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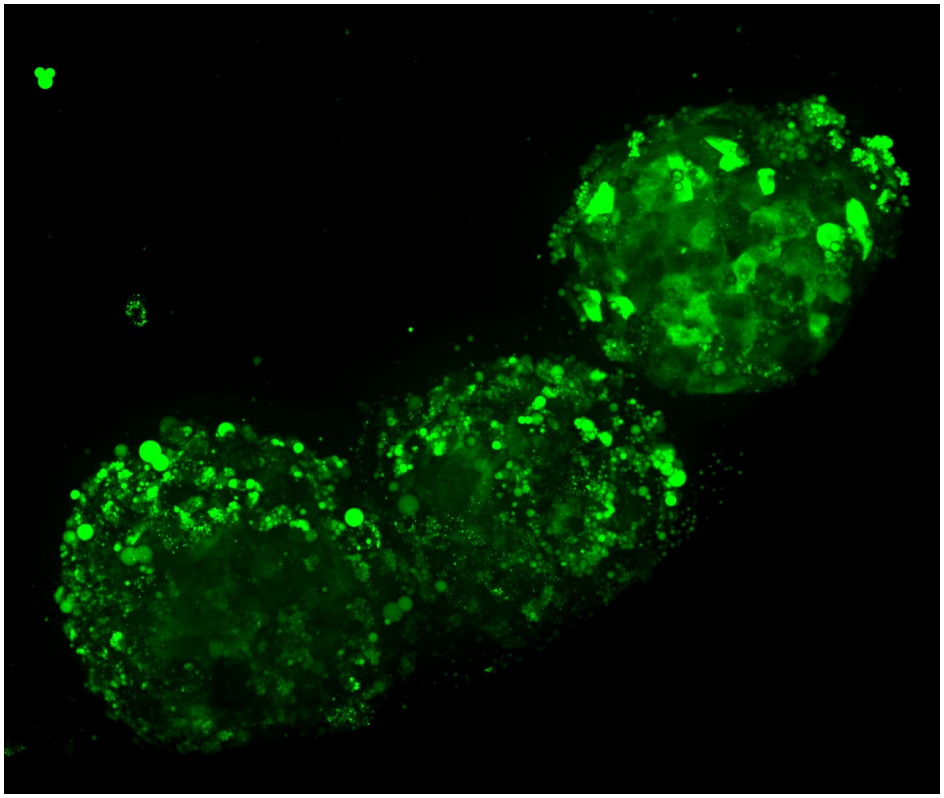

Development and characterization of human liver spheroid cultures for drug transporter studies and microphysiological systems

Master's of science thesis for MSc in Biotechnology

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Department of Physics, Division of Biological Physics
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Gothenburg, Sweden 2017

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Cover: Confocal fluorescent imaging of liver spheroids formed of primary human hepatocytes treated with a fluorescent substrate CMFDA.

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Abstract

A current problem in the drug development process today is the lack of predictable *in vitro* liver models for pre-clinical studies. Current *in vitro* methods and animal trials often fail to predict the effect of potential drugs in humans. In addition, drug-drug interactions which could lead to drug induced liver injury is often a reason for drugs not reaching the market, and it can be related to changes in efflux of metabolites from hepatocytes in liver. In this study, a two-organ-chip has been developed with aggregates formed of HepaRG cells co-cultured with stellate cells as well as pancreatic islets, and evaluated as an *in vitro* model. The focus of the present master thesis project was to evaluate functionality of liver aggregates used in the two-organ-chip system. As a comparison, liver aggregates from chip-system with liver aggregates only has been evaluated. The results showed a significant increase in albumin production from liver aggregates that were in the two-organ-chip system together with pancreatic islets. The liver aggregates showed no significant difference of glycogen production when cultured with or without pancreatic islets. As a conclusion, the chip-system with two organs used showed increased liver specific functions in liver aggregates. Another part of the study included evaluation of activity of the canalicular transporter multidrug resistance protein 2 (MRP2) in hepatic spheroids formed of primary human hepatocytes or hepatocytes co-cultured with stellate cells. The activity was evaluated using a substrate, 5-chloromethylfluorescein diacetate (CMFDA), which is converted to a fluorescent substrate inside cells and transported by MRP2 into bile canaliculi of spheroids. When using medium lacking of calcium it was possible to measure the amount of fluorescent substrate that was transported into bile canaliculi. The result indicated that MRP2 was active in spheroids, but the method used needs further development to reduce the large variation and the challenging experimental procedure.

Keywords: Two-organ-chip, primary human hepatocytes, spheroid, bile canaliculi transporter, multidrug resistance protein 2 (MRP2), 5-chloromethylfluorescein diacetate (CMFDA).

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Without all of you it would not have been possible to complete this project.

Linnea Johansson, Gothenburg, May 2017

Contents

1
Introduction

In the pharmaceutical industry, both *in vitro* and *in vivo* studies are required to ensure the safety and efficacy of a potential drug. It is very important to evaluate the specificity and toxicity of a compound *in vitro*, but *in vivo* studies are always needed to see the effect in a complete system. However, animal testing often fail to predict the effect of a drug, because of the huge difference between *in vitro* studies, animal studies and human trials. To be able to reduce the amount of animal trials, and at the same time have a better prediction of the outcome in humans, new *in vitro* methods are necessary [1]. The liver is the most important metabolic organ in the human body. It is responsible for synthesis, metabolism and detoxification of compounds. Therefore, it is important to study the biology and functionality of the liver to be able to mimic this in *in vitro* studies, an the information is very useful when developing new candidate drugs [2].

1.1 Background

In previous studies it has been shown that cultured hepatic cells in 3D liver spheroids have great similarities with human *in vivo* liver. In a study by Bell *et al.* cultivation of 3D spheroids formed of primary human hepatocytes was done to develop a system used for studying liver function, drug-induced liver injury and liver diseases. The result showed that the liver spheroids were similar to liver *in vivo* with respect to proteome analyses, morphology and viability [2]. Also, it has been shown that 3D liver spheroids can be produced in large amounts, as well as used as disease models [3]. As previously described, a huge challenge within the drug development process is to replace animal testing with *in vitro* studies that reflects the complexity of the human body. A method that has been developed to overcome this problem is a so-called organ-on-a-chip. In a study by Wagner *et al.* they present a multi-organ-chip with co-cultures of human artificial liver microtissues and skin biopsies. This method consists of a microfluidic culture chip that provides a more similar environment to *in vivo* situations due to the adjustable media fluid flow. Micropumps and the formation of separate tissue culture spaces connected through channels provides a possibility to connect different organs in the chip in a more similar *in vivo* environment. This gives a great advantage when performing pre-clinical drug development studies [4]. AstraZeneca have right now a collaboration with the company TissUse GmbH with the aim to develop a functioning multi-organ-chip device with human liver co-culture and pancreas islets. Thus, this thesis project will be a part of this larger project.

Furthermore, it is well known that transporters plays an important role in clearance of compounds in hepatic cells. It is important to study hepatic transporters to be able to understand the disposition of substances *in vitro* and to get a better prediction of drug disposition in humans. Moreover, transporters can be responsible for change in drug reactions and efficacy, which makes it important to study their biology and function for improvement in the drug discovery process [5]. Therefore, the second part of this master thesis project will be to evaluate the activity of a transporter in hepatic spheroids.

1.2 Aim

This project will be divided into two different parts. The first part is a collaboration with the company TissUse GmbH in Berlin with the task to develop a two-organ-model with liver spheroids and pancreatic islets on multi-organ-chip from TissUse. The second part of the project will be to further develop and characterize transporters in hepatic liver spheroids, which is an ongoing work at AstraZeneca.

Aim 1: Evaluate liver specific functions of liver aggregates formed of HepaRG cells co-cultured with stellate cells that has been in a two-organ-chip system together with pancreatic islets. The aim is as well to compare results with liver aggregates that has been in a two-organ-chip system in the absence of pancreatic islets.
Aim 2: Evaluate the activity of the canalicular transporter, multidrug resistance protein 2 (MRP2) in spheroids formed of primary human hepatocytes or co-cultured hepatic spheroids containing primary human hepatocytes and stellate cells.

1.3 Limitations

Due to limited time, as well as the complexity of the project, limitations are needed and these are presented below.

One limitation is that two organs are cultured in the multi-organ-chip and not more. This limitation is due to complexity of spheroid-culturing, as well as the chip complexity. It is not possible to involve more organs into the chip-system before chip with two organs are working as a functional model system. Although two organs in a closed system gives a greater understanding than one organ individually, it is not optimal. For example, the liver is not the only organ consuming glucose, and this makes the model a simplified version of the clinical situation. Due to time limitations, it is only albumin secretion, glycogen production, and CYP3A4 production in liver aggregates that is evaluated. If having the time it would be interesting to evaluate more hepatic functions like, viability, apoptosis and mRNA expression of metabolic enzymes.
The second part of the project will also have limitations such as evaluate a limited number of transporters, in this case one. In addition, the transporter that is analyzed in the present study is a canalicular efflux transporter, if having the time, it could

be interesting to also analyze a different type of transporter such as an sinusoidal transporter. Furthermore, there is only one substance and one inhibitor used in the project to evaluate the activity of the transporter. This is due to the limited amount of time and the limited access to possible substances. It would be interesting to analyze a broader range of substrates and inhibitors, for an optimal effect.

2

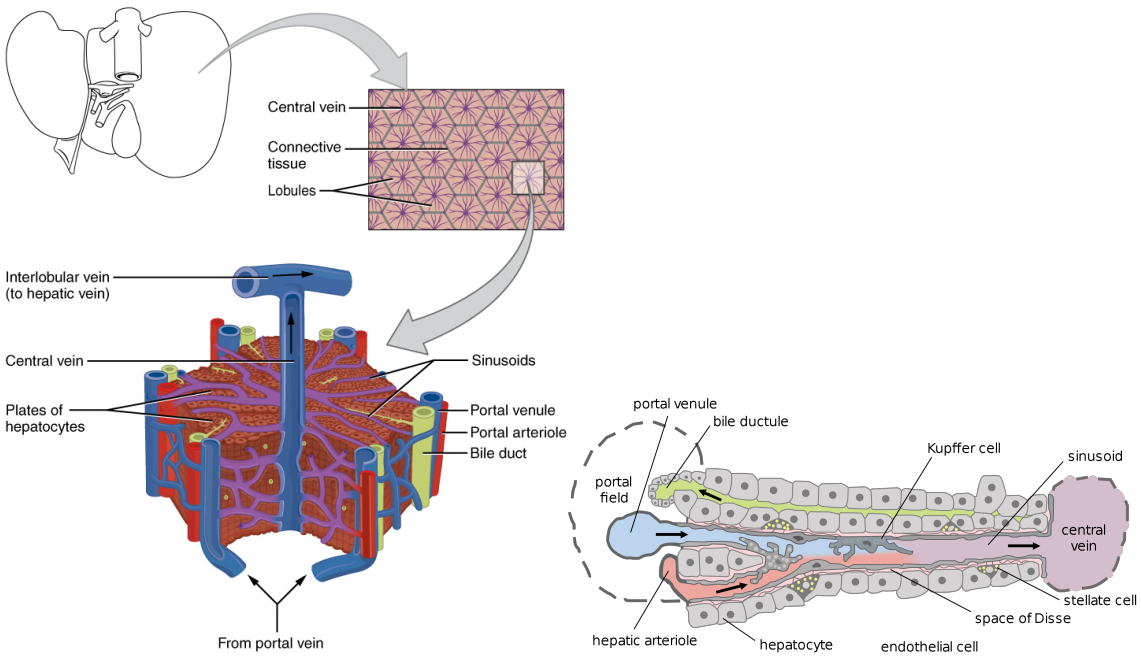
Theory

2.1 Liver

The liver is the largest organ in the human body and is responsible for many important functions such as metabolizing drugs and toxins as well as producing bile and regulating glucose and plasma protein production. The functional unit in the liver is called lobule and consist of three to six portal triads surrounding a central vein, illustrated in panel a) in figure 2.1. The portal triads consists of a branch of the portal vein, a bile duct and the hepatic artery. The portal vein and hepatic artery fuses and enters into the lobule to form the so-called sinusoid which is small blood vessels mixed with oxygen-rich blood from the hepatic artery and nutrient-rich blood from the portal vein. This is to supply the surrounding hepatocytes with oxygen and nutrients. The bile duct is the third component of the portal triad. Bile is produced by the hepatocytes and is secreted into the biliary system and flows in the opposite direction of the blood from liver to the small intestine. The three components of the portal triad are illustrated in panel b) of figure 2.1. Between the hepatocytes, bile canaliculi are located, which is the initial tubular structures of the bile ducts. Bile is a fluid consisting of inorganic and organic substances, like water, bile salts and cholesterol dissolved in alkaline solution. The main function of the bile is to help the digestion and absorption of lipids and lipid-dissolved vitamins, as well as eliminate waste products into the bile and later into feces [6].

The liver consists of several different cell types that can broadly be divided into parenchymal and non-parenchymal cells. The hepatocytes are included in the parenchymal cells and Kupffer cells, sinusoidal endothelial cells, stellate cells, periportal fibroblasts and hepatic dendritic cells are non-parenchymal cells. The hepatocytes constitutes the majority of the liver mass (80%) and forms the structure of the liver. Hepatocytes are the functional cells in the liver and are polarized with three membrane domains, sinusoidal (basal), lateral and canalicular (apical) membrane. As described above the hepatocytes produce bile that is secreted into the bile duct. The lateral domain will generate tight junction between the hepatocytes and forms a barrier between the bile duct and sinusoid. Other functions of hepatocytes, beyond producing bile is detoxification and synthesis of plasma proteins, for example albumin. The sinusoid is lined on the inside with sinusoidal endothelial cells, which as in other tissues, is an anti-thrombosis factor as well as assists in delivering oxygen and nutrients. Inside the sinusoid, the Kupffer cells are present. They are macrophaging cells that are attached to the endothelial cells. Parallel to the sinu-

soid the space of Disse is located and this separates the sinosoidal endothelial cells from the hepatocytes. The space of Disse contains blood plasma as well as the stellate cells. The stellate cells are located between the hepatocytes and the endothelial cells, or can also be located in between the hepatocytes. They contribute to 1.5% of the total liver mass and a major function is storing vitamin A in form of lipid droplets [6]. Another important function of stellate cells are their ability to store vitamin A (retinoid) as cytoplasmic droplets [7]. In addition, it has been confirmed in studies that stellate cells may be a precursor cell that synthesize ECM as well as the cell responsible for hepatic fibrosis. One other important function of the liver is to produce plasma protein to maintain the colloid osmotic pressure in plasma. Plasma proteins are almost exclusively produced by the liver. Hepatocytes produce, secret and degrades macromolecules, for example albumin to maintain the precise colloid osmotic pressure. A greatly reduced liver function will result in a reduction of plasma proteins, for example albumin [8]. Figure 2.1 illustrates an overview of a liver lobule, seen in panel a). Panel b) shows the different cell types in the liver. [6].



(a) **(b)**

Figure 2.1: Panel a) illustrates an overview of an hepatic lobule and panel b) a close-up of the hepatic structure.

2.2 Glucose regulation- Liver and pancreas

The liver has the functionality of maintaining the blood glucose level at a tightly controlled level. At high levels of glucose in blood, after food intake, the liver stores glucose in form of glycogen, a process called glycogenesis. When glucose level decreases after a few hours, the liver starts to secret glucose into the blood again to maintain a stable level. Pancreas is an organ that regulates metabolism of glucose, lipids, proteins, as well as digestive functions. The two major hormones secreted by

pancreas is insulin and glucagon. Pancreas consists of two types of tissues, islets of Langerhans and the acini. Acini secrets digestive enzymes, while the islets of Langerhans controls glucose, lipid and protein metabolism. Islets of Langerhans consists of three different cell types, α, β, δ-cells. β-cells, which constitutes to the majority of the islets-mass, secrets insulin. α-cells makes up 25% of cell mass and secrets glucagon, and δ-cells secrets somatostatin. These hormones effect one another, insulin inhibits glucagon secretion and somatostatin inhibits both insulin and glucagon. Glucagon is a hormone that increases blood glucose levels and insulin decreases blood glucose levels. In this present master thesis project, islets of Langerhans is used in the so-called organ-on-chip experiment. In a healthy person, after food intake, blood levels of glucose increases. Glucose is transported into the β-cells through glucose transporters, GLUT 2, which leads to insulin exocytosis stimulated by intracellular pathways. Insulin will bind to insulin receptors located on a wide range of different cell types, and through a cascade of phosphorylation it will affect the cell metabolism. One important example is that glucose transporters are moved to the cell membrane to facilitate glucose uptake [9].

The present master thesis project is a part of a bigger project at AstraZeneca. The goal is to develop a two-organ-chip with human liver spheroids and pancreatic islets that communicates and integrate with each other similar to how they do in the human body. The two-organ-chip should respond to high glucose, and secret insulin concentration similar to humans. Medium in the chip-system have a glucose concentration of 11 mM, which corresponds to a high glucose level in blood. This is used to be able to see a clear glucose decrease in medium in the co-cultured chip-system.

2.3 Diabetes Mellitus

Diabetes Mellitus is a group of metabolic diseases characterized by defects in insulin secretion or sensitivity. There are two types of diabetes, called type I and type II. Around 5-10% of patients with diabetes mellitus have type I diabetes. In this disease, the pancreatic β-cells are destroyed which may be due to autoimmune disorders or viral infections. The destruction of β-cells results in very low levels of insulin, as in turn leads to high blood glucose levels. Type II diabetes are far more common than type I diabetes. In type II diabetes, β-cells increases the insulin secretion due to decreased insulin sensitivity in target cells. Insulin resistance is a condition that develops over time and is closely related to overweight and obesity. There are different explanations how insulin resistance and overweight are related. Some studies suggest that there are fewer insulin receptors while others believe that signaling pathways are disrupted. At start, β-cells compensate and produces high amounts of insulin and this results in only a slightly elevated blood glucose level. Later on, β-cells can no longer produce high amount of insulin and blood glucose level increases dramatically. Diabetes mellitus is very common disease worldwide and the predictions are that it will increase. Due to this, it is a high cost for society, and the medications today are not optimal. This health problem gives a major need of functional medications and research in the field [9].

2.4 Drug metabolism

Pharmacokinetics and pharmacodynamics are two essential areas that is important to study when investigating drug disposition and efficacy of substances when developing potential drugs. Pharmacokinetics is the study of how a drug behaves in the body, time course of its absorption, distribution, metabolism and elimination. Pharmacodynamics is in contrast the study of how an organism is affected by the drug. The liver is involved in the first defense against xenobiotics dependent on drug metabolizing enzymes and transporters [10]. The metabolism of a drug is often divided into two phases, which is followed by transportation out from cells via active transport. During phase I of drug metabolism, the parent molecule is converted to a more water soluble substance by alteration, for example addition of polar groups (-OH, -SH, -NH2). Phase I metabolism is executed by enzymes in a major enzyme family called cytochrome P450 (CYP). CYPs catalyze the oxidative biotransformation for the majority of drugs and are therefore important to study during clinical pharmacology [11]. During phase II, the conjugation phase, the compounds becomes even more water soluble to be able to be excreted out of the body more easily. In the conjugation phase water soluble endogenous sugars, salt or amino acids are attached to the xenobiotic, by conjugation or synthetic enzymes. An additional phase, phase III involves the efflux system and describes how the cell excludes the xenobiotic through efflux pumps to the intestinal fluid, blood, and finally the kidney. In addition, lipophilic substances can be excreted out from the cell into the biliary system [12]. Transporters play an important role in this area and can affect the efficacy, distribution and bioavailability of a drug. Thus, the measurement and prediction of permeability over membrane for a candidate drug is important to study in the drug discovery process [13]. The optimization of a drug candidate is very much dependent on all the above described areas of drug metabolism. Increasing of bioavailability of a drug as well as controlling the time dependent mechanism of action is very important in the drug discovery process. Thus, it is important to investigate metabolism and pharmacokinetics.

2.5 Cell lines

The cells used in this present study are cryopreserved primary human hepatocytes, HepaRG cell line and primary human stellate cells. In this section, properties of these cells are described.

2.5.1 Primary human hepatocytes

Primary human hepatocytes are isolated cells originating from a human liver, and they are considered as the gold standard for xenobiotic metabolism and cytotoxicity studies [14]. Primary human hepatocytes are simple to use and remain their cytoarchitecture when cultured [15]. However, some drawbacks with primary human hepatocytes have been observed. The limited availability of fresh human liver samples is a major problem, in addition to the big individual variance between donors. Another limitation is the high cost when using primary human hepatocytes

for screening methods [14]. Furthermore, the hepatic functions decline over time in conventional cultures [15]. For example, primary human hepatocytes loose their CYP enzyme activity in culture systems [16]. To overcome these limitations, immortalized hepatic cell lines has become an alternative culture system for studying drug metabolism.

2.5.2 HepaRG

HepaRG cells is a human hepatocellular carcinoma cell line that originates from a female human with a liver tumor suffering from chronic hepatitis C infection and macronoduar cirrosis. This cell line can differentiate into two different cell types, hepatocyte-like and biliary-like cells [17, 18].The cell line has been shown to retain drug metabolizing capacity, expressing a variety of drug metabolizing genes belonging to CYP enzymes. Moreover, HepaRG have a stable expression of phase II enzymes as well as transporters. Although some metabolites have shown to have a lower concentration in HepaRG compared to cryopreserved human hepatocytes. The cell line is suitable for transporter investigations and preforming drug metabolism studies [14, 17].

2.5.3 Primary human stellate cells

It is known that human hepatic stellate cells synthesize ECM proteins in the liver, and have a regenerative role due to production of various growth mediator as hepatocyte growth factors. Stellate cells are located in the space of Disse, in direct contact with adjacent hepatocytes. Due to the direct contact as well as their specific functions, it has become an great candidate for co-culturing with hepatocytes [19]. It has also in earlier studies been shown that co-cultured spheroids with primary hepatocytes and human stellate cells have improved metabolic function as well as the maintenance of the spheroid shape and tighter cell-cell contact, compared to human hepatocyte spheroids. In recent studies, it has also been shown that the paracrine function of human stellate cells play a critical role in the improvement of liver functions such as albumin secretion and CYP reductase [20].

2.6 Spheroids

Primary human hepatocytes in 2D are considered as the gold standard *in vitro* cell-model to study biology and function of the human liver. Hepatocytes in 2D monolayer cultures lose their polarity and hepatic phenotype because of de-differentiation. Even though 2D cultures are easy to handle and cheaper to culture, 3D models are necessary to mimic the complexity of a human liver. A liver *in vivo* is based on cell-cell and ECM interactions, and many *in vitro* models have been developed to mimic this property, for example sandwich cultures and scaffold based 3D models. However, some problems like drugs binding to scaffold as well as differences in ECM substances has been identified when using these models. To overcome these problems primary human hepatocytes can be cultured in a 3D model called a spheroid,

also named 3D microtissue. It has been shown that hepatocytes in spheroids remain polarized, they have a conserved hepatic phenotype over a longer period of time and the spheroids form bile ducts [2]. There are different techniques to culture hepatic spheroids, for example culturing cells in suspension, hanging drop method or culturing cells in non-adherent microwells. Cells in suspension form aggregates while stirring a large volume of culture medium. The technique is beneficial for a mass production but it is difficult to control the size of aggregates. The hanging drop method forms aggregates with controlled size by letting droplets of cell suspension hang from a surface. In this present study a non-adherent 384-well plate have been used to form liver aggregates. This technique utilizes that cells adhere to each other to form a 3D shape instead of attaching to the plate surface in a non-adherent plate. The original technique was aimed to form aggregates in non-adherent 96-well plate, but it is now possible to use non-adherent 384-well plates for an increased quantification of spheroid production [21].

2.7 Multi-organ-chip

As described above there is an unmet need of predictable human *in vitro* methods for pre-clinical studies in the pharmaceutical industry. As of this demand, a so called organ-on-a-chip system has been developed. The organ-on-a-chip is a multi-compartment system connected with micro-channels which enables linking of different organ in addition to improvement of environment for the specific organ. This multi-organ system makes it possible to mimic an *in vivo* human system but in a miniaturized scale [4]. The micro-pump system enables a continuous circulation of media through the tissue compartments at a certain flow rate, with the physiological mechanical stress specific for the tissue used [22]. This master thesis project will be a part of a bigger project where AstraZeneca develops a multi-organ-chip containing liver aggregates formed of HepaRG-cells co-cultured with primary human stellate cells, as well as pancreatic islets. The pancreatic islets originate from cadaveric donors and are formed to spheroids by the company InSphero. During the multi-organ-chip project, many different parameters are measured and analyzed, to evaluate if liver aggregates and pancreatic islets are communicating in the chip-system and if glucose levels in medium is regulated by pancreatic islets and liver aggregates. The present master thesis project includes evaluation of functionality of liver aggregates through albumin, glycogen and CYP3A4 measurements.

2.8 Transporters in drug development

Both hepatic metabolizing enzymes and hepatic transporters have an important role in the intracellular drug distribution and the clearance of drugs [23, 24]. The movement of drugs across the hepatic membranes will be determined by the hepatic transporters [25]. Uptake transporters will increase the intracellular concentration while efflux transporters and metabolizing enzymes will decrease the concentration of a drug inside the cell [24]. Changes caused by drug transporters can therefore influence the efficacy, toxicity and disposition of a drug [26]. Thus, it is important

to study the activity of transporters that has the major role of sinusoidal uptake, biliary and sinusoidal excretion. In this project the activity of transporters in hepatic spheroids over a time of three weeks were studied to evaluate spheroids as a pre-clinical *in vitro* system.

Free unbound compounds that circulates in the blood will reach the liver through the sinusoid and are taken up by the hepatocytes either through diffusion or active transport at the basal membrane. Inside the hepatocytes the compounds can be metabolized through CYP- enzymes and/or phase II conjugating enzymes. These metabolites are then transported through carrier mediated transport to the bile or sinusoid blood for renal elimination [23]. In this thesis project activity of the bile canalicular transporter MRP2 will be investigated. MRP2 transports a wide range of compunds and is one of the most important canalicular transporters in the liver [25]. In the pharmaceutical industry *in vitro* models are very useful in pre-clinical studies to be able to give an understanding and prediction of how for example a drug behaves *in vivo*.

Drug-drug interactions on transporters can be a problem when developing drugs and is caused by inhibition of transporter activity by a co-administered drug. A functional impairment of a canalicular efflux transporter can be associated with an increased risk of drug induced liver injury (DILI) in human. This is due to an increased concentration of parent drug or metabolites inside the cell and can stop the drug candidate from reaching the market. Therefore it is important to gain more knowledge about drug candidates interactions with canalicular transporters, both as substrates and as possible inhibitors [27, 28].

2.8.1 MRP2

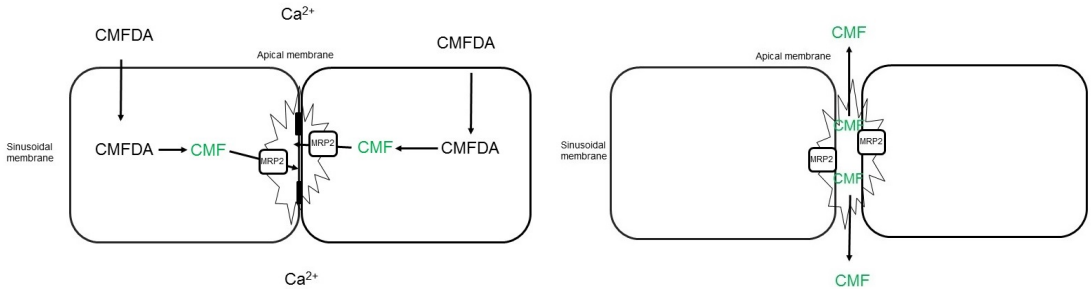
The multidrug resistance protein 2 (MRP2) is an ATP binding cassette transporter that is located in the canalicular (apical) membrane in hepatocytes. MRP2 transports a wide range of phase II metabolites, thus it has an important role in detoxification and chemo protection [29]. MRP2 transports organic anions, including bile salts and glutathione. Uncharged compound can be transported through MRP2, in co-transport with glutathione, and due to this, pharmacokinetics of a drug can be modulated. Additionally the expression of MRP2 could be altered by different drugs and disease states [28, 30].

2.9 Theory behind transporter activity experiment

In the present master thesis project investigation of the canalicular transporter MRP2 has been based on previous literature and fundamental biological concepts. Previous studies have in different ways investigated bile flow dynamics for prediction of drug induced alterations of bile flow. In a study of Deharde *et al.* they investigate bile canalicular transport of a fluorescent substance in primary human hepatocytes in different sandwich cultures using live cell imaging. They stated that further improvement of culture conditions in sandwich cultures are necessary to

increase the stability of the bile canalicular network, and for studying the biliary excretion in hepatocyes [31]. Another study of Gaskell *et al.* a fluorescent substrate, 5-chloromethylfluorescein diacetate (CMFDA) was used together with antibody staining for analyzing canalicular transports of MRP2 in hepatic spheroids and 2D mono layers formed of C3A hepatoma cells. CMFDA freely passes through membrane of hepatocytes and is converted to a fluorescent product by cytosolic esterases and is actively transported into bile through MRP2. The process is illustrated in figure 2.2. The study shows that active transporters are present in spheroids, due to higher amount of substrate inside cells when inhibiting the transporter [32]. InSphero also investigated MRP2 activity using CMFDA, but in spheroids formed of primary human hepatocytes co-cultured with primary non-parenchymal liver cells. To quantify the canalicular transport of substrate, the area and sum intensity of bile canaliculi network using Harmony High Content Imaging and Analysis Software was calculated [33].

In the present master thesis project, the aim was to developed and optimize a functional quantitative *in vitro* method used for studying MRP2 activity in hepatic spheroids using CMFDA. To be able to measure the amount of substrate transported into the bile canaliculi network, a medium lacking of calcium and magnesium were used. Tight junctions between the hepatocytes are the major barrier between the canalicular lumen and the ECM space [34]. Tight junction proteins are disassembled and dephosphorylated and cells lose their tight association by actin filaments in the absent of calcium, in monolayers [35]. Therefore, in theory, spheroids treated with medium lacking of calcium should open up their tight junction and the substrate inside the bile canaliculi networks is released and fluoresce intensity can be measured. The process is illustrated in figure 2.2. In this present master thesis project, an inhibitor for MRP2, called Probenecid was used. As described in previous section 2.8, the model used and developed in this master thesis project could be a way of evaluating if a compound is a substrate or an inhibitor for the biliary transporter MRP2. Probenecid is a known inhibitor for MRP2 and is used to be able to, in the future have the possibility to compare the inhibitory effect caused by other compounds.



(a) **(b)**

Figure 2.2: Panel a) illustration of two hepatocytes connected by tight junctions, and the formation of bile canaliculi in between hepatocytes. CMFDA, freely passes cell membrane and is converted to a fluorescent substrate for MRP2 (CMF) inside cells, and transported into bile canaliculi. Tight junctions are formed in the presence of calcium. Panel b) illustrates spheroids treated with medium lacking of calcium, leading to disruption of tight junctions and leakage of CMF into medium.

3
Methods

3.1 Materials and chemicals

Human Albumin ELISA Kit (UK) and CYP3A4 (1 mg/mL) (UK) primary antibody was obtained from Abcam. Glycogen Colorimetric Assay Kit II (USA) was obtained from BioVision. Maintenance medium and thawing/seeding additive (US) for HepaRG cells was purchased from Biopredic. HepaRG cells (HPR116189-TA08), InVitroGRO CP, Torpedo, primary human hepatocytes and Stellate cells was obtained from Bioreclamation/IVT. (USA). 24-well ULA plate, 96-well ULA plate, 384-well ULA plate and T75 collagen coated flasks were obtained from Corning. Gentamicin (UK), Insulin transferrin selen (ITS) (NY, USA), Hanks' Balanced salt solution, both with and without $CaCl_2$ and $MgCl_2$ (UK), TrypLe Select (Germany), L-glutamine (UK), Williams' medium E (UK), and was obtained were obtained from Gibco by life technologies. Diva Decloaker (10x) and Hot rinse (2X) were obtained from Biocare Medical (Concord, California, USA). Secondary antibodies, Alexa Fluor 488 Goat anti rabbit (2 mg/mL) and Alexa Fluor 555 Goat anti mouse (2mg/mL) was obtained from Life technologies (USA). Bovine Serum Albumin (BSA) (USA), Hydrocortisone hemisuccinate (USA), Penicillin/Streptomycin solution (PEST), Triton X-100 (Germany) and Trypan blue solution were gained from Sigma-Aldrich (Germany). Basal medium for stellate medium was obtained from ScienCell (USA). Albumin primary antibody (1 mg/mL) (Czech Republic), fetal bovine serum (FBS), Pierce BCA Protein Assay Kit, was purchased from Thermo Scientific. CellTracker™ Green 5-chloromethylfluorescein diacetate (CMFDA) dye, 1 mg, Hoechst 33342 Trihydrochloride, trihydrate (16.2 mM) (USA) was purchased from Thermo Fisher Scientific. Probenecid (10 mM) was prepared at AstraZeneca (Sweden). The primary human cryopreserved stellate cells used in this project were expanded at AstraZeneca and frozen at passage number 3.

3.2 Part I- Multi-organ-chip

3.2.1 Spheroid culturing

HepaRG cells were thawed in water bath and mixed with 30 mL of thawing/seeding medium, consisting of William's medium E and thawing/seeding additive. Cells were centrifuged for 3 minutes at 500 x g at room temperature. The supernatant was gently removed and cells were re-suspended in thawing/seeding medium. Cells were counted under a microscope for yield and viability using trypan blue followed by

splitting cells into two T75 collagen coated flasks for pre-culturing. Seeding density used was 0.184 million cells/cm^2. One day after seeding, medium was changed to maintenance medium ordered from biopredic, in each culture flask. The same procedure was followed for pre-culturing stellate cells, but with some exceptions. The stellate cells were centrifuged for 5 minutes at 300 x *g*, they were seeded in four T75 flasks with the density of $7.14*10^5$ cells/flask and stellate medium was used consisting of 500 mL basal medium ScienCell, 10 mL FBS, 5 mL of stellate cell growth supplement (SteCGS) and 5 mL of PEST. In addition, no medium change for stellate cells was performed one day after seeding. Flasks with HepaRG and stellate cells were incubated at 37℃, 5% CO_2. For two or one day respectively, flasks for HepaRG and stellate cells were washed with PBS, followed by addition of TrypLE Select and incubation in 37℃ until cells were completely detached. Then, chip medium consisting of William's medium E with 10% FBS, 2 mM L-glutamine, 50 µM hydrocortisone hemisuccinate, 5 µg/mL gentamicin and 5 µg/mL ITS, was added and cells were transferred to a 50 mL Falcon tube. The cells were centrifuged for five minutes at 80 x *g* at room temperature, the supernatant was removed and the cells were re-suspended in chip medium and counted for yield and viability. Each aggregate consisted of 25 000 cells, with the ratio of 24:1 HepaRG:stellate cells. The cell concentration for seeding HepaRG was 480 000 cells/mL and 20 000 cells/mL for seeding stellate cells. Cell suspension was seeded (50 µL/well) in a 384-well ultra low attachment (ULA) plate, which was centrifuged for 2 min at 100 x *g* and placed in the incubator 37℃, 5% CO_2. Two days after seeding the plates were put on a shaker. Four days after seeding, 20 aggregates were collected in 1 mL chip medium with low insulin concentration (1 nM) in a 24-well ULA plate. The plate was incubated overnight on an orbital shaker. The liver aggregates were washed twice with PBS with BSA (0.1%), and medium was changed to insulin-free chip medium and incubated for two hours on orbital shaker where after 40 liver aggregates were transferred to each chip chamber.

3.2.2 CHIP design

Two-organ-chips were used, equal to chip described in the article by Wagner *et al.*. The chips were ordered from TissUse GmbH and the layout is showed in figure 3.1 presented below. The chip contains two separate circuits with two chambers connected to three pumping tubes. Upon arrival chips were washed with PBS and 300 µL insulin-free chip medium, was added to each chamber and connected to the pump units. Pancreatic islets ordered from InSphero were washed with insulin-free chip medium. They were spun down in eppendorf tubes and media was changed. Before cells were inserted, medium in the chips were changed to 300 µL fresh insulin-free chip medium with 11 mM glucose concentration in liver chambers and 300 µL corresponding medium to pancreatic islets chambers. Thereafter 40 liver aggregates and 10 pancreatic islets formed to spheroids were transferred to each dedicated chamber. The pancreatic islets were formed to spheroids by the company Insphero.

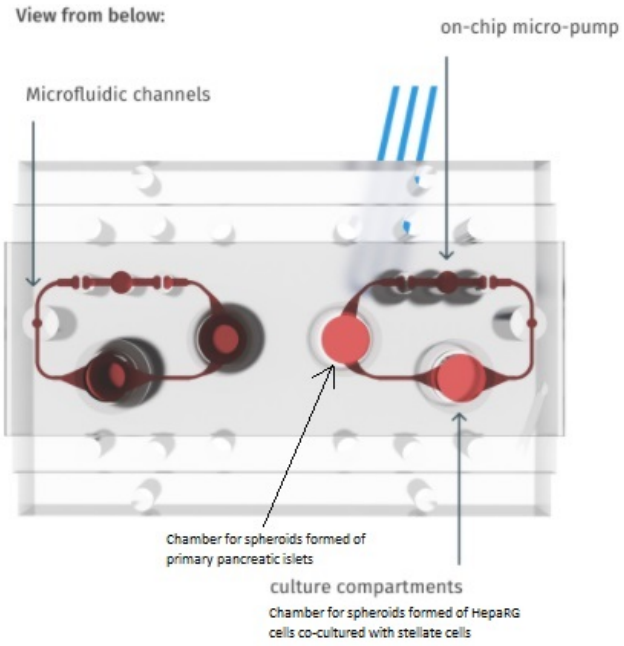


Figure 3.1: Illustration of chip used in the present study. The chip contains two replicates of two chambers with spheroids formed of primary human hepatic or pancreatic islets, respectively, connected with pumping tubes of medium. Image courtesy of TissUse GmbH.

3.2.3 Sampling

The multi-chip experiment was run for two weeks and during this time there were several time points for sampling media used for investigating the properties of spheroids. The media was changed at day 1, 3, 5, 7, 9, 11 and 13. Each time the media was removed, a sample was taken, 15 µL from both liver and islets chambers, total 30 µL sample. The sampling was taken both from the media that is changed (48 hours old), and the new media (time 0). All sampled media was stored at -20℃ until analysis. In addition to this, at day 1, 7, and 13 there was a time series of sample collection at time 0, 4, 8, 24 and 48 hours. At day 8 and 15, 3 replicates were terminated (a chip contains two replicates). Five liver aggregates were picked for analyzing glycogen content, described in section 3.2.5. Approximately 20 liver aggregates from day 8 and 15 were picked for staining and they were incubated in 4% paraformaldehfyde and sent to Histocenter for sectioning, paraffin embedding and haematoxylin and eosin staining.All media sampled was analyzed for insulin, glucagon and albumin content using ELSIA and colorimetric assay for glucose, glycogen and total protein content. During this thesis project analyzes of albumin, glycogen and total protein content has been performed, as well as immunohistochemistry for CYP3A4 and albumin.

3.2.4 Albumin secretion

Albumin analysis was performed using Human Albumin ELISA kit from Abcam according to manufacturers' instructions. In short, medium samples from chip experiment was diluted (1:150) and added to albumin microplate and incubated at 4℃ overnight. The samples were washed, antibody was added followed by 30 minutes incubation at room temperature. After washing five times with washing buffer, conjugate was added and plate was incubated 30 minutes in room temperature. Another wash was performed and substrate was added followed by 20 minutes incubation. Last step was adding stop solution and reading absorbance at 450 nm and 570 nm (background) immediately.

3.2.5 Glycogen assay

The glycogen assay was performed using Glycogen Colorimetric Assay Kit II from BioVision, according to the enclosed instructions. After sampling, liver aggregates were washed with 0.1% BSA in PBS and then lysed in 200 µL 1% Triton X-100 and kept on ice. The samples were boiled for 10 minutes and placed in -80℃ freezer. When preforming the analysis, the samples were thawed and re-suspended, centrifuged for 10 minutes at 18 000 x *g*. The supernatant was then used for the glycogen assay. The dilution used for glycogen assay was varying between 40-10 µL sample added up to 50 µL with glycogen hydrolysis buffer, depending on how much sample that was available.

3.2.6 Fluorescent staining of liver aggregates- Immunohistochemistry

Liver aggregates were sampled at day 8 and day 15 of chip experiment. From both days, 3 samples from circuits with liver and pancreatic islets were taken, as well as 3 samples from circuits with liver aggregates only. These samples were sent to Histocenter for sectioning (4 µm thick) and paraffin embedding. Unstained glass-slides were upon arrival stained for CYP3A4 and albumin. The first step was performing an epitope retrieval. Slides were immersed in DIVA anticloaking agent in a slide cuvette and two more cuvettes were filled with Hot Rinse. The cuvettes were then placed in a steamer with 750 mL dH_20 and steamed for 20 minutes followed by 20 minutes cooling. Slides were then placed in the cuvette with Hot Rinse for 5 minutes each. Afterwards the slides were rinsed in dH_20 until they looked clean, and placed in PBS for approximately 10 minutes. After this the slides were immersed in blocking buffer consisting of 10% BSA in PBS for 45 minutes. Subsequently the tissue sections were encircled with a liquid blocker pen, thus the antibody solutions were kept on the glass slide without risking it to float out. Primary antibodies for CYP3A4 and albumin were diluted in blocking buffer to a concentration of 10 µg/mL and 5 µg/mL, respectively. Approximately 150 µL of primary antibodies were then pipetted onto the slides and incubated in a humidity chamber overnight at 4℃. They were then rinsed in PBS three times for 5 minutes. Secondary antibodies for CYP3A4 (Alexa Fluor 488 goat anti rabbit) and albumin (Alexa Fluor 555 goat anti mouse) were diluted in PBS to a concentration of 5 µg/mL and 4 µg/mL respectively.

Approximately 150 µL of secondary antibodies were pipetted onto the slides and they were then incubated in a dark humidity chamber in room temperature for 45 minutes. Afterwards slides were rinsed in PBS three times for 15 minutes. The slides were then counter stained with Hoechst diluted in PBS to a concentration of 0.625 µg/µL for 1 minute and then rinsed for 5 minutes in PBS. The last step was done by placing a drop of mounting medium for fluorescent purpose on a coverslip of glass and placing it on the tissue section. The slides were then imaged in a fluorescent confocal microscope (Nikon Type 108 and H600L).

3.3 Part II-Evaluation of transporter activity

3.3.1 Spheroid culturing

InVitroGRO CP with Torpedo (InvitroGRO CP medium) was prepared and pre-heated according to Bioreclamation IVT protocol. Thereafter cryopreserved primary human hepatocytes were thawed and re-suspended in of InvitroGRO CP medium. Cells were spun down for 5 minutes at 80 x g and the supernatant was then removed. The pellet was dissolved in William's medium E with 10% FBS, 2 mM L-glutamine, 1% PEST, 100 nM dexamethasone and 1% ITS, (PHH medium). Cells were counted, using trypan blue and diluted in PHH medium. They were then seeded (80 µL/well) in a ULA round bottom 384-well plates, with the concentration 24000 cells/mL and 2000 viable cells/well. The plates were centrifuged for 2 minutes at 100 x g before put in the incubator at 37℃, 5% CO_2. After five days spheroids were formed and 40 µL medium was changed every day for five days to PHH medium without FBS. After five days the medium was changed every second to third day.

At the second occasion, both spheroids containing primary human hepatocytes and spheroids with primary human hepatocytes together with stellate cells were seeded. Primary human hepatocytes were prepared as described above. Pre-culturing of stellate cells was prepared as described in section 3.2.1. The cells were seeded (80 µL/well) in a ULA round bottom 384-well plates, with the concentration 24000 hepatocytes cells/mL (1920 cells/well), 1000 stellate cells/mL (80 cells/well), and a total volume of 2000 cells/well. The plate was centrifuged for 2 minutes at 100 x g before incubated at 37℃, 5% CO_2. After five days the spheroids were formed and 40 µL medium was changed every day for five days to PHH medium without FBS. After five days the medium was changed every second to third day.

3.3.2 Transporter activity assay

Experimental plan 1

Six days after spheroids were seeded, spheroids were picked and moved to a flat bottom ULA 96-well plate, with 10 spheroids/well in PHH medium without FBS. The inhibitor used for MRP2, probenecid, was diluted from stock concentration of 10 mM in DMSO to 30 µM, 10 µM or 5 µM in PHH medium without FBS. The second time using this experimental plan, the inhibitor concentration was increased

to 100 µM, 30 µM and 5 µM. All PHH medium without FBS was removed from the wells with spheroids and 50 µL of inhibitor was added. The plate was incubated at 37℃, 5% CO_2 overnight. After incubation, spheroids were moved to an adherent 384-well plate in PHH medium without FBS and incubated in 37℃, 5% CO_2 for approximately 3 hours. This was done to make spheroids adhere to the bottom of the wells, which facilitates the washing steps of the assay. Then, medium was removed from wells and 50 µL 5 µM CMFDA was added and the plate was incubated for 45 minutes. This was followed by a washing step using 30 µL with Hanks' Balanced Salt solution containing calcium and magnesium (HBSS ++). Thereafter 30 µL of HBSS ++ or HBSS– (HBSS lacking calcium and magnesium) was added followed by 10 minutes incubation. After incubation, 20 µL of medium was moved to a white, flat bottom 384-well plate and the fluorescence intensity was measured in a fluorometer (Infinite M200 TECAN) at excitation wavelength 492 nm and emission wavelength 535 nm. After measuring fluorescent intensity, spheroids were imaged in a fluorescent confocal microscope (Yokogawa CV7000).

Experimental plan 2

The second experimental plan is an optimization of experimental plan 1, due to problem during washing and incomplete inhibition by probenecid. Instead of incubating spheroids in probenecid over night, they were incubated for 2 hours, followed by removing half of inhibitor volume and adding substrate in combination with inhibitor. Additionally the inhibitor concentration was increased to 5 µM, 30 µM and 100 µM. During washing step, instead of removing all of the volume added of HBSS++, only half the volume was removed and the same volume was added, and repeated three times.

Experimental plan 3

The third experimental plan was a further optimization and development of the two previously described experiments. Due to incomplete attachment of spheroids to wells, the washing steps was very challenging and needed improvement. Ten spheroids in 20 µL PHH medium without FBS was mixed gently with 10 µL of ice-cold matrigel in tubes. Subsequently the mixture of total 30 µL was immediately moved to the bottom of a well in a 384-well plate. After this step the same procedure was done as previously described in experimental plan 2.

Experimental plan 4

Due to unexpected results from experimental plan 3, an optimization of experimental plan 2 was done. The CMFDA concentration was increased to 10 µM.Washing was done two times with 50 µL, and a third time with 60 µL before removing the total volume and adding 10 µL of HBSS++/- - for incubation.

4
Results and Discussion

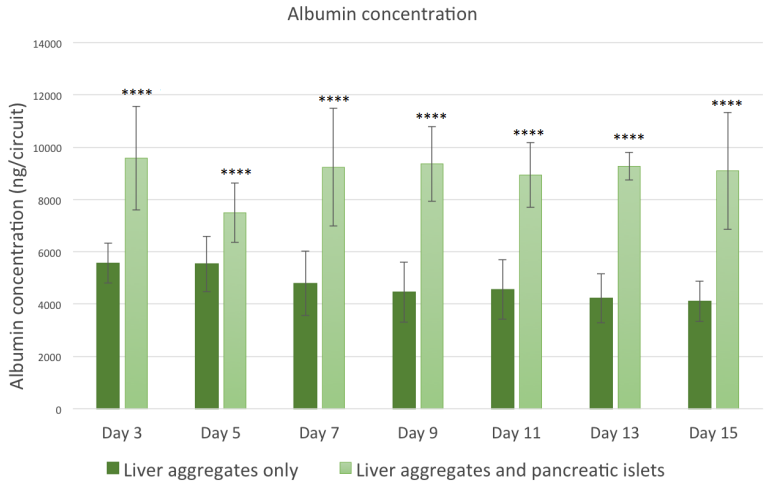
4.1 Part I- Multi-organ-chip

This section summarizes and discusses the results from the multi-organ chip experiment.

4.1.1 Albumin ELISA

Albumin is a plasma protein produced by the hepatocytes in human liver. At a state of reduced liver function, the albumin production will decrease [8]. Albumin is therefore in this project used as a marker for functional liver aggregates. Mean values, standard deviations and t-test from albumin ELISA measurements are presented in Appendix A.

Figure 4.1 shows amount of albumin in ng per circuit (a replicate of a closed system with one chamber for liver aggregates and one chamber for spheroids formed of pancreatic islets) in medium sampled from day 3, 5, 7, 9, 11, 13 and 15 in chip containing either liver aggregates only or both liver aggregates and pancreatic islets.



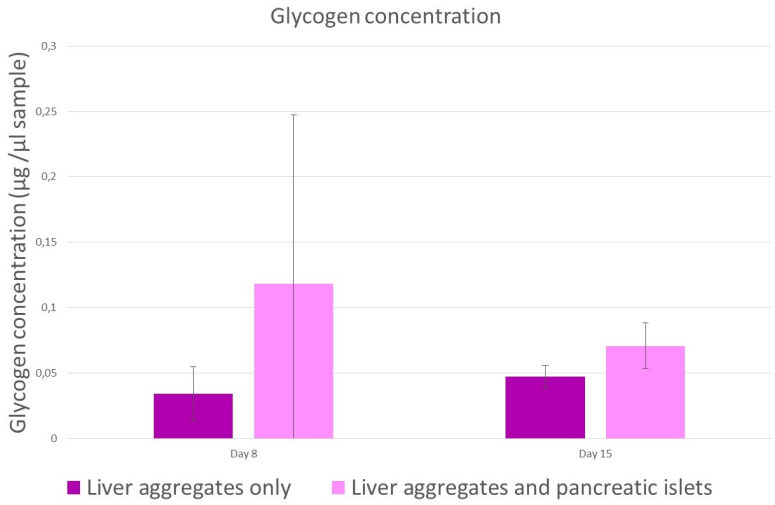
(a)

Figure 4.1: Secreted albumin from liver spheroids formed of HepaRG cells co-cultured with primary human stellate cells. Albumin concentration was measured in medium during a time course of 15 days (day 3, 5, 7, 9, 11, 13 and 15). Measurements were performed on medium from chips containing only liver aggregates, as well as on medium from chips containing both liver aggregates and pancreatic islets. Mean ± SD, n=16, ****indicates $p<0.0001$.

It is clearly shown that albumin secretion from liver aggregates in chips containing liver aggregates and pancreatic islets is the highest. This is significant for results from all days (t-test $p<0.0001$). This results indicate that the liver aggregates are healthier and more functional when communicating with the pancreatic islets. Even though levels of albumin is much higher in human blood (35-50 g/L) compared to the levels of albumin from liver aggregates in the chip experiment, the increase suggests that the liver aggregates are more functional in the co-culture system together with pancreatic islets [36]. Comparing the albumin secretion over time, it appears that the average albumin concentration in the chips with only liver aggregates slightly decreases with time in culture, however this trend is not significant. The same trend is not observed between day 0 and day 15, in the chips with both liver aggregates and pancreatic islets. Hence this further supports the interpretation that liver aggregates are more functional when cultured together with pancreatic islets in the chip system. The results as well indicates that the chip system is working and that the liver aggregates communicate with the pancreatic islets.

4.1.2 Glycogen content

At high glucose levels the human body stores, mainly in the liver, glucose in form of glycogen. This process called- glycogenesis- is regulated by insulin at high glucose levels [9]. Glycogen concentrations were investigated in this project in liver aggregates from chip-experiment containing liver aggregates only, as well as liver aggregates together with pancreatic islets, and the results are showed in figure 4.2.



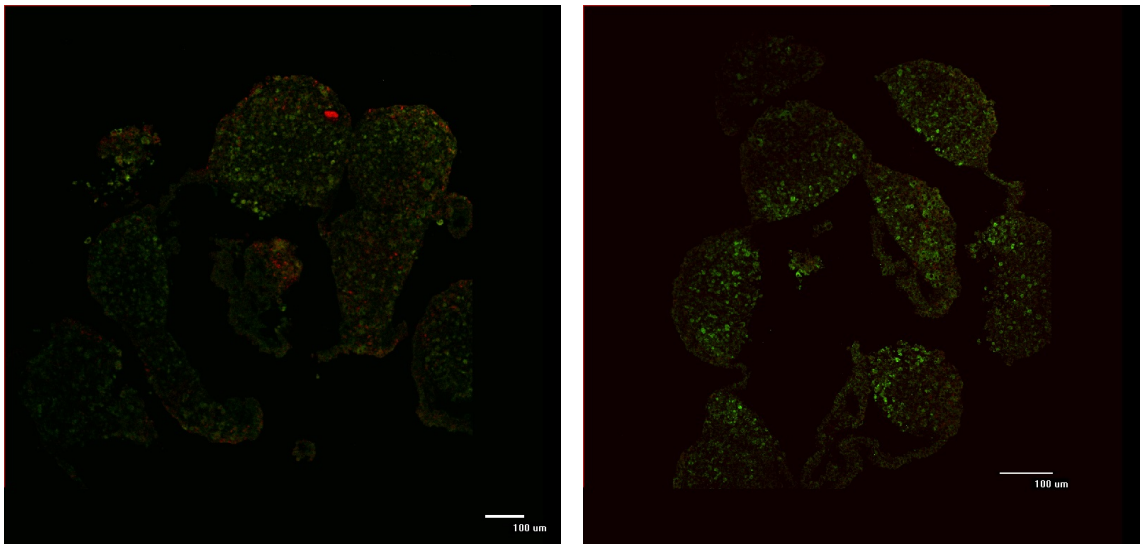
(a)

Figure 4.2: Glycogen concentration from liver aggregates in chip-system with or without pancreatic islets in the chip system. Liver aggregates were formed of HepaRG cells co-cultured with primary human stellate cells. Glycogen concentrations were measured both at day 8 and day 15 during chip experiment. Mean ± SD, n=3.

Glycogen storage is a way of evaluating how the communication between liver aggregates and pancreatic islets affects the glycogen production. Standard curve used for calculations of glycogen as well as mean values, standard deviations and t-test are presented in Appendix A. In figure 4.2 glycogen concentration in liver aggregates from chip system with liver aggregate only as well as liver aggregates together with pancreatic islets are presented. At day 8, no significant difference can be seen between the two samples, due to large variation in measurements. Even though a reduction of standard deviations can be seen in measurements from day 15, nor here can a significant difference be seen (t-test, p-value=0.1056). Due to large variation, no significant difference can be seen for any condition or over time, and the experiments needs to be repeated in order to draw conclusions.

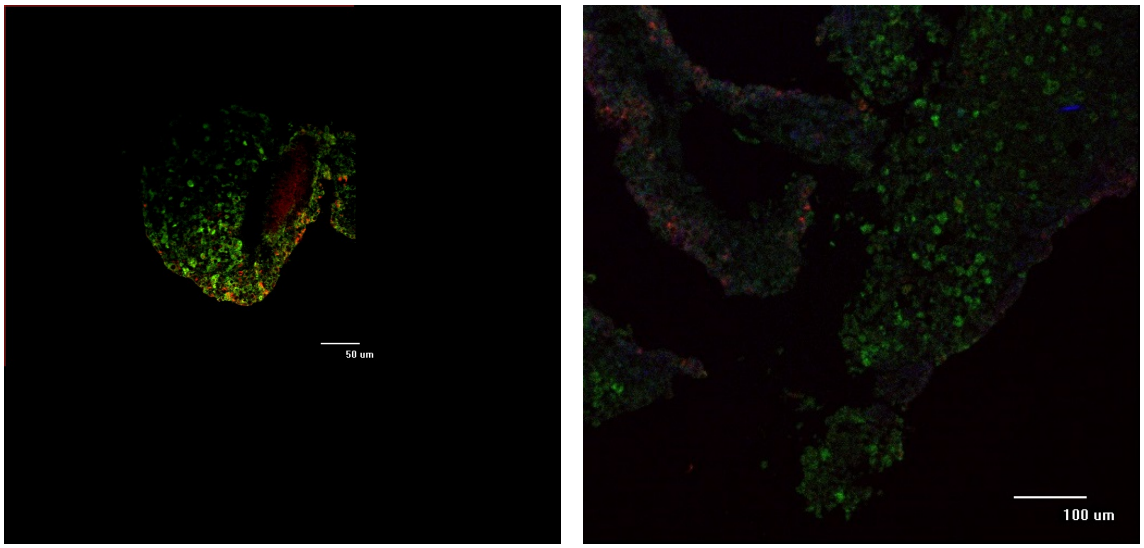
4.1.3 Immunohistochemistry

This section summarizes and discusses immunohistochemistry results in liver aggregates from chip-experiment. Both albumin and drug metabolizing enzymes are liver specific functions of hepatocytes, and have been shown to be down-regulated in primary cultures, for example in collagen sandwich cultures [37]. Nevertheless, in previous studies, it has been shown that both albumin and CYP3A4 have overall stable expression in primary human hepatocyte spheroids, over a time period of five weeks [2]. To further evaluate liver specific functions of liver aggregates from chip-system, fluorescent staining of albumin and CYP3A4 was performed. Figure 4.3 and 4.4 shows together four examples of staining of CYP3A4, displayed in green, and albumin, displayed in red, from liver aggregates terminated at day 8 and 15 of chip-experiment.



(a) **(b)**

Figure 4.3: Fluorescent staining of CYP3A4 (green) and albumin (red) in sections of liver aggregates formed of primary human hepatocytes from day 8 of chip experiment. Panel a) shows staining of liver aggregates from chip that contained only liver aggregates. Panel b) shows staining of liver aggregates from chip that contained liver aggregates as well as pancreatic islets.



(a) **(b)**

Figure 4.4: Fluorescent staining of CYP3A4 (green) and albumin (red) in sections of liver aggregates formed of primary human hepatocytes from day 15 of chip experiment. Blue color represents unsuccessful DAPI staining of cell nuclei. Panel a) shows staining of liver aggregates from chip that contained only liver aggregates. Panel b) shows staining of liver aggregates from chip that contained liver aggregates as well as pancreatic islets.

Unfortunately, DAPI staining of cell nuclei was unsuccessful, and only very weak staining can be seen in some images, for example panel b) in figure 4.4. The results evidence that both CYP3A4 and albumin are present in liver aggregates. The results also show an increased abundance of CYP3A4 in samples compared to albumin, both in liver aggregates that have been co-cultured in chip together with pancreatic islets as well as only liver aggregates in the chip. This result could be because albumin is excreted quickly into medium after production, but other quantitative measurements of CYP3A4 and albumin are needed to be able to compare their abundance. Another observation is that albumin is not equally distributed in samples, and is mostly located to borders of the slide. A potential explanation is that albumin is expressed in different cells, only in periportal hepatocytes or in perivenous hepatocytes. It has been claimed that periportal and perinenous hepatocytes have different amount of metabolizing enzymes and carbohydrate enzymes, and thus have a different metabolizing capacities. This is due to liver passage of oxygen, substrates and hormones which generates different gradients [38, 39]. A unequal distribution of CYP3A4 is not observed in this result, but it may be the reason for the undistributed albumin abundance. Another potential explanation is that cell viability in center of spheroids decreases. In a previous study [40], it has been confirmed that oxygen and nutrition supply depends on mass diffusion. Therefore in large spheroids, cells in the center can have a lack of nutrients and poor waste removal, due to limited oxygen transport. In addition, the study claims that large spheroids have an affected secretion of albumin. Large spheroids, up to

100 µm will not have oxygen limitations [40]. Spheroids used in the present master thesis project are over 100 µm, which can be seen in figures 4.3 and 4.4. This may indicate that there are cells in center of spheroids that are less healthy and decreased amount of albumin is excreted. However this may not be the case in this experiment because CYP3A4 is still equally expressed in spheroids. Therefore viability in liver aggregates needs to be further investigated.

No clear difference can be observed when comparing samples over time, neither between samples from chip-system which only contained liver aggregates nor liver aggregates together with pancreatic islets. This suggests that liver aggregates are functional over a period of 15 days in the chip system. Albumin measurements in section 4.1.1 suggestes a higher amount from aggregates co-cultured together with pancreatic islets than liver aggregates only, but unfortunately it is not supported in this experiment.

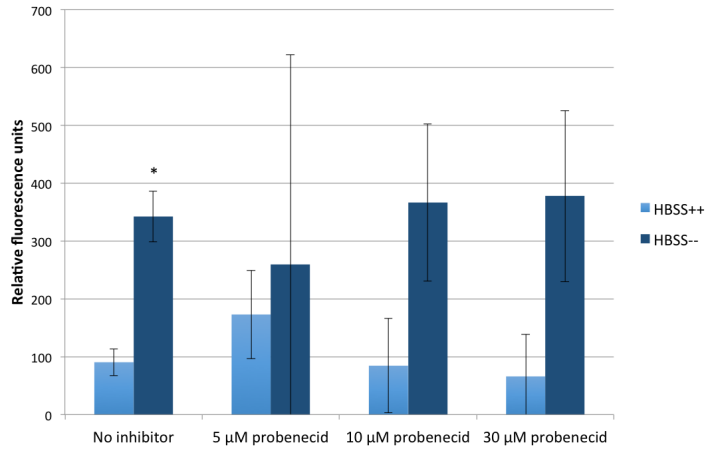
As a summary, results from part I of the project indicates that liver aggregates are more functional when cultured in chip-system together with pancreatic islets, due to a significant increase of albumin production in liver aggregates cultured with pancreatic islets. Also this indicates that the chip-system is working and that liver aggregates and pancreatic islets are communicating in the system.

4.2 Part II- Evaluation of transporter activity

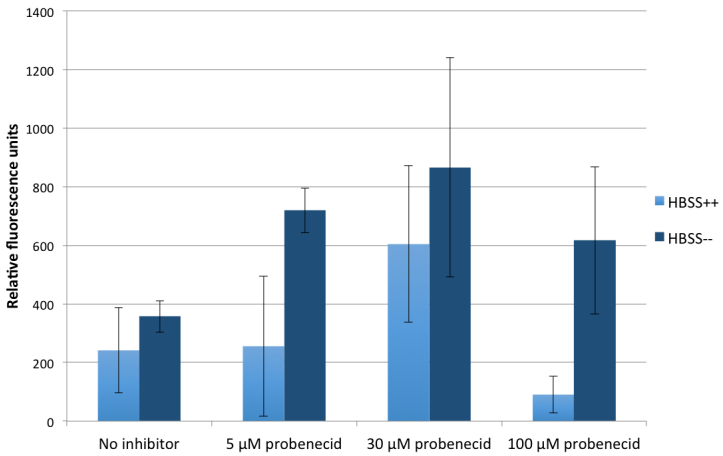
This section summarizes and discusses transporter activity in hepatic 3D spheroid cultures.

4.2.1 Transporter experiments using primary human hepatocytes

In this section results from transporter activity experiments done with spheroids formed of primary human hepatocytes are presented. Results showed in figure 4.5 are performed according to experimental plan 1, described in section 3.3.2.



(a)



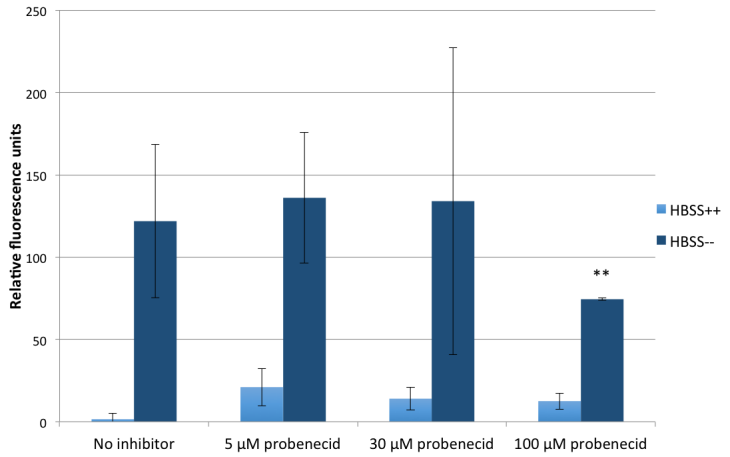
(b)

Figure 4.5: Fluorescence intensity from spheroids formed of primary human hepatocytes treated with or without inhibitor (probenecid), fluorescent substrate (CMFDA, 5 µM) and HBSS++ or HBSS- -. Panel a) shows results from experiment done 1 week after seeding and panel b) shows results from experiment done 2 weeks after spheroids were seeded. Mean ± SD, n=2, *indicates $p<0.05$.

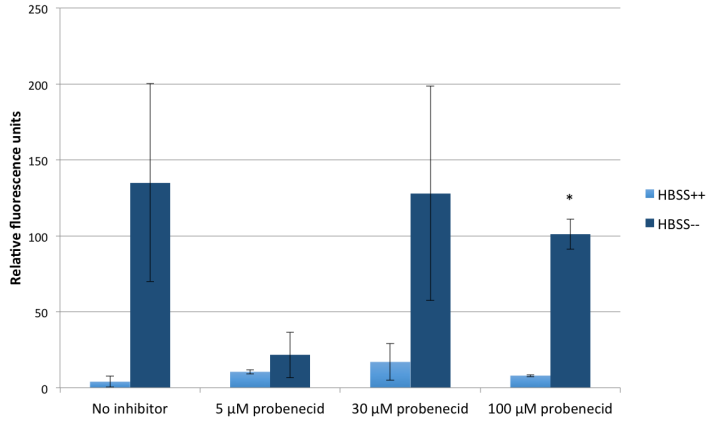
The precursor substrate for MRP2 used in this project is CMFDA, which freely passes through the cell membrane and is converted to a fluorescent substrate inside cells, CMF. The fluorescent substrate is then actively transported into the bile canaliculi in spheroids through MRP2. When treating spheroids with medium lacking of calcium and magnesium, HBSS- - , tight junctions between hepatocytes are dissociated and the fluorescent substrate leaks out into medium. Hence, it was expected to see a difference in intensity of fluorescence in medium between spheroids treated with HBSS++ and HBSS- - . The difference between spheroids treated with HBSS++ and HBSS- - is the amount of transported substrate from inside cells to the bile canalicular space, and gives an indication of how active the transporters are. In figure 4.5 a) spheroids treated with no inhibitor and HBSS++ compared to spheroids treated with HBSS- - show a significant difference with a p-value of 0.0189 (t-test, $p<0.05$) in fluorescent intensity. This indicates that MRP2 is active and transports the fluorescent substrate into the bile canaliculi in spheroids. The result also indicate that spheroids form bile canalicular space in between hepatocytes. The other conditions do not show a significant difference between spheroids treated with HBSS++ and HBSS- - , probably due to the large standard deviations. However, a trend of difference between HBSS++ and HBSS- - can be seen in all conditions except for spheroids treated with 5 µM of probenecid, which is probably due to an experimental error. Fluorescent intensity from spheroids treated with inhibitor and HBSS- - is expected to decrease with increasing concentration of inhibitor. This is due to that probenecid inhibits the efflux transporter MRP2, and less substrate should be transported into the bile canaliculi. As seen in figure 4.5 a), no decrease in fluorescence intensity is observed, at any of the three different concentrations of inhibitor. This observation suggests that the inhibition of MRP2 has not been successful.

Figure 4.5 b) displays the fluorescence intensity from spheroids treated with three different concentrations of probenecid, 5 µM CMFDA and HBSS++ or HBSS- -, two weeks after seeding. The results shows no significant difference between samples from either concentration of the inhibitor HBSS++ compared to HBSS- -. This result is probably due to the large variations, as well as due to the few replicates. The major variation between samples can be explained by the difficulties of performing the experiment. Even though spheroids were incubated in adherent plates, the spheroids tended to detach from the bottom of the well which made it very challenging to change incubation media and wash spheroids. This could result in unequal number of spheroids in each well after washing steps. Also in this experiment, no decrease in fluorescent intensity can be seen. A potential explanation for the unsuccessful inhibition could be that probenecid is washed away during the experiment. Probenecid is a competitive inhibitor [41], which means that it reversely binds to the transporter, and could have been replaced by substrate when removing it and adding CMFDA. Due to this hypothesis, next experimental plan included treating spheroids with inhibitor in combination with substrate.

Results showed in figure 4.6 are performed according to experimental plan 4, described in section 3.3.2.



(a)



(b)

Figure 4.6: Fluorescence intensity from spheroids treated with or without inhibitor (5, 30 or 100 µM probenecid), precursor substrate (CMFDA, 10 µM) and HBSS++ or HBSS- - . Panel a) shows results from experiment done 12 days after seeding, panel b) shows results from experiment performed 13 days after seeding. Spheroids used for both experiments were formed of primary human hepatocytes. Mean ± SD, n=2, *indicates $p<0.05$, **indicates $p<0.01$.

Figure 4.6 shows results from two separate experiments. The result shows fluorescent intensity from spheroids treated with CMFDA, with and without probenecid as well as HBSS++ or HBSS- - medium. To increase the signal from HBSS- -, an increased concentration of substrate (10 µM) was used. In figure 4.6 a), a significant difference of intensity between spheroids treated with HBSS++ and HBSS- - was observed in samples treated with 100 µM inhibitor (t-test, p-value=0.0032). None of the other samples evidence for significant difference between HBSS++ and HBSS- -, yet a trend of difference can be seen. This gives more demonstration of a functional method, and that cells in spheroids express functional MRP2 transporters. Regarding inhibition of MRP2, no difference can be seen, when comparing results from spheroids treated with no inhibitor, 5 µM and 30 µM probenecid. However a

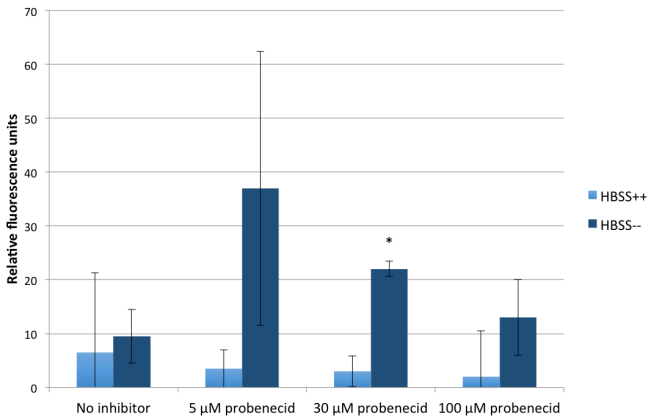
slight decrease in intensity of HBSS- - can be observed between the samples with 100 µM probenecid and the other conditions, which gives an indication that MRP2 is partly inhibited. However this is not significant and needs further investigation. A reason could be that concentrations of probenecid below 100 µM is too low for inhibiting MRP2. The same experiment was repeated to confirm the results. The results from the repeated experiment are shown in figure 4.6 b). Also in this experiment there is a significant difference between intensity from spheroids treated with HBSS++ and HBSS- - , at 100 µM probenecid (t-test, p-value=0.0056). The low intensity for HBSS- - at 5 µM probenecid is probably due to an experimental error. However, also in this experiment no clear difference can be seen between intensity of HBSS- - for any of the different conditions. To be able to draw any firm conclusions regarding inhibition of MRP2 by probenecid, further experiments are needed.

As as summary regarding the presented results, they indicate that the MRP2 is functional, but no clear inhibition of probenecid is seen. Due to large variations in both experiments, it is necessary to repeat the experiment with more replicates as well as optimize the experimental plan to reduce the large variations.

In previous studies [23, 42, 43], canalicular secretion of substrates through transporters like MRP2, has been evaluated in different culture models and different cell types. In a study of Le Vee *et al.* [23], HepaRG cells were used to analyze transporter localization and transporter efflux through fluorescent measurement, similar to experiments done in this present master thesis project. Comparison between usage of calcium free medium and medium containing calcium was performed, as well as calculations of biliary excretion index for different transporters. The study claims that MRP2 is similarly active in primary human hepatocytes and HepaRG cells in sandwich cultured hepatocytes. This results supports that the method used in this present project, using medium lacking of calcium, is applicable for studying transporter activity in hepatic spheroids [23]. Other interesting studies investigates the mechanism behind drug-induced cholestasis and state that cholestatic drugs affect the bile canalicular dynamics [42, 43]. It is described that dynamics of bile canaliculi is an essential function for bile flow. It is also stated that every 20-30 minutes human HepaRG and primary hepatocytes revealed spontaneous opening and reopening of bile canaliculi [42]. This information suggests that it could be a variation of fluorescent substrate that is transported to bile canaliculi in liver spheroids over time, due to dynamics of bile canaliculi. All spheroids used in experiments of the present master thesis study, are probably not in the exact same state of opening and reopening of bile canaliculi and this could be a contributing factor to the varying result seen in the experiment.

4.2.2 Experiments using co-cultured spheroids

In this section results from transporter activity experiments done with spheroids formed of primary human hepatocytes co-cultured with stellate cells are shown. Results showed in figure 4.7 are performed according to experimental plan 2, described in section 3.3.2.



(a)

Figure 4.7: Fluorescence intensity from spheroids formed of primary human hepatocytes co-cultured with human stellate cells, treated with or without inhibitor (probenecid), fluorescent substrate (CMFDA, 5 µM) and HBSS++ or HBSS- -. Figure shows results from experiment done 11 days after seeding. Mean ± SD, n=2, *indicates $p<0.05$.

In figure 4.7 the fluorescent intensity from spheroids treated with or without probenecid, 5 µM CMFDA as well as HBSS++ or HBSS- - medium is shown. In this experiment, probenecid was never washed away from spheroids, thus increasing the chance of it to inhibit MRP2. It was expected to see a decrease in fluorescent intensity with increasing concentration of inhibitor. Unfortunately this was not the case. Even though there are varying results and a low intensity for spheroids treated with no inhibitor and HBSS- -, a trend can be seen in decreasing fluorescent intensity with increasing inhibitor concentration. To evaluate if the inhibitor is working and to be able to draw any firm conclusions, more experiments are needed. The fluorescent intensity from spheroids treated with no inhibitor, seen in the bars to the far left in figure 4.7 show very low values. A reason for the low intensity of spheroids treated with no inhibitor and HBSS- - medium could be due to an experimental error during the experiment, for example loosing many spheroids. In spheroids treated with 30 µM probenecid, a significant difference between HBSS++ and HBSS– is observed with a p-value of 0.01357, which indicates that MRP2 is active. Non of the other conditions showed any significant difference between spheroids treated with HBSS++ medium and HBSS –.

In a study of Gaskell *et al.* [32], it was claimed that active MRP2 transporters were present in spheroids formed of C3A hepatoma cells. The same precursor substrate for MRP2 was used, CMFDA, but a different inhibitor named MK571. Through in-

hibition of MRP2, the activity of the transporter was confirmed [32]. Because of the incomplete inhibition by probenecid, it would be interesting to use other inhibitor in the project.

Results showed in figure 4.8 are performed according to experimental plan 3, described in section 3.3.2.

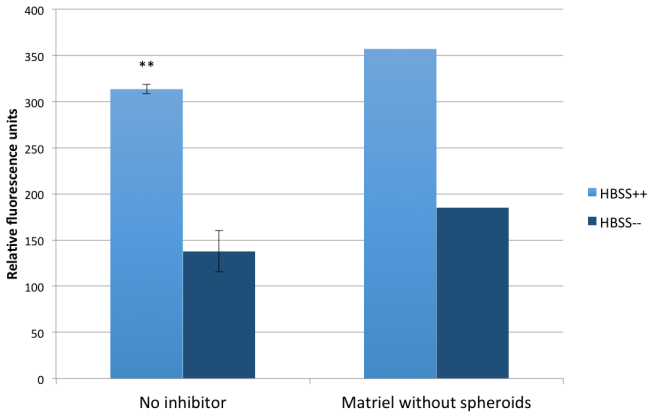


Figure 4.8: Fluorescence intensity from spheroids embedded in matrigel treated fluorescent substrate (CMFDA, 5 µM) and HBSS++ or HBSS- - . The figure shows results from experiment done 8 days after seeding. Spheroids used in this experiment were formed of primary human hepatocytes and human stellate cells. Mean ± SD, n=2, **indicates $p<0.01$.

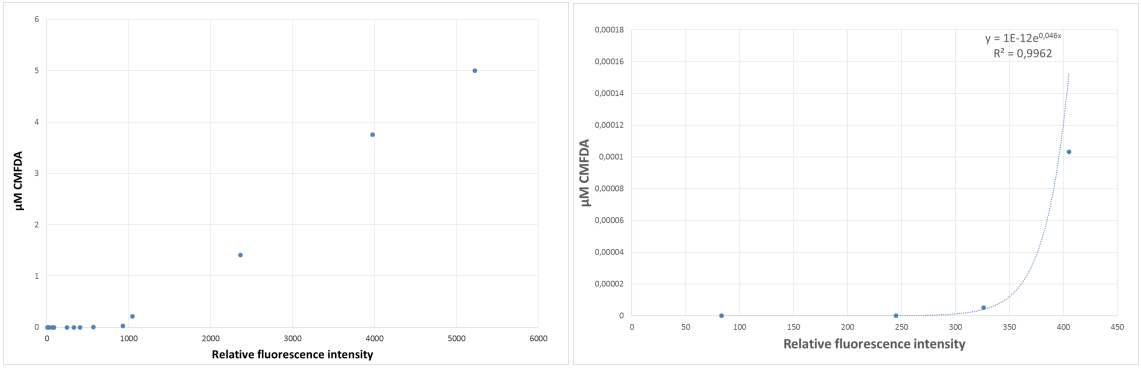
In figure 4.8, fluorescent intensity from spheroids embedded in matrigel, treated without inhibitor and with HBSS++ or HBSS- - is presented. The spheroids were embedded in matrigel to make the washing step easier. As desired, spheroids were trapped in the gel-network and the whole experiment was feasible without loosing any spheroids or leaving medium in wells. In a previous study, Huh-7 cells were used as a hepatocyte-like polarized 3D system cultured on matrigel, to investigate formation of bile canalicular structures. In the study the same precursor substrate was used as in this project, CMFDA, and no difficulties of penetration through gel was observed [44]. With this in mind, a new experimental plan was done. The method itself was remarkably easier to pursue but unfortunately the result was not as expected. As seen in 4.8, spheroids treated with no inhibitor and HBSS++ or HBSS- - , has a significant difference (p-value=0.00866, $p<0.01$), with an higher intensity of HBSS++. During the experiment, a background control was analyzed, matrigel lacking of spheroids, treated the same way as the other sample. As seen in result, fluorescent intensity is equal to the other sample, which was a surprising result. This makes it impossible to determine whether the signal comes from the spheroids or the matrigel. It should be noted that fluorescent intensity of 5 µM CMFDA itself is very high, around 5000 relative fluorescence units, even tough it has not been converted to the fluorescent substrate by esterases inside cells. A potential reason for the high fluorescent signal in in control samples could be that the substrate is trapped in the gel and is difficult to completely remove during washing steps. Result from this experiment reviled that matrigel is not the optimal choice for coating spheroids.

As a summary, the method used in this experiment made the experiment much easier to pursue, but the matrigel affected the experiment negatively, thus resulting in a very high background intensity.

A recent study of Deharde *et. al* investigated the dependence of ECM composition in sandwich cultures on biliary transport. This was executed using primary human hepatocytes in 3D cultures, a fluorescent substrate and measuring fluorescent intensity. The study claims that sandwich-cultures overlaid with collagen gave abundant bile canaliculi formation. In addition, hepatocytes cultured on matrigel and overlaid with collagen showed stable bile canaliculi [31]. This does not support results from this study where we mixed our spheroids with matrigel, and gained a huge background from the matrigel. However in the study of Deharde *et. al*, HBSS++ and HBSS- - was not used as in this current master thesis project.

4.2.3 Fluorescent intensity of CMF

When setting the protocol for fluorescent intensity measurements of CMF using fluorometer (Infinite M200 TECAN), a pre-experiment with 10 spheroids treated with 5 µM CMFDA followed by incubation for 45 minutes was done. Thereafter fluorescent intensity of medium from incubation was measured in a dilution serie. The recommended wavelengths for the substrate was 492/517 nm (excitation/emission), but when using these wavelengths, the fluorometer did not give a reliable result, due to a very high intensity in blank samples. Therefore the next step was to evaluate at what wavelength the fluorometer gave reasonable measurements. CMF have a broad range in emission curve, thus a emission span between 530-540 nm was evaluated. The result showed that at 535 nm fluorescent intensity in blank samples was very low, and therefore this emission wavelength was chosen. Another finding during the pre-experiment was that the fluorescent intensity from substrate had an exponential curve in the lower range seen in figure 4.9 panel a) and a linear curve at the higher range of intensity, seen in figure 4.9 panel b). In previous presented experiments from this present study, the intensity was below 500, which means that this is in the range of exponential increase of fluorescent. This suggests that even a slight difference in intensity between spheroids treated with HBSS++ and HBSS– corresponds to a greater difference in amount of CMF present. Because of the unknown relationship of conversion between CMFDA and CMF it is not possible to calculate the exact amount of CMF. Therefore it would be interesting to investigate further the possibility of making a standard curve of CMF for calculations of CMF located inside bile canaliculi in spheroids.



(a) **(b)**

Figure 4.9: Relative fluorescence intensity of CMF from dilutions series of CMFDA added to spheroids formed of primary human hepatocytes. Panel a) shows intensity measured from concentrations of CMFDA, and panel b) is a zoomed in figure, showing an exponential curve of intenisty.

4.2.4 Comparison

4.2.4.1 Mono-cultured spheroids compared to co-cultured spheroids

The reason for using spheroids formed of both primary human hepatocytes, as well as co-cultured spheroids with primary human hepatocytes and stellate cells was to evaluate if any differences in functionality or bile canaliculi formation could be observed. Previous studies states that spheroids formed of hepatocytes co-cultured with stellate cells have improved maintenance of spheroid structure, improved albumin secretion and improved cell-cell contact [20]. Due to the evidence for improved cell-cell contact, hepatocytes in spheroids should improve their polarization and form better structured bile canaliculi. When comparing results in figure 4.5 and 4.7, the result shows the opposite. Figure 4.5 shows in general a higher fluorescence intensity than figure 4.7. Both experiments were done with approximately the same time-interval from seeding, which should therefore not then affect the results. A reason for these results could be that more experience had been gained, and the washing procedure was done more thoroughly in the last experiment, seen in figure 4.7. Further comparing results from figure 4.6 and figure 4.7, there is also here a higher fluorescent intensity in the last mentioned experiments. However, in this case it is due to the fact that an increased concentration of CMFDA was used in experiment showed in figure 4.6. Also, as seen in all experiments, there is a lot of variation which can contribute to the differences. To be able to draw any firm conclusion regarding differences in MRP2 activity in spheroids formed in co-culture with or without stellate cells, further experiments are needed.

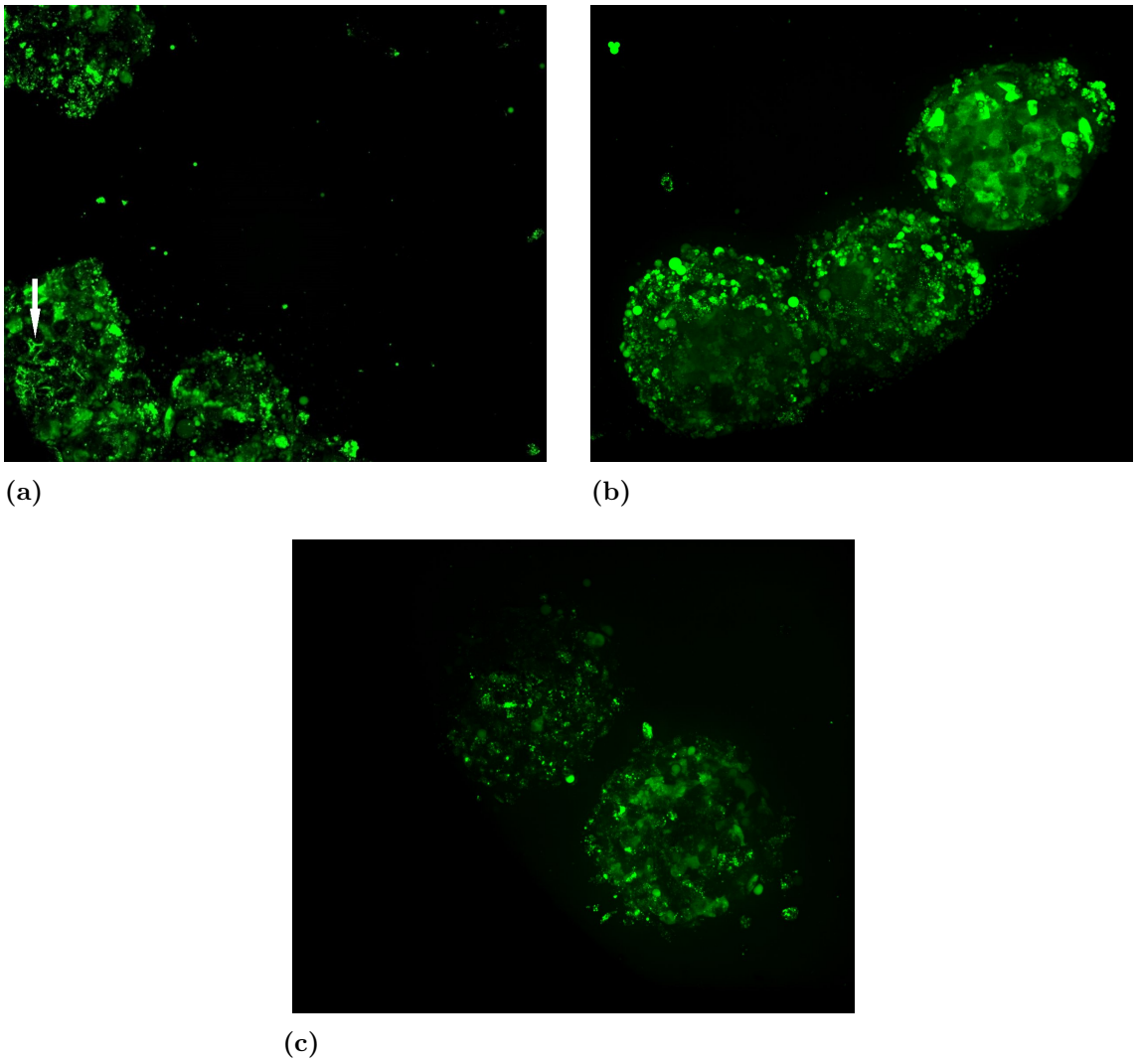
4.2.4.2 Comparison over time

When started this project, we aimed to investigate if there was a difference of transporter activity over time. However, when the project started the focus became more on developing a functional method for analyzing transporter activity. However,

when comparing results from experiments with spheroids seeded one week earlier, compared to two weeks, no clear difference in activity can be seen (see figure 4.5). Further when comparing results from figure 4.5 a) and 4.6, a decrease in fluorescent intensity can be seen. However, it is difficult to distinguish if the differences comes from the experiment itself or the state of spheroids. Further experiments are needed to evaluate any changes in transporter activity over time.

4.2.5 Spheroid imaging

In this section, images from spheroids used in the experiments described above are showed. Spheroids were imaged with a confocal microscope (Yokogawa CV7000) after measuring fluorescent intensity in medium. In figure 4.10, three examples of images are presented.



(a) **(b)** **(c)**

Figure 4.10: Panel a) Confocal fluorescent image of spheroids treated with 5 µM CMFDA, 5 µM probenecid and HBSS++ medium. Panel b) Confocal fluorescent image of spheroids treated with 5 µM CMFDA and 30 µM probenecid and HBSS++ medium. Panel c) Confocal fluorescent image of spheroids treated with CMFDA (5 µM, 100 µM probenecid and HBSS- - medium). Spheroids were formed of primary human hepatocytes. Green represents the fluorescent substrate for MRP2 (CLF), which is a product of CMFDA formed inside hepatocytes. Spheroids were imaged with a confocal microscope (Yokogawa CV7000) with magnification x20.

As seen in figure 4.10 the fluorescent substrate CMF is clearly visible inside spheroids although, it is very difficult to distinguish any clear bile canaliculi, which should be

seen as thin lines between cells. However in figure A.4 a), bile canaliculi can be observed in the spheroid seen in the lower left part of the picture, marked with a white arrow. Round bubble-like structures filled with substrate can be seen in all images, and it is difficult to distinguish their originate, thus this needs further investigation. A potential explanation could be that a few cells have detached from spheroids and floating in medium surrounding spheroids. Another reason could be that this is substrate itself forming lipophilic bubbles which is floating around in the medium. A possible reason for the difficulties of distinguishing bile canaliculi could be that it is a too high concentration of substrate for optimal imaging. When comparing panel a) and b) in figure A.4, no clear difference can be observed, even though different concentration of probenecid is used. This is not surprising since the results described above in section 4.2 did not give any evidence for a successful inhibition at these concentrations. When comparing panel a) and b) against panel c) in figure 4.10, it was expected to see a decrease in fluorescence inside spheroids due to leakage out in medium when using HBSS- - medium, but only slight decrease in fluorescence can be seen. In figure A.4 panel b), the appearance of the three spheroids look very different in the picture. In the spheroid to the right, cells are visible, but in the other two spheroids, cells are difficult to distinguish. In general, images is very varying, and only a few spheroids showed indications for bile canaliculi structures. Therefore, this protocol are not optimal for imaging, and further development and optimization is needed for improvement of imaging spheroids treated with CMFDA. More images are presented in Appendix A.

4.3 Further experiments

4.3.1 Multi-organ-chip

An additional experiment that can be performed to evaluate the functionality of liver aggregates used in chip experiment is to analyze the mRNA expression, or amount of protein of albumin and CYP3A4. This could be used as a compliment to immunohistochemistry and ELISA measurement of albumin, as well as an quantitative comparison between albumin and CYP3A4.

Spheroid viability could also be an interesting parameter to analyze. This to evaluate if the size of spheroids affect the viability of cells, and to investigate how the viability changes over time. This can be done using for example ATP assay or lactate dehydrogenase measurement. For the same reason, analysis of apoptosis could be a potential further experiment.

4.3.2 Transporter activity

As described previously, a problem during experiments was that spheroids detached from wells, which made it more difficult to accomplish a complete washing. To overcome this problem, the spheroids were mixed with matrigel, and the result is presented in section 4.2.2. However, this experiment did not give desirable results. A further experiment could be to coat spheroids with collagen, as done in previously

studies [31]. It would be interesting to evaluate how collagen affects the fluorescent intensity in the medium, and if this approach would make the washing step easier.

In a study of Sharanek *et. al* they discussed how the canalicular dynamics are essential for bile flow, and that every 20-30 minutes cells revealed spontaneous opening and closing of bile canaliculi [42]. It would therefore be interesting to evaluate the bile canaliculi dynamics in spheroids performing the experiment at different time points.

Due to incomplete inhibition of MRP2 by probenecid, it would be interesting to evaluate if a higher concentration of this inhibitor would increased the inhibition. Another approach could be to investigate other inhibitors for MRP2, for example MK71 which is used in a previous study with successful inhibition [32].

Another further property that would be interesting to evaluate is the polarization of hepatocytes in spheroids, done by staining of MRP2. Furthermore mRNA expression of MRP2 could be analyzed to see if there is a change over time, or if there is differences in mRNA expression levels in co-cultured spheroids compared to primary human hepatocytes.

5
Conclusion

5.1 Part I- Multi-organ-chip

Liver aggregates used in a two-organ-chip experiment together with pancreatic islets have shown a significant increase of albumin secretion, compared to chip-systems with liver aggregates only. The liver aggregates co-cultured with pancreatic islets showed no significant difference of glycogen production. However the results indicates that the chip-system is working and that liver aggregates and pancreatic islets are communicating in the system. Staining of albumin and CYP3A4 in liver aggregates indicates that there is a higher CYP3A4 abundance compared to albumin, but more quantitative measurements are needed to confirm this. Further, no difference over time have been observed. In addition, no difference in albumin and CYP3A4 abundance have been identified comparing liver aggregates that has been co-cultured with pancreatic islets and only liver aggregates in the chip system.
To summarize, the chip system where liver aggregates are cultured together with pancreatic islets have indications of better liver specific functions compared to chip with liver aggregates only, due to the improved glucose regulation. However, more studies have to be performed to investigate metabolic enzyme activity.

5.2 Part II- Evaluation of transporter activity

This project has optimized the experimental method for evaluating the activity of MRP2 in liver spheroids formed of primary human hepatocytes or hepatocytes co-cultured with stellate cells. The results revealed that using calcium free medium to measure leakage of fluorescent substrate from spheroids cultured in a adherent plate, gave an indication that MRP2 is active and functional. Results from three experiments showed a significant difference between fluorescent intensity from spheroids treated with medium containing calcium compared to calcium lacking medium but more experiments are needed to confirm these results. However, the method is not fully developed, and needs further improvement, due to large variation in results, challenging experimental procedure, and negative results when using matrigel to cover spheroids. In addition, no significant difference comparing intensity between HBSS – when inhibiting MRP2 with probenecid have been observed. This indicates that inhibition of MRP2 by probenecid was not successful.

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A

Appendix

A.1 Part I- Multi-organ-chip

A.1.1 Glycogen

Figure A.1 shows the standard curve and equation used in glycogen calculations.

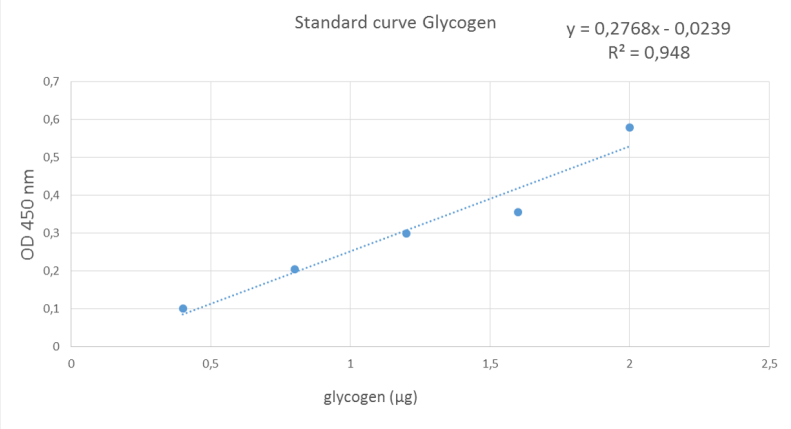


Figure A.1: Standard curve for glycogen amount in µg.

	Liver only	Standard deviation	Co-culture	Standard deviation	t-test
Day 8	0.03422	0.0208	0.1183	0.1291	0.3277
Day 15	0.0473	0.0087	0.0709	0.0176	0.1056

Table A.1: This table shows average values (n=3) from glycogen colorimetrix assay in µg glycogen/ µl sample, and the corresponding standard deviations, from liver aggregates only, and liver aggregates co-cultured with pancreatic islets. t-test was performed between liver only and co-cultured liver aggregates at day 8 and day 15.

A.1.2 Albumin

	Liver only	Standard deviation	Co-culture	Standard deviation	t-test
Day 3	5566.3647	763.9615	9581.9208	1980.0791	2.58664E-08
Day 5	5527.8	1055.2799	7493.2931	1131.1088	1.84667E-05
Day 7	4795.9481	1228.0316	9230.058	2252.9176	1.53407E-07
Day 9	4458.6036	1147.1425	9365.0535	1431.3073	1.09796E-07
Day 11	4559.202	1132.9997	8934.399	1232.9962	2.63864E-09
Day 13	4224.015	938.0466	9268.29	529.1474	1.59347E-11
Day 15	4104.9977	765.7211	9098.4434	2239.0267	2.92605E-06

Table A.2: This table shows average values (n=16) from albumin ELISA experiment in ng/circuit, and the corresponding standard deviations, from liver aggregates only, and liver aggregates co-cultured with pancreatic islets. t-test was performed between liver only and co-cultured liver aggregates at day 3, 5, 7, 9, 11, 13 and day 15.

A.2 Part II- Evaluation of transporter activity

A.2.1 Instrumental setup of fluorometer

Excitation wavelength (nm)	492
Emission wavelength (nm)	535
Gain	31
Integration time (µs)	500
Number of flashes	25

Table A.3: Instrumental setup of TECAN infinite M200. Gain sets the sensitivity of the photomuplier tube. Number of flashes is the number of time the lamp is fluorophore is excited. Integration time is the affects the signal-to-noise ratio.

A.2.2 Fluorescent intensity from experiments

	No inhibitor	5 µM	10 µM	30 µM
HBSS++	90.5	173	84.5	66
HBSS- -	342	259.5	366	377.5
St. deviation (HBSS++)	23.3345	76.3675	81.3173	72.1249
St. deviation (HBSS- -)	43.8406	362.7458	135.7645	147.7853
t-test (p-value)	0.0189	0.7728	0.1283	0.1157

Table A.4: This table shows results from experiment shown in figure 4.5 panel a). Mean fluorescent intensity measurements (n=2) with background subtracted from spheroids treated with HBSS++ or HBSS– and with or without inhibitor of three different concentrations (5, 10, 30 µM). t-test was performed on intensity between HBSS++ and HBSS– for all conditions.

	No inhibitor	5 µM	30 µM	100 µM
HBSS++	241.5	255.5	604	90.5
HBSS- -	357	718.5	865.5	616.5
St. deviation (HBSS++)	144.9569	267.2864	239.7092	144.9569
St. deviation (HBSS- -)	53.7401	75.6604	374.0595	251.02291
t-test	0.4015	0.1225	0.5099	0.0886

Table A.5: This table shows results from experiment shown in figure 4.5 panel b). Mean fluorescent intensity measurements (n=2) with background subtracted from spheroids treated with HBSS++ or HBSS– and with or without inhibitor of three different concentrations (5, 30, 100 µM). t-test was performed on intensity between HBSS++ and HBSS– for all conditions.

	No inhibitor	Matrigel without spheroids
HBSS++	313.5	357
HBSS- -	138	185
St. deviation (HBSS++)	4.9497	
St. deviation (HBSS- -)	22.6274	
t-test (p-value)	0.00866	

Table A.6: This table shows results from experiment shown in figure 4.8. Mean fluorescent intensity measurements (n=2 for "no inhibitor" and n=1 for "matrigel without spheroids"), with background subtracted from spheroids treated with HBSS++ or HBSS– and without inhibitor. t-test was performed on intensity between HBSS++ and HBSS– for all conditions.

	No inhibitor	5 µM	30 µM	100 µM
HBSS++	6.5	3.5	3	2
HBSS- -	9.5	37	22	13
St. deviation (HBSS++)	23.3345	76.3675	81.3173	72.1249
St. deviation (HBSS- -)	43.8406	362.7458	135.7645	147.7853
t-test	0.8118	0.2066	0.0136	0.2943

Table A.7: This table shows results from experiment shown in figure 4.7. Mean fluorescent intensity measurements (n=2), with background subtracted from spheroids treated with HBSS++ or HBSS– and with or without inhibitor of three different concentrations (5, 30, 100 µM). t-test was performed on intensity between HBSS++ and HBSS– for all conditions.

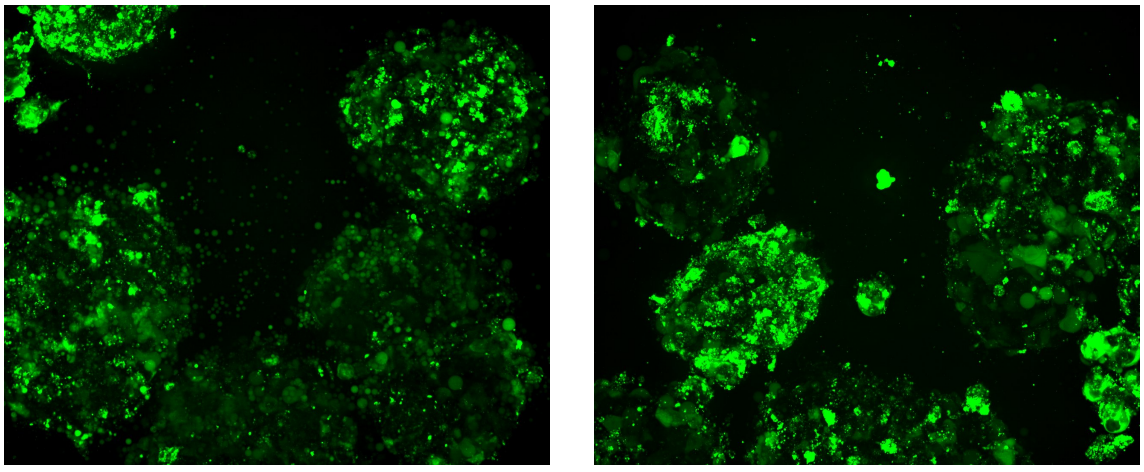
	No inhibitor	5 µM	30 µM	100 µM
HBSS++	1.5	21	14	12.5
HBSS- -	122	136	134	74.5
St. deviation (HBSS++)	3.5355	11.3137	7.0711	4.9497
St. deviation (HBSS- -)	46.6690	39.5980	93,3381	0.7071
t-test (p-value)	0.0678	0.0585	0.2115	0.0032

Table A.8: This table shows results from experiment shown in figure 4.6 panel a). Mean fluorescent intensity measurements (n=2), with background subtracted from spheroids treated with HBSS++ or HBSS– and with or without inhibitor of three different concentrations (5, 30, 100 µM). t-test was performed on intensity between HBSS++ and HBSS– for all conditions.

	No inhibitor	5 µM	30 µM	100 µM
HBSS++	4	10,5	17	8
HBSS- -	135	21.5	128	101
St. deviation (HBSS++)	3.5355	1.4142	12.0208	0.7071
St. deviation (HBSS- -)	65.0538	14.8492	70.7107	9.8995
t-test (p-value)	0.1046	0.4065	0.1601	0.0056

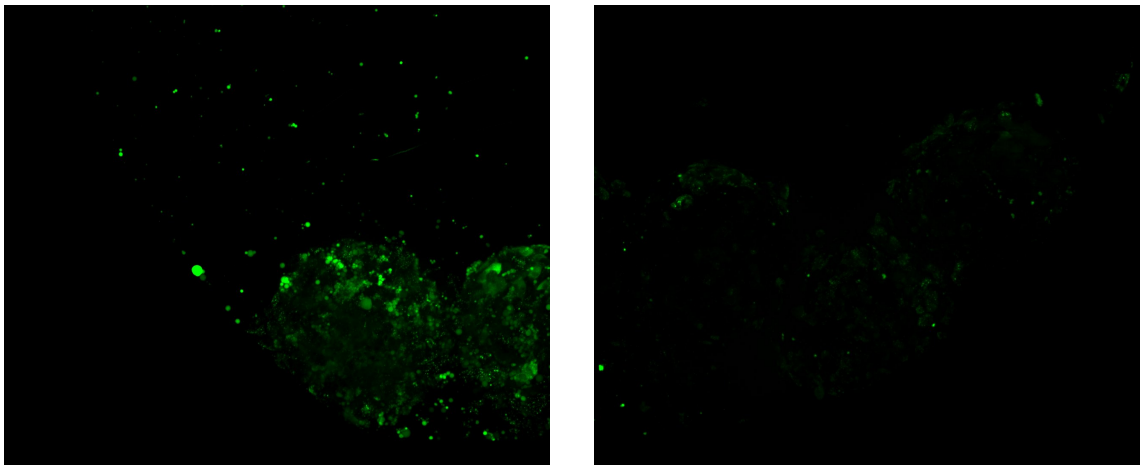
Table A.9: This table shows results from experiment shown in figure 4.6 panel b). Mean fluorescent intensity measurements (n=2), with background subtracted from spheroids treated with HBSS++ or HBSS– and with or without inhibitor of three different concentrations (5, 30, 100 µM). t-test was performed on intensity between HBSS++ and HBSS– for all conditions.

A.2.3 Spheroid imaging



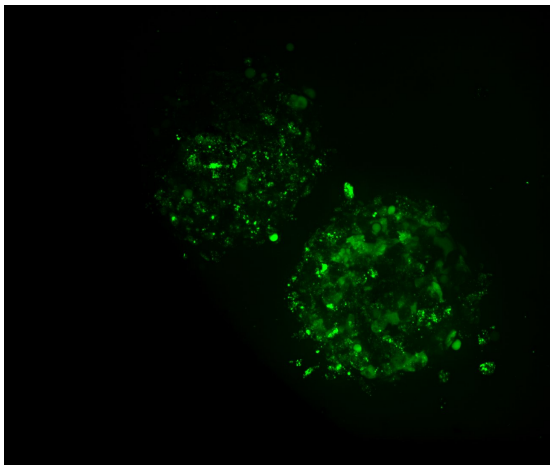
(a) **(b)**

Figure A.2: Panel a) shows confocal fluorescent image of spheroids treated with 5 µM CMFDA, no probenecid and HBSS++ medium. Panel b) shows confocal fluorescent image of spheroids treated with 5 µM CMFDA, no inhibitor and HBSS-- medium. Spheroids were formed of primary human hepatocytes. Green represents the fluorescent substrate for MRP2 (CLF), which is a product of CMFDA formed inside hepatocytes. Spheroids were imaged with a confocal microscope (Yokogawa CV7000) with magnification x20.



(a) **(b)**

Figure A.3: Panel a) shows confocal fluorescent image of spheroids treated with 5 µM CMFDA, no probenecid and HBSS++ medium. Panel b) shows confocal fluorescent image of spheroids treated with 5 µM CMFDA, no inhibitor and HBSS- - medium. Spheroids were formed of primary human hepatocytes. Green represents the fluorescent substrate for MRP2 (CLF), which is a product of CMFDA formed inside hepatocytes. Spheroids were imaged with a confocal microscope (Yokogawa CV7000) with magnification x20.



(a)

Figure A.4: Confocal fluorescent image of spheroids treated with 5 µM CMFDA, 30 µM probenecid and HBSS- -. Spheroids were formed of primary human hepatocytes. Green represents the fluorescent substrate for MRP2 (CLF), which is a product of CMFDA formed inside hepatocytes. Spheroids were imaged with a confocal microscope (Yokogawa CV7000) with magnification x20.