contained in the serum was assessed) was added to each well and cells were incubated for 30 minutes.
6. After bilirubin exposure, the cells were carefully washed with PBS to stop the bilirubin uptake.
7. **Export phase**: cells were incubated for washout in medium without serum, containing 1% BSA (1 sample (in triplicate) wasn't treated with washout solution).
8. The samples were collected immediately after uptake and after 30 min and 60 min in washout conditions.
9. **To measure cellular radioactivity**, cells of each samples were washed with PBS, detached from plated with 0.1 M NaOH, plated in a vial, mixed with 8 ml of liquid scintillation cocktail (Filter counter Nº 6013149, Packard Bioscience, Groningen, The Netherlands) and placed into liquid scintillation counter *BetaCounter* (Betamatic V, Kontron, Milan, Italy) for d.p.m. (disintegrations per minute) reading. The software program "BILI" was used with the batch n°5. The cell counts were normalized by total cell protein content.

Uptake calculation:

$$\text{Dpm} \;/\; [((\text{Specific Act.}_{UCB} \times MW_{UCB}) / 10^6) \times ((\text{mg of cell protein/mL}) \times \text{X mL of lysed cells})] = \text{pmol/mg prot}$$

[^{3}H]-UCB Specific Activity: 29300 d.p.m.
[^{3}H]-UCB concentration: 10,9 µM

2.12 REFERENCES

1 Elbashir SM, Martinez J, Patkaniowska A, Lendeckel W, Tuschl T (2001) Functional anatomy of siRNAs for mediating efficient RNAi in Drosophila melanogaster embryo lysate. EMBO J 20(23), 6877–88.

2 Wu H, Hait WN, Yang JM (2003) Small interfering RNA-induced suppression of MDR1 (P-Glycoprotein) restores sensitivity to multidrug-resistant cancer cells. Cancer Res 63, 1515-1519.

3 Fernetti C, Pascolo L, Podda E, Gennaro R, Stebel M, Tiribelli C (2001) Preparation of an antibody recognizing both human and rodent MRP1, Biochem Biophys Res Commun 288, 1064–1068.

4 Hillen W, Berens C (1994) Mechanisms underlying expression of Tn*10* encoded tetracycline resistance. Annu Rev Microbiol 48, 345-369.

5 Hillen W, Gatz C, Altshmied L, Schollmeier K, Meier I (1983) Control of expression of the Tn*10*-encoded tetracycline resistance genes: equilibrium and kinetic investigations of the regulatory reactions. J Mol Biol 169, 707-721.

6 Yao F, Svensjo T, Winkler T, Lu M, Eriksson C, Erikkson E (1998) Tetracycline repressor, tetR, rather than the tetR-mammalian cell tarnscriprion factor fusion derivatives, regulates inducible gene expression in mammalian cells. Hum Gene Ther 9, 1939-1950.

7 Gossen M, Bujard H (1992) Tight control of gene expression in mammalian cells by tetracycline-responsive promoters. Proc Natl Acad Sci USA 89, 5547-5551.

8 Anderson S, Davis DL, Dahlback H, Jornvall H, Russell DW (1989) Cloning, structure and expression of the mithocondrial cytochrome P-450 sterol 26-hydroxylase, a bile acid biosynthetic enzyme. J Biol Chem 264, 8222-8229.

9 Boshart M, Weber F, Jahn G, Dorsh-Hasler K, Fleckenstein B, Schaffner W (1985) A very strong enhancer is located upstream of an immediate early gene of human cytomegalovirus. Cell 41, 521-530.

10 Nelson JA, Reynolds-Kohler C, Smith BA (1987) Negative and positive regulation by a short segment in the 5'-flanking region of the human cytomegalovirus major immediate-early gene. Mol Cell Biol 7, 4125-4129.

11 Postle K, Nguyen TT, Bertrand KP (1984) Nuceltide sequence of the repressor gene of the Tn*10* tetracycline resistance determinant. Nuc Acids Res 12, 4849-4863.

12 Van Ooyen A, van den Berg J, Mantei N, Weissmann C (1979) Comparison of total sequence of a cloned rabbit beta-globin gene and its flanking regions with a homologous mouse sequence. Science 206, 337-344.

13 Kimura M, Takatsuki A, yamaguchi I (1994) Blasticidin S deaminase gene from *Aspergillus terreus (BSD)*: a new drug resistance gene for transfection of mammalian cells. Biochim Biophys Acta 1219, 653-659.

14 Goodwin E C, Rottman, F M (1992) The 3′-Flanking Sequence of the Bovine Growth Hormone Gene Contains Novel Elements Required for Efficient and Accurate Polyadenylation. J Biol Chem 267, 16330-16334.

15 Drocourt D, Calmels TPG, Reynes JP, Baron M, Tiraby G (1990) Cassettes of the Streptoalloteichus hindustanus ble Gene for Transformation of Lower and Higher Eukaryotes to Phleomycin Resistance. Nuc Acids Res 18, 4009.

16 Mulsant P, Tiraby G, Kallerhoff J, Perret J (1988) Phleomycin Resistance as a Dominant Selectable Marker in CHO Cells. Somat Cell Mol Genet 14, 243-252.

17 Mosmann T (1983) Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods 65(1-2), 55-63.

18 Denizot F, Lang R (1986) Rapid colorimetric assay for cell growth and survival. Modifications to the tetrazolium dye procedure giving improved sensitivity. J Immunol Methods, 89, 271-277.

19 Niks M, Otto M (1990) Towards an optimized MTT assay. J Immunol Methods 130, 149-151.

20 Bernas T, Dobrucki J (2002) Mitochondrial and nonmitochondrial reduction of MTT: interaction of MTT with TMRE, JC-1, and NAO mitochondrial fluorescent probes. Cytometry 47, 236-242.

21 Roca L, Calligaris S, Wenneberg RP, Ahlfors C, Malik SG, Ostrow JD and Tiribelli C (2006) Factors affecting the binding of bilirubin to serum albumins: validation and application of the peroxidise method. Pediatr Res 60, 724-728.

CHAPTER 3

RESULTS

3.1 RNA INTERFERENCE BY SYNTHETIC siRNA

siRNAs (small interference RNAs) can be synthesized by *in vitro* transcription with T7 RNA polymerase, providing an economical alternative to chemical synthesis of siRNAs.

In order to find out the sequences silencing *MRP1* and *MDR1*, some 21-nt RNAs were produced by *in vitro* transcription using siRNA Construction kit (Ambion, cat. 1620). The sense and antisense siRNA templates were transcribed by T7 RNA polymerase and the resulting RNA transcripts were hybridized to create dsRNA with 3' terminal uridine dimers (see *Materials and Methods* for details of synthesis, purification and quantitation).

Attempting the *MDR1* silencing, the *in vitro* transcription of a sequence published in 2003 [1] was performed and the siRNAs were introduced into cells via transient transfection. Attempting the *MRP1* silencing, 5 siRNAs were designed and synthesized, and pilot experiments were performed to determine the most effective one.

The post-transfection incubation time chosen to assess the silencing effect of the siRNAs targeting *MDR1* and *MRP1* was already used in some experiments described in literature [1-3] .

Fig. 3.1a shows a 30% reduction of *MDR1* expression detected by RT-Real Time PCR in HepG2 cells 24 hours after the transient transfection with siRNAs compared to controls (untransfected cells) ($p<0.05$). In HeLa cells (Fig. 3.1b) the reduction was higher (about 50%, $p<0.05$). The ability of the 5 double strand RNAs targeting *MRP1* to function as siRNAs was tested by 5 separate transient transfections into HepG2 cells. As it is represented in Fig. 3.2, the RT-Real Time PCR analysis, performed 48 hours after siRNA transfection, showed that none of the 5 sequences were able to modulate the *MRP1* expression ($p>0.2$), just a small reduction was detectable by the siRNA1 transfection.

These first results confirmed that the siRNA targeting *MDR1* could modulate the endogenous *MDR1* mRNA of HepG2 and HeLa cells 24 hours after transfection, while a siRNA sequence able to reduce the *MRP1* expression had still to be found.

In the meantime, Ambion Company validated some *MRP1* silencing siRNAs and one of them (*Silencer* Validated *MRP1*-siRNA, 5132-1651) was bought and tested by us in HeLa cells.

The quantitative evaluation of gene expression, performed 48 hours after siRNA transfection, showed a 50% reduction of the *MRP1* level in siRNA-transfected cells compared to controls (Fig. 3.3).

The *MRP1*-siRNA effect on the protein expression, 72 hours after siRNA transfection, was also assayed. Western Blot analysis permitted to detect an evident reduction of MRP1 expression in siRNA-transfected cells in respect of control, in every considered cell lines (HeLa, MDCK, HCT116 and SH-SY5Y) (figg. 4 and 5).

As for *MRP1* gene, the post-transfection incubation time chosen to assess the silencing effect of siRNAs on the MRP1 protein was already used in silencing studies described in literature [3].

3.2 VECTOR SYSTEM FOR INDUCIBLE EXPRESSION OF siRNA

From these preliminary experiments, the sequences able to modulate the *MRP1* and *MDR1* expression were selected.

Our aim was to apply the siRNA technology to create a model allowing us to perform protein function studies, by exposing cells showing a knockdown of MRP1 or MDR1 expression, to toxic compounds and comparing the effects caused in this cells with the ones caused in the same cells expressing endogenous MRP1 or MDR1 levels.

For this purpose, the sequences coding the selected siRNAs were put into a vector with an inducible promoter (the circular pSUPERIOR.puro vector OligoEngine, VEC-IND-0006). It should allow us to observe long-term effects of siRNA and to follow the cell behaviour after removing the agent able to trigger the siRNA transcription.

Fig. 3.6 shows the electrophoresis result of the pSUPERIOR.puro vector linearization products. They were obtained by the vector incubation with the restriction enzymes BglII and HindIII contemporaneously and separately. In order to check whether the digestion was complete or not, undigested pSUPERIOR.puro vector was also loaded into the gel.

Generally, if the digestion is complete, in the lane of the digested vector should be visualized a unique band running more slowly than undigested vector. Any other band detectable in that lane represent different supercoil forms.

Fig 6 shows that in every digested vector lanes, an unique band is present. In the gel it is located at a lower position than undigested vector. That demonstrated that the restriction enzymes BglII and HindIII digested the vector completely.

The ligation in pSUPERIOR.puro of the inserts coding the siRNA targeting *MRP1* and *MDR1* respectively was performed.

The ligation products were cloned in *E.coli Xl1.*

10 colonies were isolated on the plate of bacteria transformed by pSUPERIOR containing oligos targeting *MRP1* and 4 colonies were isolated on the plate of

bacteria transformed by pSUPERIOR, containing oligos targeting *MDR1*. No colonies were grown on the control plate.
The recombinant vectors were extracted from the colonies and linearized by EcoRI and XhoI.

Fig. 3.7 shows the electrophoresis of the products obtained by the ligation in pSUPERIOR.puro of the inserts coding the siRNA targeting *MRP1* and *MDR1* respectively, and linearized by EcoRI and XhoI.
The pSUPERIOR.puro vector without insert linearized by EcoRI and XhoI should have 248 pb, while the pSUPERIOR.puro vector with insert linearized by EcoRI and XhoI should have 281 pb (see *Materials and Methods*).
Consequently, the electrophoresis results made possible to recognize 5 vectors containing the insert targeting *MRP1* (vectors loaded in the lanes: 6, 7, 8, 11 and 12 of the agarose gel) (Fig. 3.7) and 2 vectors containing the insert targeting *MDR1* (vectors loaded in the lanes: 14 and 16 of the agarose gel) (Fig. 3.7).

Among these vectors, 2 were chosen (vector loaded in the lanes 11 and 16 of the gel represented in Fig. 3.7) and extracted from bacteria using a system that permitted to isolate high quality DNA. They were loaded into an agarose gel. The eletrophoresis results are shown in Fig. 3.8.

To be sure that the oligos coding the siRNAs targeting MRP1 or MDR1 were inserted correctly, the sequencing of the 2 recombinant vector was performed (see *Materials and Methods*).

The sequencing results analysed by BLAST (Basic Local Alignment Search Tool) showed a correct insertion of the oligos targeting *MDR1*, but not the ones targeting *MRP1*. Another recombinant vector containing oligos targeting *MRP1* (vector loaded in the lane 6 of the agarose gel, Fig. 3.7) was sequenced. The BLAST analysis confirmed that the oligos of interest were inserted correctly.
Extractions of the selected recombinant vectors were performed on large scale.

3.3 β-GALACTOSIDASE ASSAY

In such a vector system for the inducible expression of small interfering RNA, in addition to pSUPERIOR, pcDNA6/TR vector (a Tet repressor expressing vector) was required. It had to be transfected into cells of interest and the clone that expressed the highest level of repressor had to be selected by a transient tranfection of the pcDNA4/TO/*lacZ* vector. Lac Z gene encodes ß-galactosidase, an enzyme that catalyzes the hydrolysis of ß-galactosides, including the galatoside analog chlorophenol red-ß-D-galactopyranoside (CPRG). When cleaved by ß-galactosidase, catalytic hydrolysis of the colorless CPRG substrate yields a dark red water-soluble product. The levels of active ß-galactosidase in each clone could be

measured by a microtiter plate reader at λ=562 nm (β-galactosidase assay). The synthesis of ß-galactosidase was induced by adding doxycycline (3.9 μM) into the cell culture medium (see *Materials and Methods*).

In particular, our studies have been performed in SH-SY5Y neuroblastoma cells.

Tables 1, 2 and 3 report the β-galactosidase activity values of 17 SH-SY5Y clones, which proved to be positive after the transfection of the pcDNA6/TR vector and selection by blasticidin (a kill curve was established in order to determine the optimal blasticidin dose for selecting positive clones: it was 0.5 μg/ml) (see *Materials and Methods*). The β-galactosidase activity was assessed for both induced (siRNA synthesis induced by adding doxycycline) and uninduced (without adding doxycycline) sample of each clone. Blank absorbance was also assessed. The absorbance was measured every 5 minutes for 30 minutes to avoid the values of a reaction not occurred yet or already at plateau. The blank absorbance was subtracted from the each sample and the resulting absorbance was normalized by its protein concentration (table 4). The ratio of the induced sample absorbance to the uninduced sample absorbance was calculated (table 5 a, b). It shows how many times the clone β-galactosidase activity increases thanks to the induction by doxycycline. The clone showing the maximum increase was chosen. It proved to be able to synthesize the highest level of Tet repressor, in absence of doxycycline. Among the 17 clones tested, clone 81 was chosen and the transfections of the pSUPERIOR vector containing the siRNA-expressing sequence targeting *MRP1* and pSUPERIOR vector containing the siRNA-expressing sequence targeting *MDR1* were performed. As the pSUPERIOR.puro vector contains the gene coding the puromycin resistance, before performing the transfections a kill curve was estabilished to identify the lowest puromycin dose that killed non-transfected cells within approximately 5 days: it was 0.8 μg/ml.

Several positive stable SH-SY5Y clones expressing costitutively the Tet repressor from pcDNA6/TR vector and inducibly the siRNA targeting *MRP1* or *MDR1* from pSUPERIOR vector, were isolated. However, five clones containing the sequences coding the siRNA targeting *MRP1* (*MRP1*-pSUPERIOR) (clones 4, 5, 9, 10, 14) and three clones containing the sequences coding the siRNA targeting *MDR1* (*MDR1*-pSUPERIOR) (clones 2, 33, 34) were considered.

Among these clones, the one showing the highest level of the target gene/protein suppression (induced clone compared to uninduced clone) had to be found out.

Initial attempts to detect an evident reduction of *MRP1*/MRP1 and *MDR1*/ MDR1 in the mentioned clones by RT-Real time PCR and Western Blot failed. The reason was discovered later.

3.4 FUNCTIONAL ANALYSIS

In the meantime, another method for screening the clones had to be found out. As MRP1 and MDR1 are transporters able to take a variety of compounds out of the cells, we focused our attention on performing citotoxicity assays in the presence of drugs, MRP1 or MDR1 substrates. This should give us information about the functional activity level of the target proteins. In fact we expected that, after exposing to the toxic substrates of MRP1 or MDR1, the viability of the induced sample of a clone had to be lower than the uninduced sample. This is why cells expressing lower levels of MRP1 or MDR1 should have a reduced ability to extrude the substrates of these proteins and were, therefore, more susceptible to the citotoxicity effect of those compounds.

Fig. 3.9 shows the results of MTT assay performed in MRP1-pSUPERIOR clones. After a 72 hour incubation of the doxycycline-induced sample of each clone, the induced and uninduced samples were incubated with different amount of doxycycline (MRP1 and MDR1 substrate) and, separately, indomethacin (MRP1 substrate) for 24 hours. MTT reduction was assessed.

Making a comparison between the MTT metabolism of the induced sample and uninduced sample of a same clone, the results showed that:

MTT metabolism of clones 4, 9 and especially 10 was significantly (*p* values are in Fig. 3.9) affected by the induction of siRNA synthesis targeting *MRP1* (fig. 9 a, b, e, f, g, h), while MTT metabolism in the induced and uninduced samples of the clones 5 and 14 were comparable (Fig. 3.9 c, d, i, l)

Fig. 3.10 shows the results of MTT assay performed in MDR1-pSUPERIOR clones. After a 48 hour incubation of the doxycycline-induced sample of each clone, induced and uninduced samples were incubated with different amount of doxycycline (MRP1 and MDR1 substrate) and, separately, ceftriaxone (MDR1 substrate) for 24 hours. MTT reduction was assessed.

As regards as the MTT metabolism of the induced sample compared to uninduced sample of a same clone, the results showed that:

MTT metabolism of clones 2 and 33 was significantly (*p* values are in Fig. 3.10) affected by the induction of siRNA synthesis targeting *MDR1* (Fig. 3.10 a, b, c, d), while MTT metabolism in the induced and uninduced samples of the clones 34 was comparable (Fig. 3.10 e, f).

From the MTT assay results, clones 4, 9 and 10 (*MRP1*/MRP1-pSUPERIOR), and clone 2 and 33 (*MDR1*/MDR1-pSUPERIOR) showed the highest suppression of MRP1 and MDR1 level respectively.

3.5 GENE AND PROTEIN EXPRESSION ANALYSIS

Starting from these findings and focusing our attention on MRP1-pSUPERIOR clones, a quantitative evaluation of the *MRP1* expression in induced and uninduced samples of the clones 4, 5, 9, 10 and 14, was carried out. The results of the analysis by RT-Real Time PCR are represented in Fig. 3.11: after a 48 hour incubation with doxycycline, no significant differences in the *MRP1* expression between induced and uninduced samples of a same clone were detected, even in the clones 4, 9 and 10.

This observation suggested we should examine carefully the *MRP1*/MRP1 expression of the cells at different doxycycline incubation time.

Clone 10 was chosen to perform the next investigation. In order to assess the *MRP1* expression, the induced samples were incubated with doxycycline (3.9 μM) for 24, 48 and 72 hours. In addition, an induced sample was analysed 48 hours after doxycycline removing from cells previously incubated with doxycycline for 48 hours. Fig. 3.12 shows the *MRP1* expression level of the induced and uninduced sample detected by RT-Real Time PCR at every incubation time. In contrast with the results of the silencing experiments performed by synthetic siRNA in HeLa cells (Fig. 3.3), no reduction of *MRP1* expression was observed in the induced compared to uninduced sample after a 24 and 48 hour incubation with the inducing agent. Just after a 72 hour incubation with doxycycline, an evident reduction (about 45%) of the *MRP1* expression in the induced sample compared to the uninduced sample was detectable. The sample incubated with doxycycline for 48 hours and then without doxycycline for other 48 hours showed also an evident reduction (about 40%) compared to the uninduced sample. These results were confirmed by protein expression analysis performed by Western Blot (Fig. 3.13). Doxycycline (3.9 μM) was present in the medium of the induced samples for 24, 48 and 72 hours. In addition, an induced sample was analysed 72 hours after doxycycline removing from cells previously incubated with doxycycline for 72 hours. The protein analysis performed after a 24 or 48 hour incubation with doxycycline, showed a MRP1 expression of induced sample even higher than uninduced one, while the result obtained analysing the MRP1 expression after a 72 hour incubation showed an substantial change: the induced sample expressed a MRP1 level really lower than uninduced one, as visible by observing the bands and their quantification performed by Scion Image and Curver Expert softwares (Fig. 3.13).

The sample incubated in the presence of doxycycline for 72 hours and then in the absence of doxycycline for other 72 hours showed also a reduction compared to uninduced sample. In addition, a small *MRP1*/MRP1 up-regulation seemed occurred in the induced sample of the control (clone stably transfected by pcDNA6/TR vector, but not by pSUPERIOR vector, see *Materials and Methods*) compared to uninduced sample.

Fig. 3.14 shows the quantitative evaluation of *MDR1* expression in induced and uninduced samples of the clones 2, 33 and 34 (*MDR1*-pSUPERIOR). The analysis performed by RT-Real Time PCR after a 24 hour incubation of induced samples with doxycycline (3.9 μM), showed a 30% reduction of the *MDR1* expression in induced sample of clone 2 compared to uninduced sample. The clone 33 showed a 15% reduction of *MDR1* expression between induced and uninduced sample, and as regard to clone 34 even a small increment of *MDR1* expression was detectable in induced with respect of uninduced sample. The *MDR1* expression level of the control was clearly (25%) higher in the induced sample compared to the uninduced one.

In Fig. 3.15 the result of the protein expression analysis is shown. Induced samples of the clones 2, 33 and 34 were incubated with doxycycline for 48 hours before assessing the MDR1 expression. The band quantification performed by Scion Image and Curver Expert softwares showed an evident reduction of the MDR1 expression between induced and uninduced sample of the clone 33. This reduction was small in clone 2, while the clone 34 showed a MDR1 expression of the induced sample higher than the uninduced one. These results confirmed what came out from the MTT assay and RT-Real Time PCR for clone 34, while data obtained for clones 2 and 33 had to be clarified.

Clone 2 was chosen to make further investigations.

RT-Real Time PCR was used to measure the *MDR1* expression of clone 2 when the induced sample was incubated with doxycycline (3.9 μM) for 24 and 48 hours. In addition, *MDR1* expression level was assessed also when the induced sample was incubated with doxycycline (3.9 μM) for 24 hours and then without doxycycline for other 24 hours. The uninduced samples were analysed every time. In Fig. 3.16 the quantitative evaluation is represented. After a 24 hour incubation with doxycycline, induced sample showed a 30% *MDR1* level reduction compared to uninduced one. This difference halved in the analysis performed 48 hours after incubation with doxycycline, while it became higher than 50% when the induced sample, incubated with doxycycline for 24 hours and then without doxycycline for other 24 hours, was assessed.

The protein analysis (Fig. 3.17) confirmed the observation done in the gene analysis. The reduction of MDR1 expression of the induced sample compared to

the uninduced one was not significant after a 24 or 48 hour incubation with doxycycline (3.9 μM), but it became considerable if the induced sample was compared to the uninduced one after a 48 hour incubation with doxycycline followed by a 48 hour incubation without doxycycline.

3.6 [^{3}H]UCB-UPTAKE ASSAY

The confirmation that clone 10 (MRP1-pSUPERIOR) and 2 (MDR1-pSUPERIOR), induced by doxycycline, expressed a reduced level of MRP1 and MDR1 respectively, and the identification of the optimal time to observe the silencing, allowed us to perform an uptake assay to investigate the role of the MRP1 and MDR1 in the transport of unconjugated bilirubin (UCB). As shown in the Fig. 3.18, after an exposure to [^{3}H] UCB (B_f =40 nM) for 30 minutes, a clearly higher UCB amount was detected in the induced cells (after a 72 hour incubation with doxycycline) of clone 10 compared to the uninduced one ($p<0.0006$). On the contrary, after an exposure to [^{3}H] UCB (B_f =40 nM) for 30 minutes, the UCB level found out in the induced sample of clone 2 after a 48 hours incubation with doxycycline followed by a 48 hours incubation without doxycycline, was comparable to the one found out into the uninduced sample ($p>0.3$). It showed that MRP1, but not MDR1 could transport UCB having B_f =40 nM.

QUANTITATIVE GENE EXPRESSION ANALYSIS BY RT-REAL TIME PCR

Target gene: *MDR1*

Target region: 79-99

(Wu H, Hait WN, Yang JM (2003) Cancer Res 63(7), 1515-1519)

siRNA TRANSIENT TRANSFECTION

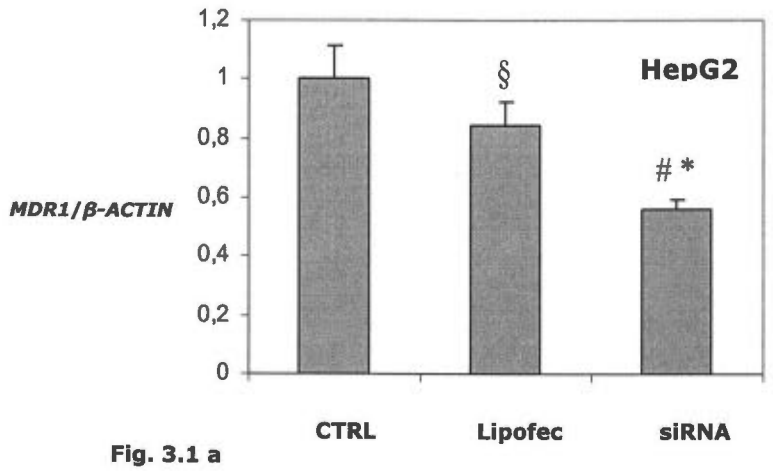


Fig. 3.1 a

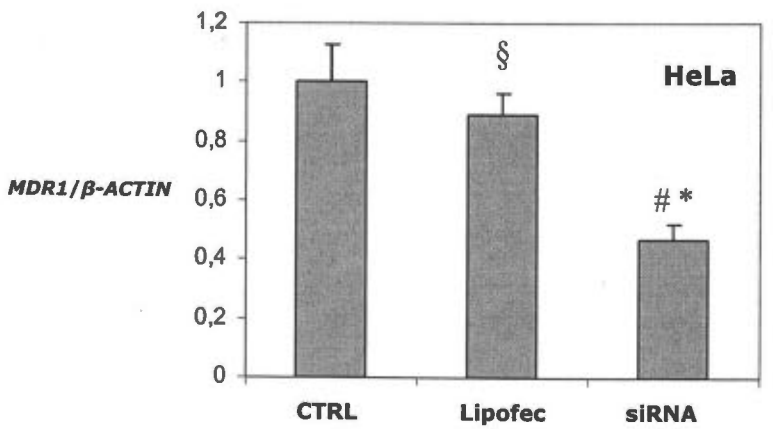


Fig. 3.1 b

Fig. 1.1 a, b **Quantitative evaluation of *MDR1* expression in HepG2 cells (fig. 1a) and Hela cells (fig. 1b) transiently tranfected by siRNA targeting *MDR1* (published sequence).** RT-Real Time PCR was performed normalizing *MDR1* expression values to housekeeping gene *β-ACTIN*. *MDR1* expression of transfected cells (siRNA) was analysed **24 hours** after transfection and compared with its controls (not transfected cells): CTRL (non treated cells) and Lipofec (cells treated with Lipofectamine 2000). *MDR1* expression was calculated relatively to *MDR1* level of CTRL, considered =1. Data are given as mean ± SD (bars). $^{\#}p < 0.05$ from CTRL; $^{*}p < 0.05$ from Lipofec; $^{\S}p > 0.4$ from CTRL.

Target gene: *MRP1*

Target regions: 299-319 (Sequence 1)
1469-1489 (Sequence 2)
1682-1702 (Sequence 3)
2867-2887 (Sequence 4)
4535-4555 (Sequence 5)

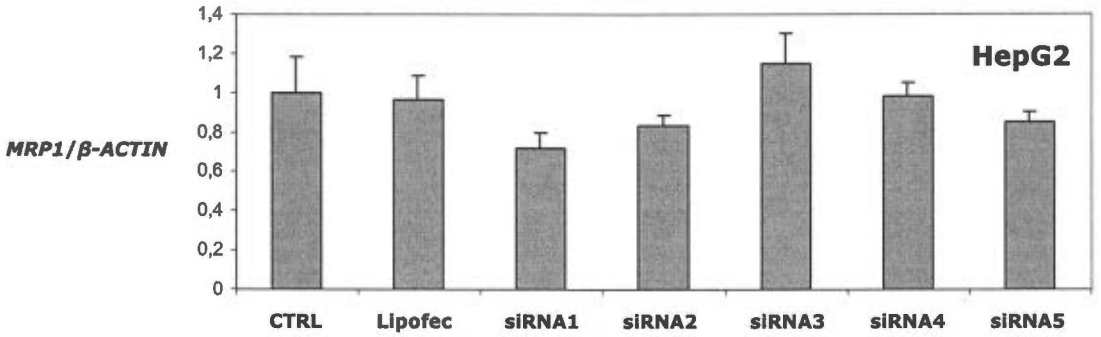


Fig. 3.2 Quantitative evaluation of *MRP1* expression in HepG2 cells transiently tranfected by siRNA targeting *MRP1* (sequences 1, 2, 3, 4, 5, designed and synthesized by myself). RT-Real Time PCR experiments were performed normalizing *MRP1* expression values to housekeeping gene *β-ACTIN*. *MRP1* expression of transfected cells (siRNA) was analysed **48 hours** after transfection and compared with its controls (not transfected cells): CTRL (not treated cells) and Lipofec (cells treated by Lipofectamine 2000). *MRP1* expression was calculated relatively to *MRP1* level of CTRL, considered=1. Data are given as means ± SD (bars). All p values > 0.2 from controls.

Silencer Validated MRP1- siRNA (Ambion 51321)

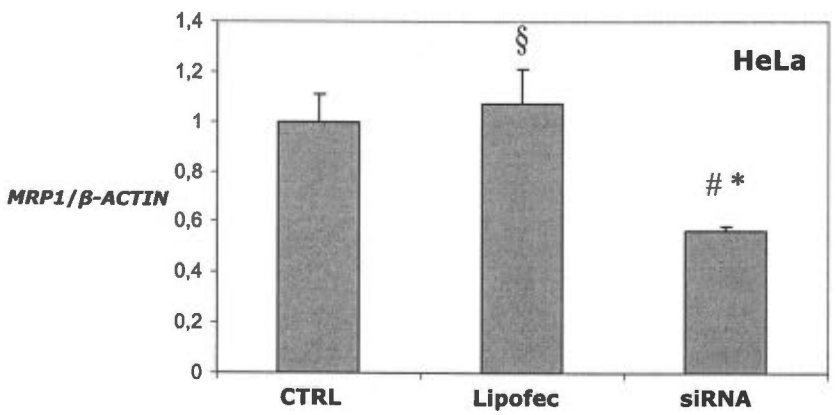


Fig. 3.3 Quantitative evaluation of *MRP1* expression in HeLa cells transiently tranfected by siRNA targeting *MRP1* (sequence designed, synthesized and validated by Ambion). RT-Real Time PCR experiments were performed normalizing *MRP1* expression values to housekeeping gene *β-ACTIN*. *MRP1* expression of transfected cells (siRNA) was analysed **48 hours** after transfection and compared with its controls (not transfected cells): CTRL (not treated cells) and Lipofec (cells treated by Lipofectamine 2000). *MRP1* expression was calculated relatively to *MRP1* level of CTRL, considered=1. Data are given as mean ± SD (bars). $^{\#}p < 0.02$ from CTRL; $^{*}p < 0.03$ from Lipofec; $^{\S}p > 0.5$ from CTRL.

PROTEIN EXPRESSION ANALYSIS BY WESTERN BLOT

Target protein: MRP1

***Silencer Validated siRNA* (Ambion, 51321)**

siRNA TRANSIENT TRANSFECTION

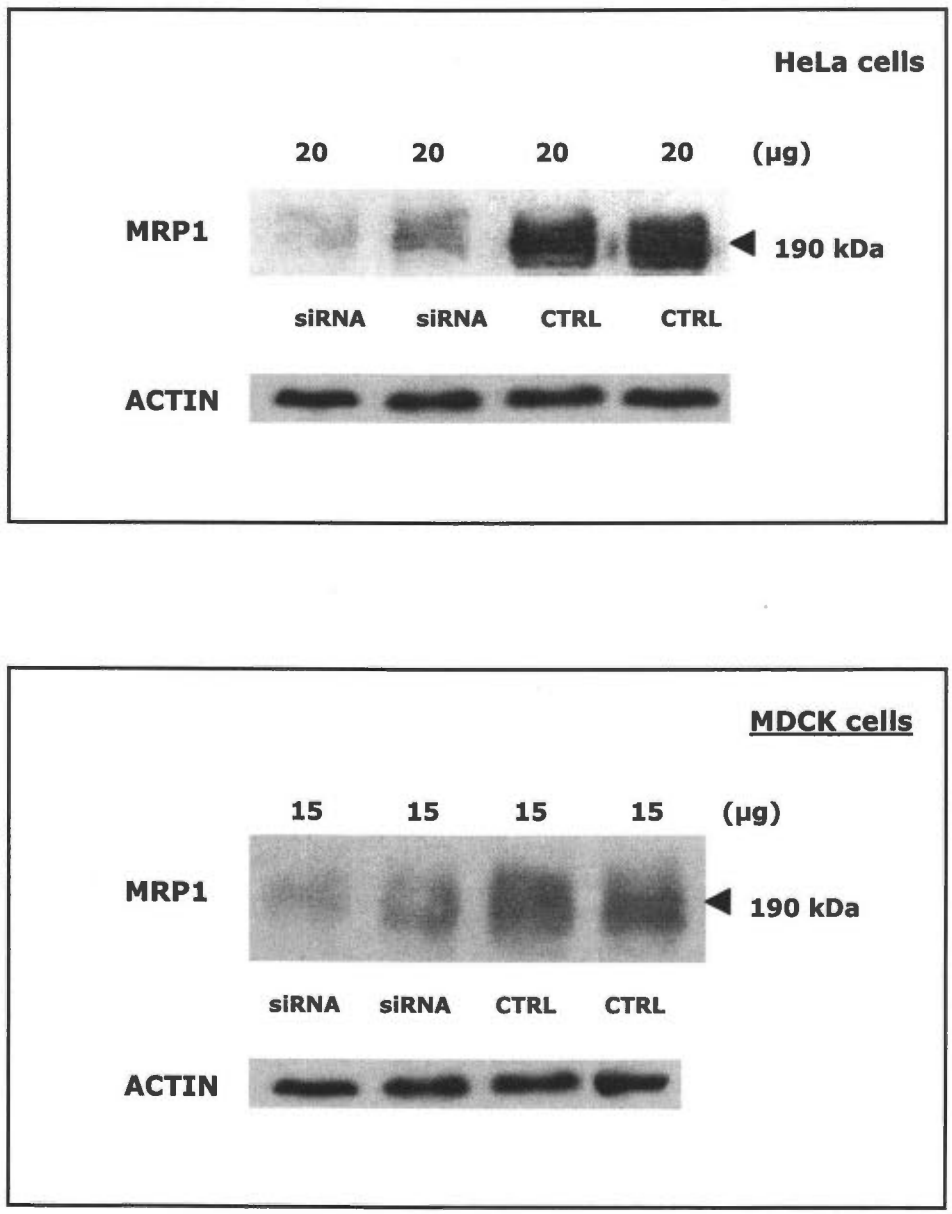


Fig. 3.4 Western Blot analysis of MRP1 protein in HeLa cells and MDCK108 cells transiently transfected by siRNA targeting MRP1 (sequence designed, synthesized and validated by Ambion). The MRP1 expression of transfected cells (siRNA) was analysed **72 hours** after transfection and compared to its control (not transfected cells) (CTRL). MRP1 was detected by a specific antibody (see *Materials and Methods*) and showed an apparent molecular weight around 190 kDa. The expression of the housekeeping protein (ACTIN) was also detected.

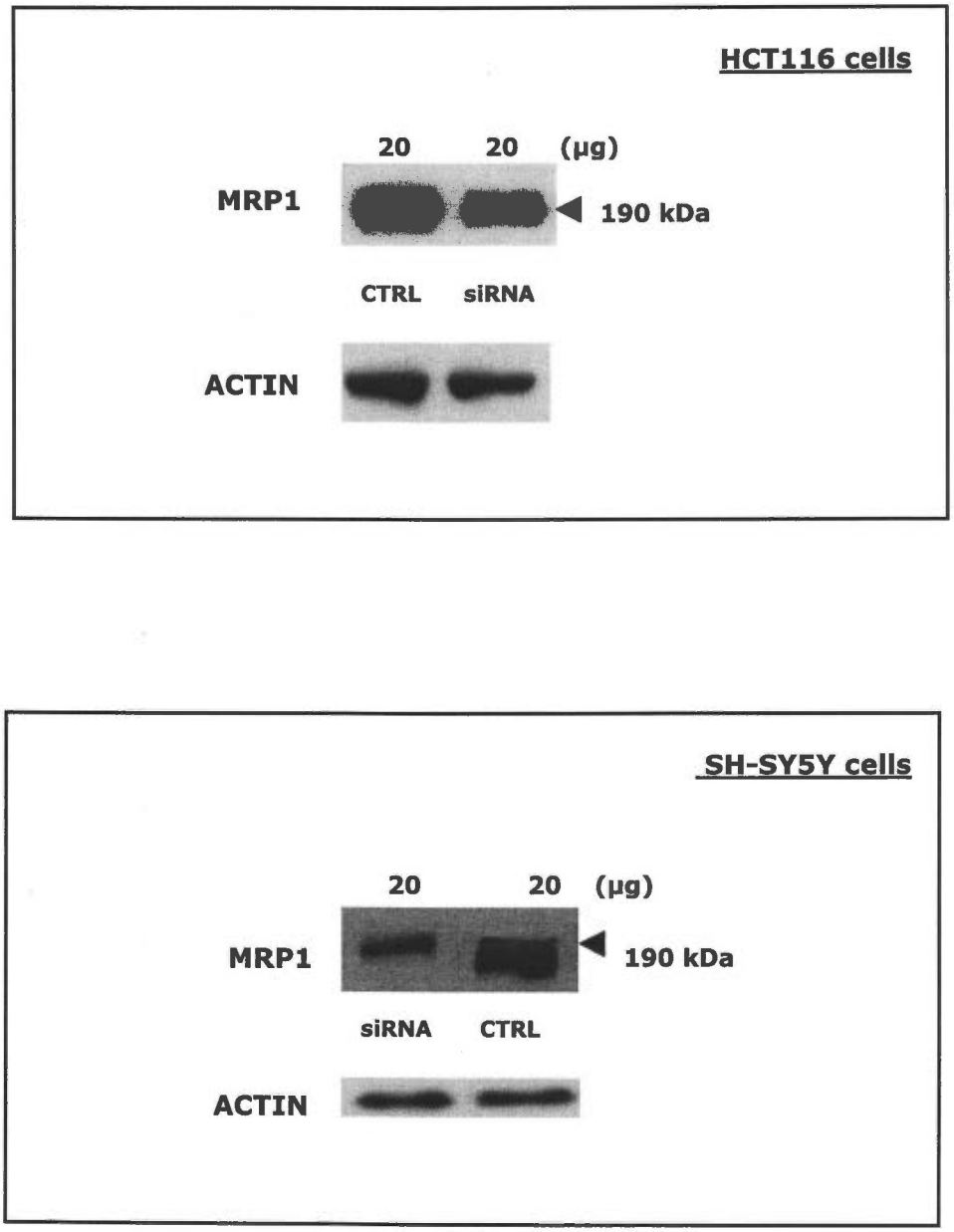


Fig. 3.5 Western Blot analysis of MRP1 protein in HCT116 cells and SH-SY5Y cells transiently transfected by siRNA targeting MRP1 (sequence designed, synthesized and validated by Ambion). The MRP1 expression of transfected cells (siRNA) was analysed **72 hours** after transfection and compared to its control (not transfected cells) (CTRL). MRP1 was detected by a specific antibody (see *Materials and Methods*) and showed an apparent molecular weight around 190 kDa. The expression of the housekeeping protein (ACTIN) was also detected.

QUALITATIVE ANALYSIS OF THE PLASMID pSUPERIOR.puro LINEARIZED BY Bgl II AND Hind III

DNA electrophoresis on 1% agarose gel

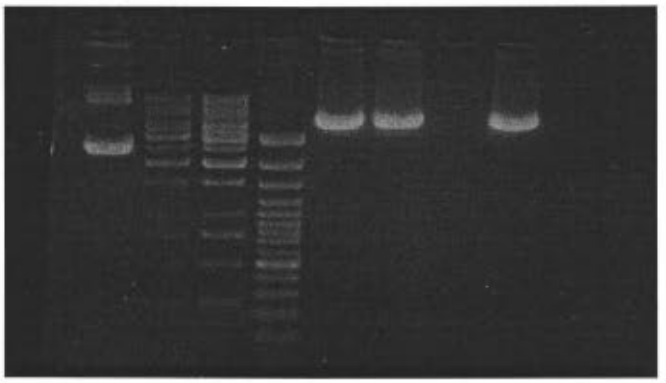


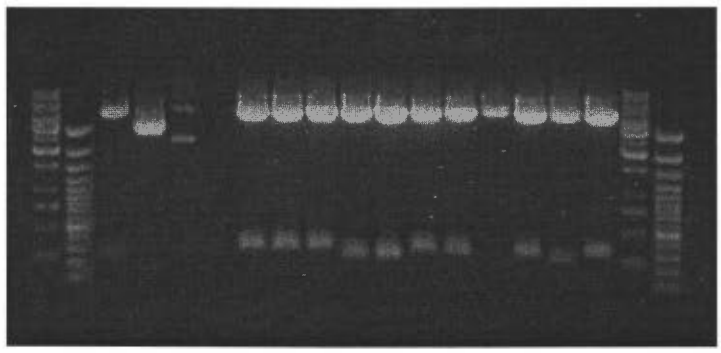
Fig. 3.6 Gel loaded with pSUPERIOR.puro undigested and digested by BglII and/or HindIII.

The samples were loaded in the following order:

1. pSUPERIOR.puro UNDIGESTED
2. MARKER 1 Kb
3. MARKER 1 Kb
4. MARKER 100 pb
5. pSUPERIOR.puro digested by BglII
6. pSUPERIOR.puro digested by HindIII
7. pSUPERIOR.puro digested by BglII e HindIII

QUALITATIVE ANALYSIS OF THE LIGATION IN pSUPERIOR.puro OF THE INSERTS CODING THE shRNAs TARGETING *MRP1* AND *MDR1*, LINEARIZED BY EcoRI e XhoI

DNA electrophoresis on 1% agarose gel



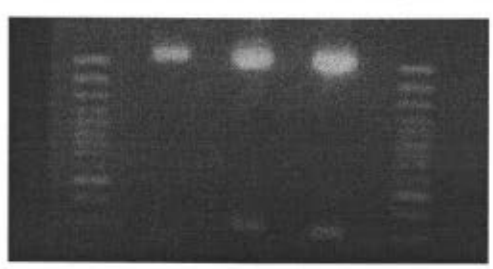
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18

Fig. 3.7 Gel loaded with the samples obtained by the ligation into pSUPERIOR.puro of the sequences coding the shRNAs targeting *MRP1* and *MDR1* and digested by EcoRI and XhoI.

The samples were loaded in the following order:

1. MARKER 1Kb
2. MARKER 100 pb
3. pSUPERIOR.puro without insert, digested by EcoRI e XhoI
4-12. pSUPERIOR.puro that should contain the insert coding the shRNAs targeting *MRP1*, digested by EcoRI e XhoI.
13-16. pSuperior.puro that could contain the insert coding the shRNAs targeting *MDR1*, digested by EcoRI e XhoI.
17. Marker 1Kb
18. Marker 100 pb

DNA electrophoresis on 2%agarose gel



1 2 3 4 5

Fig. 3.8 Gel loaded with the samples selected among the plasmids that from a first analysis (fig. 1.7) proved to contain the insert of interest.

The samples were loaded in the following order:

1. MARKER 100 pb
2. pSUPERIOR.puro without insert and digested by EcoRI e XhoI.
3. pSUPERIOR.puro containing the insert coding the shRNAs targeting *MRP1*, digested by EcoRI and XhoI (plasmid loaded into the lane 11, fig.7).
4. pSuperior.puro containing the insert coding the shRNAs targeting *MDR1*, digested by EcoRI and XhoI (plasmid loaded into the lane 16, fig. 7).
5. MARKER 100 pb.

β-GALACTOSIDASE ASSAY

ABS assay time at λ= 562 nm	Blank (ABS at λ= 562 nm)	CLONES											
		1				2				8			
		Induced		Non-induced		Induced		Non-induced		Induced		Non-induced	
		Abs	Abs - B	Abs	Abs - B	Abs	Abs - B	Abs	Abs - B	Abs	Abs - B	Abs	Abs - B
t=0	0,214	0,320	**0,106**	0,220	**0,006**	0,603	**0,389**	0,257	**0,043**	0,650	**0,436**	0,220	**0,006**
t=5'	0,215	0,374	**0,159**	0,226	**0,011**	0,806	**0,591**	0,281	**0,066**	0,883	**0,668**	0,226	**0,011**
t=10'	0,218	0,417	**0,199**	0,230	**0,012**	0,950	**0,732**	0,301	**0,083**	1,058	**0,840**	0,229	**0,011**
t=15'	0,218	0,456	**0,238**	0,232	**0,014**	1,080	**0,862**	0,319	**0,101**	1,211	**0,993**	0,234	**0,016**
t=20'	0,218	0,488	**0,270**	0,237	**0,019**	1,183	**0,965**	0,333	**0,115**	1,327	**1,109**	0,237	**0,019**
t=25'	0,218	0,522	**0,304**	0,238	**0,020**	1,289	**1,071**	0,348	**0,130**	1,445	**1,227**	0,242	**0,024**
t=30'	0,220	0,548	**0,328**	0,242	**0,022**	1,374	**1,154**	0,361	**0,141**	1,549	**1,329**	0,244	**0,024**

ABS assay time at λ= 562 nm	Blank (ABS at λ= 562 nm)	CLONES							
		81				19			
		Induced		Non-induced		Induced		Non-induced	
		Abs	Abs - B	Abs	Abs	Abs	Abs - B	Abs	Abs - B
t=0	0,214	0,494	**0,280**	0,214	**0**	0,608	**0,394**	0,775	**0,561**
t=5'	0,215	0,670	**0,455**	0,220	**0,005**	0,876	**0,661**	1,102	**0,887**
t=10'	0,218	0,808	**0,59**	0,226	**0,008**	1,059	**0,841**	1,358	**1,140**
t=15'	0,218	0,918	**0,70**	0,232	**0,014**	1,210	**0,992**	1,568	**1,350**
t=20'	0,218	1,005	**0,787**	0,236	**0,018**	1,345	**1,127**	1,752	**1,534**
t=25'	0,218	1,091	**0,873**	0,240	**0,022**	1,470	**1,252**	1,913	**1,695**
t=30'	0,220	1,167	**0,947**	0,243	**0,023**	1,578	**1,358**	2,079	**1,859**

TABLE 3.1

ABS= Absorbance
ABS-B= Absorbance - Blank

ABS assay time at λ= 562 nm	Blank (ABS at λ= 562 nm)	CLONES			
		39			
		Induced		Non-induced	
		Abs	Abs -B	Abs	Abs - B
t=0	0,268	0,914	**0,646**	0,519	**0,251**
t=5'	0,267	1,793	**1,526**	1,116	**0,849**
t=10'	0,267	2,521	**2,254**	1,602	**1,335**
t=15'	0,268	3,042	**2,774**	1,959	**1,691**
t=20'	0,270	3,468	**3,198**	2,253	**1,983**
t=25'	0,271	3,823	**3,552**	2,524	**2,253**
t=30'	0,271	4,265	**3,994**	2,745	**2,474**

β-GALACTOSIDASE ASSAY

ABS assay time at λ= 562 nm	Blank (ABS at λ= 562 nm)	SH-SY5Y CLONES											
		5				14				80			
		Induced		Non-induced		Induced		Non-induced		Induced		Non-induced	
		Abs	Abs - B	Abs	Abs - B	Abs	Abs - B	Abs	Abs - B	Abs	Abs - B	Abs	Abs - B
t=0	0,291	0,327	**0,036**	0,205	/	1,098	**0,807**	0,383	**0,092**	0,328	**0,037**	0,205	/
t=5'	0,295	0,464	**0,169**	0,206	/	2,269	**1,974**	0,634	**0,339**	0,536	**0,241**	0,211	/
t=10'	0,293	0,578	**0,285**	0,204	/	3,112	**2,819**	0,840	**0,547**	0,712	**0,419**	0,220	/
t=15'	0,296	0,688	**0,392**	0,206	/	3,769	**3,473**	1,008	**0,712**	0,853	**0,557**	0,226	/
t=20'	0,297	0,777	**0,48**	0,207	/	4,270	**3,973**	1,147	**0,85**	0,989	**0,692**	0,232	/
t=25'	0,297	0,863	**0,566**	0,209	/	4,500	**4,203**	1,269	**0,972**	1,099	**0,802**	0,239	/
t=30'	0,300	0,942	**0,642**	0,209	/	4,500	**4,2**	1,382	**1,082**	1,204	**0,904**	0,244	/

ABS assay time at λ= 562 nm	Blank (ABS at λ= 562 nm)	SH-SY5Y CLONES			
		89			
		Induced		Non-induced	
		Abs	Abs - B	Abs	Abs - B
t=0	0,291	0,322	**0,031**	0,305	**0,014**
t=5'	0,295	0,558	**0,263**	0,554	**0,259**
t=10'	0,293	0,783	**0,49**	0,765	**0,472**
t=15'	0,296	0,971	**0,675**	0,939	**0,643**
t=20'	0,297	1,152	**0,855**	1,098	**0,801**
t=25'	0,297	1,308	**1,011**	1,231	**0,934**
t=30'	0,300	1,471	**1,171**	1,336	**1,036**

TABLE 3.2

ABS= Absorbance
ABS - B= Absorbance – Blank
/: the absorbance of the induced and/or uninduced sample is = or lower than the blank absorbance.

ABS assay time at λ= 562 nm	Blank (ABS at λ= 562 nm)	SH-SY5Y CLONES											
		20				28				73			
		Induced		Non-induced		Induced		Non-induced		Induced		Non-induced	
		Abs	Abs - B	Abs	Abs - B	Abs	Abs - B	Abs	Abs - B	Abs	Abs - B	Abs	Abs - B
t=0	0,255	1,039	0,784	1,343	1,088	0,795	0,54	0,388	0,133	1,073	0,818	0,947	0,692
t=5'	0,254	1,644	1,39	2,347	2,093	1,397	1,143	0,407	0,153	2,187	1,933	2,142	1,888
t=10'	0,254	2,190	1,936	3,161	2,907	1,883	1,629	0,425	0,171	3,080	2,826	3,080	2,826
t=15'	0,255	2,658	2,403	3,731	3,476	2,277	2,022	0,442	0,187	3,766	3,511	3,799	3,544
t=20'	0,255	2,998	2,743	4,204	3,949	2,578	2,323	0,456	0,201	4,275	4,02	4,316	4,061
t=25'	0,256	3,266	3,01	4,467	4,211	2,847	2,591	0,466	0,21	4,348	4,092	4,500	4,244
t=30'	0,256	3,479	3,223	4,500	4,244	3,106	**2,85**	0,478	**0,222**	4,500	4,244	4,500	4,244

β-GALACTOSIDASE ASSAY

ABS assay time at λ= 562 nm	Blank (ABS at λ= 562 nm)	SH-SY5Y CLONES											
		6				9				32			
		Induced		Non-induced		Induced		Non-induced		Induced		Non-induced	
		Abs	Abs - B	Abs	Abs - B	Abs	Abs - B	Abs	Abs - B	Abs	Abs - B	Abs	Abs - B
t=0	0,470	0,393	/	0,365	/	0,464	/	0,335	/	1,416	/	0,885	/
t=5'	0,452	0,430	/	0,384	/	0,538	/	0,349	/	2,110	/	1,285	/
t=10'	0,443	0,461	/	0,401	/	0,602	/	0,360	/	2,625	/	1,592	/
t=15'	0,435	0,488	/	0,415	/	0,656	/	0,370	/	3,086	/	1,844	/
t=20'	0,433	0,511	/	0,429	/	0,707	/	0,378	/	3,381	/	2,049	/
t=25'	0,435	0,538	**0,103**	0,443	**0,008**	0,754	**0,319**	0,388	**0**	3,656	**3,221**	2,241	**1,806**
t=30'	0,434	0,558	**0,124**	0,455	**0,021**	0,797	**0,363**	0,394	**0**	3,976	**3,542**	2,434	**2**

ABS assay time at λ= 562 nm	Blank (ABS at λ= 562 nm)	SH-SY5Y CLONES			
		87			
		Induced		Non-induced	
		Abs	Abs - B	Abs	Abs - B
t=0	0,470	0,683	/	0,418	/
t=5'	0,452	0,984	/	0.544	/
t=10'	0,443	1,217	/	0,644	/
t=15'	0,435	1,398	/	0,723	/
t=20'	0,433	1,560	/	0,794	/
t=25'	0,435	1,703	**1,268**	0,863	**0,428**
t=30'	0,434	1,83	**1,398**	0,924	**0,49**

TABLE 3.3

ABS= Absorbance
ABS - B= Absorbance – Blank
/: the Absorbance of the induced and/or uninduced sample is = or lower than the blank absorbance.

TABLES 1, 2 and **3** show the absorbance values at λ= 562 nm of the SH-SY5Y clones transfected stably with the pcDNA6/TR vector and transiently with pcDNA4/TO/*lacZ* vector, and treated with chlorophenol red-β-D-galactopyranoside, CPRG (see *Materials and Methods*). For each clone, an induced (by 3.9 μM doxycicline) and an uninduced sample was considered. Clones were tested 24 hours after the induction.

β-GALACTOSIDASE ASSAY

	PROTEIN EXTRACT VOLUME	SH-SY5Y CLONES					
		1		2		8	
		Induced	Non-induced	Induced	Non-induced	Induced	Non-induced
Abs (λ= 562)	20 µl	**0,140**	**0,160**	**0,463**	**0,584**	**0,323**	**0,111**
Concentration		0,144 µg/µl	0,165 µg/µl	0,477 µg/µl	0,602 µg/µl	0,33 µg/µl	0,11 µg/µl
	PROTEIN EXTRACT VOLUME	**SH-SY5Y CLONES**					
		19		**81**		**39**	
		Induced	Non-induced	Induced	Non-induced	Induced	Non-induced
Abs (λ= 562)	20 µl	**0,129**	**0,078**	**0,479**	**0,410**	**0,479**	**0,410**
Concentration		0,13 µg/µl	0,08 µg/µl	0,494 µg/µl	0,423 µg/µl	0,494 µg/µl	0,423 µg/µl

	PROTEIN EXTRACT VOLUME	SH-SY5Y CLONES					
		5		14		80	
		Induced	Non-induced	Induced	Non-induced	Induced	Non-induced
Abs (λ= 562)	20 µl	**0,037**	**0,004**	**0,919**	**1,098**	**0,076**	**0,489**
Concentration		0,041µg/µl	0,005µg/µl	1,008µg/µl	1,204µg/µl	0,084µg/µl	0,536µg/µl
	PROTEIN EXTRACT VOLUME	**SH-SY5Y CLONES**					
		89		**20**		**28**	
		Induced	Non-induced	Induced	Non-induced	Induced	Non-induced
Abs (λ= 562)	20 µl	**0,042**	**0,273**	**0,122**	**0,224**	**0,309**	**0,368**
Concentration		0,046µg/µl	0,299µg/µl	0,137µg/µl	0,251µg/µl	0,346µg/µl	0,413µg/µl

	PROTEIN EXTRACT VOLUME	SH-SY5Y CLONES					
		73		6		9	
		Induced	Non-induced	Induced	Non-induced	Induced	Non-induced
Abs (λ= 562)	20 µl	**0,534**	**0,084**	**0,084**	**0,213**	**0,07**	**0,225**
Concentration		0,598µg/µl	0,094µg/µl	0,094µg/µl	0,239µg/µl	0,08µg/µl	0,25µg/µl
	PROTEIN EXTRACT VOLUME	**SH-SY5Y CLONES**					
		32		**87**			
		Induced	Non-induced	Induced	Non-induced		
Abs (λ= 562)	20 µl	**0,278**	**0,486**	**0,228**	**0,308**		
Concentration		0,31µg/µl	0,54µg/µl	0,26µg/µl	0,35µg/µl		

TABLE 3.4
The protein concentrations of induced (by doxycycline) and uninduced samples are shown.

β-GALACTOSIDASE ASSAY

Assay time	SH-SY5Y CLONES								
	1	2	6	8	9(+B)*	19	20	28	32
t=0	20,19	11,35	/	27	/	0,42	1,32	4,86	4
t=5'	16,46	11,29	/	21,21	4,96	0,45	1,33	8,81	3,48
t=10'	18,93	11,29	/	28,88	5,37	0,45	1,22	11,46	3,33
t=15'	19,43	10,94	/	21,32	5,86	0,44	1,27	12,82	3,28
t=20'	16,08	10,56	/	20,06	6,01	0,44	1,27	13,67	3,19
t=25'	17,36	10,5	45,625	17,23	6,26	0,40	1,31	14,7	1,95
t=30'	**17,08**	**10,37**	**20,95**	**18,68**	**6,5**	**0,44**	**1,39**	**15,37**	**1,94**

a.

Assay time	SH-SY5Y CLONES							
	39	73	81	87	5(+B)*	14	80(+B)*	89
t=0	2,21	0,74	/	/	0,172	10,45	10,31	14,8
t=5'	1,54	0,64	144	8	0,24	6,96	16,39	6,59
t=10'	1,44	0,62	120	5,21	0,31	6,16	20,82	6,75
t=15'	1,4	0,62	77,27	3,29	0,37	5,81	24,93	6,84
t=20'	1,38	0,62	68,57	3,12	0,41	5,61	28,26	6,95
t=25'	1,35	0,60	62,64	2,66	0,45	5,19	29,57	7,04
t=30'	**1,38**	**0,62**	**64,16**	**2,565**	**0,49**	**4,66**	**31,74**	**7,36**

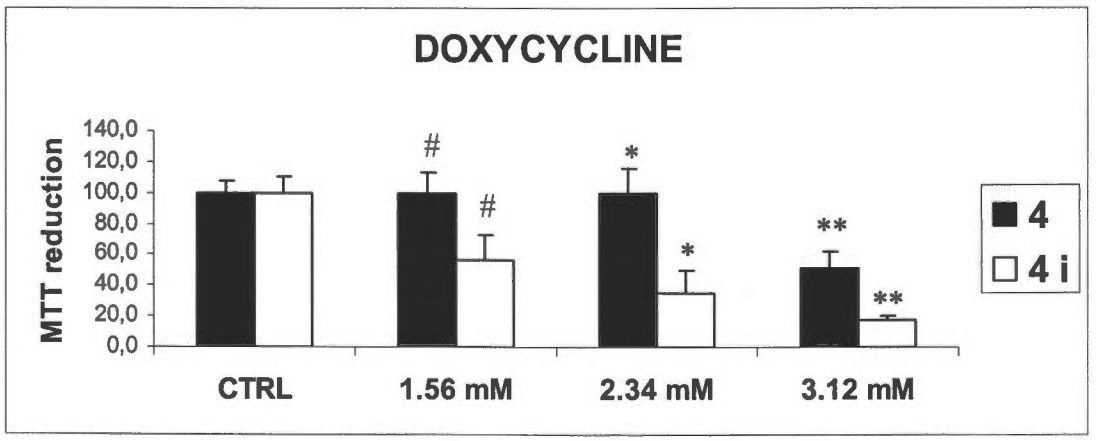
b.

TABLE 3.5 (a, b) β-galactosidase activity of the selected SH-SY5Y clones 24 hours after induction by doxycycline (3.9 μM). The absorbance values were normalized by the protein concentration of each sample and the ratio of induced sample to uninduced sample was calculated. The clones showing the highest ratio are marked.

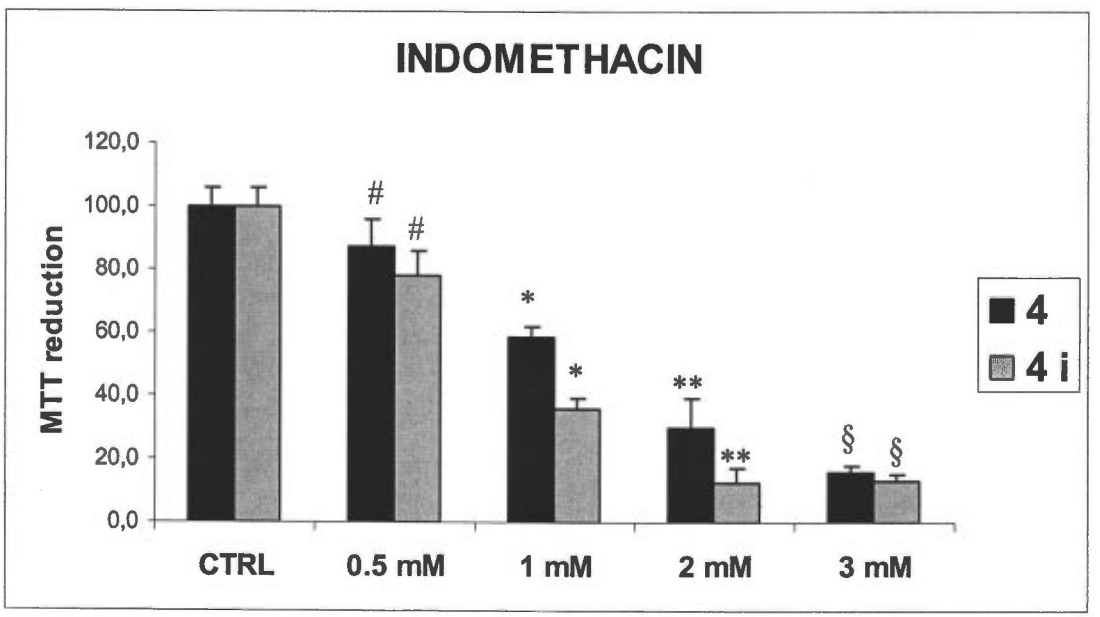
* For **clones 5, 9** and **80** the normalization and the ratio of the induced to the uninduced clone was performed without subtracting the blank, because the absorbance of the uninduced clone was lower than blank absorbance.

CITOTOXICITY EFFECT OF DRUGS (MRP1 AND/OR MDR1 SUBSTRATES) ON SH-SY5Y CLONES (pSUPERIOR-MRP1 AND pSUPERIOR-MDR1)

SH-SY5Y cells, CLONE 4 (pSUPERIOR-MRP1)

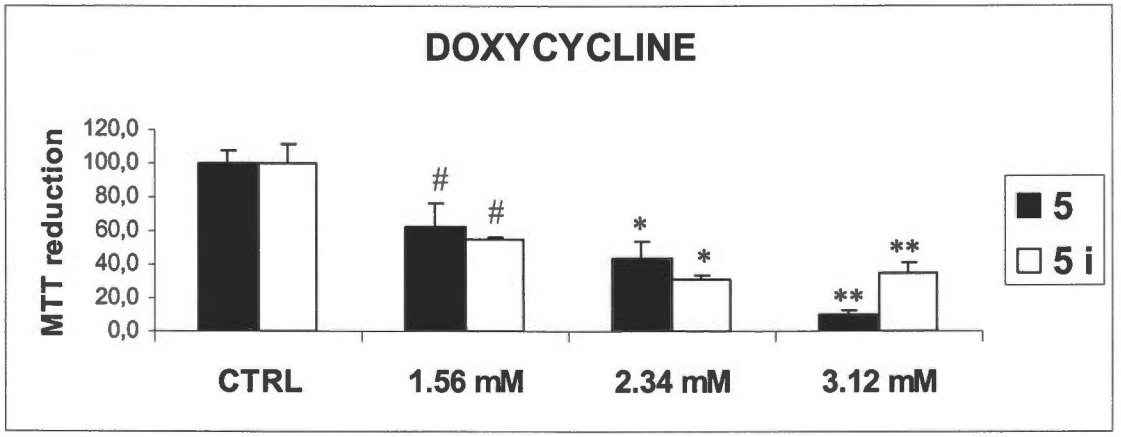


a. $^{\#}p < 0.003$; $^{*}p < 0.02$; $^{**}p < 0.002$

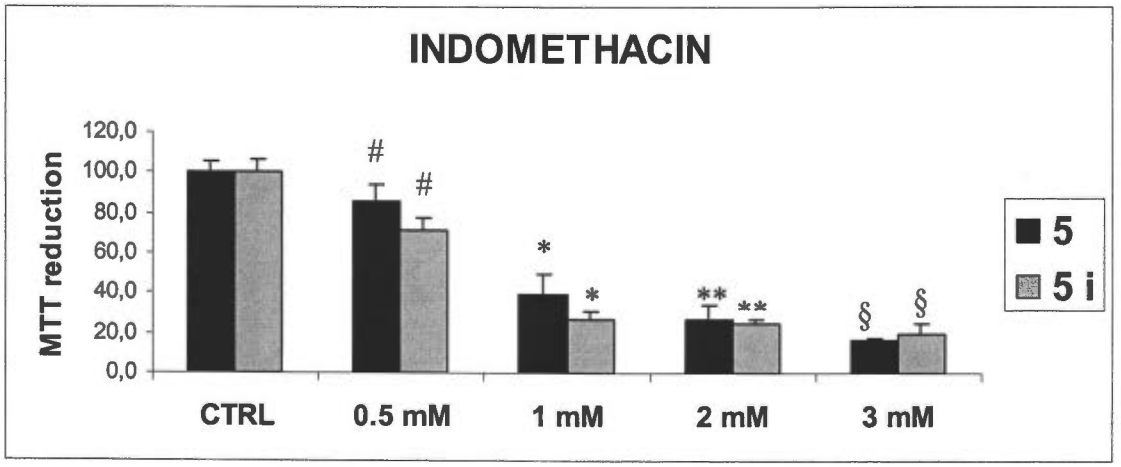


b. $^{\#}p > 0.2$; $^{*}p < 0.0001$; $^{**}p < 0.05$; $^{\S}p > 0.05$

SH-SY5Y cells, CLONE 5 (pSUPERIOR-MRP1)

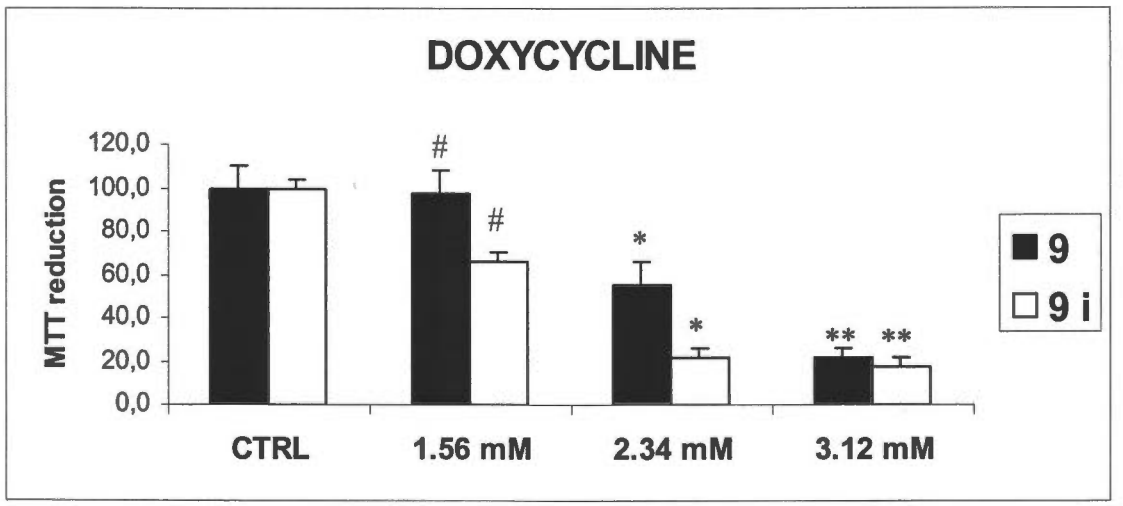


c. $^{\#}p > 0.4$; $^{*}p > 0.3$; $^{**}p < 0.02$

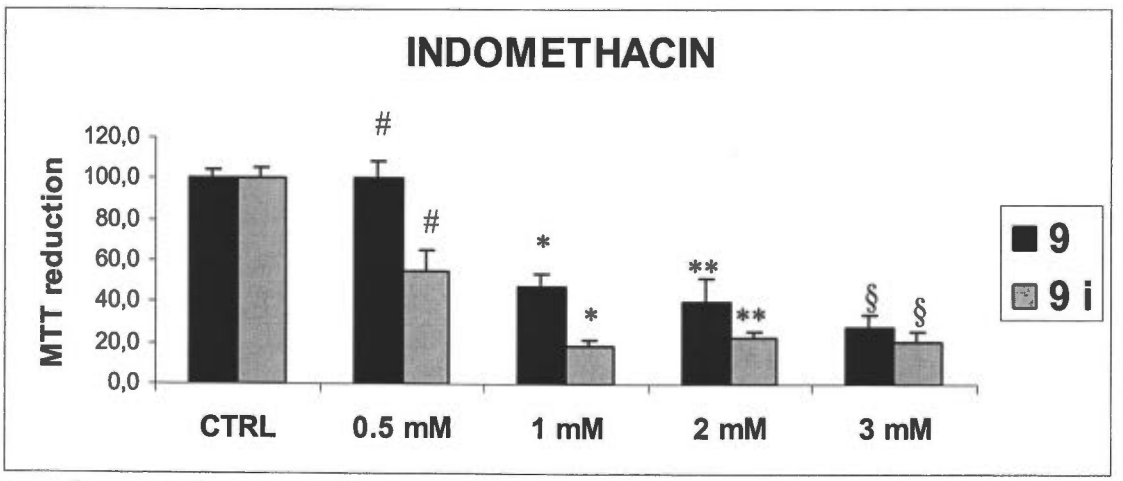


d. $^{\#}p > 0.05$; $^{*}p > 0.05$; $^{**}p > 0.6$; $^{\S}p > 0.2$

SH-SY5Y cells, CLONE 9 (pSUPERIOR-MRP1)

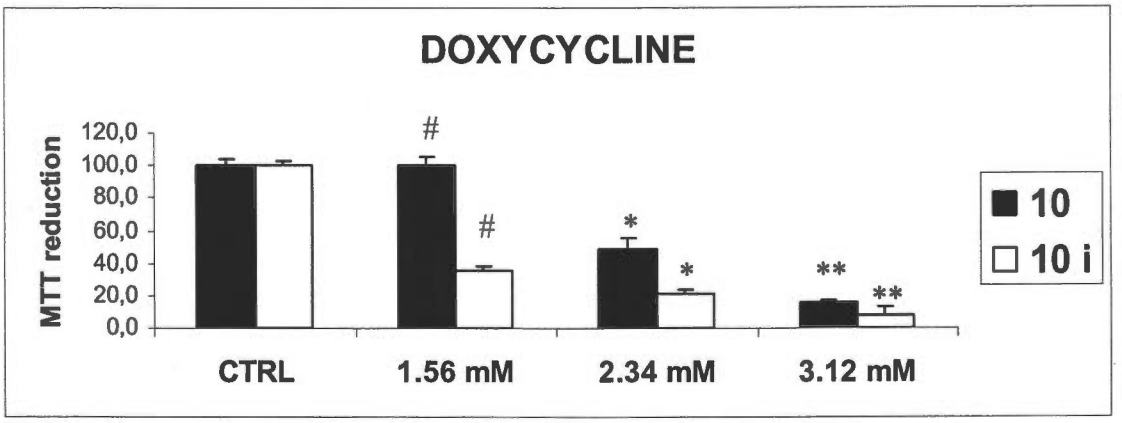


e. $^{\#}p < 0.05$; $^{*}p < 0.003$; $^{**}p > 0.2$

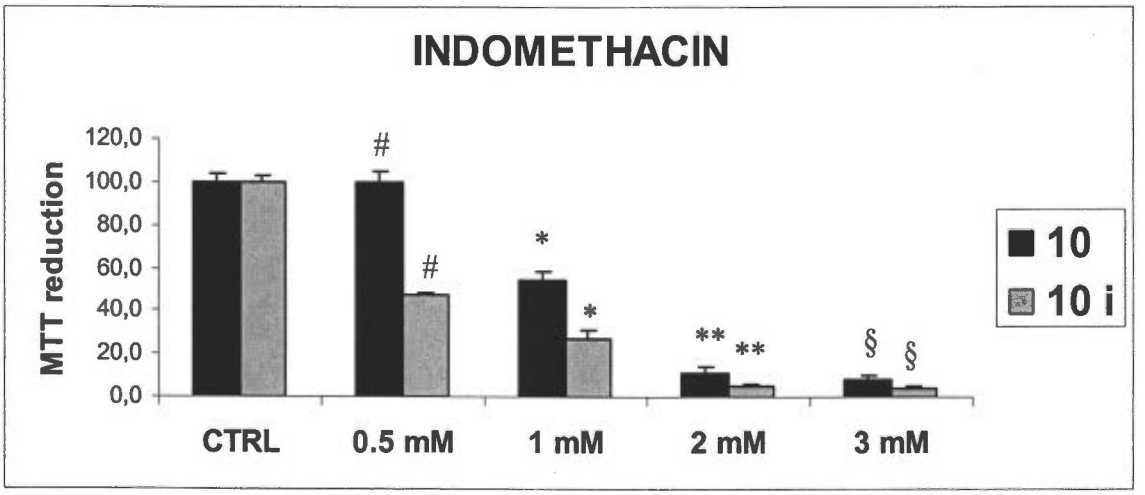


f. $^{\#}p < 0.02$; $^{*}p < 0.002$; $^{**}p < 0.03$; $^{\S}p > 0.1$

SH-SY5Y cells, CLONE 10 (pSUPERIOR-MRP1)

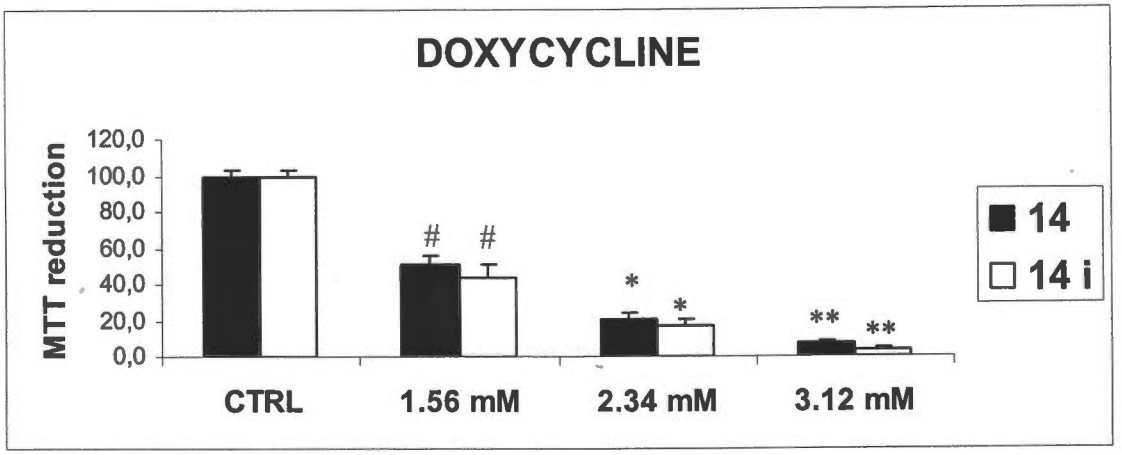


g. $^{\#}p < 0.000002$; $^{*}p < 0.0003$; $^{**}p < 0.04$

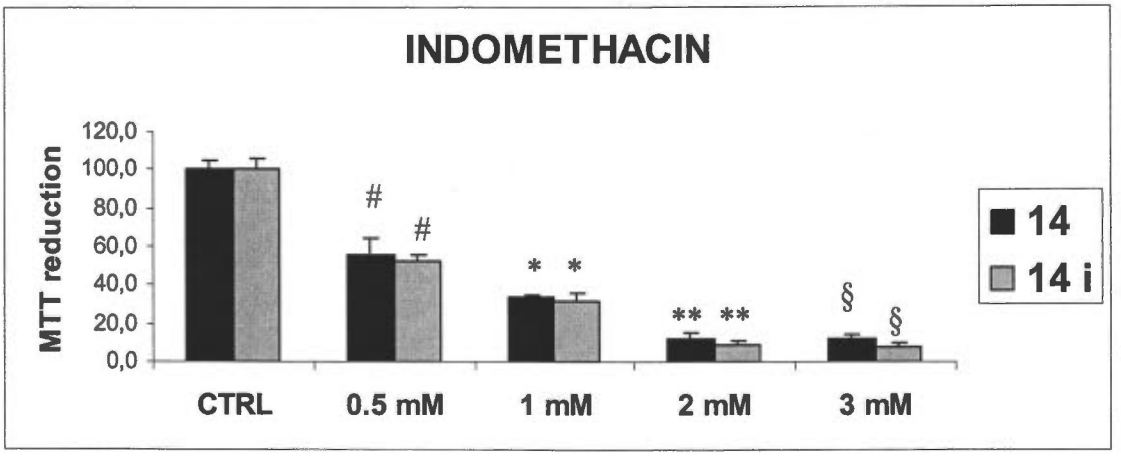


h. $^{\#}p < 0.0002$; $^{*}p < 0.002$; $^{**}p < 0.004$; $^{\S}p < 0.008$

SH-SY5Y cells, CLONE 14 (pSUPERIOR-MRP1)



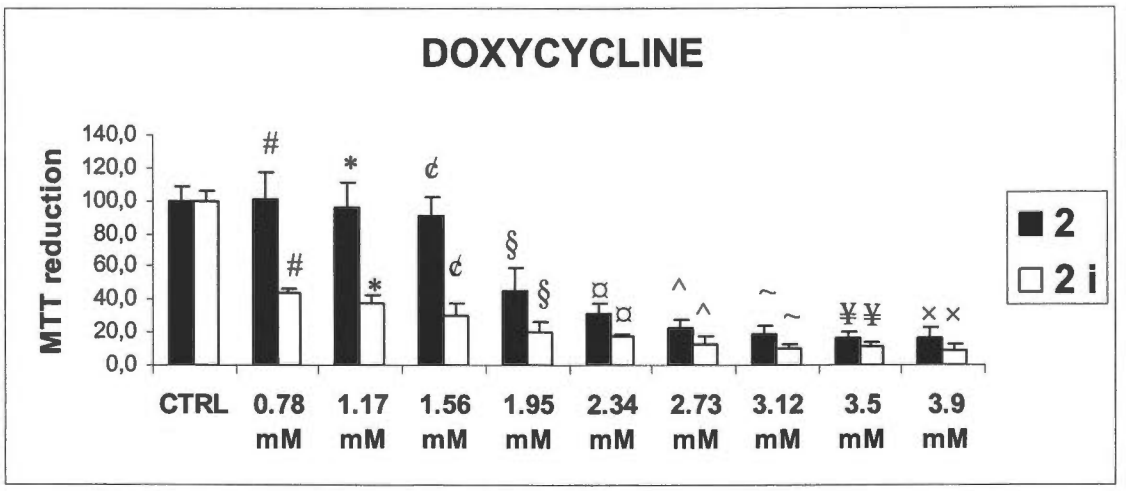
i. $^{\#}p > 0.1$; $^{*}p > 0.2$; $^{**}p < 0.008$



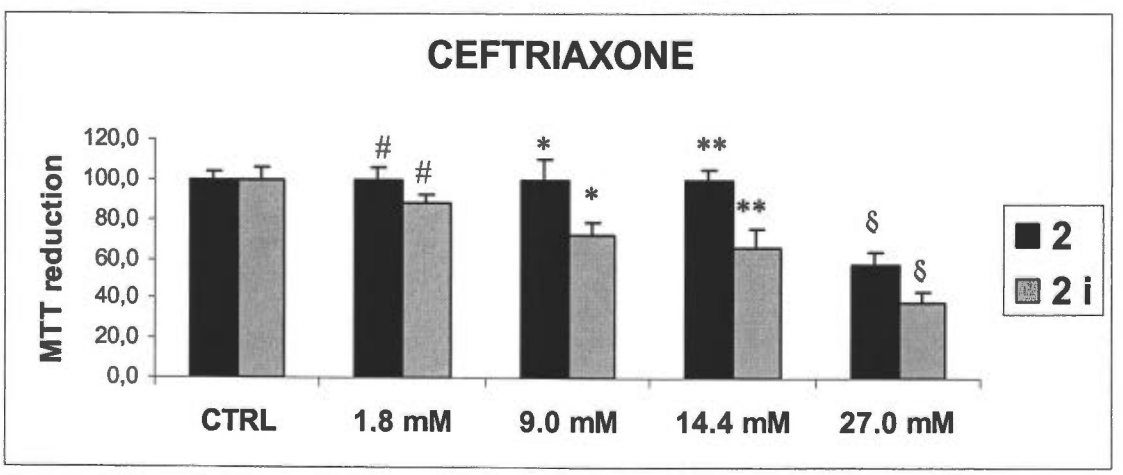
l. $^{\#}p > 0.3$; $^{*}p > 0.4$; $^{**}p > 0.1$; $^{\S}p < 0.02$

Fig. 3.9 (a, b, c, d, e, f, g, h, i, l) MTT reduction in SH-SY5Y clones exposed to different concentrations of DOXYCYCLINE and, separately, INDOMETHACIN (both MRP1 substrates) for **24 hours**. Clones expressed costitutively the Tet repressor from pcDNA6/TR vector and inducibly the **siRNA targeting MRP1** from pSUPERIOR vector. Before exposing them to these drugs, the induced samples **(4 i, 5 i, 9 i, 10 i, 14 i)** were incubated with DOXYCYCLINE (3.9 μM) for **72 hours** (see *Materials and Methods*). DOXYCYCLINE (3.9 μM) was also present in the induced sample medium during the exposure to the drugs. The uninduced samples **(4, 5, 9, 10, 14)** were incubated without DOXYCYCLINE. Controls (CTRL) represent the induced (by 3.9 μM DOXYCYCLINE for 72 hours) and uninduced clone, not treated with drugs. The MTT reduction of induced and uninduced clones, expressed as a percentage of the respective controls, is given as **means ± SD** (bars) of 4 separate experiments. *p* value is reported under each graph.

SH-SY5Y cells, CLONE 2 (pSUPERIOR-MDR1)

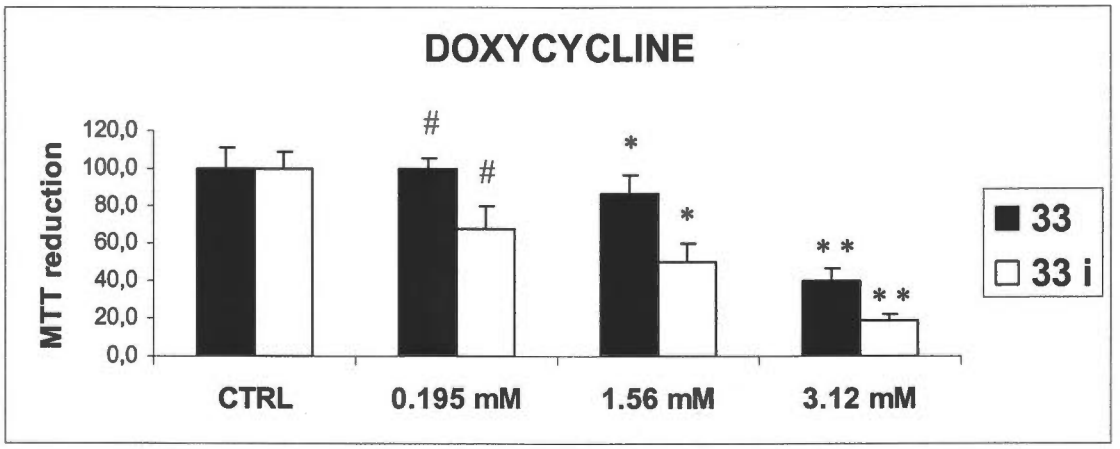


a. $^{\#}p < 0.01$; $^{*}p < 0.02$; $^{¢}p < 0.006$; $^{§}p < 0.02$; $^{¤}p < 0.02$; $^{\wedge}p < 0.04$; $^{\sim}p < 0.04$; $^{¥}p > 0.05$; $^{\times}p > 0.07$

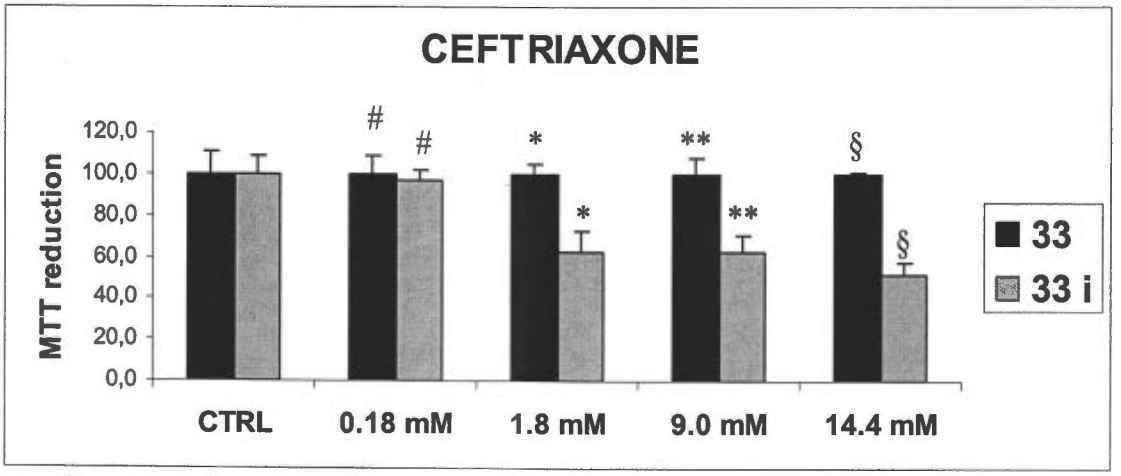


b. $^{\#}p < 0.03$; $^{*}p < 0.003$; $^{**}p < 0.002$; $^{§}p < 0.02$

SH-SY5Y cells, CLONE 33 (pSUPERIOR-MDR1)

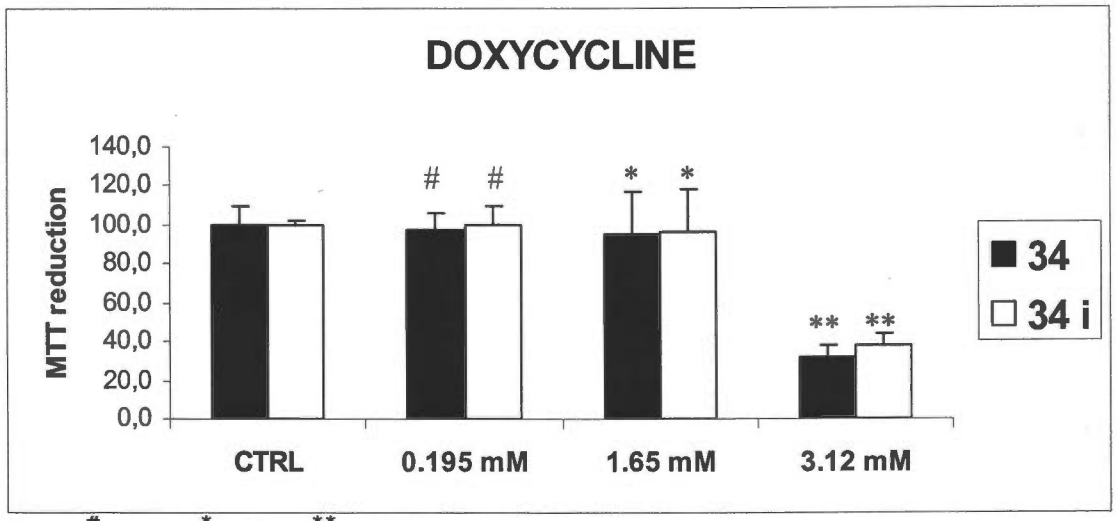


c. $^{\#}p < 0.004$; $^{*}p < 0.006$; $^{**}p < 0.002$

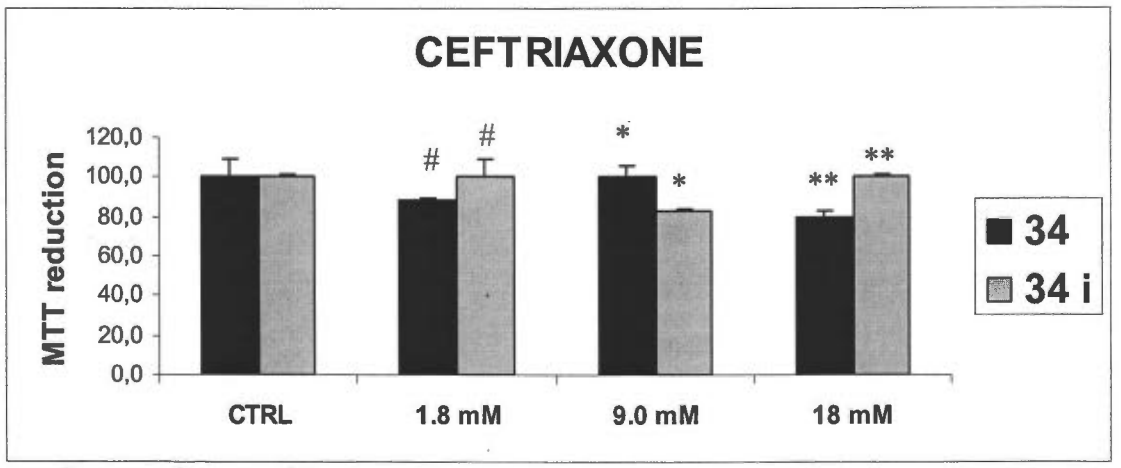


d. $^{\#}p < 0.003$; $^{*}p < 0.006$; $^{**}p < 0.005$; $^{\S}p < 0.002$

SH-SY5Y cells, CLONE 34 (pSUPERIOR-MDR1)



e. $^{\#}p > 0.4$; $^{*}p > 0.8$; $^{**}p > 0.2$

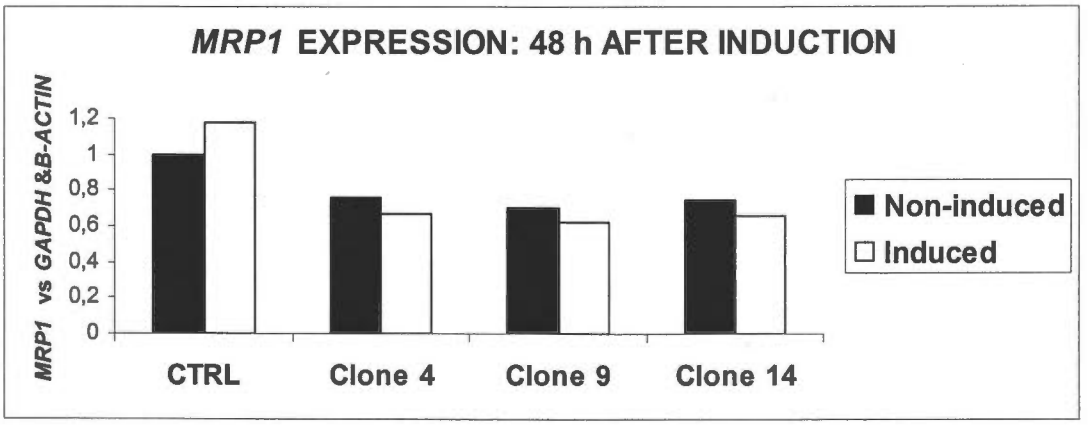


f. $^{\#}p > 0.3$; $^{*}p > 0.2$; $^{**}p > 0.4$

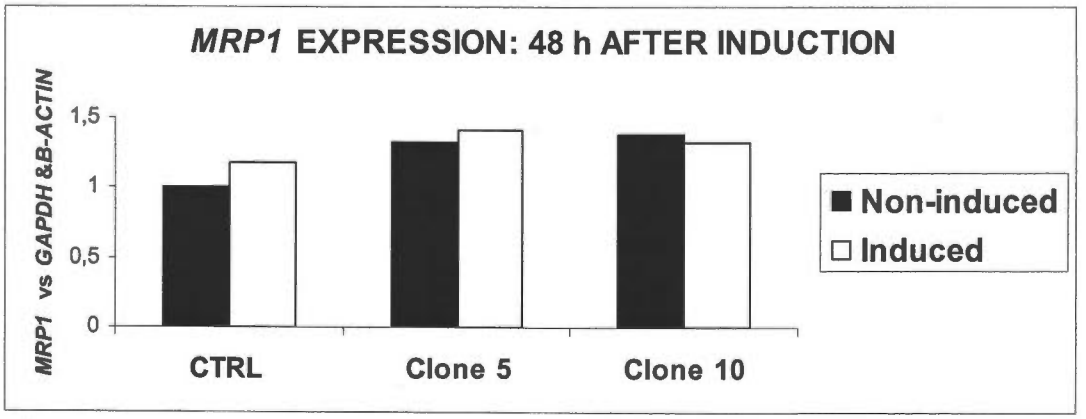
Fig. 3.10 (a, b, c, d, e, f) MTT reduction in SH-SY5Y clones exposed to different concentrations of DOXYCYCLINE and, separately, CEFTRIAXONE (both MDR1 substrates) for **24 hours**. Clones expressed costitutively the Tet repressor from pcDNA6/TR vector and inducibly the **siRNA targeting MDR1** from pSUPERIOR vector. Before exposing them to these drugs, the induced samples **(2 i, 33 i, 34 i)** were incubated with DOXYCYCLINE (3.9 μM) for **48 hours** (see *Materials and Methods*). DOXYCYCLINE (3.9 μM) was also present in the induced sample medium during the exposure to the drugs. The uninduced samples **(2, 33, 34)** were incubated without DOXYCYCLINE. Controls (CTRL) represent the induced (by 3.9 μM DOXYCYCLINE for 48 hours) and uninduced clone, not treated with drugs. The MTT reduction of induced and uninduced clones, expressed as a percentage of the respective controls, is given as **means ± SD** (bars) of 4 separate experiments. *p* value is reported under each graph.

GENE AND PROTEIN EXPRESSION IN SH-SY5Y CLONES (pSUPERIOR-MRP1)

QUANTITATIVE GENE EXPRESSION ANALYSIS BY RT-REAL TIME PCR



a.



b.

Fig. 3.11 a, b Quantitative evaluation of *MRP1* expression in SH-SY5Y cells (clones 4, 9, 14, fig. 11 a) and (CLONES 5, 10, fig. 11 b) stably tranfected by the pcDNA6/TR vector, coding the Tet repressor, and by the pSUPERIOR inducible vector, coding the siRNAs targeting *MRP1*. Before assessing *MRP1* expression, induced samples (4 i, 5 i, 9 i, 10 i, 14 i) were incubated with DOXYCYCLINE (3.9 μM) for **48 hours** (see *Material and Methods*). The uninduced samples were incubated without DOXYCYCLINE. Controls (CTRL) represent the SH-SY5Y clone stably transfected only by the pcDNA6/TR vector, from which the mentioned clones (4, 5, 9, 10 and 14) have origin (see *Materials and Methods*). The pSUPERIOR vector coding the siRNAs targeting *MRP1*. Induced (by 3.9 μM DOXYCYCLINE for 48 hours) and uninduced controls were compared. RT-Real Time PCR was performed normalizing *MRP1* expression values to housekeeping genes *GAPDH* and *β-ACTIN*. *MRP1* expression was calculated relatively to *MRP1* level of the uninduced control, considered =1. Clones with similar *MRP1* expression level put in the same graph.

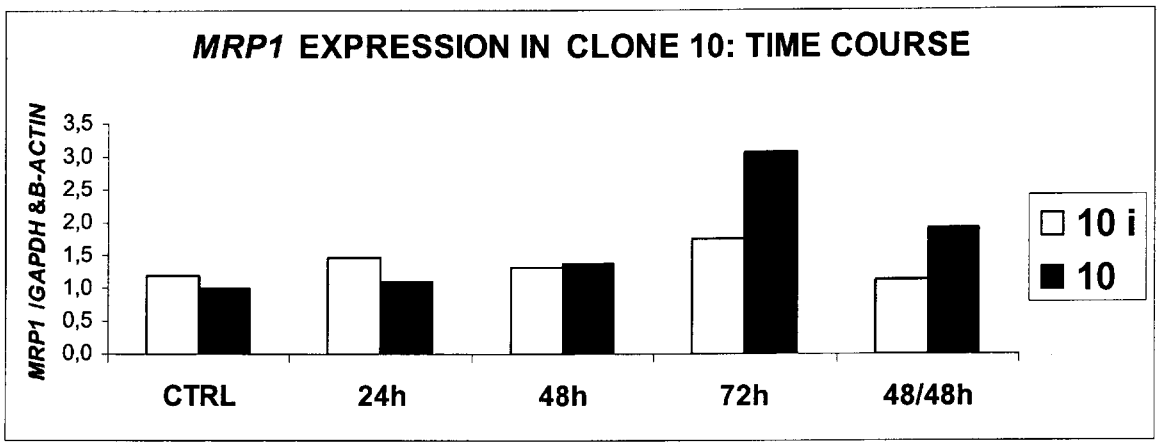


Fig. 3.12 Quantitative evaluation of *MRP1* expression in SH-SY5Y clone 10 (see above) at 24, 48, 72 hours after induction by DOXYCYCLINE (3.9 μM) and 48 hours after the DOXYCYCLINE removing from cells previously incubated with DOXYCYCLINE for 48 hours (48/48h). The *MRP1* expression of uninduced clone (10) and induced clone (10 i) was compared every time. RT-Real Time PCR was performed normalizing *MRP1* expression values to housekeeping genes *GAPDH* and *β-ACTIN*. *MRP1* expression was calculated relatively to *MRP1* level of the uninduced control, considered=1.

MRP1 EXPRESSION ANALYSIS BY WESTERN BLOT: TIME COURSE

INDUCTION	HOURS	72		24		48		72		72/72	72/72
	DOX	+	-	+	-	+	-	+	-	+/-	-/-
MRP1											
ACTIN											
µg protein		10		20		10		10		10	
% MRP1 vs CTRL(+)		100%	79%	147%	111%	159%	133%	30%	79%	56%	120%
SAMPLE		CTRL(TRex)		CLONE 10 (TRex + pSUPERIOR-MRP1)							

Fig. 3.13 Western Blot analysis of MRP1 in SH-SY5Y CLONE 10 (see above) 24, 48, 72 hours after induction by DOXYCYCLINE (3.9 µM) and 72 hours after the DOXYCYCLINE removing from cells previously incubated with DOXYCYCLINE for 72 hours (72/72). The MRP1 expression of uninduced clone (-) and induced clone (+) was compared every time. Bands were visualized by Kodak 1D image software and quantified by Scion Image software. The quantification values were analysed by the Curver Expert software and the MRP1 expression in the induced and uninduced samples were calculated as percentage of control (CTRL-) (clone stably transfected by pcDNA6/TR vector, but not by pSUPERIOR vector, see *Material and Methods*). DOX, doxycycline.

GENE AND PROTEIN EXPRESSION IN SH-SY5Y CLONES (pSUPERIOR-MDR1)

QUANTITATIVE GENE EXPRESSION ANALYSIS BY RT- REAL TIME PCR

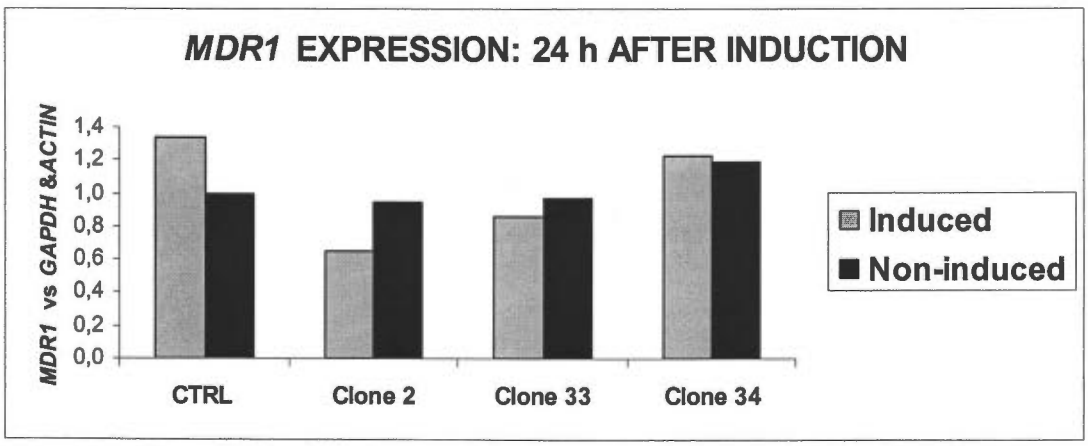


Fig. 3.14 Quantitative evaluation of *MDR1* expression in SH-SY5Y cells (clones 2, 33, 34, fig. 11 a) stably tranfected by the pcDNA6/TR vector, coding the Tet repressor, and by the pSUPERIOR inducible vector, coding the siRNAs targeting *MDR1*. Before assessing *MDR1* expression, induced samples (2 i, 33 i, 34 i) were incubated with DOXYCYCLINE (3.9 μM) for **24 hours** (see *Materials and Methods*). The uninduced samples were incubated without DOXYCYCLINE. Controls (CTRL) represent the SH-SY5Y clone stably transfected only by the pcDNA6/TR vector, from which the mentioned clones (4, 5, 9, 10 and 14) have origin (see Material and Methods). Induced (by 3.9 μM DOXYCYCLINE for 48 hours) and uninduced controls were compared. RT-Real Time PCR was performed normalizing *MDR1* expression values to housekeeping genes *GAPDH* and *β-ACTIN*. *MDR1* expression was calculated relatively to *MDR1* level of the uninduced control, considered =1. Clones with similar *MDR1* expression level put in the same graph.

MDR1 EXPRESSION ANALYSIS BY WESTERN BLOT: 48 HOURS AFTER INDUCTION

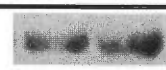
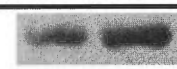
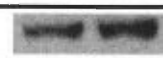
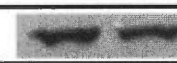
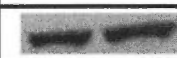
INDUCTION								
	HOURS	48	48		48		48	
	DOX	-	+	-	+	-	+	-
	MDR1							
	ACTIN							
	µg protein	50	40		40		40	
	% MDR1 vs CTRL (-)	100%	153%	179%	239%	397%	242%	181%
	SAMPLE	CTRL(TRex)	2		33		34	
			CLONES (TRex + pSUPERIOR-MDR1)					

Fig. 3.15 Western Blot analysis of MDR1 in SH-SY5Y CLONES 2, 33, 34 (see above) 48 hours after induction by DOXYCYCLINE (3.9 µM). The MDR1 expression of uninduced clone (-) and induced clone (+) was compared. Bands were visualized by Kodak 1D image software and quantified by Scion Image software. The quantification values were analysed by the Curver Expert software and the MDR1 expression in the induced and uninduced samples were calculated as percentage of control (CTRL-) (clone stably transfected by pcDNA6/TR vector, but not by pSUPERIOR vector, see Material and Methods). DOX, doxycycline.

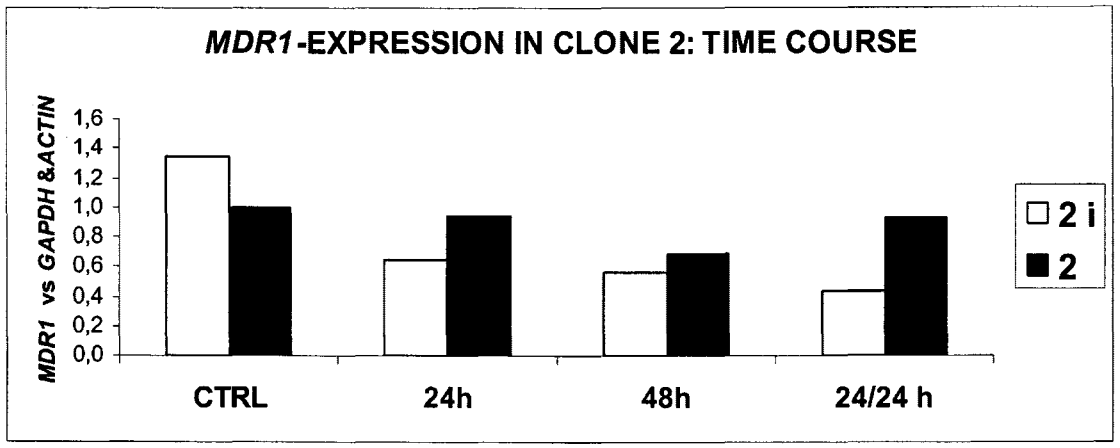


Fig. 3.16 Quantitative evaluation of *MDR1* expression in SH-SY5Y clone 2 (see above) 24, 48 hours after induction by DOXYCYCLINE (3.9 μM) and 24 hours after the DOXYCYCLINE removing from cells previously incubated with DOXYCYCLINE for 24 hours (24/24h). The *MDR1* expression of uninduced clone (2) and induced clone (2 i) was compared every time. RT-Real Time PCR was performed normalizing *MDR1* expression values to housekeeping genes *GAPDH* and *β-ACTIN*. *MDR1* expression was calculated relatively to *MDR1 level* of the uninduced control, considered=1.

MDR1 EXPRESSION ANALYSIS BY WESTERN BLOT: TIME COURSE

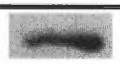
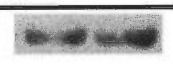
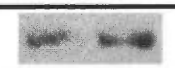
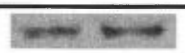
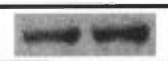
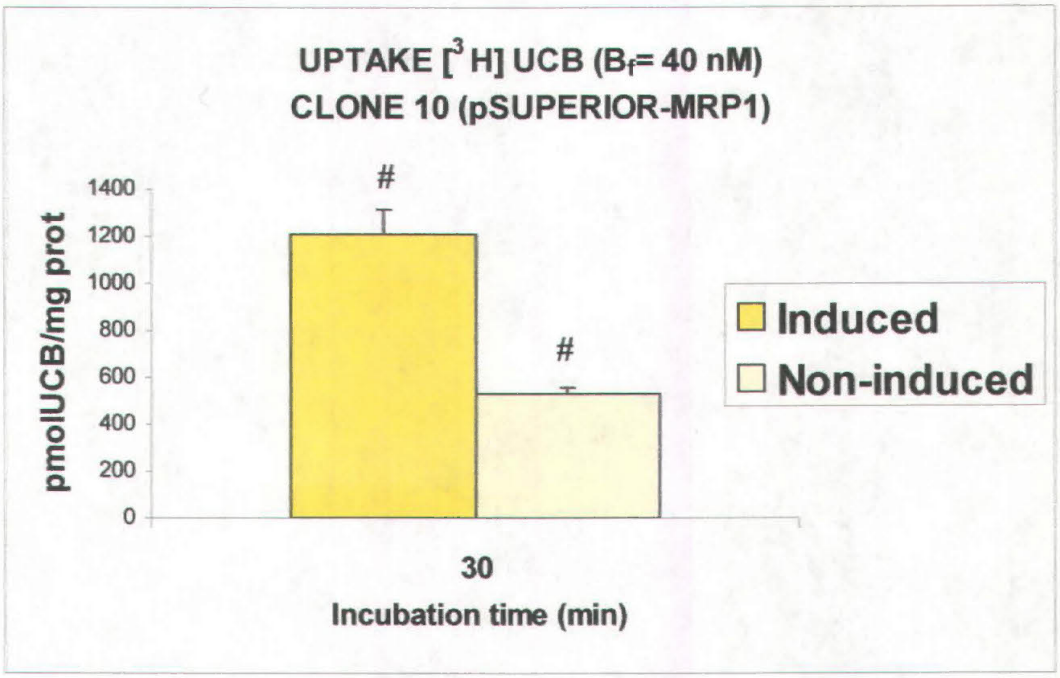
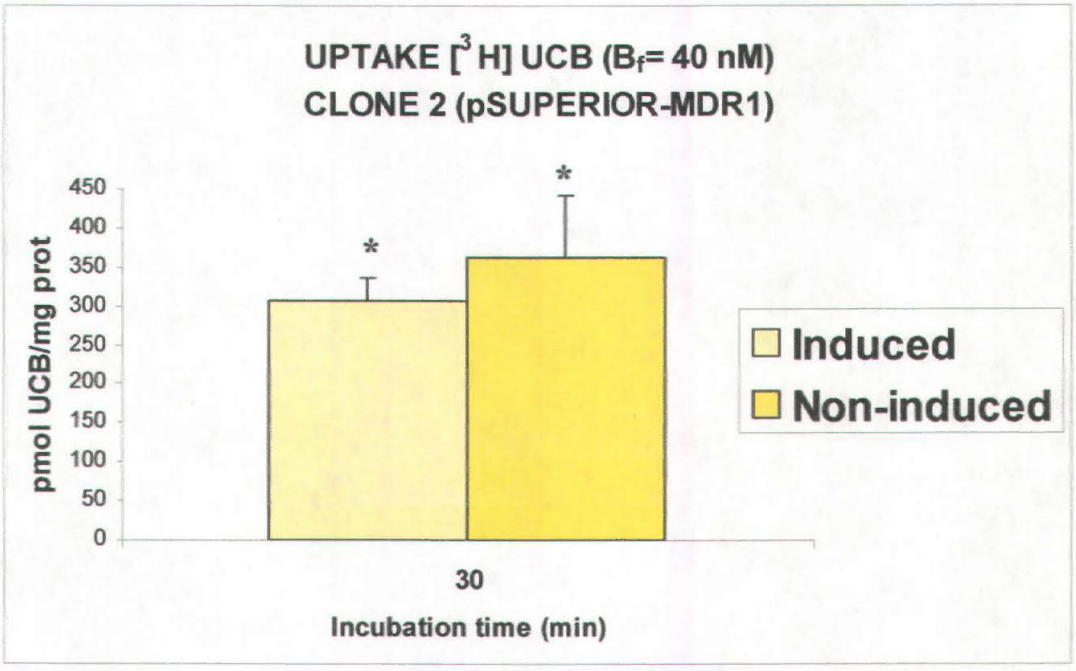
INDUCTION								
	HOURS	48	24		48		48/48	
	DOX	-	+	-	+	-	+	-
MDR1								
ACTIN								
µg protein		50	40		40		40	
% MDR1 vs CTRL (-)		100%	663%	736%	153%	179%	34%	86%
SAMPLE		CTRL(TRex)	2					
			CLONES (TRex + pSUPERIOR-MDR1)					

Fig. 3.17 Western Blot analysis of MDR1 in SH-SY5Y CLONE 2 (see above) 24, 48 hours after induction by DOXYCYCLINE (3.9 µM) and 48 hours after the DOXYCYCLINE removing from cells previously incubated with DOXYCYCLINE for 48 hours (48/48). The MDR1 expression of uninduced clone (-) and induced clone (+) was compared every time. Bands were visualized by Kodak 1D image software and quantified by Scion Image software. The quantification values were analysed by the Curver Expert software and the MDR1 expression in the induced and uninduced samples were calculated as percentage of control (CTRL-) (clone stably transfected by pcDNA6/TR vector, but not by pSUPERIOR vector, see *Material and Methods*). DOX, doxycycline.



a. $^{*}p < 0.0006$



b. $^{*}p > 0.3$

Fig. 3.18 ^{3}[H]-UCB uptake of SH-SY5Y cells: CLONE 10 (pSUPERIOR-MRP1) (a) and CLONE 2 (pSUPERIOR-MDR1) (b) after a 30 minute incubation in ^{3}[H]-UCB (B_f= 40 nM). Data are reported as mean ± SD (bars). *p* value is reported under each graph.

3.7 REFERENCES

1 Wu H, Hait WN, Yang JM (2003) Small interfering RNA-induced suppression of MDR1 (P-Glycoprotein) restores sensitivity to multidrug-resistant cancer cells. Cancer Res 63, 1515-1519.

2 Stege A, Priebsh A, Nieth C, Lage H (2004) Stable and complete overcoming of MDR1/P-glycoprotein-mediated multidrug resistance in human gastric carcinoma cells by RNA interference. Cancer Gene Therapy 11, 699-706.

3 Lee J T, Steelman L S, McCubrey J A (2004) Phosphatidylinositol 3'-Kinase activation leads to multidrug resistance protein-1 expression and susbsequent chemoresistance in advanced prostate cancer cells. Cancer Research 64, 8397-8404.

CHAPTER 4

DISCUSSION

RNA interference (RNAi), triggered by double stranded RNA (dsRNA), mediates gene silencing in a sequence specific manner.

For several years RNAi approches were not applicable to mammalian cells because the introduction of dsRNA longer than 30 nucleotides triggered a very strong interferon response that resulted in non-specific gene silencing due to an overall shutdown of protein synthesis [1]. Since Elbashir et al. [2] and Caplen et al. [3] had reported that introduction of 21-23 nucleotide double stranded siRNAs can efficiently and specifically silence gene expression from the complementary gene, RNAi has evolved into a powerful tool to study mammalian gene functions, ranging from chemically synthesized siRNA to DNA-vector-based siRNA.

In our former studies, 21-nt RNA sequences were produced by *in vitro* transcription and introduced into cells by transient transfection. Most of them were designed by ourselves, following a set of guidelines for effective selection of target sites on mRNA (see *Materials and Methods*). Although widely used, these approaches are not always successful. The *MRP1* silencing attempts performed transfecting 5 siRNA sequences designed by ourselves, failed (Fig. 3.2). The comparison between these findings and the promising result obtained by the transfection of another sequence targeting *MRP1* (Fig. 3.3) confirms that the issue of the siRNA-mediated gene knockdown is determined in large part by the ability of that particular siRNA sequence to trigger the gene silencing [4].

Besides, the gene silencing efficacy is determined also by the copy number of siRNAs present in the cell. In *Caenorhabditis elegans*, only a few molecules of siRNA per cell are required for the silencing and the effect goes through a broad part of the organism [5]; a greater number of molecules per cell may be required to obtain the desired result in mammalian cells [6]. That is due to a RNAi amplification phenomenon that appears to be restricted to worms and plants and is not observed in mammalian cells [4].

Using the optimal concentration of siRNA targeting *MRP1 or MDR1* (see *Materials and Methods*), the inhibition of the both genes, 24 hours (*MDR1*) and 48 hours (*MRP1*) after siRNA transfection in HeLa cells, reached 50% (figg. 1b and 3).

The lack of a higher gene inhibition is likely due to the limitations inside a transient application of the siRNA molecules. This approach is restricted by a possible low transfection efficiency (that could explain the reason why a greater silencing effect was observed in HeLa than HepG2 cells, Fig. 3.1 a,b) and short-term cellular persistence of the siRNA molecules. Further problems are the biological half-life times of the target transcript and its encoding protein. In the case of MDR1, a study published in 2003 using a human carcinoma cell line showed that the MDR1 mRNA had a half-life of approximately 4 hours and the

corresponding transporter protein exhibited a half-life of approximately 16 hours [7]. Accordingly, Nieth et al. showed that, 24 hours after transient siRNA transfection, a reduction of MDR1-mRNA signal started being detectable; this inhibition increased in the following 48 hours, after which the MDR1 mRNA expression level tardively increased, reaching the original mRNA expression value after 7 days [8] .

With regard to the results found by the gene and protein analysis of MRP1 expression, carried out 72 hours after the transfection of the synthetic siRNA in some cell lines, included SH-SY5Y cells (figg. 4 and 5), data in literature suggest that MRP1 protein levels were diminished over 72 hours in PC3 cells with patterns corresponding to mRNA levels [9]. Besides, in neuroblastoma cells the MRP1 expression was reduced of about 60-65% of the untreated controls, if the transfection of antisense oligonucleotides targeting *MRP1* was repeated at 48 hours and the cells were harvested 72 hours after the first transfection [10].

These observations are in agreement with the results found by the analysis of MRP1 expression carried out 72 hours after the transfection of the synthetic siRNA in some cell lines, included SH-SY5Y cells, that showed a reduction higher than 50% (figg. 4 and 5).

Thus, because of the relatively long half-life of both MRP1 (20-24 hours) [11] and MDR1 (16 hours) [7], it is evident that the use of a silencing system having a short-lived effect is restrictive. This approach permits to produce easily many siRNAs, but is limited by the transient nature of the silencing procedure. As the synthetic siRNAs are turned over by the cell, the silenced genes can recover in time, limiting this approach to analysis for short periods [4].

The application of a vector system for inducible expression of siRNA was suitable to carry out gene and protein analysis over an extended period of time [12-14], allowing us to overcome the initial obstacles and revealing some more information about the peculiarities of the proteins studied.

At the beginning, however, the quantitative evaluation of the *MRP1* expression in stable SH-SY5Y clones expressing costitutively the Tet repressor from pcDNA6/TR vector and inducibly the siRNA targeting *MRP1*, performed after a 48 hour incubation of the induced sample with doxycycline, showed that no significance differences occurred between induced and uninduced samples (Fig. 3.11 a,b). This result was in contrast with the one obtained 48 hours after the transfection of synthetic siRNAs in HeLa cells (Fig. 3.3). In fact, it showed at least a 50% reduction of *MRP1* expression. This discordance could be explained by the different system used to inhibit the target gene. In fact, results from synthetic siRNA cannot be completely transferred to vector-based siRNA construct for

unknown reason [5]. In addition, uninduced clones expressed a different *MRP1* level (Fig. 3.11 a,b). That could be caused by peculiar characteristics of each clone or by different cell confluence percentages reached by each clone. In fact, it has been observed that *MRP1*/MRP1 expression is affected by cell density [15][16]. The experiment performed to investigate the *MRP1* expression at different incubation time of the induced sample with doxycycline revealed some interesting information. A reduction in the *MRP1* expression of the induced sample compared to the uninduced one, occurred just 72 hours after the silencing induction and 48 hours after the inducing agent removing were not enough to restore the original mRNA expression level (Fig. 3.12). In fact, an evident reduction in the *MRP1* expression was still detectable 96 hours after the silencing induction even if, after 48 hours, the inducing agent was removed. These results were confirmed by the protein analysis: 72 hours after silencing induction were the best time to observe a consistent reduction of the MRP1 expression between the induced and uninduced sample (Fig. 3.13). This agreed with the results of the silencing experiments performed by synthetic siRNA in HeLa, MDCK, HCT116 and SH-SY5Y cells (figg. 4 and 5) and with the MRP1 functional analysis, performed by MTT assay in clone 10 which, after a 72 hour incubation with doxycycline, had been exposed to drugs. As an analogue phenomenon observed in the gene expression analysis, 72 hours after the inducing agent removing were not enough to restore the original protein expression level. On the contrary, 144 hours after the silencing induction still a significant reduction in the MRP1 expression was detectable even if, after 72 hours, the inducing agent was removed. In addition, it has to be considered a possible inducing effect of doxycycline on the *MRP1*/MRP1 expression. That could explain the slightly higher *MRP1*/MRP1 expression level found in the induced control compared to the uninduced one (Fig. 3.14). This phenomenon was really more evident if *MDR1* expression was analysed after a 24 hour incubation of the induced control with doxycycline (Fig. 3.16). This observation showed that doxycycline induced an overexpression of *MDR1*, as actually reported in literature [17].

The findings observed in the *MDR1* expression analysis (Fig. 3.16), were in agreement with the *MDR1* up-regulation phenomenon induced by doxycycline, mentioned above. In fact, siRNA silencing effect should be contrasted by the *MDR1* gene stimulation caused by doxycycline. Thus, after a 24 hour incubation, the *MDR1* overexpression effect should be not so strong as after a 48 hour one and the silencing was more detectable. On the other hand, the very removing of doxycycline allowed to observe the real effect of the silencing. So, the *MDR1* level reduction of the induced sample compared to the uninduced one was much higher after a 24 hour incubation with doxycycline, followed by a 24 hour incubation

without doxycycline, especially if compared with a 48 hour incubation in the presence of doxycycline. This demonstrated clearly that doxycyline made the difference.

Supporting this result, but not confirming it due to the different silencing system used, there is the finding (mentionated above) that in a silencing experiment carried out by synthetic siRNAs, a weack MDR1 mRNA signal could be detected already after 1 day of siRNA transfection and this reduction was detectable up to 3 days after the transfection [8].

The protein analysis (Fig. 3.17) points out that also the protein silencing, as the gene one, was hidden by the MDR1 up-regulation due to the doxycycline presence. In fact, as the same phenomenon observed in the gene analysis, after 96 hours from the silencing induction by the addition of doxycycline, previous removal of the inducing agent after 48 hours, not only the MDR1 expression reduction between induced and uninduced sample was still evident, but it also reached the highest level. This finding suggests that a functional assay performed in this condition, should even enhance the difference of viability between the induced and the uninduced sample found after a 48 hour incubation of the induced sample with doxycycline (Fig. 3.10). The best time to observe the maximum silencing of the protein was slightly different probably due to the different half life of the gene compared to the protein one [7].

Results of a silencing experiment obtained by using synthetic siRNAs which, as previously observed, can support the results found by vector-based siRNA construct but cannot be completely transferred to, showed that the peack of MDR1 protein reduction was reached after 3-5 days after siRNA transfection [8].

All these findings, taken together, explained the initial difficulty to detect the silencing effect by analysing the gene/protein expression for many attendant circumstances. For example, this work demonstrates that the incubation time is a critical condition for observing the silencing effect and that, as mentioned above, results from synthetic siRNA cannot be completely transferred to vector-based siRNA construct.

In its review article [5], Medema asserts that the most important criterion distinguishing a successful RNAi experiment form a failure is the time required to reduce protein expression below the thresold level that is critical to sustain normal protein function. This is in large part determined by the efficacy of the siRNA to target the mRNA of choice. But in addition, protein stability is a critical factor. The time required to reduce protein expression below the critical level, once the mRNA is degraded or translation is shut off, is primarily determined by the half-life of that protein. Silencing expression of stable proteins may require very long

incubation periods with siRNA that can only be accomplished by stable expression of the siRNA.

Further, Medema goes on considering that tipically, RNAi experiments are carried out by transfecting siRNAs into an asynchronous cell population and therefore this can be expected to introduce a major variation in timing required to impair gene function from cell to cell in that population. Moreover, the expression of the gene under investigation may vary significantly over the different cells in that population to begin with, making RNAi effects occur asynchronously. As a consequence, secondary effects, adaptation and toxicity will also occur asynchronously, making it difficult to identify the proper window of opportunity to perform an interpretable RNAi experiment. This becomes increasingly difficult if the time required to reach that critical threshold for protein function increases because of an inefficient targeting strategy or when a particularly stable protein is studied.

Another consideration coming out is that the inducible system used for the gene target silencing needs doxycycline to cause the transcription of siRNA. Doxycycline is a substrate of both MRP1 and MDR1 protein and, as mentioned above, it was proved that doxycycline induces the expression of MDR1 [17]. We found that also MRP1 expression is upregulated by doxycycline, even if the increase is less evident in MRP1 than MDR1.

These observations suggest that many phenomena have to be considered in a silencing attempt, especially if the gene target codes for transporters with affinity for a wide variety of substrates, such as MRP1 and MDR1.

This intrinsic ability of the proteins examined to confer cellular resistance to several compounds, revealed to be really helpful to investigate the reversal of MRP1 or MDR1 gene-depending multidrug resistance in clones stably transfected by a vector containing the sequences coding the siRNA targeting *MRP1* or *MDR1.*

The MTT reduction was really affected by the citotoxic substrates in clones expressing an evident reduction of the MRP1 or MDR1 expression (figg. 9 and 10), as detected by gene and protein expression analyses (figg. 12-17). Then, this approach can be really effective in RNA interference experiments targeting multidrug resistance proteins to screen clones on the basis of their functional activity.

The result of [^{3}H]UCB-uptake assay confirms the involvement of MRP1 in the transport of bilirubin already described in literature as amply reported in the *General Introduction* of this work, while excludes even a minimum role of MDR1 in the transport of this organic anion at least at a bilirubin free (B_f) value = 40 (B_f =40). Actually, this is in agreement with the nature of the two proteins. In fact, MDR1 "prefers" uncharged or slightly positively charged compounds, while MRP1

primarily transports hydrophobic anionic conjugates, but also unconjugated xenobiotics and uncharged drugs. Another aspect that comes out observing the [^{3}H]UCB-uptake assay result, is a possible high permeability of the SH-SY5Y cells to the unconjugated bilirubin (UCB). This could explain the great susceptibility of neuronal cells to the unconjugated bilirubin damages, as widely described in literature and reported in *General Introduction* of this work.

4.1 REFERENCES

1 Williams BR (1997) Role of the double-starnded RNA-activated protein kinase (PKR) in cell regulation. Biochem Soc Trans 25, 509-513.

2 Elbashir SM, Harborth J, Lendeckel W, Yalcin A, Weber K, Tushl T (2001) Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. Nature 411, 494-498.

3 Caplen NJ, Parrish S, Imani F, Fire A, Morgan RA (2001) Specific inhibition of gene expression by small double-starnded RNAs in invertebrate and vertebrate systems. Proc Natl Acad Sci USA 98, 9742-9747.

4 Medema RH (2004) Optimizing RNA interference for application in mammalian cells. Biochem J 380, 593-603.

5 Hannon GJ (2002) RNA interference. Nature (Lond.) 418, 244-251.

6 Wu H, Hait WN, Yang JM (2003) Small interfering RNA-induced suppression of *MDR1* (P-Glycoprotein) restores sensitivity to multidrug-resistant cancer cells. Cancer Research 63, 1515-1519.

7 Aleman C, Annereau JP, Liang XJ et al (2003) P-glycoprotein, expressed in multidrug resistant cells, is not responsible for alterations in membrane fluidity or membrane potential. Cancer research 63, 3084-3091.

8 Nieth C, Priebsh A, Stege A, Lage H (2003) Modulation of the classical multidrug resistance (MDR) phenotype by RNA interference (RNAi). FEBS Letters 545, 144-150.

9 Lee J T, Steelman L S, McCubrey J A (2004) Phosphatidylinositol 3'-Kinase activation leads to multidrug resistance protein-1 expression and susbsequent chemoresistance in advanced prostate cancer cells. Cancer Research 64, 8397-8404.

10 Kuss BJ, Corbo M, Lau WM, Fennell DA, Dean NM, Cotter FE (2002) *In vitro* and *in vivo* downregulation of MRP1 by antisense oligonucleotides: a potential role in neuroblastoma therapy. Int J Cancer 98, 128-133.

11 Kool M, van der Linden M, de Haas M, Sheffer GL, de Vree ML, Smith AJ, Jansen G, Peters GJ, Ponne N, Scheper RJ, Oude Elferink RPJ, Baas F, Borst P (1999) MRP3, an organic anion transporter able to transport anti-cancer drugs. Proc Natl Acad Sci USA 96, 6914-6919.

12 Kawasaki H, Taira K (2001) Discovery of functional genes in the post-genome era by novel RNA-protein hybrid ribozymes. Nucleic Acids Res Suppl, 133-134.

13 Miyagishi M, Taira K (2002) U6 promoter-driven siRNAs with four uridine 3' overhangs efficiently suppress targeted gene expression in mammalian cells. Nat Biotechnol 20, 497-500.

14 Kawasaki H, Taira K (2001) Short hairpin type of dsRNAs that are controlled by tRNA(Val) promoter significantly induce RNAi mediated gene silencing in the cytoplasm of human cells Nucleic Acid Res 31, 700-707.

15 Roelofsen H, Hooived GJEJ, Koning H, Havinga R, Jansen PLM (1999) Glutathione *S*-conjugate transport in hepatocytes entering the cell cycle is preserved by a switch in expression from from the apical MRP2 to the basolateral MRP1 transporting protein. J of Cell Sci 112, 1395-1404.

16 Pascolo L, Fernetti C, Pirulli D, Crovella S, Amoroso A, Tiribelli C (2003) Effects of maturation on RNA transcription and protein expression of four MRP genes in human placenta and in BeWo cells. Biochem and Biophys Res Comm 303(1), 259-265).

17 Mealey KL, Barhoumi R, Burghardt RC, Safe S, Kochevar DT (2002) Doxycycline induces expression of P Glycoprotein in MCF-7 breast carcinoma cells. Antimic Agents and Chemiother 46(3), 755-761.

CHAPTER 5

CONCLUSIONS AND PROSPECTIVES

The creation of stable clones that inducibly express the siRNAs targeting *MRP1* and *MDR1* and the identification of the optimal conditions to obtain the highest silencing effect provide a startup point for several *in vitro* applications. Some of them have already been carried out, but many attempts have still to be done.

The aim of this work was to find out a model to investigate the role of MRP1 and MDR1 in the transport of organic anions in neuronal cells. For this purpose an inducible silencing system was chosen. This approach revealed itself effective, even if some complications came out during the work. They are now specific recommendations and technical notes to consider in this kind of applications.

The viability decrease of SH-SY5Y neuroblastoma cells induced to express the siRNA targeting *MRP1* or *MDR1* compared to the respective uninduced cells, both exposed to citotoxic substrates, was an clear proof of a reduced functional activity of the two proteins examined, well known to protect the cell by extruding these compounds.

Further applications could be performed on these clones to test the reduced cellular resistance against other substrates of MRP1 and MDR1, such as chemotherapeutic agents.

The really higher amount of unconjugated bilirubin (UCB) found in SH-SY5Y cells induced to express the siRNA targeting *MRP1* compared to the uninduced ones, both exposed to [^{3}H]UCB, shows an evident involvement of MRP1 in the transport of this organic anion. On the contrary, the comparable amount of UCB present in the induced and uninduced state of the SH-SY5Y cells containing the siRNA targeting *MDR1*, points out that MDR1 does not transport this organic anion at least at the B_f used (B_f=40 nM). This finding suggests to perform further research to check if higher concentrations of this organic anion can induce MDR1 to take part to the transport of UCB and eventually which is the threshold UCB amount able to trigger its participation.

Observing SH-SY5Y cells which could be particularly permeable to UCB, makes us pay attention to the neuronal damages caused by bilirubin and suggests to carry out neurocitotoxicity studies on our SH-SY5Y clones (MRP1-siRNA and MDR1-siRNA). This could help clarify the importance of MRP1 and/or MDR1 in the transport of UCB, evaluating the UCB-induced effects (e.g. induction of apoptosis or necrosis) in neuronal cells subject to a modulation of MRP1 or MDR1 expression.