Exploration of the mechanism of toxicity of metallo-drugs using Precision-Cut Tissue Slices

Margot van der Zee (s1865064)

Department of Pharmacokinetics, Toxicology and Targeting, Pharmacy, University of Groningen.

Supervisors: dr. Angela Casini, dr. Ir. I.A.M de Graaf and S.J.P. Spreckelmeyer.

Date of submission: 13 November 2015

Contents

[Abstract 4](#_Toc435102832)

[1. Introduction 5](#_Toc435102833)

[1.1 Hypothesis nephrotoxic effect of Pt anticancer agents 6](#_Toc435102834)

[*i) Accumulation of cisplatin in cells/tissues* 6](#_Toc435102835)

[*ii) Formation of cisplatin metabolites in the liver* 8](#_Toc435102836)

[1.2 Effect of cisplatin on liver 10](#_Toc435102837)

[1.3 Aminoferrocene – liver 11](#_Toc435102838)

[1.4 Precision-cut tissue slices 12](#_Toc435102839)

[1.5 Quantification of platinum content in PCTS 13](#_Toc435102840)

[2. Aim of the study 15](#_Toc435102841)

[3. Results and Discussion 16](#_Toc435102842)

[3.1 *Ex vivo* viability assays 16](#_Toc435102843)

[*i) Effect of 1 and 5 µM Cisplatin (Inc. 2h) on (co-incubated) kidney* 16](#_Toc435102844)

[*ii) Effect of Acivicin on toxicity 1 and 5 µM Cisplatin on kidney* 19](#_Toc435102845)

[*iii) Effect of acivicin on toxicity 1 and 5 µM cisplatin on co-incubated kidney* 22](#_Toc435102846)

[*iv) Effect of incubation time on toxicity 1 and 5 µM cisplatin on kidney* 24](#_Toc435102847)

[*v) Effect of GSH on toxicity 1 and 5 µM cisplatin* 25](#_Toc435102848)

[*vi) Effect of 10 µM (Inc. 24h) on (co-incubated) kidney* 27](#_Toc435102849)

[*vii) Effect of cisplatin on liver* 29](#_Toc435102850)

[3.2 Quantification of platinum content in other PCTS 31](#_Toc435102851)

[3.3 BnFc-Boron toxicity on liver 32](#_Toc435102852)

[4. Conclusions 35](#_Toc435102853)

[Acknowledgements 37](#_Toc435102854)

[5. Materials and Methods 37](#_Toc435102855)

[5.1 Chemicals 37](#_Toc435102856)

[5.2 Precision cut liver and kidney slices 37](#_Toc435102857)

[5.3 Protocols for examine toxicity on slices 38](#_Toc435102858)

[5.4 Biochemical assays for viability testing 41](#_Toc435102859)

[5.5 Inductively Coupled Plasma (ICP) Mass Spectrometry 43](#_Toc435102860)

[5.6 Statistics 44](#_Toc435102861)

[6. References 46](#_Toc435102862)

[Appendix I 49](#_Toc435102863)

[Appendix II 54](#_Toc435102864)

[Appendix III 56](#_Toc435102865)

[Appendix IV 58](#_Toc435102866)

[Appendix V 59](#_Toc435102867)

[Appendix VI 60](#_Toc435102868)

[Appendix VII 62](#_Toc435102869)

[Appendix VIII 63](#_Toc435102870)

# Abstract

Metallo-drugs are widely used for anticancer therapy. Among them cisplatin is a Pt(II) compound which is proven to be effective in different cancer types alone or in combination therapy, but it’s use can also lead to severe side effects, including nephrotoxicity. Different mechanisms of cisplatin nephrotoxicity are described in the literature, among others the formation of cisplatin metabolites could be involved in nephrotoxicity of cisplatin. Suggested is that cisplatin metabolites are more toxic than cisplatin itself and that glutathione conjugates are involved in cisplatin nephrotoxicity. The assumed pathway of metabolite formation is that cisplatin is metabolized via the γ-glutamyl transpeptidase-dependent and cysteine S-conjugate β-lyase-dependent pathway in which platinum-glutathione conjugates are cleaved by GGT resulting in a conjugate which can be further metabolized resulting in the end to a reactive thiol. However liver involvement in cisplatin nephrotoxicity is not well described in literature. This project tried to determine the influence of liver metabolism of cisplatin on the toxicity of cisplatin to the kidney using Precision-cut Tissue Slices (PCTS). The main hypothesis in this project is that formation and possibly also cleavage by γ-glutamyl transpeptidase (GGT) of the cisplatin-glutathione conjugates take place in the liver. The resulting Pt-containing species are responsible for the observed nephrotoxicity. Results showed that liver slices seem to have a protective effect against cisplatin toxicity shown by an increased slice ATP content and decreased expression of Kidney Injury Marker-1 (KIM-1) (although not significant). Uptake, determined with ICP MS, of platinum is lower in kidney slices when they are incubated together with liver slices. This might explain why there is less toxicity observed when kidney slices are co-incubated with liver slices with respect to kidney slices incubated alone. Nephrotoxicity of cisplatin is examined with KIM-1 expression by using different concentrations cisplatin on PCTS. Experiments done with a GGT-inhibitor (Acivicin) did seem to have a protective effect on toxicity shown with morphology, which is expected due the supposed GGT involvement in formation of toxic metabolites. However, this effect was not observed with ATP content or KIM-1 expression. Experiments with extra added GSH to cisplatin showed that non-enzymatic platinum-glutathione conjugates indeed increased the toxicity on kidney with respect to cisplatin alone however this still does not clarify the involvement of GGT activity. Overall, the role of GGT activity on the toxicity of cisplatin is still not clear after this project.

# 1. Introduction

Metalloid-drugs are widely used for anticancer therapy. Among them cisplatin (figure 1) is a Pt(II) compound which is proven to be effective in different cancer types alone or in combination therapy, but that can also lead to severe side effects, including nephrotoxicity. Kidney damage occurs when particular proximal tubular cells are injured via both necrosis and apoptosis.

Figure 1: Structural formula of the anticancer drug Cisplatin

Concerning the mechanism of cytotoxic action of cisplatin, DNA is considered one of the main targets once the compound has entered the cell and reached the nuclei.1 The chloride ions of the cisplatin exchange with water molecules or hydroxide ions intracellularly due to lower chloride concentrations, thereby creating hydrolyzed species.1,2,3 The latter are the real active species, which can cause DNA damage. These hydrolyzed species bind to DNA at the N7 position of two guanosine residues or bind at the N3 position of two cytosine bases at N3 to form primarily 1,2-intrastand adducts between these adjacent residues.1,4 Besides intrastrand adducts also interstrand cross-links, adduct forming between two opposite residues, form although in smaller numbers than intrastrand adducts.1 X-ray structural data on cisplatin/DNA adducts (figure 2) shows that intrastrand adduct forming leads to bending of the DNA and unwinding of the helix (2a/2b). By forming an interstrand cross-link (2c) the helix is severely unwound with two cytosine bases opposite to the bound guanosines point outwards the double helix. Intrastrand cross-links formation are responsibility for cytotoxicity to cancer cells.1

Figure 2: X-ray crystal and NMR structures of double stranded DNA containing adducts of cisplatin. (a) Cisplatin 1,2-d(GpG) intrastrand crosslink. (b) Cisplatin 1,3-d(GpTpG) intrastrand cross-link. (c) Cisplatin interstrand cross-link. Figure by Todd RC (24).

Various studies have shown that formation of DNA lesions , including by Knox et al. via *in vitro* and *in vivo* experiments. They extracted DNA from different cell types including cells of Walker 256 tumor and a cisplatin resistant sub-strain, but also cells of human cancer patients treated with cisplatin.5 In this same study it is also shown that the therapy with cisplatin is dose-limiting due to its toxicity, which is also stated in more literature.6,7 Damage caused by binding of cisplatin to DNA leads to inhibiting of cancer cells transcription1 and replication1,3 and it will lead eventually also to apoptosis.1,8

In spite of the positive pharmacological effects, cisplatin presents numerous side effects, including severe nephrotoxicity. Several hypotheses have been formulated to explain the nephrotoxic effects of Pt anticancer agents, including i) increased accumulation in kidneys, ii) formation of cisplatin metabolites in the liver. Below we will give an overview of the knowledge available on the various possible mechanisms of cisplatin toxicity.

## 1.1 Hypothesis nephrotoxic effect of Pt anticancer agents

### *i) Accumulation of cisplatin in cells/tissues*

Cisplatin and other experimental anticancer metallo-drugs can bind and interact with several classes of proteins, such as transporters, antioxidants, electron transfer proteins, DNA-repair proteins and proteins used to characterize the reactivity of metallo-drugs *in vitro* and also used *in vivo.*9 Accumulation of cisplatin via certain membrane transporters in the kidney could explain the side effect of cisplatin on the proximal tubular cells. They could mediate the uptake of the compound and, therefore, cause its accumulation in certain cells. Overall, membrane transporters do not only play a role in cisplatin’s pharmacological effects in cancer tissues, but also in its side-effects in healthy organs.10

Initially, it was thought that distribution of cisplatin was mainly caused by passive diffusion. Later on it was discovered that cisplatin resistant cancer cell lines have a reduced intracellular accumulation of platinum, which supports the idea that active transport mechanisms may play a role. Thus, this effect in resistant cells could be the result of reduced presence of influx transporters or of overexpression of efflux transporters. However, it must be taken into account that not all cell lines with reduced intracellular accumulation also show a change in cytotoxicity. An overview of the possible uptake and efflux systems has been recently provided by Spreckelmeyer et al.11 Specifically, in kidneys a number of transporters have been shown to be involved in cisplatin uptake/efflux. The high expression of transporter proteins expressed in proximal and tubular cells like Organic Cation Transporters (OCTs) and Copper transporter 1 (Ctr1) could explain the severe effect of cisplatin on the proximal tubular cells. Because of these transporters there will be an accumulation of cisplatin in these cells. The Ctr1 one transporter is a transporter which is localized on the plasma membrane and is involved in Cu uptake. The intracellular form of Cu, Cu+ is toxic to the cell different pathways in the cell are involved in transport and excretion of copper to prevent damage by free Cu+. The major intracellular human Cu trafficking pathways including the Ctr1 transporter are shown in figure 3.11

Figure 3: Schematic drawing of the major intracellular human Cu trafficking pathways including the Ctr1 transporter by Spreckelmeyer.11

Ctr1 is thought to be involved also in the uptake of platinum compounds. The accumulation does not lead automatically to an increased sensitivity to cisplatin and for that reason the cisplatin must be sequestered away from its targets or exported out of the cell by ATP7A respectively or ATP7B. In the report of Kelland is written that after cisplatin enters the cell, by for example CTR1, it gets hydrolyzed due to the low chloride concentration and that these aquatic species could be exported out of the cell by ATP7A/ATP7B if they are not scavenged by glutathione or metallothionein which have high levels in platinum resistant cells.12 It must also be mentioned that cisplatin triggers loss of Ctr1 and that elevated levels of glutathione up-regulate the Ctr1, notable sensitivity to cisplatin is shown in patients with higher Ctr1 expression. This because glutathione is a Cu chelator and these chelators upregulate the expression of Ctr1, also in cisplatin resistant variants. In the review also the binding of platinum complexes to the Ctr1 is described which will mean that cisplatin is not transported by the transporter but sequestered and that the transport system might be an endocytic process.11

Besides the Ctr1 also the OCTs play a role in cisplatin transport and nephrotoxicity. Taking up of cationic substrates in the cell occurs via OCTs. The transport is independent of Na+ and the direction of transport is reversible. OCTs can transport several substrates due the fact that they have a large binding domain. On the renal proximal tubule cells mainly OCT2 is expressed. Cisplatin is thought to interact with the OCT2 transporter, which explains also the specific toxicity to the renal tubule cells. Cimetidine, an OCT2 substrate, inhibits the effect of cisplatin on the cells.11

Finally the multidrug and toxin extrusion proteins (MATEs) which are thought to play a role in excretion of organic cations, may also have a role in the excretion of cisplatin. MATE and OCTs have similar substrates, such as cimetidine.11

Overall, the mechanisms of cisplatin accumulation in cancerous and normal cells still need to be fully elucidated. In addition to the hypothesis of increased accumulation, another explanation for the nephrotoxicity of cisplatin involves the formation of cisplatin metabolites. This idea is described in more details in the following section.

### *ii) Formation of cisplatin metabolites in the liver*

In earlier work, it was suggested that cisplatin metabolites are more toxic than cisplatin itself but also less effective against tumors. The concentration of metabolites is much lower than the cisplatin concentration but it is not cleared from the plasma as quickly as cisplatin. This results in a longer residence time and, therefore, in increased toxicity.13 The mechanism of metabolites formation needs to be clarified in order to be able to reduce drug toxicity. In the study of Townsend and Hanigan, it was hypothesized that cisplatin is metabolized via the γ-glutamyl transpeptidase-dependent and cysteine S-conjugate β-lyase-dependent pathway.6 The assumed metabolism route is shown in figure 4.14 Cisplatin would first be conjugated to glutathione (GSH), via binding of one of the thiol moieties of GSH to Pt (which replaces one of the chloride ligands).2 Glutathione conjugates are known for their detoxification of toxic compounds but in this case they form the first step in becoming a nephrotoxic compound. In fact, while the Pt-GSH conjugate itself is not nephrotoxic, when it is cleaved by γ-glutamyl transpeptidase (GGT), it becomes a cysteine-glycine conjugate. Thereafter the Pt conjugate is hydrolyzed by amino-dipeptidase to a cysteine conjugate which can be taken up in the cell. Both reactions take place extracellularly before the conjugates are taken up by the proximal tubular cells in the kidney. In the cell, the conjugate is metabolized by cysteine-S-conjugate β-lyase, which forms a highly reactive thiol. 2,6,14 The results of an experiment done with rat kidney shows that β-lyase activity was only present in the proximal tubular cells.15 Moreover, the GGT is localized and expressed on the surface of proximal tubular cells as proven by immunohistochemistry.16 This could explain why cisplatin is highly nephrotoxic in tubular cells.

Figure 4: Possible metabolism route of cisplatin.14

In the study of Townsed and Hanigan, the hypothesis concerning the formation of the metabolites by the GGT and cysteine S-conjugate β-lyase-dependent pathway was confirmed by blocking this pathway with acivicin and aminooxyacetic acid (AOAA).6 Acivicin is an irreversible GGT inhibitor and found to be an inhibitor of several glutamine dependent amidotransferases.17 Instead, AOAA is a cysteine S-conjugate β-lyase inhibitor. However, it must be taken into account that other PLP-dependent enzymes can be inhibited by AOAA, which might also be involved in nephrotoxicity of cisplatin. In this study reduced nephrotoxicity was observed in mice treated with cisplatin *in vivo* in combination with the above mentioned inhibitors, without blocking the uptake of cisplatin. These results confirm the possible metabolism route of cisplatin in kidney.6 The results of acivicin and AOAA were confirmed with another study performed on isolated proximal tubule cells from pig kidney (LLC-PK1  cells).2 To check whether indeed GGT inhibition and not another acivicin related mechanism could be responsible for the reduction of the nephrotoxic effect of cisplatin , an experiment with GGT knockout mice was performed.7 Since the GGT deficient mice were less sensitive to cisplatin toxicity, it was proved that GGT is involved in the mechanism of toxicity. Evidence for the final step involving formation of a nephrotoxin via cysteine-S-conjugate β-lyase has been provided in a study performed in LLC-PK1 ­cells.14 In this study, cells were transfected with glutamine transaminase K (GTK), a PLP-dependent enzyme that catalyzes the cysteine-S-conjugate β-lyase reaction. To evaluate cisplatin toxicity on these cells, the number of viable cells after treatment was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) assay. The outcomes showed that cisplatin was more toxic to the cells with GTK, which proves that cysteine-S-conjugate β-lyase is involved in the metabolism of cisplatin to a nephrotoxin. Authors also assumed that the cisplatin-cysteine conjugate is converted by the cysteine-S-conjugate β-lyase in a more reactive Pt species because of the strong electron-withdrawing property of the cisplatin-cysteine species. Notably, the conjugate is converted in, not only the reactive cisplatin-thiol metabolite, but also in pyruvate and ammonium ions, as described in equation 1.14

(eq. 1)

It was suggested that the reactive thiol compounds bind rapidly to the sulfhydryl moieties of adjacent proteins, indicating that the location of cysteine-S-conjugate β-lyase is intracellular. Indeed, Zhang et al. proved that the cysteine-S-conjugate β-lyase is located in the mitochondria in mice. Treatment with AOAA showed a decrease in Pt-protein adducts in the mitochondria and relates to less toxicity for the mice.18 Since these findings showed that there are more enzymes other than GTK which catalyze the cysteine-S-conjugate β-lyase reaction, and because of the localization of cysteine-S-conjugate β-lyase in the mitochondria, there is evidence that a mitochondrial enzyme catalyzes the PLP dependent cysteine-S-conjugate β-lyase reaction.19 For that reason the PLP-dependent mitochondrial aspartate aminotransferase (mitAspAT) was tested. Results showed that LLC-PK1 cells which overexpressed mitAspAT were more sensitive to cisplatin nephrotoxicity than control cells. However, it should be noted that, when mitAspAT is overexpressed, some other enzymes of energy metabolism change in activity as well. Data showed that the mitochondrial enzyme α-ketoglutarate dehydrogenase complex (KGDHC) is inhibited in cells treated with cisplatin and this applies also for aconitase with higher concentrations. In this study they suggest that these enzymes bind to the platinum-thiol conjugate, resulting in contribution to mitochondrial dysfunction of energy metabolism.18

Noteworthy, no studies were performed in liver so far. It is possible that the extracellular reactions take place in the liver and thus cisplatin glutathione conjugates are produced by the liver and then taken up in kidney. In details, they could be hydrolyzed in the bile canaliculi and after re-taken up in the intestine to reach the kidneys via the blood stream. All studies mentioned above are *in vitro* studies with cell lines or *in vivo* animal studies.

## 1.2 Effect of cisplatin on liver

Hepatotoxicity of cisplatin must also be taken in account as a major dose-limiting side effect during low-dose repeated therapy of cisplatin. High dose therapy for tumor suppression can also lead to hepatotoxicity.20,21 Free radicals and Reactive Oxygen Species are involved with hepatotoxicity of cisplatin and multiple ways for prevention of hepatotoxicity are researched.21

Reactive Oxygen Species (ROS) are reactive radicals, ions or molecules due the single unpaired electron in their outer shell.22 ROS mediate different cell functions such as killing pathogens, cell migration, proliferation and differentiation.23 In normal conditions the ROS levels are controlled with scavengers. Glutathione peroxidase is one of the scavengers (antioxidant) to attenuate H2O2 molecules. It reduces hydrogen peroxide into water, which is explained in equation 2.24,25

(eq. 2)

If there is an imbalance between the ROS production and scavenging, this will results in more oxidative stress, which leads to tissue damage. In a study of Pratibha et al. decreased glutathione levels after cisplatin treatment was observed in rats, as well as reduced glutathione reductase activity. Instead, glutathione peroxidase, catalase and γ-glutamyl transpeptidase levels showed an increase. This indicates that drug treatment might cause inactivation of enzymes and other alterations which can contribute to various side effects of cisplatin.26 In a study of Waseem et al. the possible toxic effect on mitochondrial oxidative stress and electron transport pathway from isolated rat liver mitochondria were investigated. The reason for this was that previous studies showed that oxidative stress, through ROS generation, causes mitochondrial malfunction and loss of mitochondrial structure leading to inhibition of complex enzymes. A relation between oxidative stress and cell death was also mentioned. Results of their study showed a decrease in GSH and thus an induction of oxidative stress. Also a decreased activity of glutathione S-transferases (GSTs) in cisplatin treated mitochondria will lead to insufficient conjugation of electrophilic substrates and reduced detoxification of ROS. Overall, in cisplatin-induced hepatotoxicity an increase in mitochondrial metabolic stress can be found.20 Goto et al. suggested, based on studies in human colonic cancer cells, that synthesis of GSH and transport of GSH adducts are involved in cisplatin resistance. In fact, a decrease in intracellular glutathione levels leads to an increase in cytotoxicity, which is associated with resistance for cisplatin therapy.27

## 1.3 Aminoferrocene – liver

In cancer cells the ROS levels are higher than in healthy cells. The cancer cells stay viable at a higher ROS levels due their altered metabolic pathways and regulatory mechanisms.28 The elevated ROS levels can be used to develop targeted anticancer therapy. As an example, anticancer aminoferrocene-based prodrugs are activated by high hydrogen peroxide concentrations only in cancer cells. The drug becomes toxic after splitting the prodrug into an antioxidant scavenger and a catalyst for ROS-generation resulting in high concentrations of ROS. Cancer cells not only have higher ROS levels but also have a more fragile redox equilibrium, which makes them sensitive for the altered high ROS concentrations as result of the aminoferrocene drug. The strongly increased concentration of ROS will eventually lead to cell death.29,30 Earlier research in our department by Brech Aikman showed that the prodrug of BnFC-Boron is not toxic in healthy rat liver tissues depending on the pre-incubation time of the slices. In her report results show that BnFC-Boron leads to a significant toxic effect at the highest concentration (10 µM) and with pre-incubation times 0h, 1h and 5h followed by 24 h incubation with the compound. The slices which had a longer pre-incubation time of 24 hours followed by 24 h drug incubation showed less toxic effect. A high amount of ROS due to stress in the first hours could have activated the drug. It could be that the scavenging effect of glutathione doesn’t work after short pre-incubation times and that a longer recovery time is necessary to obtain normal intercellular concentration of glutathione. This could explain why there is less effect of the aminoferrocene after 24 hours pre-incubation. Also the influence of CYP involvement may be crucial because also bio-activation of BnFC-Boron by cytochrome P450 instead of activation by ROS could play a role. Using a non-specific cytochrome P450 inhibitor, 1-ABT, the influence of CYP on the toxic effects of BnFC-Boron has been tested. If CYP would be involved a decrease in toxicity would be expected. In Brechs report the results show that 1-ABT doesn’t seem to have a toxic effect itself but in these set of experiments also BnFC-Boron didn’t had any toxic effect in contrast to earlier experiments. No conclusion about CYP involvement could be drawn for that reason and further studies are necessary to confirm the initial hypothesis.30

## 1.4 Precision-cut tissue slices

Precision-cut tissue slices (PCTS) were used in this project as an *ex vivo* model to study the toxicological effect of i.e. cisplatin in the kidney. In the literature is described that the slices remain viable after culturing when they are incubated in well-plates in WME medium under 95% O2/5% CO2 atmosphere. The main advantages of this *ex vivo* method with respect to *in vitro* assays are that the slices contain all the cell types present in an organ*,* and that the intracellular and cell-matrix interactions remain intact. This is why many researchers use PCTS not only for toxicological studies but also for the study of endogenous metabolism, biotransformation and transport of drugs and other xenobiotics, as well as for assessing drug efficacy in diseased tissues.31

Moreover, using PCTS is more animal-friendly because multiple experiments can be performed on the slices collected from one animal. Specifically, benefits of PCTS include: i) multiple treatments can be performed using one rat, and ii) influences of other organs are excluded. Disadvantages can be found in that the slices do not remain viable for too long (in general few days) and that they will lose progressively viability during incubation. Therefore, studies of chronic toxicity are not possible on tissue slices. Also penetration of compounds to the inner layers of the slices is a discussed limitation of PCTS. The various phases of PCTS preparation are shown in figure 5.31

Figure 5: Preparation and incubation of liver slices.31

## 1.5 Quantification of platinum content in PCTS

As a second phase of this project, in collaboration with the group of professor Stefan Stürup at the faculty of Health Sciences, department pharmacy in Copenhagen we decided to quantify the Pt content in rat liver and kidney slices treated with cisplatin in different conditions using the Inductively Coupled Plasma Mass Spectrometry (ICP-MS) technique. In fact, ICP-MS can be used to quantify elements in any samples, including tissues. Thus, although it is not possible to determine the Pt-containing species as in other types of mass spectrometry techniques, this method is extremely important to achieve metal quantification in complex biological samples.

Before the amount of metal can be analyzed in tissue samples via ICP-MS, the tissue must be digested via acidification (mineralization). The slice must be complete digested so the elements will be in solution without loss due to precipitation, or contamination. The digestion is achieved with different mineral acids (HCl, HNO3, HF, H2SO­4, etc.), hydrogen peroxide and other liquid reagents depending on the sample.32 Specifically, for the digestion of tissue slices which contain cisplatin, sub-boiled 65% HNO­3 has been used. Once the sample is completely dissolved, it can enter the spray chamber of the ICP-MS, where also a nebulizer is present and where it is forged into an aerosol. The nebulizer uses argon gas flow as mechanical force to generate the aerosol. The solution is flowing through a capillary tube to a low-pressure region created because the argon flows rapidly passes the end of the capillary. The low pressure and the high-speed gas allow transformation of the liquid sample into an aerosol (figure 6).

Figure 6: Concentric nebulizer in an ICP-MS.

Afterwards, the aerosol sample flows through different heating sections. After drying, vaporization, atomization and ionization, the sample consists of excited atoms and ions. The route is shown in figure 7. Heating via the plasma source is used to form the ions. Once the ground-state atom receives this energy one of its orbiting electrons (e-) leaves the outer shell. This process leads to an ion with a positive charge because it has lost a negative charged electron. These positively charged ions can be detected by the mass spectrometer.33

Figure 7: Generation of positively charged ions in the plasma.33

# 2. Aim of the study

The main aim of the study was to research the influence of liver metabolism on renal toxicity of cisplatin. Thus, the role of the formation of cisplatin conjugates in the liver for kidney toxicity is examined. This is done using the precision-cut tissue slices (PCTS) of rat liver and kidney (co-)incubated in ex vivo experiments. Moreover, the relevance of reactive thiol formation for toxicity of cisplatin to the kidney is studied, as well as the mechanisms of uptake of cisplatin-GSH conjugates in the kidney. In parallel, the toxicity of cisplatin on the liver is examined.

A secondary project has involved the study of the influence of CYP-enzymes in the activation of BnFc-Boron and thus on toxicity in the liver. As previously mentioned, earlier work on this topic was performed at our department by Brech Aikman during her Master project.30

# 3. Results and Discussion

## 3.1 *Ex vivo* viability assays

To determine the toxicity of cisplatin (CisPt) in kidney and liver PCTS, different concentrations of the compound have been tested at different incubation times. The ATP content was evaluated as described in the experimental section and corrected for the amount of protein in the slices to investigate the viability of PCTS after the different treatments. Moreover, Kidney Injury Molecule-1 (KIM-1) has also been used as a marker for toxicity in kidney tubular cells. An upregulation of KIM-1 in the proximal tubule cell is visible when the kidney is injured.34

### *i) Effect of 1 and 5 µM Cisplatin (Inc. 2h) on (co-incubated) kidney*

First the ATP content of the slices was examined after 2 hours treatment with 1 µM and 5 µM cisplatin. The results are presented in figure 8, while raw data of these values can be found in Appendix I, table 5. A two-way ANOVA was conducted that examined the effect of incubation alone or co-incubation and the effect of the concentration on the ATP content. There was no statistically significant difference among treatments on the ATP content of the kidney slices incubated with cisplatin alone or in the presence of liver slices (P=0,693).

Figure 8: Relative viability of kidney slices after 2 hours treatment with 1 and 5 µM cisplatin. The average ATP of the control slice is set at 100% with a true average value of 5,60 [pmol/µg]. The standard error of the mean (SEM) is used to describe the variability (n=5).

Also the expression of KIM-1 mRNA was been determined to examine the effect of cisplatin at different concentrations over 2 hour incubation. The results are presented in figure 9, while raw data of these values can be found in Appendix I, table 6. Figure 9 shows that KIM-1 is upregulated in kidney slices treated with 1 µM CisPt and a down-regulation occurs when the slices are treated with 5 µM CisPt. With one-way ANOVA is shown that there is no significant difference in KIM-1 expression for the different treatments (P=0,222) on kidney slices. Although there is no significant difference, it seems that a trend of upregulation at 1 µM cisplatin and a downregulation at 5 µM is in place. These results correspond to earlier results found by Gerian Prins, former Master student in our department.35

In fact, Gerian Prins also showed that KIM-1 is up-regulated in kidney slices treated with 1 µM cisplatin and that down-regulation of KIM-1 could be observed when the slices are treated with 5 µM cisplatin with an incubation time of 2 hours.35 Explanation for this latter effect might be that the tubular cells are already too much damaged and apoptotic at 5 µM cisplatin, to still be able to observe upregulation of KIM-1. This theory is also examined via morphological analysis of the PCTS (related figures can be found in the next paragraph). Morphology results show indeed an increase in damage of the tubular cells as concentration of platinum increases. Interestingly, at 1 µM cisplatin the tubular cells in kidney are affected, while the measure of ATP is not decreased with respect to controls. This may be due to the fact that ATP measurements correspond to an overall value of cell viability and are not focused on tubular cells.

To test whether the incubation time is also important in this viability other incubation times were tested later on. This will be discussed in paragraph *iv.*

Thus, we continued our experiments, with kidney co-incubated with liver slices and tested using the same conditions used in Gerian’s experiments. This means that in our case, after pre-incubation, the kidney slice replaces the liver slices treated with cisplatin and are incubated in the same medium for a certain time.

The effect of incubation with and without liver and two different concentrations of cisplatin on KIM-1 expression was examined by two-way ANOVA. There was no statistically significant difference (P=0,365). The sample size is estimated with the obtained standard deviations, to compare the effect of cisplatin treatment on the means of KIM-1 expression with power analysis. This points out that with the occurring variability minimally 21 experiments would be necessary at 1 µM cisplatin treatment and 42 at 5 µM cisplatin treatment to compare the means (α=0,05, β=0,20). This could indicate that the number of experiments performed are not sufficient to take conclusions. With one-way ANOVA it is shown that there is no significant difference in KIM-1 expression for the different concentrations of cisplatin on kidney slices co-incubated with liver (P=0,953). Also statistics performed on ∆∆Ct values did not give any significant difference, data can be found in Appendix I, table 7. For this reason we chose to perform just statistics on fold induction values for the rest of the data analysis. In this way the statistics will correspond as well to the data of the graphs presented in this paper.

Figure 9: Fold induction of kidney slices after 2 hours treatment with 1 and 5 µM cisplatin. The fold induction of the control slice is set at 1. The standard error of the mean (SEM) is used to describe the variability (n=5).

The obtained data of the KIM-1 expression in kidney slices treated with 1 and 5 µM cisplatin have also been combined with data of Gerian Prins to see if we could show some statistically significant differences. The data were then merged and presented in figure 10 (raw data of Gerian Prins can be found in Appendix I, table 8).35The SEM became lower after combining the results. The tendency is that 1 µM cisplatin gives an upregulation of KIM-1 expression. With one-way ANOVA it is shown that there is still no significant difference in KIM-1 expression for the different cisplatin concentration on kidney slices co-incubated or not with liver (P=0,051).

Figure 10: Fold induction of kidney slices after 2 hours treatment with 1 and 5 µM cisplatin. The fold induction of the control is set at 1. The standard error of the mean (SEM) is used to describe the variability (n=8)

The results of the co-incubated kidney experiment are shown in figure 9. In the case of 1 µM cisplatin treatment a lower expression of KIM-1 compared to the graph of kidney incubated alone can be observed. It seems that at 1 µM CisPt treatment in the co-incubated experiment, the liver protects the kidney for tubular damage and thus toxicity appears reduced. Explanation for the fact that the KIM-1 seems to be downregulated in comparison to the incubation of kidney alone at 1 µM might be because the liver creates metabolites which are less than cisplatin itself or metabolites which are not taken up by the kidney. If the metabolites are less toxic, a decrease in KIM-1 expression is expected and an increase in ATP content since the effective cisplatin concentration decreases. If the metabolites are more toxic, a decrease in KIM-1 is expected as well as a decrease in ATP content. This also correlates with the literature.13,14 However, the fact that results are not statistically significant must be taken into account and for that reason no definite conclusions can be drawn.

However, in the case of liver slices treated with 5 µM CisPt followed by co-incubation, there is a slightly increase in KIM-1 observed compared to incubation of kidney slices alone, although not statistically significant. A reason for the increase in KIM-1 expression might be that, due the higher concentration, not all cisplatin is metabolized or taken up by the liver in the incubation time of 2 hours. Therefore, cisplatin would be still present in its original form and thus an upregulation of KIM-1 could be observed in kidney. It could also mean that the metabolites produced by the liver are toxic to the tubular cells but not that destructive as 5 µM cisplatin itself. It could be that the metabolites produced by the liver are not taken up as easy as cisplatin by the kidney due to change in hydrophilicity. This could be if the liver contributes to the hydrolysis of cisplatin and excretes the aquo species by ATP7A or ATP7B efflux transporters. Normally these species are highly reactive, especially towards sulfur containing nucleophiles in the plasma.12 Moreover, it should be mentioned that the concentration of cisplatin in the medium after incubation is not known as well as the concentrations of possible metabolites. In the set up used for the experiments performed in this project the amount of metabolites produced by the liver could be so low compared to the amount of medium in the well that they will not efficiently affect the kidney slice. Perhaps using a perfusion system instead of a static set up could help with this problem. In that case the liver slices are perfused with medium constantly and after it reaches the liver, it flows to the kidney. This set up, would better represent the way of how compounds reach the kidney *in vivo* as well. With this method only medium which contains cisplatin already metabolized or at least metabolized by the liver would get to the kidney slice. Study of the co-incubated slices via morphology might give more insight into the toxic effect as well. However, unfortunately the latter could not be performed in this project. Furthermore it would be interesting to investigate the type of cisplatin metabolites and their concentration in the medium for example via HPLC analysis.

### *ii) Effect of Acivicin on toxicity 1 and 5 µM Cisplatin on kidney*

In order to assess the influence of cisplatin-GSH conjugates on the toxic effects on kidney slices, we used an inhibitor of GGT, namely Acivicin. This molecule inhibits GGT and thereby inhibits the formation of cysteine-glycine conjugates from glutathione conjugates. 2,6,14 This may result in a lower toxicity on kidney slices. Therefore, 100 µM acivicin was added to the kidney slices 30 minutes before treatment with cisplatin. It is expected that also the level of KIM-1 expression will be lower with the 2 hours treatment of 1 µM cisplatin + acivicin, compared to the slices which are not treated with the inhibitor. In addition, the toxicity of acivicin itself was also examined to opt for the best non-toxic concentration to be tested in combination with cisplatin. Moreover, beside ATP and KIM-1, also morphology studies were conducted to further characterize the influence of 1 and 5 µM CisPt with and without acivicin.

First the ATP content of the slices was examined after 2 hours treatment with 1 µM and 5 µM cisplatin. Acivicin was added 30 minutes before treatment with cisplatin. The results are presented in figure 11 (raw data of these values can be found in Appendix I, table 9). It appears that no increase of ATP content is observed with pre-treatment of acivicin before treatment with cisplatin, compared with samples without acivicin. There was no statistically significant difference between the groups as shown by two-way ANOVA (P=0,877).

Figure 11: Relative viability of kidney slices after 2 hours treatment with 1 and 5 µM cisplatin. The average ATP of the control slice is set at 100% with a true average value of 5,60 [pmol/µg]. The standard error of the mean (SEM) is used to describe the variability (n=4).

Afterwards, the regulation of KIM-1 was determined to examine the effect of acivicin on cisplatin toxicity. The results are presented in figure 12 and analyzed by two-way ANOVA (raw data of these values can be found in Appendix I, table 10). There was no statistically significant difference (P=0,853). Indeed, figure 12 shows that slices treated with acivicin do not present any change in KIM-1 expression, the graphs are similar and even slightly overlapping.

Figure 12: Fold induction of kidney slices after 2 hours treatment with 1 and 5 µM cisplatin. The fold induction of the control slice is set at 1. The standard error of the mean (SEM) is used to describe the variability (n=4).

Conversely, some effects could be detected by morphological analysis. The untreated control is shown in figure 13. In the figure you can clearly see the glomeruli with their Bowman’s capsules. The effect of acivicin alone can be seen in figure 14. From this image it can be concluded that acivicin has some toxic effect on the kidney cells. The urinary space, the space between the Bowman’s capsule and glomerulus is larger and also the proximal tubular cells seem to be affected.

The toxic effect of 1 µM and 5 µM can be seen in figure 15 and 16, respectively. The tubular cells are clearly affected with the 5 µM treatment and also the urinary space is increased. Less marked effects can be seen at 1 µM cisplatin on the tubular cells, although the urinary space seems already be a little increased. The effect of acivicin on cisplatin-treated slices is very well visible in figure 18. The tubular cells do not seem to be affected anymore by the 5 µM CisPt and also the urinary space is not increased. Also the slices with treatment of 1 µM CisPt and acivicin look healthier (see figure 17).

Figure 13: Control slice

Figure 14: 100 µM Acivicin

Figure 10: 1 µM Cisplatin

Figure 11: 1 µM Cisplatin + 100 µM Acivicin

Figure 12: 5 µM Cisplatin

Figure 13: 5 µM Cisplatin + 100 µM Acivicin

Therefore, these latter results support the idea that acivicin has a protective effect on the toxicity of cisplatin although KIM-1 expression results and ATP data do not lead to the same conclusion. In any case, the higher dose of cisplatin tested in our experiments clearly affects the tubular cells seen in morphology which is supposed to be the reason why the KIM-1 expression is not upregulated at 5 µM: the tubular cells are too much damaged to upregulate KIM-1.

### *iii) Effect of acivicin on toxicity 1 and 5 µM cisplatin on co-incubated kidney*

To test whether liver has influence on the formation of cysteine-glycine conjugates from glutathione conjugates, acivicin has also been used as pre-treatment in the co-incubated kidney-liver set up. Therefore 30 minutes before treatment with cisplatin, 100 µM acivicin was added to the liver slices.

In a first series of experiments, the ATP content of the co-incubated kidney slices was examined after 2 hours treatment with 1 µM and 5 µM cisplatin. Acivicin was added 30 minutes before treatment with cisplatin. The results are presented in figure 19 (raw data of these values can be found in Appendix I, table 11). There was no statistically significant difference between the groups as shown by two-way ANOVA (P=0,705). Therefore, we could conclude that ATP content was not affected by incubation with acivicin, which may exclude its protective effect on kidney slices incubated with cisplatin (in the presence of liver).

Figure 19: Relative viability of kidney slices after 2 hours treatment with 1 and 5 µM cisplatin. The average ATP of the control slice is set at 100% with a true average value of 5,60 [pmol/µg]. The standard error of the mean (SEM) is used to describe the variability (n=4).

The results on the expression of KIM-1 for the same samples are presented in figure 20, raw data of these values can be found in Appendix I, table 12. The two-way ANOVA analysis on the effects of acivicin on KIM-1 expression in kidney slices co-incubated with liver in presence of cisplatin, shows no statistically significant difference (P=0,879). Although not significant, there seems to be a trend in the decrease of KIM-1 expression in the kidney slices co-incubated with liver which were pre-treated with acivicin and treated with 1 µM cisplatin compared to treatment of 1 µM cisplatin without acivicin. Therefore, a possible protective effect cannot be fully disregarded.

Figure 20: Fold induction of kidney slices incubated with liver with and without acivicin after 2 hours treatment with 1 and 5 µM cisplatin. The fold induction of the control kidney slice incubated alone is set at 1. The standard error of the mean (SEM) is used to describe the variability (n=4).

In the future, morphology studies should also be performed to detect the possible effects of specifically in tubular cells.

### *iv) Effect of incubation time on toxicity 1 and 5 µM cisplatin on kidney*

To find out whether the incubation time had influence on toxicity of cisplatin on the kidney slices, different incubation times were studied. In this experiment a pre-incubation time of 1 hour was used to allow slices to fully recover their viability after excision. Afterwards, slices were incubated with fresh medium containing cisplatin for a maximum of 4 h. It is expected that kidney ATP content would decrease during longer incubation time with cisplatin. In figure 21 the results are presented, raw data can be found in Appendix I, table 13. With a two-way ANOVA is tested if there was any statistical significant correlation between time and concentration of cisplatin. There was no statistically significant difference between the effects of time and treatment on the ATP content (P=0,640). One-way ANOVA was also conducted to observe if there was any significant difference between the different incubation times. No significant difference is found between different incubation times for both 1 µM and 5 µM treatment. Longer incubation times are necessary to see a decrease in ATP content and significant differences. Such short incubation times do not affect viability in that way that a decrease in ATP content is visible.

Figure 21: Relative viability of kidney slices after different hour’s treatment with 1 and 5 µM cisplatin. The average ATP of the control slices are set at 100% with a true average value of 7,22 , 11,30 and 23,39 [pmol/µg]. The standard error of the mean (SEM) is used to describe the variability (n=2).

Also the regulation of KIM-1 is determined to examine the effect of incubation time on toxicity of cisplatin. The results are presented in figure 22 and 23, raw data of these values can be found in Appendix I, table 14. We decided to show two separate graphs of the obtained results out of 2 experiments, because the average would not give a realistic view of the data. In both cases KIM-1 expression first seems to decrease before it starts to be upregulated. It was expected that KIM-1 expression would increase during longer incubation time, at least for 1 µM cisplatin treatment because this concentration would not be to toxic for the tubular cells and thus will not affect the upregulation of KIM-1. As can be seen in figure 22 and 23 first a decrease in KIM-1 expression seems to be the case before an increase is evolved. This increase is supposed to be the result of increased toxicity of cisplatin during longer incubation times. The decrease at the beginning could be the result of recovering from slicing before the same values are obtained as *in vivo.* Thereafter, at a later time point, the increase is similar as seen in the earlier results shown in paragraph 3.1.i. The reason that it differs from earlier experiments in the first part of the graph could be the result of the change in pre-incubation time. Here an incubation time of one hour is used and three hours are used in the earlier experiments described in this thesis.

Figure 22 /23: Fold induction of kidney slices incubated with 1 and 5 µM cisplatin for different incubation times. The fold induction of the control kidney slice for each hour is set at 1. (n=1 for both figures)

Overall it is hard to draw conclusions out of these results, and further experiments should be conducted (here we report data out of n=2). However, KIM-1 expression supports the idea of an increase of toxicity with longer incubation times as expected.

### *v) Effect of GSH on toxicity 1 and 5 µM cisplatin*

Conjugate forming of glutathione with cisplatin is expected to increase the cisplatin toxicity. These because the cisplatin conjugates are assumed to be the first step in the formation of toxic metabolites.14 Therefore, if the concentration of these conjugates is increased an increase in toxicity of cisplatin is expected as well. However it must also be taken into account that glutathione is also present in the blood circulation and that increase in toxicity could not be automatically distracted to the assumed metabolic pathway. Also an amount of glutathione is already present in the medium. Although this should not be forgotten, in these experiments extra glutathione is added to cisplatin in the wells plate to form conjugates before adding the slice to it. Described in the literature was that complex formation of glutathione and cisplatin takes place in a ratio of 2:1.36 Because of the formation of platinum-glutathione conjugates, an increase in toxicity and thus a decrease in ATP content and an increase in KIM-1 expression are expected.

In figure 24 the results of viability are presented, raw data can be found in Appendix I, table 15. With a two-way ANOVA is tested if there was any statistical significant difference. There was no statistically significant difference between the effects of time and treatment with or without GSH on the ATP content with 1 µM cisplatin (P=0,378) nor with 5 µM cisplatin (P=0,253). Also no significant difference is found between treatment with and without GSH for the different times or concentrations. Although it is not significant, slices treated with 5 µM cisplatin for 4 hours seems to be more viable than slices incubated with cisplatin and GSH. That there is no effect visible during the earlier time point could be due the fact that the incubation time is too short to obtain a significant decrease in ATP content.

Figure 24: Relative viability of kidney slices after different hour’s treatment with 1 and 5 µM cisplatin with and without GSH treatment. The average ATP of the control slices are set at 100% with a true average value of 7,22 , 11,30 and 23,39 [pmol/µg]. The standard error of the mean (SEM) is used to describe the variability (n=2).

Furthermore, the regulation of KIM-1 was determined to examine the effect of incubation with and without GSH on cisplatin toxicity on the kidney. The results are presented in figure 25 and 26, raw data of these values can be found in Appendix I, table 16. The results of KIM-1 expression our of two different experiments are presented in two separate graphs because the results differed from each other and therefore the average would not give a realistic view of the obtained data. Treatment with 1 µM cisplatin and 2 µM GSH increases the KIM-1 expression compared to treatment with cisplatin alone. This seems to be the case as well for treatment with 5 µM cisplatin and 10 µM GSH. For this reason it seems that the formation of the cisplatin-glutathione conjugates increases the toxicity of cisplatin to the tubular cells of the kidney.

Figure 25/26: Fold induction of kidney slices incubated with 1 and 5 µM cisplatin for different incubation times with and without 2 µM and 10 µM GSH. The fold induction of the control kidney slice for each hour is set at 1. (n=1 for both figures)

Although the results need to be confirmed, GSH seems to increase the toxicity of cisplatin on kidney shown in both viability and KIM-1 expression. These findings support the idea that cisplatin-GSH conjugates are supposed to be more nephrotoxic than cisplatin itself. However, with the acivicin experiment was the enzymatic GGT catalyzed metabolite formation as described in the literature ruled out.6,14 So although the non-enzymatic Pt-GSH conjugates are more toxic, no conclusion of the involvement of GGT activity on nephrotoxicity could be determined with this experiment.

### *vi) Effect of 10 µM (Inc. 24h) on (co-incubated) kidney*

To investigate the influence of liver and its produced conjugates on the toxicity on kidney, more experimental conditions where used then mentioned above, as fully described in the experimental section. For example, in this experiment liver slices were collected on day one from a rat and on the second day the kidney slices were collected from a different rat. The kidney slices replaced the liver slices and where incubated in the medium of the liver.

In figure 27 the viability results are presented of treatment with 10 µM cisplatin for 24 hours. Raw data of these experiments can be found in Appendix I, table 17. A one-way ANOVA test showed that there was no statistically significant difference between the groups treated with 10 µM cisplatin (P=0,352). However, it seems that slices co-incubated with liver are slightly protected from cisplatin toxicity which corresponds with the results described above.

Figure 27: Relative viability of kidney slices after 24 hours treatment with 10 µM cisplatin. The average ATP of the control slice is set at 100% with a true average value of 8,72 (n=5) and 6,84 (n=2) [pmol/µg]. The standard error of the mean (SEM) is used to describe the variability.

In figure 28 the results of the uptake of platinum in kidney slices treated with 10 µM CisPt are shown. Raw data of the ICP MS can be found in Appendix I, table 18. It is evident that the uptake is lower in the co-incubated kidney slices. A one-way ANOVA test showed that there was a statistically significant lower cisplatin concentration in kidney incubated with liver compared to slices that were not incubated in liver medium with a treatment of 10 µM (P=0,002)

Figure 28: Amount of platinum in the kidney slices after 24 hours treatment with 10 µM cisplatin determined with ICP MS. The standard deviation (STDEV) is used to describe the variation (n=1).

The results are also corrected for the amount of platinum inside the medium and therefore presented as percentage relative to the medium concentration in figure 29 (raw data Appendix I, table 19). In this way it can be shown that the amount of platinum is not lower inside the medium after the liver is taken out. Thus the liver does not accumulate a quantitative amount of platinum, which is taken out of the medium after replacing the slice with a kidney slice. With one-way ANOVA was a statistically significant difference between kidney incubated alone and incubated with liver with a treatment of 10 µM found (P=0,007).

Figure 29: ICP MS evaluation of percentage platinum in kidney slices relative to the platinum concentration in the medium. The standard deviation (STDEV) is used to describe the variation (n=1).

A reason for the lower uptake might be that the present form of platinum after liver incubation and thus perhaps metabolism is more difficult for the kidney to take up due to hydrophilicity. The ICP MS results also correlate with the viability shown in figure 27, although these data are not statistically significant. A higher platinum uptake in the kidney slice gives a lower ATP content. A lower platinum uptake in the co-incubated kidney slices results also in a higher ATP content. This could indicate that protection that is induced by incubating the kidney slice in liver medium could be induced by a lower intracellular exposure to cisplatin, whereas the possibly formed toxic metabolites from the liver have a minor effect on the slices.

### *vii) Effect of cisplatin on liver*

In this project also the toxicity of cisplatin on the liver was determined. It is expected that cisplatin may be also toxic to the liver in accordance with literature data.20,21,26 An increase in concentration of cisplatin in the liver is expected to lead to a lower ATP content in the liver slices. The results are presented in figure 30, raw data can be found in Appendix I, table 20. The obtained results correspond with the expectations and a treatment with a higher dose of cisplatin leads to a decrease in ATP content.

Figure 30: Relative viability of liver slices after 24 hours treatment with 5, 10 and 25 µM cisplatin. The average ATP of the control slice is set at 100% with a true average value of 11,49 [pmol/µg]. The standard deviation (STDEV) is used to describe the variation (n=1).

In figure 31 the results of the uptake of platinum in liver slices treated with three different concentrations of cisplatin are presented. Raw data of the ICP MS can be found in Appendix I, table 21. It is visible that the amount of ng platinum increases in the slices when the concentration of cisplatin increases as well. A one-way ANOVA with post hoc tests is conducted to test the differences between the concentrations. A significant difference is found between the control and treatment with 10 µM and 25 µM cisplatin (P=0,005 and P=0,000005). Also a significant difference is found between treatment with 5 µM and 25 µM cisplatin (P=000021) and between 10 µM and 25 µM (P= 000149). The higher uptake of platinum in the cell correlate with the decrease in ATP content. So a higher dose of cisplatin leads to a higher uptake of platinum and thus a lower viability.

Figure 31: Amount of platinum in the liver slices after 24 hours treatment with 5, 10, and 25 µM cisplatin determined with ICP MS. The standard deviation (STDEV) is used to describe the variation (n=1).

The results are also corrected for the amount of platinum inside the medium and therefore presented as amount in percentage relative to the medium concentration in figure 32, raw data can be found in Appendix I, table 22. A one-way ANOVA with post hoc tests is conducted to test the differences between the concentrations. A significant difference is found between the control and treatment with 5 µM, 10 µM and 25 µM cisplatin (P<0,001 in all cases). No significant difference is found between the different treatments. It can be concluded that saturation of the efflux transporters in the liver might take place at higher extracellular concentrations. This because the intercellular concentration increases if the extracellular concentration of cisplatin is higher. If saturation of the influx transporters would occur a lower percentage would be expected at higher extracellular concentrations.

Figure 32: ICP MS evaluation of percentage platinum in liver slices relative to the platinum concentration in the medium. The standard deviation (STDEV) is used to describe the variation (n=1).

It can be seen that the uptake of platinum is higher when the slices are treated with a higher concentration of CisPt. This correlates to the viability results shown with ATP content. A higher concentration CisPt gives a higher uptake in the slices and a lower viability, like expected.

Notably, the amount of Pt found in liver for slices incubated with 10 µM cisplatin is 2,4 times lower (2,1 times when relative to amount in medium) than in the case of kidney slices. This may correlate with the known nephrotoxic effect of cisplatin in vivo. The higher uptake of platinum in the kidney in comparison to the liver might indicate the role of the influx transporters, OCTs and Ctr1, which are mainly expressed in proximal and tubular cells.

## 3.2 Quantification of platinum content in other PCTS

For the analysis of the *ex vivo* experiments the uptake evaluation of platinum was planned in different tissue samples. First the difference of uptake in kidney slices treated with 10 µM CisPt in co-incubated experiment and without co-incubation was determined to evaluate the influence of the liver on platinum uptake in the kidney. Also the uptake in liver tissue treated with different concentrations CisPt is evaluated. Both results are mentioned earlier in paragraph 4.1 *vi* and *vii*.

Besides previous mentioned samples also samples of Sarah Spreckelmeyer of our department were analyzed with ICP MS during this project. Because the analysis is performed during this project a short summary of the experiments and results are described in this paragraph. More details of these experiments can be found in Sarah Spreckelmeyer her work.

To determine if the uptake of CisPt in kidney slices is active or passive the amount of Pt was also determined in slices treated with CisPt at 4°C and compared with results of slices treated at 37°C. The amount of metal inside the slices is expected to be lower compared to the 37°C when the uptake goes by active transport since the transporter (probably OCT2) is shut down at 4°C. To evaluate the influence of the OCT2 transporter in CisPt uptake slices pre-treated with cimetidine are analyzed also with ICP MS. Cimetidine is a non-selective inhibitor of the OCT2 transporter and for that reason a lower uptake of Pt is expected in slices pre-treated with cimetidine compared to non-treated slices. The final experiment analyzed with ICP MS was to evaluate the effect of BSA on Pt uptake when co-incubated together.

Earlier results of Sarah Spreckelmeyer of our department, showed that kidney slices treated with both 50 µM and 100 µM CisPt up to 60 minutes at 4°C where more viable then slices treated at 37°C. So CisPt is toxic at 37°C and not at 4°C, for this reason active transport might play a role. I must be taken into account that a temperature difference could influence toxicity not only by altering the uptake. However, results of ICP MS showed that uptake of platinum seem to be decreased at 4°C and therefore active uptake is probably responsible for the increased platinum accumulation at 37°C. No active efflux seems to be involved because then a higher accumulation would be expected at 4°C. The accumulation is also decreased in the slices treated with cimetidine. Active uptake by OCT2 is blocked by cimetidine and inhibition of the OCT2 transporter is probably responsible for decreased Pt accumulation in the slices treated with cimetidine. BSA does not seem to effect the Pt uptake when co-incubated together. The amount of platinum does not differ in the slices with BSA compared to the amount in slices untreated with BSA. This probably indicates that cisplatin does not strongly bind to BSA.

## 3.3 BnFc-Boron toxicity on liver

A side project in this Master thesis was to study the influence the CYP-enzymes in the activation of BnFc-Boron (BFB) and thus on toxicity in the liver. Earlier work was performed in our department in the project of Brech Aikman.30 It is expected that the viability of the liver slices would increase with treatment of the CYP-inhibitor 1-ABT if CYP-enzymes are involved in the metabolism of the metallodrug. Results are presented in figure 33 and 34, raw data can be found in appendix I, table 23. We chose to present results of different experiments in separate figures because the average would not give a realistic overview. In the first experiment BFB does not seem to be toxic at both concentrations and therefore the influence of 1-ABT was hard to determine. Also Brech did not have consistency with the toxicity of BFB during her project. In the second experiment the toxicity of BFB is visible. In this case, treatment with 50 µM 1-ABT seems to have a protective effect on the toxicity of 10 µM BFB, although not significant. When the slices were treated with 20 µM BFB there is no effect found of 1-ABT. It is concluded that more experiments are necessary to determine the influence of CYP-enzymes and an optimization of the procedure might be necessary.

Figure 33/34: Relative viability of liver slices after treatment with 10 and 20 µM BFB with and without 1-ABT treatment. The average ATP of the control slices are set at 100% with a true average value of 9,34 and 15,73[pmol/µg]. The standard deviation (STDEV) is used to describe the variation.

In addition, slice morphology was also studied to evidence the toxic effect of BFB and the influence of 1-ABT on the toxicity. The untreated control can be seen in figure 35. As can be seen in figure 36, 1-ABT alone does not seem to be toxic to the liver cells itself. Treatment with BFB, figure 37 and 38, seems to affect the liver cells because the nuclei appear to be condensed in comparison to the untreated slice. 1-ABT does not seem to have a protective effect on the cells when treated with BFB (figure 39 and 40) and for that reason CYP-enzymes do not seem to be involved in BnFc-Boron activation.

Figure 35: 24h control Figure 36: 50 µM 1-ABT

Figure 37: 10 µM BFB Figure 38: 20 µM BFB

Figure 39: 10 µM BFB + 50 µM 1-ABT Figure 40: 20 µM BFB + 50 µM 1-ABT

# 4. Conclusions

Cisplatin metabolites are supposed to be more toxic than cisplatin itself and glutathione conjugates are assumed to be involved in cisplatin nephrotoxicity. However liver involvement in cisplatin nephrotoxicity is not well described in literature. This project tried to determine the influence of liver metabolism of cisplatin on the toxicity of cisplatin to the kidney using PCTS. The main hypothesis in this project is that formation and cleavage by γ-glutamyl transpeptidase (GGT) of the cisplatin-glutathione conjugates take place in the liver. The resulting Pt-containing species are responsible for the observed nephrotoxicity.

The results of our experiments can be summarized as follows:

* Liver slices seem to have a protective effect against cisplatin toxicity shown with KIM-1 expression (not significant) and ATP content (10 µM, 24 hours incubation). Expected was that the liver would increased toxicity due metabolite formation, but this could not be confirmed.
* Uptake of platinum in kidney slices co-incubated with liver slices is lower than in kidney slices incubated alone. The results may be due to metabolite formation by the liver which are less toxic than cisplatin itself, or formation of metabolites which are not taken up by the kidney. This could be in the form of aquo species of cisplatin, excreted by ATP7A or ATP7B by the liver, which are more difficult for the kidney to take up.
* Kidney Injury Marker-1 (KIM-1) is (not statistically significant) upregulated in kidney slices treated with 1 µM cisplatin and downregulated with 5 µM cisplatin. These results correspond with results of Gerian Prins, former master student in the department.30 Although not significant, there seems to be a tendency that cisplatin leads to an upregulation of KIM-1 in low concentrations and to decrease in expression at high concentrations. This indicates that cisplatin leads to tubular damage (nephrotoxicity) which will lead in longer time periods as well to a decrease in viability.
* Acivicin, a GGT-inhibitor, is supposed to protect the kidney for nephrotoxicity by inhibiting the formation of toxic metabolites out of cisplatin-glutathione conjugates. Acivicin seems to have a toxic effect on the kidney slices itself, but seems to have a protective effect on toxicity of cisplatin as shown by morphology. However, this effect is not observed with KIM-1 expression or ATP viability assays.
* When GSH is added to spontaneously form conjugates with cisplatin prior incubation with the kidney slices, both ATP and KIM-1 expression assays show an increase in the toxicity on kidney with respect to cisplatin alone. This support the idea that when more Pt-GSH conjugates are formed there is more toxicity.
* With ICP MS is shown that active transport is probably responsible for the influx of platinum. The accumulation of platinum is proven to be decreased when slices are treated at 4˚C. With these conditions of low temperature the active transport is shut down. The accumulation is decreased in slices treated with an OCT2 inhibitor, cimetidine, thereby inhibiting active transport as well. Therefore, it can be concluded that the OCT2 transporter is probably responsible for the decreased platinum accumulation.

Overall, the role of GGT activity on the toxicity of cisplatin is still not clear after this project. Repeating some of the ATP essays and PCR experiments might give a better overview of the results and perhaps significant differences might be found. Also using a different inhibitor to inhibit the metabolic pathway could give more insight, this could be a different GGT inhibitor or a cysteine S-conjugate β-lyase inhibitor. Morphology of kidney slices co-incubated with liver might be interesting to see the effect of liver on the tubular cells in kidney. Furthermore also determination of the presence of conjugates and which conjugates by HPLC or another method is necessary to draw hard conclusions out of the results. Besides, a perfusion set up could give a more realistic way of how the compounds reach the kidney after passing by the liver. In this way medium containing different concentrations of cisplatin could stream through the liver and then reach the kidney. In this way there is more certainty about the fact that metabolites will reach the kidney. In the set up used in this project it could be that a low amount of metabolites is formed by the liver but that this amount is very low in comparison with the 1,3 mL medium in the well and for that reason don not reach the kidney. Finally, it would be interesting to see via ICP MS if the uptake of Pt-GSH is higher than of cisplatin alone. These results could give more insight about the toxicity of the metabolites compared to cisplatin itself.

The conclusion of the secondary project about involved of the influence of CYP-enzymes in the activation of BnFc-Boron and thus on toxicity in the liver is still not clear. BnFc-Boron does not show consistency for a decrease in viability determined with ATP. This makes it difficult to see whether the CYP-inhibitor 1-ABT has any influence on the toxicity and no conclusion can be drawn about the CYP-involvement in activation of BnFc-Boron. However, with morphology 1-ABT does not seem to have a protective effect on the BnFc-Boron toxicity. Overall, concluded can be that more experiments are necessary to determine the influence of CYP-enzymes and an optimization of the procedure might be needed.

# Acknowledgements

For this project I would like to acknowledge prof. dr. A. Casini, dr. ir. I.A.M de Graaf and S.J.P Spreckelmeyer for their help and advice. Furthermore I would like to thank prof. S. Stürup and the other people in his department for their hospitality and for their help and guidance with the ICP-MS in Copenhagen. Also I would to like to thank ing. M.H. de Jager and M.T. Merema for their guidance during all lab work and explaining me the necessary technics. Furthermore I would like to thank all people in the department Pharmacokinetics, Toxicology and Targeting for all the help offered during my master project and for the good working atmosphere.

# 5. Materials and Methods

## 5.1 Chemicals

Cisplatin and Acivicin were obtained from the firm Sigma Aldrich. The ATP Bioluminescence Assay Kit CLS II from Roche has been used (luciferase and ATP-standard, cat. No. 11 699 695 001). The protein assay Reagent A (DC™ Protein Assay Reagent A, cat# 500-0113) and Reagent B (DC™ Protein Assay Reagent B, cat# 500-0114) were obtained from Biorad. BSA (Albumin, Bovine acid free, ICN cat# 105033) was purchased at Biomedicals Inc. For the ICP MS measurements, 1000 ppm standard platinum (Standard for ICP-AES & -MS, 1000 µg/mL in 10%HCl, 125mL, PlasmaCal cat# 140-052-781,) has been used.

## **5.2 Precision cut liver and kidney slices**

To collect the precision cut liver and kidney slices the protocol of I.A.M. de Graaf has been used, and the derived protocol can be found in Appendix II.31 The organs of male Wistar rats, which had free access to food and water, were used to obtain the precision cut slices. The rat has been anesthetized with 5% isoflurane in O­2 before sacrificing. Excision of the organs has been done as quickly as possible and the organs were stored in ice-cold UW. Afterwards, liver cores were obtained with a mechanical drill containing a coring tool with a diameter of 5mm. The liver was kept wet with UW during preparation of the cores. The cylindrical cores were put in UW on ice directly after preparation. The kidney cores were prepared with a coring tool by hand with a diameter of 5mm. However, before making the cores with the drill the kidney was cut in half lengthwise with a surgical blade. The Krumdieck tissue slicer (MD6000) has been filled with ice-cold oxygenated Krebs Henseleit Buffer (pH 7.4) and connected to a refrigerated circulator bath (4°C). One core is transferred to the core holder of the Krumdieck slicer. Liver slices are required to have a weight of 5 mg and kidney slices a weight of 3 mg for optimal viability. Slices which are too thick become necrotic in the internal areas of the slice. Slices of the kidney should be obtained from the cortex side of the kidney while the side of the core which contains medulla is not used. After obtaining the slices they were stored in ice-cold UW before incubating them. Before transferring the slices, they are washed in a Petri dish with WME to remove the UW. The slices are individually transferred to pre-warmed 12 wells plates which contain 1,3 mL WME enriched with glucose and antibiotics. For the liver gentamycin(100 µL per 100 mL WME) or ciprofloxacin (500 µl per 100 mL WME) were used as antibiotics depending on the experiment, while for the kidney slices ciprofloxacin (500 µl per 100 mL WME) is used instead. The plates were maintained at least 30 minutes in the incubator at 37°C under 95%O2/5% CO2 and shaken at 90 rpm before transferring the slices. Afterwards, the slices were pre-incubated for at least one hour depending on the setup of the experiment before incubation in the incubator started.

## 5.3 Protocols for examine toxicity on slices

**Preparation of compounds solutions**

Cisplatin is dissolved in Milli-Q water to obtain a 1 mM stock solution. Afterwards, 100 µM and 400 µM cisplatin dilutions are obtained by further diluting the 1 mM solution f in Milli-Q. Acivicin is dissolved in Milli-Q to obtain a 10 mM stock solution and used as such. GSH is dissolved in Milli-Q to obtain a 1 mM stock solution, which is further diluted to 200 µM. BnFC-Boron is diluted in Milli-Q water to obtain a stock solution of 10 mM, which is then further diluted to 1 mM prior the experiment. 1-ABT is dissolved in Milli-Q water to obtain a solution of 100 mM, and a 5mM solution is obtained as well by further diluting.

**Evaluation of cisplatin toxicity in kidney and liver slices**

To examine the toxicity of cisplatin on kidney and liver slices cisplatin is pipetted to the wells plate after the slices are transferred from the pre-incubation well to the incubation well. To obtain the different concentrations in the well-plate different dilutions and amounts are used which are presented in table 1.

Table 1: Used quantities and solutions to obtain different cisplatin concentrations in the 1,3 mL well-plate

|  |  |  |
| --- | --- | --- |
| Concentration in well | Cisplatin starting solution | Quantity added |
| 1 µL | 100 µM | 13 µL |
| 5 µL | 400 µM | 16 µL |
| 10 µL | 1 mM | 13 µL |
| 25 µL | 1 mM | 32.5 µL |

**Evaluation of cisplatin toxicity in kidney slices co-incubated with liver slices**

Different approaches can be used to determine the effect of liver on the toxicity of cisplatin on kidney slices. However, the one that we considered more appropriate consist in treating first liver slices with cisplatin and then replacing the slice in the well with the kidney slices. Thus, the kidney slices were not treated with the compound directly themselves.

For the experiments where the incubation time was 24 hours this meant that the liver slices were collected on day one from a rat and on the second day the kidney slices of a different rat were used for the experiment. A schematic overview can be seen in figure 41. The kidney slices replaced the liver slices and where incubated in the medium of the liver. Because the liver consumed also medium during the first hour’s incubation, some fresh medium (300 µL) was added to the kidney slices to guarantee the best conditions for viability.

Figure 41: Schematic overview of the experimental set up with an incubation time of 24 hours for the kidney slices

In the case of co-incubation, the liver slice is treated with 10 µM CisPt and the kidney slice is put in the medium after liver incubation over 24 hour, a schematic overview can be seen in figure 42. For the experiments where the incubation time was 2 hours the slices were collected from the same rat and the kidney slices were still in the pre-incubation well during the liver incubation before replacing the liver slice.

Figure 42: Schematic overview of the experimental setup with an incubation time of 2 hours for the kidney slices

In the first experiments with the 24 hours incubation times the slices of the liver and kidney where collected from the same rat and incubated together in the same well. A schematic overview can be seen in figure 43. But in this case it was possible that the kidney was not affected by the conjugates produced by the liver but by the cisplatin directly. For this reason was decided to replace the liver slices by the kidney slices.

Figuur 43: Schematic overview of the initial setup with an incubation time of 24 hours for the kidney slices

**Evaluation of the effects of acivicin on toxicity of cisplatin in kidney slices**

To examine whether acivicin had an effect on toxicity of cisplatin a concentration of 100 µM acivicin in the wells is obtained. This means that 13 µL of the 10 mM solution is pipetted to the wells plates containing the slices. Adding acivicin to the slices was done 30 minutes before treatment with cisplatin in order to let an interaction take place between acivicin and the slice.

**Evaluation of the effect of GSH on toxicity of cisplatin in kidney slices**

To examine whether glutathione would increase the toxicity of cisplatin two concentrations are obtained in the wells plate namely 2 and 10 µM depending on the cisplatin concentration. With the 1 µM cisplatin treatment a 2 µM GSH concentration is used and with 5 µM CisPt 10 µM GSH is added. The used quantities and solutions are presented in table 2. Glutathione and cisplatin were incubated together for 1 hour in the incubation well before the slice was transferred from the pre-incubation well to the incubation well.

Table 2: Used quantities and solutions to obtain different GSH concentrations in the 1,3 mL well-plate

|  |  |  |
| --- | --- | --- |
| Concentration GSH | Solution used | Quantity added |
| 2 µL | 200 µM GSH | 13 µL |
| 10 µL | 1 mM GSH | 13 µL |

**Evaluation of the toxicity of BnFC-Boron and effect of 1-ABT in liver slices**

To examine the effect of BnFc-Boron a concentration of 10 µM as well as 20 µM is obtained in the wells plates. To test whether 1-ABT had an influence or not on toxicity of BnFC-boron a concentration of 50 µM is obtained in the wells plates. Adding 1-ABT to the slices was done 30 minutes before treatment with BnFC-Boron in order to let an interaction take place between 1-ABT and the slices. The used quantities and solutions are presented in table 3.

Table 3: Used quantities and solutions to obtain different BFB concentrations and 1-ABT concentration in the 1,3 mL well-plate

|  |  |  |
| --- | --- | --- |
| [BnFc-Boron] | Solution used | Quantity added |
| 10 µL | 1 mM BFB | 13 µL |
| 20 µL | 1 mM BFB | 26 µL |
| [1-ABT] |  |  |
| 50 µL | 5 mM 1-ABT | 13 µL |

## 5.4 Biochemical assays for viability testing

Different assays are used to test the viability of the slices. ATP is a measure for viability. More ATP shows a more viable slice. But the slice thickness influences this measure. For that reason the amount of proteins is measured to correct for the thickness of the slice. With morphology it’s possible to see the damage in the different kinds of cell types within the slice.

**ATP Assay**

The slices are put in safe lock vials containing 1 mL SONOP and one spoon minibeads after incubation in the wells plate and frozen immediately in liquid nitrogen. The protocol for ATP determination can be found in Appendix III After homogenizing and centrifuging of the samples the supernatant is added to a black 96-wells plate in duplo for measuring. The rest of the supernatant is put in an Eppendorf cup leaving the pellet behind in the safe lock vial to dry. The supernatant is stored in the -80°C fridge. A positive control is added to the plate. The positive control is made with supernatant of 8 rat liver slices. De ATP value of the control is around 405 with a protein content of 500 µg/slice. The positive control is stored in -80°C and thawed on ice before use. Besides these two also 50 µL of a calibration curve has to be added to the plate in duplo (row A and F). The calibration curve is made of the ATP-standard from the ATP Bioluminescence Assay Kit CLS II. After pipetting the samples, the positive control and the calibration curve luciferase is added to each well. The plate is measured directly after adding the luciferase and also after 5 minutes with the luminometer (mma.pf). After measuring the results are exported to an excel file. The time where the duplo’s lay most close to each other are used for the calculations.

**Protein Assay**

The protein determination is done by using the Protein Estimation by Lowry. The protocol can be found in Appendix IV. The slices are dried overnight at 37°C before the protein estimation by Lowry is performed. A calibration curve of BSA is made by dissolving BSA in 1 M NaOH. Two stocks of BSA (3.2 mg/ml, 2.4 mg/ml) are used so the amount of BSA versus NaOH is the same in each dilution (50 µL each). After pipetting the samples, calibration curve, reagent A and reagent B the plate is shake and incubated for 15 minutes in the dark at room temperature before measuring. The absorbance is measured at 650 nm. The results are exported into an excel file, and used for the correction for the ATP values.

**Morphology**

The protocol for de-hydration of the slices is reported in Appendix V. After incubation the slices are put immediately in 4% formalin in a 24-wells plate for 24h at 4°C and thereafter in 70% ethanol for 24h. Afterwards, the slices are put in a cassette with a filter in it. The slices are also covered with a filter. The cassettes are dehydrated further trough different EtOH and Histosolve steps. These steps are performed at the pathology department of the UMCG to be more accurate. Then the cassettes are placed in paraffin. The paraffin is melted and the cassettes are opened so the slices can be put in an iron cassette in new paraffin. Hereby the slices must stand up straight. Next the slices in paraffin are cut and put on a slide. The staining follows after deparaffizing and rehydration and the protocol for this can be found in Appendix VI. For the staining hematoxylin and eosin are used. The hematoxylin colors the chromatins of the nucleus blue-purple and the eosin colors the cytoplasm pink because it binds to positive charged protein molecules present in lysine and arginine. The extracellular matrix won’t be stained because it’s negatively charged as well as the eosin. After the staining the slides are dried in the air before a drop of depex mounting medium is put on top of it covered by a coverslip. After drying the slides can be observed under the microscope (Aperio scanner).

**PCR**

Three slices of the same condition are put in a RNAse free Eppendorf and immediately frozen after incubation for RNA isolation. The protocol for RNA isolation can be found in Appendix VII. To the Eppendorf’s minibeads and 220 µL homogenization solution is added before homogenization with the minibead beater for one minute. While foam is settled on ice 10 µL DNase I is added to well 4 of the cartridges. 220 µL lysis buffer is added to the samples followed by vortexing at the highest speed for 15 seconds. 400 µL of the sample is added to well 1 of the cartridges and after placing the LEV rack in the machine the program RNA, simply RNA, is started. The RNA concentration is measured with nanodrop before continuing with cDNA conversion. The RNA samples are diluted to 1,5 µg RNA in 10 µL RNase-free water. After making the master mix (3,7 µL 5x RT-buffer, 0,8 µL dNTP’s 10 mM, 0,8 µL RNasin Ribonuclease Inhibitors 10 units, 1,6 µL M-MLV Rev transcriptase 100 units and 1,6 µL random primers for 10 µL) 8,5 µL is added to each sample. The program Mastercycler MLVCDNA is used for cDNA conversion (10 min 20°C, 30 min 42°C, 12 min 20°C, 5 min 99°C and 5 min 20°C). The protocol for running the qPCR can be found in Appendix VIII. The primers s18s, GADPH, KIM-1 and KIM-2 are used. For each primer mix 0,2 µL fw and rev primer is used together with 5 µL sybr green mix for adding 5,4 µL primer mix to each well. The cDNA samples are diluted to 10 ng/µL nuclease in nuclease-free water. To the primer mix in each well 4,6 µL diluted cDNA is added. The plate is sealed with a plastic cover and centrifuged for five minutes at 2000 rpm. The cover is replaced with the ABI PRISM Optical Adhesive Cover with no plastic or adhesive on the edges. The cover is not touched with hands. The plate is read with the ABI PRISM® 7900HT.

## 5.5 Inductively Coupled Plasma (ICP) Mass Spectrometry

**Sample preparation from tissue slices**

Before the analysis by ICP-MS, the samples are prepared by adding 50 µL sub-boiled 65% HNO3 to each tissue slice. The sample is whirl mixed and digested overnight. To the clear solution 100 µL of concentrated HCl (30%) is added and again whirl mixed. Afterwards, 50 µL of samples are put in an auto sampler vial (Clean Pack vials ML 33003Vu, 2 mL 6m lysning 32\*11,6 mm, Mikrolab Aarhus A/S-8270 Hojbjerg) with 950 µL HNO3/HCl (0,65%/0,1%) and mixed by pipetting.

**Dilution medium samples**

The medium samples are diluted with acid mix HNO3/HCl (0,05%/0,1%) to bring the concentration into the range of the external standard curve.

**Analysis by ICP-MS**

The determination of metal content in the samples is performed using external calibration for the ICP-MS. The preparation of the external standard curve samples is summarized in table 4. The acid mix consist of 10 mL 65% HNO3 and 3.5mL 30% HCl diluted to 1 L with MQ.­ The calibration curve is prepared every day before analysis. The calibration solutions are all between 0-40 ppb. Argon was flowing continuously throw the ICP-MS (ELAN DRC-E, Axial field technology) during analysis. An ASX-110 FR Auto sampler was connected to the ICP-MS. In this auto sampler, 24 prepared samples fitted at a time. As rinsing fluid 0,65% sub boiled HNO3 was used with a rinsing time of 200 seconds. The data was collected with the program ELAN Instrument Control Session on a computer and exported to excel files for calculation.

Table 4: Used quantities and solutions to obtain the external standard curve samples

|  |  |
| --- | --- |
| Concentration | Solutions |
| 1000 ppb stem | 10 µL 1000ppm standard + 9,99mL acid mix |
| 100 ppb stem | 1 mL 1000ppb stem + 9mL acid mix |
| 40 ppb | 2 mL 100ppb stem + 3mL acid mix |
| 20 ppb | 1 mL 100ppb stem + 4 mL acid mix |
| 10 ppb | 2.5 mL 100ppb stem to 25mL with acid mix |
| 7.5 ppb | 375 µL 100ppb stem + 4.625mL acid mix |
| 5 ppb | 250 µL 100ppb stem + 4.75mL acid mix |
| 2.5 ppb | 125 µL 100ppb stem + 4.875mL acid mix |
| 1.5 ppb | 75 µL 100ppb stem + 4.925mL acid mix |
| 1 ppb | 50 µL 100ppb stem + 4.950mL acid mix |
| 0.5 ppb | 25 µL 100ppb stem + 4.975mL acid mix |
| 0.1 ppb | 10 µL 100ppb stem + 9.99mL acid mix |

## **5.6 Statistics**

Data are presented in graphs including the mean and the standard error of the mean (SEM). In some cases, where n=1 the standard deviation of the mean (SD) is presented. To determine if there is a difference within the variables, a two-way analysis of variance is performed in the way of two-way ANOVA by using the program IBM SPSS Statistics 23. Also one-way ANOVA (with Tukey post hoc test) results are interpreted variable differences.

Power analysis is performed once to obtain insight in the number of experiments necessary to compare the effect of cisplatin treatment on the means of KIM-1 expression. This is performed with the raw data of the results. For the power analysis the standard deviation is used with α=0,05 and β=0,20.

# 6. References

1. Todd, R. C. & Lippard, S. J. Inhibition of transcription by platinum antitumor compounds. *Metallomics* **1,** 280–291 (2009).

2. Townsend, D. M., Deng, M., Zhang, L., Lapus, M. G. & Hanigan, M. H. Metabolism of Cisplatin to a nephrotoxin in proximal tubule cells. *J. Am. Soc. Nephrol.* **14,** 1–10 (2003).

3. Douple, E. B. Cis-diamminedichloroplatinum(II): Effects of a representative metal coordination complex on mammalian cells. *Pharmacol. Ther.* **25,** 297–326 (1984).

4. Cohen, G. L. *et al.* Sequence dependent binding of cis-dichlorodiammineplatinum(II) to DNA. *J. Am. Chem. Soc.* **102,** 2487–2488 (1980).

5. Knox, R. J., Friedlos, F., Lydall, D. A. & Roberts, J. J. Mechanism of cytotoxicity of anticancer platinum drugs: evidence that cis-diamminedichloroplatinum(II) and cis-diammine-(1,1-cyclobutanedicarboxylato)platinum(II) differ only in the kinetics of their interaction with DNA. *Cancer Res* **46,** 1972–1979 (1986).

6. Townsend, D. M. & Hanigan, M. H. Inhibition of gamma-glutamyl transpeptidase or cysteine S-conjugate beta-lyase activity blocks the nephrotoxicity of cisplatin in mice. *J. Pharmacol. Exp. Ther.* **300,** 142–8 (2002).

7. Hanigan, M. H. *et al.* Gamma-glutamyl transpeptidase-deficient mice are resistant to the nephrotoxic effects of cisplatin. *Am. J. Pathol.* **159,** 1889–94 (2001).

8. Perez, R. P. Cellular and molecular determinants of cisplatin resistance. *Eur. J. Cancer* **34,** 1535–1542 (1998).

9. Casini, A. & Reedijk, J. Interactions of anticancer Pt compounds with proteins: an overlooked topic in medicinal inorganic chemistry? *Chem. Sci.* **3,** 3135 (2012).

10. Dobson, P. D. & Kell, D. B. Carrier-mediated cellular uptake of pharmaceutical drugs: an exception or the rule? *Nat. Rev. Drug Discov.* **7,** 205–220 (2008).

11. Spreckelmeyer, S., Orvig, C. & Casini, A. Cellular Transport Mechanisms of Cytotoxic Metallodrugs: An Overview beyond Cisplatin. *Molecules* **19,** 15584–15610 (2014).

12. Kelland, L. The resurgence of platinum-based cancer chemotherapy. *Nat. Rev. Cancer* **7,** 573–584 (2007).

13. Daley-Yates, P. T. & McBrien, D. C. H. Cisplatin metabolites in plasma, a study of their pharmacokinetics and importance in the nephrotoxic and antitumour activity of cisplatin. *Biochem. Pharmacol.* **33,** 3063–3070 (1984).

14. Zhang, L. & Hanigan, M. H. Role of cysteine S-conjugate beta-lyase in the metabolism of cisplatin. *J. Pharmacol. Exp. Ther.* **306,** 988–994 (2003).

15. Kim, H. S., Cha, S. H., Abraham, D. G., Cooper, a J. L. & Endou, H. Intranephron distribution of cysteine S-conjugate beta-lyase activity and its implication for hexachloro-1,3-butadiene-induced nephrotoxicity in rats. *Arch. Toxicol.* **71,** 131–141 (1997).

16. Hanigan, M. H. & Frierson, H. F. Immunohistochemical detection of gamma-glutamyl transpeptidase in normal human tissue. *J. Histochem. Cytochem.* **44,** 1101–1108 (1996).

17. Earhart, R. H. & Neil, G. L. Acivicin in 1985. *Adv. Enzyme Regul.* **24,** 179–205 (1985).

18. Zhang, L. *et al.* Cisplatin-induced toxicity is associated with platinum deposition in mouse kidney mitochondria in vivo and with selective inactivation of the alpha-ketoglutarate dehydrogenase complex in LLC-PK1 cells. *Biochemistry* **45,** 8959–8971 (2006).

19. Cooper, A. J. L., Bruschi, S. a & Anders, M. W. Toxic, halogenated cysteine S-conjugates and targeting of mitochondrial enzymes of energy metabolism. *Biochem. Pharmacol.* **64,** 553–64 (2002).

20. Waseem, M., Bhardwaj, M., Tabassum, H., Raisuddin, S. & Parvez, S. Cisplatin hepatotoxicity mediated by mitochondrial stress. *Drug Chem. Toxicol.* **00,** 1–8 (2015).

21. Naqshbandi, a., Khan, W., Rizwan, S. & Khan, F. Studies on the protective effect of flaxseed oil on cisplatin-induced hepatotoxicity. *Hum. Exp. Toxicol.* **31,** 364–375 (2012).

22. Liou, G.-Y. & Storz, P. Reactive oxygen species in cancer. *Free Radic. Res.* **44,** 479–96 (2010).

23. Bedard, K. & Krause, K.-H. The NOX family of ROS-generating NADPH oxidases: physiology and pathophysiology. *Physiol. Rev.* **87,** 245–313 (2007).

24. Cohen, G. & Hochstein, P. Glutathione Peroxidase: The Primary Agent for the Elimination of Hydrogen Peroxide in Erythrocytes\*. *Biochemistry* **2,** 1420–1428 (1963).

25. Monks, T. J. *et al.* Glutathione conjugate mediated toxicities. *Toxicol. Appl. Pharmacol.* **106,** 1–19 (1990).

26. Pratibha, R., Sameer, R., Rataboli, P. V, Bhiwgade, D. a & Dhume, C. Y. Enzymatic studies of cisplatin induced oxidative stress in hepatic tissue of rats. *Eur. J. Pharmacol.* **532,** 290–3 (2006).

27. Goto, S. *et al.* Augmentation of transport for cisplatin-glutathione adduct in cisplatin-resistant cancer cells. *Cancer Res.* **55,** 4297–4301 (1995).

28. Cairns, R. a, Harris, I. S. & Mak, T. W. Regulation of cancer cell metabolism. *Nat. Rev. Cancer* **11,** 85–95 (2011).

29. Hagen, H. *et al.* Aminoferrocene-Based Prodrugs Activated by Reactive Oxygen Species. *J. Med. Chem.* **55,** 924–934 (2012).

30. Aikman, B. The influence of oxidative stress in healthy precision- ­ ‐ cut liver slices on the toxicity evaluation of anti- ­ ‐ cancer ( metallo ) drugs. (2014).

31. De Graaf, I. A. *et al.* Preparation and incubation of precision-cut liver and intestinal slices for application in drug metabolism and toxicity studies. *Nat Protoc* **5,** 1540–1551 (2010).

32. Kotz, L, Kaiser, G, Tschopel, P and Tolg, G. Theory of Sample Preparation Using Acid Digestion , Pressure Digestion and Microwave Digestion ( Microwave Decomposition ). *Anal. Chem.* **260,** 207 – 209 (1972).

33. Thomas, R. A beginner’s guide to ICP-MS. *Spectroscopy* **16,** 4 (2001).

34. Ichimura, T. *et al.* Kidney injury molecule-1 (KIM-1), a putative epithelial cell adhesion molecule containing a novel immunoglobulin domain, is up-regulated in renal cells after injury. *J. Biol. Chem.* **273,** 4135–4142 (1998).

35. Prins, G. G. H., Spreckelmeyer, S., Jager, M. De, Casini, A. & Graaf, I. A. M. De. Development of precision-cut kidney slices for testing toxicity and disposition of platinum anticancer drugs in tubular cells. 1–79 (2015).

36. Ishikawas, T. & Ali-Osman, F. Glutathione-associated cis-Diamminedichloroplatinum (II) Metabolism and ATP-dependent Efflux from Leukemia Cells. *J. Biol. Chem.* **268,** 20116–20125 (1993).

# Appendix I

Table 5: Raw data relative viability figure 8. The average ATP of the control slice is set at 100% with a true average value of 5,60 [pmol/µg]

|  |  |
| --- | --- |
| Kidney | Kidney co-incubated liver |
| control | **1 µM** | **5 µM** | **control** | **1 µM** | **5 µM** |
| 100 | 97,56 | 124,40 | 166,39 | 111,75 | 98,51 |
| 100 | 231,61 | 174,94 | 260,78 | 202,38 | 241,77 |
| 100 | 129,27 | 97,47 | 94,80 | 68,68 | 186,21 |
| 100 | 76,03 | 122,84 | 104,75 | 101,62 | 110,32 |
| 100 | 132,69 | 119,86 | 91,93 | 96,23 | 84,95 |

Table 6: Raw data fold induction figure 9. The fold induction of the control slice is set at 1

|  |  |
| --- | --- |
| Kidney | Kidney co-incubated liver |
| control | **1 µM** | **5 µM** | **control** | **1 µM** | **5 µM** |
| 1,00 | 1,60 | 0,69 | 0,61 | 0,22 | 0,59 |
| 1,00 | 4,86 | 1,24 | 3,45 | 2,71 | 3,87 |
| 1,00 | 0,28 |  | 0,37 | 1,02 |  |
| 1,00 | 0,11 | 0,43 | 0,28 | 1,22 | 0,47 |
| 1,00 | 4,26 | 0,22 | 0,42 | 0,01 | 0,21 |

Table 7: Raw data ΔΔCT figure 9

|  |  |
| --- | --- |
| Kidney | Kidney co-incubated liver |
| control | **1 µM** | **5 µM** | **control** | **1 µM** | **5 µM** |
| 0,00 | -0,68 | 0,54 | 0,71 | 2,21 | 0,75 |
| 0,00 | -2,28 | -0,31 | -1,79 | -1,44 | -1,95 |
| 0,00 | 1,82 |  | 1,42 | -0,03 |  |
| 0,00 | 3,23 | 1,22 | 1,82 | -0,29 | 1,08 |
| 0,00 | -2,09 | 2,20 | 1,25 | 6,59 | 2,26 |

Table 8: Raw data fold induction Gerian Prins figure 10. The fold induction of the control slice is set at 1

|  |
| --- |
| Kidney |
| control | **1 µM** | **5 µM** |
| 1,00 | 1,86 | 2,08 |
| 1,00 | 2,50 | 1,10 |
| 1,00 | 2,12 | 1,01 |

Table 9: Raw data relative viability figure 11. The average ATP of the control slice is set at 100% with a true average value of 5,60 [pmol/µg]

|  |  |
| --- | --- |
| Kidney | Kidney with acivicin |
| control | **1 µM** | **5 µM** | **control** | **1 µM** | **5 µM** |
| 100,00 | 97,56 | 124,40 | 39,98 | 131,31 | 80,24 |
| 100,00 | 231,61 | 174,94 | 104,22 | 88,92 | 91,22 |
| 100,00 | 129,27 | 97,47 | 97,09 | 107,54 | 88,70 |
| 100,00 | 76,03 | 122,84 | 111,80 | 149,02 | 183,80 |
| 100,00 | 132,69 | 119,86 |  |  |  |

Table 10: Raw data fold induction figure 12. The fold induction of the control kidney slice without acivicin is set at 1

|  |  |
| --- | --- |
|  Kidney | Kidney with acivicin |
| control | **1 µM** | **5 µM** | **control** | **1 µM** | **5 µM** |
| 1,00 | 1,60 | 0,69 | 4,60 | 4,65 | 0,92 |
| 1,00 | 4,86 | 1,24 | 0,38 | 0,38 | 0,29 |
| 1,00 | 0,28 |   | 0,37 | 120,54 | 0,22 |
| 1,00 | 0,11 | 0,43 | 0,48 | 0,63 | 0,32 |
| 1,00 | 4,26 | 0,22 |   |   |   |

Table 11: Raw data relative viability figure 19. The average ATP of the control kidney slice incubated alone without acivicin is set at 100% with a true average value of 5,60 [pmol/µg]

|  |  |
| --- | --- |
| Kidney co-incubated liver | Kidney co-incubated liver with acivicin |
| control | **1 µM** | **5 µM** | **control** | **1 µM** | **5 µM** |
| 166,39 | 111,75 | 98,51 | 280,25 | 221,12 | 135,33 |
| 260,78 | 202,38 | 241,77 | 90,10 | 95,54 | 112,01 |
| 94,80 | 68,68 | 186,21 | 76,49 | 80,28 | 82,80 |
| 104,75 | 101,62 | 110,32 | 114,79 | 112,07 | 88,60 |
| 91,93 | 96,23 | 84,95 |  |  |  |

Table 12: Raw data fold induction figure 20. The fold induction of the control kidney slice incubated alone without acivicin is set at 1

|  |  |
| --- | --- |
| Kidney co-incubated liver | Kidney co-incubated liver with acivicin |
| control | **1 µM** | **5 µM** | **control** | **1 µM** | **5 µM** |
| 0,61 | 0,22 | 0,59 | 1,18 | 0,78 | 1,77 |
| 3,45 | 2,71 | 3,87 | 0,34 | 0,38 |  |
| 0,37 | 1,02 |  | 0,30 | 0,18 | 1,58 |
| 0,28 | 1,22 | 0,47 | 1,82 | 0,56 | 0,37 |
| 0,42 | 0,01 | 0,21 |  |  |  |

Table 13 : Raw data relative viability figure 21. The average ATP of the control slices are set at 100% with a true average value of 7,22 , 11,30 and 23,39 [pmol/µg]

|  |  |  |
| --- | --- | --- |
| 1h incubation | 2h incubation | 4h incubation |
| Control | **1 µM** | **5 µM** | **control** | **1 µM** | **5 µM** | **control** | **1 µM** | **5 µM** |
| 100,00 | 133,58 | 101,39 | 100,00 | 138,50 | 115,59 | 100,00 | 104,33 | 117,08 |
| 100,00 | 103,75 | 97,49 | 100,00 | 104,45 | 132,10 | 100,00 | 58,25 | 86,98 |

Table 14: Raw data fold induction figure 22/23. The fold induction of the control slices are set at 1

|  |  |  |
| --- | --- | --- |
| 1h incubation | 2h incubation | 4h incubation |
| control | **1 µM** | **5 µM** | **control** | **1 µM** | **5 µM** | **control** | **1 µM** | **5 µM** |
| 1,00 | 0,39 | 0,12 | 1,00 | 0,18 | 0,43 | 1,00 | 1,33 | 0,73 |
| 1,00 | 1,42 | 0,79 | 1,00 | 0,10 | 0,51 | 1,00 | 86,91 | 1,32 |

Table 15: Raw data relative viability figure 24. The average ATP of the control slices are set at 100% with a true average value of 7,22 , 11,30 and 23,39 [pmol/µg]

|  |  |  |
| --- | --- | --- |
| 1h incubation | 2h incubation | 4h incubation |
| control | **1 µM** | **5 µM** | **control** | **1 µM** | **5 µM** | **control** | **1 µM** | **5 µM** |
| 100,00 | 148,80 | 117,78 | 100,00 | 119,93 | 113,04 | 100,00 | 119,80 | 75,60 |
| 100,00 | 152,32 | 109,60 | 100,00 | 134,15 | 226,80 | 100,00 | 138,16 | 29,13 |

Table 16: Raw data fold induction figure 25/26. The fold induction of the control slices are set at 1

|  |  |  |
| --- | --- | --- |
| 1h incubation | 2h incubation | 4h incubation |
| control | **1 µM** | **5 µM** | **control** | **1 µM** | **5 µM** | **control** | **1 µM** | **5 µM** |
| 1,00 | 0,59 | 0,07 | 1,00 | 0,26 | 0,65 | 1,00 | 0,54 | 0,33 |
| 1,00 | 4,17 | 5,87 | 1,00 | 14,67 | 0,75 | 1,00 | 73,41 | 4,08 |

Table 17: Raw data relative viability figure 27. The average ATP of the control slice is set at 100% with a true average value of 8,72 (n=5) and 6,84 (n=2) [pmol/µg].

|  |  |
| --- | --- |
| Kidney | Kidney co-incubated liver |
| control | **10 µM** | **control** | **10 µM** |
| 100,00 | 83,72 | 100,00 | 111,99 |
| 100,00 | 69,99 | 100,00 | 99,09 |
| 100,00 | 115,83 |  |  |
| 100,00 | 58,13 |  |  |
| 100,00 | 111,62 |  |  |

Table 18: Raw data amount of platinum in the kidney slices figure 28

|  |  |
| --- | --- |
| Kidney | Kidney co-incubated liver |
| control | **10 µM** | **control** | **10 µM** |
| 0,05 | 67,86 | 0,80 | 26,62 |
| 0,02 | 91,07 | 0,05 | 28,10 |
| 0,03 | 86,28 | 0,02 | 23,03 |

Table 19: Raw data % platinum in the kidney slices figure 29

|  |  |
| --- | --- |
| Kidney | Kidney co-incubated liver |
| control | **10 µM** | **control** | **10 µM** |
| -2,05 | 2,27 | -33,72 | 1,35 |
| -0,85 | 3,17 | -2,58 | 1,42 |
| -0,77 | 3,03 | -0,89 | 1,12 |

Table 20: Raw data relative viability figure 30. The average ATP of the control slice is set at 100% with a true average value of 11,49 [pmol/µg]

|  |
| --- |
| Liver |
| control | **5 µM** | **10 µM** | **25 µM** |
| 100 | 80,27 | 67,71 | 8,89 |

Table 21: Raw data amount of platinum in the liver slices figure 31

|  |
| --- |
| Liver |
| control | **5 µM** | **10 µM** | **25 µM** |
| 0,31 | 15,74 | 37,85 | 105,56 |
| 0,35 | 19,05 | 39,52 | 92,15 |
| 0,22 | 15,18 | 24,89 | 76,34 |

Table 22: Raw data % platinum in the liver slices figure 32

|  |
| --- |
| Liver |
| control | **5 µM** | **10 µM** | **25 µM** |
| 11,14 | 1,19 | 1,56 | 2,08 |
| -12,35 | 1,40 | 1,54 | 1,73 |
| -10,23 | 1,08 | 0,94 | 1,40 |

Table 23: Raw data relative viability figure 33/34. The average ATP of the control slices are set at 100% with a true average value of 9,34 and 15,73 [pmol/µg].

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| 24h | 10 µM BFB | 20 µM BFB | 10 µM BFB, 50 µM 1-ABT | 20 µM BFB, 50 µM 1-ABT | 50 µM 1-ABT |
| 100,00 | 95,17 | 107,79 | 116,94 | 108,10 | 94,82 |
| 100,00 | 7,12 | 2,04 | 12,78 | 1,04 | 75,98 |

# Appendix II

**Precision cut liver slices protocol**

**Preparation for the slice experiment (1h)**

1. Turn the heater on in the incubation cabinet and wait until 37 °C is reached.
2. Prepare KHB (Kreb’s) for slicing of liver tissue.
	1. 10 liters of a 10x concentrated KHB stock solution (10x KHB):
		1. Solution 1: Weigh 36.7g CaCl2\*2H2O. Dissolve in 5 L ultrapure water.
		2. Solution 2: Weigh 37.3g KCl, 690g NaCl, 27.1g MgSO4\*7H2O and 16.3g KH2PO4. Dissolve in ultrapure water to 5L.
		3. Mix solution 1 with solution 2 and filter through a 0.45 µm filter.
		4. Note date and name on the 10x KHB stock solution. Store at 4°C for max. 6 months.
	2. Prepare 5L KHB (for about 300 slices):
		1. Weigh 10.5g NaHCO3, 24.75g D-glucose and 11.9 HEPES. Dissolve in 2L ultrapure water at 4°C.
		2. Add 500ml 10x KHB at 4°C.
		3. Add ultrapure water at 4°C to yield 5L. Keep solution at 0-4°C on melting ice.
		4. Oxygenate with 95% O2/5% CO2 for 30 minutes on ice using a gas dispersion tube with a fritted disc.
		5. Set pH of the solution to **7.42** by slowly adding 5N NaOH solution
		6. Reoxygenate and set pH again before use.
3. Prepare WME plus supplements for slice incubation.
	1. WME slice incubation medium:
		1. Weigh 1.375g D-glucose monohydrate.
		2. Add the D-glucose monohydrate and 500 µl gentamicin (50mg/ml) to 500ml WME (containing L-glutamine).
		3. If necessary, store at 4°C for max. 24h.
4. Transfer the incubation medium into culture plates. **(**Twelve-well plates require 1.3 ml of culture medium per well to support 3–6 mg of tissue for 24 h). One slice is incubated per well.
5. Prewarm and oxygenate the plates by placing them in the plastic boxes of the incubation cabinet at 37 °C under 95% O2/5% CO2 for at least 30 min. (Place wet tissues in the boxes to maintain a humid atmosphere to prevent evaporation of the incubation medium).
6. Assemble the Krumdieck slicer and precool it by recirculating cooled water (4 °C) through the cooling block.

**Collection of tissue (5 min)**

Performed by authorized person.

**Preparation of tissue (15-30 min)**

1. Transfer the liver to a Petri dish that has a silicone insert. Keep the surface of the liver wet by pouring UW on it.
2. Turn on drill at speed setting 2. Secure tissue by holding it down loosely by hand. Prepare cores by rapidly pressing the hollow rotating tissue coring tool perpendicularly into the tissue until it touches the silicone patch at the bottom of the Petri dish. (Cores should be cylindrical, with similar diameters at the two ends.)
3. Transfer the cores to UW using a spatula directly after core preparation.

**Preparation of liver and intestinal slices (1h for 50-500 slices)**

1. Fill the slicer with ice-cold oxygenated KHB (Kreb’s) through the glass-trap assembly.
2. Transfer the liver core from the ice-cold UW used for storage into the cylindrical core holder of the slicer with a spatula. Most flat and round end points upward to the open end of the core holder. Move the plunge downward to insert the core in the holder.
3. Cut slices using the Krumdieck slicer at a speed setting of **30–40**. Decease the speed and/or change the knife when slices quality decreases. Check the quality of the new knife before slicing. Check the wet weight of the first few slices as an indication of slice thickness after carefully blotting. (As an indication, liver slices of 5-mm diameter and **5 mg** weight)
4. After slicing one core, remove the slices from the glass trap by opening the tap, collect them in a beaker and place them immediately on ice. Select the slices on the basis of appearance. (Good slices have an equal thickness, uniform color and smooth edges. Transfer selected slices into fresh ice-cold UW with spatula.)
5. Every 15–30 min, replace the KHB slicing buffer by fresh, ice-cold and oxygenated KHB (2.2). Replace the knife when slice quality (judged by eye) decreases.

**Incubation of liver and intestinal slices**

1. **Wash liver slices quickly by gently transferring them into a Petri dish containing ice-cold red-orange WME to remove the UW.**
2. Transfer the slices (one slice per well) to a prewarmed culture plate that is placed on a surgical mattress to maintain the medium temperature at 37 °C.
3. Incubate the slices for the desired time period in the plastic boxes that are placed on a reciprocal shaker (90 cycles per minute) in the incubation cabinet.
4. Monitor the pH of the medium by checking the color of the pH indicator in the WME. Yellow or purple means pH change.
5. Monitor temperature in the boxes.
6. Refresh medium every 24h.

**Determination of ATP in slices**

1. Add 1 ml sonification solution and Mini-beads in an Eppendorf cup (safe-lock micro test tube)
2. After an experiment, place one slice in this Eppendorf cup and freeze immediately in liquid nitrogen. Store at -80°C.
3. After thawing, homogenize the sample with a sonifier or Mini-Beadbeater. Place samples in melting ice with salt during homogenization. Centrifuge homogenate for 2 min at 13,000 r.p.m. in an Eppendorf centrifuge.
4. Perform ATP assay according to the manufacturer’s instruction.

# Appendix III

**ATP Assay**

**Important!: The ATP in the slices may be breaking down by the present enzymes, therefore store samples at -80°C and keep the samples on ice during determination.**

***Chemicals and instruments*:**

1. **SONOP** (Sonification Solution), Ethanol (70% v/v) containing 2 mM EDTA (M=372.24 g/mol) with pH=10.9. Stored in fridge at lab 3.33. First dissolve EDTA in MQ-water, adjust pH at 10.9 and finally fill up with Ethanol (100%).
For 1L : Dissolve 0.744 g EDTA in ± 200 ml of mQ-water, adjust pH with 5 M NaOH to pH=10.9, add 60 ml of MQ-water and finally add 740 ml Ethanol (96%).
2. **100 mM Tris-HCl** (ISIS 410), **2 mM EDTA buffer** (pH 7.6-8.0) Stored on the bench of lab 0349
For 1L ml: Dissolve 12.0 g Tris (*M*=121.14) (Tris(hydroxymethyl)amniophen; Merck) and 0.74 g EDTA (Triplex III; *M*=372.24) in ± 600 ml MQ-water, adjust pH with 6N HCl and fill up to 1 L total volume with MQ-water.
3. Safelock vials.
4. Minibeadbeater
5. Repetative pipet with 50 µl tip.
6. Black 96-wells plate,Costar 3915.
7. ATP Bioluminescence Assay Kit CLS II, Roche. (freezer lab 3.50). cat No 11 699 695 001.

Contents: **Luciferase** – **1 week at fridge /** Dissolved luciferase is stored at 4°C, in the door of fridge. This has to warm up at RT for 30 min on the bench, before you can add it to the 96 well plate

 **ATP-standard – 4 weeks at –20˚C**

1. Lumicount microplate luminometer from Packard.

***Calibration curve:***

Dissolve the ATP-standard from the kit to **exactly** 10 mg/ml (= 1.65 x 10-2 M) with ultra-pure water. The label contains the amount of ATP-standard present in that vial. Keep the calibration curve standards at RT.

*Dilution A is only to prepare the calibration curve, it is not going to be measured.*

Table 24: Calibration curve ATP

|  |  |  |  |
| --- | --- | --- | --- |
| Dilution | Amount (µl) | Tris/EDTABuffer (µl) | Conc. (M) |
| A | 10 µl ATP-standard | 990 | 1.65 x 10-4 |
| Cal 1 | 10 µl [A] | 990 | 1.65 x 10-6 |
| Cal 2 | 100 µl [1] | 400 | 3.30 x 10-7 |
| Cal 3 | 50 µl [1] | 450 | 1.65 x 10-7 |
| Cal 4 | 100 µl [3] | 400 | 3.30 x 10-8 |
| Cal 5 | 50 µl [3] | 450 | 1.65 x 10-8 |

***Procedure:***

1. Put 1 slice in 1 ml SONOP in a safe lock vial and freeze immediately in liquid nitrogen. Store in -80.
2. Cool centrifuge to 4˚C and thaw samples/standard /pos control on ice. Luciferase at room temp
3. Homogenize the sample with minibeadbeater 45s.
4. Centrifuge 5 minutes at 13,200 rpm in an eppendorf centrifuge at 4˚C
5. Add 5 µl supernatant (samples and positive control) & 45 µl Tris/EDTA buffer in a black 96-well plate (do in duplicate) RT
6. Turn on luminometer.
7. Make a calibration curve according to the table above and pipette 50 µl into each well
8. Dissolve luciferase in 10 ml MQ-water and incubate for 5 min without stirring or shaking
9. Mix by rotating the bottle / inverting. Attention! Do not vortex the luciferase!!!!
10. Add to every well 50 µl luciferase using repetitive pipette.
11. Measure plate after 0 and 5 minutes with the luminometer (mma.pf)

|  |
| --- |
| Gain Level: 1,0, Manual PMT(volts): 1100 Read Length(sec): 0,5 Max RLU Well: None |

* Tick read settings & export the corresponding plate numbers to excel

**ATP Positive control**

* Make 8 rat liver slices (about 5mg each slice).
* Pre incubate them for 1 hour with 1.3ml WimE medium in each well.
* Put each slice in 1ml sonop, freeze them in liquid nitrogen.
* Store in -80C freezer or continue with beadbeating.
* Centrifuge and put all supernatant in one 10ml tube as the stock.
* Measure ATP from this stock with dilution 1:1 and 1:2 using sonop.
* Determine which dilution gives the right ATP value for use of positive control (ATP value should be around 4 - 5 with the protein 500 µg/slice)
* Dilute the stock to get the right ATP value. Measure ATP again from the new solution.
* When the value is ok, aliquot the solution in aliquots of 25 µl, store them in -80C, B3 drawer.

# Appendix IV

**Protein Estimation by Lowry**

**[BIO-rad DC Protein Assay]**

**On pellet of SONOP homogenate for ATP assay.**

**Chemicals and instruments**

1. Lumicount microplate luminometer from Packard.
2. Reagent A (DC™ Protein Assay Reagent A, Biorad, cat# 500-0113)
3. Reagent B (DC™ Protein Assay Reagent B, Biorad, cat# 500-0114)
4. Waterbath.
5. BSA (Albumin, Bovine acid free, ICN Biomedicals Inc, cat# 105033).
6. Mini-Beadbeater-24 (MBB), Biospec Products.
7. Assay plate (96 well flat bottom, non-treated, Costar cat# 3370).
8. Lumicount microplate luminometer from Packard.

**Preparation**

* Turn on water bath.
* Thaw BSA stocks (stored in -20).
	+ Stock A BSA: 3.2 mg/ml mQ.
	+ Stock B BSA: 2.4 mg/ml mQ.
* Make sure your slices are dry (no SONOP left in the tubes) before continuing – overnight incubation at 37°C is necessary.

**Sample preparation**

* Add 200 µl 5M NaOH (20 g in 100ml mQ) to the pellet and beads.
* Incubate 30 min at 37°C (shaking, high speed) inside the water bath.
* Add 800 µl milliQ water (5x dilution)
* Homogenize again with MBB for 40 seconds.

**Protein determination**

* Make the following standard curve diluted in 1 M NaOH at concentrations:

0 - 0.2 - 0.4 - 0.6 - 0.8 - 1.2 - 1.4 - 1.6 mg/ml.

Table 25: Calibration curve A (3.2 mg/ml)

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
|  | μl | From | μl of 1M NaOH | Conc.(mg/ml) |
| A1 | 50 | Stock A | 50 | 1.6 |
| A2 | 50 | A1 | 50 | 0.8 |
| A3 | 50 | A2 | 50 | 0.4 |
| A4 | 50 | A3 | 50 | 0.2 |

Table 26: Calibration curve B (2.4 mg/ml)

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
|  | μl | From | μl of 1 M NaOH | Conc.(mg/ml) |
| B1 | 50 | Stock B | 50 | 1.2 |
| B2 | 50 | B1 | 50 | 0.6 |

* Pipette 5 μl of calibration standard (made from BSA) or sample in 96 well plate.
* Add 25 μl of reagent A into each well of standards and samples, **with a repetitive pipet.**
* Add 200 μl of reagent B into each well of standards and samples, **with a repetitive pipet.**

Note: The end concentration of NaOH is 0.01 M.

* Keep the plate for 15 minutes at room temperature and measure the absorbance at wavelengths 750 or 650 nm depending what filter you have (Stable for 1 hour).

# Appendix V

**De-hydration of tissue/slices.**

***Equipment:***

100 % EtOH absolute AnalaR normapur order# 20821.365 campusstore,

96 % EtOH AnalaR normapur order# 20823.362 campusstore,

Histosolve (=Xylene substitute) Thermo scientific 4 L order# 9990505,

eosine-Y solution\*, embedding cassettes with lid peach Simport order#493-7,

formalin (formaldehyde solution) 4% Klinipath Baker order# 4078-9005 (lab 349)

paraffin paraclean Klinipath order# 2079A,

sponges (foam biopsy pads) Surgipath 500pcs Leica Biosystems order#01020,

2 X 1,5L beaker glass, funnel, tissues.

\*Eosine solution**:** Dissolve 0.1 g eosine Y [kleurstoffen kast] CIBA order# 22013S in 10 ml EtOH 70% (or 80%), in yellow cabinet. Add 6 ml of this solution to 1L 100% EtOH. Mark as D6. Store the solution in the yellow cabinet in lab 333.

***De-hydration of slices/tissue:***

Scoop the slices immediately after incubation in 4% formalin (in wells of 24-wellsplate). Keep them in the fridge at 4°C, at least overnight. Then place the slices in 70% ethanol for at least one night.

Describe embedding cassettes with a pencil with a sharp end. Open the cassette and put a filter in it.

**D1**: Get the slices out the 24 well filled with 70% ethanol.

Place the slices flat and apart from each other on the sponge and cover them up with a halved sponge (in case of the cores do not use the sponges). Close the cassette and put them in an iron basket that is put in a beaker filled with 80% EtOH. Put the basket through a series of EtOH and Histosolve following this scheme, all on the shaker:

Table 27: De-hydration of slices/tissue

|  |  |  |
| --- | --- | --- |
| Step | Solution (yellow cabinet, lab 333) | Time (min) |
| D2 | 80% EtOH | 45 |
| D3 | 96% EtOH | 45 |
| D4 | 96% EtOH | 45 |
| D5 | 100% EtOH | 45 |
| D6 | 100% EtOH + eosine\* (gloves) | 45 |
| D7 | Histosolve\*\* | 20 |
| D8 | Histosolve\*\* | 20 |
| D9 | Histosolve\*\* | 20 |

*Use these solutions not more than 10 times. Discard in halogenpoor organic category III.*

*\*Eosin is added to make the tissue better visible once it is embedded in paraffin:*

*\*\* This has replaced the xylol that was used before.*

Let the excess Histosolve leak out very well.

Place cassettes in paraffin (60˚C, oven lab 3.58) in glass beaker for 45 minutes. This step is to wash out the Histosolve. *Use this paraffin only once*, d*iscard in black bin*.

Place cassettes in an iron basket in new paraffin (60˚C oven lab 3.58) overnight. *Use this paraffin not more than 5 times*.

# Appendix VI

**HE Staining paraffin sections**

**Principe:**

Formalin is a non-coagulating fixative that cross-links proteins. This makes the cytoplasm an unsolvable gel that well preserves the organelles.

Haematoxylin will color the chromatin of the nucleus blue-purple.

Eosin colors the cytoplasm pink. It binds kationic groups of protein molecules that are present in lysine en arginine. Almost all proteins contain these aminoacids, so little won’t be stained. The extracellular matrix around the collagen fibers of connective tissue is negatively charged and it won’t bind to this. This also applies to many glycoproteins like mucins.

**Materials:**

▪ Coverslips, 24x50 mm

▪ Staining trays

▪ Metal rack for slides

▪ Gloves

▪ Plastic pipette for depex

▪ Funnel

▪ Marker (to note the usage of the chemicals)

▪ Depex mounting medium (firesafe, toxic!!)

▪ 4x Ultraclear (firesafe lab 333)

▪ 4x ethanol 100% (firesafe lab 333)

▪ 50% ethanol (firesafe lab 333)

▪ Haematoxylin (firesafe lab 333)

▪ 50% **acid** ethanol (firesafe lab 333)

▪ 70% ethanol (firesafe lab 333)

▪ 80% **base** ethanol (firesafe lab 333)

▪ Eosin, ready to use (firesafe lab 333)

**Procedure:**

HE Staining series: orange code bottles in firesafe.

For all solutions: Refresh after 10 X usage. Discard category III firesafe 333.

Table 28: Staining steps tissue/slices

|  |  |  |
| --- | --- | --- |
| Step | Time(min.) | Procedure |
| 1 | 5 | Ultraclear (deparaffinize) |
| 2 | 5 | Ultraclear (deparaffinize) |
| 3 | 1 | 100% ethanol (deparaffinize) |
| 4 | 1 | 100% ethanol (deparaffinize) |
| 5 | 1 | 50% ethanol (to rehydrate) |
| 6 | 10 | Stain in haematoxylin |
|  | 1 | Rinse in tapwater (ions in tapwater are necessary for the staining) |
| 7 | 1 | 50% **acid** ethanol (500 mL 50% ethanol + 1,25 ml HCL) |
| 8 | 1 | 70% ethanol |
| 9 | 1 | 80% base ethanol (500 mL 80% ethanol + 7,50 ml Ammonia (25%) ) |
| 10 | 2 | Stain in eosine solution |
| 11 | 1 | 100% ethanol **USE 5X** |
| 12 | 1 | 100% ethanol |
| 13 | 1 | Ultraclear |
| 14 | 1 | Ultraclear |

* Let the slides dry to the air, but not too long! Just until any drops are gone.
* Apply a little depex mounting medium on the slide and put a coverslip on it. Let it dry a little before

# Appendix VII

**RNA-isolation** (Maxwell®16simplyRNA Tissue Kit)

Solution preparation, cartridge preparation and instrument setup

* **Homogenization solution**: 220 μL per sample, add 20μL cold 1-Thioglycerol per milliliter of homogenization solution. Keep on normal ice. (make for n+1)
* **DNase** I: add 275 μL of Nuclease-Free Water to the vial of lyophilized DNase I. Swirl gently, no vortexing. Add 5 μL of blue dye to the mixture. Keep on normal ice.
* **Cartridges**: place in LEV rack with the label facing away from the elution tubes. Press down firmly until you feel it click. Remove the seal from top to bottom, make sure it is removed completely (including adhesive). Place LEV plungers in the wells (#8) closest to the elution tubes.
* **Elution tubes**: place the provided elution tubes in the first holes of the LEV rack. Add 35 μL of Nuclease-Free Water to the tubes, make sure this is on the bottom and not on the sides. To dilute & increase total RNA recovery increase water added (up to 50 μL).
1. Slices should be snap-frozen in RNase free Eppendorf’s, 3 slices per Eppendorf. Homogenize the tissue with 220 µL homogenization solution and add minibeads. Homogenize with Minibeadbeater for 1 minute. Let foam settle on normal ice.
2. Add 10 µL DNase to well number four of the cartridges. The well will turn green.
3. Add 220 µL Lysis buffer to the samples and vortex for 15 seconds to mix. Transfer 400 µL to well 1 of the cartridge. Avoid getting foam and minibeads in the tip of the pipet.
4. Place LEV rack in Maxwell and run the program RNA, simply RNA.

# Appendix VIII

**qPCR**

1. Reserve rtPCR machine: [www.internetagenda.nl](http://www.internetagenda.nl) username: ABI7900HT password: taqman.

2. Take primers out of the freezer. Create a Primermix for each primer for N+1 (nr of samples x triplicate + some extra):

Table 29: Primermix composition

|  |  |
| --- | --- |
|  | (μL) |
| Primer fw (50 uM) | 0,2 |
| Primer rev (50 uM) | 0,2 |
| Sybr green mix | 5 |
| Total per well | 5,4 |

3. Calculate how much cDNA mix you need ((nr of primers x triplicate +1) x 5 μL per well). Dilute cDNA samples to 10 ng/μL Nuclease-free water. If cDNA conversion went correctly, concentration should be 86,5 ng/μL. If RNA concentration was too low, adjust for this.

4. Using a stepper pipette, add 5,4 μL of the primermix to each well (triplicate).

5. Add 4,6 μL of diluted cDNA to the wells (triplicate). Total volume will be 10 μL.

6. Seal the plate with a cheap plastic cover. Centrifuge for 5 minutes at 2000 rpm. There should be no air bubbles at the bottom.

7. Replace the cover with the ABI PRISM Optical Adhesive Cover and seal tight. Make sure there is no plastic or adhesive on the edges, fit the seal nicely over the top. Do not touch the cover with your hands.

8. Take plate to the ERIBA building (you will need a pass) or store in the dark at 4°C until use.

|  |
| --- |
| **ABI PRISM® 7900HT Sequence Detection System Settings** |
| * Open SDS 2.4, open a new document
* Check: Standard curve, 384 wells clear plate, blank template, barcode 🡪 OK
* Tools – Detector Manager
* Select Jai\_sybr, copy to plate document 🡪 Done
* Select all wells
* Activate the detector
* Instrument
* Thermal Profile
* 1. 95°C for 10 mins
* 2. 95°C for 15 secs, 60°C for 25 secs (Cycle, repeat 40 times)
* 3. 95°C for 15 secs, 60°C for 15 secs, 95°C for 15 secs (dissociation stage)
* Change sample volume to 10 μL
* 9600 Emulation on
 |
| **Run immediately** | **Run overnight** |
| * Open machine, insert plate (A1 top left)
* Press start
* Name file and save
 | * Queue 🡪 Send to queue
* Save file, yes to read-only
* Send file to queue
* Close program
* Put plate in stack 1 (A1 in lower left corner)
* Start Robot (start queue)
 |