Advanced renal cell carcinoma - The role of orellanine and associated therapeutic challenges

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ABSTRACT

Orellanine is a fungal nephrotoxin selectively toxic to the human tubular epithelial cells (HTEC) of the kidney nephrons leading to kidney failure. Patients treated with renal replacement therapy after orellanine poisoning show no signs of damage to other organs in the body.

Aims: Our main aim in this thesis is to develop chronic peritoneal dialysis (PD) in anuric rodents, to better understand the pharmacokinetic properties of orellanine and to evaluate orellanine as an experimental treatment against metastasized clear cell renal cell carcinoma (ccRCC).

Methods: The first paper is an *in vivo* study of chronic automated PD in anuric rats. Orellanine was used to induce uremia. Blood, dialysis fluid, and tissue samples were examined for electrolyte profiles, inflammatory status, and morphology. The second paper is an *in vivo* study in which rats were given intravenous injections of labeled/unlabeled orellanine. The distribution of orellanine was imaged, and orellanine plasma concentrations were measured over different time points. The third paper had two parts: an *in vitro* part examining the effect of orellanine on HTEC, epithelial cells, ccRCC cells, and other cancer cell lines, and an *in vivo* part with a xenograft rat model testing the effect of orellanine on metastasized ccRCC tumors.

Results: The levels of urea and creatinine in orellanine-treated rats indicated severe uremia. The automated PD system developed in our lab provided adequate dialysis. The rats gained weight and had normal homeostasis. Orellanine was cleared renally and was mainly distributed to the renal cortex and the urinary bladder. Orellanine induced necrosis, apoptosis, and disruption of cellular functions and growth on HTEC and ccRCC cells while having no significant effect on other tested cell lines at the same doses. Finally, orellanine induced significant apoptosis and necrosis in the xenografted tumors *in vivo*.

Conclusions: Orellanine selectively causes renal failure, which is irreversible at high doses. We describe the first successful treatment of rats with severe uremia that, despite anuria, were kept healthy over a period of at least 21 days. The system can be used to improve PD and to study various aspects of uremia. The pharmacokinetic properties of orellanine were investigated and it was shown that orellanine is distributed mainly to the urinary system. Orellanine induced significant apoptosis and necrosis in metastasized xenografted tumors *in vivo* and showed no signs of affecting other organs. Therefore, we suggest that its therapeutic effects should be further examined as a treatment option for late stage ccRCC patients.

POPULÄRVETENSKAPLIG SAMMANFATTNING

Orellanin är ett svampgift som återfinns i vissa arter av släktet Cortinarius som exempelvis toppig giftspindling. Patienter som uppsöker sjukhus på grund av att oavsiktligt ha misstagit svamparna för ätliga uppvisar dosberoende skador på njurarna och kan komma att behöva dialys eller njurtransplantation för att överleva. De patienter som drabbats av orellaninförgiftning uppvisar inga skador på andra organ i kroppen vilket visar att giftet är njurspecifikt. De celler i njuren som orellanin dödar är också de celler som ger upphov till klarcellig njurcancer, de proximala tubulicellerna.

Ca 600 personer om året drabbas av njurcancer i Sverige och hälften av dem dör av sin cancer. Den vanligaste och dödligaste typen av njurcancer heter klarcellig njurcancer vilken oftast drabbar den ena njuren och om den inte har spridit sig kan tumören avlägsnas kirurgiskt. Tyvärr upptäcker man oftast denna typ av cancer när tumören redan har bildat dottertumörer i kroppens andra organ som hjärnan, benmärgen eller lungorna. Femårsöverlevnaden för spridd klarcellig cancer uppgår till ca 10%. I dagsläget finns det ingen botande behandling att tillgå.

Då orellanin dödar de celler som cancern utvecklas från är vår hypotes att orellanin skulle kunna utgöra behandling för spridd klarcellig njurcancer. För att kunna testa vår hypotes behövde vi först utveckla en bra metod för dialys på djur, då orellaninbehandling kommer skada njurarna. Dessutom ville vi studera om orellanin kan användas i en sådan dialysmodell för att introducera njurskada utan att behöva använda kirurgiska metoder. Vidare behövde vi bättre förstå orellaninets egenskaper samt distribution i kroppen innan slutligen orellanins effekt på klarcellig njurcancer kunde studeras.

Idag finns det cirka 9000 patienter Sverige som genomgår dialys eller har fått en ny njure på grund av nedsatt njurfunktion. Det finns två typer av dialys; bloddialys (HD) och peritonealdialys (PD). Vid bloddialys renas blodet utanför kroppen i en dialysmaskin och kräver att patienten är uppkopplad till denna maskin flera timmar i veckan. PD fungerar genom att man injicerar dialysvätska in i buken på patienten och denna vätska som via bukhinnan tar upp slaggprodukter och vatten till dialysvätskan. Under tiden som dialysvätskan är i buken är patienten inte bunden till några maskiner och får bättre livskvalité. Tyvärr kan bara ungefär 10 % av patienterna fortsätta med PD efter en sjuårsperiod då bukhinnan förtjockas, kan drabbas av upprepad inflammation och därför få en sämre funktion med tiden. Det finns därför ett

stort behov av utveckla metoder och dialysvätskor för peritoneladialys ytterligare. För att efterlikna PD hos människa utvecklade vi ett system där råttor utan njurfunktion dialyserades i 21 dagar. Effektiviteten och graden av inflammation undersöktes och vi fann att vår modell var mycket effektiv och kan användas för framtida studier kring PDs effekter på bukhinnan för att på så sätt förbättra situationen för patienter som erhåller peritonealdialys.

För att få veta hur länge orellanin stannar kvar i kroppen undersökte vi orellanins halveringstid i cirkulationen samt om dialys kan rena bort orellanin. Vi fann att orellanin har en halveringstid på strax under två timmar och att dialys kan rena bort orellanin från kroppen men att det tar längre tid än njurarnas eliminering av orellanin. Studier av orellanins distribution i kroppen hos råttor visade att de organ där orellanin mest ansamlades som väntat var njuren och urinblåsan.

I det tredje arbetet i avhandlingen undersökte vi orellanins effekt på tubulära njurceller samt på njurcancerceller. Vi jämförde effekten på njurcancercellerna och njurcellerna med celltyper från andra delar av kroppen. Våra resultat visade att orellanin hade en selektiv effekt på njurceller och njurcancerceller men inte på andra celltyper.

Nästa steg i att testa orellanins effekt på spridd klarcellig njurcancer var att testa behandlingen på djur. Vi inplanterade humana celler från en klarcellig cancermetastas i råttor och efter att tumören växt till startades orellaninbehandling. Dialysuppställningen från delarbete 1 användes för att ersätta njurfunktionen under behandlingen.

Resultatet var att svampgiftet minskade de inplanterade tumörmassorna signifikant och effekten på andra organ var minimal. Celldöden i de behandlade tumörerna var utbredd. Tumörmassan blev drastiskt och signifikant mindre i omkrets, vikt och antalet levande celler var kraftigt reducerat efter behandling.

Sammantaget har orellanin visat potential för att kunna vara ett framtida behandlingsalternativ för klarcellig njurcancer och vi har fått fler, nya sätt att studera hur dialys påverkar kroppen och hur vi kan förbättra nuvarande dialysprotokoll.

LIST OF PAPERS

This thesis is based on the following studies:

I. Deman Najar, Börje Haraldsson, Magnus Braide, Kerstin Ebefors, and Jenny Nyström. **Chronic peritoneal dialysis in uremic, anuric rats**.
Manuscript

II. Deman Najar, Börje Haraldsson, Annika Thorsell, Carina Sihlbom, Jenny Nyström, and Kerstin Ebefors. **Pharmacokinetic properties of the nephrotoxin orellanine in rats.** *Toxins.* 2018 Aug 17;10(8). doi: 10.3390/toxins10080333

III. Lisa Buvall, Heidi Hedman, Alina Khramova, Deman Najar, Lovisa Bergwall, Kerstin Ebefors, Carina Sihlbom, Sven Lundstam, Anders Herrmann, Hanna Wallentin, Emelie Roos, Ulf A. Nilsson, Martin Johansson, Jan Törnell, Börje Haraldsson, and Jenny Nyström. **Orellanine specifically targets renal clear cell carcinoma**.
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ABBREVIATIONS

^{3}H	Tritium
ABC	ATP binding cassette transporter
AKT	Protein kinase b
APD	Automated peritoneal dialysis
ATP	Adenosine triphosphate
BAP1	Histone deubiquitinase
CAPD	Continuous ambulatory peritoneal dialysis
Cas9	CRISPR-associated 9
ccRCC	Clear cell renal cell carcinoma
CKD	Chronic kidney disease
Cl^{-}	Chloride
CRISPR	Clustered regularly interspaced short palindromic repeats
EBM	Experimental biomedicine
ECF	Extracellular fluid
EIMS	Electron impact mass spectrometry
EMT	Epithelial to mesenchymal transition
ESDR	End-stage renal disease
ESIMS	Electron spray ionization mass spectrometry
Erk1-2	Extracellular signal-regulated kinase
ESR	Electron spin resonance

GFR	Glomerular filtration rate
GLUT1	Glucose transporter 1
gRNA	Guided RNA
HCO^{3-}	Bicarbonate
HD	Hemodialysis
HIF	Hypoxia-inducible factor
hMRP	Human multidrug resistant proteins
HPEG2	Hepatocellular cancer cell lines
HPLC	High-performance liquid chromatography
HTEC	Human tubular epithelial cells
HUVEC	Human umbilical vein endothelial cells
ICF	Intracellular fluid
IL-1β	Interleukin-1β
IL-6	Interleukin-6
ip	Intraperitoneal
iv	Intravenous
K^+	Potassium
KAT	Kidney auto-transplantation
Kt/V	Urea clearance
LD_{50}	Lethal dose 50% (median lethal dose)
MAP	Mean arterial pressure

MATE	Multidrug and toxin extrusion
MDA	Breast cancer cells of M.D. Anderson
Mg^{2+}	Magnesium
MS	Mass spectrometry
mTOR	Mammalian target of rapamycin
Na^{+}	Sodium
OAT	Organic anion transporter
OCR	Oxygen consumption rate
OCT	Organic cation transporter
PD	Peritoneal dialysis
PDF	Peritoneal dialysis fluid
P-gp	P-glycoprotein
PI	Propidium iodide
PM	Peritoneal membrane
PRM	Parallel reaction monitoring
PS	Phosphatidylserine
RCC	Renal cell carcinoma
ROS	Reactive oxygen species
RRT	Renal replacement therapy
RT	Retention time
SKRC	Sloan-Kettering-renal carcinoma

SLC	Solute carrier transporters gene superfamily
TGF-β	Transforming growth factor β
TLC	Thin layer chromatography
TPM	Three-pore-model
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labeling
UF	Ultrafiltration
UFF	Ultrafiltration failure
UPLC	Ultra performance liquid chromatography
MS/MS	tandem mass spectrometry
VEGF	Vascular endothelial growth factor
VHL	Von Hippel-Lindau
WHO	World Health Organization

1 INTRODUCTION

In 1952 in Poland, 102 patients became ill after ingesting wild mushrooms and developed kidney failure. This incident led to the discovery of the mushroom toxin orellanine, found in species of the *Cortinarius* family (1). Orellanine was found to primarily target the proximal convoluted tubular cells in the kidney (2-4). Patients that develop kidney failure due to orellanine poisoning and then receive renal replacement therapy (RRT) have shown no damage to any organs other than the kidneys (5).

In this thesis, we investigated the use of orellanine in peritoneal dialysis (PD) experimental research. Orellanine was utilized as a chemical nephrectomy tool in inducing renal failure in an *in vivo* chronic PD model developed in our lab.

Since clear cell renal cell carcinoma (ccRCC) develops from the same cells that are the target for orellanine (6), the therapeutic potential of this nephrotoxin against ccRCC was tested (7). Before using orellanine as a drug in research or therapeutically, the pharmacokinetics and distribution of orellanine were examined (8).

1.1 THE KIDNEY

The kidneys are retroperitoneally located organs in the abdomen, anatomically divided into a thin capsule, the cortex, and the medulla. The medulla tapers off into papillae that point toward the calyces that pour urine into the renal pelvis. The ureter transports urine from the renal pelvis down to the urinary bladder. Renal blood supply is maintained through the renal artery, which branches from the abdominal aorta. The renal venous return is through the renal vein, carrying blood back to the inferior vena cava. The kidneys have sympathetic and parasympathetic innervation via the renal plexus that runs alongside the renal artery (9, 10).

The function of the kidneys is to maintain the water balance in the body, keeping the electrolyte balance and the excretion of toxic waste products from the body in check. The kidneys also function as an endocrine organ, secreting active vitamin D, renin, and erythropoietin (11).

1.1.1 The nephron

The nephron is the functional unit of the kidney (see Figure 1). There are approximately one million nephrons per kidney. The nephron is divided into different segments: the glomerulus, the proximal convoluted tubule (PCT) segment, the loop of Henle, the distal convoluted tubular segment (DCT), and the collecting duct. The glomerulus has a capsule (Bowman's capsule) that is connected to the PCT and surrounds the glomerular capillary network. This network starts with the afferent arteriole and ends with the efferent arteriole that exits the capsule. The latter continues as the peritubular capillary network that surrounds the different segments of the nephrons, returning reabsorbed solutes and water to the circulation. The glomerulus is the main connection between the nephron and the circulatory system. Solute and water are filtered over the capillary wall of the glomerulus and collected in Bowman's capsule, forming the primary urine that continues through the PCT, loop of Henle, DCT, and then the collecting duct. Several collecting ducts drain urine into the calyces and then to the ureter and urinary bladder. Specialized epithelial cells line the tubular sections and different segments have different functions in terms of secretion and reabsorption of solutes and water. Each segment of a nephron is anatomically divided into a luminal brush border, a cellular part, and a basolateral border. The luminal side faces the tubular lumen of the nephron tubules. The cellular part refers to the cells comprising the nephron tubule for each segment. The basolateral side marks the contact of the tubular cells with the blood in the peritubular capillary network. Two types of nephrons are found in the kidneys: the cortical nephrons, which are characterized by short tubular segments, and the juxtaglomerular nephrons. The juxtaglomerular nephrons have their Bowman's capsules in the inner cortex, with tubular loops that are long enough to reach the papillary area. Juxtaglomerular nephrons are important for concentrating the urine (11, 12).

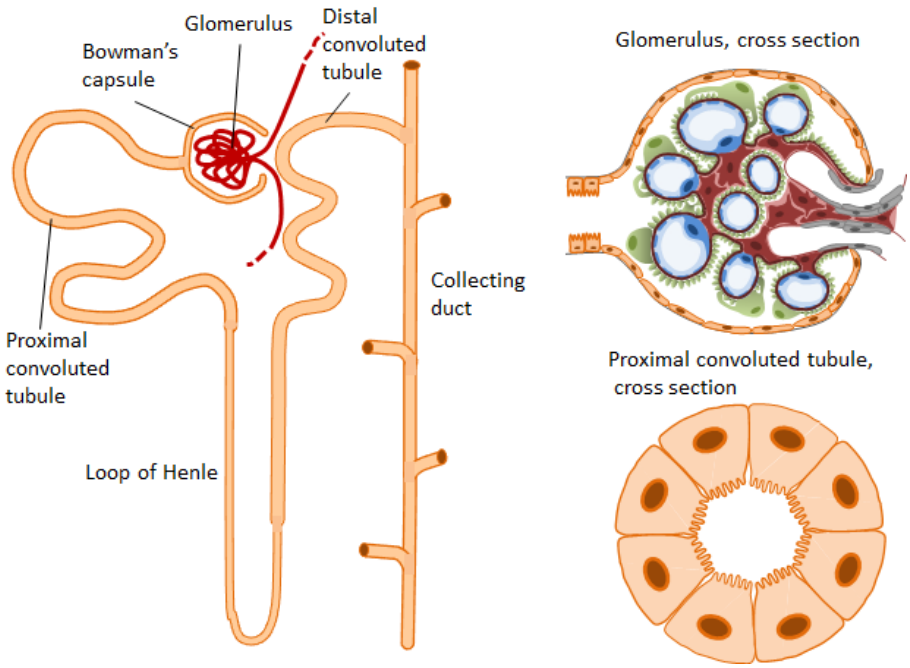

Figure 1. The nephron. Modified from Ebefors and Nyström, New insights into cross-talk in the kidney, Current Opinion in Nephrology and Hypertension (13)

1.1.1.1 The glomerulus

The first part of the nephron is the glomerulus, which is a capillary tuft forming the filtration barrier of the kidney. The hydrostatic pressure in the glomerulus is approximately 55 mmHg, compared to 18 mmHg in other capillary beds, enabling filtration over the capillary wall (glomerular filtration membrane). Small molecules like water and glucose pass freely through the membrane, whereas larger molecules pass with greater difficulty. This mechanism keeps the plasma proteins on the blood side and reduces water loss due to higher oncotic pressure in the blood (14). The inner layer of the filtration barrier, facing the blood compartment, is the endothelial cell surface layer covering the endothelium. The endothelial cell surface layer

consists of secreted and anchored proteins forming a gel-like structure covering the cell surface. The endothelial surface layer has long been debated mainly due to its complex structure and difficulty to visualize. However, there is a growing interest in research toward understanding the role of this layer and its contribution to the permselectivity of the glomerular filtration barrier (15, 16). The endothelial cells are heavily fenestrated with fenestrae of approximately 70–100 nm in diameter, covered by the cell coat (17). The second layer is the basement membrane. This layer is further divided into lamina rara externa, densa, and interna. The basement membrane is an acellular, extracellular matrix layer and accounts for approximately 50% of the hydraulic resistance (fluid restriction) of the glomerular barrier. It is composed of type IV collagen, laminins, nidogens, and proteoglycans (18). The third layer of the glomerular filtration barrier is composed of highly specialized epithelial cells called podocytes. These cells have a specialized morphology with foot processes, connecting neighboring cells with junctions that form filtration slit pores that are 25–55 nm in diameter. The slit pores are also termed slit diaphragms. They are highly important for the pemselectivity of the glomerular barrier (19, 20). All layers of the glomerular barrier are negatively charged. The negative charges are usually made up by sulfated side chains of structural proteins such as proteoglycans and collagens. These negative charges are known to repel the negatively charged plasma proteins in the blood and this is thought to make up a significant part of the permselective properties of the barrier. The barrier is generally considered to be size-, charge-, and to a certain extent, shape-restricting. This means that a small, neutral, and elongated molecule more easily passes the membrane compared to a large, negatively charged, and bulky molecule (21). Once the fluid with smaller solutes is filtered over the filtration barrier into Bowman's capsule, it is called ultrafiltrate, containing all the solutes dissolved into the blood except for blood cells, proteins, and solutes bound to plasma proteins. Therefore, the ultrafiltrate is very similar in composition to the interstitial fluid.

1.1.1.2 The tubular system

The first part of the tubules to receive the ultrafiltrate from the Bowman's capsule is the PCT. This section is approximately 15 mm long. The next section is the loop of Henle, which is similar in length. The DCT is shorter than the PCT. Four processes govern the flow of solutes and water and lead to the formation of the final urine. Filtration is the flow of fluid from the glomerular capillary network into the Bowman's capsule, thus producing the glomerular ultrafiltrate. Reabsorption is the flow of fluid back from the tubular lumen to the peritubular capillary network. Secretion is the flow of fluid and solutes out from the peritubular capillary network into the lumen of the tubuli. Excretion is the flow of urine out of the collecting duct and into the renal pelvis. Except for the descending thick limb of the loop of Henle, all the other nephron parts have a Na^+-K^+ ATPase pump on the basolateral side. This pump generates a sodium concentration gradient leading to reabsorption of solutes from various channels, co-transporters, and anti-transporters on the luminal side (11).

The PCT segment reabsorbs two-thirds of the total water and solute in the ultrafiltrate from the Bowman's capsule. This segment plays a prominent role in the reabsorption of the filtered solutes such as electrolytes, amino acids, and glucose. PCT cells are equipped with large numbers of mitochondria, making up approximately 40% of the volume of these cells (22), which is due to the high energy requirement of these cells and their abundant basolateral Na^+-K^+ ATPase pumps. The PCT segment is histologically subdivided into early PCT and late PCT sections. The early PCT section has a large number of co-transporters: Na^+-glucose, Na^+-amino acid, Na^+-phosphate, lactate or citrate co-transport, as well as the Na^+- H^+ exchanger on the luminal border. Except for the Na^+-K^+ ATPase that forces sodium into the blood against its electrochemical gradient, all other solutes exit the early PCT into the blood by facilitated diffusion. Na^+-K^+ ATPase is vital in ensuring the concentration gradient for maintaining the reabsorption of all the co-transported molecules across the luminal side of the PCT cells (23). The late PCT section has Na^+-H^+ exchangers and Cl^--formate anion exchangers, leading to the reabsorption of NaCl from the lumen into the cells. Then, Na^+-K^+ ATPase drives sodium back to the blood while chloride is reabsorbed by simple diffusion. Several transporters for drugs and xenobiotics are found in the PCT cells. The majority either belong to the ATP-binding cassette transporters (ABC) superfamily or the solute carrier transporters gene superfamily (SLC) (24, 25). The organic anion transporter (OAT) family belongs to the SLC superfamily and is responsible for handling organic anionic substances like drugs and metabolites. The main OATs in the kidneys are OAT1, 2, 3, 4, 5, 8,

and 10. Both the luminal and the basolateral sides of the epithelial cells of the PCT have these transporters. The OAT substrates are a variety of xenobiotics, drugs, and metabolites. Some of these substrates are antibiotics, antivirals, antihypertensive drugs, diuretics, H_2-antagonists, non-steroidal anti-inflammatory drugs, statins, and uricosurics (26, 27).

Human multidrug resistant proteins (hMRPs) are ATP-dependent efflux transporters that belong to the ABC superfamily and are located on the brush border side (MRP2 and MRP4) and the basolateral side (MRP6) of the PCT cells. These OATs handle the excretion of anti-cancer drugs, cAMP, cGMP, and urate into the urine (28). Organic cation transporters (OCTs) are also members of the SLC family. OCT1 is present on the brush border side and OCT2 on the basolateral side of the PCT cells (29). The substrates for these transporters are tetraethylammonium, 1-methyl-4-phenylpyridimium, endogenous monoamines, the antidiabetic drug metformin, the antihypertensive drug atenolol, the antiviral drug lamivudine, and the cytostatic drug oxaliplatin (30).

Multidrug and toxin extrusion (MATE) transporters are H^+/organic cation antiports (31). MATE1 and MATE2-k are located on the luminal brush border of the PTC cells (32). They extrude organic cations taken up by the OCTs. Other transporters of the PCT cells are the P-glycoprotein (P-gp) transporters. P-gp transporters are OCTs of the ABC superfamily and are found on the luminal border of the PCT. They are involved in the excretion of steroids and drugs into the urine (33, 34). Some of these transporters are more likely than others to be responsible for the uptake of orellanine (the nephrotoxin studied in this thesis) into the PCTs, but it is yet not known what causes a selective uptake of orellanine into the PCTs.

Collectively, the loop of Henle, the DCT, and the collecting duct reabsorb approximately 20–30% of the total solute and 15–20% of water. The loop of Henle is divided into the descending limb and the ascending limb. The descending limb does not allow reabsorption or secretion of solutes but is permeable to water. The opposite is true for the ascending limb. By the time the ultrafiltrate reaches the DCT, it contains approximately 10% of the originally filtered NaCl and 25% of the water. The DCT and the collecting duct reabsorption process are mainly regulated by hormones in response to the body's need for water, sodium, and calcium. Aldosterone regulates Na^+ reabsorption, anti-diuretic hormone regulates water reabsorption, and parathyroid hormone regulates Ca^{2+} reabsorption (11).

1.1.2 Water balance

Approximately 60% of human body weight is water, dividable in two major compartments: the intracellular fluid (ICF) compartment, having two-thirds of the body's water volume, and the extracellular fluid (ECF) compartment, containing the remaining third. The extracellular compartments are further subdivided into the intravascular compartment, comprising one fourth of the total extracellular water in the plasma, whereas the remaining three-fourths are in the interstitial extracellular compartment. Once the physiological electrolyte balance is disturbed, or when a certain compartment experiences a gain or loss of fluid, water shifts between the different compartments (35). The ICF compartment is the water inside the cells and its main solutes are the cations potassium (K^+) and magnesium (Mg^{2+}). The major anions in ICF are proteins and organic phosphates. The main cation in the ECF compartment is sodium (Na^+) and the major anions are chloride (Cl^-) and bicarbonate (HCO_3^-) (36). Alongside the proteins, the concentrations of these electrolytes dictate the tonicity of the different water compartments and thereby water volume ratios (37). The kidneys play a crucial role in keeping the homeostasis and the ratios in the fluid compartments of the body under control (11). Any disturbances in the balance of fluid distribution can lead to dramatic consequences on blood pressure, cardiac output, overall PH, and electrolyte profiles in and out of the cells of the body (38).

1.1.3 Renal clearance

The term renal clearance describes the rate at which a substance is removed from the body by renal excretion. This is stated as volume of plasma cleared of a substance per unit of time. The equation for clearance is the concentration of a substance in urine multiplied by the urine flow divided by the concentration of the substance in the plasma. The higher the clearance of a substance, the more plasma volume is cleared of that substance per unit time (39).

Inulin and organic acids are freely filtered through the glomerular membrane, while albumin is too large to pass the glomerular filtration barrier. Inulin is a small inert molecule that is filtered at the same rate as small solutes such as glucose and ions. However, glucose, sodium, urea, and other molecules cannot be used to measure clearance, as they are reabsorbed. One way to measure kidney function is by measuring the glomerular filtration rate (GFR), which is the flow of plasma from the glomerulus into Bowman's capsule over a defined period of time (40). Inulin is neither reabsorbed nor

secreted and therefore is a good molecule to use for measuring the GFR (41). A similar substance to inulin is endogenous creatinine. In clinical practice, creatinine is more commonly used to estimate the GFR than inulin. Creatinine is not as accurate as inulin, as a slight amount is excreted, which can lead to overestimation of GFR. Nevertheless, its endogenous properties, as opposed to the exogenous inulin, make creatinine an easier, cheaper, and faster way to measure GFR in the clinic (42).

1.2 THE PERITONEUM

The peritoneal membrane (PM) is a smooth thin serous membrane surrounding the abdominal cavity and organs. The PM is anatomically divided into visceral and parietal peritoneum. The parietal peritoneum lines the abdominal cavity, whereas the visceral peritoneum surrounds the viscera. The PM can be compared to a plastic bag that is vacuum-sucked to the walls and organs around it in the abdominal cavity. A fluid film separates these two layers, serving as a lubricant (43). In an adult, the PM has a surface area of 1–2 m^2. The PM also serves as a mechanical anchor via the greater and lesser omentums that hold the stomach in place and mesenteries that anchor the intestines to the posterior abdominal wall. The mesenteries are double folds of the PM. The cavity surrounded by the PM is called the peritoneal cavity. This cavity consists of the lesser sack behind the stomach and the greater sack that extends from the diaphragm down to the pelvis (44). The blood supply of the visceral peritoneum comes from the mesenteric and the coeliac arteries, with the venous return going to the portal vein and then to the inferior vena cava. Different arteries supply the parietal peritoneum: the iliac, lumbar, intercostal, epigastric, and the circumflex arteries. Lymph drained from the PM is taken to the omental and mesenteric lymph nodes. The lymph is then taken to the thoracic duct, where it joins the venous circulation (14).

Histologically, the PM consists of six layers: the capillary fluid film, the capillary endothelium, the endothelial basement membrane, the interstitium, the mesothelium, and the fluid film (45). The mesothelium consists of a continuous layer of single mesothelial cells connected by intracellular junctions (46). The cells are covered with microvilli, allowing for an increased surface area. These cells have lamellar bodies, endoplasmic reticuli, and cellular vesicles. The mesothelial cells participate in the PM barrier function. They can release vascular adhesion molecules and intracellular adhesion molecules in response to cytokines released by inflammatory cells (47). Underneath the mesothelium is a layer of collagen

and glycoproteins forming the basal lamina. The basal lamina offers mechanical support and allows for passage of inflammatory cells while preventing fibroblasts from penetrating to the mesothelial layer (48, 49). The interstitial layer of the PM is composed of collagen, elastin, proteoglycans, and a salty liquid. The components of this layer comprise the extracellular matrix of the PM. The interstitial layer, alongside the basal lamina, offers mechanical support to the mesothelium. The fibroblasts that are contained in this layer produce building components and are inflammatory active cells. This layer has macrophages and mast cells that, alongside fibroblasts, recruit leukocytes by the generation of chemokines and cytokines in case of inflammation. The PM plays an important role in keeping the peritoneal cavity aseptic. Other inflammatory active cells are the mast cells and the lymphocytes. An invasion of the integrity of the PM will lead to the activation of the macrophages, lymphocytes, mast cells, and fibroblasts. This activation then leads to the release of chemoattractants and the consequent recruitment of leukocytes to the inflammation site (50, 51).

1.3 END-STAGE RENAL DISEASE

End-stage renal disease (ESRD) is reached when the kidney function has decreased to a level where RRT is needed for survival. RRT may be either dialysis or renal transplantation. ESRD is a leading cause of morbidity and mortality worldwide, and, as of 2010, number 18 on the list of global death rates (52). An estimated 2.3–7.1 million people with end-stage kidney disease died in 2010 without access to RRT (53). Furthermore, the incidence of ESRD has increased by 8% annually, due to an aging population as well as to the increased numbers of patients with type 2 diabetes and/or cardiovascular disease leading to renal impairment (54, 55).

Renal transplantation is the first-hand choice of RRT in Sweden. Out of the 9,693 patients in Sweden who are in uremic care, 5,641 have received a renal transplant (56). However, due to the lack of donors, it is not possible for all ESRD patients to undergo transplantation, leaving dialysis as the only RRT option. Dialysis is divided into hemodialysis (HD) and PD. In 2016, there were 3,039 Swedish patients on HD and 877 on PD (56). Recently, PD has increased in popularity over HD. One of the reasons for favoring PD is its flexibility of use at home or outside, offering a better quality of life (57). Due to its ease of use compared to HD, PD is also recommended in pediatrics (58). Furthermore, diabetes patients and patients with cardiovascular problems are generally advised to undergo PD as it offers better blood sugar

control and more controlled fluid and blood pressure changes than in HD (59-62). Finally, PD is less expensive, making it advantageous for individual and population economic burdens (63, 64). Despite studies showing similar therapeutic outcomes between HD and PD, HD is still more commonly used for treating patients with ESRD (65). Taken together, this suggests that the choice of dialysis therapy is based on other facts than medical evidence.

1.4 PERITONEAL DIALYSIS

In 1896, peritoneal capillaries were used by Starling to define the fundamental principles governing fluid balance in all vascular beds (66). In 1923, the peritoneum was discovered as a filtration barrier through the work of Ganter, who was the first to use dialysis in a uremic patient (67). In the same year, Putman described the PM as a dialyzing unit (68). However, it was not until the early '60s, that PD started being used worldwide as a treatment option for uremic patients (69). Continuous ambulatory PD (CAPD) was then developed in the late 1970s, by the work of Moncreif *et al.* (70). Their method introduced 1.5–3 l of a hyperosmolar peritoneal dialysis fluid (PDF) into the peritoneal cavity through a catheter in a process called a dwell. Five dwells a day were performed, seven days a week, which can be seen as a physiologic method for waste removal compared to HD.

PD utilizes osmotic filtration that governs the blood solute and organic waste product movement from the blood into the peritoneal cavity. Dwell times range from four to eight hours, and after the diffusion of solutes is complete over the PM, the dialysate is drained out and new PDF is instilled (62). The injection and draining of PDF after a dwell are called a PD cycle.

The success of PD as an RRT is dependent on the quality and preservation of the properties of the PM. The PM is different from one individual to the other in terms of the ability to remove solutes and waste products. Variations in microvasculature and mesothelial and interstitial properties between individuals can lead to different therapeutic outcomes (71). PD has shown good outcomes in terms of survival. However, after seven years of PD, only 10% of the treated patients can remain on PD due to ultrafiltration failure (UFF) and peritonitis of the PM (72-74).

UFF is defined as having a net UF that is less than 400 ml at four hours of dwell using a hypertonic PDF in the absence of PDF leaks, catheter malfunction, or adhesion blockage (75-77). UFF has four types: high

effective peritoneal surface area (type 1), characterized by hyperpermeable PM to small solutes; low osmotic conductance to glucose (type 2), due to aquaporin dysfunction; low effective peritoneal surface area (type 3) with decreased solute and water transport (rare); and high total peritoneal fluid loss rate (type 4) with increased reabsorption of dialysate due to increased lymphatic flow (78).

Type 1 is the most common type to cause permanent UFF. It develops over time and features vascular damage and endothelial dysfunction. As a result, more protein is lost and more glucose reabsorbed from the PDF. The pathogenesis of this UFF is speculated to be due to uremia and the chronic exposure to high glucose PDF (79).

Since the abdominal cavity is penetrated in PD, there is an increased risk for infection and peritonitis. Infection can occur via the transluminal or periluminal ways. Transluminal infections may occur during PD cycles, while periluminal infections may occur due to a permanently implanted catheter (80, 81). The causative pathogens can be gram-positive or gram-negative microorganisms. Gram-positive cocci such as *Staphylococcus epidermidis*, other coagulase-negative staphylococci, and *Staphylococcus aureus* are responsible for up to 40% of all cases of infectious peritonitis worldwide (82, 83). Various other microorganisms can also penetrate to the peritoneal cavity (84).

Peritonitis is the major source of morbidity and the transfer of dialysis regimen to HD (85, 86). A cascade of inflammatory processes marks peritonitis. The interaction of macrophages, lymphocytes, fibroblasts, and mast cells in the PM with the invading organisms will lead to accumulation of various inflammatory mediators and growth factors such as transforming growth factor beta (TGF-β) and vascular endothelial growth factor (VEGF). The result of this inflammatory state is a paradoxically disadvantageous increment in the effective PM surface area due to angiogenesis and subsequent hyperpermeability (type 1 UFF). The other results are fibrotic events with thickening of the PM and further UFF (77, 87, 88).

1.5 RENAL CARCINOMA

Renal cancer is the ninth most common cancer form in men and the fourteenth most common cancer in women. In 2018, it is estimated that 403,262 new cases of kidney cancer will be reported, of which 175,098 deaths will occur (89). According to the 2016 World Health Organization's (WHO) classification of tumors of the urinary system (90), RCCs are the most common types of renal cancers and are usually unilateral. RCCs are subdivided into several types (see Table 1). The most common types are ccRCC, papillary renal cell carcinoma, chromophobe renal cell carcinoma, acquired cystic (solid) disease-associated renal cell carcinoma, collecting duct carcinoma, renal medullary carcinoma, and unclassified RCC. Together, these subtypes account for approximately 90% of all RCCs. ccRCC alone accounts for approximately 70% of all RCCs (90, 91) and originates from PCT cells (6, 92). With a ratio of 1.5:1 for men and women, it is most commonly diagnosed around the age of 40, nevertheless, any age group can develop ccRCC. The strongest non-genetic risk factors are smoking and obesity, albeit it develops sporadically in most cases (6). A triad of symptoms can present ccRCC, namely hematuria, flank pain, and a palpable mass. However, only 10% of patients present these symptoms, 40% do not show any of these symptoms, and the rest show different combinations of these and other symptoms. ccrCC is genetically characterized by mutation in genes that control cellular oxygen sensing (such as Von Hippel–Lindau (VHL)) and the maintenance of chromatin states (such as PBMR1). The inactivation of the VHL gene leads to a defect in the ability to degrade hypoxia-inducing transcription factors HIF-1α and HIF-2α, which in turn leads to increased transcription of hypoxia-inducing genes like VEGF and glucose transporter 1 (GLUT1) (93, 94). ccRCC holds the most sarcomatoid transformation potential and is the most aggressive of all RCC types (95). The lack of surveillance methods, sporadic occurrence, and vague symptoms in more than 50% of the ccRCC patients lead to late detection of this cancer form, with accidental detection in up to 60% of cases (96). Localized ccRCC can be treated with surgical removal (97). Metastasized ccRCC, however, is highly resistant to chemo- and radiotherapy (98, 99). Some of the treatment options for ccRCC include immunoregulatory drugs (100), VEGF receptor and multityrosine kinases inhibitors (101), and the mammalian target of rapamycin (102). Therapy can impede the progression of the metastases in ccRCC for approximately three to six months. However, for the overall survival, few clinical trials have offered a statistically meaningful effect (103-105). Taken together, these factors lead to ccRCC being detected at a late stage, with the highest mortality rate of the genitourinary cancers of up to 40% (106).

The ccRCC cells are divided into primary and metastasized cells. The most common metastatic locations for ccRCC are the lungs and bones, followed by the liver, lymph nodes, brain, breasts, and adrenal glands (107). Establishing RCC cell lines is important for exploring the mechanism of renal carcinogenesis and for developing therapeutic options. E. Oosterwijk created a library of RCC cells acquired from the Sloan-Kettering (SK) Memorial Cancer Center. These renal cancer cells were named SKRC cells. Forty-six of these SKRC cells were derived from primary kidney tumors and 17 were derived from metastatic sites (lung, brain, bone, and lymph node) (108).

Renal Cell Tumors
Clear cell RCC
Multilocular cystic renal neoplasm of low malignant potential
Papillary RCC
Hereditary leiomyomatosis and RCC associated RCC
Chromophobe RCC
Collecting duct carcinoma
Renal medullary carcinoma
MiT family translocation RCCs
Succinate dehydrogenase-deficient RCC
Mucinous tubular and spindle cell carcinoma
Tubulocystic RCC
Acquired cystic disease-associated RCC
Clear cell papillary RCC
RCC, unclassified
Papillary adenoma Oncocytoma

Table 1. Renal cell carcinoma subtypes by the WHO, 2016 (90). MiT, microphthalmia transcription factor; RCC, renal cell carcinoma.

1.6 ORELLANINE

Orellanine is a nephrotoxin found in mushrooms of the *Cortinariaceae* family, which includes the fool's webcap (*Cortinarius orellanus*) and the deadly webcap (*Cortinarius rubellus*) mushrooms. These mushrooms grow in the forests of Northern Europe and North America. Orellanine was discovered after the epidemic of mushroom poisoning in Poland in 1952 and has since been examined for its toxicity (109). The chemical form of orellanine is [2, 2′-bipyridine]-3, 3′,4, 4′-tetrol-1, 1′-dioxide (110). It is present in the mushroom as a di-glycoside (111, 112). Once digested, orellanine containing mushrooms can cause symptoms like flank pain, polyuria followed by oliguria, and dehydration. These symptoms are concordant with nephrotoxicity and their emergence varies in time. Symptoms may appear as early as the second day but the majority of patients develop these symptoms between one and up to two weeks after ingestion. Since there are no known antidotes for orellanine poisoning (113), treatment options that have been tested are hemodialysis, plasmapheresis, and acetylcysteine (114). The outcomes after ingestion can vary from complete remission to total kidney failure (uremia). There seems to be a dose dependency in the severity of renal impairment. Histologically, kidney lesions include tubulointerstitial nephritis with severe interstitial fibrosis, interstitial edema, and tubular epithelial necrosis (115, 116). The uremia is characterized by high blood levels of urea and creatinine (117). Once uremic, the patients will be dialysis-dependent for life. If the uremia is managed with dialysis or kidney transplantation, the patients will live a normal life as no other organs than the kidneys seem to be affected by orellanine (5). Orellanine's median lethal dose (LD_{50}) in a mouse is approximately 20 mg/kg (118). The LD_{50} in humans is not known but is estimated to be much lower (4). Orellanine seems to be taken up selectively by PCT cells and there generally are no detectable amounts of the toxin in the plasma or urine by the time the symptoms emerge (3, 119). Kidney tissue can have detectable amounts of orellanine up to 6 months after poisoning, suggesting accumulation in the PCT, but this has not been validated (120).

The molecular weight of orellanine is 252.19 g/mol and it has four pKa values of approximately 0.5, 1.0, 7.0, and 7.4. The net charge at physiological pH is close to -4. The pure form of orellanine is a colorless fine crystalline substance that, if exposed to ultraviolet light, will decompose to orellinine and subsequently to the nontoxic orelline (3).

The exact mechanism of action of orellanine is unclear. What is known so far is that orellanine induces cell damage by a rapid change from its oxidized to

its reduced form, leading to the induction of oxidative stress (4, 121). Simultaneously, orellanine shuts down the enzymatic oxidative defense features of the orellanine sensitive cells (4). The subsequently induced hypoxia renders the cells susceptible to oxidative destruction. Orellanine works mainly at the nephron tubular brush border with a variety of actions such as the inhibition of synthesis of proteins, DNA, and RNA (122), the inhibition of enzymatic activity like alkaline phosphatase and leucine aminophosphatase, and the interruption of adenosine triphosphate (ATP) production (123). Due to the oxidative stress induction features of orellanine, antioxidant therapy has shown some improvement in minor intoxications (124, 125).

2 AIMS

The general aim of this doctoral thesis is to explore the effectiveness, pharmacokinetics, and potential uses of the fungal nephrotoxin orellanine in PD research and as a potential treatment against metastasized ccRCC.

The specific aims of the papers included in this thesis were as follows:

Paper I: The first aim of paper I was to investigate whether orellanine can be used to induce chemical nephrectomy instead of nephrectomy induced by surgery. The second aim was to develop a new method for chronic automated PD (APD) in anuric rats.

Paper II: The aim of paper II was to explore the distribution and pharmacokinetics of orellanine in rats to understand how orellanine is cleared from the body.

Paper III: The first aim of paper III was to investigate orellanine's selective nephrotoxic effects on proximal tubular cells. The second aim was to explore if this toxicity extends to primary and metastasized ccRCC cells both *in vitro* and *in vivo* for orellanine to be considered as a future treatment option against ccRCC.

3 METHODOLOGICAL CONSIDERATIONS

3.1 SUMMARY OF METHODS

Paper I: The first paper in this thesis was a methodological PD study that tested a novel APD system developed by our group on female Wistar rats. Orellanine was used to induce chemical nephrectomy in order to test the system on anuric rats. Inflammatory changes on the peritoneum and the electrolytes' profiles in blood and dialysate were examined.

Paper II: The second paper investigated the pharmacokinetics of orellanine in male Sprague Dawley rats. The rats received orellanine (labeled or unlabeled) intravenously (iv) and plasma samples were collected over eight subsequent time points. Ultra performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) and beta scintillation were used to measure the halftime and clearance of orellanine in plasma. Additionally, anuric and healthy rats received labeled orellanine iv and were then sacrificed at different time points. Radioilluminography was then used to visualize the distribution of orellanine in the body.

Paper III: The third paper comprised an *in vitro* and *in vivo* part. In the *in vitro* part, human kidney tubular cells, non-kidney cells, and anaplastic cell lines were tested in terms of response to orellanine. In the *in vivo* part, human ccRCC was xenografted into immunodeficient Sprague Dawley rats. The animals were then put on dialysis and half of the animals received orellanine treatment intraperitoneally (ip). Excision of tumor masses occurred on the day of autopsy and comparisons were done on morphological, histological, and cellular levels.

3.2 ANIMALS

All the *in vivo* experiments in this thesis used PD as a RRT. Choosing the right animal species and strain is one of the challenges in PD research (126). The most commonly used animals in PD models are mice, rats, and rabbits (127). Although mice are inexpensive and easy to breed, their miniature size makes them hard to perform surgery on (128). Rabbits are able to undergo PD, with the advantage of having a PM surface area similar to humans.

However, these animals are very sensitive, expensive, and difficult to breed (129). Rats are the most commonly used animals in PD research. Rats are also affordable and easy to handle (130, 131). The disadvantages of using rats include a larger PM surface area compared to humans (132, 133) and higher amounts of amylase enzyme in the peritoneal cavity (134, 135).

All experiments in this thesis were performed on rats. Male and female Sprague Dawley and female Wistar rats were used. From our own experience, female Wistar rats formed the least adhesions in the peritoneal cavity around the implanted catheters and were therefore used for the chronic PD model. All animals were kept in a temperature-controlled environment on 12-hour light cycles and were fed a standard rodent chow and water ad libitum.

3.3 PAPER I METHODS

3.3.1 Chemical nephrectomy

In clinical settings, a patient in need of RRT will be uremic (136-138). Therefore, it is important that animal models of PD reflect this by using uremic animals. Besides, uremia itself might induce independent effects on the PM other than those from PD alone (139, 140), which adds to the need for a uremic PD animal model. There is currently a lack of totally uremic animal models of chronic PD (131). This is partly due to challenges in finding a method for inducing total uremia that is reproducible and mimics the clinical setup. Uremia in animal models is typically induced by subtotal (5/6) nephrectomy (141). This can be performed by uni-nephrectomy and subsequent ligation of two of the three branches of the renal artery on the remaining kidney (142, 143), or by the ablation method, in which uni-nephrectomy is performed and 50% (or more) of the remaining kidney is surgically removed. Pertaining to the utilization on rats, the ligation model poses a severe hypertension threat to the rat's circulation (144, 145), whereas the ablation model is hard to reproduce, causes varying grades of renal failure, and is dependent on the performer of the surgery (146). In addition, both procedures include major surgical trauma and pose an increased risk for infection, making them unreliable methods for chronic PD(147). Therefore, our model used orellanine in order to achieve total chemical nephrectomy. Orellanine specifically causes renal failure both in humans and animals (112, 114, 120). Using orellanine is non-invasive and minimizes the risk for dropouts due to surgery. To ensure anuria occurrence, three extra groups of

rats (three animals in each) were injected with an ip bolus dose of 5, 7, and 10 mg/kg orellanine without PD. Blood samples were collected at day 3 after the injection. Uremia was evident in all three groups with high serum urea and creatinine levels (see Figure 2). Therefore, the lowest dose was chosen for the chemical nephrectomy (5 mg/kg).

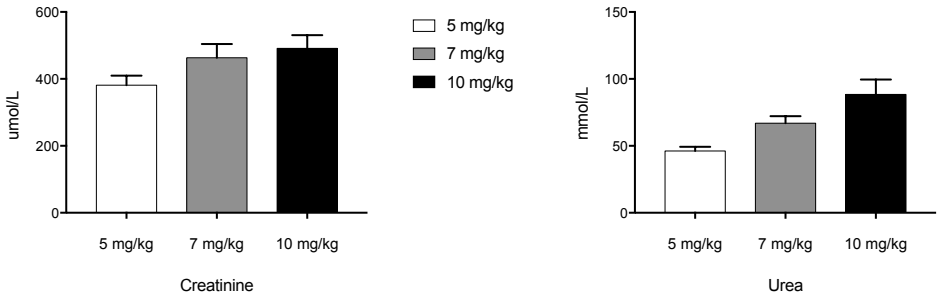

Figure 2: Serum creatinine (A) and urea (B) levels two days after three different single ip doses of orellanine.

3.3.2 PD of rats

3.3.2.1 Chronic PD

Patients on dialysis will need lifelong RRT, unless a successful transplantation occurs. Animal models aiming to improve PD treatment should, therefore, be performed chronically and on uremic animals (131). One day in an adult rat corresponds to 34.8 days in a human's lifetime (148). Hence, our 21 days of PD treatment in rats could be compared to 2 years in humans.

3.3.2.2 Peritoneal access

The literature describes three major ways to perform PD dwells (126). One method is by simply injecting the PDF into the peritoneal cavity and letting the PDF be absorbed. This method implies repeated injections with the accompanying risk of infection and trauma to internal organs. In addition,

repeated anesthesia can alter the PM permeability and kinetics (149, 150). The second method is via a temporary catheter with manual draining or leaving PDF in for absorption. The third method uses an implanted catheter with repeated dwells and emptied manually or by gravitational force. The latter method does not cause repeated trauma or anesthesia. However, the risk of peritonitis is increased with repeated handling of the implanted catheter (126). Our PD setup used the third method but instead of manual handling of the catheter, we used a computerized automated dwell and emptying valve system in order to further minimize the contamination risk.

3.3.2.3 Novel APD rat model

The PD model developed in our lab uses a catheter (CBAS-50, Instech Laboratories Inc., PA, USA) that is surgically implanted in the peritoneal cavity. The catheter is passed subcutaneously to the back of the rats' neck and fitted to a harness (SAI Infusion Technologies, USA). The harness has sealed valves that fit a swivel (custom-made in our lab) connected to the PD tubing system. The PDF used is Gambrosol® trio 10 containing 1.5% glucose. The model operates using hydrostatic pressure for filling and emptying each dwell. The tubing system is sealed and sheathed by a water bath to keep the fluids inside the tubes at body temperature before they enter the peritoneal cavity of the rats. All tubing and housing units are autoclaved before each experiment. The APD system allows for automatic dwells and emptying of the peritoneal cavity of the rats while being completely controlled from a computer program. The latest version of the APD system provides five standardized and timely regulated dwells a day. The computer software administers customized filling, dwell, emptying times, and numbers of cycles. In order to ensure that the system was running without any problems during the dwell time, monitoring was carried out daily. The rats were housed in separate cages, which were placed in a Scantainer (Scanbur Technology A/S, Denmark), providing optimized humidity, light and dark cycles, and air-flow and keeping a sanitary environment for the rats during the dialysis period (Figure 3). In order to evaluate the effectiveness and function of the dialysis, dialysate fluid was collected from the second cycle on the first day and the last day of the three weeks of the APD period. Blood and tissue samples were taken on the day of autopsy after finishing the dialysis period.

Figure 3. The Scantainer at the experimental biomedicine core facility (EBM). The rats are kept in separate cages. Cameras monitor the rats while the doors are closed. Humidity, sterile air-flow, temperature, and light cycles are standardized by the container functions.

3.3.2.4 Peritonitis

Infection and subsequent peritonitis pose the greatest threats to the integrity of the PM and its ability to yield effective PD chronically. Therefore, several precautions were taken to avoid contamination. The laboratory environment was aseptic. Animals were housed in a Scantainer that provided controlled temperature, relative humidity, air changes per hour, and positive or negative pressure under sterile conditions. All equipment entering the laboratory was autoclaved. Animals were washed twice with chlorhexidine before surgery. Before surgery and implantation of the PD catheter, the animals were covered in sterile plastic sheeting. The surgeon prepared the same way as to perform clinical surgery, wearing sterile clothing, mask, mouth protection, and sterile gloves. All PD tubing was autoclaved and connected aseptically to each animal and the sterile PD fluid. Each catheter was coated with heparin, as this method yielded efficient results by means of avoiding adhesions, fibrosis, and subsequent dropouts (151). Prophylactic antibiotics were used in the PD fluid to prevent peritonitis from the rats' own intestinal bacteria as well as external infection, thereby decreasing dropout rates evident in our own experience as well as in literature (152, 153).

In order to monitor the inflammatory profile of the PM, the following growth factors and interleukins were measured: VEGF, transforming growth factor

beta (TGF-β), interleukin-1beta (IL-1β), and interleukin-6 (IL-6). VEGF is a glycoprotein that is activated in response to hypoxia (154) and exposure to high glucose PDF (155, 156). The result is the induction of neoangiogenesis and subsequent hyperpermeability of the PM (type 1 UFF) (157). TGF-β accumulation across the PM extracellular matrix leads to increased fibrosis and epithelial to mesenchymal transition (EMT) (158, 159). VEGF and TGF-β are suggested to interplay, leading to a synergized negative effect on the outcomes of PD, which makes these cytokines significant to measure in PD testing (160). Interleukin-1β and 6 are pro-inflammatory cytokines used as predictors of peritonitis, as they are produced by inflammation-mediating cells like dendritic cells, NK cells, T cells, B cells, monocytes/macrophages, fibroblasts, mesothelial cells, and vascular endothelial cells (161, 162).

3.3.2.5 Solute profiles

The electrolytes and molecules examined to test the PD quality were urea, creatinine, sodium, potassium, phosphate, calcium, glucose, and albumin. Creatinine is the breakdown product of muscle creatinine phosphate. The kidneys remove creatinine constantly through the glomerulus and the kidney tubules. The amount of creatinine can therefore be measured in the blood to evaluate kidney and PD function in chronic kidney disease (CKD) and ESRD patients. Urea is produced by the liver and is the major end product of breakdown of amino acids. Serum urea level is utilized as an indicator of the extent of renal failure (163).

ECF volume expansion due to disturbances in sodium levels is a common problem in CKD and ESRD patients. This leads to salt-sensitive hypertension and subsequent left ventricular hypertrophy (164). Diuretic treatments have not proven to be effective (165), making sodium a central electrolyte to monitor in PD to make sure that excess is excreted through the PM. Potassium is an important intracellular electrolyte needed to maintain membrane potential over the cardiac muscle; a slight change in potassium balance may lead to arrhythmia and cardiac arrest. The kidneys play an important role in potassium balance by excreting up to 98% of the daily excess intake of potassium. Renal failure will lead to impaired potassium excretion and hyperkalemia, which is the most important and fatal complication of renal failure (166, 167). Other measured electrolytes are phosphate and calcium. These electrolytes are involved in bone mineral metabolism. Their homeostasis is orchestrated by an interplay between the kidneys, parathyroid hormone, the gastrointestinal canal, and the bone tissue. The kidneys excrete both calcium and phosphate. In addition, ESRD can lead to abnormalities in bone turnover and soft tissue calcification (168). Several

factors play a role in altering the glycemic homeostasis, making glucose a central electrolyte to monitor especially in patients with diabetes. These factors include uremic toxin accumulation, hepatic and renal impaired insulin degradation, malnutrition, low vitamin D production, secondary hyperparathyroidism, and PDF glucose load. These factors indicate the importance of close measurements of glucose profiles in patients with kidney failure on PD (169). Albumin, phosphate, and glucose can be indicators of the nutritional status of patients with ESRD on PD. ECF volume expansion, exogenous loss due to PD, and decreased albumin synthesis all contribute to hypoalbuminemia, making albumin a standard protein to test the quality of PD and the nutritional status of the patients (170).

In summary, our dialysis method is unique in three ways: first, it uses a chemical nephrectomy to induce uremia. Second, this is the first study to apply APD on rats in a fully computerized manner. Third, our dialysis method is also the first to study the changes of the PM over a chronic period of time in anuric rats while keeping normal homeostasis throughout the experiment time.

3.4 PAPER II METHODS

3.4.1 Radioiluminography

In laboratory animals, whole-body radioiluminography is used to determine the distribution and concentrations of radiolabeled drugs and compounds. This technique provides information on tissue penetration, accumulation, and retention. Briefly, animals receive a radiolabeled dose of a certain compound. At specific time points, after the administration of the dose, the animals are euthanized under anesthesia and the whole body is frozen in a carboxymethylcellulose matrix. The frozen carcasses are then cryosectioned and cross-sectional whole-body sections from different depths are obtained. The representative sections with the tissues of interest are then exposed to a phosphor-imaging scanner that produces high-quality images emphasizing the radiolabeled areas in tissues and body fluids (171, 172). The rats used for this experiment were male Sprague Dawley rats (Taconic, DK).

Orellanine was labeled using tritium (^{3}H). The radiolabeling of orellanine was performed by the Red Glead Discovery AB (Lund, Sweden), resulting in

> 95% bound ^{3}H-labeled orellanine with a specific activity of 35.5 Ci per mmol orellanine. Ten rats received a single dose of 5 mg/kg of ^{3}H-labeled orellanine iv (vena saphena). Twelve rats received a nephrotoxic dose (5 mg/kg) of unlabeled orellanine ip 72 hours prior to the introduction of 5 mg/kg of the ^{3}H-labeled orellanine. The reason for rendering the second group of rats anuric is to determine how the distribution of orellanine will be affected without kidney clearance. ^{3}H-labeled orellanine was prepared with a formula of 0.471 mCi/mL, corresponding to 1.25 mg/mL in physiological saline, while unlabeled orellanine was formulated to 1.25 mg/mL in physiological saline. The administered volume was adjusted to 4 ml/kg for each rat after weighing. Under isoflurane anesthesia, blood samples were collected from the tail veins at time points 0.5, 1, 6, 12 (last point for the nephrectomized rats group), and 24 hours after administration of orellanine. The rats were then sacrificed. The carcasses of the anesthetized animals were immersed in heptane cooled to -70°C. Each carcass was embedded in carboxymethyl cellulose and 30 µm thick sagittal sections were taken at different depths. The sections had radioactive labels and were placed on imaging plates (Fuji, Japan). Light-tight cassettes were used for exposure and had lead shielding at -20°C in order to cancel out environmental radiation.

^{3}H-radioactivity calibration standards were used in this method consisting of 10 dilutions from 6.68–22400 nCi/g for the single dose experiments and 6.60–11260 nCi/g for the nephrectomized rats experiments. All tissues were assumed to have a similar density (1 g/mL) and quench characteristics as whole blood. The mean concentration value of eight measurements for background plus triple the standard deviation values for the same measurements were used to define the limit of quantification.

The 30 µm sections and the calibration standards were put on imaging plates and exposed for 70–96 hours, then scanned with a 50 µm pixel size using BAS 2500 (Fuji Film, Sverige Ab, Sweden). Quantification was done using AIDA software, version 4.19 (Raytest, Germany). The mean value of three separate sections for each tissue was used to determine the radioactivity.

3.4.2 Detection of orellanine in plasma

Since orellanine is investigated for its quality as a chemical nephrectomy tool in a PD animal model and for its possible therapeutic effects in treating metastasized ccRCC, it is important to develop a method to detect orellanine levels in plasma. This is done in order to control the therapeutic steady-state window and to investigate the half-life. Another important clinical aspect is the need to have a reliable method for detecting orellanine in fluids and plasma of suspected cases of orellanine intoxication.

Efforts have been taken to detect orellanine levels in mushrooms, plasma, and kidney tissue before. Orellanine was discovered in mushrooms using thin layer chromatography (TLC), electrophoresis, electron spin resonance (ESR), and reversed high performance liquid chromatography (HPLC) (173, 174). HPLC with an electrochemical detector has also been used to detect orellanine in serum and kidney tissue (174, 175). Orellanine has been detected in single MS mode using electron impact (EIMS) and electrospray ionization (ESIMS), however; HPLC-ESI-MS/MS was only employed for qualitative purposes (111, 175, 176). Herrmann *et al.* optimized a quantitative HPLC-ESI-MS/MS liquid method for detecting orellanine in orellanine-spiked blood plasma (16). One of the limitations was, however, that no plasma analysis was performed *in vivo* on animals dosed with orellanine. In our study, we used a modification of the latter method using UPLC-MS/MS (112). UPLC-MS/MS combines the physical separation features of liquid chromatography with the mass analysis features of mass spectrometry, having high sensitivity and selectivity in detecting complex compounds. Since this experiment was done within a single day, there was no need to use female Wistar rats in order to avoid the formation of intraperitoneal adhesions in rats undergoing PD. Three groups of rats received an iv dose of orellanine (5 mg/kg) into the jugular vein. One group had intact kidneys and urine production, the second group had ligated kidney arteries, and the third group had ligated renal arteries and underwent PD. In this manner, the effect of PD on the elimination of orellanine could be examined. Blood was collected at seven subsequent time points: 10, 30, 45, 60, 90, 180, and 360 min. The whole experiment was conducted under isoflurane anesthesia and the rats' body temperature was kept at 37 °C with a heating pad. UPLC-MS/MS was used to measure orellanine and beta scintillation was used to detect ^{3}H-labeled orellanine and any metabolites formed.

For the UPLC-MS/MS, a standard curve was diluted from a stock solution of 200 µg/mL orellanine with human plasma to final concentrations of 0.039, 0.78, 0.16, 0.31, 0.63, 1.25, 2.5, 5, 10 and 15 µg/mL.

The quality control samples were prepared from the same stock solution and diluted with human plasma to final concentrations of 1.25 and 5 µg/mL. Human plasma was used instead of rat plasma for quality control sample preparation to enable a better comparison between the UPLC-MS/MS runs and to eliminate variations due to experimental conditions. The quality control samples were analyzed before and after each tested unknown plasma sample. As a result, large amounts of plasma were needed for the quality control samples. We opted to use human plasma as it is easier to obtain in larger volumes than animal plasma. Furthermore, in experiments performed earlier in our lab, the plasma protein binding of orellanine after 4 hours of dialysis was found to be quite low, 33.5% for rats and 42.5% for humans. The small difference makes it unnecessary to sacrifice more animals and use rat plasma.

Small molecules were separated from proteins when the samples were filtered with a 30 kDa molecular weight cut-off filter. The filters were transferred to high recovery tubes and 64 µL of 5.2% formic acid (v/v) was added to the filters. 20 µl of the rat plasma samples or the quality control samples were added to the filter for a final concentration of 4% formic acid (v/v). The samples were centrifuged for 30 min at 1200 rpm and then injected (volume 7.5 µL) into a Zorbax Eclipse plus C18 columns, with the dimensions 150 x 2.1 mm, 5 µm particle size (Waters, Milford, MA, USA). Separation temperature was at 45°C, with a flow rate of 200 µL/min. Orellanine with parent mass m/z 253.05 Da was fragmented, where fragment ion mass per charge (m/z) of 236.2 Da indicated the loss of 17 Da, corresponding to a loss of OH, which was extracted for quantification. The quality control samples were within 10% difference compared to the standard curve. The lowest detected concentration was 40 ng/ml. Orellanine was extracted in plasma with 60% efficiency. All samples were analyzed with parallel reaction monitoring on an LTQ Orbitrap Velos mass spectrometer interfaced to an UltiMate 3000 system (Thermo Fisher Scientific, Waltham, MA, USA). The results were analyzed with Xcalibur software (Thermo Fisher Scientific, Waltham, MA, USA). The average peak from two injections of each sample was then calculated. The quality control samples were analyzed before and after each tested unknown plasma sample. This allowed for a better comparison between the UPLC-MS/MS runs and to eliminate variations due to experimental conditions. In the chromatogram analysis of the quality control samples, orellanine had a retention time (RT) of 5.2 min (see Figure 4).

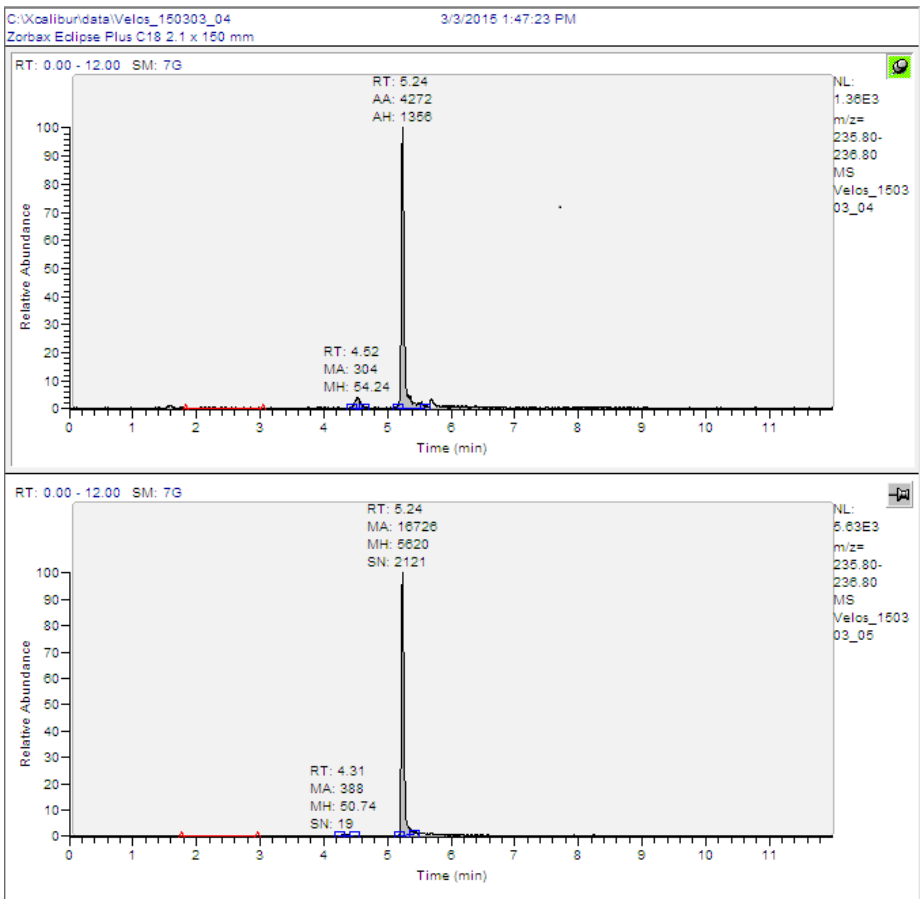

Figure 4: Chromatogram from quality control sample with a plasma concentration corresponding to 1.25 and 5 µg/mL.

3.5 PAPER III METHODS

3.5.1 *In vitro* studies

Before testing orellanine in an *in vivo* rat xerograph tumor model, the effects were examined *in vitro* on proximal tubular cells and ccRCC primary and metastasized cell lines. HTEC were prepared from tissue taken from the cortical region of the healthy part of a nephrectomy kidney. All specimens were collected after informed consent from the patient. Proximal tubular cells are the origin cells for ccRCC (6, 92). The effect of orellanine was examined on HTEC cells in comparison to other cell lines. The non-renal cell line chosen was human umbilical vein endothelial cell line (HUVEC) (Lonza LTD, Basel, Switzerland). The malignant non-renal cell lines chosen were from a metastatic region of an epithelial cell breast adenocarcinoma cell line MDA-MB-231 (ATCC® HTB-26™) and human liver carcinoma cells (HPEG2). Renal cancer cell lines were either primary ccRCC tumor cells or metastatic ccRCC cell lines. Primary cell lines were 786-O cells (ATCC® HTB-26™), 087, or SKRC-7, -10, and -21. The metastatic cell lines were SKRC-17 and -52. The SKRC cells were obtained from the work of E. Oosterwijk (Radboud University Nijmegen Medical Center, Nijmegen, The Netherlands) (108). SKRC-17 and -52 are associated with poor prognosis and strong resistance to treatment. We utilized these cells in order to test our methods on the cells most difficult to treat.

3.5.1.1 Apoptosis

Apoptosis is the sum of cellular changes leading to programmed cell death, such as cell shrinkage, DNA fragmentation, and cell membrane changes. Apoptosis is part of the normal cell life cycle but can be induced in response to toxins and cell damage (177). The signaling pathways for apoptosis are the intrinsic and the extrinsic pathway. The intrinsic pathway is activated within the cell while the extrinsic pathway is activated from outside the cell (177). Caspases are proteins with protease activity that either initiate (caspase-8, -9) or execute (caspase-1, -6, -7) the signaling pathways, leading to apoptosis (178). Caspase-3, -8, and -9 enzymes were quantified in orellanine-treated cells *in vitro*, using western blotting and caspase activity assays.

Annexin V binds to the phosphatidylserine (PS) lipids of the cells plasma membrane. In apoptosis, PS is exposed to the outer layer of the plasma membrane (otherwise restricted to the inner layer) and annexin V can bind to it. Annexin V can therefore be used to detect apoptosis. During necrosis, the

cell membrane is broken and annexin V can bind to PS and serve as an indicator of necrosis. Propidium iodide (PI) is used as a co-stain to annexin V, as it only enters necrotic cells and thereby indicates necrosis (179). This staining was used on cultured cells treated with orellanine to measure the apoptotic and necrotic profiles of the treated cells.

3.5.1.2 Reactive oxygen species

Some of the major reactive oxygen species (ROS) in living systems are superoxide, hydrogen peroxide, singlet oxygen, and hydroxyl radicals. Oxidative species are produced by cells in physiological situations during cellular metabolism. ROS are involved in anti-inflammatory functions, cell signaling, and proliferation. Uncontrolled ROS activation can cause cellular damage by mediating the oxidation of lipids, protein, and DNA (180, 181). ROS detection tests were performed using carboxy-H_2DCFDA (6-carboxy-2',7'-dichlorodihydrofluorescein diacetate). Carboxy-H_2DCFDA is a chemically reduced, acetylated form of fluorescein used as an indicator for ROS in cells. The ROS species detected are singlet oxygen, hydroxyl radicals, and/or superoxide (182). Since orellanine has been shown to increase oxidative stress and induction of free radical formation (see section on orellanine), ROS detection was used to examine orellanine's effect on cells *in vitro*.

3.5.1.3 Cellular energy metabolism

Cells generate energy in the form of ATP by oxidative phosphorylation and/or glycolysis (183). Oxidative phosphorylation is an aerobic pathway (requiring oxygen) that occurs in the mitochondria. Glycolysis is an anaerobic pathway that occurs in the cytoplasm outside the mitochondria (184). The oxygen consumption rate reflects oxidative phosphorylation, and the extracellular acidification rate reflects the glycolytic energy generation (185). These can be monitored by using a Seahorse XFp extracellular flux analyzer (Seahorse Biosciences, North Billerica, MA) on cultured cells to detect orellanine's oxidative stress induction and the shift of treated cells to the anaerobic pathways.

3.5.2 Xenograft animal model

Wistar-Lewis rats were considered as tumor models since the adenocarcinomas in these rats can develop into ccRCC tumors with metastatic potential. This tumor model shares characteristics with the human ccRCC but is not exactly the same in terms of cholesterol metabolism and

growth. In Wistar-Lewis rats, the tumor size doubling time is 2 days compared to 240–600 days in humans (186). Other difficulties with this model were the unpredictable metastatic or growth patterns and the complexity in implantation (187, 188). Since the Wistar-Lewis model was not optimal for our purpose of testing orellanine on metastasized ccRCC, it was decided to use a xenograft rat model.

Initially, a xenograft tumor model in immunocompromised rats was used. Athymic Sprague Dawley rats lacking T-cell function were chosen. We decided to implant the xenograft of ccRCC cells subcutaneously in order to be able to continuously measure the size of the tumor in the treated and control rats.

Surprisingly, the immune system in rats with functional B cells defense led to reabsorption of the tumor within one to two weeks. This led us to induce further suppression of the immune system of the athymic rats with a 4–5 Gray whole-body radiation dose, two weeks before the start of the experiment. This, in turn, initiated the successful growth of the injected ccRCC cell mass into a growing tumor within two weeks. The general growth and the appetite of the animals were not affected by this dose. The ccRCC cells implanted in the animals were SKRC-17. These cells were chosen as they are metastatic cells and they have higher resistance to treatment than the other cell lines tested. Orellanine was given ip with the PDF. During orellanine treatment, the rats underwent APD with the same setup used in paper I. At the end of the experiment time, the tumors were compared in terms of volume, weight, and assessment of *in situ* cell death with terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay. TUNEL assays can measure single- and double-stranded DNA fragmentation. Terminal deoxynucleotidyl transferase assists the incorporation of modified nucleotides into the 3’ OH of broken DNA strands due to apoptosis. These modified nucleotides are tagged with a fluorochrome or another detectable marker that can be visualized *in situ* under the microscope (189). Since DNA fragmentation can be found in necrosis as well, TUNEL assays are used to detect cell death from both apoptosis and necrosis (190). Nevertheless, this does not affect our findings, as the aim of using TUNEL assay was to detect cell death in general and not distinguish apoptotic from necrotic death.

3.6 ETHICS

All the animal experiments conducted in this thesis were granted ethical application approval. The animal ethical committee in Gothenburg, Sweden approved the animal experiments for papers I, II, and III (approval numbers 144-12 and 44-15). Lund University's animal welfare committee approved the radioiluminography tests in paper II (M53-11 and M79-13). Ethical permit for retrieving human nephrectomized kidney tissue was obtained from the regional ethical committee in Gothenburg (R110-98).

Throughout the experiments, the researchers performing the animal experiments kept a strict application of the three R´s of humane animal ethical handling (191): replacement, reduction, and refinement. Continuous clinical observation of the animals during the experiment time and care protocols were documented and supervised by veterinarians and licensed animal caretakers at the EBM core facility in Gothenburg.

4 RESULTS AND DISCUSSION

4.1 PAPER I: Chronic peritoneal dialysis in uremic, anuric rats

PD is one of two possibilities to dialyze the blood when the kidneys have reached end-stage renal failure and RRT is needed. Research on PD function, dialysis fluids, and peritoneal function is of high importance since the PD method is a great option for patients but is rarely sustainable. With a well-functioning chronic PD animal model, PD could be further examined *in vivo* and since no such model existed, we intended to develop a new model of chronic PD in uremic, anuric rats.

4.1.1 Chemical nephrectomy

When testing PD models on animals, it is important to mimic the clinical setting. This means that the model should be tested in anuric rats, as patients undergo PD due to loss of renal function. Animal models of PD have been lacking in this aspect, due to the complexity of producing such a model and the high dropout rates related to the techniques used (127, 130, 131, 143, 192). The classical methods of inducing uremia through surgery present problems, such as profound effects on the circulation, variation in achieved GFR, poor reproducibility, severe trauma, and risk of infection in the animals (144-147, 193, 194). Orellanine is known to induce irreversible renal failure in humans and rats, as shown in clinical and experimental tests of the toxin, without affecting the rest of the body's organs (114, 120). Orellanine was therefore used to induce chemical nephrectomy in our PD model.

In rats, three different levels of uremia have been defined: mild (serum urea between 6.68–13.36 mmol/l), moderate (16.7–33.4 mmol/l), and severe (> 33.4 mmol/l) (195). In order to determine the dose of orellanine needed to render the rats anuric in our experiments, three different doses of orellanine were tested (5, 7, and 10 mg/kg) (see methods section). The 5 mg/kg dose of orellanine induced severe uremia, with serum urea levels of 46.2 ± 5 mmol/L and serum creatinine values at 381.3 ± 23 μmol/L and was therefore chosen

as the chemical nephrectomy dose. This dose is in concordance with the LD_{50} of orellanine in rats described previously in the literature (4). The dialysate urea and creatinine concentrations were higher in the orellanine-treated rats than in rats with intact kidneys on the first day of the experiment, reflecting severe uremia. The same was true at Day 21 even though the urea and creatinine levels were significantly lower due to efficient dialysis. PD clearances of both creatinine and urea were nearly the same in all rats and reflecting a highly adequate PD treatment. Taken together, these data show that using orellanine is an effective way to introduce chemical nephrectomy in rats at a bolus dose of 5 mg/kg when given intraperitoneally 2-3 days prior to PD.

4.1.2 Efficacy parameters of PD

Animal models of PD have several challenges; the hardest is keeping the animals healthy for a chronic period of time while being anuric when the animals require repeated PD cycles for survival. Such a procedure is laborious and needs persistent efforts to keep the animals healthy (127, 130, 131, 192). The rats in our chronic PD model were healthy and gained weight during the experiment with a mean arterial blood pressure (MAP) being adequate for perfusion in both groups of rats. The weekly urea effective clearance (Kt/V) was higher than 3 for both intact kidney and anuric animals and over the minimum limit for PD adequacy in man ($\geq$ 2.0) (196). The precautions against peritonitis (see Methods section) and the multiple dwells played a role in obtaining a stable UF over the PM. Over the experiment time, our APD model had a steady ultrafiltration rate close to 20 ml/24h with no UFF for any of the rats. This means that neither technique failure nor peritonitis were present. Finally, as the animals were awake and only anesthetized during catheter implantation, anesthesia did not affect the PM ultrafiltration ability through increased lymphatic drainage (197) or altered PM permeability (198).

4.1.3 Inflammatory parameters and peritonitis

Peritonitis poses the greatest risk factor for rendering the PM dysfunctional in terms of performing PD (86, 199, 200). After five years on PD, 25% of anuric patients need to be transferred to hemodialysis due to peritonitis and loss of UF (201, 202). Therefore, several precautions were taken to avoid dropouts due to peritonitis in our model (see Methods section). To monitor

the inflammatory changes throughout the PD period, dialysate was collected from the second cycle on the first and last day of the experiment. Blood samples were collected during the autopsy at the end of the experiment. The inflammatory response was investigated by quantifying the expression of interleukins (IL-1β and IL-6) and growth factors (VEGF and TGF-β) in the blood, dialysate, and abdominal tissue. These cytokines are important indicators of inflammatory processes, including angiogenesis and fibrosis, and will, with time, alter the efficacy of the PM as a dialyzing barrier (203) (204). A prominent cytokine found in peritonitis is IL-1β, produced by macrophages (205). IL-1β stimulates mesothelial cells to produce IL-8, which in turn recruits polymorphonuclear cells to the PM. In rats, IL-6 elevation is found in active peritonitis and can be used to predict mortality due to inflammation (206). A similar study in humans showed that IL-1β and IL-6 are valuable predictors of systemic deterioration of patients with progressing peritonitis (207). In our experiment, IL-1β and IL-6 did not show significant elevations of expression in the peritoneal tissue, in the dialysate fluid, or in plasma from rats with chemical nephrectomy, leading to the conclusion that the model does not induce inflammation in the rats. Dialysate of rats with intact kidneys had higher IL-1β and IL-6 at day one compared to the end, due to two rats having levels that were approximately 25-fold higher than average. This could be due to these rats having a transient inflammation after the implantation of the PD-catheter that resolved before the end of the experiment.

TGF-β induces EMT of mesothelial cells in rodents and humans (208, 209). Cells having undergone EMT will induce an inflammatory response by producing more VEGF (210). VEGF in the PD setting in both rats and humans is known to increase the small solute permeability and the subsequent UFF type-1 (211-214). In the literature, peritoneal dialysis with hyperosmolar glucose-based PD fluids alone induced increased VEGF and TGF-β protein expression in the dialysate fluid of rats undergoing PD (157, 215). In our experiment, TGF-β expression in tissue and plasma showed no significant elevations. VEGF expression in tissue showed a trend of elevation in the intact kidney and the anuric rat groups compared to the controls; this observation was supported by VEGF in plasma and elevations in the two groups compared to the control (undialyzed) animals.

Pawlaczyk *et al.* showed that uremia in an acute setting (four days) could independently affect the cytokine profiles at the PM barrier (139). In another study, independent effects of chronic uremia on the PM were seen in rats being bilaterally nephrectomized (140). However, the rats in the latter study had invasive nephrectomy and were not on chronic PD and control animals

were intact kidney rats. Our study shows that uremia does not have an independent effect on any of the tested cytokines over dialysis alone – at least not to any significant degree on the biomarkers tested. A possible explanation for this could be that repeated dwells with APD over a chronic time contributed to resolving the effects of uremic toxins on the PM.

4.1.4 Solute profiles

Several parameters are used as a standard for testing the quality of PD (216). Besides urea and creatinine, we monitored the dialysate (day 1 and day 21) and the blood levels (day 21) of sodium, potassium, phosphate, calcium, and glucose. Orellanine-nephrectomized rats had higher levels of dialysate phosphate, sodium, and potassium on day 1, indicating the expected increment in these electrolytes due to the loss of kidney function (217). By day 21 of the APD period, and by virtue of the active peritoneal clearance, values of sodium and potassium in the PD fluid were similar in both groups. Serum levels of sodium and potassium were the same and confirmed the dialysate results. On day 21, phosphate levels reached the same levels in both dialysate and serum of the tested animals, both being within the normal range (1.71–2.68 mmol/L) (218, 219). Meanwhile, dialysate calcium levels remained higher in the anuric rats at day 1 and day 21. Renal failure is known to cause abnormalities in calcium and phosphate balance (168). Unfortunately, no test of the serum calcium profile was available. Glucose levels were higher in the rats with intact kidney function, with plasma being slightly in the upper range of the reference values (4.7–7.3 mmol/L) for both groups. Elevated blood glucose is commonly seen in PD of anuric patients with the use of high glucose PDF (169, 220). In general, the electrolytes profiles plus the MAP, Kt/V, and weight gain of the animals indicated adequacy in the APD system in kidney function in anuric rats.

This is the first paper to report the results of chronic APD on anuric animals with five dwells daily for three weeks. If translated to humans, this would be allometrically equivalent to approximately two years on dialysis (148). In clinical settings, ESRD patients in need of PD are uremic and will undergo treatment for a chronic period of time. Therefore, it is important to resemble the clinical setting when investigating PDF or PD quality. Taken together, it has been shown that our PD model may be a useful tool for PD research in general, and for understanding the microenvironmental physiology and pathology of the PM.

4.2 PAPER II: Pharmacokinetic properties of the nephrotoxin orellanine in rats

Clinically, mushrooms containing orellanine will induce renal failure 7 to 21 days after ingestion (5, 221). There have been no reports of the actual distribution or the pharmacokinetic profile of orellanine. Whole-body distribution and pharmacokinetics studies are important in order to acquire an understanding of which organs take up orellanine and the rate it is eliminated.

4.2.1 Radioiluminography

In order to conduct distribution tests, ^{3}H-labeled orellanine was injected intravenously in two groups of rats: one with intact kidney function prior to the ^{3}H-labeled orellanine dose, and one group that was chemically nephrectomized using a dose of 5 mg/kg of orellanine ip, two days prior to the administration of the ^{3}H-labeled orellanine. In both experiments, the radioactivity in blood gradually declined over time, from 8.6 nmol-eq/g for the single dose experiment at 0.5 h to 4.9, 2.0, 1.7 and 1.3 nmol-eq/g at 1, 2, 6, 12, and 24 h respectively. A similar pattern was seen in the nephrectomized rat experiments with values from 9.0 nmol-eq/g at 0.5 h to 12, 3.5, and 1.8 for the same time points. The quantitative evaluation of orellanine in different organs compared to heart blood in both the intact kidney and the nephrectomized rat groups are shown in Figure 5. Note that this method, like the beta scintillation, does not separate orellanine from its metabolites. Despite this fact, the kidney cortex and the urinary bladder showed the highest levels of orellanine at all time points.

This observation confirmed the distribution and clearance patterns of orellanine measured with UPLC-MS/MS and beta scintillation methods (see section below). A toxicological study with quantitative measurements of the damage to and amounts of orellanine in different organs will largely add to the knowledge of the actions of orellanine in the body but is outside the aim of this thesis.

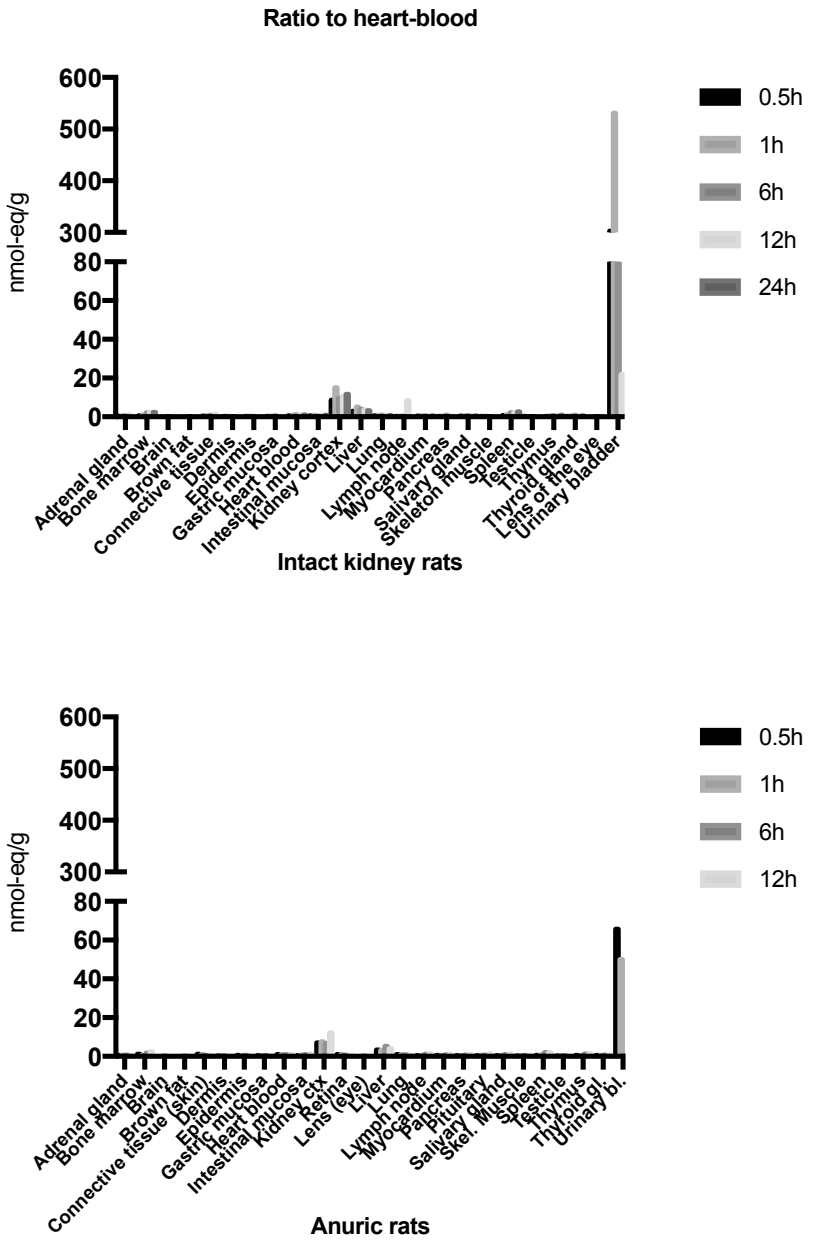

Figure 5: Tissue to heart blood ratios of the concentration of radioactivity after a single intravenous administration of ^{3}H-labeled Orellanine to the male rat (A) and after pre-treatment (chemical nephrectomy) with unlabeled orellanine (B).

4.2.2 The plasma half-life of orellanine

Orellanine is known for its complicated chromatographic behavior, and its thermo- and photo-liability, making it difficult to detect (111, 174, 222). Orellanine has been detected in plasma using various methods such as TLC, electrophoreses, ESR, HPLC, and LC/MS (3, 174, 223). Herrmann *et al.* used quadrupole time-of-flight mass spectrometry/mass spectrometry (QTOF-MS/MS) to detect orellanine in mushrooms, mushroom stew, and blood samples (112). However, there have been no reports of the measurements of the half-life or the elimination of orellanine in plasma. Such measurements are important for two reasons. Firstly, the measurement is important for diagnostics and clinical management of suspected cases of orellanine intoxication. Secondly, we are testing orellanine as a chemical nephrectomy tool (paper I of this thesis), and as a potential therapeutic option for metastasized ccRCC (paper III).

In order to investigate the half-life of orellanine, rats were given 5mg/kg of orellanine iv, and blood samples were collected 10, 30, 45, 60, 90, 180, and 360 min after administration. Three groups of rats were used in the experiment: one group with intact kidneys, one group with ligated kidneys, and one group with ligated kidneys that underwent PD. The half-life of orellanine was measured with UPLC-MS/MS and beta scintillation. The mass spectrum of orellanine (parent 253.04, fragment ion 236.2) detected by our method was in concordance with previously published spectra (176, 222, 224, 225). Orellanine was cleared from the kidneys with the lowest half-life in the intact kidney group (109 min on average) (see Figure 6). The short half-life of orellanine is in concordance with clinical experience in humans, where orellanine is not detectable in urine two days after exposure (3, 119). In animals, it was speculated that orellanine would be cleared from the body in less than two to three days (226).

In our experiment, the group of rats with ligated kidneys had the longest half-life. The group with ligated kidneys that underwent PD had a lower half-life and increased peritoneal clearance of orellanine compared to the group with ligated kidneys only (Figure 6), meaning that PD can help eliminate orellanine. ^{3}H-labeled orellanine measured with the beta scintillator showed higher half-life times in all the rat groups than measurements done with MS (Figure 6). The difference could be explained by the fact that the MS only measures orellanine, whereas the beta scintillation also picks up signals from any metabolites formed in the body. However, in our experiments, it is unknown whether these metabolites added to the toxicity of orellanine. Two metabolites of orellanine are known. One is produced by reduction of

orellanine and is toxic (orellinine), and the other is a product of orellanine reduction (orelline) and is nontoxic (223, 227, 228).

Our UPLC-MS/MC picked up metabolites with peaks at RT 3.5-4.5 min that were not present in the reference samples. They had the lowest levels in the rats with intact kidney function and highest in the rats with ligated kidneys. These metabolites fragmented in the ion source and were detected as orellanine (parent 253.04, fragment ion 236.2), which might have led to an overestimation of the intact orellanine levels with our UPLC-MS/MS method. We speculate that these metabolites can be glucuronides. Glucuronidation can result in metabolites having shorter RT than orellanine. The conjugation of hydroxyl groups in aromatic systems to glucuronic acid can form unstable metabolites in the ion source and can be detected as the parent compound (229). A previous paper published in our group showed that mono- and di-glucopyranoside are naturally occurring glucosides of orellanine in mushrooms and they were eluting with a shorter RT than orellanine (112). Acidic environments have shown to hydrolyze these metabolites to orellanine. When plasma samples were analyzed again after storage in an acidic environment, there was a further increment in orellanine levels. If such hydroxylation occurs in plasma, this will lead to an overestimated signal of orellanine in the chromatogram. In our setup, we tried to change the MS-method parameters to minimize ion-source fragmentation of such metabolites, but this led to diminished intensity of these metabolites, confirming that they are unstable during the ionization process. A suggestion for improvement of our UPLC-MS/MS method would, therefore, be to separate these metabolites at baseline, in order for their fragmentation products not being mistaken for intact orellanine. Such separation is hard to achieve in our setup for two reasons. Firstly, glucuronides are highly hydrophilic, and therefore tend to co-elute near the peak of the solvent they are in. Secondly, we speculate more than one glucuronide may be present in the plasma samples, leading to further complication of the chromatographic separation (230).

Nevertheless, UPLC-MS/MS detected orellanine levels as low as 0.04 µg/ml. Doses given with therapeutic purpose for ccRCC in rats were 10 mg/L in PDF, which counts for 10 µg/ml. Thus, our UPLC-MS/MS is suitable for measuring the future therapeutic window of orellanine.

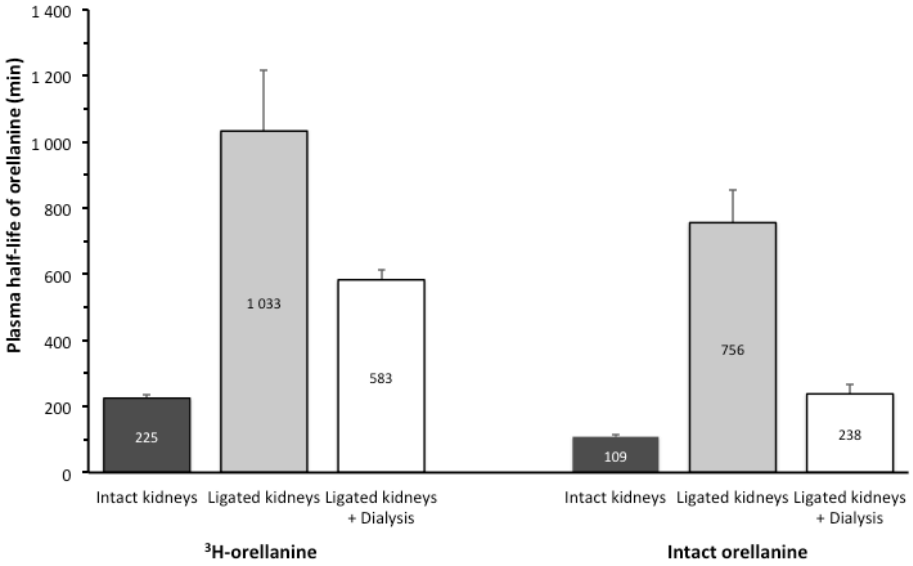

Figure 6: Plasma half-life of intact and ^{3}H-orellanine in intact kidneys, ligated kidneys, and ligated kidneys in rats on PD.

4.3 PAPER III: ORELLANINE SPECIFICALLY TARGETS RENAL CLEAR CELL CARCINOMA

Seventy percent of RCC are ccRCC, notorious for their metastatic frequency and high resistance to conventional cancer therapies (99, 231). Emerging therapies directed against specific molecular targets are not curative in ccRCC (98). We wanted to investigate the toxicity of orellanine in tubular and cancer cells. Since ccRCC originates from proximal tubular cells, we hypothesized that if orellanine is toxic to the tubular cells, it may also be toxic to the cells in the metastases of ccRCC.

4.3.1 *In vitro* tests

Although the mechanism of orellanine toxicity is yet not fully understood, the outcome of toxicity and selectiveness for PCT are well described (4, 109, 122, 221, 226, 232). The results in our study confirmed the findings in the literature: orellanine selectively and irreversibly damages HTEC while having a minimal and reversible effect on other tested cell lines, such as HUVEC, HPEG2, and MDA cells. Once orellanine was removed from the cultured cells, all cell lines recovered and continued to grow, except for HTEC. This is in line with the orellanine poisoning pattern, in which after the accidental intake of the mushroom, no other organ damage except for kidney failure has been observed in patients (5, 221). When investigating the effect of orellanine on different ccRCC primary cell lines (786-O, SKRC-7, SKRC-10, SKRC-21, 087), and metastatic cell lines (SKRC-17, SKRC-52), orellanine showed a dose-dependent effect with decreased viability of the cells. This effect increased with longer incubation times but not by repeated low dosing, which could be useful in the future planning of dosage regimens in treating metastasized ccRCC. Earlier studies of orellanine's mechanisms for cell damage have indicated ROS activation as a part of the mechanism (4) and our data confirmed these findings. Orellanine-treated cells showed a decreased ability to shift to the glycolysis pathway for generating energy once the mitochondrial pathway was shut down. Orellanine damaged the cellular metabolism through an increase in proton leakage, a decrease in ATP synthesis, and a global decrease in the respiratory capacity (basal, maximum, and spare capacity).

Apoptosis of the cells was investigated by studying the activation of cleaved caspase-3, as well as both the intrinsic (caspase-9) and the extrinsic (caspase-

8) pathways. Apoptosis and necrosis were evident in the aggressive and resistant metastatic ccRCC cell lines SKRC-17 and SKRC-52 after orellanine treatment. This was shown by western blot results and confirmed with activity assay results. Flow cytometry analysis of annexin V and PI staining of treated metastatic ccRCC cells showed a shift toward necrosis and apoptosis after 72 hours of cell incubation with orellanine. Western blot analyses of the phosphorylated protein kinase B, also known as AKT, showed decreased levels. AKT is a downstream effector of signaling pathways involved in glucose metabolism, apoptosis, cell proliferation, transcription, and cell migration. The decreased phosphorylation of AKT indicated a loss of ability to inactivate apoptotic pathways in treated cells. Extracellularly regulated kinase-1 and -2 (ERK1 and 2) are important proteins for cell survival functions, including the regulation of meiosis, mitosis, and post mitosis in differentiated cells. The expression of both ERK1 and 2 was found to be down-regulated after orellanine treatment. Proteomic analyses showed a battery of up-regulated apoptosis and necrosis activator proteins as well as loss of protein synthesis, cell migration, and protein transcription catalyzing proteins. Taken together, this shows that orellanine toxicity is not limited to proximal tubular cells but affects tumors and metastasis originating from these cells.

4.3.2 A xenograft rat model for metastasized ccRCC

Since our *in vitro* experiments showed the potential of orellanine to damage ccRCC-derived cells, we investigated the effect *in vivo* using a xenograft model for metastasized ccRCC in rats. In order to further immunosuppress athymic rats, they were subjected to a radiation dose of 4 Gray before xenograft implantation (details in the methods section). This led to the suppression of B-cell function without affecting the rats' general health in terms of appetite and growth. SKRC-17 cells were injected into the rats subcutaneously and allowed to form a tumor. When the cell mass had turned into a solid tumor (approximately after seven to eight days), intraperitoneal dosing of orellanine in PDF (10 mg/L) was administered with each dwell. Orellanine levels in the rats' blood reached a steady state after the first day. Since orellanine renders the rats anuric, the kidney function during treatment with orellanine was replaced using the APD system developed in paper I. Plasma levels of creatinine and urea raised to uremic levels with polyuria at day two and three, and anuria at day six. Thirty minutes after starting APD, the dialysate creatinine and urea levels approached those in plasma, indicating an efficient PD. The APD system functioned in replacing the

kidney function in all orellanine-treated rats. Eight days after the implantation of the SKRC-17 cell mass, 10 mg/L orellanine given for eight days shrunk the tumors and increased necrosis in the tumors significantly compared to untreated tumors. When given at day 4 after implantation of the ccRCC cell mass, orellanine at 10 mg/L for five days or 20 mg/L for three days led to an even more successful outcome. Extreme damage to the tumor mass was seen and a complete resolution of the tumor cell mass in two of the treated rats. The tumor masses collected from the treated rats were morphologically dark, soft, significantly lighter in weight, and 90% smaller in mass than the control rat tumors (Figure 7). Positive TUNEL staining was observed in 23 ± 7% in the 10mg/L dose group and 24 ± 5% in the 20 mg/L dose group. It is furthermore worth mentioning that gross morphological evaluation of the other organs by an independent pathologist revealed no damage on the brain, lung, heart, liver, spleen, intestine, or skeletal muscle tissue from the treated rats.

These results confirm that orellanine is highly toxic to human ccRCC cells even after metastases.

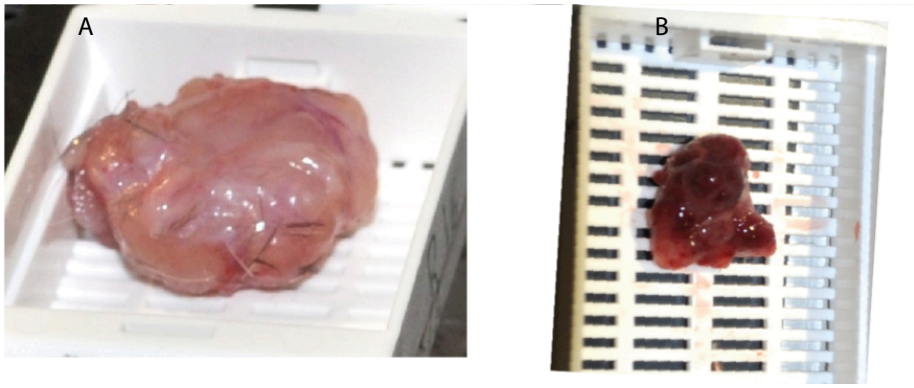

Figure 7: Representative pictures of matching SKRC-17 tumors from untreated (A) and orellanine-treated rats 24 h after treatment (B). Note: treated tumors are purple-colored and small, while untreated control tumors are hard, pink, and considerably larger.

5 CONCLUSIONS

Paper I

Orellanine can be considered as a chemical nephrectomy tool to induce anuria in experimental PD models. Our new APD method is efficient in maintaining anuric rats healthy and with normal homeostasis over a chronic period of time. Uremia did not have an independent effect on the dialysis procedure itself, did not induce peritonitis or PM damage, and did not alter the electrolyte profiles of the treated animals compared to rats undergoing APD with intact kidneys. The APD model developed is reproducible, can be applied to other animals, and may be useful in both PD research and in understanding the physiology and pathology of the PM in ESRD.

Paper II

Orellanine injected intravenously in rats *in vivo* is eliminated via the kidneys. The kidney cortex and the urinary bladder receive the majority of orellanine and its metabolites after administration. Orellanine forms metabolites in the blood that are eliminated with the same pattern as the intact parent molecule, albeit at a slower pace. PD and/or urinary excretion clear orellanine and its metabolites from the body.

Paper III

Orellanine selectively targets HTEC and ccRCC primary and metastasized cell lines both *in vitro* and *in vivo* in ccRCC bearing rats. Orellanine interferes with the energy metabolism, protein synthesis, and cellular growth, leading to decreased survival and induction of apoptosis and necrosis in treated cells. Orellanine, therefore, holds a promising capacity to be used as a potential therapeutic option for patients with late stage, metastasized ccRCC.

6 FUTURE PERSPECTIVES

This thesis examined the nephrotoxic and therapeutic effects of orellanine, highlighting the use of orellanine as a nephrectomy tool for PD research, as well as a therapeutic option for patients with metastasized ccRCC. Although this thesis has added to our understanding of orellanine and uses of the toxin, there is still more that needs to be investigated. Several challenges are ahead of us in order to take full advantage of the therapeutic potential of this nephrotoxin. Here follows some suggestions for future studies that would be valuable additives to our understanding of the subject.

6.1 TRANSPORTER STUDIES

Both literature and experiments in this thesis indicate that orellanine is selectively taken up by PCT cells, but the exact mechanism behind how orellanine enters the PCT cells is still unknown. Therefore, it is of great importance to study the uptake of orellanine into tubular and renal cancer cells. Since PCT are highly active cells with many different transporters involved in reuptake of filtered solutes from primary urine to circulation, it is difficult to test all transporters. One way to approach the large number of transporters and proteins important for orellanine toxicity is by utilizing a CRISPR-Cas9 library (233). A clustered regularly interspaced short palindromic repeats (CRISPR) library consists of approximately forty thousand guided genes (gRNAs) that can be incorporated into cells using viral transfection. These gRNAs will then connect to the CRISPR-associated 9 (Cas9) enzyme and create a complex that will target DNA and cleave a certain gene coding for a protein in the cell. If incorporated into orellanine sensitive cells, and the gene knocked out codes for the protein responsible for orellanine uptake, the hypothesis is that these cells will survive orellanine treatment. Surviving cells can, via deep sequencing, be screened for the knocked gene responsible for the cellular resistance to orellanine treatment. Possible transporters for orellanine will then need to be evaluated both *in vitro* and *in vivo* as the toxic effect of orellanine should be abolished by blocking the selective uptake mechanism. Finding the transporter and the signaling pathways for orellanine action will provide knowledge of its specific mechanism of action.

6.2 TOXICOLOGY STUDIES

Whether researching the use of orellanine in PD or as a drug against metastasized ccRCC, it is vital to evaluate the possible side effects of orellanine. Patients ingesting orellanine by mistake have not expressed any signs of organ damage other than kidney failure (5), but the amounts ingested are difficult to determine. Our gross pathological evaluation of the orellanine-treated rats confirmed the specific renal toxicity as well. Only one study in literature on mice has shown hepatic and splenic damage plus kidney damage (225). The only way to evaluate and quantify the potential damage is by running a toxicological *in vivo* test. Organ damage should be evaluated in combination with quantitative tissue LC/MS to analyze traces of orellanine in all the organs of the body. This will give a more complete picture of the elimination and toxicity of orellanine and will complement the distribution tests in paper II.

6.3 COMPUTATIONAL MODELING OF THE PERITONEAL MEMBRANE USING THE THREE-PORE MODEL

In order to further examine the PD model developed in paper I, peritoneal dialysis capacity can be measured in terms of the transperitoneal passage of water and solutes during PD with the three-pore model (TPM) described by Rippe and Haraldsson (234, 235). The TPM describes the transport of water and solutes from the capillaries in the peritoneum from three different pore sizes: Large (diameter 10–20 nm), small (4–6 nm), and ultra-small pores (mainly aquaporin-1, 0.4–0.6 nm). This technology is validated with clinical observations (236). With the aid of this method, a standardized measurement of the nutritional status, UF, residual renal function, total peritoneal clearance, and PM transport can be achieved. To analyze the properties of the peritoneum, we will use MathCad Prime 4.0 and Delphi Professional 10.2 Tokyo.

6.4 CHRONIC EFFECTS OF ORELLANINE ON METASTASIZED CCRCC

In paper III, we explored the effect of orellanine on metastasized ccRCC in an *in vivo* rat model. The treatment period was not more than eight days. This was due to the complexity of protecting immunocompromised anuric animals from infection after exposure to surgery and APD tubing. Several experimental sets needed to be terminated ahead of experimental study plans due to confirmed infections from intestinal *Enterococci*. Since orellanine had a dramatic effect on the metastasized SKRC-17 tumor masses, we hypothesize that a longer period of treatment will further increase the apoptosis and necrosis of these tumors and further reduce viability of the tumor mass. A xenograft mouse model could be utilized, in which instead of PD, the mice will be terminated before signs of uremia start. The implanted tumors will then be moved to the next mouse. Repeated xenograft transplantation will show the effect of orellanine chronically. An example of how such an experiment could be set up is shown in Figure 8.

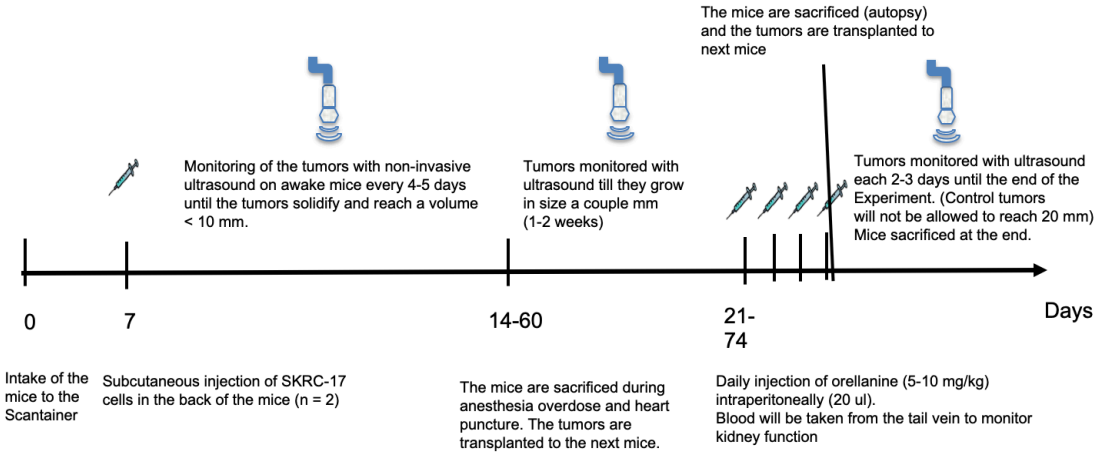

Figure 8: The working plan of a xenograft mouse model of ccRCC allowing for a longer exposure of orellanine.

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