Preclinical Evaluation of Gold-Based Chemotherapeutic Candidates for the Treatment of Metastatic Renal Cancer

Benelita Elie

The Graduate Center, City University of New York
PRECLINICAL EVALUATION OF GOLD-BASED CHEMOTHERAPEUTIC CANDIDATES FOR THE TREATMENT OF METASTATIC RENAL CANCER

by

BENELITA TINA ELIE

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This manuscript has been read and accepted by the Graduate Faculty in Biology in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

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Date

Maria Contel, Brooklyn College
Chair of Examining Committee

__________________________

Date

Cathy Savage-Dunn
Executive Officer

Supervisory Committee:

Mara Schwarzstein, Brooklyn College
Guillermo Gernoa-Navarro, Brooklyn College
Karen Hubbard, City College
Joe Ramos, University of Hawaii

THE CITY UNIVERSITY OF NEW YORK
ABSTRACT

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by

Benelita Tina Elie

Advisor: Dr. Maria Contel, Ph.D.

Renal cell carcinomas (RCCs) remain a significant health concern in the United States because of a lack of effective treatment. To date, RCCs account for 2.4% of all cancer deaths in the United States, and about 14,970 people will die from the disease in 2018, mostly of advanced or metastasized RCCs\(^1,2\). Mutations in metabolic pathways are characteristics of RCCs, including changes in redox metabolism, deregulation of proteolytic factor expression, and inflammation. These molecular events promote tumor vascularization, growth, invasion, and metastasis, which often correlate with a poor prognosis. Many pharmaceutical avenues have been explored, including cytotoxic chemotherapeutics, biologics targeting cellular pathways, and immune therapies administered as mono- or combination therapies; all with very modest success in treatment of the advanced disease, and most are associated with significant adverse events\(^3,4\).

The absence of viable treatment options has led to a resurgence of interest in unconventional metal-based drugs that have shown some potential in the treatment of RCCs\(^5-8\). The conception of molecules containing two different metals with known anticancer properties to potentiate their cytotoxic effect is recent, and studies suggest that this approach may be
successful\cite{9,10}. In our group, a series of heterobimetallic compounds containing gold-titanium or gold-ruthenium were synthesized and progressively modified to optimize their stability, bioavailability, and cytotoxicity\cite{11–13}. After screening a series of novel bimetallic complexes and their monometallic moieties against an array of cancerous cell lines, we were able to substantiate our assertion that gold drives the potent efficacy of the complexes. From these screens, three promising candidates emerged: (gold-titanium) Titanocref and Titanofin and (gold-ruthenium) RANCE-1. For those compounds, mechanistic and efficacy studies were carried out \textit{in vitro} and in a mouse model of RCC. We found that our novel gold-titanium and gold-ruthenium compounds were highly cytotoxic to ccRCC cells, were selective for tumor cells, and inhibited molecular markers associated with advanced ccRCC malignancies such as invasion and angiogenesis at subtoxic concentrations. \textit{In vivo} the complexes induced significant tumor shrinkage in the case of Titanocref and Titanofin or exhibited chemo-static effects in the case of RANCE-1. We also obtained pharmacokinetic and pathology reports indicating that the compounds were deposited in tumors and that they caused no chronic systemic toxicity. This body of work from my doctoral research, indicates that these three novel metallodrug complexes are viable candidates for further preclinical development.
Bibliography

DEDICATION

To my father.
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Our commitment to developing drugs against kidney cancer has a personal dimension. In 2015 Dr. Roberto Sanchez-Delgado succumbed to a decade-long fight against kidney cancer. And, it is in part inspired by his struggle that the Contel lab began research geared towards kidney cancer. He is missed.
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To my friends.

To my family of friends.

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What I will take with me, in addition to the intellectual rigor of my doctoral training, are lessons about a scientist’s role in civil society.
In these times of relentless attacks on knowledge and reason, the future of scientific progress in the United States is in peril. Scientists cannot be apolitical because knowledge is never apolitical, since it is by relying on a people’s ignorance that despots retain power. We, scientists, have the moral imperative to take a stand, get involved, do our part to implement concrete changes to counter this climate of anti-intellectualism, anti-facts, and anti-science. We must do so in the classroom and at times in the streets, always advocating for the integrity of scientific knowledge and facts, from evolution to climate change. There is no such a thing as an apolitical educator or citizen: silence is consent. We must resist.

RESIST.

"Scientific literacy may likely determine whether or not democratic society will survive into the 21st century."

-L. M. Lederman

"In a democracy, it is very important that the public have a basic understanding of science so that they can control the way that science and technology increasingly affect our lives." - Stephen Hawking
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ABBREVIATIONS AND ACCRONYMS

5-FU: 5-fluorouracil
A/G ratio: Albumin/Globulin Ratio
ADAMs: A Disintegrin and Metalloproteinases
AF: Auranofin
ALB: albumin
ALP: alkaline phosphatase
ALT: Alanine Transaminase
ANCE-1: [AuCl(IMes)]
AR: drug accumulation ratio
ASK1: Apoptosis Signaling Kinase 1
AST: Alanine Aminotransferase
ATCC: American Type Culture Collection
Au: gold
AUC0–72h: area under the plasma concentration–time curve from time zero to 72 h post dose
BASO: basophils
bFGF: basic Fibroblast Growth Factor
BUN: blood urea nitrogen
Ca: calcium
Caki-1: human clear cell renal cell carcinoma
CAPG: macrophage-capping protein
CBC: Complete blood count
CCL7/MCP3: Chemokine (C-C motif) Ligand 7/ Monocyte-Chemotactic Protein 3

CCLs: chemokine (C-C motif) ligands ccRC: clear cell renal cell carcinoma

CD31: Cluster of Differentiation 31, also known as Platelet endothelial cell adhesion molecule (PECAM-1)

CHOL: cholesterol

CK: Creatine Phosphokinase

Cl: Chloride

Cl/F: apparent total clearance from plasma

Cmax: maximum observed plasma concentration

Cox-2: Cyclooxygenase-2

CPPD: cisplatin (CPPD)

CREA: creatinine

CTLA-4: Cytotoxic T-Lymphocyte-Associated Antigen 4

Cts: Cathepsin

DBIL: direct bilirubin

DFS: disease-free survival

DPPF: 1,1′-Ferrocenediyl-bis(diphenylphosphine)

dppm: diphenylphosphanylmethyl(diphenyl)phosphane

DTNB: 3,3′-Disulfanediylbis(6-nitrobenzoic acid)

EC50: half maximal effect/response concentration

ECM: Extracellular Matrix

ECs: Endothelial Cells

EMT: Epithelial-Mesenchymal Transition
EO: eosinophils
ERK: Extracellular Signal-Regulated Kinase
FAK: Focal Adhesion Kinase
FDA: The United States Food and Drug Administration
FGF: Fibroblast Growth Factor
FLS: Fibroblast-Like Synovial
FOXC2: Forkhead Box C2
G-CSF: Granulocyte Colony-Stimulating Factor
GGT: gamma-glutamyl transferase
GLOB: globulin
GLU: glucose
GM-CSF: Granulocyte-Macrophage Colony-Stimulating Factor
HCT 116: Human Colorectal Carcinoma
HEK-293T: Human embryonic kidney cells 293 Transformed
HGB: Hemoglobin
HIF-1α: Hypoxia-Induced Factor 1 alpha
HUVEC: Human Umbilical Vein Endothelial Cells
i.p.: intraperitoneal
IAP: inhibition of apoptosis proteins
IBIL: indirect bilirubin
IC₅₀: half maximal inhibitory concentration
ICAM-1: Intercellular Cell Adhesion Molecule-1
IFN-α: Interferons alpha
IFNγ: Interferon gamma
ILs: Interleukins
IMes: 1,3-bis(2,4,6-trimethylphenyl)imidazol-2-ylidene
IMR90: human foetal lung fibroblast
K: Potassium
ke: elimination rate constant
LD50: median lethal dose
LT: Lymphotoxin
LYMPH: lymphocytes
M-CSF: Macrophage Colony Stimulating Factor
mAb: monoclonal antibodies
MAPKAPKs: Mitogen-Activated Protein Kinase-Activated Protein Kinases
mccRCC: metastatic ccRCC
MCH : mean corpuscular hemoglobin
MCHC: mean corpuscular hemoglobin concentration
MCV: mean corpuscular volume
MDR-1: Multidrug Resistance-1
MDSC: myeloid-derived suppressor cells
mEq/L: milliequivalents per liter
MMPs: Matrix Metalloproteinases
MONO: monocytes
MPV: mean platelet volume
MTD: maximum tolerated dose mTOR:
mammalian target of rapamycin
Na: Sodium
Neut: neutrophil
NHC: N-Heterocyclic Carbene
NK: Natural Killer cells
nM: nanomolar
P: Phosphate
p-cymene: 4-Isopropyltoluene
PAI-1: Plasminogen Activator Inhibitor-1
PBS: Phosphate Buffered Saline
PD-1: programmed death-1
PD-L1: Programmed Death Ligand-1
PD-L1: programmed death-ligand-1
PDFF: platelet-derived growth factor
PDGF-R β: Platelet-Derived Growth Factor Receptor beta
PDGF: Platelet-Derived Growth Factor
PDW: platelet distribution width
PGE2: Prostaglandin E-2
PGF: placental growth factor
Pgp: permeability glycoprotein
PI3K: Phosphatidylinositol-3-Kinases
PK: Pharmacokinetic
PLT: Platelets
R: [Ru(p-cymene)Cl2(dppm-κP)]
RANCE-1: [Ru(p-cymene)Cl2(η1-dppm)]Au(IMes)]ClO4

RBC: Red blood cell count

RCC: renal cell carcinoma

RDW-CV: red cell distribution width coefficient of variation

RDW-SD: red cell distribution width - standard deviation

RET: Reticulocyte Count

ROS: Reactive Oxygen Species

RTK: receptor tyrosine kinase

Ru: ruthenium

t1/2: terminal elimination half-life

TBIL: Total bilirubin

TCO2: Total Carbon Dioxide

TDC: titanocene dichloride

TGFβ1: Transforming Growth Factor β1

Ti-Y: Titanocene-Y

Ti: titanium

Tie2: Tyrosine kinase with Immunoglobulin-like and EGF-like domains 2

TIMPs: Tissue Inhibitors of Metalloproteinases

Tmax: time to reach Cmax

TNB2-: 5-thio-2-nitrobenzoic acid

TNF-α: Tumor Necrosis Factor-alpha

TP: Total Protein

Treg: regulatory T cells
TRIG : Triglygerides
Trx: Thioredoxin
TrxR: Thioredoxin Reductase TXNIP:
Thioredoxin-Interacting Protein
uPA: urokinase Plasminogen Activator
VCAM-1: Vascular Cell Adhesion Molecule-1
Vd/F: apparent volume of distribution
VE-cadherin: Vascular Endothelial cadherin
VEGF: Vascular Endothelial Growth Factor
VEGFRs: VEGF receptor
VHL: Von Hippel-Lindau
WBC: White blood cells
μL: microlitre
μM: micromolar
1.1 Kidney cancer

There are multiple types of kidney cancer. In adults, renal cell carcinoma (RCC) is the most common, making up about 85% of diagnoses, and in that population, 70% clear cell RCC (ccRCC) phenotype; papillary, chromophobe, ductal, and medullary kidney cancers make up less than 15% of adult diagnoses. Approximately 15000 people (10,000 men and 5,000 women) are expected to succumb to kidney cancer in 2018, and an approximate 63,340 new cases will be diagnosed, largely in individuals over 64 years of age or with known risk factors (Table 1.2); in about 30% of new diagnoses, the cancer will have already metastasized. ccRCC are renal cortical tumors distinguishable by hyperproliferating cancerous epithelial cells with clear cytoplasm in a compact mass enlaced by an extensive vasculature network. These cancers develop in the proximal renal tubules of the kidney, through which blood is filtered. Clear cells are stratified by rate of growth from slow growing (grade 1) to fast growing (grade 4), as well as their location relative to the kidneys boundary (Figure 1.1).

Table 1.1. Kidney cancer

- Lifetime risk of kidney cancer is approximately 1:48 for men, 1:83 for women.
- Projected deaths due to kidney cancer for 2018 is 14,970 people (10,010 men and 4,960 women).
- Predicted kidney cancer diagnosis for 2018 is 63,340 (42,680 in men and 22,660 in women).
- RCC death rates dropped by about 1% annually due to early detection because of advanced imaging tests including CAT scans and MRI, which detect cancers that would have previously not been detected.
- The most common kind of kidney cancer is the ccRCC which form in the cells lining the small ducts in the kidney which filter the blood and produce urine.
- There are two subtypes of renal cell carcinoma, clear cell carcinoma the most common type representing approximately 92 percent of renal cell carcinoma and papillary carcinoma which accounts for about 8 percent of cases.
Figure 1.1. Diagram of the kidney indicating stages of disease progression and treatment options.
Table 1.2 Risk factors

<table>
<thead>
<tr>
<th>Risk factors</th>
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</thead>
<tbody>
<tr>
<td>Old age. The incidence of kidney cancer is highest in individual 64 year and older.</td>
</tr>
<tr>
<td>Tobacco Smoking.</td>
</tr>
<tr>
<td>Obesity.</td>
</tr>
<tr>
<td>Hypertension.</td>
</tr>
<tr>
<td>Treatment for kidney failure such as chronic dialysis.</td>
</tr>
<tr>
<td>Inherited genetic syndromes such as von Hippel-Lindau disease, Birt-Hogg-Dube syndrome, hereditary papillary renal cell carcinoma or a family history of renal cancer.</td>
</tr>
<tr>
<td>Family history of kidney cancer. Unrelated to genetically inherited syndrome.</td>
</tr>
<tr>
<td>Ethnicity. In the United States, African Americans and American Indians/Alaska Natives have higher rates of kidney cancer than whites.</td>
</tr>
<tr>
<td>Occupational exposure such as exposure to asbestos, cadmium, or certain herbicides.</td>
</tr>
<tr>
<td>Chronic misusing of some pain medicines prescribed or over the counter.</td>
</tr>
</tbody>
</table>

1.1.1 Treatment options

When detected early, most cases of kidney cancer can be cured with an approximate 5-year disease-free survival (DFS) rate of about 95 percent \(^4,8,9\). However, because efficient treatment options for advanced stage and metastatic RCC (mRCC) are lacking, the rates of 5-year DFS is about 12 percent when the cancer has spread beyond the kidney capsule and into surrounding and distal tissues \(^4,10,11\). Hence, the recommended courses of treatment are stage specific. Surgery is usually the preferred intervention for small and localized tumors \(^3\). The introduction of pharmacological interventions such as chemotherapy, targeted therapy, or immunotherapy is most often reserved for advanced stages of ccRCC (Figure 1.1). Also, participation in clinical trials is greatly encouraged for patient with metastatic disease \(^12\).
1.1.2 HIF-α, VEGF, and the PI3K/Akt/mTOR pathway

Initially, tumors are not vascularized and thus rely only on the diffusion of oxygen from the surrounding healthy blood vessels. Angiogenesis, the emergence of de novo vasculature from adjacent blood vessels, is an adaptive trait in response to low oxygen conditions, which is promoted by cancer cells to initiate oxygen supply essential for the continued growth of solid tumors. Hypoxia-inducible factors (HIFs) are principal regulators of angiogenesis by controlling the expression of genes associated with angiogenic signaling, amongst which VEGF-A, angiopoietin, placental growth factor (PGF), and platelet-derived growth factor (PDFF). The synthesis of cell cycle proteins such as cyclin D and HIF-α are increased when their upstream regulator, mTOR, is activated. In this context, the activation state of mTOR determines whether the tumor undergoes proliferation, survival, and angiogenesis. Because the activation of HIF-α, VEGF, and the PI3K/Akt/mTOR pathways consists of multiple diverging yet interconnected kinase cascades, they present a target-rich druggable therapeutic ecosystem that has driven RCC therapy development for the past twenty years.

1.2 Pharmacological interventions

1.2.1 Chemotherapy

Chemotherapy broadly serves to kill tumor cells through cytotoxic arrest of growth or division, thus priming the cells for apoptotic death. However, healthy cells are also affected indiscriminately. While cytotoxic chemotherapy is usually the standard of care for cases of renal sarcoma and ductal renal cell carcinoma, it has limited efficacy against ccRCC. In the last two decades, treatment of advanced ccRCC has undergone a shift, distancing from non-specific therapies such as cytotoxic chemotherapies in favor of therapies that target cancer-specific or
tumor microenvironment-specific markers, or activate the immune system in the tumor microenvironment, or a combination therein.\textsuperscript{18}

Though ccRCC is often characterized by resistance to most chemotherapy, we know now that there is merit to each form of intervention and most often, combining agents from each of those classes potentiates treatment efficacy. The United States Food and Drug Administration (FDA) has a list of drugs approved for the treatment of RCC (Table 1.3) and the rationale behind each treatment choice lies in the etiopathology of ccRCC.\textsuperscript{19}

**Table 1.3. FDA Approved Drugs for Kidney Cancer Treatment**  

<table>
<thead>
<tr>
<th><strong>Immunotherapy</strong></th>
<th>Lenvima (Lenvatinib Mesylate)</th>
<th>Bevacizumab (Avastin®)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yervoy (Ipilimumab)</td>
<td>Inlyta (Axitinib)</td>
<td>Pazopanib (Votrient®)</td>
</tr>
<tr>
<td>Opdivo® (Nivolumab)</td>
<td>Nexavar (Sorafenib Tosylate)</td>
<td><strong>Chemotherapy</strong></td>
</tr>
<tr>
<td>Avastin (Bevacizumab)</td>
<td>Pazopanib Hydrochloride</td>
<td>Gemcitabine (Gemzar)</td>
</tr>
<tr>
<td>Proleukin/Interleukin-2/IL-2(Aldesleukin)</td>
<td>Sorafenib Tosylate</td>
<td>Doxorubicin</td>
</tr>
<tr>
<td>Afinitor (Everolimus)</td>
<td>Sutent (Sunitinib Malate)</td>
<td>Capecitabine (Xeloda)</td>
</tr>
<tr>
<td>Torisel (Temsirolimus)</td>
<td>Cabometyx (Cabozantinib-S-Malate)</td>
<td>fluorouracil (5-FU, Adrucil)</td>
</tr>
<tr>
<td>Votrient (Pazopanib Hydrochloride)</td>
<td><strong>Angiogenesis Inhibitors</strong></td>
<td>Vinblastine</td>
</tr>
</tbody>
</table>

| | Axitinib (Inlyta®) |

Thus far, clinical chemotherapy regimens are administered as mono- or combination therapies. The most extensively studied monotherapies (single organic agent chemotherapies) for ccRCC include vinblastine, gemcitabine, capecitabine, floxuridine, and 5-fluorouracil (5-FU).\textsuperscript{20} Vinblastine in monotherapy did not yield promising results; However, responses to vinblastine...
were not improved with the addition of p-glycoprotein efflux channels inhibitors which aim at prevent the cancerous cells from expelling the chemotherapeutic agents from the cytoplasm \(^{21}\). Gemcitabine, with a response rate of only \(~10\%\), demonstrated modest activity in monotherapy with a progression free survival period of about four months and a 12 month average overall survival \(^{22,23}\). 5-FU was the most effective monotherapy, with a response rate of \(~20\%\), and a 15 months average overall survival. Monotherapy response to the pro-drug capecitabine was similar to that of 5-FU \(^{22}\). Thus, 5-FU and capecitabine are the most effective monotherapies within their limited spectrum.

Although chemotherapy is effective against many cancers, ccRCC are often chemoresistant \(^{17}\). To circumvent resistance, combinations of chemotherapeutic agents have often been used in the clinic. In some patients, the combination of standard of care cytotoxic chemotherapeutic agents such as gemcitabine with capecitabine or 5-FU has temporarily shrunk tumors \(^{22}\). A combination of a prodrug 5-FU called Tegafur, along with an inhibitor of dihydropyrimidine dehydrogenase, a prognostic marker for 5-FU therapy response, yielded a response rate of 24%, and a 2-year DFS in 67% of patients \(^{24}\). The moderate response rates observed following fluoropyrimidine treatment prompted investigation of the efficacy of a combination of fluoropyrimidine with gemcitabine and 5-FU in individuals with metastasized ccRCC (mccRCC) \(^{22}\). The combination of capecitabine and gemcitabine led to similar results \(^{22}\) in that no increase in disease free survival (DFS) was observed. In an attempt to increase response Taxol/taxanes, thalidomide, or metal based chemotherapeutics such as cisplatin, oxaliplatin, were then added to the combination of gemcitabine and 5-FU, however, all failed to improve outcomes and resulted in significantly increased toxicity and side-effects/ adverse effect (AEs) \(^{22,25}\). Though we must note that AEs of chemotherapy are often dose dependent, and do not outlast the treatment; they include increased
risk of infection, gastrointestinal distress, and hair loss. In sum, both mono- and combination therapies of cytotoxic chemotherapy agents have proven only modestly effective with mcccRCC and currently do not represent a viable treatment option for most patients.

1.2.2 Immunotherapy

1.2.2.1 Interferons (IFN-α) and interleukins (e.g. IL-2)

Immunotherapy seeks to exploit natural immune-modulating agents to potentiate the anti-cancer immune response of the patient. The cytokines interleukin-2 (IL-2) and interferon-α (IFN-α) are two such agents that are intimately associated with ccRCC therapy. IL-2 also called T-cell growth factor, is a cytokine produced by activated T cells known to increase proliferation and activate cytotoxic T-cells, natural killer (NK) cells, and monocytes, as well as stimulating hematopoiesis. IL-2 also promotes B cell proliferation and antibody production to further assault cancer cells. The antitumor activity of IFN-α is mediated by a series of mechanisms including immunomodulation, promoting anti-proliferation signals, inhibition of angiogenic signaling, regulation of differentiation, crosstalk with growth factors, and other gene expression modulation. IL-2 and IFN-α have been the most evaluated immunotherapies for treatment of ccRCC, but have yielded very modest overall response rates and low DFS advantages, and are coupled with significant toxicity, which renders them less than ideal for clinical use.

1.2.2.2 Additional immunomodulators

In addition to cytokines, there are cell-surface modulators of immune activation that can affect anti-cancer response. For example, the programmed death-1/programmed death-ligand-1
(PD-1/PD-L1) pathway, is involved in the interaction between the immune system (specifically T cells) and tumor cells, and has been found to be a key player in the ability of tumor cells ability to avoid host immune responses. The approach of immune checkpoint blockade is to reestablish natural tumor-specific T-cell-mediated immunity. The immune checkpoint inhibition rationale for anti-PD-1 therapeutics such as nivolumab in ccRCC is that the tumor antigens will be presented by MHC Class I to T cells, resulting in their activation and the successful killing of the tumor cell. The FDA has approved nivolumab in combination with ipilimumab and immunostimulatory monoclonal antibody, as a first-line treatment in cases of advanced ccRCC whose disease have poor prognosis.

One of the most appealing features of immunotherapy is the ability to enhance the patient's natural antitumor response. This relies on blocking the tumorigenic immunoregulatory apparatus. One such critical inhibitory checkpoint is cytotoxic T lymphocyte antigen-4 (CTLA-4), which is a crucial in the negative regulation of the antitumor T-cell response. Ipilimumab is an immunotherapeutic agent that exhibits potent immune-related anti-cancer toxicities, and functions by blocking CTLA--4. The average overall survival in response to PDL-1 and ipilimumab is between ten and 20 months.

Finally, Bevacizumab is a recombinant humanized monoclonal antibody (mAb) proposed as an immunotherapeutic that blocks angiogenesis by inhibiting VEGF-A. Bevacizumab combined with IFN-α is now approved to treat of naïve ccRCC. Its average overall survival of nine to ten months is comparable to the clinical first-line multityrosine kinase inhibitors that is sunitinib and pazopanib.
1.2.3 Chemotherapy in combination with immunotherapy

Traditional chemotherapy in combination with immunotherapy has yielded a modest improvement in patients treated with immunotherapy such as IL-2 or IFN-α. However, this course of treatment induces significant AEs and is rarely used in the clinic. There are very few trials reporting studies of combination between chemotherapeutic agents and the new wave of immunotherapy.

1.2.4 Targeted therapies

A paradigm shift away from non-specific chemotherapy, towards tumor specific therapeutics has led to the advent of targeted therapy, that is the design of agents to specifically identify and disable cancer cells without harming healthy cells. Because ccRCC are a highly vascularized cancer due to overactivated VEGF signaling and mTOR deregulation, the these pathways have been identified as therapeutically druggable targets. Current clinical guidelines for ccRCC patients with poor prognoses recommend targeted therapies with FDA approved multi-targeted receptor tyrosine kinase (RTK) inhibitor agents such as sunitinib, pazopanib, or the mTOR-specific inhibitor temsirolimus as the standard of care for first-line treatment. Sunitinib, similar to other multi-targeted RTK inhibitors, inhibits cellular signaling by targeting several RTKs, including the PDGF receptors (PDGFRs) and VEGF receptor (VEGFRs) known to drive tumor proliferation and angiogenesis. The simultaneous inhibition of these targets curbs tumor vascularization and apoptosis and has been reported to lead to tumor shrinkage. Sunitinib also inhibits CD117, a RTK, which drives the malignant progression of many gastrointestinal tumors when dysregulated. One of the downsides of sunitinib is that it also binds other receptors.
which leads to AEs such as advanced dermatologic toxicities \cite{10,27}. Pazopanib is a potent and selective RTK inhibitor that hinders tumor growth and inhibits angiogenesis. Its main targets are c-KIT, fibroblast growth factor receptor (FGFR), PDGFR, and VEGFR \cite{26,40}. Temsirolimus is a clinically used mTOR-specific inhibitor that modulates proliferation, growth and survival regulating proteins in tumor cells. Temsirolimus leads to G\textsubscript{1} cell cycle arrest, and inhibits tumor angiogenesis by downregulating VEG.

While targeted therapeutics for mRCC constitute two different mechanistic classes, they often have overlapping functions \cite{17}, in particular the inhibition of VEGF upregulation. Anti-VEGF agents such as bevacizumab (directed against the ligand with mAb) or, sunitinib, sorafenib or pazopanib (directed at the receptor, TKI). On the other hand, temsirolimus and everolimus act through inhibition of intracellular mTOR kinase signaling, leading to cell-cycle arrest, enhanced apoptosis, and angiogenic inhibition \cite{17,42}. To enhance the efficacy of the targeted approach to ccRCC therapy, a combination of agents capable of targeting different points in the VHL–hypoxia-inducible gene pathway has been proposed.

Though it has been determined that the VEGF and mTOR pathways are up-regulated by various tumor specific factors such as hypoxia, select cytokines, the inactivation of tumor-suppressor genes, and angiogenic enhancement, their targeted inhibitors exhibit different antitumor activities and dissimilar AEs. These differences are important in composing the best clinical intervention regiment limited outcome improvement.
1.2.5 Chemotherapy in combination with targeted therapies

A deepening of our understanding of the molecular biology of ccRCC and great progress in rational drug design have rendered metastasized ccRCC amenable to treatment with targeted therapies using multi-TKI inhibitors, mTOR inhibitors, and VEGF targeting agents as the current standard of care for mccRCC. In monotherapy, mTOR inhibitors such as everolimus and temsirolimus are reported to slow tumor growth, but clinical response to such therapy is very modest, and most cases, the tumor does progress and necessitates second, or thirst line therapies. As with other chemo- and immune-therapies, combination of several agents or agents from different classes have been studied in attempts to improve efficacy. The hypothesis being that there might be synergy that would potentiate each agent against their distinct targets while avoiding resistance and reducing AEs. Combination therapy using cytotoxic chemotherapy with targeted therapy have shown promising results in ccRCC clinical trials. For example, the VEGF inhibitor antibody bevacizumab combined with capecitabine and gemcitabine yielded modest overall survival rate improvement with no significant side effects in ccRCC. Other antiangiogenics agents such as thalidomide were tested in combination with gemcitabine and 5-FU but cause significant AEs while yielding little clinical outcome improvements.

1.2.6 The proposed biology of cytotoxic chemotherapy resistance in ccRCC

The mechanisms of chemoresistance in ccRCC are not completely understood. The mechanism most frequently proposed postulates that the overexpression of an ATP-binding cassette group of transporter efflux pumps drives resistance by limiting the intracellular accumulation of the cytotoxic agent. The permeability glycoprotein (Pgp) is an example of such
an efflux pump system. However, the use of Pgp antagonists in clinical trials yielded modest results, challenging the assertion that Pgp is an essential mediator of chemo-resistance \(^{49,50}\). It has also been proposed that there might be alterations in the efflux pumps characteristic of ccRCC due to mutations of the VHL gene. The disabling of VHL results in intracellular accumulation of HIF, a microtubule dependent transcription factor involved in angiogenesis, thereby deregulating/removing the breaks on tumor cell proliferation and survival \(^{51}\). It has been suggested that VHL, HIF, and the cytoskeletal microtubules are druggable targets that are key to the pathogenesis of ccRCC. However, notwithstanding the fact that there are no observed mutations in the taxane binding site, the use of taxanes which is an anti-microtubule chemotherapeutic has been unsuccessful in the treatment of ccRCC, although very effective in treating other solid tumors such as breast cancers \(^{51}\). Taxanes are anti-mitotic agents that work by suppressing the dynamic of microtubules therein preventing proliferation and killing tumor cells\(^{51,52}\). This suggests that anti-microtubule therapy resistance might rely on the expression of non-standard tubulin isoforms. Finally, it has also been proposed that low levels of tumor suppressor phosphatase and tensin in ccRCC contributes to chemoresistance by activation of the key regulator of the tumor suppressor signaling pathway Akt/HDM2 \(^{53}\).

### 1.2.7 In defense of chemotherapy

The clinical advantage of targeted multi-kinase inhibitors, mTOR inhibitors, and VEGF specific agents has fueled the expansion of therapeutic options for advanced and mCCRCC. However, these advantages have only provided modest increases in the DFS window. Immunotherapy has also provided only modest improvements in clinical outcome, and causes significant toxicity and AEs, however, it still plays a central role in the treatment of metastasized
ccRCC. FDA approved antibodies such as bevacizumab have been shown to slow tumor growth in patients mcccRCC; and in combination with IFN-α, slow tumor growth and metastasis but are not curative.29 Axitinib, cabozantinib, pazopanib, sorafenib, and sunitinib are also FDA approved TKIs for the treatment of ccRCC; but their efficacy is similarly limited.17,40,54 Often, patients must discontinue immunotherapy treatment because of drug related toxicity or because the treatment fails to hinder disease progression.27,55 Also, about a quarter of patients with advanced ccRCC treated with targeted therapies have VEGF refractory disease, requiring second- and third-line therapies, and even with these multiple therapies and have poor prognosis.17,56,57 However, it has been reported that for some patients with tumors refractory to anti-VEGF there is a response cytotoxic chemotherapy regiments, more specifically with treatment regimens of gemcitabine and capecitabine after a failed targeted therapies regimen.57 Several chemotherapy regimens have yielded modest efficacies in patients whose tumors grow following treatment with cytokines or targeted therapy, suggesting that the first treatment course might have sensitized the tumor.58,59 Combination therapy using cytotoxic chemotherapy and targeted therapy have shown promising results in clinical trials.45,57,58 These results make a case that cytotoxic chemotherapy may be beneficial in patients with ccRCC refractory to standard therapy. Consequently, given the relatively high prevalence of metastatic ccRCC that do not respond to immunotherapy or targeted therapies, there is an urgent need for novel effective, non-toxic clinical alternatives to the currently available pharmaceutical arsenal to extend survival and improve quality of life.

For the sake of over 14,000 patients whose lives will be lost due to our pharmaceutical shortcomings, we must rethink how we treat and ccRCC and perhaps consider going back to basics. Harnessing the benefits of chemotherapies and improving on their success. Such efforts are underway, and the use of organometallic compounds has recently been proposed to meet that critical need with great preclinical success.
1.3 Chemotherapy beyond cytotoxicity

1.3.1 Context of novel metal-based approach.

Organometallic compounds have been offered as a potential chemotherapeutic alternative or complementary option to the current clinical regimens in the treatment of ccRCC. While many cancer chemotherapeutics are highly toxic to both cancerous and normal cells, and cause debilitating AEs, there are now reports of metallodrugs, specifically titanium-based and gold-based complexes, that are highly selective for tumor cells while minimally toxic to healthy cells at low doses. Some such compounds are also reported to inhibit growth factors, cytokines, and proteases key to the malignant progression of ccRCC. Molecules made of two different metal centers (heterobimetallic), each with distinct known chemotherapeutic properties, are proposed as a novel single-drug approach to multi-targeted anticancer therapy.

1.3.2 Metallodrugs

The platinum based drug cisplatin (CPPD in Chart 1.1) was the first metallodrug oncotherapy approved by the FDA in 1978. To date, cisplatin is still the first line of treatment for many ovarian and testicular carcinomas, small cell lung cancer, bladder, and cervical cancers. However once metastasized, most cancers do not respond to cisplatin. Despite tremendous successes, the coordination compound cisplatin and its offshoot compounds carboplatin and oxaliplatin face three main obstacles: 1) existence of cisplatin-resistant tumors, 2) severe secondary effects such as neuropathy, myelotoxicity, and nephrotoxicity, and 3) lack of specificity as conventional platinum anticancer drugs target DNA, which is ubiquitous among normal and cancerous cells.
Therefore there is a need for alternative metal compounds with greater efficacy and lower non-specific-toxicity than cisplatin \(^74\).

Organometallics offer great structural variety, along with greater stereochemical diversity than organic compounds \(^74\)-\(^76\). Furthermore, organometallic compounds allow for rational ligand design, providing control over electrochemical properties and important kinetic properties, specifically their rate of ligand hydrolysis. Many organometallic drugs are lipophilic, are most uncharged, and are contain a kinetically stable metal atom in low oxidative state \(^77\). Because of these assets not found in classical (inorganic) coordination metal complexes, organometallics offer a large area for the design of a novel realm of chemotherapeutics characterized by their metal specific biological properties.

Organometallics containing different metals [e.g. titanium (Ti), gold (Au) and ruthenium (Ru)] such as metallocenes, containing arene-, NHC-carbene-, or phosphine ligands are among the most reported for medicinal applications \(^74,78,79\). For example, Ti complexes such as titanocene dichloride (TDC) and Titanocene-Y (Ti-Y) have exhibited significant antitumor activity. Gold coordination and organometallic complexes such as Au-NHC-carbenes and Au-phosphines, have demonstrated outstanding cytotoxic and chemosensitizing properties against many cisplatin resistant tumors. Recently, ruthenium-based compounds, including Ru-arene, have shown great promise in animal models and in the clinical setting in the treatment of metastatic tumors \(^72,80,81\).

1.3.3 Titanium

In the late 1980’s, titanocene dichloride (Cp\(_2\)TiCl\(_2\) also known as TDC, Chart 1.1) was shown to have potential cancer- chemotherapeutic properties both \textit{in vitro} and in mouse models \(^82\).
TDC’s cytotoxicity was credited to interactions with the iron transport protein, transferrin, via which it is shuttled into the cell.\textsuperscript{83}

\textbf{Chart 1.1.} Platinum, titanium, gold and ruthenium metallodrugs that have entered clinical trials, or that have been part of advanced preclinical studies (Titanocene Y and RAPTA-C).

One notable asset of TDC and many subsequent (monometallic) titanocenes, is that it does not affect bone marrow proliferation (key to immune health), a frequent dose-limiting secondary-effect of organic drugs.\textsuperscript{64} Owing to promising preclinical success, in 1993 TDC became the first non-platinum coordination complex and the first organometallic drug to enter clinical trials.\textsuperscript{84} However, TDC clinical trials against breast and renal-cell carcinoma (RCC) were unsuccessful due to poor aqueous solubility and hydrolytic instability.\textsuperscript{64,84} Ti-Y (Chart 1.1) was a more stable and more lipophilic descendent of TDC, and like TDC, it is highly cytotoxic to cisplatin resistant cancers.\textsuperscript{85} The proposed mechanism of Ti-Y activity suggests that it diffuses via serum albumin
to the cancer cells where it is internalized through interaction with transferrin. Titanocene activity requires pH dependent hydrolysis of the Ti-Cp ligands. Ti(IV) species can be kept in a lipophilic milieu long enough to permit uptake of Ti by transferrin due to its slow hydrolysis. We must mention that cytotoxic Ti derivatives, with more stable Cp-Ti bonds, may exhibit distinct biochemical interaction than TDC. Over the last decade, new cytotoxic Cp-derived and diverse coordination Ti complexes have been shown to be active against breast, prostate, and kidney cancers that are refractory to cisplatin, though much is still to be optimized in their design to increase stability and efficacy.

1.3.4 Gold

Gold (Au) complexes were found to be highly antiproliferative, a property for which Au-complexes have garnered great attention. Au complexes comprise a large variety of different ligands (i.e. thiolates, phosphines, heterocyclic NHC-carbenes, and aryl-containing moieties) attached to Au in the oxidation state I or III. The inhibition of tumor growth by Au complexes has been linked to a potent and specific inhibition of the mitochondrial enzymes thioredoxin (Trx) and thioredoxin reductase (TrxR). Trx is upregulated in many malignant solid tumors and high levels of expression correlate to chemoresistance and aggressive tumor invasion and metastasis. It has been reported that Trx mediates hormesis against oxidative stress-induced apoptosis. The pharmacodynamics and pharmacokinetics of Au based metallodrugs is distinct from that of cisplatin. One key point of dissimilarity with cisplatin is that Au(I) compounds only weakly bind to DNA, thus are unlikely to cause DNA damage. Because of the great structural diversity of ligands, and the unlikelihood of a single biochemical or pharmacological profile for all gold complexes, it is expected that not all pharmacodynamic
effects of Au-complexes are based on TrxR inhibition. Very recently, the phosphine-gold(I)-thiolate compound auranofin (AF) (Chart 1.1), an FDA approved anti-arthritic drug in use for over 25 years, has entered clinical trials as an anticancer agent\textsuperscript{94,95}. AF is undergoing trials for the treatment of leukemia, small cell lung cancer and ovarian cancer\textsuperscript{94,96}. AF has also proven very efficacious against bacterial and parasitic infections. AF’s efficacy in killing bacteria, parasites, and cancer cells, was also correlated to a strong inhibition of TrxR\textsuperscript{97}.

1.3.5 Ruthenium

In the last three decades, numerous ruthenium (Ru) containing complexes have been evaluated with notable success against primary and metastatic breast cancers, and are therefore being studied and developed as building blocks for new antiproliferative and antimetastatic metallodrugs\textsuperscript{98}. Though Ru is a transition metal in the Pt group, Ru compounds’ means of efficacy against cancer differ from that of Pt drugs\textsuperscript{80,99}. Ru(III) drugs can accumulate in cancer cells with limited accrual in healthy tissue, which curbs one of the main shortcomings of cisplatin\textsuperscript{87,100}. Such selectivity may be explained in part by Ru’s use of transferrin to enter tumors\textsuperscript{80,101}. Also, Ru(III) is a fairly inactive oxidation state prior to its reduction to the active Ru(II) form. Such activation is driven by a reduction pH and occurs most likely inside cells or in the tumor microenvironment, where it may be stimulated by hypoxia\textsuperscript{79,80,98,99,101,102}. The oxidative mechanism was suggested to account in part for the innoxiousness of Ru(III) drugs NAMI-A, and KP1019 (Chart 1.1) to healthy cells; perhaps a key feature for their entry into clinical trials\textsuperscript{79,103–105}. Ru(II) organometallic complexes such as RAPTA-C (Chart 1.1) or RAPTA-T were reported to inhibit metastatic invasion, proliferation, and angiogenesis, though the complexes were inactive against primary tumors\textsuperscript{99,106}. NAMI-A, and RAPTA-C have also
been reported to drive down the expression of TGF-β and VEGF, and inhibit vascular cell assembly into blood vessel precursor tubes \(^{80,81}\).

Recently, two Ru(II) polypyridyl complexes TLD-1433 and RuPOP have garnered great interest; in 2016, TLD-1433 entered phase I clinical trials against non-muscle invasive bladder cancers \(^{102,107}\), and RuPOP, which was reported to inhibit growth and metastasis of triple negative breast cancer cells while enhancing apoptosis, is being evaluated \textit{in vivo} in animal models \(^{108}\). The selective intracellular uptake and cytotoxicity of TLD-1433 and RuPOP is associated with either transferrin receptor, or integrin mediated endocytosis receptor activity \(^{109}\). Moreover, RuPOP was reported to induce remedial alteration in the expression of proteins that regulate metastasis such as urokinase-type plasminogen activator (uPA) or MMP-2/-9, while inhibiting VEGF \(^{109–111}\). The oxidative properties of some Ru-arenes result in complex interactions with double helical DNA, which was demonstrated to induce radio-sensitization of cancer cells otherwise refractory to irradiation therapy \(^{79,112,113}\). Thus, Ru-based complexes are evidently a promising line of antitumor and antimetastatic therapeutics. However, notwithstanding the data gathered in the past decade, the causal mechanisms of the antitumorigenic potential of Ru-based oncotherapies are not fully understood.

1.3.6 Heterobimetallic compounds

Not many polymetallic complexes (homometallic: a complex with one species of metal centers; heterometallic: a complex with two or more different species of metal centers) with anticancer potential have been evaluated, in contrast with monometallic complexes.
Chart 1.2. Some heterometallic Ti-Au and Ru-Au compounds with anticancer activities reported by the Contel group\textsuperscript{88,111}.

In 2011, the Contel research group and the Casini group reported on titanocene-gold(I) complexes with cyclopentadienyl-phosphine linkers that showed a synergistic effect in ovarian and prostate cancer cell lines\textsuperscript{88,111}. However, like titanocene dichloride (TDC), the compounds were not very stable at physiological pH (7.4). In 2015, the Contel group reported on the first arene Ru(II)-Au(I) compounds based on the structure of RAPTA-C and gold(I)-thiolate or chlorophosphines similar to auranofin (Chart 1.2). These new complexes displayed more promising activity toward colon cancer cells \textit{in vitro} than the individual Ru and Au species, these bimetallic compounds were also more cytotoxic or more selective. The Ru(II)-Au(I) compounds showed no interaction with plasmid (pBR322) DNA, but did induce inhibition of TrxR, which may be attributed to the gold moiety. Ru(II)-Au(I) compounds also inhibited cysteine proteases cathepsin B ($CtsB$), known to be inhibited by Ru-containing compounds\textsuperscript{111}. $CtsB$ is a poor prognostic marker in many cancers and is associated with the recruitment of blood-vessels as well as protumorigenic immune cell recruitment to the tumor microenvironment\textsuperscript{114–116}. It has therefore become evident that heterometallic compounds possess multifunctional features, all valuable assets for cancer treatment.\textsuperscript{111}
There seems to be no conserved narrative of how a set of given metals affect cancer cell behavior, in part because there has not been systematic study of the phenotypes and pathways involved. Our limited understanding of the mechanisms of the antitumorigenic potential of Ti, Au-, and Ru-based compounds has hindered rational design of novel compounds and hampers their clinical application. There is empirical evidence that some Ti-Au and Ru-Au compounds are highly toxic to ccRCC while minimally toxic to healthy cells, there are also some broad indications of the molecular pathways affected in response to treatment with some of the Contel Research Group Ti-Au and Ru-Au compounds. 89,117.

1.4 Molecular signaling and drug efficacy (Markers of interest)

Tumor progression impinges on both inherent tumor characteristics and crosstalk between tumor cells and stromal1 cells in the tumor microenvironment. Fibroblasts, macrophages, and adipocytes are central components of the tumor microenvironment. A variety of soluble factors serve to connect stromal cells and tumor cells. Those factors include pro-inflammatory cytokines and chemokines secreted by stromal and tumor cells as well as growth factors. It is therefore critical to validate in vitro findings obtained from cancer cells in mono culture, to in vivo data obtained from a mammalian animal models since, the protein expression profile observed in vitro in cancer cell monoculture might not reflect the signaling complexity and expression profile of in vivo tumor samples. To this end, to gain essential mechanistic insights as to how the Titanocref,

1 In immunology stromal cells are defined as non-hematopoietic cells, for our purposes this term will be used to refer to all non-cancerous cells of the tumor microenvironment.
Titanofin and RANCE-1 achieve their efficacies, we interrogated, key pathways *in vitro* and *in vivo* known to drive proliferation, angiogenesis, migration, metastasis, cell death and death resistance.

**1.4.1 Inflammation**

**1.4.1.1 Inflammation at the intersection between immunity and angiogenesis**

The link between cancer and inflammation has been extensively studied, and cytokines are known to be important mediators of many cancers and inflammatory diseases. In fact, as previously noted, the FDA approved Au-organometallic drug Auranofin (AF) is prescribed to treat rheumatoid arthritis, a chronic inflammatory disease, is now also being tested for cancer treatment. The efficacy of Au complexes in palliating inflammation is credited to their effects on the signal transduction of immune cells, specifically macrophages, and the inhibition of inflammation promoting cytokines, including interleukin-6 (IL-6), Cyclooxygenase-2 (Cox-2), and focal adhesion kinase (FAK). Several Ru compounds that inhibit angiogenesis have also been observed to either inhibit or lessen the expression of IL-6 or VEGF. AF has been reported to inhibit angiogenesis via cytotoxic effects on endothelial cells and by decreasing proangiogenic factor secretion (e.g. IL-6) by cancer cells. p38 MAP kinases, which were shown to be inhibited by some Ti-Au complexes, were also found to promote inflammatory cytokines such as IL-4, IL-6, and tumor necrosis factor-α (TNF-α). It has also been reported that treating cancer cells with p38 MAPK inhibitors diminished the expression of both uPA and its receptor uPAR through which tumor invasion and metastasis was shunted.
COX-2 is a membrane bound enzymes that serves as the catalysis of the synthesis of the inflammatory mediator prostanoid \(^{127}\). Its expression is intimately regulated by inflammatory modulators such as growth factors, cytokines, and endotoxins \(^{128}\). AF has been shown to shunt pro-inflammatory eicosanoid formation (in particular prostaglandins, leukotrienes, and eoxins) in human serum via the inhibition of COX-1/2 \(^{129}\). It was shown that AF inhibits the synthesis of the COX-1 baseline enzyme product prostaglandin E-2 (PGE2) in human synovial fibroblast-like (FLS) cells through the inhibition of TrxR, while also suppressing COX-2 expression, which produces PGE2 through inflammatory stimulation \(^{130}\).

TrxR activity is also known to increase inflammation in lung and joint tissue, is significantly inhibited by several gold complexes \(^{131}\). Trx may induce the expression of hypoxia-induced factor 1 (HIF-1α) and upregulate expression of VEGF, promoting neoangiogenesis in tumors \(^{132}\). Finally, the antiangiogenic properties of Ru-based compounds have also been linked to the inhibition of zinc-containing and calcium-dependent endopeptidases matrix metalloproteinases (MMPs) and the inhibition of HIF-1α \(^{108,133}\). We note that HIFs also participate in the modulation of proliferation and metastasis.

What is evident in this pathologic orchestra, is that many players such as HIF, TrxR, MMPs, and IL-s to name a few, are promiscuous and contribute to multiple disease phenotypes depending in their interaction partner(s) or environmental factors.

1.4.2 Angiogenesis

Angiogenesis, the sprouting of neovascularure a budding pre-existing blood vessels, plays a key role in tumorigenesis and in cancer treatment \(^{134}\). To initiate angiogenesis, emerging solid
tumors secrete factors that induce the production and chemotaxis of endothelial cells (ECs) \(^{135}\). The oxygen-poor environment of the inner tumor induces the expression of HIF-1α, which then activates the expression of an angiogenic gene cascade that includes VEGF and Fibroblast Growth Factor (FGF) basic that respectively stimulate vascular permeability and EC growth \(^{136}\). FGF drives angiogenic branching morphogenesis, which is critical for the proper outlay of vasculature in healthy tissue, but in tumors can serve to fulfill the vascular promoting functions much like VEGF \(^{137}\). While VEGFs is the most studied and most discussed angiogenesis promoting protein family, the initiation of angiogenesis by the EC can be triggered via several other growth factors such as angiopoietins, FGF, TGFs, and PDGF, thereby enabling resistance to anti-VEGF therapy \(^{137–139}\). In response, tumor cells can bypass VEGF’s contribution to angiogenesis by up-regulating FGF-2

In addition to angiogenesis, FGF basic signaling regulates cell growth, differentiation, and wound healing, and in those capacities to promote to proliferation of vascular cells has been proposed to play a central role in the resistance to anti-VEGF therapy \(^{137,140}\). PDGF and angiopoietin-1 facilitate chemotaxis, while ephrin guides newly formed blood vessels and maintains cell-cell separation and together they also compensate for the absence of VEGF. Considering the VEGF compensatory response by FGF, agents targeting FGF signaling are being explored as an additional target of antiangiogenic therapies. Targeting both VEGF and FGF pathways has great potential and may translate into improved patient outcomes.

Trx has also been reported to play a role in HIF mediated angiogenesis \(^91\). Trx and TrxR are of interest because they have been reported to be significantly inhibited by gold compounds. Trx is overexpressed in a variety of cancers, and correlate enhances vascular networks and increases VEGF levels \(^{91,123,141,142}\). The Trx inhibitor AW464 is reported to impair Trx1 as well as
HIF1 activity and lessen EC which contributes to hypoxia-driven vascular remodeling that facilitates tumor extravasation\textsuperscript{143,144}. Other Trx1 signaling inhibitors such as pleurotin and PX-12, decrease Trx1 expression and activity, thereby decreasing HIF1\(\alpha\), and reducing VEGF levels, leading to the formation of poorly perfused vasculature\textsuperscript{145–148}. Blockade of Trx1 using PX-12 has shown promise in clinical trials as it induces a significant decrease in plasma concentrations of VEGF\textsuperscript{148}.

Equally important to the vascular dynamic of the tumor microenvironment are the negative regulator of angiogenesis such as Tie2 (a tyrosine kinase receptor) and endogenous inhibitors such as endostatin and thrombospondin-1. The signaling promiscuity extend to angiogenic proteins as well, such as angiopoietins which can regulate the survival and apoptosis of endothelial cells through interactions with their receptor, Tie2 (a tyrosine kinase receptor)\textsuperscript{149,150}. Tie2 activation in turn enhances the normalization of tumor vessels and decreases angiopoietin expression and facilitates local drug delivery as blood vessels with more integrity will best deliver to the tumor\textsuperscript{151}. Endostatin, inhibits angiogenesis by down regulating many signaling cascades like ephrin, inflammatory cytokine TNF-\(\alpha\), and NF\(\kappa\)B signaling, as well as coagulation and adhesion cascades that are involved in angiogenic initiation and local recruitment of vascular cells\textsuperscript{152,153}. Thrombospondin-1 is an antiangiogenic factor that is enhanced by IL-18. Therefore, in designing novel therapeutic agents, the potentiation of those antiangiogenic factors, while also blocking their natural inhibitor remains of interest.
1.4.3 Immunomodulators

1.4.3.1 CCL-7, M-CSF and GMCSF

The immune system can be a powerful weapon against cancer, however it can also be coopted by the tumor microenvironment as a tumor promoting phenotype \(^{154,155}\). Upon the acquisition of pro-tumorigenic phenotype, macrophages are known to play a key role in the malignant progression of tumors via a large orchestra of signaling molecules. In this molecular arsenal are chemokine (C-C motif) ligands (CCLs), CCL-7 [also known as macrophage chemotactic protein-3 (MCP3)] and macrophage colony stimulating factor (M-CSF). CCL-7 is a key regulator of the interplay between stroma and tumor, an increase in CCL7 expression recruits monocytes to the tumor periphery and establishes a protumorigenic ecosystem. M-CSF is the chief regulator of macrophage differentiation, proliferation, and survival. It is also critical to macrophage-related functions such as vascular development and maintenance, and innate immunity. Both M-CSF and its receptor can be expressed by tumor cells, thus M-CSF may serve as an autocrine growth factor for cancer cells. An increase in CCL7 or M-CSF in the tumor microenvironment fosters the recruitment of tumor associated macrophages which provide proteolytic factors to the tumors cells, thereby allowing them to extravasate into the vasculature or degrade the ECM to promote angiogenesis.

Granulocyte-macrophage colony-stimulating factor (GM-CSF) is much higher in many primary tumors than healthy tissue. Along with its receptor in RCC and cancerous intestinal tissues high GM-CSF levels modulated by IL-3 and IL-5 correlate with greater survival \(^{156}\). Recent clinical studies demonstrated that first line therapy combining inhibition of cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4) and GM-CSF-based immunotherapies can potentiate the antitumor efficacy \(^{157}\).
1.4.3.2 ICAM-1

Adhesion is critical to immune cell to tumor cell as well as immune cell to immune cell communication. The intercellular cell adhesion molecule-1 (ICAM-1) plays an important role in both acute and chronic inflammatory responses \(^{158–160}\). ICAM-1 is a transmembrane signal transducer, and modulates leukocyte-leukocyte, leukocyte-endothelial cell, and leukocyte-epithelial cell interactions, as well as cell motility and adhesion \(^{161}\). ICAM-1 expression can help determine the malignant potential of cancer and is positively correlated with metastatic potential and is therefore of therapeutic interest.

1.4.4 Metastasis

Tumor metastasis is a sequential process in which cancer cells migrate from the primary tumor site (extravasation), survive vascular or lymphatic dissemination, relocate in distant secondary niches (intravasation), and continuously divide \(^{162,163}\). Communication between intracellular signaling proteins and the extracellular matrix (ECM) is a critical axis in driving successful metastasis. Cell-ECM communication is a critical process that regulates cell morphological dynamics, survival, differentiation, proliferation, and migration. The activation of MMPs is regulated through the serine protease uPA and its receptor uPAR \(^{108,115}\). uPA dysregulation leads to overexpression of MMP-2 and MMP-9, which are linked with the initiation of metastasis of many types of solid tumors \(^{108,110,164–167}\). Importantly, Ru compounds have been reported to be inhibited by MMP-9 \(^{74,105,108,168,169}\).

In fibroblasts and macrophages, MMP-9 is upregulated by transforming growth factor \(\beta_1\) (TGF\(\beta_1\)), TNF-\(\alpha\), FGFbasic, IL-1\(\beta\), and lymphotoxin (LT) \(^{170–172}\). TNF-\(\alpha\) serves as an
endogenous tumor promoter that enhanced tumor invasion, while FGF overexpression correlates with poor prognosis.\textsuperscript{173,174} Following upregulating MMP-9 production in fibroblasts and macrophages, these cells then contribute to the peritumoral proteolytic activity that cuts through the ECM and creates a path for the tumor cells to invade.\textsuperscript{175–177} Blocking the FGF receptor has been reported to inhibit tumor growth, lymphangiogenesis, and metastasis.\textsuperscript{137,178} IL-17A can also increase tumor cell motility by upregulating MMP-2 and MMP-9 via activation of NF-κB.\textsuperscript{166,179,180} IL-17A is associated with and hormone receptor negative breast tumor hyper proliferation and poor prognosis. IL-17 has been reported to induces a HIF-1α-dependent invasive phenotype in both tumor cells as well as synoviocytes. This is noteworthy because IL-17 in part regulates migration and invasion of synoviocytes in joints affected by rheumatoid arthritis, plays a key role in the malignant development of this inflammatory disease for which Auranofin is already a clinically approved treatment.\textsuperscript{179,181,182}

A disintegrin and metalloproteinases (ADAM) (ADAM-8 and ADAM9 in particular) are also modulated by MMPs, and are known to be expressed in in breast or lung cancers that have metastasized to the brain.\textsuperscript{183–185} The apparent brain homing metastatic phenotypes suggest that there may be a zip-code like property to ADAM-8 and ADAM-9. Also it has been reported to serve as a metastatic disease progression marker.\textsuperscript{184–188} Also ADAM9 expression is used in the clinic as a prognostic relapse in prostate cancer.\textsuperscript{189} The ability to block ADAM expression holds a great deal of therapeutic potential. Another protein with metastatic site-specific features is the macrophage-capping protein (CAPG). It serves as a predictive biomarker for the metastasis of breast cancer to the bone, and in that capacity is used to guide therapeutic interventions.\textsuperscript{190,191}

Forkhead Box C2 (FOXC2) can also induce the migration of ccRCC by upregulating MMP-2 and MMP-9.\textsuperscript{192,193} However, FOXC2’s contribution to metastasis is not only associated
with MMP activity. Independently of MMPs, FOXC2 expression is induced when ECs undergo EMT and take on a which protects the cells from immune detection and facilitates the metastatic process. Overexpression of FOXC2 is a feature of many highly metastatic breast cancers 194,195.

E-cadherin is a transmembrane protein that facilitates homophilic cell–cell critical in cell motility. Not unlike FOXC2, E-cadherin contribute to tumor through its ability to modulate EMT. Tumor progression is often accompanied by the loss of E-cadherin function and the transition to a more invasive phenotype 196,197. When E-Cadherin levels decrease, tumor cells undergo the pathological EMT process and migrate. E-cadherin is released as a soluble factor that augments cancer cell motility and EGFR-dependent survival and proliferation 197. Finally, E-cadherin has been reported to interact with FOXC2 in the process of tumor EMT 198.

CCL8, like CCL7, plays an important role in tumor-stroma, tumor-immune cell and tumor-tumor cross-talk. It is most important in the early stages of metastasis when dermal fibroblasts increase the production of CCL8 to facilitate proteolysis by stimulating the production of pro-metastatic proteolytic factors such as MMP-9 and MMP-2 by tumor cells and peritumoral stromal cells 199–201. The levels of CCL8 in the periphery tumor microenvironment modulate the initial protrusion of the tumor cells and the initiation of metastasis 199.

**1.4.5 Survival**

Cell survival relies on the activation of inhibition of apoptosis (IAP) proteins, which is occurs through the expression of pro-apoptotic factors and the enhanced expression of anti-apoptotic factors. Survivin is a member of the IAP family 202. Increased survivin expression in fully differentiated tissues, often leads to unregulated cell survival, resulting in cancer 203. Survivin has been reported to shield cells from caspase-induced apoptosis activated in mitosis.
through Caspase3, 7, or 9 binding. If overexpressed in ccRCC and other solid tumors, survivin is of prognostic relevance as its expression has been significantly associated with poorly differentiated, advanced stage and more aggressive ccRCCs. Bcl-2 is critical to mitochondrial mediated apoptosis. Bcl-2 family members down-regulate apoptosis by directing mitochondrial permeability following death signaling, Bc-l2 promotes the release of cytochrome c resulting in apoptosis. Lastly, in addition to all the functions of Trx previously discussed, it also serves to evade cell death. In its capacity as a ROS scavenger, Trx is overexpressed in cancer cells in order to escape oxidative damage induced apoptosis, thereby overcoming cytotoxic assault.

1.5 Therapy resistance

Cancers are characterized by their ability to evade death and develop chemoresistance. There are several known markers of chemoresistance, and knowing if they are expressed or not in a given tumor type can help inform treatment decisions. The drug resistance process can be regulated by an increase in the apoptotic threshold through a variety of cytokines and growth factors, hypoxia levels and high activity of drug-efflux transporters. Recent reports have suggested that the emergence of drug resistance may be caused during the EMT stage in which the differentiated tumor cells gain stem-cell like molecular signatures. Most drug-resistant cancers have populations with stem-cell like phenotypes that are resistant to chemotherapy. For example, CD31 is an immunoglobulin member expressed on the surfaces of circulating platelets, neutrophils, and lymphocytes and is involved in cell-cell contact. When CD31 expression is low or lacking cells lose part of their endothelial properties, undergo EMT, and are more tumorigenic and chemoresistant. FOXC2 also contributes to chemoresistance by activating EMT, and by
eliminating the epithelial phenotype of tumor cells. Furthermore, the inhibition of FOXC2 reinstates epithelial phenotype and drug sensitivity 195.

HIF-1 enhances the expression of IL-6, IL-8, and multidrug resistance (MDR)-1, and in so doing, mediates the cellular efflux of chemotherapeutic agents thus increasing cell survival 217–219. It has been shown that co-administration of HIF inhibitors, like digoxin or acriflavine, countered paclitaxel- or gemcitabine-dependent initiation of IL-6, IL-8, and MDR-1 expression and led to tumor resorption 212,220,221. It’s been shown that acriflavine also prevents acquired chemoresistance by inhibiting EMT 212.

The mitochondria isoform of TrxR is a target of several gold containing drugs including the Au(I)-thiolate AF 130,131,222,223. TrxR and its substrate, Trx, are overexpressed in many malignant carcinomas, and are known to protect cells from ROS induced apoptosis. Upregulation of Trx expression contributes to malignant cell growth, tumor proliferation, as well as chemoresistance 121,130,224. It has been reported that upon inhibition of Trx, cancer cells regain their chemosensitivity to agent such as cisplatin for instance 225. Au-containing complexes inhibit TrxR in mitochondria which causes a reduction in membrane potential that initiates the caspase-9 and caspase-3 dependent apoptotic cascade 91,96,123,130,226.

1.6 In search of pathways

The markers of oncological interest discussed here were selected for analysis guided by our own preliminary work and insights from the literature. The behaviors that are modulated by those factors are critical to several aspects of tumor malignancy, therefore compounds capable of modulating their function hold therapeutic benefits. We utilized these markers to investigate the
mechanism by which novel heterobimetallic potential chemotherapeutic agents achieve their anticancer efficacy *in vitro* and in mouse models.

Understanding which compounds affect which factor can be informative for several aspects of therapy. First, such knowledge can inform further rational design of novel gold-based compounds aimed at a malignant phenotype or marker pathway by providing some indications of how design affect function. Second, a better mechanistic understanding of the compounds’ effects can be invaluable in determining potential responsiveness to treatment, in planning treatment regiments in monotherapy or the optimal combination therapy partner drug. Third, because so little is known on the mechanism of nontraditional metallodrugs, we chose a broad array as to explore members of many families of malignant phenotype markers.
1.7 Bibliography


34. Freeman, G. J. et al. Engagement of the PD-1 immunoinhibitory receptor by a novel B7 family


177. Hemmerlein, B. *et al.* The balance between MMP-2/-9 and TIMP-1/-2 is shifted towards MMP in renal cell carcinomas and can be further disturbed by hydrogen peroxide. *Int. J. Oncol.* (2004).


1401 (2012).

Lacey, D. et al. Control of Fibroblast-Like Synoviocyte Proliferation by Macrophage Migration Inhibitory Factor These data suggest that MIF may regulate RA synovial hyperplasia by acting directly and via involvement in the effects of IL-1 and TNF. In addition, the effects of MIF on FLS activation are independent of NF-B, and dependent on ERK MAP kinase. These data suggest an important therapeutic potential for MIF antagonism in RA. *ARTHRITIS Rheum.* 48, 103–109 (2003).


216. Biswas, S. et al. CD31 angiogenesis and combined expression of HIF-1α and HIF-2α are prognostic in primary clear-cell renal cell carcinoma (CC-RCC), but HIFα transcriptional products are not: implications for antiangiogenic trials and HIFα biomarker studies in primary CC-RCC. *Carcinogenesis* **33**, 1717–1725 (2012).


CHAPTER 2
THE GENESIS OF THE PRECLINICAL EVALUATION OF GOLD-BASED CHEMOTHERAPEUTIC AGAINST ccRCC

This dissertation is centered on the preclinical evaluations of three gold containing heterobimetallic compounds as anti-renal cancer chemotherapeutic candidates, the gold-titanium compounds Titanocref and Titanofin, and the gold-ruthenium compound RANCE-1. This chapter will serve first, to briefly position the compounds in the current landscape of heterobimetallic chemotherapeutic candidates (2.1). Second, it will serve to present the experimental genesis of this work and what led to the choice of those three compounds (2.2). Third, this chapter will serve to state the aims of this body of work (2.3).

2.1 Positioning Titanocref, Titanofin, and RANCE-1 in the current landscape of heterobimetallic chemotherapeutic candidates.

2.1.1 Heterometallic Compounds Containing Titanocenes

The heterometallic compounds gold- titanium complexes discussed here have a titanocene motif (that is a titanium (IV) metallic center attached to two cyclopentadienyl Cp rings). Titanocene dichloride (TDC, [C₅H₅]₂TiCl₂) was the first organometallic compound to undergo clinical assessment for the treatment of breast and renal cancers, following significant preclinical success in vitro and in vivo.¹⁻⁴ In phase II clinical trials patients exhibited modest reduction in tumor size, or the inhibition of metastatic proliferation of RCC or breast cancer.¹⁻⁴ Since this trial, titanocene derivatives have been developed with varied degrees of efficacy in vivo against RCC.¹⁻⁴
Chart 2.1 Structures of three generation of novel Ti-Au compounds and their predecessors.

2.1.1.1. Titanocene-Ruthenium Compounds

The first heterometallic complexes based on titanocene dichloride (TDC) and the Ru-p-cymene derivative RAPTA-C, were developed by the Casini laboratory in 2010$^{5,6}$. RAPTA-C is a highly selective antimetastatic compound with no systemic toxicity when tested *in vivo* $^6$. The Casini research group described a series of neutral (like compounds 1 and 2 in Chart 2.1) and cationic bimetallic complexes (titanocene(IV)-ruthenium(II)) that were selectively cytotoxic *in vitro* to human ovarian cancer cell lines. The novel bimetallic Ti-Ru complexes displayed strikingly improved efficacy over TDC and RAPTA-C, their monometallic counterparts $^6$. 

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Chart 2.1 Structures of three generation of novel Ti-Au compounds and their predecessors.
2.1.1.2 Titanocene-Gold Compounds

In 2011, the Contel and Casini research groups developed gold (I) titanocene compounds containing gold-phosphane (titanocene(IV)-gold(I) compounds) \(^7\)–\(^9\) (Chart 2.1). The cyclopentadienyl rings in TDC can be modified to contain phosphane ligands able to coordinate gold(I) fragments such as gold-chloride \(^7\),\(^8\). Such modification lead to an increased efficacy against ovarian and/or prostate cancer cell lines with respect to TDC, in particular cationic compound \(3\) \(^7\) and neutral compound \(4\) \(^8\). Compounds of that class, referred to as zero generation in Chart 2.1, showed synergistic effects between the two metal fragments on the same molecule \(^7\),\(^8\). Because these compounds did not bind to DNA (plasmid DNA; pBR322) \(^7\), or exhibited only weak electrostatic interactions with DNA (calf thymus DNA) \(^8\), their mode of action was evidently different from that of cisplatin which achieves efficacy by creating DNA covalent adducts. The difference between these compounds interaction with DNA and that of cisplatin was attributed to their gold(I) fragments (known not to interact with DNA but affect other biomolecular targets such as mitochondrial TrRx) \(^8\). Though the complexes were more suitable for potential medicinal application as they are more stable in physiological pH and less acidic than TDC, their stability was still suboptimal \(^8\). This may be because TDC breaks down at neutral pH (hydrolysis) providing titanium oxo species and cyclopentadiene. If that hypothesis hold true, zero generation Ti-Au compounds would break into distinct titanium and gold monometallic species in physiological media or \textit{in vivo} before reaching the tumor site, thereby failing to function as a single mutimetallic molecule. We hypothesized that incorporating the second metal to a ligand strongly bound to the titanium (IV) center would ensure that heterometallic Ti-M species remain after the Ti-Cp hydrolysis takes place under physiological pH conditions. Since Ti-O bonds are considerably stronger (\(\Delta H_{298} = 662(16) \text{ KJ/mol}\)) than Ti-C (\(\Delta H_{298} = 439 \text{ KJ/mol}\)) or Ti-Cl (\(\Delta H_{298} = 494\)
KJ/mol) bonds we envisioned a carboxylate as the ideal group to bind titanium(IV) centers. This gave rise to first generation compounds that were subsequently modified and that we will describe in sections 2.2.2-2.2.4).

2.1.2 Ruthenium-Gold Compounds

The Contel research group in collaboration with that of Dr. Messori (in Florence, Italy) reported on the first, non-ferrocene-based heterometallic complexes bearing a p-cymene ruthenium(II) motif (Chart 2.2). Arene-ruthenium(II)-gold(I) complexes such as compounds 10 and 11, which are neutral and incorporate a [Ru(p-cymene)Cl₂(dppm-kP)] fragment (R) with known cytotoxic and potential antimetastatic attributes, and a gold(I) chloride or a thiolate moiety were synthesized and exhibited cytotoxic activity in vitro against the colon cancer cell line, HCT116. These novel complexes were more cytotoxic and more selective to cancerous cell lines than individual ruthenium and gold species. Additionally, DNA (plasmid DNA, pBR322) was determined not to be their primary target, unlike cisplatin. Micromolar quantities of compound 11 inhibited the cysteine protease CtsB, known to play a critical role in the malignant progression of many solid tumors, specifically in the formation of new tumor vasculature and facilitation of metastasis. Our group improved on the design and efficacy of those compounds by developing second generation cationic complexes such as 12-14 and RANCE1 (Chart 2.2), which incorporate gold(I)-N-heterocyclic (NHC) carbene ligands because cationic gold NHC complexes possess antimitochondrial and anti TrxR properties. Indeed, the combination of the ruthenium monometallic compound (R) with the [Au(NHC)]⁺ fragment, resulted in highly physiologically stable cationic Ru-Au complexes with selective cytotoxicity towards renal and colon cancer cell lines. We were able to demonstrate the synergistic potentiation of the bimetallic complex, as the
co-administration of the two monometallic moieties did not achieve the efficacy of the monometallic structures \(^{11}\). These compounds achieve efficacy by similar means to other Au(I) derivatives containing phosphane (such as Auranofin) and NHC ligands, and are unlike cisplatin in their mechanism of action \(^1\). The compounds did not interact with DNA and inhibited mitochondrial TrxR in the metastatic human clear cell renal carcinoma cell line (Caki-1) \(^{11}\).

![Chart 2.2](chart2.2.png)

**Chart 2.2** Structures of first and second generation ruthenium-gold compounds with selected bimetallic compound RANCE-1 and monometallic compounds ruthenium (R) and gold (ANCE-1).

### 2.2.1. Preliminary studies

The ultimate goal of my doctoral work was to identify the tumor-malignancy phenotypes affected by a selected set of novel titanium-gold (Ti-Au) and ruthenium-gold (Ru-Au) compounds, and to elucidate some of the underlying mechanisms of their efficacies. In order to identify viable candidates for mechanistic and eventual *in vivo* preclinical evaluation, a series of approximately thirty compounds were tested against several tumor cell types (breast, colon, kidney, and prostate)
from which three promising compounds were identified with activity against renal cancer: two
titanocene-gold Titanocref and Titanofin (Chapter 3) and one ruthenium-gold RANCE-1 (Chapter
4) compounds. The preliminary studies described here gave rise to four publications and one patent
(years 2014-2017). These studies, and the follow-up investigation contained a synthetic
component (with synthesis, characterization, and stability studies of new compounds) conducted
by members of the Contel lab and a biological component (in vitro and in vivo evaluation of the
compounds and preliminary mechanistic studies) conducted by me.

We first evaluated a series of bimetallic compounds focusing on Ti-Au titanocene(IV)-
gold(I) and on Ru-Au arene-ruthenium(II)-gold(I) derivatives as well as their monometallic
counterparts, which were used as control compounds (Chart 2.1 and 2.2). In addition, a few
published reference compounds such as cisplatin, titanocene dichloride (TDC), titanocene Y (Ti-
Y), auranofin, or ruthenium dimer starting material (Ru-d) were also evaluated for comparative
purposes.

2.2.2 First Generation Organometallic Titanocene-Gold Compounds as Potential
Chemotherapeutics in Renal Cancer.

We first assessed the antitumor potency of a class of Ti-Au compounds with a carboxylate-
phosphane ligand, which we named “first generation” (Chart 2.1), as they were improved and
more stable versions of the compounds previously described by our group (zero generation or
compounds 3 and 4 in Chart 2.1.)

From this work, we observed that compound 5 and compound 6 (Charts 2.1 and 2.3) were more stable in physiological media and more cytotoxic than the
previously reported zero generation Ti-Au-Phosphane. Compounds 5 and 6 were found to be
highly cytotoxic to renal cancer cell lines with IC<sub>50</sub> values in the low micro- and nano-molar-range (Table 2.1).

**Chart 2.3.** Structures of heterometallic first generation titanocene–gold complexes 5 and 6 and corresponding gold monometallic complexes 19 and 18.
Table 2.1. IC\textsubscript{50} (\textmu M) of Heterometallic TiAu\textsubscript{2} Compounds 5 and 6 (Chart 2.1), Monometallic Au 18 and 19, Cisplatin, Titanocene Dichloride, and Titanocene Y in Human Cell Lines.\textsuperscript{a} All compounds were dissolved in 1\% of DMSO and diluted with water before addition to cell culture medium for a 24 or 72 h incubation period. Cisplatin and titanocene dichloride were dissolved in H\textsubscript{2}O.

<table>
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<th>Compound</th>
<th>A498 (24 h)</th>
<th>UO31 (24 h)</th>
<th>Caki-1 (24 h)</th>
<th>HEK-293T (24 h)</th>
<th>PC3 (24 h)</th>
<th>DU145 (24 h)</th>
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<td>6.0 ± 1.8 (24 h)</td>
<td>24 ± 0.73 (24 h)</td>
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<td>3.2 ± 0.38 (72h)</td>
<td>1.4 ± 0.1 (72h)</td>
<td>2.2 ± 0.99 (72h)</td>
<td>6.9 ± 2.4 (72h)</td>
<td>27.1 ± 4.4 (72h)</td>
<td>35.5 ± 1.4 (72h)</td>
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<td>\textsuperscript{18}</td>
<td>58 ± 11.0 (24 h)</td>
<td>27.3 ± 2.7 (24 h)</td>
<td>33.8 ± 2.1 (24 h)</td>
<td>36.5 ± 3.3 (24 h)</td>
<td>38.2 ± 2.2 (24 h)</td>
<td>34.7 ± 1.8 (24 h)</td>
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<td>6.9 ± 2.2 (24 h)</td>
<td>10.3 ± 4.1 (24 h)</td>
<td>39 ± 4.1 (24 h)</td>
<td>51 ± 4.2 (24 h)</td>
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<td></td>
<td>6.8 ± 0.2 (72h)</td>
<td>0.3 ± 0.06 (72h)</td>
<td>1.0 ± 0.29 (72h)</td>
<td>20.1 ± 1.6 (72h)</td>
<td>37.7 ± 7.1 (72h)</td>
<td>6.6 ± 1.8 (72h)</td>
</tr>
<tr>
<td>\textsuperscript{19}</td>
<td>36.1 ± 6.3 (24 h)</td>
<td>38.3 ± 4.1 (24 h)</td>
<td>28.4 ± 5.9 (24 h)</td>
<td>34.2 ± 3.7 (24 h)</td>
<td>&gt;200 (24 h)</td>
<td>32 ± 2.7 (24 h)</td>
</tr>
<tr>
<td></td>
<td>21 ± 2.5 (72h)</td>
<td>1.2 ± 0.8 (72h)</td>
<td>19.2 ± 2.9 (72h)</td>
<td>31 ± 0.9 (72h)</td>
<td>78 ± 18.1 (72h)</td>
<td>39 ± 5.7 (72h)</td>
</tr>
<tr>
<td>Cisplatin</td>
<td>74.7 ± 6.0 (24 h)</td>
<td>&gt;100 (24 h)</td>
<td>68.8 ± 0.14 (24 h)</td>
<td>64.4 ± 7.9 (24 h)</td>
<td>&gt;200 (24 h)</td>
<td>44.5 ± 0.33 (24 h)</td>
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<tr>
<td></td>
<td>37.2 ± 4.6 (72h)</td>
<td>8.9 ± 2.7 (72h)</td>
<td>29 ± 4.1 (72h)</td>
<td>92 ± 18 (24 h)</td>
<td>&gt;200 (24 h)</td>
<td>12.1 ± 3.9 (72h)</td>
</tr>
<tr>
<td>\textsuperscript{titanocene dichloride}</td>
<td>&gt;200 (24 h)</td>
<td>&gt;200 (24 h)</td>
<td>&gt;200 (24 h)</td>
<td>&gt;200 (24 h)</td>
<td>&gt;200 (24 h)</td>
<td>&gt;200 (24 h)</td>
</tr>
<tr>
<td></td>
<td>&gt;200 (72h)</td>
<td>&gt;200 (72h)</td>
<td>&gt;200 (72h)</td>
<td>&gt;200 (72h)</td>
<td>&gt;200 (72h)</td>
<td>&gt;200 (72h)</td>
</tr>
<tr>
<td>\textsuperscript{Titanocene Y}</td>
<td>&gt;200 (24 h)</td>
<td>&gt;200 (24 h)</td>
<td>&gt;200 (24 h)</td>
<td>&gt;200 (24 h)</td>
<td>&gt;200 (24 h)</td>
<td>&gt;200 (24 h)</td>
</tr>
<tr>
<td></td>
<td>&gt;29.6 ± 2.8 (72h)</td>
<td>&gt;29.4 ± 4.2 (72h)</td>
<td>&gt;58.1 ± 11.2 (72h)</td>
<td>&gt;55.2 ± 7.9 (72h)</td>
<td>&gt;200 (24 h)</td>
<td>&gt;200 (24 h)</td>
</tr>
</tbody>
</table>

\textsuperscript{a}Data are expressed as mean ± SD (n = 3).

Compound 6 was on average 20-fold more potent than cisplatin and over 20-fold more cytotoxic than Ti-Y against ccRCC cells (Caki-1, UO-31) after 72 hours of incubation (Table 2.1).\textsuperscript{8} Two specific aspects of the cytotoxic evaluation are salient for this investigation: 1) the heterobimetallic compounds were far more toxic to tumor cells than they were to healthy human embryonic kidney cells transformed (HEK-293T) which indicates that these Ti-Au compounds were highly selective; 2) the monometallic Ti fragment (TDC) and Au fragments (compounds 18 and 19) of the bimetallic compounds were significantly less cytotoxic than in their bimetallic forms. Even when both monometallic fragments were added to the cells together (1:1 ratio), they...
were still significantly less cytotoxic than the heterobimetallic compound; this finding points to cytotoxic synergism between the metals.

Compound 5 induced necrotic cell death and no binding to DNA, while compound 6 induced apoptotic cell death while also exhibiting no interaction with plasmid (pBR322) DNA (Figures 2.1 and 2.2), suggesting that primary target(s) implicated in cell death was not DNA.

**Figure 2.1.** Cell death assays on Caki-1 cells induced by 5 and 6 (10 μM) measured by using two-color flow cytometric analysis, after 6 h of incubation. 1% DMSO is vehicle alone control, and staurosporine is a known inducer of apoptosis as positive control.
Figure 2.2 Electrophoresis mobility shift assays for cisplatin, titanocene dichloride, heterometallic compounds 5 and 6. DNA refers to untreated plasmid pBR322. Letters a, b, c, and d correspond to metal/DNAbp ratios of 0.25, 0.5, 1.0, and 2.0, respectively.

In our study of the organometallic titanocene-gold compounds as potential chemotherapeutic agents in ccRC, we also evaluated their protein kinase inhibitory properties. From a study of the inhibition of kinases with oncological interest, it was observed that this set of compounds includes strong inhibitors of molecules key to tumor migration such as Akt(s) and MAPKAPKs in the p38 pathway, with a higher inhibitory selectivity towards MAPKAPK3, with IC$_{50}$ values of 91 nM and 117 nM for compounds 5 and compound 6 respectively (Figure 2.3). While titanocene dichloride inhibits exclusively protein kinases of the PI3 kinase family at nanomolar concentrations, Compounds 5 and 6 inhibited protein kinases Akt and MAPKAPK2 at nanomolar concentrations.

To further investigate the ability of these compounds to impair MAPKAPK2/3 activity in cancer cells in vitro, we examined the ability of the heterometallic compound 5 and compound 6 to inhibit IL6 secretion, which can be activated by MAPKAPK2/3.
Figure 2.3. IC\textsubscript{50} (μM values) for compound 5, and compound 6 against selected protein kinases of the AKT and MAKPAPK families.

We found that compounds 5 and 6 significantly reduced secretion of pro-migratory and proangiogenic IL-6 in Caki-1 cells (Figure 2.4). Inhibition of MAPKAPK2/3 and IL-6 is likely to play a role in the onco-efficacy of the compounds. This study additionally provided clues for further rational design of novel Ti-Au compounds with greater target specificity by rational modification of the ligand scaffolds \textsuperscript{8}.

Figure 2.4. Compounds 5 and 6 may affect downstream targets of MAPKAP2/3 in renal cancer cells. Inhibition of IL6 secretion over the course of 6 h in Caki-1 cells treated with compound 3 or 5 (10 μM). Analysis was done using a capture ELISA approach (human IL-6 ELISA kit).
2.2.3 Second Generation Titanocene-Gold Compounds as Potential Chemotherapeutics in Renal Cancer: Preliminary in vivo studies

We then set out to demonstrate that other heterometallic titanium-gold complexes inhibit renal cancer cell growth in vitro and in vivo\(^\text{13}\). Therefore, we evaluated a series of compounds containing titanocene(IV)-methyl fragments and gold(I)-phosphine fragments linked by a dual carboxylate-thiolate ligand where one carboxylate group is attached to the titanium center and the thiolate is attached to the gold center (“second generation” of Ti-Au compounds, seeking to model AF like Titanocref and compound 7 in Chart 2.4). We chose the most active first generation compound 5 and second generation Titanocref for further studies since second generation compound 7 with the phoshane DPPF was less active and selective.

**Chart 2.4.** Structures of heterometallic second generation titanocene–gold complexes Titanocref and 7.

![Chart 2.4](image)

Compound Titanocref, closely related to first generation compound 5, was more cytotoxic than compound 5 and yet still selective. We found that one compound in particular, titanocref, was able to block ccRCC growth both in vitro (with an IC\(_{50}\) of 0.12 µM ± 0.003 after 72 hours) (Table 2.2) by pathway(s) that involve the inhibition of TrxR (Figure 2.5), and that it exhibited notable anti-invasive properties (Figure 2.6).
Table 2.2. IC₅₀ values (µM) in human cell lines were determined with heterometallic Ti-Au compounds 5 and Titanocref, cisplatin, TDC, and titanocene Y. All compounds were dissolved in 1% of DMSO and diluted with cell culture media before addition to cell culture for a 72 h incubation period. Cisplatin and titanocene dichloride were dissolved in H₂O.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Caki-1</th>
<th>HEK-293T</th>
</tr>
</thead>
<tbody>
<tr>
<td>Compound 5</td>
<td>1.0 ± 0.29</td>
<td>20.1 ± 1.6</td>
</tr>
<tr>
<td>Titanocref</td>
<td>0.12 ± 0.003</td>
<td>0.49 ± 0.008</td>
</tr>
<tr>
<td>Cisplatin</td>
<td>29 ± 4.11</td>
<td>3.27 ± 0.13</td>
</tr>
<tr>
<td>[(η⁵-C₅H₅)₂TiCl₂]</td>
<td>&gt; 200</td>
<td>&gt; 200</td>
</tr>
<tr>
<td>Titanocene Y</td>
<td>29.42 ± 4.18</td>
<td>&gt; 200</td>
</tr>
</tbody>
</table>

Figure 2.5. Comparison of thioredoxin reductase activity in compound 5, Titanocref or auranofin treated Caki-1 cells. Activity of Caki-1 thioredoxin reductase from soluble whole cell lysates following incubation with 5 µM of compound 5, Titanocref, or auranofin for 5 hours.
Figure 2.6. A. Scratch assay showing that compounds 5 and Titanocref interfere with Caki-1 migration. Panels show representative images of control (0.1% DMSO treated) cells at time points up to 12 hours (top row), or cells treated with 5 (middle row), and Titanocref (bottom row). The left column shows a representative image of untreated Caki-1 cells immediately post-scratch (t=0 h). B. Migration assay showing that 5 and Titanocref interfere with Caki-1 migration. The bar graph shows the percentage of the wound healed surface. Each bar represents one treatment group, 0.1% DMSO control treated cells (blue bar), 5 treated cells (blue bar) and Titanocref treated cells (red bar). The graph represents results from measurements of the area of the scratch from 4 separate and random fields of view.

Compound 5 and Titanocref also downregulated protein kinases implicated in modulating cell migration such as p90-RSK, AKT, and MAPKAPK-3 (Figure 2.7). Furthermore, we
demonstrated in the case of Titanocref that both metals were localized inside Caki-1 cells, with Ti and Au metals at a 1:1 ratio in a dose dependent manner (Figure 2.8).

**Figure 2.7.** Decreased expression of p90-RSK, AKT, and MAPKAPK-3 in Caki-1 cells in response to compounds Titanocref and 5. Cells were incubated with either Titanocref or 5 or 0.1% DMSO for the indicated times, followed by cell lysis, and western blot analysis. Blots were probed with anti-β-Actin antibody as a control for protein loading. Samples from cells treated with second generation Titanocref are shown in panel A, and samples from cells treated with first generation compound 5 are shown in panel B.

From these two studies, we determined that heterometallic titanium-gold compound first generation 5 and second generation Titanocref inhibit ccRCC growth *in vitro* and *in vivo* with some mechanistic difference. As we had done previously with first generation compound 5, Titanocref was then tested for activity against 35 protein kinases commonly associated with cancer.
Figure 2.8. Cellular uptake of Titanocref in CAKI-1 cells. The concentration of Titanocref was calculated based on Ti and Au content in the cell lysates was similar suggesting that the compound was robust and that both elements were co-localized within in the cell.

We found that compound 5, and Titanocref inhibited MAPKAPK3, kinases regulated through direct phosphorylation by p38 MAP kinase. p38 kinases are known to be pivotal to many cellular processes including inflammatory responses, gene expression regulation and cell proliferation, and are highly expressed in cancerous cells. From immunoblotting experiments, we also found that both compounds exhibited strong inhibition of MAPKAPK 3 activity, and given that MAPKAPK 3 expression is increased in certain cancers, novel inhibitors such as compound 5 and Titanocref are therefore of particular interest.

Compound 5 and Titanocref were both tested in vivo in NOD SCID mice bearing subcutaneous Caki-1 tumors. Following 28 days of treatment (14 doses) in mice treated with Titanocref (at 3mg/kg/48h), we observed a 67% reduction in tumor burden. In mice treated with compound 5 (5mg/kg/48h) under the same conditions, we observed 90% tumor growth, though the growth was 15% lower than that of control mice. Thus although modest, compound 5 may play an inhibitory role in tumor growth. (Figure 2.9)
Figure 2.9. A. Percent reduction of tumor burden with compound 5 or Titanocref in a cohort of 18 female NOD.CB17-Prkdc SCID/J mice inoculated subcutaneously with $8 \times 10^6$ Caki-1 cells. The treatment was initiated when tumors were palpable (6 mm diameter). 6 mice were treated with Compound 5 or Titanocref (red bars), and 6 were treated with the vehicle 100μl normal saline (0.9% NaCl) (black bars). Compound 5 was administered at 5mg/kg/48h, Titanocref was administered in the amount of 3 mg/kg/every other day.

Such results warrant further in vivo studies to optimize effective dosage and to determine the compound’s pharmacokinetics. In addition, these results corroborate the notion of multifunctional efficacy of heterometallic Ti-Au complexes and highlight their chemotherapeutic potential.

2.2.4 Third generation Titanocene–gold compounds as potential chemotherapeutics for prostate, renal and colon cancer.

We then proceeded to explore the inhibitory properties of titanocene–gold complexes containing N-Heterocyclic Carbene (NHC) ligands against prostate, renal, and colon cancers in vitro $^{12}$. Such modifications were made in the hopes that perhaps replacing the phosphane with a carbene might improve efficacy as recent publications had suggested N-
herocyclic carbenes confer an anticancer advantage. We therefore tested Ti-Au compounds containing gold(I)–N-heterocyclic carbene moieties (instead of Au(I)-phosphane fragments) (9a-d in Chart 2.1 and Chart 2.5). The design of this “third generation” of Ti-Au compounds was intended to improve or modify the pharmacological profile of the described “second generation” compounds.

**Chart 2.5.** Structures of heterometallic third generation titanocene–gold complexes 9a-d and corresponding gold monometallic complexes 8a-d.

Monometallic NHC–Au(I) complexes are usually more stable than Au(I)–phosphane compounds and allow for further synthetic modification of the ligand (to for instance improve hydrophilicity, luminescence, etc.). However, the cytotoxicity of the new compounds in renal cancer cell lines was found to be much lower than the “second generation” Ti-Au compounds containing phosphanes as ligands. Their greatest cytotoxicity was found against prostate and colon cancer cells. We also observed considerably higher selectivity (than both the first and second generation compounds) with respect to noncancerous cell lines (Table 2.3). Two selected
compounds (9a, 9b) were found to potently induce apoptosis and to inhibit TrxR in prostate PC3 cancer cell lines (Figure 2.10). Moreover, as with the previously studied Ti-Au phosphane compounds, both metal fragments were co-localized inside of the cells (Figure 2.11). These complexes also displayed strong antimigratory properties (Figure 2.12).

**Table 2.3.** IC$_{50}$ values (µM) in human cell lines were determined with heterometallic Ti-Au compounds 9a-d, monometallic Au compounds 8a-d and titanocene Y used as control. All compounds were dissolved in 1% of DMSO and diluted with water before addition to cell culture medium for a 72 hour incubation period. Data are expressed as mean ± SD (n =3).

<table>
<thead>
<tr>
<th>Compound</th>
<th>PC3</th>
<th>DU-145</th>
<th>Caki-1</th>
<th>DLD1</th>
<th>MDA-MB-231</th>
<th>HEK-293T</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ti-Y</td>
<td>58.1 ± 11.2</td>
<td>55.2 ± 7.9</td>
<td>29.4 ± 4.2</td>
<td>56.2 ±9.8</td>
<td>18.0 ± 3.6</td>
<td>&gt;200</td>
</tr>
<tr>
<td>9a</td>
<td>9.8 ± 2.2</td>
<td>11.8 ± 3.0</td>
<td>21.0 ± 1.9</td>
<td>13.9 ± 1.7</td>
<td>&gt;100</td>
<td>58.8 ± 6.7</td>
</tr>
<tr>
<td>9b</td>
<td>10.3 ± 2.8</td>
<td>18.9 ± 2.9</td>
<td>51.5 ± 3.7</td>
<td>30.4 ± 4.1</td>
<td>&gt;100</td>
<td>&gt;100</td>
</tr>
<tr>
<td>9c</td>
<td>17.1 ± 2.9</td>
<td>13.76 ± 2.7</td>
<td>29.11 ± 4.1</td>
<td>19.9 ± 4.1</td>
<td>&gt;100</td>
<td>69.7 ± 9.9</td>
</tr>
<tr>
<td>9d</td>
<td>11.8 ± 1.6</td>
<td>16.7 ± 2.0</td>
<td>42.9 ± 5.8</td>
<td>21.5 ± 2.0</td>
<td>&gt;100</td>
<td>77.1 ± 9.1</td>
</tr>
<tr>
<td>8a</td>
<td>66.3 ± 6.4</td>
<td>74.8 ± 4.4</td>
<td>81.4 ± 2.9</td>
<td>78.2 ± 6</td>
<td>&gt;100</td>
<td>&gt;100</td>
</tr>
<tr>
<td>8b</td>
<td>70.4 ± 6.8</td>
<td>60.9 ±5.2</td>
<td>79.2 ±11.7</td>
<td>82.6 ± 5.9</td>
<td>&gt;100</td>
<td>&gt;100</td>
</tr>
<tr>
<td>8c</td>
<td>57.1 ± 5.1</td>
<td>67.6 ± 7.1</td>
<td>97.2 ± 8.6</td>
<td>73.1 ± 9.6</td>
<td>&gt;100</td>
<td>87.9 ± 6.4</td>
</tr>
<tr>
<td>8d</td>
<td>65.1 ± 4.4</td>
<td>59.9± 4.7</td>
<td>88.9 ± 5.1</td>
<td>77.5 ± 8.1</td>
<td>&gt;100</td>
<td>97.2 ± 5.1</td>
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**Figure 2.10.** Thioredoxin reductase activity in monometallic Au 8a, 8b, 9a, or bimetallic Ru-Au 9b treated PC3 cells: (Left) activity of endogenous PC3 thioredoxin reductase from soluble whole cell lysates following incubation with 30 μM of 8a or 8b for 5 and 24 h (values normalized against DMSO control); (Right) activity of endogenous PC3 thioredoxin reductase from soluble whole cell lysates following incubation with 5 μM of 9a or 9b for 5 h and 24 h (values normalized to DMSO control).

**Figure 2.11.** Cellular uptake of compound 9a in PC3 cells. The concentrations of compound 9a calculated on the basis of Ti and Au content in the cell lysates are similar, suggesting that the compound is robust and that both elements are colocalized in the cells.
Figure 2.12. Cell migration in 9a or 9b treated PC3 cells. Migration of PC3 cells was assessed using a wound-healing assay following treatment with 15 μM titanocene Y or 5 μM of 9a or 9b incubated for 24 h (values normalized against 0.1% DMSO control). Both mono— and bi—metallics lead to significant reduction in migration.

The focus of my research broadened to include ruthenium containing compounds because there was suggestion that they could confer antimetastatic properties to the complex. We thought that it might be a potentiating agent to the observed cytotoxic properties observed with gold based compounds. The Contel lab had demonstrated that indeed ruthenium-gold compounds were indeed more cytotoxic and more selective to cancerous cell lines than individual ruthenium and gold species 9. As with the third generation of Ti-Au described before, we decided to incorporate gold containing N-heterocyclic carbene ligands to obtain second generation compounds that we expected to have a better pharmacological profile. These compounds were synthesized in the Contel lab and evaluated against renal and colon cancers 10.
2.2.5 Second generation heterobimetallic ruthenium–gold compounds as potential anticancer agents for renal and colon cancer

As described before, we went on to evaluate new cationic heterobimetallic ruthenium-gold compounds containing a N-heterocyclic carbene–gold(I) fragment (second generation). Building on work reported by the Contel group in 2015 on neutral ruthenium-gold Ru(II)-Au(I) compounds (first generation 10 and 11, in Chart 2.2), improved heterobimetallic cationic Ru-Au derivatives were synthesized incorporating the same linker (dppm) but also incorporating NHC-carbene ligands coordinated to Au\(^{10}\). Gold NHC-carbene complexes exhibit great antimitochondrial properties attributable to both their cationic nature and their lipophilicity. Joining the potential antimetastatic/cytotoxic properties of Ru to the TrxR inhibitory effects of Au species may give rise to potent cancer chemotherapeutics.

This novel series of heterometallic compounds [second generation Ru-Au depicted in Chart 2.2, compounds 12-14 and RANCE-1] have been shown to be highly cytotoxic to ccRCC Caki-1 and colon cancer DLD1 cell lines while also more selective than their monometallic counterparts (Table 2.4), which suggests that there is synergism between the two metal moieties\(^{10}\). They are also very stable in physiological media, more so than their Ti-Au-NHC counterparts with half lives of 24h to 72h.
Table 2.4. IC50 values (μM) in human cell lines were determined with monometallic [(NHC)AuCl] 115-17 and ANCE-1 monometallic [Ru(p-cymene)Cl2(dppm-kP)] R, and new heterometallic Ru–Au compounds (12–14) and RANCE-1.

<table>
<thead>
<tr>
<th></th>
<th>Caki-1</th>
<th>HEK-293T</th>
<th>HCT 116</th>
</tr>
</thead>
<tbody>
<tr>
<td>[(NHC)AuCl]</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>27.1 ± 2.0</td>
<td>61.5 ± 5.1</td>
<td>39.7 ± 4.9</td>
</tr>
<tr>
<td>ANCE-1</td>
<td>21.2 ± 1.6</td>
<td>&gt;100</td>
<td>31.2 ± 3.2</td>
</tr>
<tr>
<td>16</td>
<td>58.8 ± 3.9</td>
<td>&gt;100</td>
<td>59.1 ± 5.8</td>
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<td>17</td>
<td>17.5 ± 2.2</td>
<td>&gt;100</td>
<td>27.7 ± 4.9</td>
</tr>
<tr>
<td>[Ru(p-cymene)Cl2(η1-dppm)] R</td>
<td>35.6 ± 3.7</td>
<td>81.6 ± 3.0</td>
<td>18.2 ± 2.2</td>
</tr>
<tr>
<td>New cationic Ru-Au</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>5.2 ± 0.9</td>
<td>73.2 ± 2.5</td>
<td>8.1 ± 1.8</td>
</tr>
<tr>
<td>RANCE-1</td>
<td>14.1 ± 1.9</td>
<td>&gt;100</td>
<td>5.22 ± 0.7</td>
</tr>
<tr>
<td>13</td>
<td>3.8 ± 0.6</td>
<td>&gt;100</td>
<td>6.4 ± 1.0</td>
</tr>
<tr>
<td>14</td>
<td>12.7 ± 2.7</td>
<td>&gt;100</td>
<td>9.6 ± 3.1</td>
</tr>
<tr>
<td>cisplatin</td>
<td>29 ± 4.11b</td>
<td>3.27 ± 0.13b</td>
<td>---</td>
</tr>
</tbody>
</table>

aAll compounds were dissolved in 1% of DMSO and diluted with water before addition to cell culture medium for a 72 h incubation period. Cisplatin was dissolved in H2O.

As mentioned in chapter 1, changes in cell anti-oxidant capacity are a characteristic of many chemo-resistant cancers, and overexpression of thioredoxin reductase (TrxR) is among the key defense and survival mechanisms of cisplatin-resistant cells. It has been reported that Auranofin, some heterometallic titanocene– gold as well as Au–NHC compounds are inhibitors of TrxR, with varying degrees of potency13. We wanted to know if the addition if a Ru moiety would preclude or potentiate the Au-driven inhibition of TrxR (Figure 2.13)
Figure 2.13. Inhibition of mitochondrial protein TrxR in Caki-1 renal cancer cells by bimetallic Ru-Au compound 12, the monometallic ruthenium R, and gold 15 compounds and Auranofin (1 μM) for 24h (A) and 72h (B). The values for indicate the percentage of reduction of TrxR activity relative to 1% DMSO treated control. (C) Thioredoxin reductase activity of 12 treated Caki-1 cells. Activity of endogenous Caki-1 thioredoxin reductase from soluble whole cell lysates following incubation with 5 μM of compound 12 or 0.1% DMSO for 5, 12 and 24 hours.

Therefore, we measured the activity of thioredoxin reductase in Caki-1 cells, following incubation with compounds bimetallic Ru-Au 12, its monometallic Au moiety 15, Ru moiety R and Auranofin as a control. We found TrxR activity to be significantly lowered in cells treated with 1 μM of Auranofin (-61% after 24h and -85% after 72h of incubation) and 12 (-51% after 24h and -77% after 72h of incubation), while not significantly affected by R (-3% after 24h and -8% after 72h of incubation) or 15 (-5% after 24h and -6% after 72h of incubation). It appears that the inhibition of TrxR is time dependent, at least for Au-containing compounds, as the activity increases remarkably from 24h to 72h. 12 anti TrxR activity is also dose dependent since when treated with 5 μM of 12, TrxR activity is reduced by 63% after 24h incubation (Figure 2.13) while treated with 1 μM for 24h TrxR inhibition is of 51%.
We have determined that the incorporation of a Au-NHC moiety and the change from neutral to cationic compounds seem to improve anticancer activity. From this work we identified RANCE-1 (containing the NHC ligand = IMes) which we went on to study further in vivo and in vitro (Chapter 4). We chose compound RANCE-1, because though it is less potent than other compounds, it is more stable, and because in later evaluations not reported in the publication at hand, at sub-cytotoxic doses it was most effective at reducing migration and invasion, and also a better inhibitor of tube formation.

2.2.6 Formulation of research objectives.

From this body of work we identified Ti-Au Titanocref and Ru-Au RANCE-1 as candidates worth exploring further. As mentioned in Chapter 1, after assessing the literature, one determines that there is no conserved narrative of how a set of given metals affect cancer cell behavior, in part because there has not been systematic study of the phenotypes and pathways affected. It has been shown that gold compounds, ours included, inhibit mitochondrial enzyme Thioredoxin reductase (TrxR), which in part induces chemoresistance, and this was confirmed for our compounds. There is little or fragmented knowledge on mode of cell death, and molecular pathways by which heterobimetallic complexes achieve anti-cancer efficacy. We found that Titanocref is an excellent inhibitor of MAPKAPK3, and know that it leads to a reduction in tumor burden, not much else was known. During my thesis an analogue of Titanocref was synthesized in our lab. This analogue (Titanofin in Chart 2.1) contains a gold-phosphane fragment (AuPEt₃) that is also present in the drug Auranofin (AF). As described in chapter 1, AF has been used in the clinic for the treatment of rheumatoid arthritis but it is currently being investigated in clinical trials in cancer as a potential anticancer chemotherapeutic.

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16–19
To gain essential insights we have studied the complexes effect on cellular behaviors linked to tumor malignancy, then interrogated protein of oncological interest, particularly those involved in angiogenesis, metastasis and cell death to obtain a cleared understanding of the compounds efficacy. These studies constitute chapters 3 and 4 of this thesis.
2.3. OBJECTIVES

2.3.1 The main goal of the work described in this thesis

The aim of my doctoral work has been to identify the tumor-malignancy phenotypes affected by a selection of novel Ti-Au and Ru-Au compounds (three in total) and to elucidate some of the underlying mechanisms of their \textit{in vitro} and \textit{in vivo} efficacies.

2.3.2 Hypothesis

The individual metals in Ti-Au and Ru-Au complexes contribute to multiple aspects of their anticancer efficacy, 1) direct cytotoxicity 2) hindrance of tumor cell malignancy phenotypes, and/or 3) inhibition of protumorigenic stromal cellular behavior. Furthermore, distinct mechanisms are engaged by these compounds to bring about these anti-cancer phenotypes.

2.3.3 Specific Aims of this thesis

2.3.3.1 Aim 1. To refine our understanding of the cytotoxic and antiproliferative profile of a series of Ti-Au and Ru-Au compounds \textit{in vitro}. This aim was designed as an exploration wherein novel compounds were tested for activity against multiple cell lines in order to identify compounds and tumor types for more in depth investigation (Chapter 2).
2.3.3.2 Aim 2. To evaluate the effects of Titanocref, Titanofin and their gold monometallic moieties on metastatic and antiangiogenic phenotypes and related pathways in vitro and in vivo in a mouse model of RCC. Based on the results of Aim 1, two gold-titanium compounds (Titanocref and Titanofin) were selected for a deeper investigation of their mechanism of action based on their inherent chemical characteristics, their efficacy, and an indication of synergism between the two metals in the compound. Their specificity, cytotoxicity, and mechanism of action were assessed using in vitro models, and their potential for development and clinical trials was assessed using an in vivo model (Chapter 3).

2.3.3.3 Aim 3. To evaluate the effects of RANCE-1 and its monometallic moieties on metastatic and antiangiogenic phenotypes and related pathways in vitro and in vivo in a mouse model. Similar to Aim 2, but focused on a selected gold-ruthenium complex (RANCE-1). The specificity, cytotoxicity, and mechanism of action were assessed using in vitro models, and their potential for development and clinical trials was assessed using an in vivo model (Chapter 4).
2.4 Bibliography


18. NIH, M. C. Sirolimus and Auranofin in Treating Patients With Advanced or Recurrent Non-Small Cell Lung Cancer or Small Cell Lung Cancer, or, PKCγ and mTOR Inhibition for Patients With Advanced or Recurrent Lung Cancer (NSCLC and SCLC) Without Standard Treatment Options. Available at: https://clinicaltrials.gov/ct2/show/NCT01737502. (Accessed: 23rd August 2018)

CHAPTER 3

EFICACY AND MECHANISTIC STUDIES OF GOLD-TITANOCENE COMPLEXES IN VITRO AND IN VIVO.

Part 1


3.1 INTRODUCTION

The potential of heterometallic compounds in medicinal chemistry and particularly in cancer therapy has been described and discussed in the earlier chapters\textsuperscript{1}. Importantly, a single molecule with two or more distinct biologically active metallic centers can potentiate oncotherapeutic efficacy\textsuperscript{2}. This may derive from synergism (combined action of the different metals) or cooperation (beneficial change in the physicochemical properties) between the two metal entities. In the past eight years a number of heterometallic compounds have been reported as anticancer agents\textsuperscript{1–17}. However, there are very few publications reporting the comparison of anticancer properties of heteronuclear compounds to that of their monometallic fragments (alone or in combination)\textsuperscript{1,5,7,8,10,18–20}. Our group has focused on heterometallic compounds with gold fragments as one of the metallic centers. Gold(I) compounds bearing lipophilic ligands such as phosphanes (PR\textsubscript{3}) and N-heterocyclic carbene (NHC) have displayed significant antitumor, antimicrobial, and antiparasitic effects mostly mediated by inhibition of the thioredoxin/thioredoxin reductase Trx/TrxR systems\textsuperscript{21–23}. In this context, we have recently
reported on the preparation of complexes containing titanocenes\textsuperscript{7,8,19,24,25} [TiCp\textsubscript{2}] or ruthenium(II) arene derivatives\textsuperscript{5,18} [Ru(p-cymene)Cl\textsubscript{2}(dppm)] and gold(I)-phosphone or gold(I)-NHC fragments (2, a and b in Chart 1) and their potential as chemotherapeutics against renal, colorectal and prostate cancers.

![Chemical structures](image)

**Chart 3.1.** Select heterometallic Ti-Au Titanocref, Ti-Au-NHC and Ru-Au (b) compounds with relevant *in vitro* and *in vivo* activity reported by our group\textsuperscript{5,7,18,25}. Structure of monometallic cref, AF and TDC also employed in this work.

Preliminary mechanistic studies indicated that these compounds achieve toxicity through mechanisms different than that of cisplatin, as is the case for many other metallodrugs\textsuperscript{5,19,25}. In this section, we report on the comparative *in vitro* mechanistic evaluation of the efficacy of bimetallic Titanocref and Titanofin with that of AF and the monometallic gold compounds cref and fin\textsuperscript{2−}. All compounds are depicted in Chart 3.1. We studied their cytotoxicity, mode of cell death induction, cell cycle disruption, as well as their anti-migratory and anti-angiogenic properties and their inhibitory effects on 84 markers of oncological interest. We have recently reported on
the mechanism of action of a ruthenium-gold derivative in vitro (Caki-1 cancer cells), in which we also used AF as control in a number of similar experiments 5.

3.2 RESULTS AND DISCUSSION

3.2.1 Chemical profile

This data was obtained from Dr. Jacob Fernández-Gallardo and Dr. Natalia Curado

Titanofin was more soluble than Titanocref in DMSO/PBS. While the half-life for Titanocref in neat DMSO-d₆ was 8 hours, the half-life of new Titanofin was 24h (three times higher). In addition, Titanofin was soluble in mixtures DMSO:D₂O (3:2) and the stability in these solutions was higher (4 days). Both Titanocref and Titanofin are soluble in DMSO:PBS (1:99) in the micromolar range (necessary for the biological assays).

3.2.2 Cytotoxicity, Selectivity, Cell Death and Cell Cycle Arrest

The cytotoxicity of bimetallic Titanocref (compound 2) and Titanofin (compound 4), the two monometallic gold derivatives, cref (compound 1) and fin (compound 4), and monometallic TDC were evaluated. For comparative purposes, the cytotoxic profile of AF was also determined. In this assay, human clear-cell renal carcinoma Caki-1 cells and non-tumorigenic human fetal lung fibroblasts (IRM-90) were incubated with the Titanocref, cref, Titanofin, fin, AF, or TDC for 24h and 72 h. The results are summarized in Tables 3.1 and 3.2. The heterometallic compounds were considerably more toxic to the renal cancer cell line (Caki-1 cells) than titanocene dichloride and AF (in the sub-micromolar range as already reported for Titanocref for different renal cancer cell lines) 7,25. In addition, Titanocref was far more toxic in the nanomolar range than the monometallic
gold compound, cref on these cells. Titanofin was three times more toxic than the monometallic compound PEt₃ fin. This supports the hypothesis that the presence of two biologically active metals improves cytotoxicity.

Table 3.1. Cell viability IC₅₀ values (µM) for Caki-1 cells and IMR-90 fibroblasts treated with bimetallic Titanocref and Titanofin, or monometallic gold cref and fin compounds. Monometallics AF and TDC were used as controls. The IC₅₀ are reported with the standard deviation of the sample mean.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Caki-1</th>
<th>IMR90</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ti-Au (PPh₃)</td>
<td>Titanocref</td>
<td>0.097 ± 0.019</td>
</tr>
<tr>
<td>[Au(Hmba)(PPh₃)]</td>
<td>cref</td>
<td>2.70 ± 0.052</td>
</tr>
<tr>
<td>Ti-Au (Pet₃)</td>
<td>Titanofin</td>
<td>0.273 ± 0.043</td>
</tr>
<tr>
<td>[Au(Hmba)(Pet₃)]</td>
<td>fin</td>
<td>0.794 ± 0.002</td>
</tr>
<tr>
<td>[Au(SR)(Pet₃)]</td>
<td>AF</td>
<td>2.8 ± 0.6ᵇ</td>
</tr>
<tr>
<td>[Ti(Cp)₂Cl₂]</td>
<td>TDC</td>
<td>&gt;100</td>
</tr>
</tbody>
</table>

ᵃAll compounds were dissolved in 1% of DMSO and diluted with media before addition to cell culture medium for a 72 hour incubation period. Cisplatin was dissolved in H₂O. Structures of the compounds are provided in Chart 1. Values reported in reference 30.
Table 3.2. Cell viability IC_{10}, IC_{20}, IC_{50} values (µM) for Caki-1 cells and IMR-90 fibroblasts treated with bimetallic Titanocref, Titanofin and monometallic gold cref and fin compounds for 72h; and Cell viability IC_{20}, IC_{50} values (µM) for Caki-1 cells and IMR-90 fibroblasts treated with bimetallic Titanocref, Titanofin and monometallic gold cref and fin compounds for 24h. Monometallics AF and TDC were used as controls\textsuperscript{a}. The IC_{s} are reported with the standard deviation of the sample mean.

<table>
<thead>
<tr>
<th></th>
<th>IMR90</th>
<th>IMR90</th>
<th>Caki-1</th>
<th>Caki-1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Titanocref cref</td>
<td>1.62 ± 0.006</td>
<td>0.97 ± 0.019</td>
<td>0.05 ± 0.006</td>
<td>0.03 ± 0.002</td>
</tr>
<tr>
<td>Titanofin fin</td>
<td>3.13 ± 0.072</td>
<td>0.27 ± 0.043</td>
<td>0.18 ± 0.086</td>
<td>0.08 ± 0.002</td>
</tr>
<tr>
<td>Aurano(fin AF)</td>
<td>2.99 ± 0.006</td>
<td>0.79 ± 0.002</td>
<td>0.39 ± 0.01</td>
<td>0.29 ± 0.05</td>
</tr>
<tr>
<td>Titanocene dichloride (TDC)</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>&gt;100</td>
</tr>
</tbody>
</table>

\textsuperscript{a}All compounds were dissolved in 1% of DMSO and diluted with media before addition to cell culture medium for a 24h or 72 h incubation period. Cisplatin was dissolved in H\textsubscript{2}O. Structures of the compounds are provided in Chart 1 \textsuperscript{b}. Values previously reported\textsuperscript{5}.

Importantly, both heterometallic compounds were more selective to renal cancer cells than AF or the monometallic gold compounds, especially Titanocref. In view these results and the very high concentration of TDC required to observe cytotoxic effects, we decided not to explore the effects of titanocene dichloride in Caki-1 cells any further.

In addition to measuring the IC_{50} at 72h, we also assessed the IC_{50} after 24h of incubation. Because we sought to determine the changes in cellular behavior as well as the associated molecular signaling events, it was essential that the cells were viable throughout the experiment, therefore we used concentrations at which we know that after 72h incubation 80% (IC_{20}) or 90%
of the cells would be viable. Some assays were carried out after 24h of incubation with the 72h IC<sub>20</sub>, which is the lowest dose at which we were able to observe detectable changes in the expression levels of the proteins of interest after 24h. For changes in cellular behaviors such as tube formation, we reported on the IC<sub>10</sub> at 72h since we observed changes at that concentration in a trial dose response experiment. Finally, the 24h IC<sub>50</sub> evaluation was conducted to demonstrate that the 72h IC<sub>20</sub> of the compound did not induce significant cytotoxicity after 24h incubation.

Following the evaluation of the cytotoxicity of bimetallic Titanocref or Titanofin and monometallic gold AF, we proceeded to evaluate how the cells died. For this assay Caki-1 cells were incubated with the indicated compound at the IC<sub>50</sub> concentration for 72 hours. We observed that all the compounds induced apoptosis to some degree at their IC<sub>50</sub> concentration. For the Ti-Au compounds Titanocref and Titanofin (at nanomolar concentration) 58% and 76% of cells died by apoptosis (Figure 3.1).

AF and other gold (I) compounds are known to induce apoptosis in several cancer cell lines. TDC also induces apoptosis albeit at much higher concentrations than the aforementioned compounds. Thus the apoptotic behavior of bimetallic Titanofin and Titanocref may be due to the presence of both the Ti and Au fragments. We also determined the apoptotic EC<sub>50</sub> of the monometallic and bimetallic compounds, and degree of necrosis in a triple assay (Figure 3.2).

Next, we evaluated the effects of bimetallic Titanocref and Titanofin and monometallic AF on cell cycle arrest (Figure 3.1). Titanocref and Titanofin decreased G<sub>1</sub>/G<sub>0</sub> by 32% and 33% respectively and increased the G<sub>2</sub>/M population by 38% and 36% respectively compared to the DMSO control.
Figure 3.1. Effects of Titanocref, Titanofin, and AF on apoptotic index and cell cycle distribution on Caki-1 cells. (A) Cell apoptosis in bimetallic Titanocref, Titanofin and monometallic AF treated cells was detected by Annexin V and propidium iodide (PI) labeling combined with flow cytometry after 72h of incubation with IC_{50}. The level of apoptosis was measured by the percentage of the total PI positive cells reported in each quadrant 2 (Q1= dead cells, Q2=Apoptotic cells, Q3= Alive cells). Treatment with of bimetallic Titanocref, Titanofin and monometallic AF for 24h caused cell cycle arrest. Cell-cycle analysis was conducted by FxCycle staining and flow cytometry 24 h after treatment with bimetallic Titanocref, Titanofin, monometallic AF or DMSO (0.1%) control. Numbers in the bar-graph (C) correspond to histograms (D) show the percentage of cells in the Sub G_{1}, G_{0}-G_{1}, S and G_{2}-M phases of the cell cycle (values below 3% were not reported on the bar-graph).
**Figure 3.2.** Effects of Titanocref, Titanofin, and AF on caspase 3/7 activity. Caspase 3/7 was used as an indicator of caspase dependent apoptosis. Apoptosis induced by Staurosporine treatment was used as control. Necrosis was studied by measuring dose-dependent increase in cytotoxicity in the absence of caspase-3/7 activation a cellular behavior associated with primary necrosis, and necrosis induced by Ionomycin treatment was used as control. The cells were treated with doses ranging from 0.001 µM to 10 µM and incubated for 72h.

AF increased G₁/G₀ by 27% and induced an arrest of 76% of the cell population at G₁/G₀ (as we recently reported), while also generating a peak at SubG₁ in 20% of the cells arrested, consistent with apoptosis. This suggests that the AF induced complete G₁/G₀ arrest, as has already been reported for other cells lines treated with AF, or other anti-inflammatory drugs. The cell cycle shifts of Titanocref and Titanofin were strikingly similar, and unlike that of AF,
suggesting that perhaps the Ti moieties account in part for the distribution in G₂/M and the prevent the complete switch to G₁/G₀ observed with AF.

3.2.3 Inhibition of migration and invasion

Increased local cell migration followed by distal invasion are hallmarks of metastasis. We therefore evaluated the anti-migration and anti-invasive properties of bimetallic Titanocref and Titanofin and monometallic gold cref, fin, and AF. The effect of the compounds (IC₂₀) on migration was determined using a wound-healing 2D scratch assay on a collagen-coated plate (Figure 3.3A). We chose the IC₂₀ because it was determined that at that concentration about 80% of the cells (78% ± 2.31 depending on the compound) remained viable. Migration was quantified by measuring the space in the wound gap that is occupied by cells 24h after treatment. We observed that heterometallic Titanocref and Titanofin as well as AF significantly reduced migration (89%, 83% and 81% respectively), while gold monometallic cref and fin inhibited migration to a lesser degree (40% and 33% respectively). While the cytotoxicity and apoptotic properties of AF on different cancer cell lines are well known, the efficacy of AF as an anti-migratory and perhaps anti-metastatic compound has only recently been identified. All the compounds studied interfered with invasion and followed a similar trend, with AF and Ti-Au compounds being most effective (Figure 3.3B). There was also a clear trend that the monometallic gold compound fin was a better inhibitor of invasion than cref, although their ability to inhibit of migration was similar. This may be because proteolysis is necessary for the cells to infiltrate though the 3D matrix, but is not needed for 2D migration.
Figure 3.3. Cell Migration and Invasion Inhibition Assays for bimetallic Ti-Au Titanocref and Titanofin and monometallic Au cref, fin, and AF. A. Inhibition of migration (2D wound-healing scratch assay). Scratch assay showing that Titanocref, Titanofin, and monometallic cref, fin, and AF interfere with Caki-1 migration. Panels show representative images of untreated cells at time points T₀ (top row) when the compound is added to the assay up to 24 hours (bottom row). B. Inhibition of invasion (3D transwell assay Geltrex) showing that Titanocref, Titanofin and monometallic cref, fin, and AF interfere with Caki-1 invasion. The invasion assay was performed using an ECM-like Reduced Growth Factor Basement Membrane Matrix (Geltrex) 3:1. Panels show representative images of cells 24 hours after treatment with the 72h IC₂₀ concentration at which we observe less than 8% cell death after 24h of incubation. The data reported in the graph are the mean and standard deviation of the mean from two independent trials averaging quantitation from five fields of view per trial.
Indeed fin which is a better inhibitor of invasion, was also a stronger inhibitor of MMP-9 and MMP-2 (see next sections), which are proteolytic agents known to be key players in migration and metastasis. As explained above, the most efficient compounds in these experiments were bimetallic Titanocref and Titanofin.

3.2.4 Inhibition of angiogenesis

Neovascularization plays an essential role in tumor malignancy.

**Figure 3.4.** Instigation of endothelial cell reorganization into 3D vessel assembly. Human umbilical vein endothelial cells (HUVEC) were seeded in plates coated with Geltrex® matrix using complete HUVEC media and incubated at 37°C and 5% CO₂. At post-seeding the 72h IC₁₀ of bimetallic Ti-Au Titanocref or Titanofin, or AF or 0.1% DMSO was added. (A) Representative phase-contrast images captured 24h after the compounds were added. (B) Quantitation of tube formation was performed using Image-J with the Angiogenesis plugin.
We therefore chose to measure potential effects of our compounds on neovascularization by examining the formation of tube-like structures by HUVEC cells in an \textit{in vitro} extracellular matrix \cite{34}.

In this assay, the endothelial tube formation of human umbilical vein endothelial cells (HUVECs) on an ECM-like matrix was determined as a function of length of the uninterrupted tubes (TL), and number of branching point or nodes in the tubes (TN) (Figure 3.4). The number of tubes and nodes was counted using Image-J with the Angiogenesis plugin. The greater the inhibition of tube formation [lower length of tube (TL) and the lower the number of nodes (TN)], the higher the anti-angiogenic properties of a compound.

Both Titanocref and Titanofin, as well as control AF induced similar disruption in tube integrity with an average of 50% in disruption tube length (TL) and 45% disruption in tube node (TN) formation. This indicated that the three compounds are good disrupters of vascular formation, which is a key attribute of many antiangiogenic compounds and can prevent tumor growth and hinder metastasis.

3.2.5 \textit{Inhibition of targets associated to cancer cisplatin resistance, metastasis and angiogenesis}

3.2.5.1 \textit{Inhibition of Thioredoxin Reductase}

Changes in intracellular oxidation states are a distinctive feature of many chemo-resistant cancers. We previously reported on the significant inhibition of TrxR in Caki-1 cells by AF and heterometallic titanocene-Au and Ru-Au complexes \cite{5,7,25}. Here, we measured the activity of (TrxR) in Caki-1 cells, following incubation with bimetallic Ti-Au and monometallic Au complexes (Figure 3.5). After 72h (but not at 24h) of incubation, Caki-1 TrxR activity was significantly reduced by AF (86\%) \cite{5,25}. After 72 h of incubation, the inhibition of TrxR by the
bimetallic Ti-Au Titanocref and Titanofin was very similar (Titanocref, 87%; Titanofin 79%) and was larger than the inhibition displayed by monometallic gold compounds cref and fin (54% and 57% respectively).

3.2.5.2 Inhibition of VEGF

![Graphs showing inhibition of TrxR and VEGF](image)

**Figure 3.5.** Inhibition of proangiogenic factors TrxR and VEGF in Caki-1 cells by bimetallic Ti-Au Titanocref, Titanofin and monometallic Au cref, fin and AF occurs in a time and dose dependent manner. (Left) Inhibition of proangiogenic anti-apoptotic mitochondrial protein TrxR following treatment with IC_{20} concentrations of each compound for 24 h and 72h. The values indicate the percentage of TrxR activity relative to DMSO treated cells. (Right) Inhibition of proangiogenic protein VEGF, following treatment with IC_{20} concentrations of each compound for 24 h and 72h. The values indicate the percentage of VEGF expression relative to DMSO treated cells. The data shown, and standard deviation of the sample mean, result from two independent trials.

Given the key tumor promoting properties of VEGF, we set out to evaluate the inhibitory effects of bimetallic Ti-Au Titanocref and Titanofin and monometallic Au cref, fin and AF on VEGF. We found that VEGF secretion is inhibited by both bimetallic Ti-Au Titanocref and
Titanofin, and AF after 72h incubation with IC$_{20}$ of each compound (41% , 59% and 55% (AF) reduction respectively), while for monometallic Au cref and fin there was no significant inhibition of VEGF secretion (22% and 17% respectively). In this case, the strong inhibition of VEGF could be correlated to the anti-angiogenic effects we found since VEGF is known to be a key regulator of angiogenesis and its downregulation is often correlated with reduced angiogenesis.

3.2.5.3 Inhibition of Cytokines (TNF-α and interleukins)

We then investigated the inhibition of TNF-α and interleukins (ILs) by our bimetallic and monometallic compounds after 72 hours of incubation at IC$_{20}$ concentrations (Figure 3.6). The 72h timepoint was selected because no significant effect was observed after 24h of incubation at the same concentrations (Figure 3.6B). We found that the bimetallic compounds, gold monometallic compounds, and AF inhibited IL-6 expression almost completely. AF and Titanocref also inhibited IL-5 expression while the inhibition by Titanofin was more modest (89% Titanocref, 59% Titanofin and 88% AF). AF was a better inhibitor of IL-8 while both Titanocref and Titanofin were much stronger IL-4 and IL-17A inhibitors. Titanocref, Titanofin, and monometallic Au cref and fin were particularly strong inhibitors of IL-17A. Because IL-17A expression is associated with estrogen receptor negative (ER(-)) and triple negative tumor hyper proliferation and poor prognosis in breast cancer, and not only promotes tumor cell survival and invasiveness, but also contributes to the promotion of tumor angiogenesis, IL-17 inhibitors are of significant clinical interest. AF inhibited IL-6, as previously reported $^5$, but we also observed that it also inhibited expression of IL-5 and IL-8, all key players in inflammatory signaling $^{5,35,36}$.
Figure 3.6. Bimetallic Ti-Au Titanocref, Titanofin and monometallic Au cref, fin and AF induced changes in the expression levels of prometastatic cytokines (TNF-α, and interleukins) and matrix proteases (matrix metalloproteinases) in Caki-1 cells. Analysis of 150 ng of protein extracted from cell lysate collected from cell treated with IC_{20} of the compounds for 72h. The data shown result from two trials, the standard deviation of the sample mean is reported.
3.2.5.4 Inhibition of matrix metalloproteases

We next studied the inhibition of several MMPs of oncological interest (MMP-1, MMP-2, MMP-3, MMP-7, MMP-8, MMP-9) by bimetallic Ti-Au Titanocref and Titanofin (4), and monometallic Au cref, fin, and AF. The protein expression of all the selected MMPs was strongly inhibited by the bimetallic compounds except MMP-2 which was most inhibited by the monometallic compounds. For MMP-3, both the bimetallic and monometallic compounds achieved inhibition greater than 50%. The inhibition of MMP-7 was significant, and similar between the bimetallic compounds, while treatment with the monometallic compounds caused no inhibition (Figure 3.7). AF and Titanofin (to a slightly higher degree) inhibit the expression of MMP-9, while Titanocref and the monometallic cref and fin did not have a significant effect.

It is evident from the changes in expression we observed as a result of treatment with Titanocref, Titanofin and monometallic cref, fin and AF, that while bimetallic compounds are most often more effective inhibitors than monometallic compounds, the latter do have a non-negligible IL and MM inhibitory profile, rendering them promising anti-cancer candidates (see fin).

Because there is limited understanding of the pathways by which metallodrugs achieve efficacy, we performed a protein expression screen of our compounds’ effects on 84 cancer-related proteins for 72h at IC

From this screen, we identified that our compounds had a significant effect on nine markers of oncological interest in addition to the fourteen reported earlier in this chapter. In an attempt to determine if there was any discernable pattern in the inhibition of expression of oncological proteins by bimetallic compounds, we generated a heat map (Figure 3.7) comparing the effects of bimetallic Ti-Au (Titanocref and Titanofin) and monometallic Au (cref and fin) as well as AF shown in Figures 3.5 and 3.6.
Figure 3.7. Heat map visualization of protein expression data. Each row represents a protein of oncological interest and each column represents a specific compound incubated for 72h at IC$_{20}$. Markers features whose levels vary significantly (p < 0.05) between at least two treatment groups (bimetallic Titanocref and Titanofin, monometallic cref, fin and AF) are projected on the heat map and used for clustering. The scaled expression value of each feature is plotted in red-green color scale. The red color of the tile indicates high abundance and green indicates low abundance. The color gradients indicate the degree of expression relative to the DMSO control.

Markers whose levels varied significantly (p < 0.05) between at least two treatment groups (Titanocref, Titanofin, cref, fin, and AF) were projected on the heat map and used for clustering.
In the heat map (Figure 3.7), we included seven additional markers of interest in cancer progression that were inhibited by either bimetallics, monometallics, or both classes of compounds.

One particular salient point arose from this analysis, there seems to be some stratification of markers that are affected by the bimetallic and not the monometallic compounds and vice versa. This stratification warrants future exploration.

These findings are informative not only for guiding further rational drug design, but also in adding mechanistic insights to an already FDA approved drug that is actively being explored as a potential anticancer agent. Broadening our understanding of the mechanism by which AF achieves its efficacy is a valuable contribution to the study of its potential as safe, clinically available, and efficient anticancer agent. What has also become evident from our evaluations is that however effective the monometallic compounds might be, overall the bimetallic compounds are more potent and affect a broader spectrum of molecular targets and cellular behaviors than any single monometallic as observed in their inhibition of migration, invasion, and angiogenesis, as well as their inhibition of VEGF, MMP(s), and IL(s).

3.3 CONCLUSIONS

This study expands on the discovery of the high in vitro efficacy of bimetallic titanocene-gold derivatives against a human clear-cell renal carcinoma cell line. One of the compounds (Titanocref) had been previously described as extremely efficacious in vitro and in vivo. In this study, we report a more complete mechanistic view on Titanocref, and on a second related compound, Titanofin that contains the same gold-phosphane fragment as AF. Comparisons were made with AF as control and with the monometallic gold precursors necessary to prepare these bimetallic
titanocene-gold derivatives. While we have used TDC as titanocene monometallic control, the high concentrations (well over 100 micromolar) needed to observe effects have precluded us to use it for relevant comparisons in most assays. The bimetallic compounds Titanocref and Titanofin strongly inhibited migration, invasion, and angiogenic assembly along with molecular markers associated with these processes such as prometastatic IL(s), MMP(s), TNF-α, and VEGF. Bimetallic titanocene-gold compounds have thus emerged as potential chemotherapeutics for renal cancer as they hindered three of the most harmful behaviors of the tumor microenvironment, local migration of tumor cells, invasion into the vasculature for metastasis, and the formation of de novo blood vessels. We also evaluated AF and observed that it had similar effects to the bimetallic compounds in the renal cancer cell line Caki-1 in terms of its antiproliferative and antimetastatic properties and inhibited most notably VEGF and IL(s).

These results along with those previously published for Titanocref, indicate that further investigations of their efficacy, pharmacokinetic (PK), and histopathological studies and mechanistic exploration in vivo for bimetallic titanocene-gold derivatives are warranted. In addition, the relevant results found for AF also warrant further exploration of this drug for renal cancer treatment.
Part 2

Preclinical evaluation of heterobimetallic titanocene-gold complexes unveils great tumor suppressive and angiosuppressive efficacy in a mouse clear cell renal carcinoma xenograft

3.4 INTRODUCTION

A previous study, briefly discussed in Chapter 2, of the *in vivo* efficacy of Titanocref yielded a decrease in tumor volume of 67% after a 28 day treatment course with doses separated by 48h. However, that study lacked the pretrial PK study which lead to an under-dosing of the mice with too narrow of a window between doses. The dose range component of that study was performed in a different mouse strain (C57BL/6) than that used for the efficacy trial (NOD.CB17-Prkd SCID/J), which might also have skewed the dose choice. Therefore, despite these limitations, given the promising *in vivo* results of this first trial, and in light of the impressive *in vitro* data obtained for both Titanocref and Titanofin outlined in part 1, we chose to test both compounds following rigorous preclinical design parameters informed by an expert clinical pathologist and oncology pharmacologist. Therefore, to obtain further clinically relevant insights into Titanocref and Titanofin’s anticancer efficacy, the biological response, and organ distribution, an *in vivo* study was performed in NOD.CB17-Prkd SCID/J mice bearing a subcutaneous clear cell renal carcinoma (Caki-1) xenograft, an established preclinical model of ccRC. We report here that measurements of the acute and chronic toxicity, pharmacokinetics, efficacy, and pathology of treatment with Titanocref and Titanofin generated promising results, and suggest that our compounds are viable preclinical candidates.
3.5 RESULTS AND DISCUSSION

3.5.1 Dose range finding study.

First, we report the acute toxicity and maximum tolerated dose (MTD) of Titanocref and Titanofin in naïve NOD.CB17-Prkdc SCID/J mice. This study was carried out to assess the toxicological profile of Titanocref and Titanofin following 6 intraperitoneal (i.p.) doses between 10mg/kg/48h and 30mg/kg/48h Titanocref and 30mg/kg/48h to 50mg/kg/48h for Titanofin followed by a two-week recovery period. Vehicle solution (0.5% DMSO + 99.5% normal saline) treated mice were used as controls. Lung, liver, kidney, spleen, and heart were collected, weighed, and visually evaluated during a cross necropsy. Parameters, such as mortality, bodyweight, posture, grooming, and clinical and gross pathologies, were monitored throughout the study. The acute toxicity study indicated that the median lethal dose (LD\textsubscript{50}) of Titanocref was 30mg/kg/48h and of Titanofin was 45mg/kg/48h (Figure 3.8). The MTD study indicated that male and female mice tolerated three i.p. injections at doses of 10mg/kg/48h to 15mg/kg/48h of Titanocref and 30mg/kg/48h to 40mg/kg/48h of Titanofin without notable signs of toxicity or changes pathological parameters in the treated animals (Figure 3.9). These findings informed the rationale for selecting the doses of 5mg/kg/48h for Titanocref and 10mg/kg/48h for Titanofin for the PK and histopathological analysis. It was concluded that administration of seven consecutive doses of 5mg/kg/48h Titanocref and 10mg/kg/48h Titanofin via intraperitoneal injection in female and male NOD.CB17-Prkdc SCID/J mice would be well tolerated under these study conditions since no changes were observed in mean body weight compared to control and no signs of distress were observed at three times these doses. Although some residue of precipitated compound was observed in the peritoneal cavity, no obvious signs of local toxicity were observed. These dose
range finding toxicity studies demonstrate that Titanocref and Titanofin are rather safe compounds within a two week treatment at doses of less than 20 mg/kg i.p.

**Figure 3.8.** Median lethal dose (LD$_{50}$) was estimated by injecting i.p. five increasing doses of Titanocref and Titanofin into two mice (weighing between 18 and 22 g). Deaths occurring during the first two doses were recorded and LD$_{50}$ was calculated.
Figure 3.9. Titanocref and Titanofin are best tolerated at doses below 15 mg/kg/48h and 40 mg/kg/48h respectively in non-tumor bearing female and male NOD.CB17-Prkdc SCID/J mice. A. Survival curves associated with MTD determination of Titanocref and Titanofin. B. Body weights at the end of the course of treatment with various doses during the MTD determination. Error bars represent SEM for each group.

We then proceeded to evaluate the biodistribution and bioavailability of the compounds. To that end we performed a PK study in a preclinical mouse xenograft model of kidney cancer.

3.5.2 Pharmacokinetics and Biodistribution (PK analyses were performed by the Laboratory of Swayam Prabha, at the University of Minnesota Department of Experimental and Clinical Pharmacology)
Second, we determined the pharmacokinetics of Titanocref and Titanofin upon intraperitoneal (i.p.) administration of one single dose followed by time lapse blood collection and terminal tissue collection in xenograft Caki-1 (renal cell carcinoma) tumor bearing NOD.CB17-Prkdc SCID/J mice to provide insights into critical preclinical criterion such as absorbance time clearance rate.

We observed that though the bimetallic compounds Titanocref and Titanofin are closely related, their kinetic profiles were quite different (Figure 3.10). The compounds were absorbed slowly, as indicated by a peak plasma concentration of Ti reached at 12 h for Titanocref, and 24 h for Titanofin reached after dosing. Compounds were eliminated slowly from the blood compartment with an elimination half-life ($t_{1/2}$) of 32.38 h for Titanocref and 47.16 h for Titanofin (Table 3.3). The plasma concentration of Au was not measured due to experimental constraints. This informed the choice of a 72 h window between doses, as it allowed sufficient time for clearance of the compound. In our previous study of Titanocref, the doses were spaced by 48 h which might have contributed to significant accumulation of the metals in the kidney and liver, in this investigation, the accumulation of Ti was in the order kidney > liver > tumor, and was accompanied by notable loss in body weight. In this new tissue distribution study (Figures 3.11 and 3.12 and Tables 3.13-3.15), a significant amount of both Ti and Au were found in the tumor tissue for each compound, however while the accumulation of Titanocref was in the order liver > tumor > kidney, for Titanofin it was liver > kidney > tumor, (Figure 3.11, Table 3.5). It is important to note that we observed close to a 1:1 colocalization of both metals (Ti and Au) in the tissue, which can be reconciled with previous observation that *in vitro* (Figure 3.12), both metals colocalized inside the cells in a dose dependent manner. These data indicate that both metals in
the compounds are delivered to the tissue at a near 1:1 ratio, suggesting stability of the bimetallic compounds in the course of biodistribution.

Table 3.3. Pharmacokinetic\(^b\) parameters of Titanocref and Titanofin after single intraperitoneal injection in tumor-bearing mice.

<table>
<thead>
<tr>
<th>Pharmacokinetic Parameters</th>
<th>Titanocref</th>
<th>Titanofin</th>
</tr>
</thead>
<tbody>
<tr>
<td>( T_{\text{max}} ) (h)</td>
<td>10.50±3.00</td>
<td>21.00±6.00</td>
</tr>
<tr>
<td>( C_{\text{max}} ) (µg/mL)</td>
<td>57.20±1.77</td>
<td>29.73±7.56</td>
</tr>
<tr>
<td>( \text{AUC}_{\text{last}} ) (µg.h/mL)</td>
<td>2473.9±223.4</td>
<td>1346.6±76.8</td>
</tr>
<tr>
<td>( \text{AUC}_{\text{Inf}} ) (µg.h/mL)</td>
<td>3247.04±380.50</td>
<td>2660.79±614.24</td>
</tr>
<tr>
<td>ke (h(^{-1}))</td>
<td>0.0215±0.0031</td>
<td>0.0140±0.0058</td>
</tr>
<tr>
<td>( t_{1/2} ) (h)</td>
<td>32.73±4.44</td>
<td>56.02±1.58</td>
</tr>
<tr>
<td>( V_d/F ) (mL/Kg)</td>
<td>72.65±4.64</td>
<td>298.83±71.37</td>
</tr>
<tr>
<td>( C_l/F ) (mL/h/Kg)</td>
<td>1.55±0.19</td>
<td>3.93±0.93</td>
</tr>
<tr>
<td>( C_{\text{min ss}} ) (µg/mL)</td>
<td>20.83±3.45</td>
<td>25.61±9.40</td>
</tr>
<tr>
<td>( \text{Time to reach steady state} ) (h)</td>
<td>163.6±22.2</td>
<td>280.1±107.9</td>
</tr>
</tbody>
</table>

\(^b\) Pharmacokinetic parameters determined include the maximum observed plasma concentration (\( C_{\text{max}} \)), the time to reach \( C_{\text{max}} \) (\( T_{\text{max}} \)), area under the plasma concentration–time curve from time zero to 72 h post dose (\( \text{AUC}_{0-72h} \)), elimination rate constant (ke), terminal elimination half-life (\( t_{1/2} \)), apparent total clearance from plasma (\( C_l/F \)), and apparent volume of distribution (\( V_d/F \)).
Figure 3.10. Plasma concentration of the Ti metal of Titanocref and Titanofin at various intervals after single intraperitoneal injection. Data represent mean ± SD (n = 4).

Table 3.4. Plasma concentration of the Ti metal of the Titanocref and Titanofin bimetallic at various intervals after single intraperitoneal injection. Data represent mean ± SD (n = 4).

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>Titanocref Conc. (µg/mL)</th>
<th>Titanofin Conc. (µg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>42.51 ± 3.78</td>
<td>13.93 ± 1.43</td>
</tr>
<tr>
<td>2</td>
<td>45.47 ± 4.42</td>
<td>13.71 ± 1.32</td>
</tr>
<tr>
<td>6</td>
<td>49.47 ± 5.71</td>
<td>18.80 ± 8.07</td>
</tr>
<tr>
<td>12</td>
<td>56.83 ± 1.70</td>
<td>21.62 ± 10.64</td>
</tr>
<tr>
<td>24</td>
<td>45.26 ± 1.78</td>
<td>27.17 ± 5.49</td>
</tr>
<tr>
<td>48</td>
<td>22.92 ± 10.23</td>
<td>19.56 ± 2.51</td>
</tr>
<tr>
<td>72</td>
<td>16.19 ± 1.80</td>
<td>13.42 ± 2.10</td>
</tr>
</tbody>
</table>
Figure 3.11. Tissue distribution of both titanium and gold metals from Titanocref, Titanofin at the end of the PK study (72 h post-injection). Below the x-axis, Ti:Au represents the ratio of the two metals in each tissue. Data represent mean ± SD (n = 4).

Figure 3.12. Cellular uptake of Titanocref in Caki-1 cells. The concentration of Titanocref calculated based on Ti and Au content in the cell lysates after 72h of incubation is similar suggesting that both the elements are co-localized in the cells. Modified from 25)
Table 3.5. Tissue distribution of the Ti and Au metals of the bimetallic Titanocref and Titanofin 72h after single intraperitoneal injection. Data represent mean ± SD (n = 4).

<table>
<thead>
<tr>
<th></th>
<th>Titanocref</th>
<th></th>
<th>Titanofin</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ti (µg/gm)</td>
<td>Au (µg/gm)</td>
<td>Ti (µg/gm)</td>
<td>Au (µg/gm)</td>
</tr>
<tr>
<td>Liver</td>
<td>18.92 ± 3.32</td>
<td>15.06 ± 1.97</td>
<td>14.96 ± 14.9</td>
<td>17.16 ± 13.87</td>
</tr>
<tr>
<td>Kidney</td>
<td>4.94 ± 1.14</td>
<td>6.33 ± 1.51</td>
<td>3.25 ± 1.29</td>
<td>5.28 ± 1.82</td>
</tr>
<tr>
<td>Tumor</td>
<td>6.42 ± 5.60</td>
<td>6.36 ± 4.00</td>
<td>2.89 ± 1.46</td>
<td>4.60 ± 3.82</td>
</tr>
</tbody>
</table>

Table 3.6. Ratio of Ti metal present between tissue and plasma of mice treated with Titanocref and Titanofin 72h after single intraperitoneal injection. Data represent mean ± SD (n = 4).

<table>
<thead>
<tr>
<th></th>
<th>Titanocref</th>
<th></th>
<th>Titanofin</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Tissue : Plasma</td>
<td>Tissue : Plasma</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Liver</td>
<td>1.19 : 1</td>
<td>1.47 : 1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kidney</td>
<td>0.31 : 1</td>
<td>0.24 : 1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tumor</td>
<td>0.39 : 1</td>
<td>0.22 : 1</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 3.7. Ratio of Ti and Au metals present in tissue of mice treated with Titanocref and Titanofin 72h after single intraperitoneal injection. Data represent mean ± SD (n = 4).

<table>
<thead>
<tr>
<th></th>
<th>Titanocref</th>
<th></th>
<th>Titanofin</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ti : Au</td>
<td>Ti : Au</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Liver</td>
<td>1.26 : 1</td>
<td>0.87 : 1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kidney</td>
<td>0.78 : 1</td>
<td>0.62 : 1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tumor</td>
<td>1.01 : 1</td>
<td>0.63 : 1</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
3.5.3 Efficacy study

Third, we report on an intervention trial to determine the \textit{in vivo} anticancer efficacies of Titanocref and Titanofin in xenograft Caki-1 (renal cell carcinoma) tumor bearing NOD.CB17-Prkdc SCID/J mice in response to intraperitoneal (i.p.) administration of seven doses spaced by 72h followed by a 72h recovery period before sacrifice. The previous \textit{in vivo} efficacy study of Titanocref performed yielded an increase in tumor volume of 67\% after a 28 day treatment course with doses separated by 48h. However, as noted, this study did not contain an accurate PK analysis. Therefore, we chose to re-test the \textit{in vivo} efficacy of the compound, this time following more rigorous preclinical design parameters. Hence, following our most recent PK study, it was determined that doses of one quarter of the MTD administered once every 72h would be appropriate to evaluate \textit{in vivo} the anti-cancer efficacy of Titanocref and Titanofin.

In mice treated with Titanocref, we observed a decrease (shrinkage) in tumor size of 51\% from the starting tumor burden recorded at the beginning of the trial, while mice treated with Titanofin had a reduction in tumor burden of 60\% after 21 days of treatment (a total of 7 doses) (Figure 3.13). This result can be reconciled with data obtained in our previous study (Chapter 2 Figure 2.9), where after 28 days of treatment with Titanocref every 48h we observed a tumor shrinkage of 67\%. In comparison, we observed a growth in tumor volume of 138\% in the vehicle treated mice.

No significant change in weight or any decline in the wellbeing of the Titanocref and Titanofin treated mice were observed during this trial. These results are remarkable, as they indicate the compounds not only prevented tumor growth, but additionally shrank the primary tumor.
Figure 3.13. Titanocref and Titanofin treatment reduces tumor growth in a xenograft renal cancer intervention trial (Top) Representative histological samples of tumors resected from each treatment group. (Bottom) The average tumor volume after 21 days of treatment was decreased by 51% in Titanocref treated mice and 60% in the Titanofin treated mice compared to the vehicle (Veh) control-treated group where tumor volume increased by 138%, ** p<0.01. The progressive change in tumor burden became evident 7 days after the first injection. Mice were treated with Titanocref (5 mg/kg/72h), Titanofin (10 mg/kg/72h), or Veh by intraperitoneal injection from for 21 days. Titanocref :n=6 mice, Titanofin: n=6 mice, Veh: n=6 mice.

We then sought to determine the biodistribution of each metal after the 21-day efficacy study. Using the same methods as the PK study (Figure 3.14, Table 3.8), we observed that the biodistribution of Titanocref was in the order liver>kidney>tumor; whereas that for Titanofin it
was liver>kidney>tumor. Which is a small shift from the biodistribution of observed in the PK analysis the Titanocref accumulated more in the tumor than in the kidney. These changes cannot be clearly attributed to a specific metabolic or clearance change over the course of treatment.

**Figure 3.14.** Tissue distribution of Titanocref, Titanofin at the end of efficacy study (i.e. 72 h after the last injection of a 21 day trial (Titanocref (5mg/kg/72h), Titanofin (10mg/kg/72h). Below the x-axis, Ti: Au represents the ratio of the two metals in each tissue. Data represent mean ± SD (n = 4).

We observed that, compared to the tissue distribution results obtained at the PK study, at the end of the efficacy trial, significantly less metal was retained in the tissue at the end of the PK study. For mice treated with Titanocref, 18.92 µg Ti/gm and 8.19 µg Ti/gm respectively were found in the liver. This accounted for almost half the total metal injected, suggesting that perhaps, during the trial, the liver adjusted to more effectively dispose of the metals (Table 3.9). The same is true for mice treated with Titanofin, where significantly less metal was retained in the tissue at the end of the PK study (14.96 µg Ti/gm, and 8.13 µg Ti/gm of liver respectively).
**Table 3.8.** Tissue distribution of the Ti and Au metal of bimetallic Titanocref and Titanofin at the end of efficacy study (i.e. 72 h after the last injection of a 21 day trial (Titanocref (5mg/kg/72h), Titanofin (10mg/kg/72h). Data represent mean ± SD (n = 4).

<table>
<thead>
<tr>
<th></th>
<th>Titanocref</th>
<th></th>
<th>Titanofin</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ti (µg/g)</td>
<td>Au (µg/g)</td>
<td>Ti (µg/gm)</td>
<td>Au (µg/g)</td>
</tr>
<tr>
<td>Liver</td>
<td>8.19 ± 1.55</td>
<td>6.45 ± 1.26</td>
<td>8.13 ± 3.42</td>
<td>6.45 ± 2.94</td>
</tr>
<tr>
<td>Kidney</td>
<td>5.12 ± 1.89</td>
<td>4.86 ± 1.96</td>
<td>2.99 ± 1.58</td>
<td>2.46 ± 1.72</td>
</tr>
<tr>
<td>Tumor</td>
<td>3.55 ± 1.60</td>
<td>3.12 ± 1.36</td>
<td>2.79 ± 1.14</td>
<td>2.45 ± 1.42</td>
</tr>
</tbody>
</table>

Au accumulation followed a similar trend. Though there is also a reduction in the amount of metal retained by kidney and tumor tissue at the end of the efficacy study, the decrease was less drastic than that of the liver. There were striking differences between the tissue accumulation of Titanocref and Titanofin in tissue 72h following a single dose of compound as compared to 72h after a 21 day treatment course. We first observed that in both Titanocref and Titanofin treated mice, the ratio of Ti to Au in the tissue was higher than in the PK study indicating that more Ti was retained in the tissues than Au during the course of treatment. Second, we observed that the absolute concentration of metal per tissue weight was two-fold lower in liver tissue after the 21 day trial suggesting that over the course of treatment, the liver increased its ability to excrete the compounds (Table 3.9).

We then compared the biodistribution profiles of two metallodrugs currently in clinical use, Cisplatin and AF, reported in the literature to our results (Figure 3.15). The available reported
tissue distributions for Cisplatin are 48h after 6mg/kg (i.v.) in colorectal xenograft mice and for AF 24h after a single oral dose of 1mg/kg in non tumor bearing mice.

Figure 3.15. Tissue distribution of A. Cisplatin 48h after 6mg/kg (i.v.) after a single dose 20mg/kg (i.v.) in colorectal xenograft mice. Data represent mean ± SD (n = 4) B. AF 1 mg/kg 24h after a single oral dose in non-tumor bearing mice. Data represent mean ± SD (n = 6), C. Titanocref mg/kg 72h after a single dose. Data represent mean ± SD (n = 4). D. Titanofin 10 mg/kg 72h after a single dose. Data represent mean ± SD (n = 4).

Both studies were dissimilar to ours in dose, means of administration, and the timepoint of data collection. However, we may gain some insight from them as to the trend of distribution in the short term 24h to 72h and long term. The relative tissue concentration of AF at 24h was 0.427µg Au/g in the liver and 21.1µg Au/g in the kidney. The relative tissue concentration of Cisplatin at 48h was 1µg Pt/g in the liver and 0.5µgPt/g in the kidney. Both Titanocref and Titanofin accumulated at significantly higher levels than either AF or cisplatin relative to the administered dose. Importantly, despite the high levels of Ti accumulation in the liver and kidney, we observed...
no signs of hepatic or renal pathology as a result of treatment, therefore it is not of critical clinical concern.

**Table 3.9.** Fold difference in tissue distribution of the Ti metal of the bimetallic Titanocref and Titanofin between the PK study (i.e. 72 h after a single injection of Titanocref (5mg/kg), Titanofin (10mg/kg) and the efficacy study (i.e. 72 h after the last injection of a 21day trial [Titanocref (5mg/kg/72h), Titanofin (10mg/kg/72h)]. Data represent mean ± SD (n = 4).

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Titanocref</th>
<th>Titanofin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver</td>
<td>2.29</td>
<td>1.84</td>
</tr>
<tr>
<td>Kidney</td>
<td>0.97</td>
<td>1.10</td>
</tr>
<tr>
<td>Tumor</td>
<td>1.81</td>
<td>1.04</td>
</tr>
</tbody>
</table>

**3.5.4 Mechanism of action in vivo**

Given the substantial reduction in tumor size in Titanocref and Titanofin treated mice, we next sought to determine the cellular mechanisms driving this decrease. We sought to interrogate the effects of Titanocref and Titanofin on the proliferating to apoptotic cell ratio in clear cell renal carcinoma xenograft tumors. We first examined cell proliferation profile of the tumors using the proliferation marker Ki67, and because tumor growth can also reflect changes by in cell death, we compared the proliferation rate to apoptotic rates using apoptotic marker cleaved caspase 3. The average of proliferating cells in individual tumors of the vehicle (Veh.) treated mice control group was 36%, which was reduced to 16.2% in and 17.3% in tumors treated with Titanocref and Titanofin respectively, while the average of apoptotic cells in individual tumors of the Veh. treated
mice was control group was 2%, which was increased to 6.2% in and 7.9% in tumors treated with Titanocref and Titanofin respectively (Figure 3.16).

Figure 3.16. Titanocref and Titanofin treatment affects proliferation and apoptosis at the 21-day trial endpoint. Titanocref and Titanofin treatment reduces tumor cell proliferation by 47% and 38% respectively and increases apoptosis by 270% and 312% respectively in a xenograft clear cell renal cancer intervention trial. The tumor proliferation and apoptosis were analyzed by staining tumor tissue with a cell proliferation specific marker Ki67 (green) and an apoptosis specific marker Cleaved caspase 3 (red) (n=4 mice per group (apoptosis Titanocref and Titanofin ***p< 0.001, proliferation Titanocref and Titanofin **p <0.01 ).
Figure 3.17. Titanocref and Titanofin treatment does affect tumor angiogenesis at the 21-day trial endpoint. (A, B) The tumor vasculature was analyzed by intravenous perfusion with Dextran (Texas Red) and endothelial specific cell surface marker Lectin (n=2 mice per group). The percentage of Lectin-labeled blood vessels over the total tumor area is graphed in (B- green), showing significant decrease in blood vessel coverage between the Titanocref, Titanofin and vehicle treated tumors, ** p<0.01. The percentage of dextran-stained surface over the total tumor area is graphed in (B -red), showing significant difference in vessel leakiness (measured by dextran dispersion from blood vessels) between Titanocref, Titanofin and vehicle treated tumors, ** p<0.01.
We determined, as reported in Part 1 of this chapter, *in vitro* that both Titanocref and Titanofin disrupt vascular assembly and noted that they were potent inhibitors of IL-6 and VEGF. It has been shown that inhibition or deletion of IL-6 and VEGF in mice induced a significant decrease in tumor invasion and angiogenesis, and thus sought to determine if the use of our compounds disrupted angiogenesis and VEGF and IL-6 levels were affected. By staining tumors extracted from mice following the efficacy study, we observed that Titanocref and Titanofin treatment resulted in a 38% and 54% reduction of vascular distribution respectively as indicated by the decrease in lectin positive staining while also increasing vascular integrity as evidenced by the reduction dextran positive surface correlated to vessel leakiness (Figure 3.17).

### 3.5.5 Proteomic analysis

Given the significant decrease in angiogenesis in Titanocref and Titanofin treated mice, as well as the drastic reversal in the proliferating to apoptotic cell ratio, we next aimed to identify some of the molecular signaling changes that may underlie this phenotype. We interrogated the effects of treatment with Titanocref and Titanofin on clear cell renal carcinoma in culture as well xenograft tumors on an array of proteins of oncological relevance by immunoblotting (Figure 3.18) and compared those results to data obtained *in vitro*.

We observed from the histological analysis (Figure 3.17A) that in response to Titanocref treatment there was a striking reduction in angiogenesis, which could be mediated by a change in balance of angiogenic factors. There was indeed an increase in expression of antiangiogenic angiopoietin-1, endostatin, thrombospondin-1, and a decreased in VEGF at the end of the efficacy trial which would correlate with the reduced vasculature. We also observed an increase in
antitumorigenic immune factors IL-2 R and GM-CSF which are clinically associated with good prognosis \(^{39-41}\). The expression of the macrophage related CapG protein was suppressed, which usually correlates with reduced migration \(^{42,43}\). Those antitumorigenic phenotype changes were observed both \textit{in vitro} and \textit{in vivo}. There were however, changes in protein expression observed \textit{in vivo} that were not observed in the \textit{in vitro} samples. The \textit{in vivo} efficacy was accompanied by a significant decreased in prometastatic factors Cathepsin S (CtsS), FoxC,2 and MMP-3 \(^{44-47}\). We also observed a decrease in CCL7 levels upon Titanocref treatment, which could decrease the tumor’s ability to recruit monocytes, thus rendering the tumor ecosystem more hostile to tumor progression. The decrease in Tie-2 observed would be expected to indicate decrease in tumor vascular normalization, however, there was no evidence from the histological samples that the vasculature lacked integrity, suggesting that perhaps other factors were at play. Those changes were not observed in the \textit{in vitro} samples perhaps because \textit{in vitro} monoculture the signaling dynamic is limited to tumor-tumor cells communication while \textit{in vivo} the tumor microenvironment ecosystem is rich and factors can be contributed by different cell types. It was thus predictable that the most significant differences were in the expression of factors primarily produced by or in response to immune cells \textit{in vivo}. Also, the 3D nature of the \textit{in vivo} model lends itself to behaviors such at EMT which would not readily accruing in 2D monoculture. This might perhaps explain why we observe a decrease in FOXC2 expression which is induced when epithelial cells undergo EMT which facilitates the metastatic process.

In response to Titanofin treatment \textit{in vivo} and \textit{in vitro}, we observed an increase in antiangiogenic Angiopoietin-1, Endostatin, Thrombospondin-1 and VEGF both \textit{in vitro} and \textit{in vivo} which can be reconciled with the antiangiogenic phenotype observed both in vivo and in vitro following Titanofin treatment. These data suggest that the constellation of factor involved in
inhibiting angiogenesis in response to Titanocref is dissimilar to that of Titanofin, *in vivo*. The decrease in FGF basic, MMP-2, and u-PA can be reconciled with their compounds’ reported antiinvasive and antimigratory properties. What we found of great interest is that the antitumorigenic immune factor GM-CSF was very highly expressed which would indicate a positive clinical outcome. It is unsurprising given the role of immune cells in the production of GM-CSF that no changes in expression levels are observed *in vitro*. All these changes suggest a potent tumor inhibiting phenotype. *In vivo*, Titanofin significantly decreases the expression of Cathepsin S, CCL8, E-Cadherin, HIF-1a, ICAM, M-CSF, VE-Cadherin, all of which when overexpressed promote tumor progression, however, *in vitro*, those proteins were either unaffected or they are overexpressed. This difference might be due to the lack of stromal cell contribution in the in vitro system. For instance, CCL8 is expressed by dermal fibroblasts to modulate tumor-stroma and tumor-tumor cross-talk in the initiation of metastasis, while ICAM and M-CSF are mainly produced and modulated by immune cells or in response to immune cell signal which are absent in 2D monoculture. Also, solid tumors are a hypoxic environment where high levels of HIF-1a would be expected and in response to treatment that shrinks the tumor mass one would expect increased oxygenation thus decrease in HIF-1a, whereas hypoxia is a lesser phenomenon in 2D culture. Cts levels are often lowered in the absence of angiogenesis or active invasion both processes in which their proteolytic capacities are necessary. It is unclear if the change in Cts is causative of angiogenic disturbance.

IL-6 and Survivin were inhibited *in vitro* in response to both Titanocref and Titanofin, but expression was increased *in vivo*, which may be due to the contribution of stromal cells contribution in the case of IL-6 in particular to the overall protein levels. Survivin however is a member of the inhibitor of apoptosis (IAP) family which given the high level of apoptosis observed
in vivo, we would expect to be significantly reduced. There might be compensatory or competing signals driving apoptosis, despite high Survivin activity.

It is also notable that Titanocref and Titanofin did not necessarily affect the expression levels of the same molecules in vivo, as we observed that Titanofin affected expression levels of u-PA/Urokinase, Survivin, M-CSF, ICAM-1/CD54, CCL8/MCP-2, FGF basic, and HIF-1alpha, while Titanocref did not. Conversely, Titanocref affected the expression levels of CCL7, FoxC2, Tie-2 and MMP-3, while Titanofin did not. This suggests a greater effect or reliance on immune factors for the efficacy of Titanofin than Titanocref, which appears to rely on other factors known to drive metastasis and angiogenesis. We observed no changes of systemic presence of immune cells from circulating blood as a percentage of blood circulating neutrophils (NEUT) lymphocytes (LYMPH) and monocytes (MONO) were unchanged between control and treated mice and fell within the normal range. This would indicate that any change in immune cell population is either localized to the tumor, or systemically too small to be detected in complete blood work (Table 3.12).

Thinking of these results in the context of potential anticancer therapeutic agents these results are of great interest. Both compounds promoted the expression of Angiopoietin-1, which is known to disrupt vascular connections while promoting vascular regression and cell death, and when deficient, increases tumor metastasis. Vascular Endothelial Cadherin (VE- Cadherin) a critical regulator of VEGF expression was downregulated in response to treatment with either compound, suggesting that these compounds are viable antiangiogenic candidates. We have also observed a significant increase in GM-CSF expression, which can inhibit cancer growth and metastasis by initiating an anti-angiogenic cascade in cooperation with tumor associated macrophages.
Figure 3.18. Titanocref and Titanofin treatment induce tumor changes in expression angiogenic regulators as metastasis and apoptosis modulators in vitro and in vivo. In vitro changes in expression levels of proteins of oncological interest, following treatment of Caki-1 cells in vitro with IC_{20} concentrations of each compound for 72h. In vivo changes in expression levels of proteins of oncological interest, following a 21-day efficacy trial in Caki-1 xenograft tumor bearing NOD.CB17-Prkdc SCID/J mice. Analysis of 150µg of protein extracted from cell or tumor lysate. The values indicate the percentage of change in expression relative to DMSO treated cells.
Figure 3.19. Titanocref and Titanofin treatment induce changes in expression of cell stress markers in vivo. In vivo changes in expression levels of stress proteins, following a 21-day efficacy trial in Caki-1 xenograft tumor bearing NOD.CB17-Prkdc SCID/J mice. Analysis of 150µg of protein extracted from cell or tumor lysate. The values indicate the percentage of change in expression relative to DMSO treated cells.

This is particularly exciting because GM-CSF, which is a potent cytokine stimulator myeloid cells production, is being used, in the clinic, as an immuno-adjuvant to draw antitumorigenic immunity. In response to treatment, we also observed a significant reduction in expression of factors whose overexpression is linked to metastatic progression of many solid tumors in vivo, such as CCL7 and CCL8.

The protein expression analysis revealed that in most cases, proteins affected in vitro were also affected in vivo, and followed similar trends for both drugs. It is not unusual for in vitro and in vivo protein expression prolife to retain a lot of similarities but not be identical. Two notable
exceptions being Cathepsin and Fox2, which were (decreased in vivo and increased in vitro) in response to Titanocref treatment, and IL6 in response (increased in vivo, decreased in vitro) to Titanofin treatment. These changes might be in part driven by the contribution of stromal cells to those protein levels. In vivo Titanocref or Titanofin are potent inhibitors of stress markers known to drive tumor metastasis and drug resistance such as HIF know to drive hypoxia, Trx known to drive chemo resistance and Bcl2 known to inhibit apoptosis. Both complexes affected tumor metabolism as they substantially induced the phosphorylation of Heat shock protein 27 (Hsp27) and reduced the activity of two inhibitors of apoptosis HSP60, and HSP70. Though both bimetallic do reduced the expressions of those proteins, they did so to different degrees (Figure 3.19).

3.5.6 Pathology

Finally, we report on our study to determine any pathological effects that might be caused by treatment with Titanocref or Titanofin. Access to expertise in cancer histopathology is critical in making a definitive determination of the safety, and therefore clinical viability of a compound for potential use in humans. A complete pathology study was performed in collaboration with Dr. Adam Michel from Memorial Sloan Kettering Cancer Center. This study revealed no significant adverse effects related to the Titanocref or Titanofin treatment were observed after evaluation of the histology (Table 3.10), in clinical indicators of liver, kidney and spleen function (Table 3.11) complete blood counts (Table 3.12), and clinical chemistry (Table 3.13) of all mice treated with either test compound when compared to vehicle treated mice. The levels of ALP <100 which is below normal (Table 3.13), however lowered ALP is not a critical health concern and because all groups included vehicle treated mice have ALP below 100, we cannot associate this pathological manifestation to the test compounds.
We found no notable difference in total weight or organ weight between the treated and control mice at the end of the trial, the data in Table 3.10 suggest there were no enlargements or atrophy as a result of treatment. From stained histological samples we can appreciate the absence of any lesion, or signs of pathologic tissue distension, discoloration, necrosis or structural abnormalities (Figure 3.20). The histological data is supported by hematologic data on the levels of key clinical indicators of liver, kidney through clinical chemistry (Table 11A) and spleen function though complete blood count (CBC) (Table 11B).

Liver function is clinically assessed by measuring tests including aspartate aminotransferase (AST), Alanine transaminase (ALT), and bilirubin (BILI). The table reports total bilirubin (TBil) which is the sum of direct bilirubin (DBil) and indirect bilirubin (IBil) \(^{53,54}\). Abnormal liver function are defined as AST> 100U/L, or total bilirubin level > 3 mg/dL. The liver function indicators are within the norm as reported by bilirubin levels for the Veh group as well as the Titanocref and Titanofin treated mice, there appears to be no notable difference between treatment group. The AST is however very elevated > 100 U/L in the Veh. Groups as well as the Titanocref and Titanofin treated mice. We did verify that neither DMSO nor normal saline administered i.p. are reported to induce increase in AST levels. Though in isolation those numbers are concerning, we can appreciate that the increase is not caused by the compounds since the vehicle treated mice seem to have as high levels as the test groups. Therefore we did not observe any liver pathology be associated with Titanofin or Titanocref treatment.

Kidney damage is often identifiable clinically by changes in creatinine (CR) clearance and blood urea nitrogen (BUN) \(^{55–57}\). High serum creatinine levels in the blood indicate that the kidneys aren't functioning properly. There too, we observed that Veh group as well as the Titanocref and Titanofin treated mice have kidney function profiles within the healthy norm.
The spleen is the largest lymphoid organ in the body and is critical for immune homeostasis. Its rich and diverse population of immune cells such as T and B cells, dendritic cells, and macrophages. In the event of splenic dysfunction, circulating thrombocyte and leukocyte counts can be altered if there is enlargement or atrophy of the spleen. Furthermore, an increase in abnormal circulating red blood cells is often an indicator of spleen malfunction. Again, as evident in Table 3.11B, there were no abnormal changes in immune cell levels, confirming that there are no detectable signs of spleen pathology in response to chronic treatment with either compounds.

The most common agents causing chemotherapy-associated nephrotoxicity include cisplatin, methotrexate, mithramycin semustine, gemcitabine and streptozocin. Cisplatin and gemcitabine are known to cause severe nephrotoxicity, dangerously high CR levels, and induce debilitating decrease in red blood cell production, leading chemotherapy induced anemia. Chemotherapy is also known to reduce the production of white cells in your blood, which severely diminishes the immune system of patients. We did not observed differences between the mice treated with Titanocref, Titanofin, or the vehicle control in the blood count report (Table 3.12), and all three cohorts had complete blood count profiles that fell within the NSG MICE Reference ranges. Finally, we found that no differences in the clinical chemistry of the treated or control animals were determined (Table 3.13), indicating normal production and excretion of physiological fluids and metabolic markers whose deregulation are indicators of pathology or drug side effects.
Table 3.10. Summary of histological data

<table>
<thead>
<tr>
<th></th>
<th>Titanocref</th>
<th>Titanofin</th>
<th>DMSO</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (g)</td>
<td>23.78 ± 3.58</td>
<td>22.96 ± 2.48</td>
<td>22.82 ± 2.14</td>
</tr>
<tr>
<td>Liver weight (g)</td>
<td>1.24 ± 0.21</td>
<td>1.26 ± 0.17</td>
<td>1.17 ± 0.28</td>
</tr>
<tr>
<td>Spleen weight (g)</td>
<td>0.042 ± 0.004</td>
<td>0.05 ± 0.003</td>
<td>0.05 ± 0.01</td>
</tr>
<tr>
<td>Heart weight (g)</td>
<td>0.17 ± 0.05</td>
<td>0.14 ± 0.02</td>
<td>0.13 ± 0.02</td>
</tr>
<tr>
<td>Average Kidney weight (g)</td>
<td>0.19 ± 0.04</td>
<td>0.19 ± 0.04</td>
<td>0.17 ± 0.05</td>
</tr>
</tbody>
</table>

Figure 3.20. Titanocref and Titanofin treatment do not induce histological changes in liver, spleen or kidney tissue of mice at the end of the 21 day efficacy trial. Histopathology on H&E staining of paraffin sections magnification 20x. Sections are representative of 3 mice of each treatment.
Table 3.11. Summary of key clinical indicators of (A) liver, kidney through clinical chemistry and (B) spleen function though complete blood count (CBC).

A.

<table>
<thead>
<tr>
<th>Clinical chemistry</th>
<th>Titanocref</th>
<th>Titanofin</th>
<th>DMSO</th>
<th>NSG Reference Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kidney</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BUN (mg/dL)</td>
<td>22.33 ± 1.53</td>
<td>21.5 ± 2.12</td>
<td>23 ± 2.65</td>
<td>5.0-28</td>
</tr>
<tr>
<td>CREA (mg/dL)</td>
<td>0.17 ± 0.02</td>
<td>0.17</td>
<td>0.17 ± 0.04</td>
<td>0.2-0.5</td>
</tr>
<tr>
<td>ALB (g/dL)</td>
<td>2.7 ± 0.1</td>
<td>2.95 ± 0.07</td>
<td>2.83 ± 0.11</td>
<td>2.4-4.3</td>
</tr>
<tr>
<td>AST (U/L)</td>
<td><strong>135.67 ± 91.31</strong></td>
<td><strong>374.5 ± 41.79</strong></td>
<td><strong>372.33 ± 27.94</strong></td>
<td>54-77</td>
</tr>
<tr>
<td>TBIL (mg/dL)</td>
<td>0.167 ± 0.06</td>
<td>0.2</td>
<td>0.2 ± 0.003</td>
<td>0.2-0.6</td>
</tr>
<tr>
<td>DBIL (mg/dL)</td>
<td>0.03 ± 0.06</td>
<td>0.05 ± 0.07</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td>IBIL (mg/dL)</td>
<td>0.13 ± 0.06</td>
<td>0.15 ± 0.07</td>
<td>0.2 ± 0.04</td>
<td>-</td>
</tr>
<tr>
<td>ALT (U/L)</td>
<td>36.33 ± 26.63</td>
<td>167 ± 198</td>
<td>163.33 ± 156.69</td>
<td>± 27-195</td>
</tr>
</tbody>
</table>

B.

<table>
<thead>
<tr>
<th>Complete Blood Count</th>
<th>Titanocref</th>
<th>Titanofin</th>
<th>DMSO</th>
<th>NSG Reference Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spleen</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RBC (M/uL)</td>
<td>9.02 ± 0.10</td>
<td>9.38 ± 0.01</td>
<td>8.11 ± 2.51</td>
<td>7.84-10.84</td>
</tr>
<tr>
<td>WBC# (K/ul)</td>
<td>3.21 ± 0.98</td>
<td>2.3 ± 0.60</td>
<td>2.39 ± 0.30</td>
<td>0.94-4.68</td>
</tr>
<tr>
<td>Neut# (K/uL)</td>
<td>2.18 ± 0.83</td>
<td>1.49 ± 0.48</td>
<td>1.37 ± 0.19</td>
<td>0.54-3.16</td>
</tr>
<tr>
<td>LYMPH# (K/uL)</td>
<td>0.7 ± 0.22</td>
<td>0.52 ± 0.21</td>
<td>0.57 ± 0.30</td>
<td>0.23-1.56</td>
</tr>
<tr>
<td>MONO# (K/uL)</td>
<td>0.24 ± 0.07</td>
<td>0.15 ± 0.11</td>
<td>0.36 ± 0.25</td>
<td>0.03-0.26</td>
</tr>
</tbody>
</table>
Table 3.12. Complete blood count report

<table>
<thead>
<tr>
<th></th>
<th>DMSO</th>
<th>Titanocref</th>
<th>Titanofin</th>
<th>NSG Reference Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>RBC (M/uL)</td>
<td>8.11 ± 2.51</td>
<td>9.02 ± 0.10</td>
<td>9.38 ± 0.01</td>
<td>7.84-10.84</td>
</tr>
<tr>
<td>HGB (g/dL)</td>
<td>12.6 ± 3.81</td>
<td>13.83 ± 0.23</td>
<td>14.30 ± 0.28</td>
<td>11.8-17.6</td>
</tr>
<tr>
<td>HCT (%)</td>
<td>40.93 ± 14.32</td>
<td>45.66 ± 0.67</td>
<td>48.35 ± 0.07</td>
<td>44.1-58.3</td>
</tr>
<tr>
<td>MCV (fL)</td>
<td>49.93 ± 2.72</td>
<td>50.67 ± 0.21</td>
<td>51.55 ± 0.07</td>
<td>51.1-58.6</td>
</tr>
<tr>
<td>MCH (pg)</td>
<td>15.57 ± 0.23</td>
<td>15.33 ± 0.15</td>
<td>15.25 ± 0.35</td>
<td>13.7-17.2</td>
</tr>
<tr>
<td>MCHC (g/dL)</td>
<td>31.27 ± 2.04</td>
<td>30.30 ± 0.17</td>
<td>29.6 ± 0.57</td>
<td>25.1-31.3</td>
</tr>
<tr>
<td>RDW-SD (fL)</td>
<td>29.77 ± 3.23</td>
<td>29.23 ± 0.67</td>
<td>30.65 ± 0.49</td>
<td>-</td>
</tr>
<tr>
<td>RDW-CV (%)</td>
<td><strong>21.30 ± 3.49</strong></td>
<td><strong>22.23 ± 0.15</strong></td>
<td><strong>22.5 ± 0.28</strong></td>
<td>17.3-20.3</td>
</tr>
<tr>
<td>RET# (K/ul)</td>
<td>440.95 ± 4.88</td>
<td>318.47 ± 124.46</td>
<td>427.55 ± 127.63</td>
<td>294-444</td>
</tr>
<tr>
<td>PLT (K/ul)</td>
<td>626.67 ± 60.07</td>
<td>1244 ± 76.02</td>
<td>1327.5 ± 284.96</td>
<td>651-2055</td>
</tr>
<tr>
<td>PDW (fL)</td>
<td>6.85 ± 0.49</td>
<td>6.67 ± 0.06</td>
<td>6.65 ± 0.07</td>
<td>-</td>
</tr>
<tr>
<td>MPV (fL)</td>
<td>6.15 ± 0.35</td>
<td>6.10 ± 0.1</td>
<td>6.05 ± 0.07</td>
<td>4.2-6.3</td>
</tr>
<tr>
<td>WBC# (K/ul)</td>
<td>2.39 ± 0.30</td>
<td>3.21 ± 0.98</td>
<td>2.3 ± 0.60</td>
<td>0.94-4.68</td>
</tr>
<tr>
<td>Neut# (K/ul)</td>
<td>1.37 ± 0.19</td>
<td>2.18 ± 0.83</td>
<td>1.49 ± 0.48</td>
<td>0.54-3.16</td>
</tr>
<tr>
<td>LYMPH# (K/ul)</td>
<td>0.57 ± 0.30</td>
<td>0.7 ± 0.22</td>
<td>0.52 ± 0.21</td>
<td>0.23-1.56</td>
</tr>
<tr>
<td>MONO# (K/ul)</td>
<td>0.36 ± 0.25</td>
<td>0.24 ± 0.07</td>
<td>0.15 ± 0.11</td>
<td>0.03-0.26</td>
</tr>
<tr>
<td>EO# (K/ul)</td>
<td>0.1 ± 0.03</td>
<td>0.09 ± 0.02</td>
<td>0.14 ± 0.03</td>
<td>0.00-0.39</td>
</tr>
<tr>
<td>BASO # (K/ul)</td>
<td>0.01</td>
<td>0.007 ± 0.01</td>
<td>0.005 ± 0.01</td>
<td>0.00-0.15</td>
</tr>
<tr>
<td>NEUT (%)</td>
<td>58.3 ± 14.01</td>
<td>66.63 ± 6.38</td>
<td>64.35 ± 4.03</td>
<td>44.21-79.92</td>
</tr>
<tr>
<td>LYMPH (%)</td>
<td>22.57 ± 9.14</td>
<td>21.8 ± 0.87</td>
<td>22.05 ± 3.18</td>
<td>13.51-42.61</td>
</tr>
</tbody>
</table>

Complete Blood Count (CBC). NOD scid gamma mice (NSG mice)
Table 3.13. Clinical chemistry

<table>
<thead>
<tr>
<th></th>
<th>Titanocref</th>
<th>Titanofin</th>
<th>DMSO</th>
<th>NSG Reference Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>BUN (mg/dL)</td>
<td>22.33 ± 1.53</td>
<td>21.5 ± 2.12</td>
<td>23 ± 2.65</td>
<td>5.0-28</td>
</tr>
<tr>
<td>CREA (mg/dL)</td>
<td>0.17 ± 0.02</td>
<td>0.17</td>
<td>0.17 ± 0.04</td>
<td>0.2-0.5</td>
</tr>
<tr>
<td>BUN/CREA ratio</td>
<td>128.99 ± 2.49</td>
<td>126.47 ± 12.48</td>
<td>143.14 ± 32.51</td>
<td>-</td>
</tr>
<tr>
<td>ALP (U/L)</td>
<td>37 ± 14.73</td>
<td>42.5 ± 19.1</td>
<td>62 ± 19.97</td>
<td>105-370</td>
</tr>
<tr>
<td>ALT (U/L)</td>
<td>36.33 ± 26.63</td>
<td>167 ± 198</td>
<td>163.33 ± 156.69</td>
<td>27-195</td>
</tr>
<tr>
<td>AST (U/L)</td>
<td>135.67 ± 91.31</td>
<td>374.5 ± 417.90</td>
<td>372.33 ± 279.43</td>
<td>54-77</td>
</tr>
<tr>
<td>GGT (U/L)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td>TBIL (mg/dL)</td>
<td>0.167 ± 0.06</td>
<td>0.2</td>
<td>0.2 ± 0.003</td>
<td>0.2-0.6</td>
</tr>
<tr>
<td>DBIL (mg/dL)</td>
<td>0.03 ± 0.06</td>
<td>0.05 ± 0.07</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td>IBIL (mg/dL)</td>
<td>0.13 ± 0.06</td>
<td>0.15 ± 0.07</td>
<td>0.2 ± 0.04</td>
<td>-</td>
</tr>
<tr>
<td>TP (g/dL)</td>
<td>5.03 ± 0.12</td>
<td>5.3</td>
<td>5</td>
<td>4.8-7.2</td>
</tr>
<tr>
<td>ALB (g/dL)</td>
<td>2.7 ± 0.1</td>
<td>2.95 ± 0.07</td>
<td>2.83 ± 0.11</td>
<td>2.4-4.3</td>
</tr>
<tr>
<td>GLOB (g/dL)</td>
<td>2.33 ± 0.21</td>
<td>2.35 ± 0.07</td>
<td>2.17 ± 0.06</td>
<td>1.7-2.2</td>
</tr>
<tr>
<td>A/G ratio</td>
<td>1.17 ± 0.15</td>
<td>1.26 ± 0.07</td>
<td>1.31 ± 0.08</td>
<td>-</td>
</tr>
<tr>
<td>P (mg/dL)</td>
<td>8.87 ± 1.60</td>
<td>9.95 ± 1.91</td>
<td>10.1 ± 1.68</td>
<td>7.3-14.5</td>
</tr>
<tr>
<td>Ca (mg/dL)</td>
<td>9.33 ± 0.21</td>
<td>9.6 ± 0.14</td>
<td>9.43 ± 0.42</td>
<td>9.5-12.5</td>
</tr>
<tr>
<td>GLU (mg/dL)</td>
<td>166.33 ± 26.50</td>
<td>170 ± 4.24</td>
<td>190 ± 33.51</td>
<td>172-372</td>
</tr>
<tr>
<td>CHOL (mg/dL)</td>
<td>85 ± 9.17</td>
<td>88 ± 11.31</td>
<td>82.67 ± 9.81</td>
<td>55-169</td>
</tr>
<tr>
<td>TRIG (mg/dL)</td>
<td>109 ± 22.72</td>
<td>76.5 ± 19.09</td>
<td>93.67 ± 34.59</td>
<td>67-289</td>
</tr>
<tr>
<td>CK (U/L)</td>
<td>278.67 ± 120.79</td>
<td>196.5 ± 17.68</td>
<td>689 ± 471</td>
<td>428-1609</td>
</tr>
<tr>
<td>TCO2 (mEq/L)</td>
<td>22.67 ± 3.79</td>
<td>21.5 ± 0.71</td>
<td>18.67 ± 2.31</td>
<td>-</td>
</tr>
<tr>
<td>Na (mEq/L)</td>
<td>157 ± 2</td>
<td>155 ± 1.41</td>
<td>155 ± 3.46</td>
<td>145-181</td>
</tr>
<tr>
<td>K (mEq/L)</td>
<td>8.13 ± 1.36</td>
<td>9.3 ± 0.85</td>
<td>9.73 ± 1.96</td>
<td>7.3-11.1</td>
</tr>
<tr>
<td>Cl (mEq/L)</td>
<td>110.67 ± 1.53</td>
<td>112 ± 1.41</td>
<td>114.33 ± 1.53</td>
<td>111-134</td>
</tr>
<tr>
<td>Na/K ratio</td>
<td>19.66 ± 3.20</td>
<td>16.74 ± 1.68</td>
<td>16.37 ± 3.39</td>
<td>-</td>
</tr>
<tr>
<td>Anion Gap</td>
<td>31.8 ± 1.31</td>
<td>30.8 ± 0.14</td>
<td>31.73 ± 2.1</td>
<td>-</td>
</tr>
</tbody>
</table>
3.5.7 Considerations about the xenograft model used for this study.

The cells used are Caki-1 ccRC which are adherent epithelial cells. The Caki-1 cells express wildtype von Hippel-Lindau (VHL) tumor-suppressor protein and are known to form tumors in immunocompromised mice. These cells were extracted in 1971 from a metastatic site (skin) in a 49-year-old Caucasian male with ccRCC. While exploring changes in immune populations in the tumor microenvironment might have been very informative, because the NOD genetic background contains alleles that reduce the function of the innate branch of the immune system, macrophages and dendritic cells are defective. Thus, readouts of immune changes in the immunocompromised NOD SCID model would not necessarily be highly informative as when studying an immune competent organism.

3.5.8 CONCLUSIONS

What we have shown is that the two heterobimetallic titanocene-gold complexes lead to drastic reduction in tumor burden upon seven doses administered over the course of 21 days in mice bearing clear cell carcinoma xenograft tumors. The reduction in tumor burden coincided with a significant increase in apoptotic cells and drastic decrease in the number of proliferating cells in the Titanocref and Titanofin treated tumors. These cellular phenotypes were accompanied by a remarkable reduction in tumor vascularization, and an improvement in vascular integrity, which are features that may hinder tumor metastasis.

We have identified changes in expression levels of eleven factors known to modulate tumor growth, resistance to apoptosis, and angiogenesis, changes that may drive the in vitro and in vivo efficacy observed. To date, there is a relatively high prevalence of primary and metastasized
ccRCC that do not respond to immunotherapy and targeted therapies such as Ipilimumab, Vinblastine, Proleukin or Bevacizumab or current chemotherapeutic regimens of Gemcitabine, Capecitabine 5-FU. Those treatment options are most often associated with debilitating side effects. Therefore, there is an urgent need for novel effective, non-toxic clinical alternatives to the currently available pharmaceutical arsenal to improve survival and quality of life. This study shows that two novel metallodrugs are not only efficacious at reducing a tumor type that is difficult to treat, but they do so without any prediction of clinical side effects as indicated by the pathology study. Moreover, at dose below that of cytotoxicity, the novel compounds significantly inhibit TrxR, IL-6 and VEGF which are all key drivers of drug resistance, tumor proliferation, angiogenesis and metastasis. Therefore, we believe that we have done due diligence in making a case for exploration of those compound towards clinical trials in human. We also conclude that the compounds’ synergy may be due to the simultaneous targeting of multiple non-overlapping pathways involved in tumor growth and progression, given our protein profiling results. We have found a distinct changes in expression levels specific subset of factors whose changes that can be reconciled with changes in cellular behavior observed such as reduction in proliferation, migration, invasion, angiogenesis and increased in apoptotic cell death.
3.6 Bibliography


CHAPTER 4

Preclinical Study of a Second Generation Ruthenium-Gold Compound:
RANCE-1 in Renal Cancer

Part 1
Mechanistic studies in vitro in Caki-1 clear renal cell carcinoma.

4.1.1. INTRODUCTION

From previous work briefly discussed in chapter 2, we identified several heterobimetallic ruthenium-gold-NHC complexes that were cytotoxic against and selective for clear Caki-1 cells in vitro\(^1,2\). In light of their cytotoxic potential, and because ruthenium-based complexes have been reported to be potent antimetastatic agents, have demonstrated antiangiogenic efficacies, and are suggested to act through molecular mechanisms distinct from gold alone,\(^1,3\) we sought to further explore our novel Ru-Au-NHC’s effect on malignant phenotypes such as proliferation, migration, and angiogenesis. To that end, we chose the most selective and stable of the Ru-Au-NHC, RANCE-1 (Chart 4.1) and evaluated its anticancer properties in detail. We report here on the mechanism of RANCE-1 efficacy in ccRCC Caki-1 cells. These studies indicate that the bimetallic compound RANCE-1 is significantly more cytotoxic than the Ru or Au monometallic derivative moieties. RANCE-1 significantly inhibited migration, invasion, and angiogenesis in vitro, which are essential for metastasis. RANCE-1 was found to disturb pericellular proteolysis by inhibiting cathepsins, and the metalloproteases MMP and ADAM, which play key roles in the etiopathogenesis of cancer. RANCE-1 also inhibits the mitochondrial protein TrxR that is often overexpressed in cancer cells and facilitates apoptosis evasion. We found that while Auranofin perturbed migration and invasion
to similar degrees as RANCE-1 in Caki-1 renal cancer cells, RANCE-1 also inhibited antiangiogenic formation and VEGF expression. We further determined that Auranofin and RANCE-1 have distinct proteolytic profiles. In summary, RANCE-1 constitutes a very promising candidate for further preclinical evaluations in renal cancer.

Chart 4.1. Compounds used in this study: bimetallic [Ru(p-cymene)Cl₂(η¹-dppm)]Au(IMes)]ClO₄ (RANCE-1)¹, monometallic [Ru(p-cymene)Cl₂(η¹-dppm)] R⁴, and [AuCl(IMes)] ANCE-1⁵, and Auranofin.

4.1.2. RESULTS AND DISCUSSION

4.1.2.1 Cytotoxicity, Selectivity, Cell Death and Cell Cycle Arrest

The cytotoxicity of the bimetallic Ru-Au compound RANCE-1 (Chart 4.1), and monometallic ruthenium R, and gold ANCE-1 compounds was evaluated. The cytotoxic profiles of Auranofin and cisplatin (for comparative purposes) were also determined. In this assay, human renal Caki-1 and non-tumorigenic human fetal lung fibroblast (IRM-90) cells were incubated with the individual compounds for 72 hours and compared to incubation with cisplatin or Auranofin. The results are summarized in Table 4.1.
Table 4.1. Cell viability IC$_{50}$ values ($\mu$M) in Caki-1 cells and IMR-90 fibroblasts for bimetallic RANCE-1 and monometallic Ru R Au ANCE-1 compounds. Auranofin and cisplatin were used as controls. The values are also plotted in a bar graph for a better visualization of the selectivity profiles.

<table>
<thead>
<tr>
<th></th>
<th>Caki-1</th>
<th>IMR90</th>
</tr>
</thead>
<tbody>
<tr>
<td>RANCE-1</td>
<td>8.7 ± 0.9</td>
<td>17.3 ± 0.8</td>
</tr>
<tr>
<td>R</td>
<td>27.8 ± 2.9</td>
<td>35.9 ± 2.3</td>
</tr>
<tr>
<td>ANCE-1</td>
<td>19.8 ± 1.6</td>
<td>37.6 ± 2.1</td>
</tr>
<tr>
<td>R + ANCE-1</td>
<td>16.6 ± 1.3</td>
<td>28.1 ± 3.4</td>
</tr>
<tr>
<td>Auranofin</td>
<td>2.8 ± 0.6</td>
<td>3.7 ± 0.4</td>
</tr>
<tr>
<td>Cisplatin</td>
<td>23.9 ± 2.4</td>
<td>3.9 ± 0.5</td>
</tr>
</tbody>
</table>

$^a$All compounds were dissolved in 1% of DMSO and diluted with media before addition to cell culture medium for a 72 hour incubation period. Cisplatin was dissolved in H$_2$O.

RANCE-1 was more cytotoxic to the Caki-1 cells than cisplatin, monometallic ruthenium R, or monometallic gold ANCE-1. Also, RANCE-1 is a selective compound as it was considerably less toxic to the non-tumorigenic fibroblast IMR-90 cells than cisplatin, R or ANCE-1. While Auranofin was also cytotoxic with a low IC$_{50}$ value in Caki-1 cells, it was only moderately selective. We then studied the effect of the combination of monometallic ruthenium R and gold ANCE-1 compounds (1:1 equivalents) on Caki-1 cells using the same conditions as for the bimetallic compounds. The resulting IC$_{50}$ values of the combination treatments were larger than those of RANCE-1, while also remaining selective. This fact supports the idea that there is indeed a synergistic effect for RANCE-1 on the renal cancer cells as described for other Ru-Au and Ti-Au compounds described by our group $^2,^6-^8$. In view of the results obtained, we decided not to explore the effects of cisplatin in Caki-1 cells any further.
Following the evaluation of the cytotoxicity of bimetallic RANCE-1 and monometallic ruthenium R, gold ANCE-1, and Auranofin, we proceeded to evaluate the mechanism of cell killing for RANCE-1 and Auranofin. For this assay Caki-1 were incubated with the indicated compound at the IC$_{50}$ concentration for 72 hours. We observed that RANCE-1 and Auranofin induced apoptosis in 82% and 86% of cells killed respectively (Figure 4.1). Auranofin and other gold(I) compounds are known to be apoptotic in several cancer cell lines$^{9,10}$, and p-cymene Ru derivatives (as the classic RAED described by Sadler and RAPTA-C described by Dyson) are also mainly apoptotic (ca 80%) on the ovarian cancer cell line A2780$^{11}$. Thus the apoptotic behavior of bimetallic Ru-Au RANCE-1 in terms of cell death may be due to the presence of both the Ru and Au fragments.

**Figure 4.1.** Cell death assays on Caki-1 cells induced by IC$_{50}$ concentrations of RANCE-1, and Auranofin measured by using two-color flow cytometric analysis, after 24 h of incubation. (A) Flow cytometry histogram of RANCE-1 induced apoptosis in 32% while Auranofin induced apoptosis in 76% of cell population (B) Bar-graph representation of from flow cytometry histogram quantifying cell death.

Next we evaluated the effects of the most cytotoxic compounds, which are RANCE-1 and Auranofin, on cell cycle arrest. We observed that cells treated with RANCE-1 had the greatest
percentage of cells in Sub G1 (26%) and more G1/G0 (37%) the fewest cells in S phase (16%), and similarly few cells in G2/M (20%). Similarly for other p-cymene Ru derivatives such as RAED and RAPTA-C it was reported that greatest percentage of cells were in G1/G0 (60%) and fewer cells in S phase (approximately 15%) and G2/M (5%) for ovarian cancer A278 cell lines.  

Figure 4.2. Cell Cycle Arrest induced by RANCE-1, and Auranofin. Cells were treated with or without IC<sub>20</sub> concentrations of RANCE-1, and Auranofin for 24 h. (A) Flow cytometry histogram of RANCE-1 increased G1/G0 (37%) and Sub G1 (26%) population accumulation while Auranofin increased G1/G0 (82%) population accumulation. (B) bar-graph representation of from flow cytometry histogram.
Auranofin treated cells were almost exclusively in G0/G1 (82%), suggesting that the compound induced complete G1/G0 arrest, as has already been reported for AF in other cell lines \cite{12-15}, and also in cancer cells treated with anti-inflammatory drugs \cite{16}.

### 4.1.2.2 Inhibition of migration and invasion

Increased cell migration and invasion are hallmarks of metastasis in advanced tumors \cite{17,18}. We therefore evaluated the anti-invasive properties of RANCE-1, the monometallic Ru R, and Au ANCE-1 compounds. The effect of the compounds on migration was determined using a wound-healing 2D scratch assay (Figure 4.3A). RANCE-1 and Auranofin significantly reduced migration by 82% and 88% respectively. While the cytotoxicity and apoptotic properties of Auranofin on different cancer cell lines are well known \cite{10}, the efficacy of Auranofin was unexpected and has not been previously reported. The monometallic Au compound ANCE-1 reduced migration by 26% while the monometallic Ru derivative R reduced migration by 68%. The antimetastatic attributes of R as it prevents \textit{in vitro} cell invasion had been previously described for the triple-negative MDA-MB231 cancer cells \cite{4}, and this potential metastatic phenotype was one of the reasons we choose the Ru fragment of the RANCE-1 heterometallic compound.

RANCE-1 was observed to inhibit invasion in a 3D Transwell assay fitted with Geltrex® matrix an extracellular matrix analogue (Figure 4.3B). RANCE-1 reduced invasion by 66%, and the monometallic Auranofin was also an excellent inhibitor of invasion (54% of invasion is inhibited).
A.

Figure 4.3. Cell migration and Invasion Inhibition Assays for bimetallic Ru-Au compound RANCE-1, the monometallic Ru R and Au ANCE-1 compounds and Auranofin. A) Inhibition of migration (2D wound-healing scratch assay). Scratch assay showing that RANCE-1, R, and Auranofin interfere with Caki-1 migration. Panels show representative images of untreated cells at time points T₀ (top row) when the compound is added to the assay up to 24 hours (bottom row).

B) Inhibition of invasion (3D Transwell Assay). Cells were seeded onto a transwell migration chambers containing filters coated on with Geltrex® matrix, then incubated for 24h with IC₂₀ concentrations of RANCE-1, R, ANCE-1, or Auranofin. The transwell assay shows that RANCE-
1, R and Auranofin interfere with Caki-1 invasion. Panels show representative images of treated cells at time points T_{24}. Error bars indicate standard deviations.

The inhibition of invasion by the Ru monometallic compound R at 30% was quite robust (as previously described for another cancer cell line) \(^4\), higher even than that of RANCE-1 or Auranofin. The monometallic Au compound ANCE-1 on the other hand only inhibited 18% of migration in agreement with the data obtained in the 2D scratch assay.

4.1.2.3 Inhibition of angiogenesis

Neovascularization plays an essential role in the pathology of tumor growth.

**Figure 4.4.** Induction of endothelial cell reorganization into 3D vessel structures. Human umbilical vein endothelial cells (HUVEC) were seeded in plate coated with Geltrex® matrix using completer HUVEC media and incubated at 37°C and 5% CO2. At post-seeding IC10 concentrations of RANCE-1, A, ANCE-1, Auranofin or 0.1% DMSO was added. (A) representative phase-contrast images captured 24h after the compounds were added. (B) Quantitation of tube formation.
We chose the formation of tube-like structures by HUVEC cells on an extracellular matrix as a mean to assess the effects on angiogenesis of RANCE-1, R, ANCE-1, and Auranofin.

The assay measures the endothelial tube formation of Human umbilical vein endothelial cells (HUVECs) (Figure 4.4). The number of tubes and nodes was counted as described by DeCicco-Skinner et al. The greater the inhibition of tube formation results in a decrease of the length of tubes (TL) and a decreased number of nodes (TN), the higher the antiangiogenic properties of a compound.

The ruthenium containing derivatives RANCE-1 and R were observed to inhibit all tube formation in a 3D assay on Geltrex® matrix an extracellular matrix analogue (Figure 4.4). The gold monometallic compounds ANCE-1 and Auranofin caused mild disturbances in tube arrangement but there was still viable tube formation. This indicates that the ruthenium component of the compound may be responsible for the antiangiogenic properties. However RANCE-1 displayed an impressive antiangiogenic effect (higher than that of the monometallic compound R).

4.1.2.4 Inhibition of targets associated to cancer progression, cisplatin resistance, metastasis, and angiogenesis

4.1.2.4.1 Inhibition of Thioredoxin Reductase

Changes in cellular anti-oxidant capacities are a characteristic of many chemo-resistant cancers. Overexpression of thioredoxin reductase (TrxR) is a critical component of cisplatin-resistant cancer cell survival, thus making this enzyme an important anti-cancer target. We have reported on the relevant inhibition of TrxR in Caki-1 cells by Auranofin and heterometallic
titanocene-Au $^{2,6}$ and Ru-Au complexes $^{1}$. Therefore, we measured the activity of thioredoxin reductase in Caki-1 cells following incubation with compounds RANCE-1 Ru R, and Au ANCE-1. Auranofin was used as a positive control [we have already reported, after 72 h of incubation Caki-1$^{6}$ TrxR activity is significantly reduced by Auranofin (86%)] (Figure 4.5). We observed that inhibition of TrxR activity by all the compounds did not significantly improve between 24h and 72h. The inhibition of TrxR by RANCE-1 and monometallic Au(I) compound ANCE-1 was almost identical (RANCE-1, 47%; ANCE-1 (40%). While R inhibited TrxR by less than 10% in that same time frame. This indicates that the Au(I) fragment present in heterometallic RANCE-1 compound is most likely responsible for the TrxR inhibition effect.

![Figure 4.5](image_url)

**Figure 4.5.** Inhibition of mitochondrial protein TrxR in Caki-1 renal cancer cells by bimetallic Ru-Au compound RANCE-1, the monometallic ruthenium R and gold ANCE-1 compounds and Auranofin (1 $\mu$M) for 72 hours. The values indicate the percentage of reduction of TrxR activity relative to 1% DMSO treated control.
4.1.2.4.2 *Inhibition of VEGF*

Also, ruthenium compounds have been reported to inhibit VEGF \(^{24}\). Given this, and the key tumor promoting properties of VEGF, we set out to evaluate the inhibitory effects of the RANCE-1, the monometallic ruthenium R and gold ANCE-1 compounds, and Auranofin on VEGF. We found that VEGF secretion was significantly inhibited by both RANCE-1 and Auranofin after 72h of incubation (70% and 55% reduction respectively), while R and ANCE-1 lead to no notable inhibition of VEGF secretion (Figure 4.6). Angiogenesis is a complex process driven by diverse activities and an intricate sequence of factors. While VEGF is known to be a key regulator of angiogenesis and its downregulation is often correlated with reduced angiogenesis, such correlation is not absolute.

![Figure 4.6. Inhibition VEGF. Caki-1 cells were incubated with bimetallic Ru-Au RANCE-1, monometallic ruthenium R or gold ANCE-1 compounds, and Auranofin (IC\(_{20}\) concentrations) for 24 hours. The values for indicate the absolute concentration (pg/ml) of VEGF following treatment, 0.1% DMSO control.](image-url)
As our data indicate, while there is evident inhibition of tube formation by RANCE-1 and significantly less inhibition of tube formation by Auranofin, they are both potent inhibitors of VEGF, this suggests that there might be more at play in RANCE-1 inhibition of VEGF and its effect on tube formation.

4.1.2.4.3 Inhibition of Interleukins

ILs also play an important role in tumor promotion and metastasis through various proteolytic interaction and through control of matrix metalloproteinases (MMP) expression and the expression of angiogenic proteins growth factors such as VEGF 25. We studied the inhibition of ILs by our most active compounds, bimetallic RANCE-1 and Auranofin after 72 hour of incubation at IC20 concentrations (Figure 4.7). We found that RANCE-1 inhibited IL-6 expression by 60%, inhibited IL-5 expression by 37%, had no effect on IL-13 expression, and resulted in a complete inhibition of IL-17A expression. The inhibition of IL-17 is of great clinical interest because increased IL-17A expression is associated with ER (−) and triple negative tumor hyper proliferation and poor prognosis in breast cancer. IL-17 is also known to drives several pathogenic processes during breast cancer progression to metastasis, IL-17 not only promotes tumor cell survival and invasiveness, it also contributes to the promotion of tumor angiogenesis. Auranofin, as previously reported, inhibited IL-6, but also completely inhibited expression of IL-5, IL-8 and IL-13, all key players in inflammatory signaling pathways 26. Given the potent inhibition of IL expression observed upon treatment with RANCE-1, this compound might be a good candidate for combination therapy with a more cytotoxic agent. The great value of IL inhibitors is further increased because ILs are known inducer of MMPs which are critical in metastasis 25,27.
Figure 4.7. RANCE-1 and Auranofin induced changes in the expression levels of Interleukins in Caki-1 cells. Analysis of 150ng of protein extracted from cell lysate collected from RANCE-1 or Auranofin (IC$_{20}$) treated cells. The data shown are representative of triplicates.

4.1.2.4.4. Inhibition of metalloproteases and other pro-cancer proteins

Many members of the MMP family are involved in tumor induced inflammation signaling and angiogenesis in cooperation with members of the IL family including IL-6$^{28-30}$. The secretion of 9 MMPs was significantly inhibited by RANCE-1: MMP-1 (72%) MMP-3 (74%) MMP-7 (100%), MMP-8 (97%) MMP-9 (50%), MMP-10 (100%), MMP-12 (89%), and MMP-13 (100%) (Figure 4.8). Such inhibition is particularly robust and in combination with the VEGF and IL inhibition by RANCE-1, indicating that this compound presents a molecular target profile that would potentially make it a very efficient antimetastatic and antiangiogenic agent.

Accumulating evidence demonstrates the crucial role of proteolytic enzymes such ADAMs, which are closely related to MMPs facilitate tumor cell invasion and metastasis. In particular, ADAM-9 is reported to be highly expressed in invasive renal cell cancer while ADAM8 is considered a
robust hemo—histo—chemical marker for lung cancer and correlated with poor prognosis for patients with pancreatic ductal adenocarcinoma $^{31,32}$.

**Figure 4.8.** RANCE-1 and Auranofin induced changes in the expression levels of matrix metalloproteases in Caki-1 cells. Analysis of 150ng of protein extracted from cell lysate collected from RANCE-1 or Auranofin (IC$_{20}$) treated cells. The data shown are representative of triplicates.

From our study, we observed that treatment with RANCE-1 ablated expression of ADAM-8, and inhibited ADAM-9 expression by 37%, while Auranofin had no effect on the expression levels of those two proteases (Figure 4.9).
Figure 4.9. RANCE-1 and Auranofin induced changes in the expression levels of a disintegrin and metalloprotease (ADAM) family members in Caki-1 cells. Analysis of 150ng of protein extracted from cell lysate collected from RANCE-1 or Auranofin (IC\textsubscript{20}) treated cells. The data shown are representative of triplicates.

Cysteine cathepsin proteases (Cts) are known regulators of cancer progression and therapeutic responses. We have previously reported on the inhibition of cathepsin B (purified) by a neutral ruthenium-gold compound\textsuperscript{27}. Here, we observed that RANCE-1 ablated CtsB and CtsD expression (100% suppression), while inhibiting CtsL, CtsS and CtsX/Z by 38%, 35% an 53% respectively. Auranofin had no inhibitory effect on the expression of the five Cts evaluated (Figure 4.10).

From these studies, RANCE-1 has emerged as a potent pan-MMP and pan-cathepsin inhibitor with additional notable inhibition of members of the ADAM protease family. These observations may explain the mechanism by which RANCE-1 inhibits tumor growth, invasion, and angiogenesis (Figure 4.10).
Figure 4.10. RANCE-1 and Auranofin induced changes in the expression levels of members of the cathepsin proteases family in Caki-1 cells. Analysis of 150 ng of protein extracted from cell lysate collected from RANCE-1 or Auranofin (IC\textsubscript{20}) treated cells. The data shown are representative of triplicates.

4.1.3. CONCLUSIONS

We have demonstrated the synergism of a heterometallic Ru-Au (RANCE-1) compound designed to harness the cytotoxic and apoptotic effects of Au(I) lipophilic cations as well as their TrxR inhibition properties with the potential antimetastatic effects of a Ru p-cymene derivative containing a phosphine. In addition to cytotoxicity and pro-apoptotic activity, RANCE-1 inhibited migration, invasion, and angiogenesis. The molecular targets inhibited include different interleukins, metalloproteases, and cathepsins, which are all involved in tumor metastasis and angiogenesis, and to an even higher degree, the angiogenic factor VEGF. It is noteworthy that the inhibition observed was in general higher than that of the individual monometallic fragments present in the heterometallic compound. In some cases, this inhibition can be correlated with a particular metallic
fragment of the bimetallic compound (reinforcing the idea of the positive synergistic effect caused by the two distinct metals).

During this study, we also evaluated Auranofin and observed it had very similar effects to RANCE-1 on the renal cancer cell line Caki-1 in terms of its antiproliferative and antimetastatic properties as well as in some of the sets of targets inhibited. RANCE-1 however was a stronger VEGF inhibitor than Auranofin, and a much better pan-MMP and pan-cathepsin inhibitor. Moreover, RANCE-1 blocked all angiogenic formation while Auranofin merely induced branching disturbances of de novo angiogenesis in our in vitro model.

These results are highly relevant in the search for multi targeted therapies that would best avert target specific induced resistance, and thus warrant further evaluation of the efficacy and mechanism of RANCE-1 in renal cancer in vivo. In addition, the relevant results found for Auranofin (an old anti-rheumatic drug being currently ‘repurposed’ as a chemotherapeutic for additional diseases, including cancer) also warrant further exploration of this agent in renal cancer treatment.
Part 2

Preclinical evaluation of a heterobimetallic gold-ruthenium complex unveils great tumor suppressive and angiosuppressive efficacy in a mouse clear cell renal carcinoma xenograft

4.2.1 INTRODUCTION

The results obtained in vitro were very encouraging as we determined that RANCE-1 has anti-proliferative, anti-metastatic, and anti-angiogenic properties. We also found that RANCE-1 was a strong inhibitor of proteolytic factors critically associated with tumor metastasis such as cathepsins, ADAMs, and MMPs, all markers of clinical interest. We thus sought out to further explore the efficacy of RANCE-1 in vivo and obtain clinically relevant insights into the biological response. We observed organ distribution of RANCE-1 in NOD.CB17-Prkdc SCID/J mice bearing a subcutaneous clear cell renal carcinoma (Caki-1) xenograft, an established preclinical model of ccRC. We report here on the measurements of the acute and chronic toxicities, pharmacokinetics, efficacy, and pathology of treatment with RANCE-1 generated interesting results. These experiments also demonstrated that RANCE-1 blocked all tumor growth.

4.2.2 RESULTS AND DISCUSSION

4.2.2.1. Dose range finding study.

First, we report the acute toxicity and maximum tolerated dose (MTD) of RANCE-1 in naïve NOD.CB17-Prkdc SCID/J mice. This study was carried out to assess the toxicological profile of RANCE-1 following 6 intraperitoneal (i.p.) doses between 30mg/kg/48h and 50mg/kg/48h followed by a two-week recovery period. Vehicle solution (0.5% DMSO + 99.5%
normal saline) treated mice were used as controls. Lung, liver, kidney, spleen, and heart were collected, weighed and visually evaluated during a gross necropsy. Parameters such as mortality, bodyweight, posture, grooming, and clinical and gross pathologies were monitored throughout the study. The acute toxicity study indicated that the lethal dose (LD$_{50}$) of RANCE-1 was 45 mg/kg/48h (Figure 4.11). The MTD study indicated that male and female mice tolerated three i.p. injections at doses of 30mg/kg/48h to 40mg/kg/48h of RANCE-1 without notable signs of toxicity or changes pathological parameters in the treated animals (Figure 4.12). These findings informed the rationale for selecting the doses of 10mg/kg/48h for RANCE-1 for the subsequent pharmacokinetic (Pk) and histopathological analyses. It was concluded that administration of seven consecutive doses of 10mg/kg/48h via i.p. injection in female and male NOD.CB17-Prkdc SCID/J mice, as we had initially envisioned for the efficacy trial, would be well tolerated under these study conditions since no concerning changes were observed in mean body weight compared to control and no signs of distress were observed at three times these doses. There were, upon gross-necropsy, no obvious signs of local toxicity were observed in the peritoneal cavity. This dose range and toxicity study demonstrates that RANCE-1 is a relatively safe compound within a two week treatment at doses at or below 35 mg/kg i.p.
Figure 4.11. Median lethal dose (LD$_{50}$) was estimated by injecting i.p. five increasing doses of RANCE-1 into two mice (weighing between 18 and 22 g). Deaths occurring during the first two doses were recorded and LD$_{50}$ was calculated.

Figure 4.12. RANCE-1 is best tolerated at doses below 30 mg/kg/48h in non-tumor bearing female and male NOD.CB17-Prkdc SCID/J mice. A. Survival curves associated with MTD determination of RANCE-1s. B. Body weights at the end of the course of treatment with various doses during the MTD determination - . Error bars represent SEM for each group.
4.2.2.2. Pharmacokinetics and Biodistribution

(Pk analyses were performed by the Laboratory of Swayam Prabha, at the University of Minnesota Department of Experimental and Clinical Pharmacology)

We then proceeded to evaluate the biodistribution and bioavailability of the compound as to obtain the optimal time between doses in preparation for an efficacy trial. To that end we performed a Pk study in a preclinical mouse xenograft model of kidney cancer. We determined the pharmacokinetics of RANCE-1 upon intraperitoneal (i.p.) administration of a single dose followed by time lapse blood collection and terminal tissue collection in xenograft Caki-1 tumor bearing NOD.CB17-Prkdc SCID/J mice to provide insights into critical preclinical criterion such as absorbance time clearance rate. The compound was absorbed slowly, as indicated by a peak plasma concentration of Ru reached at 12 h after dosing. RANCE-1 eliminated slowly from the blood with an elimination half-life (t1/2) of 40.8h (Table 4.2 and Figure 4.13). The plasma concentration of Au was not measured due to experimental constraints. This informed the choice of a 72h window between doses, as this allowed sufficient time for clearance of the compound.

We then determined the tissue distribution of RANCE-1 72h after a single i.p. injection. In this study, the tissue distribution of Ru and Au was in the order kidney>tumor>liver (Figure 4.14 and Table 4.4). Notwithstanding that more compound accumulated in the kidney than in the tumor, there was a significant amount of both Ru and Au were found in the tumor tissue (Figure 4.14, Table 4.4). It is important to note that we observed close to a 1:1 colocalization of both metals (Ru and Au) in the tissue (Table 4.6). These data indicate that both metals in the compound are delivered to the tissue at a near 1:1 ratio, suggesting stability of the bimetallic compounds in the course of biodistribution.
Table 4.2 Pharmacokinetic parameters\(^b\) of RANCE-1 after single intraperitoneal injection in tumor-bearing mice.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>RANCE-1</th>
</tr>
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<tbody>
<tr>
<td>(T_{\text{max}}) (h)</td>
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<tr>
<td>(C_{\text{max}}) (µg/mL)</td>
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<td>(\text{AUC}_{\text{last}}) (µg.h/mL)</td>
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<td>(\text{AUC}_{\text{Inf}}) (µg.h/mL)</td>
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<td>(k_{\text{e}}) (h(^{-1}))</td>
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<tr>
<td>(t_{1/2}) (h)</td>
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<td>(V_d/F) (mL/Kg)</td>
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<tr>
<td>(C_{\text{min}}) ss (µg/mL)</td>
<td>7.17±3.91</td>
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<tr>
<td>Time to reach steady state (h)</td>
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</tbody>
</table>

\(^b\) Pharmacokinetic parameters determined include the maximum observed plasma concentration (\(C_{\text{max}}\)), the time to reach \(C_{\text{max}}\) (\(T_{\text{max}}\)), area under the plasma concentration–time curve from time zero to 72 h post dose (\(\text{AUC}_{0-72}\)), elimination rate constant (\(k_{\text{e}}\)), terminal elimination half-life (\(t_{1/2}\)), apparent total clearance from plasma (\(C_l/F\)), and apparent volume of distribution (\(V_d/F\)).

Figure 4.13. Plasma concentration of the Ru metal of RANCE-1 at various intervals after single intraperitoneal injection. Data represent mean ± SD (n = 4).
Table 4.3. Plasma concentration of the Ru metal of the RANCE-1 bimetallic at various intervals after single intraperitoneal injection. Data represent mean ± SD (n = 4).

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>Conc. (µg/mL)</th>
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<tbody>
<tr>
<td>1</td>
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</tr>
<tr>
<td>2</td>
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</tr>
<tr>
<td>6</td>
<td>16.53 ± 3.11</td>
</tr>
<tr>
<td>12</td>
<td>21.39 ± 4.62</td>
</tr>
<tr>
<td>24</td>
<td>13.88 ± 7.93</td>
</tr>
<tr>
<td>48</td>
<td>8.35 ± 4.72</td>
</tr>
<tr>
<td>72</td>
<td>5.16 ± 2.98</td>
</tr>
</tbody>
</table>

Figure 4.14. Tissue distribution of both Ruthenium and gold metals from RANCE-1 at the end of the Pk study (72 h post-injection). Below the x-axis, Ru:Au represents the ratio of the two metals in each tissue. Data represent mean ± SD (n = 4).
Table 4.4. Tissue distribution of the Ru and Au metals of the bimetallic RANCE-1 72h after single intraperitoneal injection. Data represent mean ± SD (n = 4).

<table>
<thead>
<tr>
<th>Tissue</th>
<th>RANCE-1 Ru (µg/gm)</th>
<th>RANCE-1 Au (µg/gm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver</td>
<td>4.86 ± 3.94</td>
<td>3.14 ± 3.96</td>
</tr>
<tr>
<td>Kidney</td>
<td>14.59 ± 10.88</td>
<td>15.24 ± 10.69</td>
</tr>
<tr>
<td>Tumor</td>
<td>7.70 ± 5.81</td>
<td>9.98 ± 7.27</td>
</tr>
</tbody>
</table>

Table 4.5. Ratio of Ru metal present between tissue and plasma of mice treated with RANCE-1 72h after single intraperitoneal injection. Data represent mean ± SD (n = 4).

<table>
<thead>
<tr>
<th>Tissue : Plasma</th>
<th>RANCE-1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kidney</td>
<td>4.00 : 1</td>
</tr>
<tr>
<td>Liver</td>
<td>0.95 : 1</td>
</tr>
<tr>
<td>Tumor</td>
<td>2.17 : 1</td>
</tr>
</tbody>
</table>

Table 4.6. Ratio of Ru and Au metals present in tissue of mice treated with RANCE-1 72h after single intraperitoneal injection. Data represent mean ± SD (n = 4).

<table>
<thead>
<tr>
<th>Ru : Au</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver</td>
</tr>
<tr>
<td>Kidney</td>
</tr>
<tr>
<td>Tumor</td>
</tr>
</tbody>
</table>

4.2.2.3. Efficacy study

Third, we report on an intervention trial to determine the in vivo anticancer efficacy of RANCE-1 in xenograft Caki-1 tumor bearing NOD.CB17-Prkdc SCID/J mice in response to
intraperitoneal (i.p.) administration of seven doses spaced by 72h followed by a 72h recovery period before sacrifice. Following our most recent Pk study, it was determined that doses of one quarter of the MTD (10mg/kg/72h) administered once every 72h would be appropriate to evaluate \textit{in vivo} the anti-cancer efficacy of RANCE-1.

In mice treated with RANCE-1, we observed neither tumor growth nor shrinkage from the starting tumor burden recorded at the beginning of the trial. RANCE-1 had a chemo-static effect on tumor growth. Mice treated with RANCE-1 had no change in tumor burden after 21 days of treatment (a total of 7 doses) (Figure 4.14).

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{RANCE-1.png}
\caption{RANCE-1 treatment inhibits tumor growth in a xenograft renal cancer intervention trial (A) Representative histological samples of tumors resected from each treatment group. (B) The average tumor volume after 21 days of treatment did not significantly change in RANCE-1 treated mice, average tumor growth 3.87\% (p=0.082) compared to the vehicle (Veh) control-treated group where tumor volume increased by 138\%, ** p<0.01. Mice were treated with RANCE-1 (10 mg/kg/72h), or Veh by intraperitoneal injection from for 21 days. RANCE-1: n=6 mice, Veh: n=6 mice.}
\end{figure}
This result was rather unexpected given that the compound was cytotoxic in vitro. In contrast, we observed a growth in tumor volume of 138% in the vehicle treated mice. No significant change in weight or any decline in the well-being of the RANCE-1 treated mice were observed during this trial. These results are interesting, as they indicate the compound has growth inhibiting properties.

We then sought to determine the biodistribution of each metal after the 21-day efficacy study. Using the same methods as the Pk study (Figure 4.15, Table 4.7), we observed that the biodistribution of RANCE-1 was in the order kidney>tumor>liver following a single dose, however the order of accumulation changed to liver> kidney > tumor at the end of the 21 day intervention trial. There is evidently a shift in tissue between the 72h Pk study and the 21 day efficacy study. Importantly, there was far less metal in the tissue after the 21 day trial, in fact, we observed a 30% reduction in Ru accumulation in the liver, and 80% reduction in metal accumulation in the kidney, but also a significant decrease in the amount of Ru accumulated in the tumor. These changes in metal retention cannot be clearly attributed to a specific metabolic or clearance change over the course of treatment.

![Figure 4.16](image.png)

**Figure 4.16.** Tissue distribution of Ru and Au metal of bimetallic RANCE-1 at the end of efficacy study (i.e. 72 h after the last injection of a 21 day trial RANCE-1 (10mg/kg/72h). Below the x-axis, Ru :Au represents the ratio of the two metals in each tissue. Data represent mean ± SD (n = 4).
**Table 4.7.** Tissue distribution of the Ru and Au metal of bimetallic RANCE-1 at the end of efficacy study (i.e. 72 h after the last injection of a 21day trial with RANCE-1 (10mg/kg/72h). Data represent mean ± SD (n = 4).

<table>
<thead>
<tr>
<th></th>
<th>RANCE-1</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ru (µg/gm)</td>
<td>Au (µg/gm)</td>
</tr>
<tr>
<td>Liver</td>
<td>5.17 ± 0.71</td>
<td>4.03 ± 0.34</td>
</tr>
<tr>
<td>Kidney</td>
<td>3.86 ± 1.02</td>
<td>3.07 ± 0.85</td>
</tr>
<tr>
<td>Tumor</td>
<td>1.73 ± 0.47</td>
<td>1.47 ± 0.45</td>
</tr>
</tbody>
</table>

We observed that, compared to the tissue distribution results obtained at the Pk study, at the end of the efficacy trial, significantly less metal was retained in the tissue at the end of the Pk study. We first observed that in RANCE-1 treated mice, the ratio of Ru to Au in the tissue was higher than in the Pk study, indicating that more Ru was retained in the tissues than Au during the course of treatment. Second, we observed that the absolute concentration of Ru metal per tissue weight was 30% and 80% lower in liver and kidney tissue respectively after the 21 day trial suggesting that over the course of treatment, the liver increased its ability to excrete the compound (Table 7).

**Table 4.8.** Percentage of change in tissue distribution of the Ru metal of the bimetallic RANCE-1 between the Pk study (i.e. 72 h after a single injection of 10mg/kg and the efficacy study (i.e. 72 h after the last injection of a 21day trial [RANCE-110mg/kg/72h]. Data represent mean ± SD (n = 4).

<table>
<thead>
<tr>
<th></th>
<th>RANCE-1 (%)</th>
<th>Change relative to Pk</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver</td>
<td>33.19</td>
<td>decrease</td>
</tr>
<tr>
<td>Kidney</td>
<td>80.8</td>
<td>decrease</td>
</tr>
<tr>
<td>Tumor</td>
<td>83.6</td>
<td>decrease</td>
</tr>
</tbody>
</table>
4.2.2.4. Mechanism of action in vivo

Given the substantial dramatic inhibition of tumor growth in treated mice treated with RANCE-1, we next sought to determine the cellular mechanisms driving that static effect. We sought to interrogate the effects of RANCE-1 on the proliferating to apoptotic cell ratio in clear cell renal carcinoma xenograft tumors.

Figure 4.17. RANCE-1 treatment affects the proliferation and apoptosis ratio/equilibrium at the 21-day trial endpoint. RANCE-1 reduces tumor cell proliferation by 18 fold (from 36% for the veh. treated to 2% for the RANCE-1 treated) and does not significantly affect apoptosis (from 2% for the veh treated to 1.6% for the RANCE-1 treated- mice) in a xenograft of ccRCC in an intervention trial. The tumor proliferation and apoptosis were analyzed by staining tumor tissue with a cell proliferation specific marker Ki67 (green) and an apoptosis specific marker Cleaved caspase 3 (red) (n=4 mice per group (apoptosis RANCE-1 p= 0.71, proliferation RANCE-1 ***p <0.001 ).
We first examined cell proliferation profile of the tumors using the proliferation marker Ki67, and because tumor growth can also reflect changes in cell death, we compared the proliferation rate to apoptotic rates using the apoptotic marker cleaved caspase 3. The average of proliferating cells in individual tumors of the vehicle (Veh.) treated mice control group was 36%.

We determined in vitro, as reported in Part 1 of this chapter, that RANCE-1 disturbed vascular assembly and RANCE-1 is a potent inhibitor of IL-6 and VEGF. It has been shown that inhibition or deletion of IL-6 and VEGF in mice induced a significant decrease in tumor invasion and angiogenesis, and thus sought to determine if the use of our compounds disrupted angiogenesis and VEGF and IL-6 levels were affected. Of the mice injected with dextran and lectin the dye could not be detected thus we were unable to image vasculature in this treatment group. This might be because the tissue was significantly less dense with high presence of connective tissue and as it was not perfused with PFA, the tissue was fragile and retaining morphologic integrity was difficult. These findings are interesting because, the tumors did not seem to gain volume, as compared to vehicle treated mice, and this is reflected by the difference in proliferation to apoptosis ratio of the two treatment groups. Perhaps, as fewer cells are proliferating and more cells are dying, the cell density of the tumor mass is reduced, but that volume is unchanged because connective tissue fill in that void. One could posit that at that size there is no vasculature in the tumors. However, we know from another study of bimetallic compounds containing gold and titanium (Titanocref and Titanofin) that tumors that are even smaller than at the starting volume can be vascularized, we also know from those studies that smaller tumors do not necessarily have lower cells density (Chapter 3). Thus, the apparent lack of vasculature and tissue integrity might be a phenotype driven by RANCE-1 itself and later studies reported later on in this chapter seem to provide some insights.
into the mechanism that might drive this phenotype. RANCE-1 treatment induces a significant
decrease in growth factors involved both in the croissance of the tumor and the development of
vasculature.

4.2.2.5. Proteomic analysis

Given the drastic decrease in proliferating in tumor tissue, we next aimed to identify some of the
molecular signaling changes that may underlie this phenotype. We interrogated the effects of
treatment with RANCE-1 on ccRCC in culture as well in xenograft tumors on an array of proteins
of oncological relevance by immunoblotting (Figures 4.18-4.19).

We observed from the histological analysis (Figure 4.17) that in response to RANCE-1
treatment, there was a striking reduction in proliferation, which could be mediated by changes in
balance of proliferative factors. There was indeed a decrease in expression of many growth factors
including VEGF, PDGF, FGF, EGFR, and HGRF, all key to the proliferation of tumor cells, but
also to the stromal cells serving protumorigenic purposes. We noticed that while PDGF, FGF,
EGFR, and HGRF we all significantly decreased in the \textit{in vivo} sample, their expression was
moderately increased \textit{in vitro}. Perhaps the mono-culture nature of \textit{in vitro} study and the lack of
cellular diversity contributed to this difference. We also observed an increase in antitumorigenic
immune factors IL-2R and GM-CSF, which are clinically associated with improved prognoses \textsuperscript{33–35}. The expression of the macrophage related CapG protein was suppressed \textit{in vivo}, which usually
correlates with reduced migration \textsuperscript{36,37}. Levels of the prometastatic cytokines IL-5, IL-6 were
increased in response to RANCE-1 treatment \textit{in vivo}, though they were markedly decreased \textit{in
vitro}, which might be the result of prolonged treatment, local selection of tumor cells with robust
production of IL-5 and IL-6, or contribution by stromal cells. We observed an increase in M-CSF
expression *in vivo*, which is the primary regulator of macrophage survival, proliferation, and differentiation. M-CSF is also critical to macrophage-related functions such as vascular development and maintenance, and innate immunity. Despite this upregulation, the tumors did not grow, suggesting that the depletion of key growth factors might be a main driver of the absence of proliferation and absence of tumor growth observed. *In vivo*, RANCE-1 significantly decreased the expression of, HIF-1α, ICAM, VE-Cadherin, and vascular cell adhesion molecule-1 (VCAM-1), while *in vitro*, those proteins were either unaffected as was the case for VCAM, ICAM and VE-Cadherin, or increased as was the case with HIF-1α.

The *in vivo* efficacy was accompanied by a significant decrease in the prometastatic factors cathepsin B (CtsB), cathepsin S (CtsS), FoxC2, MMP-2, MMP-3, MMP-8, ADAM8, and ADAMP9, all of which are peritumoral proteolytic factors known to drive angiogenesis, migration, invasion, and metastasis \(^{38-42}\). We also observed a decrease in CCL7 and CCL8 levels upon RANCE-1 treatment, which could decrease the tumor’s ability to recruit monocytes, thus rendering the tumor ecosystem more hostile to tumor progression. CCL8 is expressed by dermal fibroblasts to modulate tumor-stroma and tumor-tumor cross-talk in the initiation of metastasis, while ICAM is mainly produced and modulated by immune cells or in response to immune cell signal which are absent in monolayer monoculture.

We believe the most important differences were those observed *in vivo*. There are multiple potential explanations for differences observed between *in vitro* and *in vivo* samples. Specifically, signaling in the *in vitro* monoculture is limited to tumor-tumor cell communication, while the *in vivo* tumor microenvironment is significantly more complicated and involves multiple cell types. However, in most cases the protein expression changes followed similar trends *in vitro* and *in vivo*, with the notable exception being genes involved in tumor immunity.
Figure 4.18. RANCE-1 treatment induces changes in the expression of growth factors and cancer-immune interface regulators in vivo. In vitro changes in expression levels of proteins of oncological interest, following treatment of Caki-1 cells in vitro with IC<sub>20</sub> concentrations of each compound for 72h. In vivo changes in expression levels of proteins of oncological interest, following a 21-day efficacy trial in Caki-1 xenograft tumor bearing NOD.CB17-Prkdc SCID/J mice. Analysis of 150µg of protein extracted from cell or tumor lysate. The values indicate the percentage of change in expression relative to DMSO treated cells.
Figure 4.19. RANCE-1 treatment induces changes in the expression of proteolytic factors in vivo. *In vivo* changes in expression levels of pericellular proteolytic factors or activators of proteolytic factors, following a 21-day efficacy trial in Caki-1 xenograft tumor bearing NOD.CB17-Prkdc SCID/J mice. And, *in vitro* changes in expression levels of proteolytic factors of oncological interest, following treatment of Caki-1 cells *in vitro* with IC<sub>20</sub> concentrations of each compound for 72h. Analysis of 150µg of protein extracted from cell or tumor lysate. The values indicate the percentage of change in expression relative to DMSO treated cells.

In the context of potential anticancer therapeutic agents, these results suggest that RANCE-1 would be a good antimetastatic candidate as it inhibits a series of key proteolytic factors (MMPs, ADAMs, Cts) associated with metastasis whose inhibitions have been reported to curb metastasis. Also, the compound potentiated the expression of immune factors, IL-2-R and GM-CSF, known to be associated with optimal host anti-cancer immune responses and their presence is correlated with good prognosis. RANCE-1’s inhibitory capacities towards five key growth factors also adds to its therapeutic potential. It is a single molecule multi-drug of sorts, as it promotes
antitumorigenic immune responses, blocks prometastatic proteolytic factors and inhibits key growth factors.

4.2.2.6. Pathology

Finally, we report on our study to determine any pathological effects that might be caused by treatment with RANCE-1. Access to expertise in cancer histopathology is critical in making a definitive determination of the safety, and therefore clinical viability of a compound for potential use in humans. A complete pathology study was performed in collaboration with Dr. Adam Michel from Memorial Sloan Kettering Cancer Center. This study revealed that no significant adverse effects were observed after histological evaluation related to the RANCE-1 treatment (Table 4.9, Figure 4.20). We found no notable difference in total body weight or organ weight between the treated and control mice at the end of the trial, the data in Table 4.9 also suggest there were no enlargements or atrophy as a result of treatment. While there were no significant or visible changes to the spleen, we observed a drastic increased in percentages of blood circulating neutrophils (NEUT), white blood cells (WBC) and monocytes (MONO) that were above the normal range which would indicate changes in splining activity which can be reconciled with the observed increase in GM-CSF (Table 4.10). Because this change was only observed in RANCE-1 treated animals, the effect was drug induced or the result of a response to an unrelated infection.

Clinical indicators of liver, kidney and spleen function (Table 4.10) complete blood counts (Table 4.11), and clinical chemistry (Table 4.12) of all mice treated with RANCE-1 were compared to vehicle treated mice. While levels of liver function indicators such as aspartate aminotransferase (AST) (indicative of either an inflamed or injured liver (REF)) and Alanine transaminase (ALT) were elevated in RANCE-1 treated mice, they were also elevated in the Veh treated mice,
suggesting the effect did not result from the drug treatment \( ^{44} \) (Table 4.10). The pathology report also indicated extramedullary hematopoiesis in the liver, which is the formation of blood cells outside of the bone, and results from microvacuolar changes. The pathology report gave no indication that these responses were a serious health concern.

From stained histological samples, we observed no lesions, or signs of pathologic tissue distension, discoloration, necrosis, or structural abnormalities (Figure 4.20). The histological data is supported by hematologic data on the levels of key clinical indicators of liver, kidney through clinical chemistry (Table 4.10A) and spleen function as measured by complete blood count (CBC) (Table 4.10B). All kidney health indicators were within normal values. Finally, we found that no differences in the clinical chemistry of the treated or control animals (Table 4.14), indicating normal production and excretion of physiological fluids and metabolic markers whose deregulation are indicators of pathology or drug side effects.

**Table 4. 9. Summary of histological data**

<table>
<thead>
<tr>
<th></th>
<th>RANCE-1</th>
<th>DMSO</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (g)</td>
<td>21.31 ± 5.1</td>
<td>22.82 ± 2.14</td>
</tr>
<tr>
<td>Liver weight (g)</td>
<td>1.15 ± 0.37</td>
<td>1.17 ± 0.28</td>
</tr>
<tr>
<td>Spleen weight (g)</td>
<td>0.04 ± 0.017</td>
<td>0.05 ± 0.01</td>
</tr>
<tr>
<td>Heart weight (g)</td>
<td>0.11 ± 0.05</td>
<td>0.13 ± 0.02</td>
</tr>
<tr>
<td>Average Kidney weight (g)</td>
<td>0.17 ± 0.06</td>
<td>0.16 ± 0.06</td>
</tr>
</tbody>
</table>
**Figure 4.20.** RANCE-1 treatment does not induce histological changes in liver, spleen or kidney tissue of mice at the end of the 21 day efficacy trial. Histopathology on H&E staining of paraffin sections magnification 20x. Sections are representative of 3 mice of each treatment.
Table 4.10. Summary of key clinical indicators of (A) liver, kidney through clinical chemistry and (B) spleen function through complete blood count (CBC).

A.

<table>
<thead>
<tr>
<th>Clinical chemistry</th>
<th>RANCE-1</th>
<th>DMSO</th>
<th>NSG Reference Range</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Kidney</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BUN (mg/dL)</td>
<td>18.33 ± 5.51</td>
<td>23 ± 2.65</td>
<td>5.0-28</td>
</tr>
<tr>
<td>CREA (mg/dL)</td>
<td>0.12 ± 0.04</td>
<td>0.17 ± 0.04</td>
<td>0.2-0.5</td>
</tr>
<tr>
<td>ALB (g/dL)</td>
<td>2.56 ± 0.02</td>
<td>2.83 ± 0.11</td>
<td>2.4-4.3</td>
</tr>
<tr>
<td><strong>Liver</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AST (U/L)</td>
<td>394.66 ± 456</td>
<td>372.33 ± 27.94</td>
<td>54-77</td>
</tr>
<tr>
<td>TBIL (mg/dL)</td>
<td>0.23 ± 0.06</td>
<td>0.2 ± 0.003</td>
<td>0.2-0.6</td>
</tr>
<tr>
<td>DBIL (mg/dL)</td>
<td>0.03 ± 0.06</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td>IBIL (mg/dL)</td>
<td>0.2</td>
<td>0.2 ± 0.04</td>
<td>-</td>
</tr>
<tr>
<td>ALT (U/L)</td>
<td>170.66 ± 248.03</td>
<td>163.33 ± 156.69</td>
<td>27-195</td>
</tr>
</tbody>
</table>

B.

<table>
<thead>
<tr>
<th>Complete Blood Count</th>
<th>RANCE-1</th>
<th>DMSO</th>
<th>NSG Reference Range</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Spleen</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RBC (M/uL)</td>
<td>10.31 ± 1.05</td>
<td>8.11 ± 2.51</td>
<td>7.84-10.84</td>
</tr>
<tr>
<td>WBC# (K/ul)</td>
<td>9.39 ± 7.06</td>
<td>2.39 ± 0.30</td>
<td>0.94-4.68</td>
</tr>
<tr>
<td>Neut# (K/µL)</td>
<td>8.21 ± 7.18</td>
<td>1.37 ± 0.19</td>
<td>0.54-3.16</td>
</tr>
<tr>
<td>LYMPH# (K/µL)</td>
<td>0.52 ± 0.32</td>
<td>0.57 ± 0.30</td>
<td>0.23-1.56</td>
</tr>
<tr>
<td>MONO# (K/µL)</td>
<td>0.57 ± 0.45</td>
<td>0.36 ± 0.25</td>
<td>0.03-0.26</td>
</tr>
</tbody>
</table>
Table 4.11. Complete blood count report

<table>
<thead>
<tr>
<th>Parameter</th>
<th>DMSO</th>
<th>RANCE-1</th>
<th>NSG Reference Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>RBC (M/uL)</td>
<td>8.11 ± 2.51</td>
<td>10.31 ± 1.05</td>
<td>7.84-10.84</td>
</tr>
<tr>
<td>HGB (g/dL)</td>
<td>12.6 ± 3.81</td>
<td>15.8 ± 1.55</td>
<td>11.8-17.6</td>
</tr>
<tr>
<td>HCT (%)</td>
<td>40.93 ± 14.32</td>
<td>52.8 ± 4.45</td>
<td>44.1-58.3</td>
</tr>
<tr>
<td>MCV (fL)</td>
<td>49.93 ± 2.72</td>
<td>51.3 ± 1.84</td>
<td>51.1-58.6</td>
</tr>
<tr>
<td>MCH (pg)</td>
<td>15.57 ± 0.23</td>
<td>15.37 ± 0.057</td>
<td>13.7-17.2</td>
</tr>
<tr>
<td>MCHC (g/dL)</td>
<td>31.27 ± 2.04</td>
<td>29.9 ± 0.96</td>
<td>25.1-31.3</td>
</tr>
<tr>
<td>RDW-SD (fL)</td>
<td>29.77 ± 3.23</td>
<td>31.03 ± 1.46</td>
<td>-</td>
</tr>
<tr>
<td>RDW-CV (%)</td>
<td>21.30 ± 3.49</td>
<td>23.5 ± 0.72</td>
<td>17.3-20.3</td>
</tr>
<tr>
<td>RET# (K/uL)</td>
<td>440.95 ± 4.88</td>
<td>192.67 ± 214.33</td>
<td>294-444</td>
</tr>
<tr>
<td>RET (%)</td>
<td>4.62 ± 0.001</td>
<td>2.03 ± 2.43</td>
<td>2.56-4.56</td>
</tr>
<tr>
<td>PLT (K/uL)</td>
<td>626.67 ± 60.07</td>
<td>1056.66 ± 149.94</td>
<td>651-2055</td>
</tr>
<tr>
<td>PDW (fL)</td>
<td>6.85 ± 0.49</td>
<td>7.5 ± 0.44</td>
<td>-</td>
</tr>
<tr>
<td>MPV (fL)</td>
<td>6.15 ± 0.35</td>
<td>6.53 ± 0.38</td>
<td>4.2-6.3</td>
</tr>
<tr>
<td>WBC# (K/ul)</td>
<td>2.39 ± 0.30</td>
<td>9.39 ± 7.06</td>
<td>0.94-4.68</td>
</tr>
<tr>
<td>Neut# (K/uL)</td>
<td>1.37 ± 0.19</td>
<td>8.21 ± 7.18</td>
<td>0.54-3.16</td>
</tr>
<tr>
<td>LYMPH# (K/uL)</td>
<td>0.57 ± 0.30</td>
<td>0.52 ± 0.32</td>
<td>0.23-1.56</td>
</tr>
<tr>
<td>MONO# (K/uL)</td>
<td>0.36 ± 0.25</td>
<td>0.57 ± 0.45</td>
<td>0.03-0.26</td>
</tr>
<tr>
<td>EO# (K/uL)</td>
<td>0.1 ± 0.03</td>
<td>0.08 ± 0.04</td>
<td>0.00-0.39</td>
</tr>
<tr>
<td>BASO # (K/ul)</td>
<td>0.01</td>
<td>0.006 ± 0.01</td>
<td>0.00-0.15</td>
</tr>
<tr>
<td>NEUT (%)</td>
<td>58.3 ± 14.01</td>
<td>77.37 ± 21.06</td>
<td>44.21-79.92</td>
</tr>
<tr>
<td>LYMPH (%)</td>
<td>22.57 ± 9.14</td>
<td>13.93 ± 18.8</td>
<td>13.51-42.61</td>
</tr>
</tbody>
</table>

Complete Blood Count (CBC)

NOD scid gamma mice (NSG mice)
Table 4.12. Clinical chemistry

<table>
<thead>
<tr>
<th></th>
<th>RANCE-1</th>
<th>DMSO</th>
<th>NSG Reference Range</th>
</tr>
</thead>
<tbody>
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<td>BUN (mg/dL)</td>
<td>18.33 ± 5.51</td>
<td>23 ± 2.65</td>
<td>5.0-28</td>
</tr>
<tr>
<td>CREA (mg/dL)</td>
<td>0.12 ± 0.04</td>
<td>0.17 ± 0.04</td>
<td>0.2-0.5</td>
</tr>
<tr>
<td>BUN/CREA ratio</td>
<td>183.33 ± 117.87</td>
<td>143.14 ± 32.51</td>
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</tr>
<tr>
<td>ALP (U/L)</td>
<td>39.33 ± 7.37</td>
<td>62 ± 19.97</td>
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</tr>
<tr>
<td>ALT (U/L)</td>
<td>170.66 ± 248.03</td>
<td>163.33 ± 156.69</td>
<td>27-195</td>
</tr>
<tr>
<td>AST (U/L)</td>
<td>394.66 ± 456</td>
<td>372.33 ± 279.43</td>
<td>54-77</td>
</tr>
<tr>
<td>GGT (U/L)</td>
<td>0</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td>TBIL (mg/dL)</td>
<td>0.23 ± 0.06</td>
<td>0.2 ± 0.003</td>
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</tr>
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<td>DBIL (mg/dL)</td>
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<td>0</td>
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</tr>
<tr>
<td>IBIL (mg/dL)</td>
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<td>5</td>
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<tr>
<td>ALB (g/dL)</td>
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<td>2.83 ± 0.11</td>
<td>2.4-4.3</td>
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<td>GLOB (g/dL)</td>
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<td>2.17 ± 0.06</td>
<td>1.7-2.2</td>
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<td>1.31 ± 0.08</td>
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</tr>
<tr>
<td>P (mg/dL)</td>
<td>10.37 ± 2.92</td>
<td>10.1 ± 1.68</td>
<td>7.3-14.5</td>
</tr>
<tr>
<td>Ca (mg/dL)</td>
<td>9.67 ± 0.56</td>
<td>9.43 ± 0.42</td>
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</tr>
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<td>GLU (mg/dL)</td>
<td>165 ± 69.47</td>
<td>190 ± 33.51</td>
<td>172-372</td>
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<td>CHOL (mg/dL)</td>
<td>87.67 ± 10.26</td>
<td>82.67 ± 9.81</td>
<td>55-169</td>
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<td>TRIG (mg/dL)</td>
<td>116 ± 48.51</td>
<td>93.67 ± 34.59</td>
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<td>CK (U/L)</td>
<td>368.67 ± 174.37</td>
<td>689 ± 471</td>
<td>428-1609</td>
</tr>
<tr>
<td>TCO2 (mEq/L)</td>
<td>18 ± 7</td>
<td>18.67 ± 2.31</td>
<td>-</td>
</tr>
<tr>
<td>Na (mEq/L)</td>
<td>150.67 ± 4.04</td>
<td>155 ± 3.46</td>
<td>145-181</td>
</tr>
<tr>
<td>K (mEq/L)</td>
<td>10.97 ± 1.43</td>
<td>9.73 ± 1.96</td>
<td>7.3-11.1</td>
</tr>
<tr>
<td>Cl (mEq/L)</td>
<td>106 ± 6.56</td>
<td>114.33 ± 1.53</td>
<td>111-134</td>
</tr>
<tr>
<td>Na/K ratio</td>
<td>13.89 ± 1.89</td>
<td>16.37 ± 3.39</td>
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</tr>
<tr>
<td>Anion Gap</td>
<td>37.63 ± 9.41</td>
<td>31.73 ± 2.1</td>
<td>-</td>
</tr>
</tbody>
</table>

4.2.2.7. Considerations about the xenograft model used for this study.

The cells used are Caki-1 ccRC which are adherent epithelial cells. The Caki-1 cells express wildtype von Hippel-Lindau (VHL) tumor-suppressor protein and are known to form tumors in immunocompromised mice. These cells were isolated in 1971 from a metastatic site (skin) in a 49-
year-old Caucasian male with ccRC. While exploring changes in immune populations in the tumor microenvironment might have been very informative, because the NOD genetic background contains alleles that reduce the function of the innate branch of the immune system, macrophages and dendritic cells are defective\(^{45}\). Thus, readouts of immune changes in the immunocompromised NOD SCID model would not necessarily be highly informative as when studying an immune competent organism.

### 4.2.3. CONCLUSIONS

We have shown that the heterobimetallic ruthenium-gold complex RANCE-1 drastically inhibits growth in an efficacy trial using mice bearing xenografted metastasis-derived clear cell renal carcinoma tumors without prediction of grave clinical side effects as indicated by the pathology study. In vivo, the inhibition of tumor growth coincided with a significant decrease in proliferation, and a reduction in the expression of growth factors known to drive malignant tumor progression phenotypes including angiogenesis such as VEGF, PDGF, FGF, EGFR, and HGRF. Overall, we have also identified changes in expression levels of 30 factors some known to modulate tumor growth, immune recruitment (i.e. CCL7, CCL8, ICAM) resistance to death (i.e. TrxR, Trx, HIF-1), and metastasis (i.e. MMPs, ADAMs, ILs, Cts) and others, changes that may drive the efficacy observed.

Moreover, in vitro at doses below that of cytotoxicity, the novel compound significantly inhibited proteolytic factors critical to metastasis such as members of the ADAM, MMP, and Cts families and other associated malignant factors. While we observed great inhibition of angiogenic tube formation in vitro, were unable to detect blood vessels in vivo.
There is a salient distinction between *in vivo* RANCE-1 efficacy profile *in vitro* and *in vivo*, which is their effect on cancer cell behavior. Cytotoxic drugs act by stimulating cell death by triggering apoptosis, which is the efficacy profile of RANCE-1 *in vitro*. Whereas, cytostatic drugs act by inhibiting the hyperproliferation of cancerous cells thus blocking cell growth, which seems to be the *in vivo* profile of RANCE-1. Because, notwithstanding the great complexity of cancer its core definition is often simplified as the abnormal and deregulated and uncontrollable proliferation of cells. Since RANCE-1 seems able to block this phenotype and inhibit tumor growth, it still holds clinical potential as effective, curative, alternatives are lacking. An added asset of RANCE-1 in addition to tumor growth inhibition is its favorable pathology profile which would suggest no significant clinical side effects. The efficacy of RANCE-1 seems to be linked to reduction of activity of protumorigenic growth factors, the modulation of immune cell markers and the inhibition of proteins whose expression is associated with chemoresistance all of those features might make RANCE-1 a good candidate for combination therapy. Most interestingly, since RANCE-1 inhibit factors that often drive chemoresistance, it might be a valuable addition to therapies effective in other cancers but for which ccRCC are resistant such a cisplatin or other compounds to which ccRCC are non-responsive. The inhibition of key growth factors might be a potentiating addition to other growth factor inhibitors as to create a pan-growth-factor inhibitor cocktail. In the event of inoperable cancers, controlling the growth of is critical, thus an agent such as RANCE-1 might be a good candidate. The tumor model used is one of metastasis derived cells Caki-1, thus RANCE-1’s effect are against a cell line with inherent metastatic phenotype, and because ccRCC are often diagnosed after the tumor has spread to the liver or lung, in such scenario, a drug that can stop growth would be of great interest in the absence of drug alternatives that can kill the tumors and metastasis.
It is worth reiterating that, the indications that RANCE-1 is likely to cause no or minimal side effects as predicted by the pathology report make is an appealing compound to potentiate drugs whose efficacy is associated with significant AE.

The landscape of ccRCC treatment is terse. Currently available pharmaceutical interventions are associated with limited efficacy since neither cytotoxic chemotherapies, nor targeted or immunotherapies have to date successfully cured advance renal cancer or brought DFS past 30 months and that with significant side effects. Therefore, there is an urgent need for novel effective, non-toxic clinical alternatives to the currently available pharmaceutical arsenal to improve survival and quality of life. Life can be extended by inhibiting further tumor growth. Therefore, we are making a case for exploration of this compound towards clinical trials in humans perhaps in combination therapy. As we work toward an ideal cure, there is merit to clinical interventions that can prevent malignant evolution of solid tumors.
4.3 Bibliography


8. Dean, T. C. *et al.* Human Serum Albumin-Delivered [Au(PEt 3 )] + Is a Potent Inhibitor


29. Nagase, H. Substrate Specificity of MMPs.


CHAPTER 5
MATERIALS AND METHODS
(for work described in Chapters 3 and 4)

Unless otherwise indicated, the experiments were carried out in the Contel (Brooklyn College, CUNY) or Hubbard (City College of New York, CUNY) laboratories.

5.1 In vitro assays

5.1.1 Metallic compounds

Compounds used in these studies: Cisplatin, titanocene dichloride, and auranofin (AF) were purchased from Strem Chemicals (Newburyport, MA) and used without further purification. [Ru(p-cymene)Cl₂(dppm-kP)] Ru-dppm \(^1\); [Cl₂(p-cymene)Ru(μ-dppm)Au(IMes)]ClO₄ (RANCE-1), [AuCl(IMes)] ANCE-1 \(^2\), [(η-C₅H₅)₂TiMe(μ-mba)Au(PPh₃)], cref [Au(Hmba)(PR₃)] (PR₃ = PPh₃) \(^3\), fin [Au(Hmba)(PET₃)] \(^3\) and were prepared as described previously. Titanofin [(η-C₅H₅)₂TiMe(μ-mba)Au(PR₃)] is a new compound described in a recently submitted manuscript \(^4\). (Chart 5.1)

Chapter 3
Chapter 4

Chart 5.1. Structure of compounds studied in Chapter 3 *in vitro* and structure of compounds studied in Chapter 4 *in vitro.*

5.1.2 Cells

Caki-1, a human epithelial clear cell renal cell carcinoma (ccRCC) line derived from a metastasis to the skin was newly obtained for these studies from the American Type Culture Collection (ATCC) (Manassas, VA) and cultured in Roswell Park Memorial Institute (RPMI-1640) (Mediatech Inc., Manassas, VA) media containing 10% fetal bovine serum (FBS, Life Technologies, Grand Island, NY), 1% Minimum Essential Media (MEM) nonessential amino acids (NEAA, Mediatech) and 1% penicillin-streptomycin (PenStrep, Mediatech) and incubated at 37°C and 5% CO₂ in a humidified incubator. IMR90 (human fetal lung fibroblast) cells were purchased from ATCC and maintained in Dulbecco's modified Eagle's medium (DMEM) (Mediatech) supplemented with 10% FBS, 1% NEAA and 1% penicillin-streptomycin. HUVEC (human umbilical vein endothelial) cells were obtained from ATCC and cultured in Medium 200PRF (Gibco, Gaithersburg, MD)
5.1.3 Cell Viability Analysis

Cytotoxic profiles (IC\textsubscript{50}) of bimetallic, monometallic, and control compounds (Chapters 3 and 4) were obtained by assessing the viability of Caki-1 and IMR90 control cells treated with the appropriate culture medium containing 0.01 μM, 0.1 μM, 1 μM, 10 μM and 100 μM of compound for 24h and 72h using the colorimetric cell viability assay PrestoBlue (Invitrogen, Carlsbad, CA). All compounds were dissolved in DMSO, except Cisplatin, which was dissolved in H\textsubscript{2}O with a final DMSO concentration of 1%.

5.1.4 Caspase 3 and 7 activity

Cells were treated with the bimetallic test compounds Titanocref and Titanofin as well as Auranofin at doses ranging from 0.001 μM to 10 μM and incubated for 72h (Chapter 3). To evaluate apoptosis and necrosis induced because of treatment, we used an ApoTox-Glo Triplex Assay kit (Promega, Madison, WI). Apoptosis studies were conducted by measuring caspase 3/7 activity. Caspase 3/7 activity was analyzed using a bioluminescent indicator and detected by a BioTek Microplate Reader (BioTek U.S., Winooski, VT) according to the manufacturer’s protocol. Apoptosis induced by Staurosporine treatment was used as control. Necrosis was studied by measuring dose-dependent increase in cytotoxicity in the absence of caspase-3/7 activation a cellular behavior associated with primary necrosis.

5.1.5 Cell Death Assay

The cell death profile was determined in Caki-1 cells cultured in appropriate media containing IC\textsubscript{50} concentrations of bimetallic Ti-Au Titanocref or Titanofin, or monometallic Au
control AF (Chapter 3); or IC_{50} concentrations of bimetallic Ru-Au RANCE-1 or monometallic Au control AF (Chapter 4) for 24h was analyzed by flow cytometry stained with Annexin V (FITC) dye (Invitrogen). Staurosporine and Ionomycin treated cells were also stained with Annexin V dye and served as positive controls for apoptosis and cell death. FITC fluorescence intensity was detected by flow cytometry analysis using a BD LSR II flow cytometer (Franklin Lakes, NJ). 10*10^5 events per sample were recorded. The flow cytometer was calibrated prior to use.

### 5.1.6 Cell Cycle Profile

The cell cycle profile was determined in Caki-1 cells cultured in appropriate media containing IC_{20} concentrations of bimetallic Ti-Au Titanocref, or Titanofin, or monometallic Au control AF (Chapter 3); or IC_{50} concentrations of bimetallic Ru-Au RANCE-1 or monometallic Au control AF (Chapter 4) for 24h was analyzed by flow cytometry wherein total DNA was stained with FxCycle Violet (FCV; DAPI) dye (Invitrogen). DAPI fluorescence intensity was detected by flow cytometry, the analysis was conducted using BD FACSDiva 8.0.2 10*10^5 events per sample were recorded. The flow cytometer was calibrated prior to use.

### 5.1.7 Cell Migration and Invasion Analysis

The anti-migratory profile of bimetallic Ti-Au Titanocref, or Titanofin, or monometallic Au fin, cref, or Au control AF (Chapter 3); or that of bimetallic Ru-Au RANCE-1, monometallic Au ANCE-1, monometallic Ru control R or monometallic Au control AF (Chapter 4) was assessed by an in vitro scratch assay using Caki-1 cells cultured in appropriate media and treated under serum-free conditions with IC_{20} concentration of the compounds. The diluting agent (0.1% DMSO)
served as a control. Briefly, cells were seeded in 6-well collagen-coated plates for 24h, followed by 4 hours of serum starvation, and scratching with 200 μL pipette tips. 24 hours after injury, cells were photographed using a Moticam (Kowloon, Hong Kong) camera mounted on a Zeiss inverted microscope (Oberkochen, Germany) at 20X magnification. The area invaded was measured then averaged in five randomly selected segments from three photos from each sample. Data were collected from two independent experiments.

The anti-invasion profiles of bimetallic of bimetallic, monometallic, and control compounds (Chapters 3 and 4) were assessed by transwell assay by determining the invasion of Caki-1 cancerous cells treated with the appropriate serum-free culture medium containing IC_{20} concentration of the compounds. The diluting agent (0.1% DMSO) served as a control. Briefly, Geltrex® a Reduced Growth Factor Basement Membrane Matrix (Invitrogen) was thawed and added to a 24-well transwell insert and solidified in a 37°C incubator for 30 minutes to form a thin gel layer. 5\times10^5 cells in serum-free media were then added on top of the Geltrex® coating to simulate invasion through an extracellular matrix. The insert was then mounted into a well containing complete media. 24 hours later, the membrane was fixed and stained with hematoxylin and eosin to assess the extent of cell invasion. The invaded cells were captured with a Moticam camera mounted on a Zeiss inverted microscope at 20X magnification. Cell numbers were counted from views randomly selected field of views from three photos for each sample then averaged. Data were collected from at least two independent experiments.
5.1.8 Angiogenesis Analysis

96 well plates were coated with Geltrex®, Reduced Growth Factor Basement Membrane Matrix (Invitrogen) (50μl/well) and incubated at 37°C for 30 minutes to allow gelation to solidify. HUVECs were added and the antiangiogenic profiles of bimetallic, monometallic, and control compounds (Chapters 3 and 4) were determined by assessing their effect on endothelial tube formation by Human umbilical vein endothelial cells (HUVECs). We measured the ability of HUVECs to form tubes and reach the top of the gel at a density of 6,000 cells/well in the presence or absence of the bimetallic test compounds or monometallic control (1 μM) 1% DMSO served as a control. Cells were incubated at 37°C with 5% CO₂ for 24h and pictures were captured with a Moticam camera mounted on a Zeiss inverted microscope at 10X magnification. Quantification of tube formation was assisted by SCORE, a web-based image analysis system (S.CO BioLifescience). Tube formation was quantified by determining the number of branching points (tube nodes, TN) and total length of skeleton (tube length, TL). Data presented are an average of three wells per treatment condition.

5.1.9 Thioredoxin reductase activity assay

Caki-1 cells treated in vitro with IC₅₀ concentrations bimetallic, monometallic, and control compounds (Chapters 3 and 4) or 0.1% DMSO were lysed after 24 hours of treatment. TrxR activity was measured using a kit (Abcam ab843463). Briefly, lysate was incubated for 20 minutes with DTNB (5, 5′-dithiobis (2-nitrobenzoic) acid), mixed with Thioredoxin Reductase Assay buffer, then TrxR levels were detected according to the manufacturer's instructions using a BioTek Microplate Reader (BioTek U.S., Winooski, VT) at λ = 412 nm. Tests were conducted in duplicate.
TrxR activity was measured relative to the linear quantity of TNB produced per mg of total protein. Background activity from non TrxR enzymes in the lysates were adjusted.

5.1.10 VEGF protein blotting assay

Caki-1 cells treated \textit{in vitro} with either IC$_{20}$ concentrations of bimetallic, monometallic, and control compounds (Chapters 3 and 4) or 0.1% DMSO were lysed after 72 hours of treatment, cell culture supernatant was collected, and VEGF expression was assessed by a VEGF human ELISA kit (100663 Abcam) following manufacturer’s instructions.

5.1.11 Cytokine Expression

Caki-1 cells treated \textit{in vitro} with IC$_{20}$ concentrations of bimetallic, monometallic, and control compounds (Chapters 3 and 4) or 0.1% DMSO. After 72 hours of treatment, cell culture supernatant was collected, and interleukin expression was determined using a Multi-Target ELISA array kit (PathScan Cytokine Antibody Array Kit, Cell Signaling, Danvers, MA). The relative expression levels were determined according to the manufacturer’s protocol, and signal intensities were compared using HLImage$^{++}$ software (Western Vision Software, Layton, UT).

5.1.12 Protein array

Caki-1 cells treated \textit{in vitro} with IC$_{20}$ concentrations of bimetallic, monometallic, and control compounds (Chapters 3 and 4) or 0.1% DMSO were lysed after 72 hours of treatment. Before application to the array, protein concentration was determined by BCA. 150 $\mu$g of lysate was
then incubated for 24 h with the Proteome Profiler Human Protease Array Kit (ARY025) \textit{[in vitro samples]} or Proteome Profiler Human XL Oncology Array (ARY026) \textit{[in vitro and in vivo samples]} (R&D Systems, Minneapolis, MN). The relative expression levels of the proteases were determined according to the manufacturer’s protocol, and signal intensities were compared using HLImage\textsuperscript{++} software.

5.1.13 Data collection and statistical analysis

Results for all experiments were expressed as mean ± Standard Error of the Mean. Experiments were conducted in duplicate or triplicate. For all other parameters, statistical significance was determined using an independent two-tailed Student t-test and one-way ANOVA for independent data. $p < 0.05$ was considered as significant for all statistical analyses. All statistical analysis was done using GraphPad Prism\textsuperscript{®} software (La Jolla, CA).

5.2 \textit{In Vivo} Assays

5.2.1 Animals

Five to eight-week-old male and female non-obese diabetic/severe combined immunodeficient (NOD/SCID) mice NOD.CB17-Prkdc SCID/J mice were purchased from The Jackson Laboratory (Bar Harbor, ME). The NOD.CB17-Prkdc SCID/J strain of mice is routinely used in safety evaluation studies, is recommended for xenograft studies, and is often used for preclinical efficacy studies of novel cancer chemotherapeutic leads \textsuperscript{5,6}. The mice weighed 18–20 g when they arrived to vivarium of the City University of New York (CUNY), City College of New York (CCNY), where they were housed in pathogen-free rooms in clear polycarbonate plastic
cages and acclimated for at least 72h prior to use. Mice were observed daily for signs of illness. The vivarium housing the mice was set to a cycle of 12 hour of darkness and 12 hours of light.

The number of animals, animal use protocols and experimental design, were evaluated and approved by the CUNY, CCNY Institutional Animal Care and Use Committee (IACUC). All procedures of this study followed the CCNY IACUC and the USDA Animal Welfare Act. Treatment to prevent or assuage pain, including the administration of analgesics or recommendation for euthanasia was the responsibility of the veterinary staff in consultation with the principal investigator and study coordinator. In the event of toxicity, the decision to proceed with treatment or euthanize was made by the veterinary staff. Mice were randomly assigned to dose treatment group, or assigned based on body weight, sex, and tumor size where applicable.

5.2.2 Xenograft

Naïve male and female NOD.CB17-Prkdc SCID/J mice received 5x10^6 tumor cells via subcutaneous injection. Exponentially growing human clear cell renal carcinoma Caki-1 cells were suspended at a 1:1 ratio in 50 μL phosphate-buffered saline (PBS; pH 7.4) plus 50 μL of MMatrigel (BD Biosciences) were injected subcutaneously on both the left and right flanks. Within two weeks, the cells grew into a solid tumor, the diameter of which was measured twice a week using an electronic digital caliper. Tumor volume (TV) was calculated according to the empirical equation \( TV = (a)(b^2) \times \pi/6 \) where \( a \) = longest dimension; \( b \) = largest dimension orthogonal to \( a \). The median volumes for each group were normalized to the initial tumor volume to determine the relative tumor volume. Xenograft mice were then used for pharmacokinetic, efficacy and pathology studies; treatment began once the tumors reached a volume of \( \geq 93 \text{mm}^3 \), and throughout the studies the tumor burden was measured at least once a week. Based on weekly external digital
caliper measurements, tumor burden was determined for each mouse and averaged for each treatment. Sex-matched mice were randomized to treatment groups [Titanofin, Titanocref or vehicle control (Chapter 3); RANCE-1 or vehicle control (Chapter 4)] based on their starting tumor burden to ensure equivalent distribution between the three groups.

5.2.3 Test and control articles.

5.2.3.1 Test articles

Titanocref (MW: 807.1g/mol) and Titanofin (MW: 660.1g/mol) (Chapter 3) and Ru-Au RANCE-1 (MW: 1291.4g/mol) (Chapter 4) were synthesized in the Contel Laboratory at Brooklyn College, CUNY as previously reported. The purity of the compounds was greater than 99%. Data on handling and disposal instructions along with any available safety information were provided by the Contel Laboratory and approved by the CCNY laboratory animal study director.

Chart 5.2. Structure of compounds studied in Chapters 3 (Titanocref and Titanofin) and 4 (RANCE-1) in vivo.
5.2.3.2 Control articles

The control agent was the vehicle (Veh.) solution of 0.5% Dimethyl sulfoxide (DMSO) 99.5% (GC) (Sigma-Aldrich St. Louis, MO) and 99.5% normal saline (NS) (0.9% NaCl) (G-Biosciences, St. Louis, MO). (Table 5.1)

5.2.4 Route of administration

All the compounds were administered by intraperitoneal (i.p.) injection. The location of the i.p. injection was alternated between the lower left and lower right abdomen.

Table 5.1. Compound information

<table>
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<tr>
<th>Test articles</th>
<th>Control</th>
</tr>
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<td>MW (g/mol) Yellow powder</td>
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</tr>
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</tr>
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<td>Resuspension solution</td>
<td>-</td>
</tr>
<tr>
<td>Preparation</td>
<td>-</td>
</tr>
<tr>
<td>Titanofin Dark orange powder</td>
<td>660.1</td>
</tr>
<tr>
<td>Description</td>
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</tr>
<tr>
<td>Resuspension solution</td>
<td>-</td>
</tr>
<tr>
<td>Preparation</td>
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<tr>
<td>RANCE-I (r) Rusty</td>
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</tr>
<tr>
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<td>-</td>
</tr>
<tr>
<td>Handling</td>
<td>-</td>
</tr>
<tr>
<td>Resuspension solution</td>
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<tr>
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<tr>
<td>0.5% DMSO + 99.5% NS*</td>
<td>0.5% DMSO + 99.5% NS*</td>
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<td>0.5% DMSO + 99.5% NS*</td>
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<tr>
<td>0.5% DMSO + 99.5% NS*</td>
<td>0.5% DMSO + 99.5% NS*</td>
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</tr>
<tr>
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*NS = normal saline

5.2.5 Acute Toxicity and MTD Study Design

22 naïve males and 22 naïve female NOD.CB17-Prkdc SCID/J mice were assigned to either the acute toxicity (Part A) or MTD study (Part B). The animals received dose formulations containing Titanocref, Titanofin, or RANCE-I suspended in vehicle solution (0.5% DMSO + 99.5% normal saline) at various dosages by i.p. injection (Part A), then six sub-acute doses spaced by 48h followed by a two-week recovery period (Part B). The location of the i.p. injection was alternated between lower left and lower right abdomen.
Table 5.2. Detailed design of dose range finding study (A) Titanocref and Titanofin (Chapter 3) (A) Titanocref and Titanofin (Chapter 3). (B) RANCE-1 (Chapter 4)

<table>
<thead>
<tr>
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<td></td>
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<td>Male</td>
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<td></td>
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<td>2</td>
</tr>
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(A) RANCE-1

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<thead>
<tr>
<th>Compound</th>
<th>No. of animals</th>
<th>Dose (mg/kg/48h)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Female</td>
<td>Male</td>
</tr>
<tr>
<td>RANCE-1</td>
<td>Cohort 1</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>Cohort 2</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>Cohort 3</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>Cohort 4</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>Cohort 5</td>
<td>2</td>
</tr>
<tr>
<td>Veh.</td>
<td>Cohort 6</td>
<td>2</td>
</tr>
</tbody>
</table>

* Dose level was determined based on the result in consultation with drug trial pharmacologists.

** NS = normal saline.
The MTD dose for this study was defined as the highest dose that tolerated without inducing significant debilitating side effects for the 24 days of the study. The starting dose for the dose range finding study was 15 mg/kg/48h, which was been selected based upon the findings of previous studies (Table 5.2).

The dose range finding study (escalating dose design) was used to determine acute toxic dose and MTD. The following parameters and end points were evaluated in this study: mortality, clinical signs of distress, body weight; post mortem procedures included, gross examination of the i.p. cavity, liver, kidney, and spleen for each animal at the terminal necropsy.

5.2.6 Pharmacokinetics

The pharmacokinetics of Titanocref, Titanofin (Chapter 3), and RANCE-1 (Chapter 4) were investigated in a xenograft mouse model. Female (n=2/compound or control) and male (n=2/compound or control) NOD.CB17-Prkdc SCID/J mice bearing subcutaneous Caki-1 tumors (≥ 93mm^3 in volume at start of trial) (Table 5.3). Following a single injection of Titanocref (5mg/kg), Titanofin (10mg/kg), RANCE-1 (10mg/kg), or vehicle control [40μl (0.5% DMSO + 99.5% normal saline)], mice were gently anesthetized by isoflurane, and 200 μL of blood was collected from the submandibular vein over the course of 72h (at 1h, 2h, 6h, 12h, 24h, 48h, and 72h post-infection) into blood collection tubes coated with sodium heparin and mixed briefly by gentle inversion three to four times, then kept on ice. Within 1 hour after collection, whole blood was centrifuged for 10 minutes at 4000 rpm at 4 °C to separate the plasma, it was then stored at −80 °C.
Table 5.3. Design of pharmacokinetic study

<table>
<thead>
<tr>
<th>Compound</th>
<th>No. of animals</th>
<th>Dose (mg/kg/once)</th>
<th>Sample collection post dosing (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Female</td>
<td>Male</td>
<td></td>
</tr>
<tr>
<td>Titanocerf</td>
<td>Cohort 1</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Titanofin</td>
<td>Cohort 2</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>RANCE-I</td>
<td>Cohort 3</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Veh.</td>
<td>Cohort 4</td>
<td>2</td>
<td>2</td>
</tr>
</tbody>
</table>

*NS = normal saline

(The samples were then sent for pharmacokinetic analysis were performed by the Laboratory of Swayam Prabha, at the University of Minnesota’s Department of Experimental and Clinical Pharmacology).

Gold and titanium content was determined using inductively coupled plasma mass spectrometry (ICP-MS). Standards were prepared in untreated mouse plasma prepared identically to that of treated animals. In brief, 50μl of plasma was transferred into a glass vial and 1ml of concentrated acid mix (comprising of 75% of 16 N nitric acid and 25% of 12 N hydrochloric acid) was added. The mixture was heated at 90°C for 5 hours. After cooling, the samples were diluted with water, 40 ppb of Indium internal standard was added, and then samples were analyzed in a Thermo Scientific XSERIES 2 ICP-MS with ESI PC3 Peltier cooled spray chamber with SC-FAST injection loop and SC-4 autosampler. All the elements were analyzed using He/H₂ collision-reaction mode. Plasma from control mice was spiked with the test compounds to determine the extraction efficiency of gold and titanium.

For measurement of metal content in organs (liver, kidney, and tumor), tissues were harvested, weighed, and transferred into glass vials. One ml of water was added to each sample and subjected to ultrasonic tissue disruption at 15W power for 1 minute. The tissue homogenates were frozen at -80°C for 2 hours and lyophilized. The lyophilized product was heated at 90°C with the concentrated acid mix (described above) for 5 hours, cooled, diluted with water and analyzed.
for titanium and gold by ICP-MS. Pharmacokinetic estimates were obtained from the plasma concentration–time profiles by non-compartmental analysis.

5.2.7 Pharmacokinetic parameters

The pharmacokinetic parameters quantified were the maximum plasma drug concentration ($C_{\text{max}}$), the time to reach $C_{\text{max}}$ ($T_{\text{max}}$), the area under the plasma concentration–time curve from 0 h to last measurable concentration ($\text{AUC}_{\text{last}}$), elimination rate constant ($ke$), plasma half-life ($t_{1/2}$), apparent total clearance of the drug from plasma ($\text{Cl/F}$), and apparent volume of distribution ($Vd/F$). Pharmacokinetic parameters were obtained from the plasma concentration–time profiles by noncompartmental analysis using Phoenix WinNonlin 6.4 version 6.4 (Pharsight Corporation, Mountain View, CA). All the measurements were normalized to blood and tissue samples from Veh. treated mice.

5.2.8 Termination

5.2.8.1 Unscheduled sacrifices and deaths

Animals exhibiting signs of acute pain or distress, which were unable to eat, walk, or groom normally, or were moribund, or exhibited other signs of severe systemic toxicity were euthanized immediately. Animals found dead were refrigerated and necropsied. Animals sacrificed at an unscheduled point were weighed and euthanized followed by a gross necropsy.
5.2.9 Scheduled sacrifice

5.2.9.1 Dose range finding study.

The endpoint of Part A was death because of acute toxic effects at some point during the six rounds of doses of the test compound spaced by 48h. This was followed by a gross necropsy. For Part B, following 6 doses spaced by 48h and a two-week recovery period, blood was collected from each surviving animal; then all surviving animals were euthanized, tissues were collected, and a gross necropsy was performed.

5.2.9.2 Efficacy and pathology study.

The preclinical experimental endpoint is to determine therapeutic responses to drugs. In this investigation, a reduction in tumor growth after seven doses within a 21-day period was used as a study end-point. At trial endpoint, mice were sacrificed, and tissue and blood were collected.

5.2.10 Tumor analysis

Mice were anesthetized, followed by heart perfusion using 10 ml of 1x PBS, then 10 ml 4% Paraformaldehyde Solution (Affymetrix, In., Santa Clara, CA). The tumors, liver, and spleen of the mice were collected. The diameter of the tumors was measured once weekly, and after excision from the euthanized mice using an electronic digital caliper, the tumor volume (TV) was calculated according to the equation $TV = (a)(b^2) \times \pi/6$ where $a =$ longest dimension; $b =$ largest dimension orthogonal to $a$. 
5.2.11 Preparation of samples for pathology

The clinical pathology core facility at Memorial Sloan Kettering Cancer Center (New York, US) carried out the histology, clinical chemistry and complete blood count analysis of mice treated with Titanofin, Titanocref, RANCE-1, or vehicle control.

5.2.12 Preparation of histological samples and immunohistochemistry

Tissues were prepared by embedding in OCT (Thermo Fisher Scientific, Waltham, MA) followed by freezing at −80°C. Vessel leakiness was evaluated following tail-vein injection of 100 μL of Dextran Texas Red (Invitrogen). Vessel integrity was assessed after tail-vein injection of 50 μL of lectin labelled with FITC (Vector Labs, Burlingame, CA). The Dextran and Lectin labeling was visualized using a fluorescent microscope at 20X magnification (CCNY). Apoptotic cells were visualized using an anti-rabbit cleaved caspase 3 primary antibody (Cell Signaling Technology, Danvers, MA) and an goat-anti-rabbit Alexa Fluor 594 secondary antibody (Cell Signaling Technology); proliferating cells were visualized using an anti-mouse Ki67 primary antibody (Cell Signaling Technology) and an goat-anti-mouse Alexa Fluor 488 secondary antibody (Cell Signaling Technology); DAPI containing ProLong Gold Antifade Mounting Medium (Cell Signaling Technology) was used to visualize the nuclei and mount the slide.

5.2.13 Analysis of cell proliferation, apoptosis, angiogenic coverage.

For all histological analyses, tumors from treated mice were compared to those of the vehicle control of the intervention trial. Samples from four mice per treatment group were analyzed. Stained samples were imaged at 20X magnification (ZEISS LSM 700); samples injected with Dextran and Lectin were imaged using a fluorescent microscope at 20X magnification (ZEISS...
Axioplan-2). Cell proliferation and apoptosis were quantified one channel at a time using ImageJ and were calculated as the percentage of Ki67 positive or cleaved caspase 3 positive cells per DAPI positive cells per field of view. Vessel area as defined by lectin-covered area and vessel leakiness was determined by dextran-covered area and was determined using ImageJ imaging software (NIH, open access software). All the image analyses were performed treatment-blind.

5.2.14 Analysis of changes in protein expression

Whole tumors were extracted at the trial endpoint and lysed. Before application to the array, protein concentration was determined by BCA. Then 150 µg of lysate was incubated for 24 h with the Proteome Profiler Human XL Oncology Array (ARY026, R&D Systems) and Human Cell Stress Array (ARY018, R&D Systems). The relative expression levels of the proteases were determined according to the manufacturer’s protocol, and signal intensities were compared using HLImage++ software.

This section contains methods from four publications of which I was the contributing author for all the biological assay 3,7–9
5.3 Bibliography


CHAPTER 6

CONCLUSIONS AND FUTURE DIRECTIONS

6.1 Conclusions

We have identified three potent cytotoxic and selective agents against metastatic renal cancer cell line Caki-1 in vivo and in a xenograft mouse model of ccRCC.

Figure 6.1. Visual summary of in vitro trend of Titanocref, Titanofin and RANCE-1. After incubation with the IC20 concentration of the respective compound, in all instances there is a significant reduction in proliferation and angiogenesis and a marked increase in apoptosis (see Chapter 3 and 4 for details).

The initial assessment of a large series of gold based heterobimetallic titanium-gold and ruthenium-gold compounds and the preliminary investigation of anticancer-profile beyond
cytotoxicity led to the selection of Titanocref, Titanofin and RANCE-1 for further studies. Prior to the selection of these three compounds, the initial screen revealed two important patterns. First, that the compounds were most effective against urogenital cancer cell lines (kidney and prostate cancer). Second, that heterobimetallic complexes outperformed their individual monometallic moieties or metal matched pairs (i.e. 1:1, gold moiety + titanium moiety or gold moiety + ruthenium moiety), which suggested that indeed there might be synergistic potentiation of each metal moiety when mounted onto a single molecule. This initial work (introduced in Chapter 2) has been published and strongly suggests that our gold-based heterobimetallic compounds have potential medicinal relevance.

We then evaluated Titanocref, Titanofin and, RANCE-1 in vitro, and in vivo in a mouse model to determine their efficacy and their mechanism of action. We observed that the all three compounds at subtoxic doses inhibited proteolytic factors and growth factors key to angiogenesis and metastasis in vitro and in vivo.

Though all three compounds were cytotoxic in vitro, in vivo, Titanocref and Titanofin appear to retain their cytotoxic profile wherein they induced apoptotic cell death which lead to a drastic decrease in tumor size (Figures 6.2, 6.3), while RANCE-1 exhibited cytostatic properties and inhibited tumor growth by curbing cell proliferation without increasing the rate of apoptosis (Figure 6.4). The rate of increase in apoptosis and the concurrent decrease in proliferation in response to Titanocref and Titanofin can explain why we observe such significant shrinkage of tumor mass: while fewer cells are dividing twice as many cells are dying.

The staticity in tumor size of mice treated with RANCE-1 does not void RANCE-1 of clinical potential because owing to its lack of systemic toxicity, and its inhibition of many key tumorigenic growth factors, RANCE-1 might be well suited for combination therapy.
**Figure 6.2.** In response to treatment with Titanocref the tumor burden decreased by 51% from its volume at the beginning of the trial, the number of apoptotic cells increased by 270%, the number of proliferating cells dropped by 47% while angiogenesis decreased by 28%.

**Figure 6.3.** In response to treatment with Titanofin the tumor burden decreased by 60% from its volume at the beginning of the trial, the number of apoptotic cells increased by 312%, the number of proliferating cells dropped by 38% while angiogenesis decreased by 54%.
Figure 6.4. In response to treatment with RANCE-1 the tumor burden did not change from its volume at the beginning of the trial, though re observed the tumor became less dense upon histological analysis. This compound seems to possess chemo static properties wherein it inhibits growth (100%).

Also, in a proteomic profile study, aimed at detecting changes in expression levels of proteins of oncological interest in response to treatment, it appeared that RANCE-1 lead to a significant decrease in the production of five growth factors, which was not the case for Titanocref or Titanofin.

From the studies presented in this thesis we can conclude that ruthenium(II)-gold(I) compounds display a synergistic effect for biological effects and mode of action due to the combination of both metallic fragments. For titanocene-based heterometallic complexes it seems that the mode of action is mainly due to the second metal (gold). However, in these heterometallic
complexes the titanocene fragments seem to have a non-negligible role in the modification of some of the chemicophysical properties of the compounds with remarkable improvement of their pharmacological profile. Assessing the effects of each individual metal in the mode of action is not an easy task and more in-depth follow-up studies will be necessary in the future. It can be asserted that the addition of a second metal to the monometallic Ti compounds does significantly improve efficacy, and the addition of a second metal to the monometallic Au compounds also improves anticancer properties.

Titanocref, Titanofin, and RANCE-1 caused no changes in tissue or blood in the mouse model that would be indicative of serious side effects in humans. Though preclinical animal data is not an absolute predictor of human response, the results obtained here are encouraging, especially given the bare landscape of viable non-toxic agents.

6.2 Future directions

The results of this body of work gave rise to questions that have yet to be addressed and will be the object of the next experimental venture on this project.

As previously discussed, the tumor microenvironment’s diverse and unique cellular composition plays a key role in the malignant progression of cancer. This investigation did not include a determination of the composition of the cellular ecosystem of the tumor microenvironment. Some of the drastic changes in protein levels were suggested to result from the presence of immune cells (such as the increased immune marker presence or factors associated with increased immune presence) or from other cell types in the tumor microenvironment. Such information could be obtained through detailed flow cytometric analysis of tumor samples and/or
through histological analysis using immunological markers. These experiments will indicate if there are any differences in intratumoral cell populations in response to treatment with a given compound, as compared to the vehicle treated mice. There are however some considerations to be given to the meaning of such results given the animal model used is immunocompromised and thus will have a tumor microenvironment populated in a manner drastically dissimilar to that of an immunocompetent individual. While much is learned from preclinical trials carried out in immunocompromised mice, this model is rather artificial and, the lack of immunocompetence is an important consideration especially when our observations indicate immune markers are chiefly modulated by our three compounds. Also, the fact that tumors are implanted subcutaneously creates another dimension of artificiality because in this model the tumor does not interact with the kidney tissue’s microenvironment removing which is a signaling rich variable. Therefore, it would be informative to test the compound in a spontaneous ccRCC mouse model that is immunocompetent.

The proteome profiling data was informative but failed to capture more profound changes that might be induced at the gene level in response to treatment. Much could be learned from investigating gene expression changes in cells responsive to our compound (e.g. Caki-1 cells) in comparison to non-responsive cells (e.g. MDA-MB-231 triple negative breast cancer cell line). Gene expression analysis in combination with the protein profile will help to generate more refined picture of the compounds' mechanisms of action.

Finally, to advance the compounds’ clinical relevance, a univariate genomic analysis will be performed on wide array of cancerous cells from different tissue types to identify and prioritize predictive genomic biomarkers from drug response data by analysis of several hundreds of genomic features clustered by cancer subtypes and tissue classification. This work will be
performed by a commercial service. The genomic attributes that will be analyzed include mRNA over expression, amplifications, deletions, coding and non-coding mutations, and gain/loss of function lesion categories.

The mechanistic understanding of the mode of action of the compounds will help the synthetic research group in the optimization of lead drugs.

There is still lot of progress to be made in search for a cure for ccRCC. With the work presented in this thesis, the Contel laboratory is contributing to that search, and we believe our findings hold great potential for further preclinical exploration and potential clinical applications.
7.1 Publications


7.1.1 Submitted Manuscript


7.1.2 Manuscripts in preparation


7.1.3 Other publication from the past four years non-related to this thesis


7.2 CONFERENCE PRESENTATIONS

7.2.1 Posters


7.2.2 Oral communications


Elie BT, Fernández-Gallardo J, Contel M. Preclinical studies of two unconventional anticancer agents as promising candidates for breast and renal cancers. 1st International Symposium on Clinical and Experimental Metallodrugs in Medicine: Cancer Chemotherapy (CEMM) University of Hawaii Cancer Center (NCI designated center), December 2015, Honolulu, Hawaii.

7.3 US PATENTS
