Defining orellanine as treatment of advanced renal cell carcinoma

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Cover illustration: Photo of *Cortinarius Rubellus* modified with the chemical structure of orellanine in the upper left corner. Photo: Michael Krikorev. Reproduced with permission.

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ABSTRACT

Renal cancer causes over 100,000 annual deaths worldwide, and the incidence is increasing. Clear cell renal cell carcinomas (CCRCC), constituting 75% of renal cancer, are known for their high metastatic frequency and resistance to conventional therapies. Metastases are encountered in over half of patients with renal cancer, drastically reducing their life expectancy. Even with new specifically directed molecularly targeted therapies, the median survival of metastasizing CCRCC is less than one year.

Orellanine is a nephrotoxin found in fungi, and sometimes ingested accidentally. The ingestion of the fungi leads to renal failure and disruption of the proximal tubular cells. Interestingly, CCRCC originate from this cell type. Our hypothesis is that since proximal tubular cells in the kidney selectively take up orellanine, cancer cells in metastases of the same origin would also do so. This may give rise to a potentially curative therapy against metastasizing CCRCC. The aim of the thesis is to 1) Determine the efficacy of orellanine as a targeted therapy against clear cell renal cell carcinoma *in vitro* and *in vivo* 2) Establish a robust technique for detection of orellanine in plasma 3) Evaluate the long-term effects in patients after accidental intake of mushrooms containing orellanine.

We could demonstrate that orellanine induces dose-dependent cell death in a number of CCRCC cells while cells from other areas of the body remained unharmed. When we treated human CCRCC xenografts in nude rats with orellanine, the tumor cell mass was significantly reduced within a few days, featuring large apoptotic and necrotic areas. We could also detect orellanine with our newly developed analysis method in minute concentrations. This is necessary for monitoring orellanine concentrations in the body in a possible future clinical trial. The specificity for renal cells was evident in our study of the long-term outcome after accidental intake of orellanine-containing mushrooms. In these patients, we could not detect any difference in mortality or morbidity compared to age- and sex-matched controls.

In conclusion, this thesis shows that orellanine is indeed highly toxic to CCRCC cells both *in vitro* and *in vivo*. Orellanine seems to be highly kidney-specific with no long-term effects other than renal failure, which can be well dealt with using dialysis or renal transplantation. Orellanine thus has potential to become a new potentially curative treatment of metastatic CCRCC.

POPULÄRVETENSKAPLIG SAMMANFATTNING

Njurcancer drabbar över 100000 personer i världen och runt 1000 personer i Sverige varje år. Det finns flera olika sorters njurcancer, och klarcellig njurcancer är den vanligaste sorten (ca 75%). Om tumören hittas i tid kan den opereras bort från njuren med god prognos, men om den hunnit bilda dottersvulster i andra delar av kroppen (ca 60% av patienterna drabbas) är prognosen kraftigt försämrad. I dagsläget finns det ingen botande behandling för dessa patienter och hälften av dem dör inom ett år, trots behandling med nya molekylära målsökande terapier.

Klarcellig njurcancer har sitt ursprung i en specifik celltyp av njuren som kallas proximala tubuli. Svampgiftet orellanin, som återfinns i toppig giftspindling (som ibland förväxlas med trattkantareller), dödar specifikt dessa celler. Frågeställningen i avhandlingen är om orellanin också kan döda cellerna i metastaserna då de är av samma ursprung. Då skulle svampgiftet kunna användas som botande behandling av denna cancerform.

Våra studier visar att orellanin är starkt toxiskt mot klarcellig njurcancer både i cellodling och i en experimentell tumörmodell av human klarcellig njurcancer. Behandling av tumörerna med orellanin minskar tumörens vikt och andelen levande tumörceller minskar drastiskt. För att eventuellt kunna utnyttja orellanin terapeutiskt måste man kunna följa halten av orellanin i människa och djur noggrant. Därför etablerade vi en ny och mycket känslig metod för mätning av orellanin, baserad på masspektrometri, där vi kan mäta koncentrationer av orellanin som är lägre än en promille av den effektiva dos som förstör njurcancerceller. Orellanin tycks inte orsaka skador i några andra organ utan drabbar specifikt njurceller. Vi såg till exempel inte någon ökad sjuklighet hos patienter upp till 30 år efter oavsiktlig förgiftning med orellanin-innehållande svamp jämfört med ålders- och könsmatchade kontroller. Detta tyder på små eller inga biverkningar på andra organ än njuren vid cancerbehandling med orellanin.

Sammantaget har orellanin potential för att vara en botande behandling av spridd klarcellig njurcancer. Den njursvikt som orellanin skulle ge som biverkan kan framgångsrikt behandlas under lång tid med modern dialys eller njurtransplantation.

LIST OF PAPERS

This thesis is based on the following studies, referred to in the text by their Roman numerals.

I. Orellanine, a renal toxin, as novel targeted treatment of advanced renal cancer

Hedman H, Buvall L, Najar, D, Herrmann A, Roos E, Nilsson U, Johansson M, Nyström N, and Haraldsson B.

Manuscript

II. Analysis of the Mushroom Nephrotoxin Orellanine and Its Glucosides

Herrmann A, Hedman H, Rosén J, Jansson D, Haraldsson B, and Hellenäs K-E.

Journal of Natural Products 2012; 10: 1690-1696.

III. What is the long-term prognosis for patients poisoned by Cortinarius mushrooms?

Hedman, H, Holmdahl J, Nyström J and Haraldsson B.

Submitted

TABLE OF CONTENTS

ABBREVIATIONS

CCRCC	Clear Cell Renal Cell Carcinoma
CNT	Cortinarius NephroToxicity Index
Cy	Cyclophosphamide
DISC	Death Inducing Signaling Complex
ESL	Endothelial Cell Surface Layer
FADD	Fas-ligand Associated Death Domain protein
GBM	Glomerular Basement Membrane
GLUT-1	Glucose Transporter 1
HIF	Hypoxia Inducible Factor
IFN-α	Interferon α
MDR	Multi drug resistant
mRCC	Metastatic Renal Cell Carcinoma
mTOR	Mammalian Target of Rapamycin
NAC	N-Acetyl Cystein
NSS	Nephron Sparing Surgery
OAT	Organic anion transporter
OCT	Organic cation transporter
OS	Overall Survival
PD	Peritoneal dialysis
PDGF	Platelet Derived Growth Factor

PFS	Progression Free Survival
PFS	Progression Free Survival
P-gp	P-glycoprotein
RCC	Renal Cell Carcinoma
RN	Radical Nephrectomy
SGLT-2	Sodium Glucose Transporter 2
TAA	Tumor-associated Antigen
TNF	Tumor Necrosis Factor
TNFR	Tumor Necrosis Factor Receptor
TGF-α	Tumor Growth Factor α
TGF-β	Transforming Growth Factor β
TRAILR	TNF-related apoptosis-introducing ligand receptor
VEGF	Vascular Endothelial Growth Factor
VEGFR	Vascular Endothelial Growth Factor Receptor
VHL	Von Hippel-Lindau
VLDL-R	Very Low Density Lipoprotein Receptor

1 INTRODUCTION

Cancer induces 12.6 million new cases per year in the world and further causes 7.6 million deaths annually, thus rendering it as the second most common cause of mortality, after heart and cardiovascular events.[1] The hallmarks of cancer are; growth stimulation, resistance to growth-inhibitory signals, unlimited ability to divide, promoted angiogenesis, resistance towards apoptotic signals and metastatic capacity.[2] The prognosis varies for different cancer types as well as different subgroups within a distinct cancer form. Overall, the prognosis is impaired when the cancer has metastasized. Renal cancer claims 116,000 deaths per year worldwide[1] and is notorious for its high metastatic frequency and high resistance to chemo-[3] and radiotherapy.[4] Hence, patients with metastatic renal cancer have a truly poor prognosis. The lack of curative treatment results in a median survival of only 10 months.[5]

This thesis describes the initial evaluation of a fundamentally new experimental treatment against renal cancer, with actual curative potential. It originates from the discovery that low concentrations of the fungal nephrotoxin, orellanine, occurring naturally in several Webcap species, has a remarkable, previously unknown ability to kill cells of a specific subtype of renal cell carcinomas and metastases. Other cell types are unaffected. In the introduction section, various aspects of renal cancer, as well as the mode of action of orellanine, will be covered in some detail.

1.1 The kidney

The kidneys are the organs in the body responsible for long-term acid-base balance, maintenance of water and electrolyte balance, osmolarity regulation, hormonal production including erythropoietin, elimination of metabolic waste products, and excretion of exogenous water-soluble compounds. The urine volume produced daily through the capillaries in the glomerulus is 180 liters. Due to the reabsorption in the tubular compartments, the final urine leaving the body is approximately 1.5 liters. All of the essential procedures in the kidney take place in the nephron, which is the smallest functional unit of the kidney. 90% of the nephrons are located in the cortical, outer part of the kidney. The nephron consists of a capillary tuft, called the glomerulus, surrounded by Bowman's capsule, which is connected to a convoluted tubular part. The glomerular cell components represent approximately 5% of the cortex in kidney, whilst the remaining 95% have a tubular origin. Each kidney contains approximately one million nephrons.

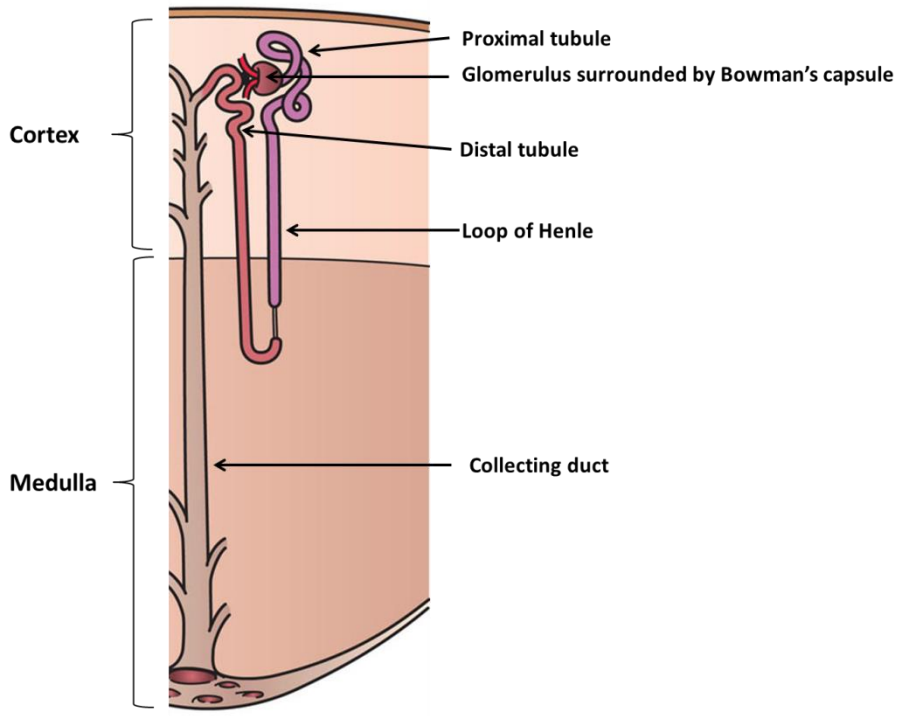

Figure 1. Illustration of a nephron. Modified from University of Michigan (open.michigan,http://open.umich.edu, Urinary tract)

1.1.1 The glomerular filtration barrier

The glomerular filtration barrier is similar to a highly selective sieve, highly permeable for water, electrolytes and small molecules, while normally completely preventing blood cells and proteins from passage. The glomerular barrier is composed of four specific layers from blood to urine: The endothelial cell surface layer (ESL), the fenestrated endothelial cell, the glomerular basement membrane (GBM) and the podocyte with its slit membrane.[6]

The ESL is a gel-like structure lining the endothelial walls in the glomerulus. It consists of two components; the glycocalyx, which is covalently bound to the endothelial cells, and the more loosely attached endothelial cell coat.[7] The ESL is composed of a variety of molecules (e.g. proteoglycans and glycoproteins). It is highly negatively charged, and the charge is believed to be a requirement for the permselective properties of the filtration barrier.[8]

The endothelial cells of the glomerular capillaries are distinguishable from other endothelial cells, due to their abundant fenestrations. The fenestrae cover 30-50% of the glomerular endothelial surface.[9] They are 60-80 nm in diameter and would be permeable for plasma proteins, like albumin, if the endothelial cells and the fenestrae were not covered by the ESL, which acts like a plug.[10,11] The glomerular basement membrane provides adjacent cells with attachment support. On the vascular side, the endothelial cells are attached to the GBM and the podocytes are attached on the other side, facing the urinary space. The GBM also functions as a barrier to prevent filtration of macromolecules.[12,13] Interestingly, the GBM is built up by components produced by the endothelial cells and the podocytes, like laminin and collagen type IV.[12,14]

The "outermost" layer in the glomerulus is made up by epithelial cells (podocytes). This specialized cell has a large cell body with foot processes that encircle the GBM and the entire glomerulus. The small gaps between the foot processes are called slit diaphragms. The slit diaphragm is stabilized by several proteins important for the filtration integrity of the glomerulus, like nephrin, podocin and CD2AP. [15]

1.1.2 The tubular compartment

The tubular compartment is anatomically divided into the proximal tubular region, the loop of Henle, the distal tubulus and finally the collecting duct, discharging the urine into the renal pelvis. The proximal region and the loop of Henle are each about 15mm in length, while the distal tubules are shorter (3-4 mm). The majority (2/3) of the urine produced by glomerular filtration is reabsorbed across the proximal tubular cells, amounting to 120 liters per day. Water moves through water channels (aquaporins), or paracellularly driven by the crystalloid-osmotic pressure created by the Na-K-ATPase situated on the basolateral side of the proximal tubular cells, facing the peritubular capillaries. A multitude of vital solutes are taken back from the urinary space together with sodium through selective transporters driven by the sodium concentration gradient. Several other ions are readily reabsorbed in the proximal tubular segment, like Cl^- and Ca^{2+}.[16] Other substances being reabsorbed to more than 99% are glucose, amino acids, and bicarbonate. Other inorganic ions (e.g. phosphate) and organic acid metabolites are also reabsorbed to a large extent.[17] Some selective transporters will be described in detail below. The proximal tubular cells also reabsorb sodium via the Na^+/H^+-exchanger 3 (NH3).[16] The sodium reabsorbed into the proximal tubular cells must exit the cell on the abluminal side, against its electrochemical gradient, to maintain the sodium concentration gradient

across the cell membrane and hence the sodium-dependent luminal reabsorption. This is made possible by abundant quantities of Na^+-K^+-ATPase, requiring constant and abundant supply of energy, which is why the proximal tubular cells are densely populated with mitochondria. Thus, the proximal tubular cells are metabolically very active and have a high oxygen consumption.

In this context, it is not surprising that the proximal tubular cells are highly susceptible to ischemia. It has been shown that loss, malfunction, or death of these cells is the main cause behind acute kidney injury (AKI). When proximal tubular cells are dying or malfunctioning, they lose their polarity, Na-K-ATPase becomes evenly distributed all over the cell surface, directional sodium transport ceases and fluid cannot be reabsorbed. [18]

Several pathophysiological conditions involve the proximal tubular cells. One example is the Fanconi syndrome, which is a generalized concept for the dysfunction of the reabsorption in the proximal tubules.[19] A patient with Fanconi syndrome loses glucose, vitamins, amino acids, proteins and other important substances via the urine due to impaired tubular re-uptake.

Transporters within the tubular compartment

In this thesis, I have studied a substance that is thought to be specifically and selectively taken up by proximal tubular cells. It is therefore prudent to discuss in more detail some of their known transporters. There are two main superfamilies of transporter proteins: the ATP binding cassette (ABC) transporters and the solute carriers (SLC). [20] Within the latter family, a huge variety of substrates are being transported; organic and inorganic cations and anions, amino acids, vitamins, glucose, urea and lipids among several others. [21] The proximal tubular cells are rich in a variety of transporters. Below, a few are mentioned.

Glucose transporters

Glucose reabsorption and transportation back to the blood is performed by sodium glucose transporters (SGLT) and glucose transporters (GLUT). Glucose is filtered freely through the glomerulus and with a plasma concentration of 5 mM, or 1 g/l, about 180 grams is filtered each day. Due to the effective reabsorption of glucose in the proximal tubules, almost no glucose is eliminated via the urine. Glucose is mainly reabsorbed in the first segment (S1) through a sodium co-transporter, sodium glucose transporter 2 (SGLT-2), which reabsorbs 90% of the glucose. Further down the tubular segment, SGLT-1 reabsorbs the remaining 10%. [22] Glucose transporter 2

(GLUT-2) excretes glucose from the proximal tubular cell to the interstitium on the blood-side. [23]

Organic anion transporters

Organic anion transporters (OAT) family

The organic anion transporter (OAT) family belongs to the SLC superfamily of transporters. They are responsible for uptake and elimination of a variety of organic anionic substances, (e.g. drugs and metabolites) in the proximal tubular cells in the kidney. They are localized either on the apical or basolateral membrane of the proximal epithelial cell and are often working in cooperation. OAT1 and OAT3 are situated on the basolateral side, transporting drugs from the blood stream into the proximal tubular cells and dicarboxylate in the opposite direction.[24,25] They are also responsible for uptake of glutathione.[26] On the apical membrane, OAT4 and NST/URAT eliminates substances from within the cell in combination with reabsorption of certain substances from the urine.[24] OAT4 exchanges organic anions from the urine with dicarboxylate, while URAT1 reabsorbs urate from the lumen and elimintates anions.[25] Hence, the proximal tubular cells eliminate drugs by extracting them from the blood and extruding them into the urine and thus eliminating them from the body. Drugs that are thought to be eliminated in this manner are numerous, like penicillin, statins, antiviral drugs, loop diuretics, and non-steroid anti-inflammatory drugs (NSAIDs).[24,27] Examples of metabolites are p-aminohippurate (PAH) and prostaglandin E2.[28]

MRP (Multi drug resistant proteins)

The multidrug resistant proteins were initially discovered in cancer cells, highly resistant to chemotherapeutic agents [29]. They belong to the ABC superfamily. MRP2[30] and MRP4 have been localized to the apical membrane of the proximal tubules while MRP6 is found basolaterally.[31] MRP2 is responsible for excretion of anti-neoplastic drugs like vinblastine, adriamycin, antiviral compounds and other organic anions into the urine.[25] MRP4 transport cAMP, cGMP and urate into the lumen. The function of MRP6 remains elusive.[31,32]

Organic cation transporters

OCT (Organic cation transporter) family

The organic cation transporters (OCTs) are members of the SLC family. There are several members in the OCT family, but so far OCT1 has been shown to be present on the apical membrane of human proximal tubular cells[33] and OCT 2 on the basolateral membrane.[34] OCT2 transports cations

from the interstitium into the proximal cells while OCT1 performs the same task on the apical membrane and it is thought to among other substances reabsorb metmorfin from the urine. Both transporters mediate the uptake of tetraethylammonium (TEA) and 1-methyl-4-phenylpyridinum (MPP) among other organic cations.[34]

MATEs

Another family within the SLC family is the multidrug and toxin extrusion (MATE) transporters. MATE1 and MATE2-K are found on the apical membrane in proximal tubular cells. They are H+/organic cation antiporters, transporting H^+ along its concentration gradient into the cell and extruding organic cations into the lumen. They extrude the TEA and MPP, taken up by the OCTs. They also transport creatinine, guanidine and estrone sulfate to the urinary space.[35]

P–Glycoprotein/MDR1

An additional organic cation transporter is P-Glycoprotein (also known as MDR1), a member of the ABC super family.[29] Like the MRP family, P-glycoprotein (P-gp) was first identified as a drug extruder in cancer cells. It is located on the apical membrane of proximal tubular cells,[36] where it excretes drugs such as taxol and steroids into the urine and other organic cations. [31]

Megalin and Cubilin

Although the glomerular filtration restricts proteins from being filtered into the urine, small amount of plasma proteins such as albumin actually do pass into the urine. Most of these proteins are however recovered in proximal tubular cells by megalin[37] and cubilin[38] receptor endocytosis. The receptors are present at the apical membrane and bind a diversity of different substances. [37] Megalin in combination with cubilin binds to practically all proteins, like albumin, insulin, apolipoproteins and vitamin-associated proteins. [39] Megalin is the transmembrane protein of the two and hence responsible for endocytosis of the bound compounds of cubilin. Another protein, amnioless, is required for the normal function of cubilin.[37] When internalized, the vesicle is transported to the lysozyme, in which proteins are degraded to amino acids and are returned to the circulation with the vitamins. The megalin and cubilin receptors are transported back to the apical membrane in a process requiring a specific chloride channel ClC-5.[37,39,40]

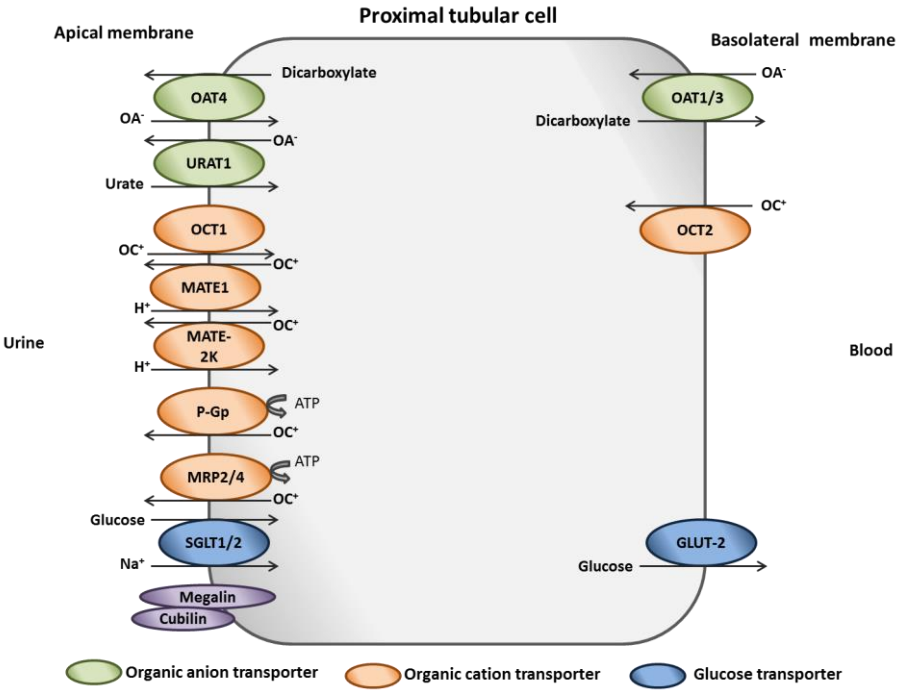

Figure 2. Some of the transporters of the proximal tubular cell in the kidney.

1.2 Renal cancer

With an incidence of about 2 % of all solid tumors, cancer of the kidney is not as common as breast or prostate cancer. However, it still claims 116,000 lives every year out of the 274,000 new cases diagnosed worldwide.[1] The most common form of renal cancer is the conventional (clear cell) renal cell carcinoma (CCRCC). It constitutes 75% of all renal carcinomas. The remainder is distributed between papillary renal carcinoma (10-15%), chromophobe renal cell carcinoma (5%), collective duct carcinoma (1%) and unclassified renal carcinoma (3-5%), according to the Heidelberg classification from 1996[41]. Sarcomatoid appearance may be present in all of the subtypes of renal cancer, and is a hallmark for high transformation[41] Furthermore, it is associated with a worse prognosis.[42,43] The absolute majority of renal cancers are formed from cells in the tubular system in the kidney. Wilms' tumor is an exception, since it seems to originate from the glomerular podocyte that express the same Wilms' tumor (WT) antigen.

1.2.1 Incidence, risk factors and prognosis

The global distribution of renal cancer varies, with the highest incidence in Europe, North America and Australia. In Sweden, the trend is a slight decrease over the last decade.[44] In contrast, incidence is rising in the United States.[45] In general, there is a male predominance of 2:1. Smoking is an established, dose-dependent risk factor for renal cell carcinoma (RCC).[46-48] Obesity and hypertension have also been shown to be risk factors for RCC[49-51], while fruits and vegetables are believed to have a protective effect.[52] The average age at diagnosis and surgery is 61 years.[53] Overall survival varies with tumor status at diagnosis. Patients with local tumors at diagnosis, status T1 or T2 (please see Appendix for tumor staging), have a 5-year survival of over 80%.[54 55] Furthermore, the prognosis and survival also vary with specific subtypes of RCC, with a worse prognosis for CCRCC compared to papillary RCC for T1 and T2 tumors.[56] If the cancer has metastasized, the prognosis is significantly impaired.

1.2.2 Clinical manifestation

Renal carcinoma is generally asymptomatic and the disease is typically discovered incidentally. The most common clinical manifestation is macroscopic hematuria, which is present in approximately 40% of the patients.[57] Previously, a classic triad of symptoms was expected by the clinicians namely hematuria, flank pain and a palpable tumor. However, these three symptoms combined occur only in a minority of the patients, less than 10%, and usually at an advanced state of the disease.[58] With the evolution of sophisticated detection techniques (e.g. computed tomography), tumor discovery has improved during the last decades.[59] This leads to tumors being detected at an earlier stage, and concomitantly less than 1% of cases are currently diagnosed by the classic triad.[60] The symptoms of renal carcinoma differ notably between patients and are often diffuse. Examples of clinical indications displayed during renal carcinoma include hematuria, fever, flank pain, weight loss, hypercalcemia, anemia, hypertension, loin pain and hepatic dysfunction, all of which could be indicative of a variety of disorders.

Renal cancer, including renal cell carcinoma, is classified according to the TNM staging system. This system serves as a guide for treatment as well as for the prediction of patient outcome. Please refer to Appendix for details of the classification.

1.2.3 Primary tumor treatment

The sole curative treatment of renal cell carcinoma available today, is surgical removal of the entire tumor mass if it is localized to the kidney.[61] This can be achieved either by radical nephrectomy (RN), i.e. removal of the entire kidney, or by nephron-saving surgery (NSS). Nephron-saving surgery has been shown to be equally good in terms of overall survival for renal cancers for tumors fully localized to the kidney and smaller than 4 cm. Thus NSS is the recommended procedure for this tumor size.[62]

1.2.4 Metastatic tumor

The prognosis for patients with metastasis at diagnosis is significantly reduced; the 5-year survival is less than 10%.[54,55] Approximately 30% of the patients have an advanced disease at diagnosis and furthermore one third with localized tumor initially will subsequently develop metastases.[54 63] The most common metastatic site is the lungs, followed by bone, lymph node, liver, adrenal gland and brain (see Table 1).[64]

If the metastatic lesion is limited, it can be excised as adjuvant therapy. This has mostly been applied to lung metastases, where the 5 year survival is 40% after complete resection.[65] However, in most patients, metastatectomy is performed mainly for musculoskeletal integrity or pain control.[66]

Two prospective randomized trials have shown an improvement of median survival time in patients with metastatic disease submitted to cytoreductive nephrectomy, (i.e. removal of the primary tumor), followed by interferon-α (IFN-α) treatment, compared to patients receiving IFN-α treatment alone. In the studies, the median survival in the surgery plus IFN group was 17.4 versus 11.7 months and 17 versus 7 months, respectively.[67,68] In combination, these trials showed an improvement of median survival of 13.6 over 7.8 months, which was statistically significant.[69] This has however, not been studied in other systemic treatment strategies, (e.g. targeted therapies) of mRCC.[70] In less than 1%, metastases (lung metastases most frequently) have gone through spontaneous remission, typically after the excision of the primary tumor.[71,72]

Metastatic sites	
Site	**%**
Lung	45
Bone	30
Lymph node	22
Liver	20
Adrenal	9
Brain	8
Other	8

Table 1. Distribution of renal cancer metastases sites.

1.2.5 Systemic treatments of advanced disease

Standard treatments of cancer (i.e. chemotherapy [3] and radiation therapy), are generally ineffective against renal cell carcinoma.[4,73] Immunotherapy have been the standard care of mRCC for the last decades. Targeted therapies have emerged during the last 10 years, providing an increase in progression free survival (PFS) and a few months extended overall survival (OS). They are discussed briefly below.

Immunotherapy

In immunotherapy using cytokines, the object is to stimulate tumor-infiltrating lymphocytes and natural killer cells to react against the tumor cells.[74] The two main candidates used as treatment for mRCC are interferon-α (IFN- α) and interleukin-2 (IL-2).

IFN–Y

IFN-alpha was explored early as treatment in combination with vinblastine chemotherapy. The combination of IFN-alpha and vinblastine compared to vinblastine alone had a significant effect in median survival (67.6 versus 37.8 weeks).[75] Subsequent studies, comparing IFN-alpha alone against a

combination of IFN-alpha and vinblastine showed no increased benefit for the combination; hence IFN-alpha was considered solely responsible for the survival benefit. A Cochrane review, from 2005, showed increased overall survival in IFN-alpha treated patients compared to control. The one-year mortality odds ratio (OR) was 0.56 [0.40, 0.77] for the pooled studies of 615 patients.[76] However, survival benefits were generally modest for IFN-alpha.

IL–2

Interleukin 2 (IL-2) was the first treatment approved by the food and drug administration (FDA) for treatment of mRCC. In a clinical trial with 255 patients, high dose IL-2 induced a complete response in 5% of the patients and partial response in 9%. However, performance status was a predictive factor of the response to IL-2 and severe side effects were reported. In total, 4% of the patients died, likely due to the treatment, reflecting a relatively high toxicity for high dose Il-2.[77] When reducing the dose to 90% of the high dose, reduction of severe side effects was observed. The response rate was significantly higher in the high-dose patients compared to low-dose (21% vs. 13%). However, in terms of overall survival no significant results were obtained. Using low dose, interleukin 2, administered subcutaneously, instead of intravenously, further reduced the side effects observed, with similar response rates.[78] In the Cochrane review from 2005, a slight benefit for IFN-alpha over low dose IL-2 was seen (0.93 [0.66, 1.31]).[76] However, there are no studies comparing the two cytokines, using high IL-2, in analysis in overall survival.

VEGF, VEGFR and mutityrosine kinase inhibitors

Since the detection of the von-Hippel Lindau (VHL) protein in clear cell renal cell carcinoma (please see section below), therapies targeting the molecular pathways activated during VHL-deficiency have emerged. Mainly, these therapies affect neo-vascularization by inhibiting either vascular endothelial growth factor (VEGF) or its receptor VEGF-receptor (VEGFR).

Sorafenib

Sorafenib acts by inhibiting the VEGFR-2 and 3 and platelet derived growth factor receptor (PDGFR). It also targets RAF signaling, thus inhibiting both angiogenesis together with proliferation.[79] It has been shown to induce longer progression-free survival in IFN-a drop out patients, but has so far not been able to produce increased overall survival (OS). Combinatory studies, using Sorafenib and IFN-alpha at low doses, have also been ineffective.[80]

Sunitinib

Sunitinib targets the VEGFR and PDGFR.[81] In a clinical phase III trial, Sunitinib increased progression free survival compared to IFN-alpha with 6 months (11 vs. 5) and overall survival with 4.6 months (26.4 vs. 21.8). Both Sunitinib and Sorafenib have significant side effects, including hypertension, fatigue, and diarrhea.[82]

Bevacizumab

Bevacizumab is a monoclonal antibody, directed against soluble VEGF.[83] In a phase III trial, combining bevacizumab with IFN-alpha versus IFN-alpha and placebo, a progression free survival benefit was observed in the bevacizumab arm (10.2 vs. 5.4 months). OS was not possible to assess. Known side effects of bevacizumab include proteinuria, bleeding and hypertension.[84]

Axitinib

Axitinib is an inhibitor of the VEGF-receptors.[85] In a phase III trial, axitinib was compared to sorafenib, as a second line treatment. Results showed increased progression free survival in the axitinib arm (6.7 vs. 4.7 months). Side effects of axitinib are diarrhea, fatigue and hypertension.[86]

Pazopanib

Pazopanib blocks the VEGRF, PDGFR and c-Kit.[87] A phase II trial of pazopanib versus placebo showed a tendency to increase in OS for pazopanib (22.9 vs. 20.5 months). This was not significant, though, probably due to crossover from the placebo arm to the treatment arm. Side effects reported where diarrhea, hypertension and liver abnormalities.[88]

Tivozanib

Tivozanib inhibits the VEGFR.[89] In a phase III study, tivozanib was evaluated as a first or second line treatment versus sorafenib. Previous treatment with VEGF/VEGFR or mTOR inhibitors was not allowed. PFS was significantly longer in the tivozanib group (11.9 vs. 9.1 months). In terms of overall survival, sorafenib did show slightly increased survival (29.3 vs. 28.8), but it did not reach statistical significance. Side effects for tivozanib (e.g. hypertension) were more common compared to sorafenib.[90]

Mammalian target of rapamycin (mTOR) inhibitors

The mammalian target of rapamycin (mTOR) is participating in proliferation in cells by activating AKT/PI3 kinase pathway. By inhibiting mTOR, proliferation is impaired and it has been shown that blocking of mTOR activity also reduces the amount of HIF-α and VEGF.[91]

Temsirolimus

Temsirolimus efficacy on renal cell carcinoma was assesses in untreated, poor risk patience, versus IFN-α and IFN-α and temsirolimus. Temsirolmus alone had the best OS with 10.9 months versus 7.3 months for IFN-α and 8.3 for IFN- α plus temsirolimus. In the combination group, both the IFN-α and the temsirolimus dose were lower than in the single treatment groups. Reported side-effects for temsirolimus are rash, peripheral edema, hyperglycemia and hyperlipidemia.[92]

Everolimus

Everolimus versus placebo was evaluated in a phase III trial, as secondary treatment after progression post VEGRF treatment. PFS was 4.9 months for everolimus versus 1.9 months for placebo. Overall survival did not show any significant effect (14.8 vs. 14.4 months). This might be due to 80% cross-over of the patients from the placebo arm to the everolimus arm. Side effects reported were infection, dyspnea and fatigue.[93]

Vaccine therapies

Vaccine therapy is a new type of therapy explored within the cancer field during the last decade. There are several different types of vaccines including whole tumor vaccines, peptide vaccines, dendritic cell vaccines, DNA-vaccines[94] and viral vector vaccines.[95] In general, they exploit the fact that tumors express antigens not present on normal tissues. The vaccine directs the body's immune system to destroy these tumor-associated antigen-presenting cells. Several vaccines' efficacy has been explored in clinical trials. For a dendritic cell vaccine, AGS-003, a phase III study is planned for mRCC patients, in combination with sunitinib.[96] Two additional different vaccines are discussed below.

Viral vector vaccine, MVA–5T4

In a phase II trial on mRCC patients using a viral vector, the modified vaccine Ankara (MVA-5T4), with or without IFN-α, the vaccine treatment alone showed promising results in an increased OS (18.3 vs 5.9 months).[97] However, in a following phase III trial, with combination of MVA, plus IL-2, no difference in OS was achieved 20.1 vs. 19.2 months.[98] The vaccine was well tolerated and the only adverse side effect reported was soreness at the injection site.[99]

Multi–pepetide vaccine, IMA901

The use of a multi-peptide vaccine, IMA901, consisting of 10 different tumor-associated peptides, was investigated in a phase II trial of mRCC. Patients, progressing on prior systemic treatment, were treated with IMA-901

with or without a single-dose cyclophosphamide (Cy). Cy was used with the intention to reduce the amount of regulatory T-cells. Results showed a trend towards increased OS in the combinatory group, (23.5 vs. 14.8 months). The effect could, however, not be contributed to Cy alone, since in the non-responder patients, the OS did not differ between the two treatments.[100] A phase III trial on mRCC patients is ongoing where IMA901 is combined with sunitinib.[101]

1.2.6 Conventional (Clear cell) renal cell carcinoma

The most common form of renal cancer is called conventional, or clear cell, renal cell carcinoma (CCRCC). It originates from the proximal tubular epithelial cells in the kidney[102,103], although a more distal origin has been proposed.[104] The histologic appearance is characterized of a clear cytoplasm, due to a high lipid and glycogen content.[105,106]

Staging and prognosis

Clear cell renal cell carcinoma form has the worst prognosis of the three most common RCCs; i.e. clear cell, papillary and chromophobe RCC.[56] This is true especially for smaller tumor sizes (T1 and T2). The metastatic frequency is also higher for clear cell carcinomas. The 5-year overall survival is 50% for clear cell carcinoma [56] and for the patient groups presenting an advancement of the disease at diagnosis it is less than 5% with a median survival of 6-10 months for this patient group.[55] When a sarcomatoid component is present, the prognosis is even further impaired, since sarcomatoid CCRCC has been shown to be substantially more proliferative and displays a mesenchymal phenotype.[107]

Von-Hippel Lindau protein

Several cancer forms, among them CCRCC, is common in familial von Hippel-Lindau (VHL) disease. A molecular marker for the disease is a germ line mutation harbored in the tumor suppressor VHL gene.[108,109] The gene was first identified in 1993 on chromosome 3p25-p26.[110] In sporadic forms of CCRCC, the VHL-protein (pVHL) is frequently inactivated.[111-113] Loss of pVHL have not been shown to be sufficient for development of cancer on its own, it rather requires other genetic modifications.[114,115] However, patients with VHL syndrome develop renal cancer or cysts at a median age of 39 years.[116]

The pVHL is involved in degradation of the α-subunit of hypoxia inducible factor (HIF-α). At normoxia, HIF-α is hydroxylated, marking it for

ubiquitination by pVHL and hence degradation. At hypoxic conditions, or when pVHL is inactivated, hydroxylation does not take place, thus stabilizing HIF-α and promoting its dimerization with HIF-β. The active HIF-complex translocates into the nucleus, acting as a transcription factor to promote gene expression for a variety of genes including vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), tumor growth factor alpha (TGF-α) and glucose transporter 1 (GLUT-1). This promotes angiogenesis, cellular progression, metastasis and increased glucose metabolism. Loss of VHL thus has a considerable impact on the tumor genesis of CCRCC and gives rise to highly vascularized tumors.[109,117]

Abnormal levels of HIF-1α have also been shown to be partly responsible for the high lipid accumulation in clear-cell renal cancer cells, due to increased expression of very low-density lipoprotein receptors (VLDL-R). A partial knock-down of HIF-1α or VLDL-R, reduced the lipid accumulation.[118]

Defects or loss of VHL leads to increased secretion of transforming growth factor β (TGF-β) and sensitivity to TGF-stimuli in clear cell renal cancer cells. Augmented TGF-signaling correlates with worse prognosis. Interestingly, patients with metastatic CCRCC present with increased TGF-activity.[119] Genetic mapping have shown extensive overlapping of the HIF and TGF-β signaling pathways, indicating a cross-talk between the two pathways.[120]

1.3 Orellanine

Orellanine is the toxin responsible for the intoxication subsequent to ingestion of fool's webcap (*Cortinarius Orellanus*) or deadly webcap (*Cortinarius Rubellus* formerly called *Cortinarius Speciossiumus*). The toxicity of these toadstools was first detected by Grzymala[121] after a mass poisoning in Poland in 1952, where over 100 people were affected. Grzymala also isolated, characterized and named the toxin in 1962.[122] The chemical structure was revealed in 1979, by Antkowiak and Gessner,[123] to be 3,3',4,4'-tetrahydroxy-2-2'-bipyridine-N-N'-dioxide. Further, it was synthetsized in 1985.[124] Orellanine is photosensitive and decomposes when exposed to UV-light into orellinine and orelline. The former is also nephrotoxic whilst the latter is non-toxic.[125] Orellanine-containing mushrooms are distributed both in Europe and North America, with *Cortinarius Rubellus* being more frequent in Northern part of Europe (i.e. Scandinavia) and *Cortinarius Orellanus* in the central and Southern parts.[126]

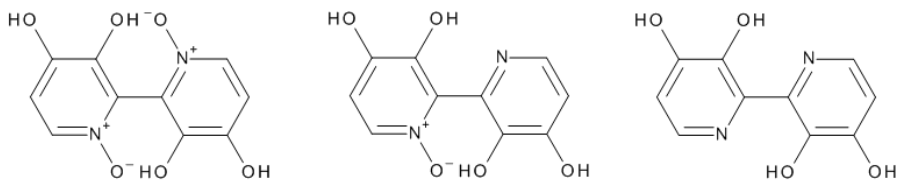

Figure 3. The structures of orellanine, orellinine and orelline, respectively.

1.3.1 Clinical manifestations of orellanine poisoning

The latency phase from ingestion to clinical manifestation varies from 12h to14 days. In general, a longer incubation phase is associated with a better prognosis. Of 90 intoxication cases, reviewed by Danel et al, about one third of the patients developed chronic renal insufficiency. The remaining patients regained their kidney function within weeks to months.[127] The toxin effect is dose-dependent, but there is considerable individual variation in sensitivity.[128] This has been confirmed also in animal studies.[126,129] Holmdahl developed an index to predict the prognosis, based on the creatinine level in relation to the days since the mushroom ingestion.[126] For patients with end stage renal failure, lifelong dialysis will be necessary, unless kidney transplantation is an option.

1.3.2 Histological and cellular effects of orellanine

The clinical symptoms of patients suffering from orellanine intoxication are similar to those seen in animal studies. Characteristics are tubulo-interstitial damage with edema and infiltration of immune cells.[126,130-132] More careful histological examinations have revealed that it is the proximal tubular cells in the kidney that are affected by orellanine.[132,133] The toxin is believed to be taken up selectively by the proximal tubular cells and is thought to be sequestered there.[134] Orellanine has been reported in kidney biopsies as long as 6 months after ingestion.[131] However, orellanine cannot be detected in plasma or urine and after the manifestation of clinical symptoms it is found exclusively in the kidneys.[134]

The mode of action of orellanine remains somewhat elusive. It is clear that orellanine causes increased oxidative stress, both in vitro[135] and in vivo,[136] and markedly down-regulates the oxidative stress defense.[136] Also, inhibition of protein synthesis have been reported.[137] Antioxidant therapy using N-acetylcystein (NAC) in patients after orellanine intoxication has been tried in

a few patients.[138,139] They presented with mild (n=2) to intermediate (n=1) intoxication symptoms when scored according to the Cortinarius NephroToxicity Index (CNT).[126] All of the three patients recovered, two where dialysis dependent during a treatment period and one never required dialysis. Hence, the evidence regarding the possible benefit of NAC after orellanine intoxication is inconclusive.

1.4 Reactive oxygen species

Reactive oxygen species (ROS) is produced in the cells normally as a by-product of cellular metabolism.[140] It may also act as a messenger in signaling pathways.[141] There is a strict balance of the ROS-levels and the antioxidant defense in the cell. Uncontrolled ROS levels, produces oxidative stress and may induce severe damage. This may induce oxidation of lipids, proteins and DNA.[142] Also mitochondrial DNA is susceptible to oxidative damage. [143] The damages may induce cell death in form of apoptosis or necrosis.[144] Interestingly, in cancer cells, the oxidative stress is increased together with anti-oxidative defense.[143] Hence, the oxidative level in these cells is higher compared to the reducing level, which is the contrary in normal cells. The most common oxidative stress radical is the superoxide anion (O_2^-), formed from oxygen by an electron addition. The anti-oxidant enzyme, superoxide dismutase (SOD) catalyzes the reaction of the superoxide anion to the non-radical but still a ROS-molecule; hydrogen peroxide (H_2O_2). This is further converted to water by another important anti-oxidant enzyme, namely catalase (CAT).[141]

1.5 Apoptosis

Induction of apoptosis has a central role in cancer treatment strategies as an effort to kill the tumor and overcome the resistance to apoptosis which is typical in cancer.[145] Apoptosis has a normal role in cells during development and homeostasis.[146] However, it may also be induced as a response to severe stress or damage of the cell, like elevated ROS-levels.[144] Apoptosis is characterized by cell shrinkage, chromatin condensation, DNA-fragmentation and membrane blebbing. The cell forms apoptotic bodies, containing organelles and nuclear fragments surrounded by plasma membrane, that are phagocytized by macrophages, parenchymal cells or neoplastic cells and subsequently degraded.[147]

Apoptotic signaling is divided into two pathways: the intrinsic and the extrinsic pathway. The previous is activated within the cell whilst the latter is

activated from the outside. The main players in apoptotic signaling are the caspases. These are proteins with protease activity that cleave a number of target proteins.[148] There are two types of caspases: initiator and effector caspases, taking part in the initial activation and the finalization of apoptosis respectively. There are distinct caspases for the intrinsic and the extrinsic pathways. However the two pathways converge in the activation of the effector caspases 3, 6 and 7. Below, the two pathways will be described.

1.5.1 The intrinsic pathway

The intrinsic pathway is activated as a response to severe intracellular stress like DNA-damage, hypoxia and oxidative stress. This triggers the activation of the tumor suppressor protein, p53, which activates proteins that trigger the release of SMAC/DIABLO and cytochrome C from the mitochondrion into the cytosol. SMAC/DIABLO binds to and thus prevents inhibitor proteins of apoptosis (IPA) to inhibit caspases. Cytochrome C binds to an adaptor protein, APAF1, and this complex, called the apoptome, activates one of the initiator caspases, caspase-9. Caspase-9 initiates a caspase cascade by the activation of the effector caspases 3, 6 and 7 that will execute apoptosis. [149]

1.5.2 The extrinsic pathway

The extrinsic pathway is activated by ligand binding to a death receptor at the cell's plasma membrane. There are several receptor members in this tumor necrosis factor (TNF) family. The most well-known are: Fas, TNF-receptor (TNFR) and TNF-related apoptosis-inducing ligand receptor (TRAIL).[150] The activation of the receptor (e.g. Fas) initiates a formation of the death-inducing signaling complex (DISC) at the cytosolic part of the receptor. The DISC-complex, in this case, contains Fas-associated death domain protein (FADD) and caspases 8 and 10, which become activated. These may now activate the effector caspases 3, 6 and 7. Alternatively, caspase-8 may cleave inhibitor proteins of the intrinsic pathway, inducing activation of casapse-9. Caspase-9 activates caspase-3, which further triggers caspase-8 activation, hence producing a positive feedback loop.[151]

2 AIM AND HYPOTHESIS

The overall aim for this thesis is to determine the potential of the specific nephrotoxin, orellanine, as a curative treatment for advanced clear-cell renal cell carcinoma.

It stems from the observation that orellanine apparently is toxic only towards the proximal tubular cells in the kidney, and is shown to accumulate in kidney cortex and is thought to accumulate in the proximal tubular cells. Therefore, we hypothesized that there is a selective and specific uptake mechanism of orellanine in these proximal tubular cells. Since clear cell renal cell carcinoma is thought to originate from the proximal tubular cells, we believed that also these cells would retain the same uptake mechanism for orellanine and hence also inherit the sensitivity for orellanine's toxicity.

2.1 Specific aims

Paper I:

To elucidate if orellanine is toxic for clear cell renal cell carcinoma (CCRCC) cells *in vivo* and *in vitro*. Further we wanted to study the cellular effects of orellanine.

Paper II:

To establish a technique for the quantification of orellanine in solution and in plasma.

Paper III:

To evaluate long-term effects in patients post orellanine intoxication.

3 PATIENTS AND METHODS

3.1 Patients

All patients were diagnosed with orellanine poisoning and medical consent was given for each patient intoxicated during the period of 1979-2012. In total there were 28 patients who agreed to participate. They were admitted to hospitals in Borås, Gothenburg, Jönköping, Skövde, Trollhättan or Örebro.

3.2 The cancer model

We reviewed the literature for a suitable model for CCRCC research. Since the animal will be dialysis dependent, the minimum size of the animal is confined; hence the mouse is too small for this purpose. The spontaneous RC model, the Eker rat,[152] was found not to be suitable due to histological character of the tumors, not being CCRCC rather of a chromophobe subtype.[153] In the Wistar-Lewis rat,[154] adenocarcinoma arose spontaneously in a male rat and the tumor has been transplanted into syngeneic animals to keep it alive. The tumor develops into CCRCC carcinomas with metastatic potential to the lungs.[154,155] Although this tumor shares several characteristics with human CCRCC, some features could still be lost or not transferable to hCCRCC. Therefore we decided to establish a human xenograft model of CCRCC in rat.

The RNU-rat (strain NIH-Foxn1^{rnu}, Charles River, Germany) was established at National Institute of Health in the 1979-1980 and came to Charles River in 2001.[156] This rat is an athymic nude rat that lacks T-cells and is immune-deficient, making it suitable for xenograft research. We initially tried to establish a subcutaneous CCRCC model in these rats, to be able to follow the tumor growth easily in each individual.

At first, hCCRCC were injected at the right flank of the animals. However, tumor growth in these rats ceased after 1-2 weeks, followed by a rapid reabsorption of the tumor. We attributed this effect to mobilization of the animals' B-cell defense since the RNU-rat, while being T-cell deficient, has normal bone marrow-derived B-cell populations. In order to achieve continuous tumor growth for at least 5-6 weeks, which was required in order to study any antitumor effects of orellanine, we administered whole body irradiation in a dose range of 4-5 Gy. This suppressed B-cell mediated immune responses without apparent side-effects on appetite or general

behavior. Lower doses were inefficient, and significantly higher doses were lethal.

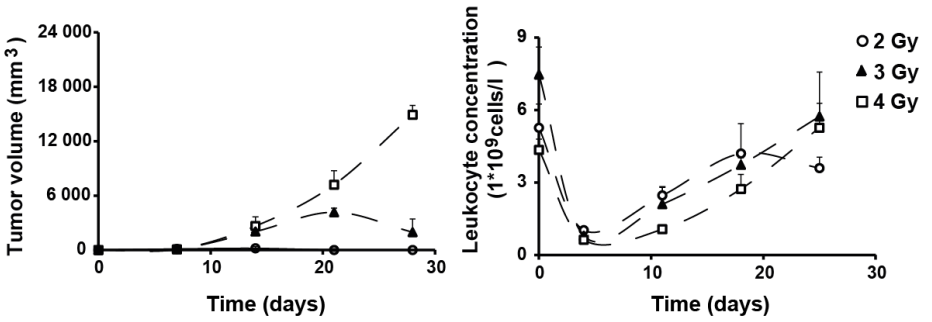

Figure 4. Tumor volume and leukocyte concentration in irradiated RNU-rats

3.3 The dialysis of rats

Since orellanine is strongly toxic, not only to CCRCC but also to proximal tubular cells in the kidney, administration of orellanine will inevitably lead to kidney-failure, killing the animals within a few days. Therefore a dialysis system for rats was constructed to simultaneously replace the renal function of up to twelve animals for several weeks.

In Sweden, one-third of all patients requiring dialysis receive peritoneal dialysis.[157] The underlying principle is to use the abdominal cavity for dialysis fluid, the peritoneum as a dialyzer membrane, and glucose as the osmotic agent to remove excess of fluid. Briefly, the abdominal cavity is filled with a sterile PD-solution with physiological electrolyte composition, a buffer and hypertonic glucose content. The high glucose concentration will produce an osmotic driving force, causing water to leave the blood and enter the abdominal cavity. Waste products, like urea and creatinine, diffuse across the peritoneal membrane from the blood to the PD-solution due to the favorable concentration gradient. After the dwell-time, the solution in the abdominal cavity is drained and hence excess water and waste products are eliminated. The process is repeated several times per day to achieve adequate dialysis.

3.3.1 The dialysis system

The general design of the system is illustrated in Figure 5. It operates completely without pumps, relying on hydrostatic pressure for filling as well as for draining the dialysate from the rats. It is a sealed system, which is kept sterile and autoclaved before each experiment and where the dialysis solution is warmed up before entering the rat. It is automated and computerized, and all fluid displacements are carried out by the opening and closing of valves. Fill times, dwell times and draining times can be set as desired in the customized software. It soon became apparent that the rat ideally requires constant dialysis during the majority of the day and night. This is likely due to its high metabolic activity and the correspondingly higher Kt/V number compared to humans. Although the system is fully automated, continuous monitoring is required, occasionally with rapid intervention, e.g. in the case of a valve failing to open or close properly. Typically we have performed dialysis over a period of 12-14 hours of the day for up to 2-3 weeks.

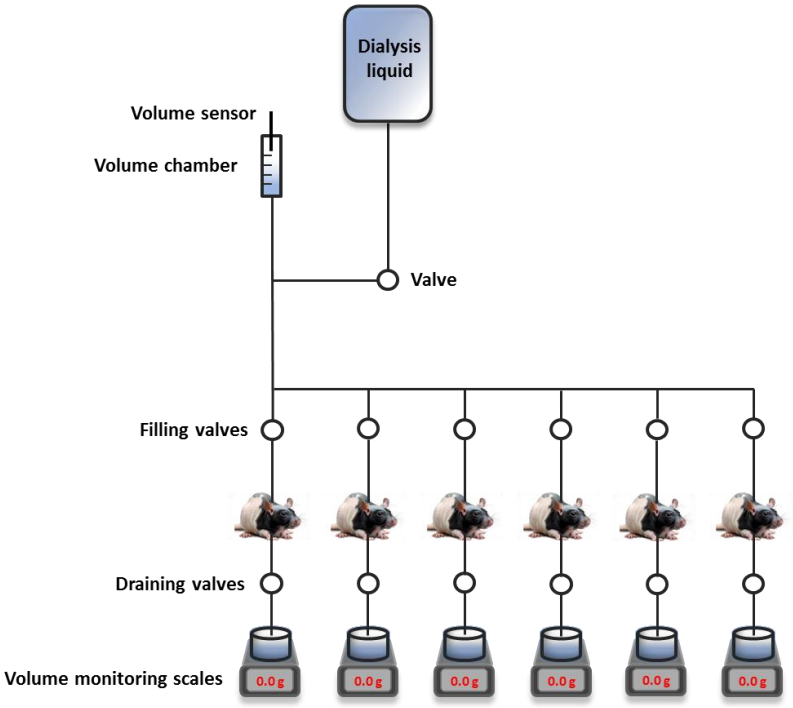

Figure 5. Schematic illustration of the dialysis system for rats

The dialysis procedure at a glimpse:

1. The valve from the dialysis liquid reservoir is opened, allowing filling of the volume chamber with warm dialysis solution until the desired volume has been reached and the valve closes.

2. The filling valve to the rat is opened, allowing the dialysis solution to pass from the volume chamber into the rat.

3. Dialysis takes place for the set dwell time, usually 1 hour.

4. The draining valve from the rat is opened and it is allowed to drain, typically for 30 minutes.

5. The next cycle is initiated.

3.3.2 PD-catheter insertion

It is extremely crucial to maintain an aseptic environment throughout the instrumentation procedure, since the animals are devoid of immunological capability and thus virtually defenseless against bacterial invasion.

The day before the procedure, the rat is anesthetized using inhalation of isofluran (2-4 % v/v Schering-Plough, Stockholm, Sweden) and thoroughly washed with DesCutan 4% (Fresenius Kabi AG, Bad Homburg, Germany), allowing the disinfectant to work for 5 minutes. The rat is rinsed with water, dried in sterile paper and placed in an autoclaved cage with autoclaved bedding and food.

On the day of catheter insertion, anesthesia is induced and maintained using isofluran and the rat's body is disinfected with chlorhexidine. Next, the rat is embedded in sterile coverings. A small incision is made in the neck and the animal is placed on its back on a heating plate covered with a sterile cloth. The rat is given Temgesic (0.3mg/kg, Schering-Plough, Stockholm, Sweden) for pain relief, and a bolus dose of 4 mg antibiotics (Ciprofloxacin) is administered sub-cutaneously. A 2 cm incision is made through the skin along the nodal line of the belly. Using a 20G needle, two holes, spaced

approximately 0.5 cm apart, are made along the white line of the peritoneum to allow access to the abdominal cavity without unnecessary bleeding or damage. A 1.5cm tip of a 5 Fr heparine-coated polyurethane catheter (Instech Laboratories, Inc., Plymouth Meeting, PA USA) is inserted into each hole and secured by suturing to the peritoneum. The catheters are tunneled subcutaneously on the right hand side of the animal up to the neck and out via the incision and attached to a sterile dual luer harness (SAI infusion technologies, Libertyvill, IL, USA). Finally, the rat is placed in a new autoclaved cage and a swiveled metal tether-embedded tubing is attached to one of the harnesses' valves via a luer connection. The animal is now ready for dialysis, but is left to recover for 48 hours before initiation of dialysis.

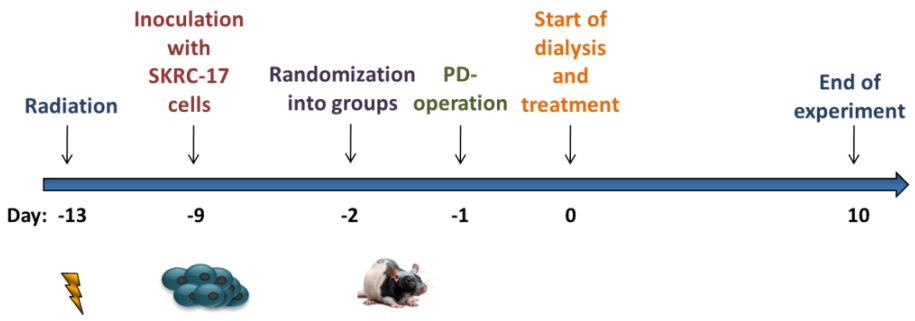

Figure 6. Schematic overview of the in vivo studies in hCCRCC inoculated RNU-rats treated with orellanine.

3.4 TUNEL staining

For detection of apoptosis in tumor sections, the ApopTag® Fluorescein In Situ Apoptosis Detection Kit (Millipore, Billerica, MA) was used. Formalin fixed and paraffin embedded tumor sections, 5µm thick, were de-paraffinized in xylene followed by an ethanol dilution series. For improved antigen retrieval, the sections were pressure boiled in a DIVA decloacker with DIVA solution (Biocare Medical, Concord, CA) for 30 minutes and dipped 10 times in Hot Rinse (Biocare Medical, Concord, CA). Sections were washed twice in PBS for 5 minutes at room temperature (RT) and then permeabilized with proteinase K (Millipore, Billerica, MA; 20 µg/ml in PBS) for 15 minutes at RT. After a 5 minutes wash in PBS, the sections were incubated in equilibration buffer at RT for 10 minutes. Next, the terminal deoxynucleotide

transferase (TdT) mixture was prepared and incubated for 1 hour at 37°C before the reaction was terminated by washing slides for 10 minutes in Stop/Wash buffer. Slides were then washed in PBS 3times for 1 minute before applying the Anti-Digoxigenin-conjugate and incubating for 1 hour at RT. Sections were finally washed 4 times for 2 minutes in PBS and mounted in ProLong Gold Antifade with DAPI nuclear staining (Life Technologies, Stockholm, Sweden).

3.5 The cells

3.5.1 Preparation and characterization of human proximal tubular cells

Human proximal tubular cells (HTEC) were prepared from a nephrectomy. An unaffected lobe was used and the cortex was cut out. In the cell culture, the cortex was minced carefully and transferred to a 15ml falcon tube. Next the minced tissue was washed three times with PBS and spun down in between at 500 rcf and the supernatant was taken off. After the last wash, the pieces were treated with sterile collagenase IV (1mg/ml medium) for 1 hour at 37°C. They were vortexed every 15 minutes for 10 seconds. The supernatant was then filtered through a 40μm cell strainer and spun down 5 min at 500 rcf, the supernatant was taken off and cells were re-suspended in DMEM/Ham's F12 medium with 10% FBS. This centrifugation re-suspension step was repeated three times. Finally, the pellet was dissolved in DMEM/Ham's F12 media supplemented with 10% FBS and 5ml PSA. The cells were seeded into two rat tail I collagen (5 ug/cm^2, Becton Dickinson, Frankling Lakes, NJ) coated 25cm^2 flasks.

HTEC were seeded onto rat tail Collagen I coated cover slips and fixated in 4% PFA in PBS for 15 minutes. They were stored in PBS in 4°C over-night. Next, the cells were blocked in 2%FBS and 2% BSA in distilled water for 1 hour in room temperature (RT). Blocking solution was taken off and the primary antibody, neprilysin (Chemicon, Millipore, Billerica, MA) diluted 1:100 in blocking buffer was applied to the cells and allowed to work for 1 hour at RT. Cells were then washed with PBS for 70 minutes and then incubated with a secondary antibody, anti-rabbit Alexa 488 (Life Technologies, city, country), dissolved in blocking buffer, for 1 hour at room temperature. The slides are then washed with PBS for 1 hour at RT, rinsed with water and mounted with ProLong® antifade Gold with DAPI (Life Technologies, Stockholm, Sweden). About 90% of the cells were neprilysin positive.

3.5.2 Cancer cells

The 786-O cells are a cell line available from the American Type Culture Collection (ATCC). It originates from a 58 year old male with clear cell renal cell carcinoma. It is a double mutant of the VHL-gene.[158] The cells were cultured in DMEM High glucose, supplemented with 10% FBS and 5 ml PSA. The SKRC-cell lines (SKRC-7, -10, -17, -21 and -52) were all prepared and described by Ebert et al.[159] SKRC-7, -10 and -21 are from primary tumors while SKRC-17 and -52 are from metastatic lesions, in soft tissue and mediastinum respectively. They were cultured in RPMI supplemented with 10%FBS and 5 ml PSA or Antimycotic-antibiotic solution. All of them are clear cell renal cell carcinomas and they are either mutated or methylated in the VHL-gene, and hence do not express pVHL.[160] The cells prepared from a primary CCRCC tumor (087) were from a female of age 48 years and were cultured as the SKRC-cells.

3.5.3 Control cells

The human umbilical vein endothelial cells (HUVEC) were purchased from Becton Dickinson (Becton Dickinson, Stockholm, Sweden) and cultured in EBM-2 media with bullet kit (Lonza, Basel, Switzerland). MDA-MB-231 cells, from ATCC, are breast cancer epithelial cells derived from a metastatic lesion. They were cultured in DMEM high glucose, supplemented with 10% FBS and 5ml PSA.

3.6 Alamar Blue

Alamar Blue (Life Technologies, Stockholm, Sweden) is a widely used assay to assess cytotoxic stress in cells. It has an advantage over the MTT assay in that the fluorescent compound is excreted by the cells, whilst in MTT the cells have to be lyzed to monitor the cytotoxicity. Especially during evaluation of toxicity with respect to time and/or dose, Alamar Blue has a great advantage over MTT, and was making it our choice when studying any toxic effects that orellanine exerts on various cell types.

An initial Alamar Blue measurement was performed before exposing the cells to orellanine, controlling for any inequalities pre-treatment. Alamar Blue (1 ml) was mixed with 10 ml warm complete medium. 600 µl was then added to each well of a 12-well plate containing cells, and into 3 wells of an empty 12-well plate, serving as blanks. The plates were incubated for 2 hours at 37°C. Next, 200 µl of the alamar blue solution from each well was pipetted to a black 96-well plate. The fluorescence in the wells was monitored at 530-560 nm excitation and 590 nm emission wavelengths, using a fluorescence

plate reader (Spectra Max Gemini XS, Molecular Devices, Sunnyvale, CA). The cells were washed twice with PBS and orellanine-containing medium was transferred to the wells at final orellanine concentrations of: 0, 4 20, 60, 100 and 200 µg/ml. Cells were incubated for 24 hours at 37°C. Then orellanine containing medium was removed, cells were washed twice with PBS and then restored in 37°C. The Alamar blue measurement was done at 48, 72, 96 and 144 hours respectively post orellanine treatment.

3.7 Caspase cleavage and activity

3.7.1 Western blotting

Western blotting was used for monitoring of differences in caspase cleavage in vehicle or orellanine treated SKRC-52 cells. The cells were lysed on ice using a cell scraper and a lysis buffer containing: 150 mM NaCl, 100 mM Tris HCl, 2 mM EDTA, dH_2O and 100 mM TritonX-100. Equal concentration of whole cell protein lysates were separated on NuPAGE Bis-Tris gels 4-12% (Novex, San Diego, CA). They were transferred to polyvinylidene difluoride (PVDF) membranes and blocked in 5% nonfat dry milk (BioRad) in tris buffered saline with Tween 20 (TBS-T) at room temperature for 1 hour and incubated with primary antibody (1:500) (Cell Signaling Technology, Inc., Danvers, MA), in 5% milk in TBS-T at 4°C over-night. The membrane was washed 4*15 minutes in TBS-T and was then incubated with secondary antibody (1:1000) at room temperature for 1 hour followed by an hour washing in TBS-T. Incubation with Immun-Star WesternC kit and CCD-camera (Molecular Imager Chemidoc XRS+ Systems, Bio-Rad Laboratories Inc., Hercules, CA) was used to visualize immunoreactive bands.

3.7.2 Caspase activity assay

Caspase activity assay kits (Abcam, Ltd, United Kingdom), were used to detect apoptosis induction in orellanine treated SKRC-17 and SKRC-52 cells. The caspases investigated were caspase 3, 8 and 9.

SKRC-17 and SKRC-52 cells were lysed on ice using a cell scraper and a lysis buffer containing: 150 mM NaCl, 100 mM Tris HCl, 2 mM EDTA, dH_2O and 100 mM TritonX-100. The lysates were aliquoted and were frozen immediately in -20°C except for one aliquote from each sample, which was used for protein measurement according to manufacturer's protocol (Pierce BCA Protein assay kit, Pierce Biotechnology, Rockford, IL). At the day for activity measurement, samples were thawed and diluted with dH_2O to yield

an equal protein concentration of each sample. 50 µl of equal amount of protein were pipetted into a 96-wells plate. Next, 50 ul Reaction buffer containing DTT, according to manufacturer's protocol, were pipetted into all the samples of the 96-well plate. Finally, 5 µl of the substrate for each caspase was added to the wells, the plate was shaken lightly and was then incubated at 37°C for 2 hours in the dark. The fluorescence of the cleaved substrates and hence the caspase activity, was determined at an excitation wavelength of 400 nm and an emission wave length of 505 nm on a fluorescence plate reader (Spectra Max Gemini XS, Molecular Devices, Sunnyvale, CA).

4 RESULTS AND DISCUSSION

This thesis is based on three papers. The aim of paper I was to elucidate the effects of orellanine on clear cell renal cell carcinoma *in vitro* and further study its effect *in vivo* in a model of human CCRCC in rat. In paper II, an LC-MS model was outlined for the detection of orellanine in solution (e.g. plasma). In paper III, the long term effects of orellanine in patients were investigated in a case-controlled study.

4.1 Orellanine toxicity towards HTEC and CCRCC cells (Paper I)

Orellanine induces kidney failure by shutting down the proximal tubular cells in the kidney. In paper I, we confirm the toxicity of orellanine towards human proximal tubular cells (HTEC) in terms of reduced viability over time (Figure 7). The sensitivity of these cells to orellanine is evident also for the lowest dose tested (4µg/ml). In contrast, CCRCC cells are only slightly affected when treated with 4µg/ml for 144 hours (Figure 8). Interestingly, the two cell lines from metastatic lesions (SKRC-17 and -52) require higher doses to be affected as is evident at the concentration of 20µg/ml. The 087 cells, prepared fresh from a primary tumor, and the primary CCRCC cell lines (786-O and SKRC-10) are more sensitive to orellanine. SKRC-52 has been histologically identified as having a high sarcomatoid component[107] and the SKRC-17 tumors from our animal studies, were shown to be poorly differentiated (anaplastic). Both of these characteristics are associated with a worsened prognosis and also treatment resistance. Note however, that all renal cancer cells tested show low viability at doses of orellanine of 60µg/ml and higher.

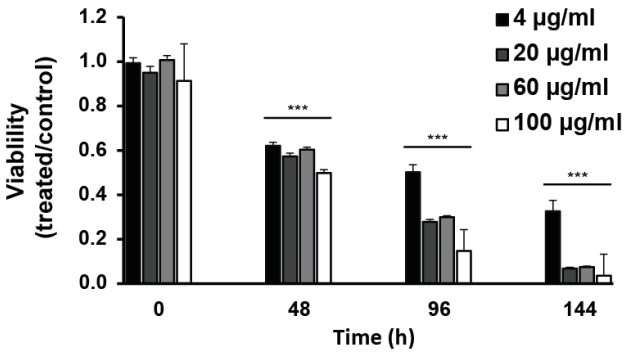

Figure 7. Reduction in viability in HTEC after 24 hours treatment with orellanine at the indicated doses and the time-points specified.

The control cell lines, human umbilical vein endothelial cells (HUVEC) and MDA-MB-231 (noted MDA), cells from a metastatic breast cancer, were both unaffected by orellanine except at the highest concentrations. This suggests that there is a therapeutic window where orellanine effectively kills all clear cell renal cancer cells while leaving other cells substantially unaffected.

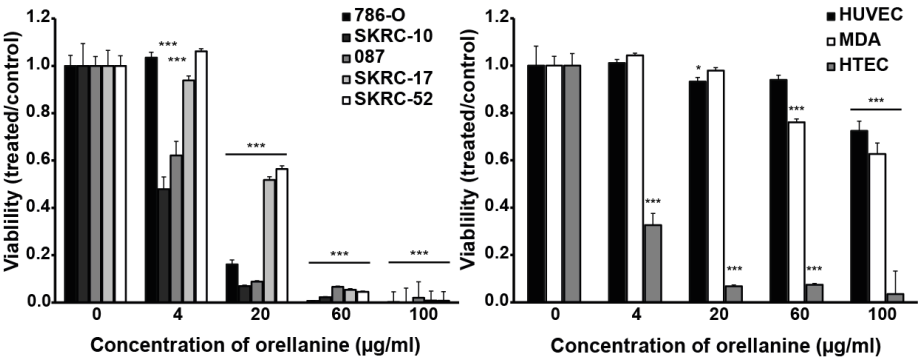

Figure 8. Viability 144 hours post 24 hours orellanine treatment at the indicated doses.

4.1.1 Active transportation of orellanine into HTEC and CCRCC cells

Orellanine seems to be taken up by the proximal tubular cells in the kidney.[134] To address the nature of orellanine uptake into HTEC and CCRCC, cells were cooled down to 8°C to stop all active transport,[161,162] allowing only diffusion to take place. Cells treated with orellanine at 37°C had a reduced viability, while the cells incubated with the toxin at 8°C were protected completely. The same pattern was observed in all of the three cell lines investigated (HTEC, SKRC-17 and SKRC-52). This demonstrates that the uptake mechanism for orellanine is an active process. Most importantly, the active transport mechanism is present also in the poorly differentiated renal cancer metastases, such as SKRC-17 and SKRC-52 cells.

4.1.2 The transporter of orellanine

The exact mechanism for the transportation of orellanine into proximal tubular cells is still unknown. We hypothesize that there is a specific

transporter that transports orellanine into the proximal tubular cell, either from the apical side or from the interstitial side. Orellanine is present mainly as a diglucoside, both in the fruit body of the fungus and when analyzed in plasma samples (Paper II). A hypothesis raised by Rohrmooser et al, is that orellanine is taken up as a glucoside into the proximal cell.[134] Once within the cell, the glucose groups are cleaved off, and orellanine, which is unable to diffuse across the cell membrane, would then rapidly accumulate into toxic concentrations. Orellanine may also be bound to, for example, a protein and co-transported into the cells.

Our initial candidate transporter was SGLT-2, found abundantly, but not exclusively, on the apical membrane of proximal tubular cells in the kidneys[163] Phlorizin, a non-selective inhibitor of SGLT-2,[164] resembles orellanine structurally. Therefore, we speculated that while phlorizin binds to and inhibits SGLT-2, orellanine is actually transported into the cells by the same transporter. However, a specific SGLT-2 blocker did not protect the cells from the toxicity of orellanine, indicating that SGLT-2 is not the orellanine transporter that we are looking for.

To further analyze the properties of orellanine transport into proximal epithelium, sodium-dependence was studied. Indeed, most substances reabsorbed from the urine enter the proximal tubular cells by one of several sodium-co-transporters. Therefore, we wanted to study if orellanine could be prevented from entering the cell and hence exert its toxicity by reducing the extracellular sodium concentration from145mM to sub-cellular concentration of sodium (7mM),[165] with choline added instead of sodium to maintain identical osmolality. However, the toxicity of orellanine-treated cells incubated at low or normal sodium concentration was similar. This strongly indicates a sodium-independent transport of orellanine.

Next, we sought to identify sodium-independent transporter candidates present on proximal tubular cells. Some of these are mentioned in the introduction, like OAT1, 3 and 4, OCT 1 and 2, and MATE1. This is still ongoing work.

4.1.3 Apoptosis and ROS in CCRCC cells

Since we observed a pronounced decrease in viability of renal cancer cells treated with orellanine, our aim was to investigate whether this was due to induction of apoptosis. Indeed, we could detect an activation of caspase-3, an executor of apoptosis, in SKRC-52 cells when exposed to orellanine. Next we sought to identify which of the apoptotic pathways that were activated;

the intrinsic or the extrinsic pathway. To do this, we studied caspase-9 and caspase-8 activation, respectively. With western blotting, only caspase-8 activation was detected (Figure 9a). To further confirm caspase activation, we used caspase activity kits. Results showed an activation of caspase-3 and caspase-9 in SKRC-52 cells (Figure 9 b,d). A tendency towards caspase-8 activation was observed, but this was not statistically significant (p=0.09) (Figure 9c). This shows that the caspase activity measurement might be a more sensitive analysis than Western blotting in terms of caspase activation. The caspase-9 levels of activity were very low, which may explain why it was difficult to identify the activation by western blotting. In SKRC-17 cells, all the three caspases were significantly activated. Thus, orellanine induces apoptosis in the two CCRCC cell lines, probably via both the intrinsic and extrinsic pathways.

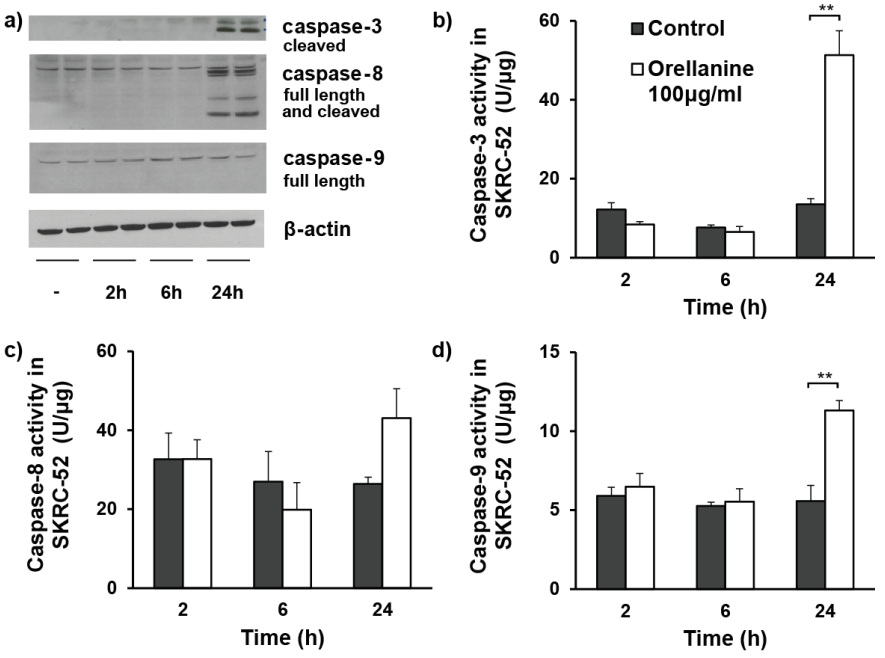

Figure 9. Caspase activation in SKRC-52 cells after treatment with orellanine, 100 µg/ml for 2, 6 or 24 hours.

We have shown that orellanine reduces the oxidative stress defense in HTEC and induce ROS in both HTEC and the CCRCC cell line SKRC-52. This is consistent with previous observations *in vivo*, where increased ROS-levels were observed in tubular cells in kidney sections of orellanine treated rats.[166]

Also a decrease in mRNA-level for several antioxidant enzymes was detected in kidney cortex from the same animals. The exact mechanisms behind the observed effects remain unclear. However, it is a highly interesting phenomenon that oxidative stress is observed in concert with decreased oxidative stress defense, which may be one explanation of orellanine's toxicity. However, further investigation in terms of molecular steps and pathways is required for this to be fully elucidated.

4.2 Effects of orellanine on CCRCC tumors *in vivo* (Paper I)

To further investigate if the results *in vitro* were reproducible *in vivo,* and could be used in the clinical setting, a tumor model of human CCRCC in rat was developed, as described in section 3.2. Furthermore, a peritoneal dialysis system for rats was constructed (see section 3.3).

4.2.1 Impact on weight, necrosis and apoptosis

For the tumor model, a cell line from a metastatic CCRCC lesion (SKRC-17) was used to mimic a metastatic model of human CCRCC.

In animals, treated with 10mg/l orellanine, for 2 days, 9 days after tumor cell implantation, the tumor weight was significantly smaller in treated rats compared to control animals (1.0g versus 4.5 g). The control rats did not receive dialysis and tumors were assessed after a mean of 9 days post-treatment initiation. Necrosis in the remaining tumor was also increased in the treated animals (65%) compared to control (15%). In another setting, animals were treated early on, 4 days after inoculation, with the same (10mg/l) or a higher dose of orellanine (20mg/l), with treatment for 5 days or 3 days, respectively. In this study, the control rats received one cycle of dialysis per day. The effect on tumor weight was even more pronounced (control 0.98g, 10mg/l: 0,11g and 20mg/l: 0.07g) with $p< 0.001$ for both treatment groups, assessed 5 days post treatment initiation. In tumors from animals treated with 20mg/l, necrosis was significantly increased. Apoptosis was increased in both treatment groups compared to control ($p< 0.01$ 10mg/l and 0.001 20mg/l) (Figure 10). Taken together, a more pronounced effect was seen on tumor volume, apoptosis and necrosis when treating animals with higher doses of orellanine for a shorter time period.

Although we have chosen to use the more anaplastic, fast-growing SKRC-17 cells in our tumor model, we still observe pronounced effects on tumor volume, induction of apoptosis and necrosis. As discussed earlier in section

4.1, the SKRC-17 was one of the CCRCC cell lines with the lowest sensitivity for orellanine. This supports the hypothesis that orellanine has a significant effect in the clinical setting, where generally, the metastatic lesions resemble the primary tumors histologically. Even if this was not the case, our data implies that even highly resistant tumors, with a sarcomatoid component or with anaplastic appearance, are likely to respond to the treatment.

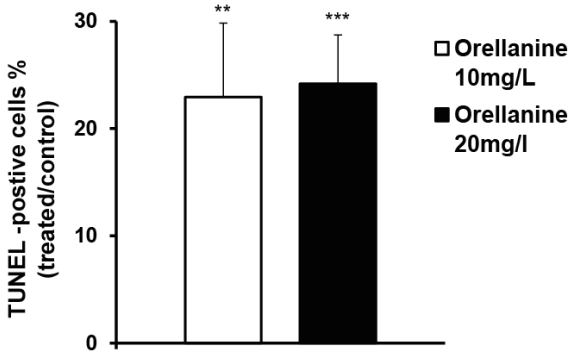

Figure 10. Apoptosis in tumor sections from RNU-rats treated with orellanine.

4.3 Establishing a technique for quantitative detection of orellanine in plasma and solution (Paper II)

In order to be able to measure the orellanine distribution and uptake we wanted to establish a new and highly sensitive method. Methanol (MeOH) has been previously used as an extraction solvent for orellanine.[133,167,168] Here we investigated four different extraction solvents for orellanine: MeOH, MeOH/3M HCl (10:1), water and 3M HCl. Of these, 3M HCl was the superior extraction solvent, followed by water, acidified MeOH and MeOH. An interesting finding was that in both water and 3M HCl, any glucosides of orellanine were rapidly hydrolyzed to orellanine. In acidified MeOH, this hydrolyzation took place, consistent with previous data,[167] but to a smaller and slower extent while in the MeOH, no hydrolyzation was identified and hence less free orellanine was detected. It was also observed that the majority of orellanine in the mushroom extract initially appeared to be in the diglucoside and monoglucoside forms, as has been suggested previously.[167]

In patients suffering from accidental orellanine poisoning, the toxin is not detectable in urine or plasma after onset of symptoms, and orellanine is only to be found in kidney biopsies at that time. However, in a clinical setting using orellanine as a treatment of patients with mCCRCC, a careful monitoring of the serum concentration of orellanine will be crucial to provide a satisfactory treatment paradigm. For this purpose, a technique using high-performance liquid chromatography combined with electrospray ionization tandem mass spectrometry (HPLC-ESIMS/MS) for quantitative measurements was established. This method has previously been used only for qualitative detection of the diglucoside of orellanine[167] and was now optimized. The technique displayed high sensitivity and could detect orellanine concentrations down to 5ng/ml or less. In the treatment of animals with CCRCC (Paper I), the orellanine concentration most frequently used was 10mg/l (10µg/ml), which would be the maximum theoretical plasma concentration in these animals. This is 2000 times higher than the limit of detection with the method established, hence the technique will be suitable for detection of serum concentrations of orellanine within the treatment range.

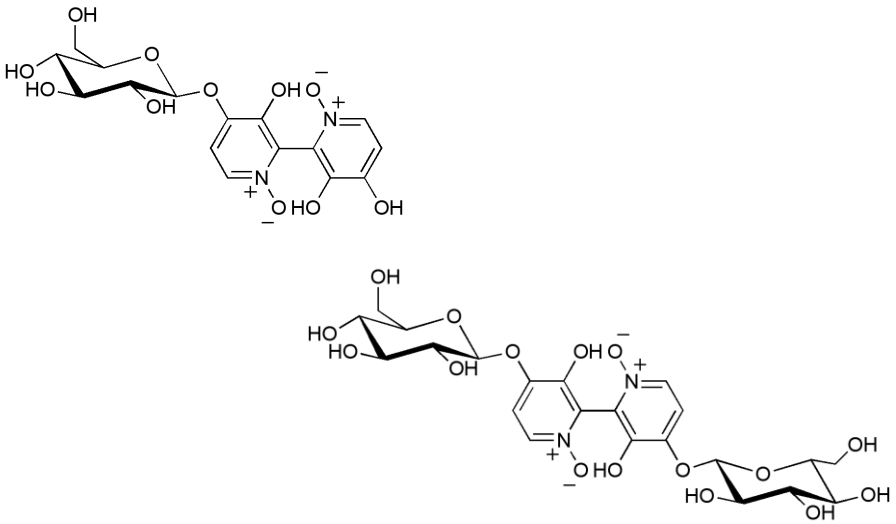

Figure 11. Chemical structures of monoglucoside of orellanine at the top and diglucoside of orellanine at the bottom.

4.4 Clinical effects in patients after accidental intoxication with orellanine containing mushrooms (Paper III)

Orellanine is known to induce renal failure after ingestion of *Cortinarius* mushrooms. Depending on the amount of mushroom and hence orellanine ingested, the outcome for the patient differs. Some patients never require dialysis and will recover most of their renal function, while others will need dialysis for a transient period, or for life. Acute symptoms due to orellanine intoxication have been well studied. [128,130,169-171] We wanted to investigate whether orellanine-induced end-stage kidney failure (CKD5), leading to life-long dialysis dependence or transplantation, would lead to increased mortality or other non-kidney related effects/morbidity, mortality or higher cancer frequency. Furthermore, additional clinical parameters were also investigated in the *Cortinarius*-group. We included all patients agreeing to participate in the study with a clinical diagnosis of mushroom poisoning from 6 hospitals.

In the *Cortinarius*-group of patients, other than renal failure, liver function in terms of enzymatic activity and blood values were examined. All values were in the normal range, and hence no abnormalities were observed. As expected, both urea and creatinine serum levels were higher in patients being dialysis-dependent after intoxication compared to patients whose remaining kidney function was sufficient, and hence not needing any dialysis (Figure 12).

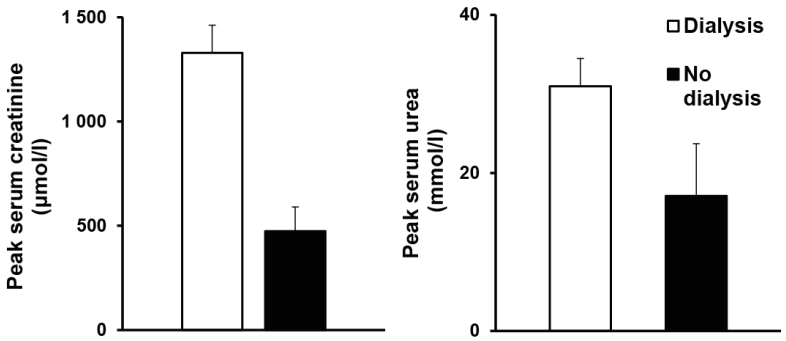

Figure 12. Peak creatinine and serum levels in patients needing dialysis or not, post intoxication with orellanine containing mushrooms.

Next, patients with CDK5 were age and sex matched with control patients that were transplanted or initiated on dialysis the same year, in a randomized and blinded manner. Of the 21 patients in the orellanine group, 20 were matched, 7 on dialysis and 13 of the 14 transplanted patients. Results showed 5 deaths in the *Cortinarius*-group and 6 in the case control population and hence there was no statistical significance in deaths between the two populations. Similarly, in terms of cancer, 4 patients in each group were diagnosed with cancer. However, cancer was the cause of death only in one patient in the entire population, belonging to the case control group.

Hence the patients in the *Cortinarius* and the case control group do not significantly differ between the groups. This indicates that orellanine intoxication *per se*, is not a cause for increased mortality or higher cancer frequency. Furthermore, we could not detect any other clinical symptoms, except for renal failure, in orellanine-intoxicated patients. These are important findings in terms of using orellanine as a treatment for mCCRCC.

4.5 Final remarks

In the pathophysiological setting of mushroom intoxication, orellanine is taken up by cells in the intestine, circulated in blood, eliminated by glomerular filtration and/or tubular secretion, and is probably accumulated in the proximal tubular cells in the kidneys. The toxin can no longer be detected in body fluids after 3-6 days, when the patient experiences their uremic symptoms.[134] As seen in Paper I, control cells exposed to orellanine *in vitro* are insensitive to orellanine at the concentrations used in the *in vivo* settings for killing renal cancer cells (20μg/ml). Furthermore, in Paper III, liver values were normal and no side-effects, other than kidney failure, were reported. Additionally, no increased mortality was observed due to acute orellanine toxicity. During treatment of a patient with metastatic CCRCC, orellanine will not only be taken up by the kidneys but also by the metastatic lesion, and its toxicity will hypothetically eliminate the tumor. This is what we observed in our rat model of CCRCC. The serum concentration of orellanine will be carefully monitored with the improved technique that we developed in Paper II, to be able to optimize the treatment regime. In all, by these investigations we have taken the first crucial steps towards an essentially new therapy for metastatic clear cell renal carcinoma with a true curable potential. However, important questions remain unanswered, especially in terms of how orellanine is taken up by the cells and its intracellular mechanisms. Furthermore, animal toxicity studies also need to be performed before orellanine can be tested in the clinical setting.

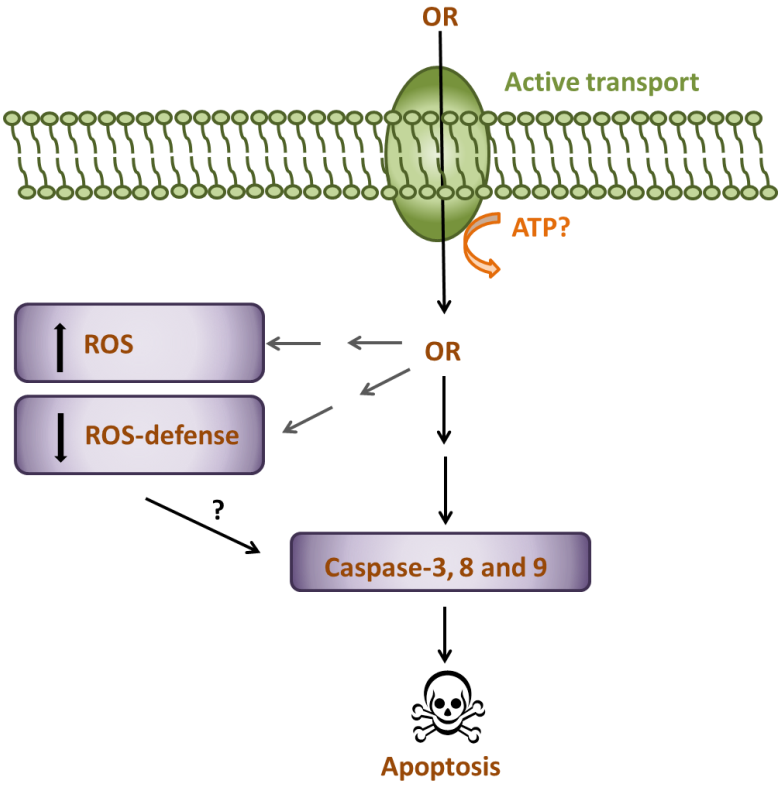

Figure 13. Schematic illustration of the signaling pathways induced by orellanine in HTEC and/or CCRCC. The gray arrows indicate previously described findings in renal cortex. OR=orellanine, ROS=reactive oxygen species, ATP=adenosine triphosphate.

5 CONCLUSIONS

The major findings in this thesis are:

1. The nephrotoxin, orellanine, is highly toxic to human CCRCC cells while other cell types are unaffected at therapeutic concentrations.

2. In a proof of concept study in rodents, human CCRCC are effectively destroyed and the animals appear unharmed and survive on dialysis.

3. Orellanine can be quantitatively detected down to 5ng/ml with our optimized HPLC-ESIMS/MS system. This technique is stable and sensitive and will give excellent measurements of serum concentrations in patients treated with orellanine in the clinical setting.

4. Patients suffering from orellanine intoxication do not differ from the case-control group in terms of survival or cancer incidence. Liver status and blood values were normal, thus renal failure remains the solely known outcome of orellanine intoxication.

Taken together, our results show that orellanine does affect aggressive human CCRCC both *in vitro* and *in vivo* and does not have any other effects apart from renal failure, which can be well managed with dialysis or kidney transplantation. Patients with metastatic CCRCC lack curative treatment to date and have a bad prognosis with a 5-year survival of less than 10%. We conclude that orellanine has potential to become a novel curative treatment for these patients and that further research into the matter is highly motivated.

6 FUTURE PERSPECTIVES

We have shown orellanine's potential as a curative treatment of mCCRCC. There are several interesting and challenging areas that should be further explored.

6.1 Increased ROS and decreased oxidative defense the trigger of apoptosis?

We have observed reduced levels of anti-oxidative enzymes in concert with increased oxidative stress in proximal tubular cells treated with orellanine. Increased ROS was also detected in SKRC-52 cells after orellanine treatment. Additionally, apoptosis was detected due to orellanine treatment in the two cancer cell lines studied (SKRC-17 and SKRC-52). This led us to hypothesize that orellanine treatment, which reduces the ROS-defense and concomitantly increases ROS, in this way triggers apoptosis of the cells. It would be interesting to study if ROS indeed is the trigger of the apoptosis observed. To address this, different ROS-scavengers (e.g. Tempol® and NAC) could be used, to see if the cells can be rescued from apoptosis with this treatment. It would also be interesting to investigate whether the ROS induced is especially severe on different compartments of the cell, like the mitochondrion. The mitochondrion specific ROS-scavenger, mito-Tempol, could be used to examine this.

6.2 Transporter study

To further prove that orellanine is selectively and actively taken up by HTEC cells and CCRCC cells, we have made tritium-labeled orellanine, which can be used for this purpose. After orellanine treatment, cells are washed carefully and run in a radiometer for the detection of radioactivity. This should also be applied on control cells (e.g. HUVEC), in which we hypothesize that no radioactivity will be detected. To further prove orellanine's cellular localization within the kidney, rats may be injected with the radiolabeled orellanine and kidney tissue sections can be examined for radioactivity.

As mentioned in the results and discussion section, we are currently searching for the transporter(s) of orellanine. This is important for several reasons, i.e. to understand the molecular transportation and uptake-mechanism. If we find the transporter, the metastatic lesion or mother tumor

might be biopsied and screened for the receptor, so only patients that will respond to treatment will undergo orellanine therapy. Finding the transporter could further help to potentiate orellanine, in terms of using targeted radiation therapy connected to orellanine, directing it specifically to the cancer cell.

6.3 Orellanine as treatment of papillary RCC?

Orellanine has been shown to be toxic to both proximal tubular cells as well as CCRCC cells, which are thought to evolve from the former cell type. The second most common RCC, papillary renal carcinoma, is considered to originate from distal tubular cells.[172] However, in a study published 2010, the similarity of papillary RCC and the stem cell of proximal tubular cells was indicated.[173] Perhaps this cancer cell type, if evolved from a proximal tubule stem cell, still might have the transporter for orellanine present on its cell surface and hence share the sensitivity for orellanine? It would of course be very interesting to investigate if orellanine could be used as a treatment for the two most common forms of RCC.

6.4 Clinical trial on patients

Results from our studies have further encouraged the evolvement of orellanine into a treatment of mCCRCC. Toxicity studies in rats will be performed during summer 2014. Presently, orellanine toxicity is evaluated *in vitro* on several primary CCRCC, CCRCC cell lines as well as a range of control cells, like human glomerular endothelial cells and mesangial cells. If these results are promising, this is of course our main goal, to transfer our findings into the clinical setting and hopefully offer a new and curative treatment for these patients.

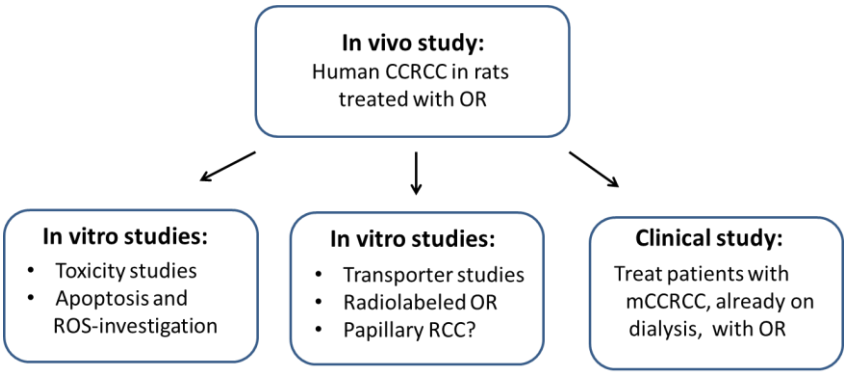

Figure 14. Schematic summary over future perspectives. OR=orellanine

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APPENDIX

Staging of renal cell carcinoma

The TNM-staging gives a guideline for both treatment and patient outcome. For renal cell carcinoma, the system was revised in 2010 and the guidelines are as follows:

Primary tumor (T)	
TX	Primary tumor cannot be assessed
T0	No evidence of primary tumor
T1	Tumor ≤7 cm in greatest dimension, limited to the kidney **T1a** Tumor ≤4 cm in greatest dimension, limited to the kidney **T1b** Tumor >4 cm but not >7 in greatest dimension, limited to the kidney
T2	Tumor >7cm in greatest dimension, limited to the kidney **T2a** Tumor>7 cm but ≤10 cm in greatest dimension, limited to the kidney **T2b** Tumor >10 cm, limited to the kidney
T3	Tumor extends into major veins or perinephric tissues not beyond Gerota fascia **T3a** Tumor extends into major vein or its segmental branches or invades perirenal and/or renal sinus fat but not beyond Gerota fascia **T3b** Tumor grossly extends into vena cava below diaphragm **T3c** Tumor grossly extends into vena cava above the diaphragm or invades the wall of vena cava
T4	Tumor invades beyond Gerota fascia

Regional Lymph Nodes (N)

NX	Regional lymph nodes cannot be assessed
N0	No regional lymph node metastasis
N1	Metastases in regional lymph node(s)

Distant Metastasis (M)

M0	No distant metastasis
M1	Distant metastasis

Stage Grouping

Stage I	T1	N0	M0
Stage II	T2	N0	M0
Stage III	T3	N0	M0
	T1-T3	N1	M0
Stage IV	T4	Any N	M0
	Any T	Any N	M1