



Protéine-kinases et cancer du rein : Découverte et validation d'une nouvelle combinaison d'inhibiteurs ciblant les protéine-kinases ATM et CK2

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THÈSE

Pour obtenir le grade de

**DOCTEUR DE LA COMMUNAUTE UNIVERSITE
GRENOBLE ALPES**

Spécialité : **Doctorat CSV/Biologie Cellulaire**

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Présentée par

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Préparée au sein du **Laboratoire Biologie du Cancer et l'Infection**
dans l'École Doctorale Chimie et Sciences du Vivant

Protéine-kinases et cancer du rein : Découverte et validation d'une combinaison d'inhibiteurs ciblant les protéine-kinases ATM et CK2.

**Protein kinases and renal carcinoma: discovery and validation
of a novel combinational target therapy through co-inhibition
of CK2 and ATM kinases**

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Résumé:

L'incidence du cancer du rein et sa mortalité associée se sont accrues au cours des dernières années. Le type de cancer rénal le plus fréquent est celui nommé Cancer Rénal à Cellules Claires (CRCC) où le plus souvent, le gène suppresseur de tumeur Von Hippel Lindau (VHL) est inactivé. Malgré une détection plus précoce, l'évolution de la pathologie demeure incertaine, en particulier quand les patients développent des métastases ou acquièrent une résistance au traitement (25-30% des patients). De nouvelles thérapies ciblant des kinases (Sunitinib, Sorafenib ou Temsirolimus) bien que très prometteuses conduisent très souvent à l'acquisition de résistance. Dans ce contexte, il est urgent de développer de nouveaux modèles prédictifs de la réponse des patients aux traitements et d'identifier de nouvelles cibles thérapeutiques.

Ma thèse de science visait trois objectifs complémentaires : 1) Identifier par criblage chimio-génomique des kinases comme cibles thérapeutiques combinées. 2) Etablir deux modèles de culture 3D de cancer du rein qui intègrent le microenvironnement d'une tumeur: les sphéroïdes et la culture organotypique de coupe de tissus. 3) Etudier la chimio-sensibilité de ces modèles à une combinaison de molécules identifiées dans le criblage.

Un criblage cellulaire a été réalisé sur la plateforme de Criblage de Molécules BioActives (CEA- Grenoble). Il a consisté à tester 80 molécules inhibitrices de protéine-kinases en combinaison avec l'extinction génique par interférence ARN (shRNAs lentiviraux) de 36 cibles potentielles connues pour leur implication dans divers cancers. La lignée cellulaire choisie (786-O) est dérivée d'une tumeur rénale à cellules claires radio et chimio-résistante et dépourvue de VHL. Parmi les touches qui compromettent la viabilité des cellules 786-O, la combinaison choisie pour son efficacité cible deux kinases importantes dans le contrôle de la survie cellulaire et de la réparation de l'ADN : CK2 et ATM. Le statut VHL des cellules module de façon dramatique leur sensibilité à cette combinaison, l'association de ces deux inhibiteurs étant plus efficace sur les cellules 786-O (VHL-) que sur les mêmes cellules dans lesquelles VHL a été réintroduit (VHL+). Au sein d'une tumeur, les différents niveaux d'oxygénation constituent une variation environnementale supplémentaire créant des susceptibilités ou des résistances aux traitements thérapeutiques. Pour déterminer l'impact de nos molécules dans ce contexte, nous avons testé la viabilité des cellules 786-O VHL+ et VHL- dans des conditions normoxiques (21% O₂) ou hypoxiques (1,5% O₂), en présence des molécules seules ou en combinaison. En normoxie, une diminution synergique de la viabilité des cellules 786-O VHL- est observée en présence de la combinaison, alors que cet effet n'a pas lieu sur les cellules 786-O VHL+. Cette synergie est potentialisée en condition hypoxique. Au niveau mécanistique, les voies de signalisation de stress cellulaires sont d'avantage activées dans les cellules VHL- en présence de la combinaison de molécules comparé au traitement avec chacune des molécules seules. Dans les sphéroïdes tumoraux multicellulaires reproduisant l'organisation d'une micro-tumeur, nos résultats montrent que notre combinaison de molécules induit d'avantage l'apoptose des cellules VHL- que les molécules seules, alors que les cellules VHL+ ne sont sensibles à aucun des traitements.

Ces résultats montrent que l'action de nos molécules combinées est clairement plus efficace dans un modèle 3-D. Ils démontrent également qu'il est possible d'objectiver une pharmaco-modulation de la viabilité de cultures organotypiques de tumeur du rein par des combinaisons d'inhibiteurs chimiques de protéine-kinases. Les perspectives de ce travail sont la validation de cette combinaison sur des tumeurs humaines et l'exploitation des cultures organotypiques comme test personnalisé de réponse aux traitements.



Abstract :

Renal cell carcinoma accounts for 3% of all malignant diseases in adults making it the 10th most common cancer in France. The most frequent type of Kidney cancer is Clear Cell Renal Cell Carcinoma (CCRCC). Almost all CCRCC show an inactivation of the Von Hippel Lindau tumour suppressor gene (VHL). Between 25-30% of the patients will develop metastatic renal cell carcinoma (mRCC) by the time they are diagnose or become unresponsive to all treatments and in these cases, the disease has a rapid progression. Over the past years, kinase-targeted therapies (Sunitinib, Sorafenib, Temsirolimus) have become the mainstay of treatment for mRCC, however, most, if not all, patients acquire resistance to these approaches over time.

In this context my PhD had 3 goals: a) to find a new combinatory targeted therapy through a High Throughput Screening; b) to establish 3D models mimicking the real environment of the tumours (spheroids, Tissue Slice Culture); c) to validate the Hits through different molecular and cellular biology studies.

We conducted a synthetic lethal screen on the CMBA platform (CEA-Grenoble), choosing 36 potential genes targets and 80 kinases inhibitors drugs. Each of the target gene was silenced by a transduction with shRNA Lentivirus into the 786-O cell line derived from ccRCC that lacks the tumour suppressor VHL, is radio- and chemo-therapy resistant, has increased mobility and is highly metastatic. Among the hit combinations that affect cell viability, one of them was chosen because it targets two important kinases involved in cell survival and DNA repair: CK2 and ATM. Moreover, this combination is specifically more active in the 786-O VHL- cells than in 786-O VHL+ cell line. We evaluated the effect of our drugs on the viability of our 786-O VHL+ and VHL- cells in normoxic (21% O₂) or hypoxic (1.5% O₂) conditions that reflect different environments that are present in a tumour. Surprisingly, in normoxia, we found a synergetic effect of the drug mix only on the 786-O VHL- cells but not on 786-O VHL+ cells. Furthermore, this effect was even stronger in conditions of Hypoxia (up to 20% of synergism).

Mechanistically, an up-regulation of the stress pathways was much stronger in the VHL- cells in the presence of the combination than with the drugs alone. No apoptosis was detected in this 2D models. In Multi-Cellular Tumour Spheroid (MCTS) where the organization of a micro-tumour is reproduced, our drugs are even more effective in inducing cell apoptosis than in 2D monolayers of 786-O VHL- cells. These results also demonstrate that pharmaco-modulation of viability of renal tumour organotypic culture by chemical combination targeting protein kinases can be studied. Perspectives of this work are the validation of this drug combination on human renal tumours and the use of organotypic culture as a test for personalized treatment response.



Je voudrais remercier les rapporteurs de ce jury de thèse Dr. Bernard Ducommun et Dr. Gilles Pages ainsi que les autres membres du jury Dr. Yannick Arlot-Bonnemais, Pr. Sylvie Negrier et Dr. Pierre Hainaut d'avoir accepté d'évaluer mon travail.

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LIST OF ACRONYMS

- (AKT) protein kinase B
- (ATM) Ataxia telangestasia mutated kinase
- (BEV) Bevacizumab
- (ccRCC) Clear cell renal cell carcinoma
- (CK2) CK2 previously named Casein kinase 2
- (COPD) chronic obstructive pulmonary disease
- (CTDA) C- terminal activation domain
- (CUL2) Cullin 2
- (DDR) DNA damage response
- (DSBs) Double Strands Breaks
- (Elongin C) transcription elongation factors C
- (Elongin B) transcription elongation factors B
- (EMT) Epithelial to Mesenchymal transition
- (FDA) Food and drug administration
- (FIH1) asparaginyl hydroxylase
- (GBM) glioblastoma
- (HIF) Hypoxia inducible factor
- (HR) Hazard ratio
- (HRE) Hypoxia Response Elements
- (IFN α) Interferon α
- (IL-2) Interleukin-2
- (IR) Ionizing radiation
- (MAPK) mitogen activated protein kinases
- (MPPs) Matrix Metalloproteinases
- (mRCC) Metastatic renal carcinoma
- (mTOR) Mammalian target of rapamycin
- (NK) natural killer cells
- (NNK) 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone

(NNKOA) 4-[(acetoxymethyl) nitrosamino]-1-(3-pyridyl)-1-butanone

(NTAD) N-terminal transactivation domain

(ORR) Objective response rate

(ORR) objective response rate

(OS) Overall survival

(PDGF) Platelet-derived growth factor

(PDK1) phosphoinositide –dependent-kinase

(PFS) Progression Free survival

(PI3K) phosphatidylinositol 3-kinase

(RET) Receptor Tyrosine Kinase

(ROS) Reactive oxygen species

(RXB1) RING finger protein

(siRNA) small interfering RNAs

(shRNA) short hairpin RNA

(SSBs) single strand breaks

(TAM) Tumor associated macrophages

(TCEB1) Transcription elongation factor polypeptide 1

(TRAIL) TNF-related apoptosis-inducing ligand

(TSC2) tuberin

(VEGF) Vascular endothelial growth factor

(VHL) von Hippel-Lindau tumor suppressor

(AKT) protein kinase B

(ATM) Ataxia telangestasia mutated kinase

(BEV) Bevacizumab

(ccRCC) Clear cell renal cell carcinoma

INTRODUCTION

CHAPTER 1

1. WHY IS YOUR KIDNEY SO IMPORTANT?

1.1 Kidney Physiology.

Kidneys are pair of bean-shape organs that are localized on the upper level at the back of the abdominal cavity, side by side the vertebral column. They show a dissymmetry in their position due to that right kidney is “pushed down” by the liver compared to the left kidney that is free in peritoneal area, nevertheless both kidneys are protected by the lower ribs. The kidney itself is enclosed in the fibrous capsule and surrounded by the perinephric and the paranephric fat. As well, there are renal glands that are on the outside part of the kidney and have extreme important functions as hormones production.

In a transversal cut section (figure 1) we can observe the different areas of a kidney. The white area is the cortex, just below the renal capsule, where the nephron bodies are located. Lower we have a pink section called medulla, where we find the tubules forming a renal triangle shape structure called renal pyramid. Between the cortex and the medulla, there are the renal columns that will collect the filters coming through the cortex section and will take them to the renal papilla. Renal papilla is formed by minor calix that together form the major calix and address the filter to the renal pelvis and finally to the ureter.

The renal artery enters the kidney in the hilum, crosses the renal lobule (formed by the renal pyramid and the cortex) and arches around the kidney changing its name interlobular renal and collecting the filter through branches. The renal vein has the same structure as the renal artery but it will “leave” the kidney at the hilum.

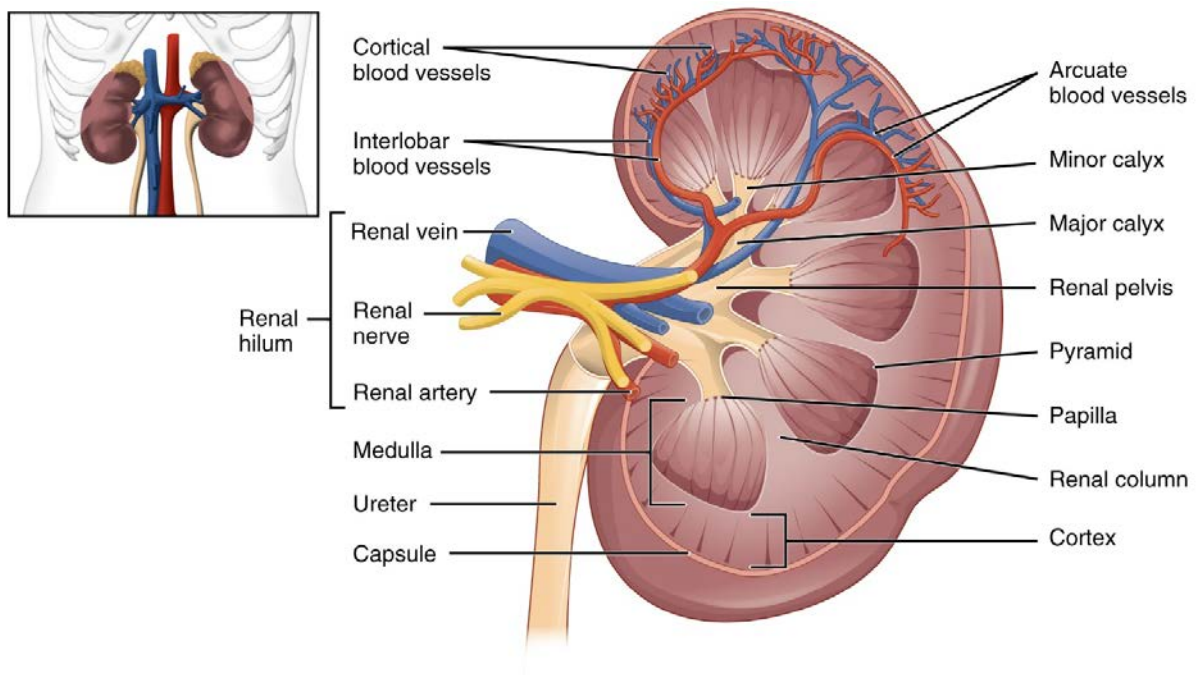


Figure 1: Internal Anatomy of a human kidney.

Inside the kidney it can be distinguished 4 main sections: The renal capsule (light pink), the cortex that is just below the capsule where the nephron bodies are inserted, the medulla where the nephron is located and the renal hilum containing the renal artery and renal vein. Picture taken from ([Openstack College 2016](#))

Nephrons are part of the microstructures of the kidney and are the main responsible elements for the blood filtration (Figure 2). Nephrons consist in two main structures, the renal corpuscle and the renal tubule. In the renal corpuscle the blood will enter and will be filtrated by the glomerulus, leaving through the proximal renal convoluted tubules. Part of this filter will be absorbed (as glucose, electrolytes, etc.) at this level and the other part will continue towards the distal tubule, then to the renal papilla until the ureter. ([Bard 2003](#)) ([Openstack College 2016](#))

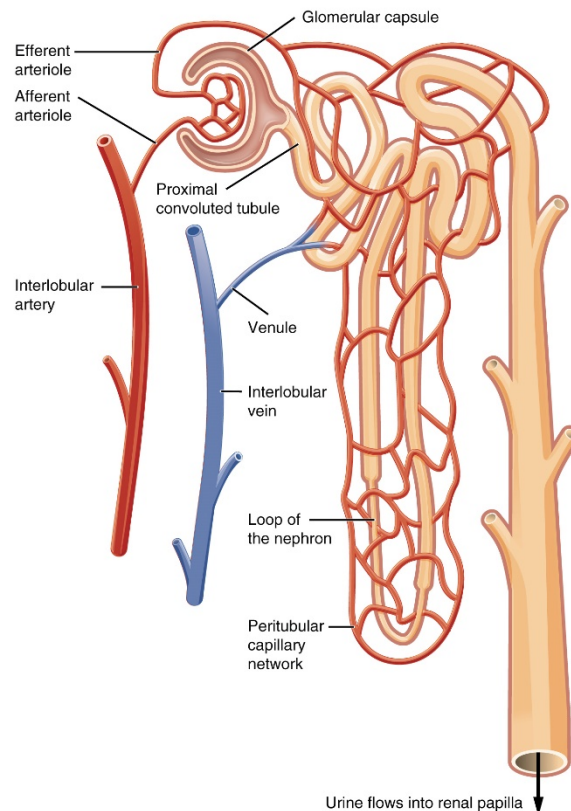


Figure 2: Scheme of a kidney nephron.

The renal corpuscle is at the beginning of the filtering process by separating the solutes from the blood to then send the small solutes to the renal corpuscle for reabsorption and secretion (Picture taken from ([Openstack College 2016](#))).

1.2 Kidney Functions

Kidneys have several functions ([Greger 1996](#)) ([Tanner 2009](#)) ([Owen 1969](#))

- I. Regulation of extracellular flux volume and blood pressure by adjusting sodium excretion and producing various substances, for example renin, that can affect blood pressure.
- II. Regulation of osmotic pressure (osmolarity) of the body fluids by excreting osmotically dilute or concentrated urine.
- III. Production of hormones including erythropoietin and 1, 25-dihydroxy vitamin D₃.
- IV. Homeostatic regulation of PH: Regulation of acidosis is done by reabsorbing bicarbonate through the tubular cells and generating ammoniogenesis (formation of

NH₃ buffer) by the collecting duct cells. Lowering rates of glutamine metabolism and ammonium secretion makes regulation of alkalosis.

- V. Gluconeogenesis when starvation: In short term starvation, kidney's main substrate for gluconeogenesis is glutamine together with other amino acids being converted into it by transamination. As a result, ammonia product will be excreted by the urine and partly reutilized for protein production.
- VI. Excretion of waste: Kidneys eliminate the products of metabolism, including urea (the main nitrogen-containing end product of protein metabolism in humans), uric acid (an end product of purine metabolism), and creatinine (an end product of muscle metabolism).

2. KIDNEY CANCER

2.1 Frequency and mortality

Renal Cell Carcinoma represents the 12th most frequent type of cancer and the 2% of all adult malignancies ([Ferlay 2015](#)). In 2012, worldwide, there were 338.000 new cases diagnosed, with a mortality reaching 20-40% of the patients. As well, the predictions for 2020 for the medium and more developed countries are 23.000 new patients per year, compared with 2.200 new patients in the less developed countries, counting both sex ([GLOBOCAN PROYECT](#)).

The frequency of kidney cancer is still 2.1 in men versus women; one of the reason could be that men tends to smoke more and to be exposed to cancer-causing chemicals at work. Although due to the increase stress factors added to the everyday life, this ratio is getting more equal ([Znaor 2015](#)).

It is encouraging that, due to the use of imaging techniques that allows better and earlier detection of small renal masses, RCC shows decrease mortality and the 5 years survival after RCC treatment has increased ([Pierorazio 2012](#)) ([Cairns 2011](#)).

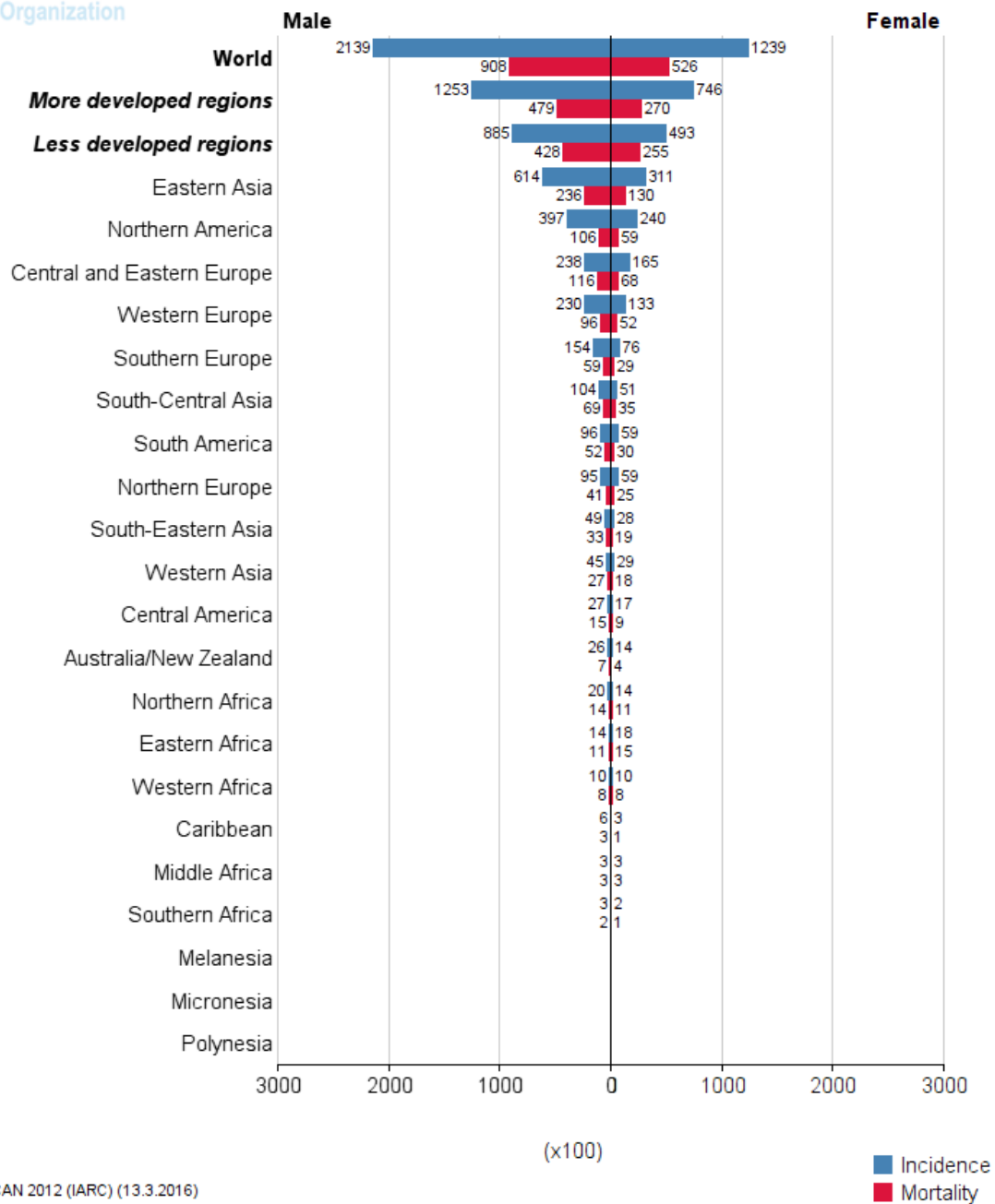
Kidney
all ages

Figure 3: Incidence and mortality of kidney cancer worldwide

Although RCC incidence is still increasing in most countries, there is a marked trend in increase of RCC mortality in countries with lower human development. Picture taken from GLOBOCAN.

2.2 Causes of kidney cancer

A) Non genetic associated factors

Smoking

It is considered as a risk factor in the RCC. (International Agency for Research Cancer IARC) In non-smokers and smokers populations, the possibility of getting RCC increases by 50% in men and 20% in women that smoke regularly. The reasons why smoking increases the risk of having RCC could be tissue chronic hypoxia as a result of the exposure to carbon monoxide, as well as the smoking-related diseases such as chronic obstructive pulmonary disease (COPD) ([Sharifi 2006](#)). In addition, several studies suggest cigarette smoke is a major risk factor for RCC ([Hunt 2004](#)) ([IARC 2004](#)). 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) is one of the most abundant carcinogenic *N*-nitrosamines present in cigarette smoke. As show by ([Clague 2009](#)) lymphocytes derived from patients with RCC showed higher levels of DNA damage compare to the samples coming from control that were treated with a NNK precursor 4-[(acetoxymethyl) nitrosamino]-1-(3-pyridyl)-1-butanone (NNKOAc). These studies suggest that patients that have higher sensitivity to NNKOAc-induced DNA damage exhibited a higher risk of developing RCC.

Obesity

It is now well accepted that overweight is implicated in more than 40% of RCC cases in USA and in 30% of the cases in Europe ([Calle 2004](#)). Several studies world wide, have shown that patients that had overweight or obesity at the beginning of a prospective study, had an increase risk to develop RCC (24% for men and 34% for women for 5 kg/m² in their body mass index ([Reeves 2007](#)), ([Pischon 2006](#)), ([Adams 2008](#)).

The global rise in obesity likely has contributed to the upward RCC incidence trends, but does not explain the recent levelling of RCC in some countries. Several mechanisms could explain the increased risk for an obese person to acquire RCC, among them it has been described chronic tissue hypoxia, insulin resistance and a compensatory hyperinsulinemia, altered endocrine milieu and production of adipokines, obesity-induced inflammatory response, and lipid peroxidation and oxidative stress ([Drabkin 2010](#)).

Other risk factors

In the next table you will find a summary of the life-style related RCC risk factors.

Risk factors for renal cell carcinoma		
Risk Factors	Association	Comment
Established*		
Cigarette smoking	Positive	<ul style="list-style-type: none"> ▪ Dose-response with pack-years ▪ Smoking cessation reduces risk
Excess body weight	Positive	<ul style="list-style-type: none"> ▪ Dose-response with usual adult BMI ▪ Effect of weight change on risk unclear
Hypertension	Positive	<ul style="list-style-type: none"> ▪ Dose-response with blood pressure ▪ Control of hypertension may reduce risk ▪ Effect independent of body weight
Suspected§		
Diabetes mellitus	Positive	<ul style="list-style-type: none"> ▪ Independent effect from obesity and hypertension not yet established
Parity in women	Positive	<ul style="list-style-type: none"> ▪ Dose-response with number of births
Physical activity	Inverse	<ul style="list-style-type: none"> ▪ Dose-response with activity level
Alcohol consumption	Inverse	<ul style="list-style-type: none"> ▪ Dose-response with amount consumed
Trichloroethylene exposure	Positive	<ul style="list-style-type: none"> ▪ Dose-response with exposure level

Table 1: Summary of life-style related RCC risk factors. Taken from ([Chow 2010](#))

B) Genetic associated risk factors

Inherited renal cancer is known to be part of a certain number of familial cancers, mostly in the case of the von Hippel-Lindau (VHL) syndrome disease. The disease is named after the German ophthalmologist Eugen von Hippel, who identified and described characteristic retinal manifestations and the Swedish pathologist Arvid Lindau, who discovered the frequent co-occurrence of retinal and cerebellar hemangioblastoma with tumors and cysts in visceral organs ([Gläsker 2015](#)).

This syndrome is characterized for alteration of the VHL gene and a higher susceptibility to develop several types of tumors as pheochromocytomas, hemangioblastomas of the

cerebellum, and spinal cord, retinal angiomas, pancreatic cysts, and bilateral renal carcinoma including the RCC subtype Clear cell renal cell carcinoma (ccRCC). Only a small portion of renal cancer is known to occur in patients with this disease (estimated birth incidence of one in 36000 people ([Maher 1991](#)) ([Latif 1993](#)), indicating that in most cases RCC have a sporadic origin ([Glasker 2015](#)).

Nevertheless, ([Claque 2009](#)) showed that there is up to 2.2 fold increase on the risk to acquire sporadic RCC between the persons that have close relative family diagnosed with RCC than those who doesn't. In conclusion, it is believed that the different exposures to environmental risk factors and genetic susceptibility of exposed individuals can influence the risk of developing sporadic renal cell cancer.

3. CLEAR CELL RENAL CELL CARCINOMA (ccRCC)

3.1 Histopathology and frequency.

Renal carcinoma are separated in two groups: 1) “Non- papillary renal carcinoma” have a loss of heterozygosity in the chromosome 3 containing the VHL gene, due to somatic mutation (in 50% of the cases). In 10 % of the cases there is a inactivation of the VHL gene by epigenetic changes as hypermethylation in its promoter); 2) those named “Papillary Renal Carcinoma” that are characterized with chromosomal abnormalities at loci other than chromosome 3. Genetically, these tumors are characterized by trisomies (chromosomes 3q, 7, 12, 16, 17, and 20) and loss of the Y chromosome ([Störkel 2000](#)) ([Zar 1994](#)). According to the [World Health Organization](#) , from all subtypes of RCC, non-papillary ccRCC is diagnosed in the 70-85% of cases versus only 10-15% papillary RCC and 4-5% chromophobe RCC.

Non-papillary clear cell of Renal carcinoma is considered to belong to the malignant group of kidney neoplasm. In histology this renal cortical tumor is typically characterized by epithelial cells with clear cytoplasm, because it is filled with lipids and glycogen that are dissolved during the histologic processing, creating the distinct clear cytoplasm ([Haddad 1993](#)). It is frequent as well, to find foci in which cells have an eosinophilic staining, often in the case of high grade tumors and adjacent to areas with necrosis or haemorrhage. These tumors cells have an acinar or very compact –alveolar growth ([Storkel 2000](#)).



Figure 4: Picture of a Clear cell renal cell carcinoma

The clear cell renal cell carcinoma is typically golden yellow due to the rich lipid content of its cells; cholesterol, neutral lipids, and phospholipids are abundant. Tumor extension can be observed along the renal vein. Picture taken from [Pathology of Clear Cell Carcinoma-emedicine.](#)

3.2 Somatic Genetics

As previously described, the majority of ccRCC does not have an origin in the von Hippel Lindau disease. Nonetheless, deletions in the short arm of the chromosome 3 leading to partial or total inactivation of the VHL gene has shown to be a constant in the sporadic clear cell Renal Carcinoma ([Brouch 2010](#)) ([Foster 1994](#)) ([Gnarra 1994](#)). In the von Hippel Lindau disease, mutation in the tumor suppressor gene is inherited and the malignant kidney cancer arises due to a second sporadic mutation in the wild type allele gene. In the case of sporadic ccRCC, the mutations occur on the same cell type after somatic inactivation of both alleles of the VHL gene by allelic deletion, mutation, or epigenetic silencing in 70% or more of the cases. ([Gnarra 1994](#)) ([Knudson 1985](#)) ([Herman 1994](#)) (Latif 1993) ([Schram 2002](#)). These data suggest that the VHL gene is most likely a tumor suppressor gene in sporadic clear cell RCC.

3.3 Kidney cancer stages, treatment and prognosis

At first basis, the type of treatment that will be applied to a patient with RCC is determined by the probability of cure, that in most cases is directly related on how early the patient is diagnosed, the state of the tumor and the degree of tumor dissemination ([American Cancer Facts 2015](#); [Sachdeva 2016](#); RCC is mostly a silenced disease, probably because kidneys are localized deep in the body and there may not be any symptoms until the tumor size is large, only 10% of patients will approach their doctor due to classic triad of flank pain, hematuria, and flank mass.

Chart N°1 reveals the different sizes and stages of RCC. Chart N°2 describes the 5 years survival prognosis. More than 50% of patients with early stage renal cell carcinoma are cured, but according to the [Cancer Center treatments of America](#) and [the Surveillance, Epidemiology and End Results \(SEER\) database of the National Cancer Institute](#) in USA, the 5 years survival outcome for stage IV disease is poor (around 10% of patients).

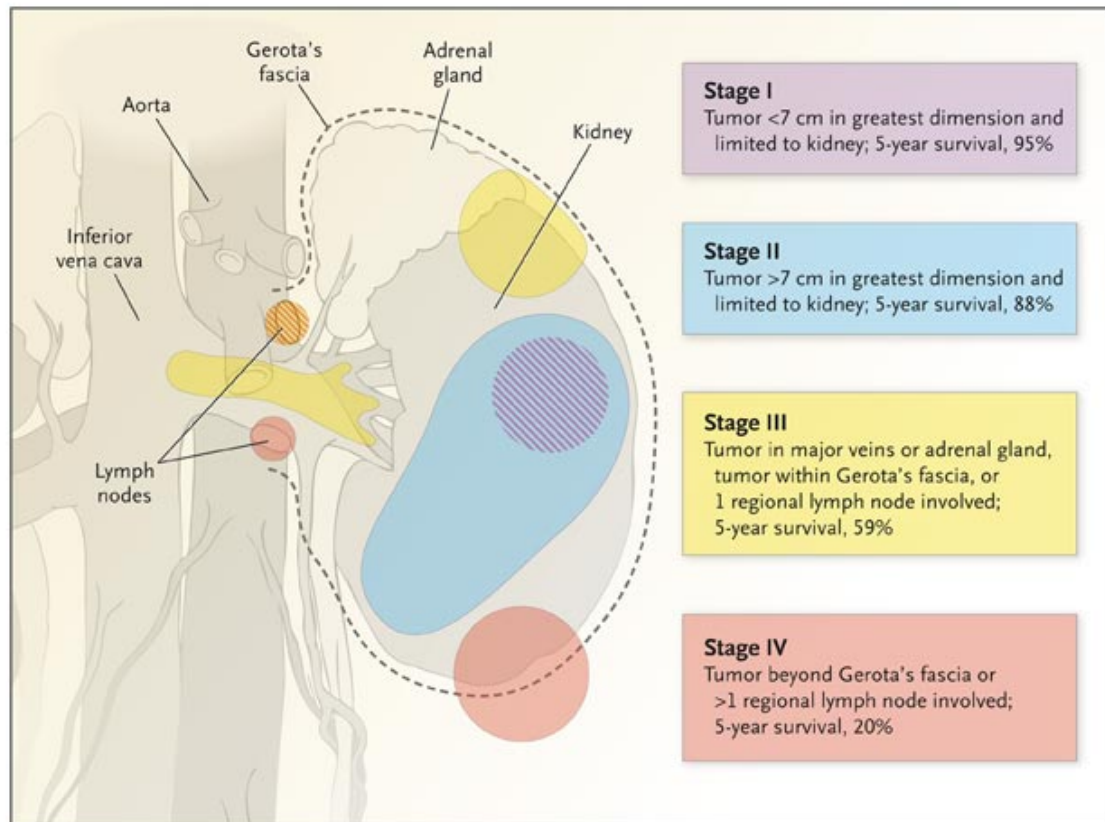


Figure 5: Kidney cancer stages and 5 years survival rates.

RCC is often an asymptomatic disease in early stages. Often, patients are diagnosed in an advanced stage of the disease, greatly decreasing their chances of survival.

Picture taken from http://www.aboutcancer.com/renal_survival_section_0808.htm

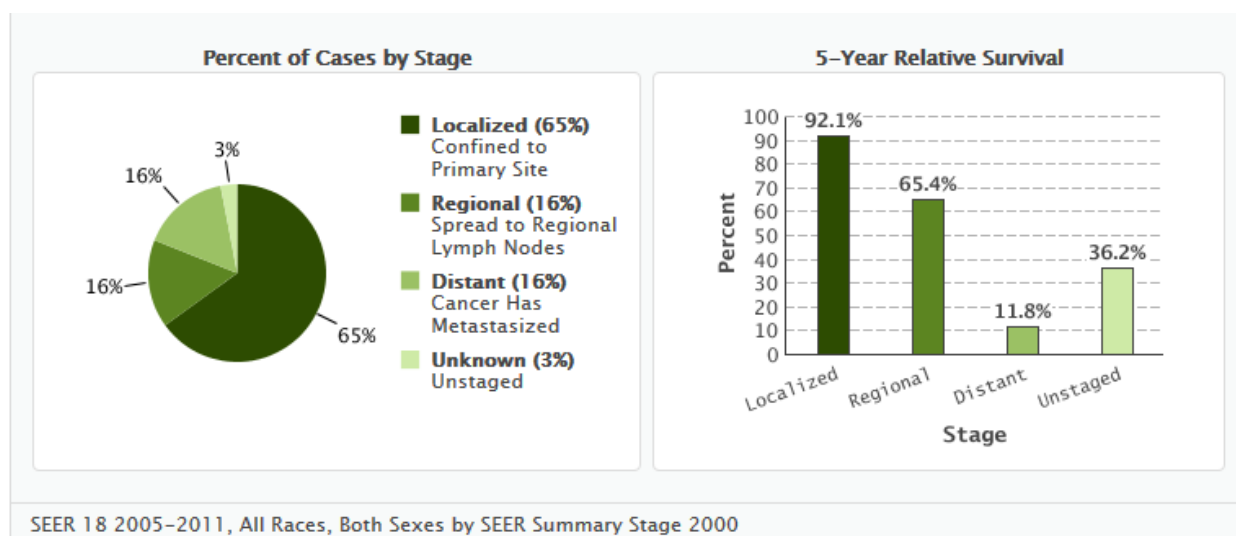


Chart 1: Percent of cases & 5-year relative survival by stage at diagnosis in kidney and renal pelvis cancer. Taken from the Surveillance, Epidemiology and End Results (SEER) database of the National Cancer Institute (USA).

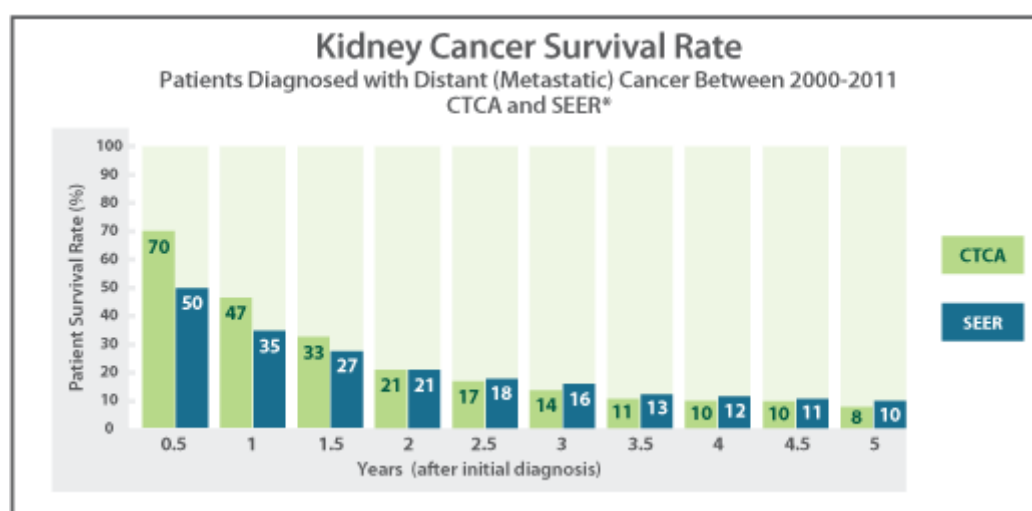


Chart 2: Patients survival rate in patients with distant metastatic cancer according to [Cancer Center treatments of America](#) and [the Surveillance, Epidemiology and End Results \(SEER\) database of the National Cancer Institute](#) (USA).

4. TREATMENT OF METASTATIC RCC

4.1 Surgery

Partial nephrectomy has been so far the first line of treatment for organ-confined ccRCC (tumors smaller than 4 cm), and in some cases a total nephrectomy can be applied in larger tumors. In the last years there have been an increased interest in including studies that will evaluate not only the appearance of local recurrence after surgery but also the way metastasis respond to drug therapies in presence or absence of the primary tumor ([Coppin 2011](#); [Lams 2006](#)).

4.2 Adjuvants therapies

Adjuvants therapies are the treatments that are given in addition to the primary or initial therapy to maximize their effectiveness. Usually, in different types of cancer, the adjuvant is the treatment given after surgery where the detectable disease has been removed but when there is still risk of relapse due to non-detectable residual disease ([Pal 2014](#); [National Cancer Institute: Dictionary of cancer terms](#)).

In this section I will discuss the therapies that are used in a monotherapy or as an adjuvant in the systemic treatment of mRCC.

- Immunotherapies

- A) *Interferon α (IFN α)*

Interferon α was the first cytokine therapy approved for the systemic treatment of mRCC. This natural glycoprotein expressed by leukocytes, stimulates natural killer cells (NK), decreases cell proliferation through the inhibition of cyclin kinases, increases immunogenicity of tumor cells and inhibits angiogenesis ([Fossa 2001](#); [Goldstein 1988](#); [Coppin 2000](#); [Oliver 1989](#)).

- IFN- α as monotherapy

As reviewed by ([Canil 2010](#)) although IFN- α had a promising start in the treatment of advanced or metastatic clear cell carcinoma, comparison analyses of 8 randomized trials in patients with inoperable renal cell carcinoma, have proven that there is limit in the response rate (up to 20%) in patients treated with IFN- α versus control (placebo).

Moreover, there is a high toxicity of this treatment including lack of appetite (51%), tiredness (68%), nausea/vomiting (26%/9%), lack of energy (65%), dry mouth (41%), shivering (23%) and depressed mood (25%) after 4 weeks of treatment with IFN- α . ([Medical Research Council Renal Cancer Collaborators 1999](#)) has led to limit the use of this treatment or to try several combinations with others agents as first or second-line therapy for metastatic renal cell carcinoma. This last subject will be later discussed.

- IFN- α as adjuvant treatment

Surgery in the case of mRCC has been subject of several studies. As showed by [Flanigan 2001](#) the median overall survival of patients that were treated with IFN- α after cytoreductive nephrectomy went up to 11.1 months survival compared to the 8.1 months when treated with IFN- α only. This was also proved in another study by ([Mickisch 2001](#)) where they observed an increase of median overall survival up to 17 months when there was a treatment with IFN- α compared to the 7 months of patients that were treated with α -interferon only. This suggest that IFN- α should be considered for the treatment of patients with high risk of relapse after surgery, although this is not possible in all the cases due to the high toxicity associated with this agent.

B) Interleukin-2 (IL-2)

IL-2 is a T-cell growth factor and activator of T cells, monocytes, macrophages and natural killer (NK) cells. IL-2 affects tumor growth by activating lymphoid cells *in vivo* without affecting tumor proliferation directly.

- IL-2 as monotherapy

Several studies performed by ([Law 1995](#) , [Rosemberg 1993](#)) have shown that high-dose of IL-2 can induce durable long term remission but in a very small portion of patients (1 of 36 and 4 of 48 patients respectively). In the study performed by ([Fisher 1997](#)) and colleagues a median of response duration for all partial responses was 20 months. As well, 20% of patients were estimated to be alive, 5 to 10 years following treatment.

The main limitation in this treatment was that serious adverse effects occur. Toxic effects associated with high-dose of IL-2 are related to increased vascular permeability and secondary cytokine secretion (eg, IL-1, interferon gamma, tumor necrosis factor, nitric oxide). The management of high-dose toxicities requires in patient monitoring, often in an intensive care unit. ([Rosemberg 1993](#); Law 1995; Fisher 1997). All these studies had mix number of patients that had gone under nephrectomy so they cannot be evaluated as either monotherapy or surgery adjuvant therapies.

- IL-2 as adjuvant treatment

The role of IL-2 after nephrectomy, remains controversial ([Belledgrun 2000](#) and [Fligin 1997](#)). Studies showed that the combination of nephrectomy with high-doses of IL-2 significantly improves survival outcomes in patients with metastatic renal cell carcinoma.

In contrast, the results obtained by [Clark 2013](#) showed no difference in the overall survival (OS) after one cycle of IL-2 high-dose when added as adjuvant in patients with locally advanced and metastatic disease that had been completely resected.

- Targeted Therapies

Overall, the systemic therapy of advanced RCC has not been satisfactory since, as discussed before, cytokine treatments have shown limited efficacy as well as high toxicity.

Since then, the better understanding of the signaling pathways involved in the development of ccRCC has led to the development of novel therapies that target specifically the dysregulated pathways inside the tumor cell and/or the cells that are in the tumor environment.

As described before, the inactivation of the VHL gene leads to the accumulation of the transcription factor HIF, over-expression of vascular endothelial growth factor (VEGF) and platelet-derived growth factor (PDGF) ([Wiessener 2001](#); [Cookman 2000](#); [Iliopoulos 1996](#)). These proteins are known to promote angiogenesis, a key process in tumor development and progression ([Takahashi 1994](#)).

As well, the mammalian target of rapamycin (mTOR), a protein kinase, is in the upstream pathway regulating not only HIF but also other factors as angiogenesis, cell proliferation, metabolism that are critical for the pathogenesis of mRCC ([Hudson 2002](#)).

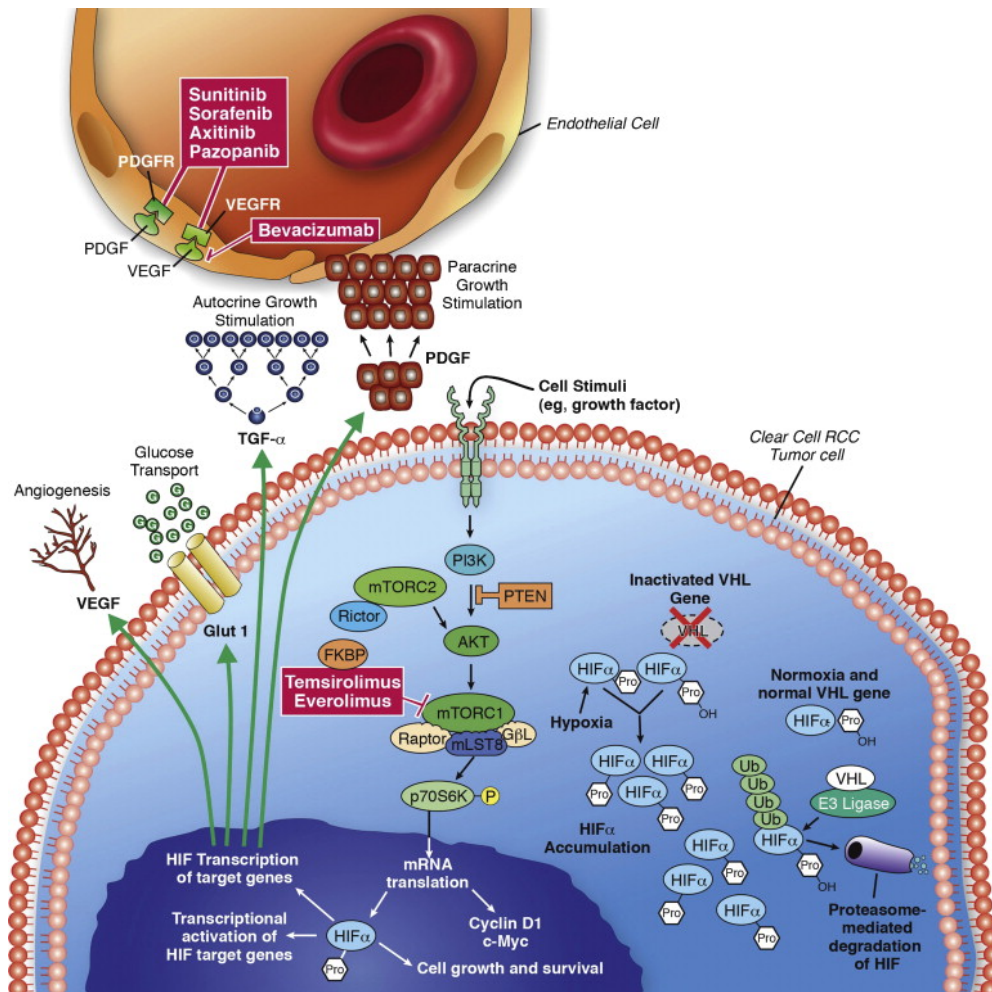


Figure 6: Dysregulated pathways of ccRCC and targeted therapies mechanism of action.

The inactive form of VHL in the ccRCC leads to stabilization and accumulation of the hypoxia inducible factor HIF α . This accumulation can also result as a consequence of the activation of mTOR and the PI3-K/AKT pathway due to cell stimuli. Increased levels of HIF induce activation of hypoxia inducible genes as for example vascular growth factor (VEGF), platelet-derived growth factor (PDGF), GLUT1, that are implicated in several processes as neovascularization, glucose uptake, and others cell growth survival processes. Temsirolimus and Everolimus inhibit the kinase activity of the mTOR complex 1 (mTORC1); Bevacizumab is a VEGF ligand-binding antibody; sunitinib, sorafenib, axitinib, and pazopanib are small molecule inhibitors of multiple tyrosine kinase receptors including VEGF-R and PDGFR (Picture taken from [Shuch 2015](#)).

During the last 8 years, targeted therapies against these different dysregulated pathways have been developed and tested in different trials either alone or in combination (figure N°6).

Note: Definitions to understand trials outcomes (According to [National Cancer institute dictionary](#))

Progression Free survival (PFS): The length of time during and after the treatment of a disease, such as cancer, that a patient lives with the disease but he does not get worse. In a clinical trial, measuring the PFS is one way to evaluate how a new treatment works.

Overall Survival (OS): The length of time from either the date of diagnosis or the start of treatment for a disease, that patients diagnosed with the disease are still alive. In a clinical trial, measuring the overall survival is one way to see how well a new treatment works.

Hazard ratio (HR): A measure of how often a particular event happens in one group compared to another group, over time. In cancer research, hazard ratios are often used in clinical trials to measure survival at any point in time in a group of patients who have been given a specific treatment compared to a control group with another treatment or a placebo. A hazard ratio of one means that there is no difference in survival between the two groups. A hazard ratio of greater than one or less than one means that survival was better in one of the groups.

Objective response rate (ORR): Objective response means either a partial or complete response. Rate is expressed in percentage of each case.

A) VEGF/VEGF Receptor (VEGFR) inhibitors

- Bevacizumab

Bevacizumab (BEV) is a humanized monoclonal antibody that interacts with circulating VEGF and blocks it's binding to its receptor (VEGFR) thus inhibiting the angiogenesis pathways implicated in ccRCC ([Presta 1997](#)).

This novel targeted agent was tested in ccRCC in a randomized phase II trial with intravenous treatment every 2 week with 10 mg/kg of BEV. Results showed that, compared to the

control (placebo), the PFS was extended in median 4.8 vs 2.5 months. However the response rate was low (10%) ([Yang 2003](#)).

More recently, the AVOREN trial ([Escudier 2007](#)) and the CALGB 90206 trial ([Rini 2008](#)) compared the BEV + IFN α versus IFN α alone. The objective response rate (28.4 vs 12.9% for BEV + IFN α vs IFN α or IFN α + placebo), and the progression free survival (10.2 months vs 5.4 months for BEV + IFN α vs IFN α or IFN α + placebo) were substantially improved when there was a combination of BEV+ IFN α versus the IFN α alone or in combination with placebo.

In 2009, the FDA approved the use of BEV+ IFN α for the treatment of advanced RCC. ([FDA](#))

- Sunitinib

Sunitinib is an orally administered multi-kinase tyrosine kinase that blocks VEGFR-1, -2, platelet derived growth factor receptor- α and β (PDGFR- α/β), and related tyrosine kinases (RTKs) ([Roskoski 2007](#)).

The pivotal phase 3 clinical trial NCT83889 ([Motzer 2007](#)) including patients with previously untreated mRCC and mainly favourable or intermediate prognostic risk, showed that median progression free survival (PFS) was significantly longer with Sunitinib than IFN α (11 months versus 5 months). A higher objective response rate (ORR) was also reported with Sunitinib versus IFN α (independent review, 39% vs 8%; $p < 0.000001$). The OS gave a very small statistical significance ($P = 0.051$). Further analysis revealed that the Sunitinib's group of patients that did not received any treatment after the trial, extended their overall survival compared to the IFN α group (28.1 months versus 14 months) ([Motzer 2009](#)) . Sunitinib is today, a standard of care in the first line treatment of mRCC.

- Sorafenib

Sorafenib is another multi-kinase inhibitor targeting VEGFR-1, VEGFR-2, VEGFR-3, PDGFR- α and - β and Receptor Tyrosine Kinase (RET) although it was found originally as a Ras-Raf-MEK-ERK pathway inhibitor ([Wilhelm 2004](#) ; [Carlomagno 2006](#)).

As the first studies to determine its efficacy, showed little difference between the PFS reported for Sorafenib alone (5.7 months) versus IFN α alone (5.6 months), Sorafenib was not approved as a first line of treatment.

In another study that compared groups of patients treated with INF- α or Sorafenib alone, where after disease progression both were switch to sorafenib (dose escalation in the second case), authors observed tumor size reduction, better quality of life, and improved tolerability ([Escudier 2007](#)). Results showed better outcome in these patients that were cytokine refractory with an improved PFS as compared with that of placebo (5.5 versus 2.8 months) ([Escudier 2009](#)).

In conclusion, Sorafenib is recommended to be use in cytokine-refractory patients with advanced or metastatic renal cell carcinoma, although there are very high toxicities associated. Some of them are diarrhea (48%), rash and desquamation (41%), alopecia (31%), hand-foot syndrome (33%), and fatigue (29%) ([Escudier 2009](#)).

- Pazopanib

Pazopanib is a multi-kinase inhibitor of VEGFR-1, -2, and -3, PDGFR- α and - β , and c-Kit (Sonpavde 2007).

Its activity over mRCC was demonstrated in a phase III randomized trial ([Sopavde 2008](#)). In this study, two arms were examined: a population of patients that were naïve for any other treatment versus placebo and another arm with a group who had pre-cytokine-based treatment versus the naïve group treated with Pazopanib. In the treatment-naïve subpopulation, the PFS for Pazopanib was 11.1 months versus the 2.8 months with placebo. In the case of the cytokine-pre-treated subpopulation, the PFS was of 7.4 months compared to the 4.2 months of placebo.

The toxicity of Pazopanib is similar to, if not less severe than, that of similar molecules such as Sunitinib and Sorafenib, becoming a promising alternative treatment against mRCC.

- Axitinib

Axinitib emerged as an alternative for patients who had failed in the response to prior systemic therapy. This VEGFR-1, VEGFR-2, and VEGFR-3 tyrosine kinase inhibitor, has been

shown to be 450 times more potent to target these receptors than the first generation of VEGF inhibitors.

The AXIS trial corresponding to therapies in patients with mRCC refractory to Sunitinib plus Sorafenib cytokines and Sorafenib (group 2; n = 29), or Sorafenib alone (group 3; n = 15), showed a benefit of 2 months in the PFS compared to Sorafenib (6.7 months versus 4.7 months), as well, the ORR survival was increased. For groups with mRCC refractory to Sunitinib and Sorafenib, the ORRs were 7% with PFS of 7.1 months, whereas, in patients with mRCC refractory to cytokines, the ORR was 28% with PFS of 9 months and in those refractory to Sorafenib the ORRs were 28% with PFS of 7.7 months. Toxicity and adverse events included fatigue (13%), hypertension (11%), and hand–foot syndrome (11%) ([Cella 2013](#); [Motzer 2013](#); [Escudier 2014](#); [Rini 2011](#); [Ueda 2013](#)). These results establish Axitinib as a second-line treatment option for patients with metastatic renal cell carcinoma.

B) mTOR inhibitors

- Temsilotimus

Temsilotimus is an inhibitor of the mammalian target rapamycin mTOR. Treatment of cancer cells with this agent induces cell cycle arrest and inhibition of angiogenesis through regulation of VEGF that is under the regulation of the HIF pathway ([Hudson 2002](#); [Faivre 2006](#)).

Its efficacy was investigated in a randomized phase-III study ([Hudes 2007](#)) that included patients that had poor prognosis factors according to the Memorial Sloan-Kettering Cancer Center (MSKCC) prognostic criteria ([Motzer 2002](#)). The comparison was done between three arms: The first one, treatment with only IFN α ; the second one, treatment with Temsilotimus and the third one, the combination of both. The results of ORR did not differ significantly between arms: INF α (4.8%), Temsirolimus (8.6%), and combination therapy (8.1%). PFS showed a benefit of 2 months for the second group (Temsilotimus alone, compared to the first group of INF- α alone). OS was higher as well in the second group (10.9 versus 7.3 months). However, when patients on the combination regimen were compared with the interferon- α group, PFS and OS were similar, being 4.7 and 8.4 months, respectively.

Nevertheless, there are high toxicities associated to this treatment. In 11% of the patients the most common grade 3 and 4 side effects are asthenia and hyperglycemia.

Based on positive survival and PFS results, Temsirolimus is recognised in recent guidelines as a first-line treatment option for patients with mRCC who have poor MSKCC prognostic factors.

- Everolimus

Compared to Temsirolimus, this mTOR inhibitor is administered orally and has a different active form. In RECORD-1 trial ([Motzer 2008](#)), patients with metastatic renal cell carcinoma, which had progressed on Sunitinib, Sorafenib, or both, were randomly assigned into a group treated with Everolimus versus a group treated with placebo. The outcome of this study showed no difference in the median OS between groups at the end of double-blind analysis. PFS was evaluated in an unblinded analysis, since at disease progression patients with placebo were offered to turn into Everolimus. Median PFS was significantly improved on this late group compared to the placebo group (4.9 versus 1.9 months). Lack of increased OS was likely the result of the crossover design of the study ([Motzer 2010](#)).

Toxicities associated to this treatment are mostly of grade 3 and are observed in 15% of patients. These toxicities, stomatitis (44%), infections (37%), asthenia (33%), fatigue (31%), diarrhea (30%), rash (29%), nausea (26%), anorexia (25%), peripheral edema (25%), vomiting (24%), thrombocytopenia (23%), hypercholesterolemia (77%), hypertriglyceridemia (73%), and hyperglycemia (57%) are comparable to those induced by the Temsirolimus treatment (Hudson 2007). In addition, Everolimus-induced pneumonitis is a side effect in 4% of patients ([Motzer 2010](#)).

Based on the data from this trial, Everolimus is now the recommended therapy in patients who have progressed on prior VEGF-targeted therapy.

C) MET inhibitors

- Cabozantinib

Cabozantinib has arisen as a novel alternative and second line treatment for patients that have failed to VEGFR therapy. This inhibitor of tyrosine kinases including MET, VEGFR, and

AXL has shown to improve the median overall survival (21,4 months) compared to everolimus (16,5 months).

Cabozantinib treatment also resulted in improved progression-free survival (7.4 months in patients treated with cabozantinib compared to 3,8 months in patients treated with everolimus). The most frequent type of adverse effects were grade 3 or 4. hypertension (49 [15%] in the cabozantinib group vs 12 [4%] in the everolimus group), diarrhoea (43 [13%] vs 7 [2%]), fatigue (36 [11%] vs 24 [7%]), palmar-plantar erythrodysesthesia syndrome (27 [8%] vs 3 [1%]), anaemia (19 [6%] vs 53 [17%]) , between others. ([Choueiri 2016](#), [Grassi 2016](#))

Based on these results, cabozantinib has been approved for the treatment of mRCC as a second line therapy.

4.3 Sequential and Combinational therapy

Often, mRCC become recurrent due to the acquisition of resistance to the first line treatment. Sequential therapy, allows the treatment of this cancer as a chronic disease. Table N°2 summarises some of the several efforts that have been done to establish the best strategy of sequencing targeted therapies after cytokine pre-treated disease progression.

The main treatments for mRCC are molecular target therapies focused in two axes: The VEGF based therapies and the mTOR inhibitor therapies.

The current paradigm in mRCC treatment is sequential monotherapy where patients are treated with first line therapy in naïve patients until the disease progress and then a second line therapy is applied. As discussed before, VEGF-based therapies are the most standard first treatment for mRCC, although complete response and durable treatments are not frequent. Resistance to VEGF-TKIs appears around 6 to 11 months after treatment ([Motzer 2007](#); [Escudier 2007](#)).

Combinational therapies

Numerous combinations of cytokines, with or without other systemic therapies, have been tried, but no regimen has been found to be consistently superior. In addition, it appears that

only a relatively small proportion of highly selected patients with mRCC benefit from cytokine therapy.

In the next two tables (Table N°2 and Table N°3), I will resume the several trials that have been performed to obtain the best systematic and combinational therapies in cytokine and VEGF inhibitor refractory patients.

Clinical Trial name	Reference	Combination	n	PFS (months)	OS (months)
Cytokine preteated					
TARGET	Escudier 2009	Sorafenib vs placebo	903	5,8 vs 2,8	17,8 vs 14,3
NCT00334282	Sternberg 2013	Pazopanib vs placebo	405 (prior cytokines n=202)	post cytokines (7,4 vs 4,2)	22,9 vs 20,5
AXIS	Motzer 2013	Axinitib vs Sorafenib	723 (prior cytokines n=251)	post cytokines (12,2 vs 6,5)	20,1 vs 19,9 (OP)
NN	Yang 2003	Bevacizumab HD vs Bevacizumab LD	116 (post IL-2 n=107)	4,8 vs 3	NS
		Bevacizumab HD vs placebo		4,8 vs 2,5	
VEGF inhibition refractory					
RECORD-1	Motzer 2008	Everolimus vs placebo	416 (OP)	4,6 vs 1,8	18,8 vs 14,4 (OP)
AXIS	Motzer 2013	Axinitib vs Sorafenib	723 (sunitinib pretreated n=389)	post sunitinib: 4,8 vs 3,4	20,1 vs 19,2
INTORSECT	Hudson 2014	Temsirolimus vs Sorafenib	512	4,3 vs 3,9	12,3 vs 16,6
RECORD-3	Motzer 2014	Sunitinib/ Everolimus vs Everolimus/Sunitinib	n = 243 and n= 271 for each group	10,7 vs 7,9	32 vs 22,4
SWITCH	Eicherlberg 2015	Everolimus vs placebo	n =208 and n=256 for each group	NS	NS
METEOR	Choueri 2015	Cabozatinib vs Everolimus	658	7,4 vs 3,8	20 vs 15
Overall population (OP) number of patients (n)					

Table 2: Retrieved phase 2 and 3 studies from systematic research in the cytokine- refractory and in the post-vascular endothelial growth factor inhibition setting. PFS: Progression Free survival, OS: Overall survival, n: number of patients. Table adapted from ([Albiges 2015](#))

Clinical Trial name	Reference	Combination	n	RR%	PFS (months)
INF based					
AVOREN	Escudier 2007	IFN and Bevacizumab vs IFN placebo	306/289	31/13	10,2/5,4
	Escudier 2010	IFN and Bevacizumab vs IFN	366/349	26/13	8,5/5,2
ARCC	Hudes 2007	IFN vs Temsilorimus alone	207/209	4,8/8,6	3,1/5,5
		IFN vs IFN and Temsilorimus	207/210	4,8/8,1	3,1/4,7
NN	Jonasch 2010	Sorafenib and IFN vs Sorafenib alone	40/40	25/30	7,6/7,4
IL-2 based					
ROSORC	Procopio 2011	Sorafenib and IL-2 vs Sorafenib	66/62	27/15	7,6/6,9
Bevacizumab based					
TORAVA	Négrier 2011	Bevacizumab and Erlotinib vs Bevacizumab and placebo	53/50	14/13	9,9/8,5
		Bevacizumab and Temsilorimus vs Sunitinib	88/42	27/29	8,2/8,2
		Bevacizumab and Temsilorimus vs Bevacizumab and INF	88/41	27/46	8,2/16,8
INTORAC	Rini 2013	Bevacizumab and Temsilorimus vs Bevacizumab and INF	400/391	27/27,4	9,1/9,3
RECORD-2	Ravaud 2012	Bevacizumab and Everolimus vs Bevacizumab and INF	182/183	N/A	9,3/10
BEST	McDermontT 2013	Bevacizumab vs Bevacizumab and Temsilorimus	89/91	12/28	8,7/7,3
		Bevacizumab vs Bevacizumab and Sorafenib	89/90	12/30	8,7/11,3
		Bevacizumab vs Sorafenib and Temsilorimus	89/91	12/27	8,7/7,7
Sorafenib based					
NCT00467025	Rini 2012	Sorafenib and AMG 386 (3mg/kg) vs or (10mg/kg)	51/50	37/38	8,5/9
		Sorafenib and AMG386 (10 mg/kg) vs placebo	51/51	37/25	8,5/9

Table 3: Retrieved studies from systematic research of cytokine-based combination and targeted therapy-based combination PFS: Progression Free survival, OS: Overall survival, n: number of patients, RR: response rate [Albiges 2015](#)

5. MECHANISM OF ACQUIRED RESISTANCE IN THE TREATMENT OF ccRCC

5 Resistance to Tyrosine Kinase Inhibitors

According to the Response Evaluation Criteria in Solid Tumors or RECIST criteria, resistance is defined as a progression of the disease despite treatment application. The progression itself corresponds to at least 20% increase in the sum of the diameters of target lesions, taking as reference the smallest sum on study. As well the appearance of new lesions is considered as progression ([Rini 2009](#); [Eisenhauer 2009](#)).

The inhibitory effect of a certain targeted agent relies on how much the tumor is dependent on the particular pathway that is inhibited, to survive. So, resistance comes when the tumor becomes independent from the activity of drug-targeted pathway.

This independence can have two origins:

1. An intrinsic resistance, due to genetic alterations that pre-exist inside the tumor and that don't allow proper drug binding as a consequence of the lack of expression of crucial proteins.
2. An acquired resistance, defined as progression of the disease after the patients had have a benefit from a first treatment. This progression is due to the activation of alternative pathways or increased expression of specific molecules as an answer to the inhibition, this could be defined as a compensation ([Rosa 2013](#)).

Key players in signaling pathways are tyrosine protein kinases that can be divided in two major groups: Receptor Tyrosine kinases that have a crucial role in the transduction of extracellular signals into the cell, and the non-receptor tyrosine kinases that are implicated in intracellular communications. Tyrosine kinases targeted antiangiogenic inhibitors as sunitinib, sorafenib, pazopanib and axitinib, were actually designed as multi-target inhibitors (targeting both VEGF and PDGF pathways) since their effects could be broader than a single-targeted inhibitor ([Gotink 2010](#)). Unfortunately during the last years, there has been several reports of acquired resistance to these anti-angiogenic tyrosine kinase inhibitors ([Motzer](#)

[2007](#); [Escudier 2007](#); [Karashima 2013](#)). The response to anti-angiogenic targeted therapy can activate several mechanisms to induce resistance as described in the next Table N°4 and represented in Figure N°7 and reviewed in [Giuliano 2013](#)).

Process name	Cell type involved	Mechanism of resistance	Results	References
<i>EMT (Epithelial to Mesenchymal transition)</i>	Epithelial cells	Protein accumulation take place after long exposure of cells to extra-cellular stimuli.	Polarized cells convert into motile mesenchymal cells, allowing escape from their biological structure. B-catenin translocates to the nucleus activating the Wnt canonical pathway. Matrix Metalloproteinases (MPPs) are upregulated and decrease cell-cell interaction.	Harada2012 ; Hugo2007 ; Klymkoswky 2009 ; Chaffer 2011 ; Chuang 2008
<i>EMT induced CSCs (Cancer Stem cells)</i>	Separated population	Inducers of EMT such as TGF- β , Wnt or Notch cause cells to acquire cellular markers of CSCs.	EMT induces cell to transform into CSCs associated with reversible epigenetic changes. Then these cells will form a new tumor population (metastasis) with chemo refractory features.	Huang 2013 ; Hollier 2009 ; Singh 2010
<i>TAM (Tumor associated macrohages)</i>	Tumor associated macrophages	M2 are highly present in infiltrate ccRCC. TAMs triggers EMT through regulation of TLR4/IL-10, Versican V1, TGF- β 1, Gas6/Ax1, miR488, FoxQ1 and NF κ BIL-10. Increases overproduction of PDGF, TGF β , VEGF.	Through increase of proangiogenic factors, there is increase vessel formation and tumor adaption to hypoxia. EMT is increase. Patients having TAMs'high levels in their serum have poor prognosis outcome	Ikemoto 2003 ; Zhang 2015 ; Santoni 2013 ; Dannennman 2013
Process name	Cell type involved	Mechanism of resistance	Results	References

<i>Lysosomal Sequestration</i>	ccRCC cells	sunitinib is hydrophobic, weak base drug and thus is sequestered in acidic lysosomes via ion trapping mechanism.	Antiangiogenic inhibitors affect not only endothelial cells but also tumor cells, although this is dependent of the drug concentration. Inside the human or experimental tumors the drug concentration correspond to the one that can inhibit tumor cell proliferation. Drug concentration in serum are 10 times lower than inside the tumor. Continuous exposure to sunitinib induces lysosomal sequestration in ccRCC cells, so, therapeutic concentrations are not achieved.	Gotink 2011 ; Logan 2013 Giuliano2015 Santoni 2013
<i>Increase Pericyte coverage of tumor cells</i>	Perivascular cells/ vascular smooth muscle cells	In tumoral conditions, endothelial cells activate pericytes that induce newly highly differentiated vessels. Stabilizing process of abnormally complicated vascular system formation. Pericytes depletion leads to EMT associated with increase hypoxia conditions.	Excessive angiogenesis of the ccRCC, more aggressive tumor type with increase number of metastasis, poor prognosis.	Ribatti 2011 ; Armulik 2011 ; Hall 2006 ; Cao 2013 ;
<i>Angiogenic switch</i>	Vascular cells	Elevated expression of IL-8 (potent proangiogenic factor). Expression of independent VEGF	ccRCC progression, neo-angiogenesis after tyrosine kinase treatment.	Huang 2010 ; Mizukami 2005 ; Porta 2013

		angiogenic factor as cytokines ie: basic fibroblast growth factor (bFGF), hepatocyte growth factor (HGF), and interleukin (IL)-6		
<i>Accumulation of bone marrow derived cells</i>	Vascular progenitor cells pro-angiogenic monocytes VEGFR-1 hemangiocytes, myeloid cells	Bone marrow derived cells accumulation inside and around the tumor; new blood vessels supplying the arising tumor.	ccRCC adaptation to hypoxia conditions through neovascularization, tumorogenesis.	Paèz-Ribes 2009 ; Dewhirst 2013 ; Loges 2010 ; Ahn 2009

Table 4: Mechanism of resistance to Tyrosine kinases inhibitors in ccRCC.

(Adapted from [Bielecka 2014](#))

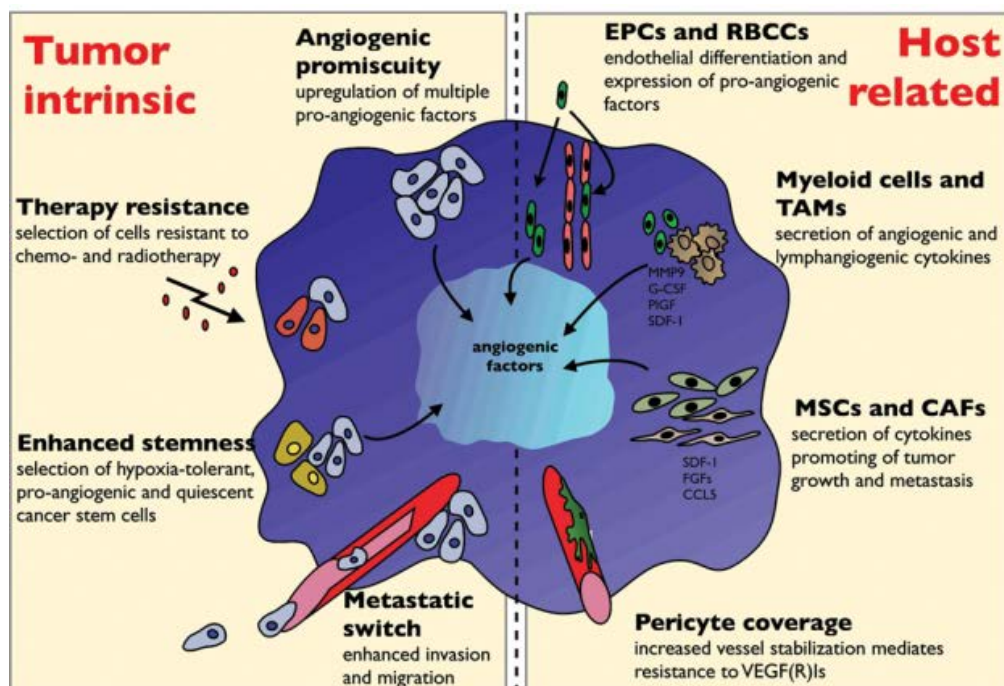


Figure 7: Mechanisms of resistance to anti-angiogenic targeted therapies. Several processes are activated after long exposure to inhibitors that induces hypoxia in the tumor. These responses can have a tumor intrinsic origin as for example

augmentation of pro-angiogenic factors, increased number of cells that have become resistant to chemo- and radiotherapy, increased selection of hypoxia-tolerant and pro-angiogenic cancer stem cells (CSCs), and enhanced invasion and migration properties of tumor cells. Host-related mechanisms of resistance: hypoxia increases recruitment of endothelial progenitor cells (EPCs) and of bone marrow-derived circulating cells (RBCCs) toward the tumor vasculature, where they induce endothelial differentiation by secretion of angiogenic cytokines. Tumor-associated macrophages (TAMs) can infiltrate the tumor and mediate resistance by secreting tumor-promoting, angiogenic, and lymphangiogenic cytokines. Cancer-associated fibroblasts (CAFs) support tumor growth and angiogenesis by secreting fibroblast growth factors (FGFs). Increased coverage with pericytes enhances resistance by stabilization of the newly formed vessels. Picture taken from [Sonjas 2010](#).

5.2 Resistance to mTOR inhibitors

The discovery of the molecular basis in the ccRCC has led to design more targeted therapies against it. As discussed before, the Hypoxia Inducing Factor HIF has a determinant role in the tumorigenesis as mediator of the angiogenesis due to the mutation of the VHL tumor suppressor. Angiogenesis inhibitors prevent the formation of blood vessels rather than inhibiting proliferation of tumor cells ([Dordevic 2009](#); [Nicol 1997](#)).

In the case of the mTOR pathway, its targeting becomes crucial since its activation leads to constitutive HIF-1 α expression, indicating then that inhibiting this pathway could not only affect tumor angiogenesis but also cell proliferation as this pathway is hyperactivated both in epithelial and cancer cells. ([Salvadori 2012](#))

There are two main mTOR inhibitors that are now frequently used, Temsirolimus and Everolimus alone or in combination (see Treatment of RCC section), however they do not offer long lasting response and almost all patients show disease progression during the treatment. ([Batelli 2012](#))

To better understand the mechanism of resistance to the mTOR, first we will take a look to Figure N°8, that represents the complexity of the mTOR pathway.

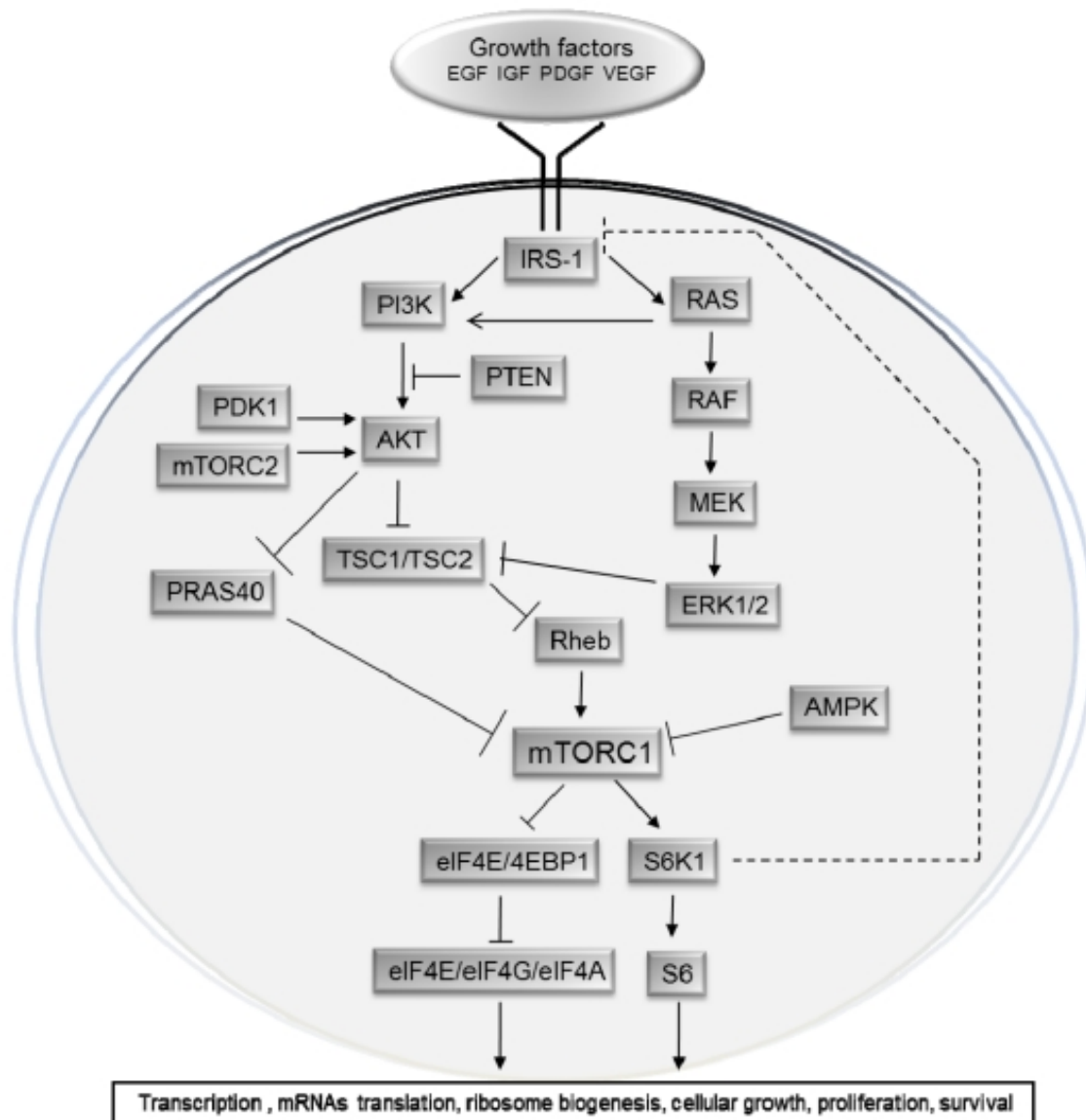


Figure 8: Scheme of the mTOR pathway

mTOR is involved in a complicated network of interactions linking growth factors receptors and intracellular regulatory factors in the process of angiogenesis, tumorigenesis and cellular metabolism. Picture taken from ([Populo 2012](#))

It is useful to differentiate two basic signalling cascades leading to mTOR activation. In the first pathway, insulin binds to its Insulin receptor substrate (IRS1) inducing its activation. The signal is then transduced by the phosphatidylinositol 3-kinase (PI3K), which subsequently will activate phosphoinositide –dependent-kinase (PDK1) and then AKT (protein kinase B) ([Laplane 2012](#); [Taniguchi 2006](#); [Wang 2006](#)).

In a second pathway, after Ras activation, there will be a cascade of activation through Raf and the MEK 1 /2 to mitogen activated protein kinases (MAPK) and ribosomal s6 kinases (RSKs). Together these two signal cascades will increase phosphorylation of tuberin (TSC2) inactivating the harmatin-tuberin complex formed with TSC1 ([Carrière 2008](#); [Chang 2003](#)).

Inactivation of TSC1/TSC2 leads to mTORC1 activation, although another mechanism can also activate mTORC1 independently of TSC1/TSC2 ([Laplane 2009](#), [Wang 2007](#)).

There are crosstalks between elements of PI3K/AKT, leading to activation of mTORC1 through activation of not only PDK1 but also mTORC2 that phosphorylates and fully activates AKT. Indicating that interaction between mTOR complexes are bidirectional ([Sarbasov 2005](#)).

There are two main downstream targets of mTORC1 activity, the eukaryotic initiation factor 4E (IeF4E)-binding protein 1 (4E-BP1) and the ribosomal p70 S6 kinase 1 (S6K1). ([Khaleghpour 1999](#)) Stimulation of this last one, can lead to activation of HIF-1 α and cell cycle regulators as c-myc and cyclin D1 ([Choo 2008](#)).

In ccRCC, VHL inactivation impedes HIF-1 α and/or HIF-2 α degradation. Upregulated levels of HIF's subunits should inhibit mTORC1 through the upregulation of REDD1. However, this is not the case, since it was reported that mTORC1 is often activated in ccRCC ([Rini 2009](#)).

There are several genes that are mutated in the PI3K signalling pathway regulating mTORC1 and its inhibition. Table N°6, represents some of the mutated genes and their implications in drug resistance.

Gene	Gene Function	Abnormality in resistance	Impact on mTOR network	References
VHL	Tumor suppressor gene	Inactivating mutation	Defective proteolysis of HIF α that is not recognized by E3 complex.	Kong 2006 Hasse 2009
			MAPK upregulation (due to increase PI3K pathway)	Pal 1997 Hasse 2009
			IGFR upregulation	Cardillo 2013 Yuen 2007
			AKT upregulation (due to increase PI3K pathway)	Hasse 2009
mTORC2	Proliferation, angiogenesis, cell metabolism	Activating mutation	When inhibition of mTORC1 up-regulates PI3K/AKT, resistance is driven by mTORC2 through activation of AKT and HIF-2 α .	Harada 2013
TSC2	Tumor suppressor gene (possible)	Inactivating mutation	Under hypoxia conditions, TSC2-deficient cells fail to down-regulate S6K phosphorylation (and activity) and accumulate HIF in excess.	Deyoung 2008 Brugos 2004
REDD1	Tumor suppressor gene (possible)	Inactivating mutation	REDD1 mutants fail to elicit TSC2/14-3-3 dissociation leading to TSC2/TSC1 inactivation and failing to inactivate mTORC1 in response to hypoxia.	Kucejova 2011 Deyoung 2008

Table 6: Mutations in the mTOR pathway and its implication in mTOR inhibitors drug resistance in ccRCC. Adapted from [Kornakiewicz 2014](#).

In the case of Tyrosine Target Therapies (TKI's), there are reports that confirmed that the increased overall survival of patients that underwent cyto-reductive nephrectomy compared with those that did not, was not associated to their treatment with or without vascular endothelial growth factor targeted agents ([Choueiri 2011](#)). The COMPARZ trial showed that both the Pazopanib (PDGFR inhibitor) and Sunitinib (PDGFR and VEGFR inhibitor) extended the progression free survival in equal manner, in patients that did not have any other treatment before, although most patients had been submitted to nephrectomy. ([Motzer 2013](#)) So the real influence of nephrectomy could not be correctly evaluated. To address this question more precisely, the TARIBO trial classifies patients to study the statistical significance of patients that had or not nephrectomy before TKIs' treatment (Pazopanib or Sunitinib). First results indicate that there is a benefit on the overall survival cyto-reductive nephrectomy with TKIs (Sunitinib or Pazopanib) compared to single TKIs treatment in subjects with mRCC.

CHAPTER 2: THE ROLE OF HYPOXIA IN ccRCC

As described before, the majority of ccRCC have somatic mutation of both alleles of VHL leading to an inactive form of VHL. The identification of this mutation has allowed the characterization of important ccRCC-associated pathways. Importantly, absence of this tumor suppressor induce stability and increase levels of hypoxia inducible factors (HIF). The two main HIF- α isoforms have opposing effects on RCC biology, possibly through distinct interactions with additional oncogenes; In this chapter I will describe the roles of pVHL and the how its dysregulation affects HIF pathways been one of the main drive forces that promote kidney cancer.

1.VON HIPPEL LINDAU TUMOR SUPPRESSOR GENE (VHL)

1.1 VHL gene products

The VHL gene encodes for 2 different mRNA variants that will give place to 3 isoforms of the VHL gene product (pVHL) ([Gnarra 1994](#)). Variant number 1 (V1) encodes for a protein of 213-aminoacid (pVHL₃₀) that is primarily found in the cytoplasm with some expression in the nuclear and the membrane compartment, and a 160-amino-acid protein (pVHL₁₉) that is equally distributed in the nucleus and in the cytoplasm ([Iliopoulos 1998](#)). Both pVHL₂₁₃ and pVHL₁₆₀ are functional tumor suppressors. Indeed, reintroduction of either isoform in *VHL*-defective ccRCC cells suppresses their ability to form tumors when xenografted in nude mice ([Schoenfeld 1998](#)).

The second mRNA variant generates a protein of 172-amino-acids (pVHL₁₇₂), that has been described to be present in several organs during human embryogenesis and in sporadic ccRCC with specific VHL point mutations ([Martella 2006](#); [Taylor 2012](#)). New tools have been developed to detect this isoform that sometimes is the only one present in the ccRCC samples, suggesting that its presence is not sufficient to act as a tumor suppressor or even could induce and maintain tumorigenesis ([Chesnel 2015](#)).

1.2 VHL and the HIF axis

VHL is part of the “VCB” complex together with the transcription elongation factors C and B (elongin C and B from now on) ([Duan 1995](#); [Kibel 1995](#)) . As well, it is bound to the Cullin 2 (CUL2) and the RING finger protein (RXB1) forming the VCB-CR complex ([Kamura 1999](#); [Pause 1997](#)).

The entire VCB complex is resistant to proteasomal degradation ([Shoenfeld 2000](#)); Through an exhaustive whole genome and whole exome RNA sequencing in more than 100 ccRCC samples, Sato and colleagues found that tumorigenesis in ccRCC can be link to mutations in the Transcription elongation factor polypeptide 1 (TCEB1) that encodes for the elongins ([Sato 2013](#)). The fact that elongins are in charge of linking pVHL to the heterodimers of CUL2 and RXB1, (Shoenfeld 2000) could indicate that the tumorigenic effects seen in renal carcinoma due to hypoxic conditions are more related to dysfunctions in the VCB complex than the pVHL alone ([Hakimi 2015](#); [Brugarolas 2014](#)).

pVHL consist in two highly coupled domains: The domain α that directly interacts with the elongins ([Lonergan 1998](#);) and the domain β where the Hypoxia Inducible Factors (HIF α) (HIF-1 α or HIF-2 α (also called EPAS1)) bind. This binding is dependent on the hydroxylation by the 3 prolyl hydroxylases (PHD1, PHD2 and PHD3) of two conserved prolines residues within HIF α ([Bruick 2001](#); [Min 2002](#)). This hydroxylation requires oxygen as a co-substrate and is active under normoxic conditions ([Ivan 2001](#); [Jaakkola 2001](#)).

As explained in the Figure N°9, under physiological conditions (normoxia), HIF α is recognized by the VCB-CR complex due to its prolyl-hydroxylation by the PHDs. Then HIF α is ubiquitinated by the E3 ligase and sent to degradation through the 26S proteasome ([Jaakkola 2001](#); [Ohh 2000](#)).

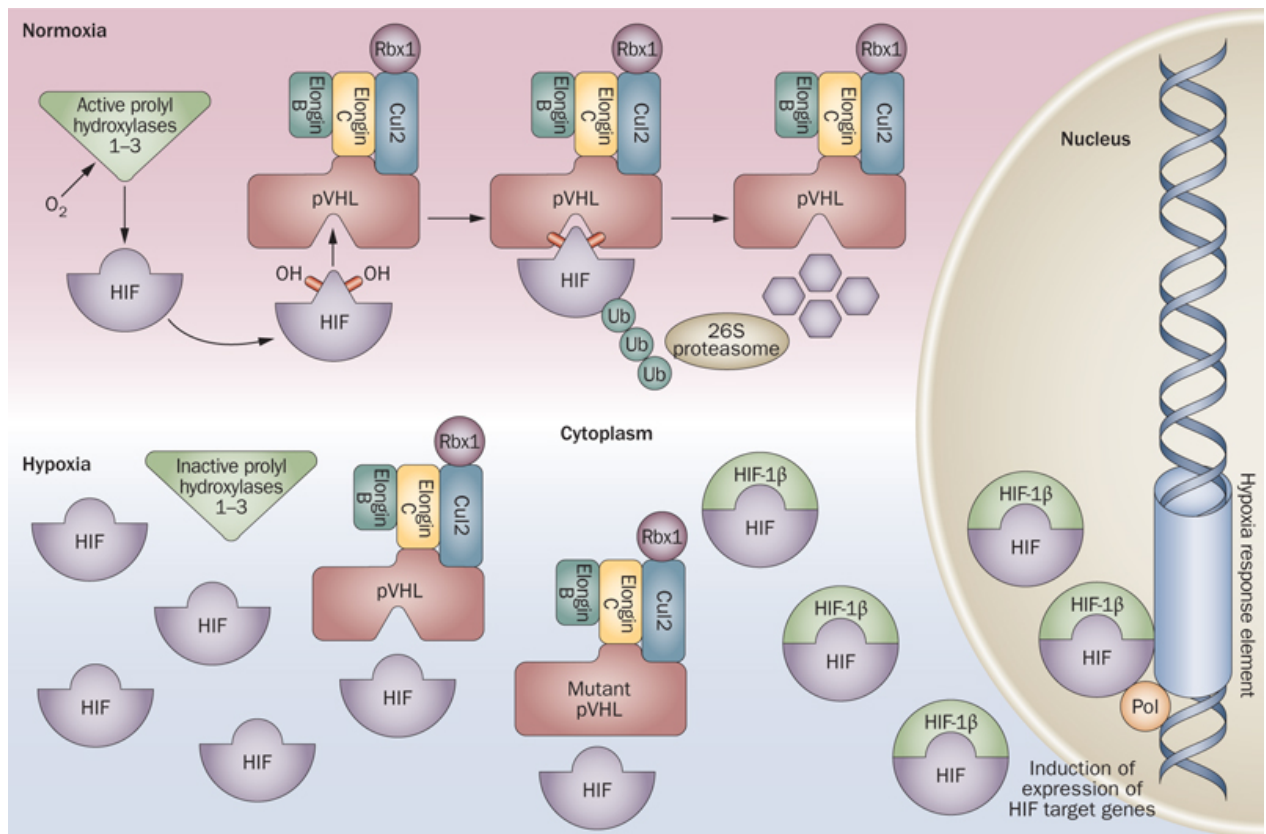


Figure 9: HIFα Oxygen-dependent regulation.

In normoxic conditions, prolyl hydroxylases 1–3 hydroxylate HIFs-1α and -2α, which are recognized by the ubiquitin ligase complex pVHL-E3-ElonginC-B-Cul2-Rbx1 and sent towards proteasomal degradation. In hypoxic conditions, the prolyl hydroxylases are inactive, so non-hydroxylated HIF-1α and -2α accumulate and form heterodimers with HIF-β. These heterodimers translocate to the nucleus, bind to hypoxia response elements and initiate transcription of genes involved in adaptations to hypoxia. These adaptations involve increased matrix metabolism, proliferation, expression of VEGF and carbonic anhydrase IX, differentiation and viability.

Abbreviations Cul2, cullin 2; HIF, hypoxia-inducible factor; Pol, DNA polymerase II and other transcriptional co-activators; Rbx1: RING finger protein; pVHL, von Hippel–Lindau protein; Ub, ubiquitin. Taken from [Gossage 2010](#).

Under hypoxic conditions, the PHD are inactive since oxygen is an essential co-factor, HIF forms a heterodimer with HIF-1 β and translocates to the nucleus where it binds to the Hypoxia Response Elements (HRE) ([Wang 1995](#); [Semenza 2001](#)) to initiate the transcription of over 800 HIF target genes, allowing cellular adaptation to low oxygen levels (Reviewed by [Semenza 2012](#)).

Although HIF-1 α and HIF-2 α share almost 48% of homology on their amino-acid sequences, they have been shown to target different sites of transcription. HIF-1 α –HIF-1 β heterodimers preferentially induce transcription of genes associated with apoptosis and gluconeogenesis, whereas HIF-2 α –HIF-1 β heterodimers mostly activates the transcription of genes associated with angiogenesis and erythropoiesis. When pVHL is mutated, it becomes unable to bind to the hydroxylated sites, resulting in accumulation of HIFs and increased transcription of HIF target genes similar to that caused by hypoxia ([Gossage 2010](#); [Rankin 2008](#)).

As mentioned before, people that harbour a defective VHL tumor suppressor gene are predisposed to have Clear cell carcinoma. VHL gene is located in the short arm of the chromosome 3 (3p25). In the case of the von Hippel Lindau disease, one allele is deleted and, in most cases, tumor development is due to somatic inactivation of the remaining wild-type allele in the cell ([Maher 1997](#)). This disease is not frequent and it has been largely shown that most patients with clear cell renal carcinoma have origin with spontaneous bi-allelic inactivation of the VHL gene, either by loss of heterozygosis (LOH) (98% of cases) or hypermethylation (5-30% of cases) ([Glasker 2011](#); [Gnarra 1994](#)).

The reported incidence of somatic VHL mutations in sporadic ccRCC highly varies and includes a large list of genes ([Catalogue of Somatic Mutation COSMIC](#)), as PBRM1 (30%) (Tumor suppressor that encodes for BAF180 protein, implicated in chromatin remodeling and gene expression) ([Varela 2011](#); [Burrows 2010](#)); BAP1 (15%) (Deubiquitinase related to regulation of the monoubiquitinated histone 2A lysine 119 (H2AK119ub1); mTOR signaling implicated in cell proliferation ([Pena-Llopis 2012](#)) and SETD2 (15%) (Histone methylase that controls chromatin structure and transcription) ([Hakimi 2013](#)), between others. These studies suggest that PBRM1 and SETD2 genes are mutated together with VHL in ccRCC, otherwise they could constrain transformation driven by VHL.

This inactivation compromises the function of pVHL, where HIF α stabilization and degradation is impaired even in conditions of normoxia. This promotes persistent translocation of HIF α to the nucleus and activation of the downstream genes that would normally be activated only in conditions of hypoxia. The accumulation of these genes directly contributes to tumorigenesis (See table N°5).

pVHL has also several non-HIF dependent functions that are resumed in the same table N°5. How these functions cooperate with HIF dependent functions in ccRCC is not yet very clear.

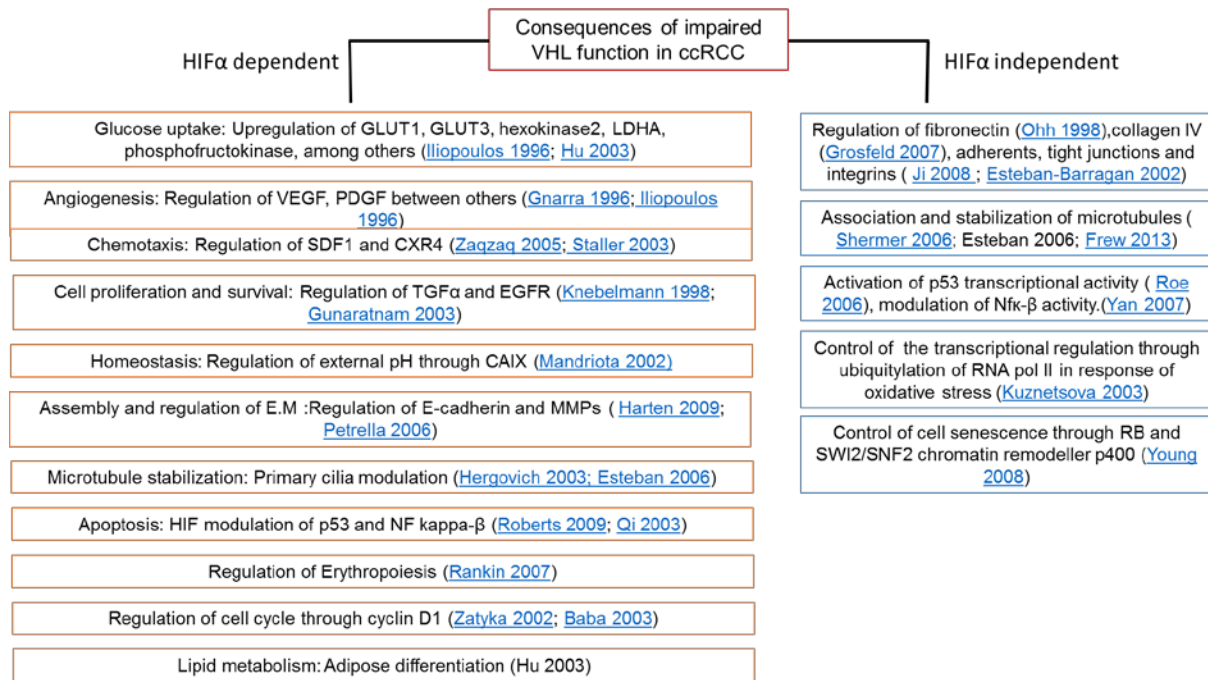


Table 5: pVHL functions in HIF α -dependent and independent fashions. Modified from [Gossage 2015](#); [Gossage 2010](#)). CAIX, carbonic anhydrase IX; CXCR4, CXC-chemokine receptor 4; EGFR, epidermal growth factor receptor; GLUT, glucose transporter; HIF, hypoxia-inducible factor; LDHA, lactate dehydrogenase A; MMPs, matrix metalloproteinases; pVHL, von Hippel–Lindau protein; NF- κ B, nuclear factor- κ B; PDGF, platelet-derived growth factor; SDF1, stromal-cell derived factor 1 (encoded by CXCL12); TGF α , transforming growth factor- α ; VEGF, vascular endothelial growth factor.

2. HYPOXIA INDUCIBLE FACTORS

6 Structures and tissue expression level.

The HIF proteins are part of a family with 3 members (HIF-1 α , HIF-2 α and HIF-3 α) and two HIF β members (HIF-1 β and HIF-2 β). HIF-1 β is also called Aryl hydrocarbon receptor nuclear translocator (ARNT). Both HIF-1 α and HIF-2 α have two transcriptional activation domains, the N-terminal transactivation domain (NTAD) and the C-terminal activation domain (CTDA) that activates transcription when bound to the DNA ([Kaelin 2008](#)).

As discussed before, the degradation of the HIF-1 α and HIF-2 α isoforms is dependent on their recognition by pVHL of one or two hydroxylated prolyl residues within their NTAD, generated by PHD. Oxygen dependent post-translational modifications will lead to ubiquitination and later degradation through the proteasome ([Ivan 2001](#); [Jaakkola 2001](#); [Ju 2001](#)). This is described in the next figure N°10:

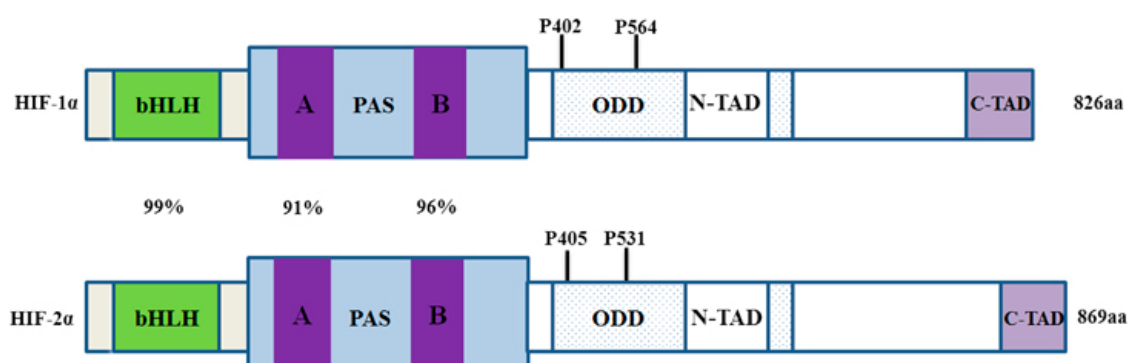


Figure 10: The structural domains of HIF-1 α and HIF-2 α . Both of them contain basic helix-loop-helix (bHLH), per-Arnt-SIM (PAS), oxygen-dependent degradation domain (ODD) and C-terminal transactivation domains (CADs). Prolyl hydroxylases (PHDs) hydroxylate proline residues 402 and 564, and 405 and 531, respectively, in ODD of HIF-1 α and HIF-2 α , under normoxic conditions, targeting it for degradation by the proteasome, respectively. Numbers refer to amino-acid similarity between human HIF-1 α and HIF-2 α in the defined domains. Picture taken from ([Zao 2015](#)).

An alternative oxygen dependent gene regulation is given by hydroxylation of a specific asparagyl residue within the CTAD by the asparaginyl hydroxylase (FIH1), which results in reduced transcriptional activity of HIF α . The CTAD of the HIF-2 α isoform is less sensitive to be targeted and degraded by the FIH1 than its HIF-1 α counterpart ([Lando 2001](#); [Bracken 2006](#)). As well there are different affinities of the PHD prolyl hydroxylases and the FIH1 to oxygen, been the last one more active at lower oxygen levels and expanding the range of HIF activation ([Koivunen 2004](#)).

HIF-1 α and HIF-2 α are expressed differentially in different tissues and even between cells types in some organs. In kidney, for example, HIF-1 α expression is detected mostly in tubular cells. In contrast, endothelial cells, fibroblast and glomerular cells show higher expression of the HIF-2 α isoform ([Schietek 2012](#); [Rosenberger 2002](#)).

The different roles of HIF subunits in ccRCC can be explained on how these subunits are regulated. After loss of function of the VHL gene, HIF-1 α is stabilized and releases HIF-2 α with increased expression of their target genes. This is possibly due to the fact that HIF-2 α is less sensitive to FIH1 degradation in conditions of profound hypoxia. Nonetheless, to overcome senescence and to promote tumor progression, other events dependent and independent from HIF α are necessary.

In summary, HIF- α is mainly an activator of a widespread list of genes that regulate angiogenesis, glycolysis, chromatin remodelling, cell cycle, and even the oxygen-sensing pathway itself ([Elvidge 2006](#); [Warnecke 2004](#)). In the ccRCC context, HIF-1 α and HIF-2 α show some overlap in the transcription of their targets while others are controlled only by one isoform (See table N° 8).

<i>Gene</i>	<i>Reported function</i>	<i>HIF isoform target</i>	<i>Reference</i>
VEGFA	Angiogenesis	HIF-1/HIF-2	Hu 2003
CXCR4	Chemotaxis	HIF-1/HIF-2	Staller 2003
CAXII	pH regulation	HIF-1/HIF-2	Hu 2003
GLUT1	Glucose transport	HIF-1/HIF-2	Hu 2003
MIR210	Mitochondrial function	HIF-1/HIF-2	McCormick 2013
GAS6/AXL	Metastasis	HIF-1/HIF-2	Rankin 2014
CDCP1	Metastasis	HIF-1/HIF-2	Razonerova 2011
KDM3A	Histone methylation	HIF-1/HIF-2	Krieg 2010
JARID1C	Histone methylation	HIF-1/HIF-2	Niu 2012
BNIP3	Apoptosis	HIF-1	Raval 2005
HK2	Glycolysis	HIF-1	Hu 2003
PFK	Glycolysis	HIF-1	Hu 2003
ALDOA	Glycolysis	HIF-1	Hu 2003
PGK1	Glycolysis	HIF-1	Hu 2003
LDHA	Glycolysis	HIF-1	Hu 2003
FBP1	Gluconeogenesis	HIF-1	Li 2014
SPAG4	Cell migration	HIF-1	Knaup 2014
CAIX	pH regulation	HIF-1	Grabmaier 2004
FYN	Metastasis	HIF-1	Razonerova 2011
HIG2	Lipid storage	HIF-1	Gimm 2010
PLIN2	Lipid storage	HIF-2	Hu 2003
CCND1	Cell cycle control	HIF-2	Raval 2005
MMP1	Matrix remodelling	HIF-2	Petrella 2005
TGF α	Inflammation	HIF-2	Raval 2005
SK1	Invasion, angiogenesis	HIF-2	Salama 2015

Table 6: List of genes in the ccRCC context that are regulated either by both HIF-1 α and HIF-2 α or only one of them.

Specifically, HIF-1 α regulates mostly genes that are implicated in the regulation of glycolysis and apoptosis, while HIF-2 α regulates genes related to invasiveness

(Adapted from Schödel 2016). Abbreviations: VEGFA: Vascular endothelial factor; CXCR4: CX chemokine Receptor 4; CAXII Carbonic anhydrase 12; GLUT1: Glucose transporter I; MIR210: MicroRNA 210; GAS6/AXL Growth arrest specific gene/ Tyrosine receptor; CDCP1: CUB Domain Containing Protein1; KDM3A: lysine demethylase 3A; JARID1C: Histone demethylase; BNIP3 BCL2/adenovirus E1B: 19 kDa protein-interacting protein 3; HK2: Hexokinase 2; PFK: Phosphofructokinase; ALDOA: Aldoase A; PGK1: Phosphoglycerate Kinase 1; LDHA: Lactate dehydrogenase A; FBP1: fructose-bisphosphatase 1; SPAG4: sperm associated antigen 4; CAIX: carbonic anhydrase 9; FYN: Proto-oncogene tyrosine-protein kinase; HIG2: Hypoxia-inducible protein 2; PLIN2: Lipid droplet associated protein; CCND1: cyclin D1; MMP1: Matrix metallo protease 1; TGF α : Transforming growth factor α ; SK1: Sphingosine kinase-1.

2.2 Implications of HIF-1 α and HIF-2 α in the ccRCC.

Although the implication of the different isoforms during the early stages of the development of kidney cancer is not very clear, there are data suggesting that in early lesions in patients with VHL disease, both isoforms are expressed together with overexpression of other HIF targets as CAIX and GLUT1, whereas in the normal tubular cells only HIF-1 α is expressed, suggesting that sustained expression of HIF-2 α is an early event in tubular cells lacking pVHL (Rosemberg 2002; [Mandriota 2002](#); [Hanckenbeck 2009](#)).

It is known that in many pVHL null ccRCC cell lines only HIF-2 α (and not HIF-1 α) is expressed. Depletion of HIF-2 α or restoration of the pVHL functions in these cell lines can suppress their ability to form tumors in nude mice. In addition, HIF-2 overexpression can override pVHL's tumor suppressor activity in the same mice model, (Raval 2005; [Kondo 2002](#)) and enhance c-myc transformation in embryonic fibroblast (MEFs) ([Gordan 2007](#)).

All these data indicate that HIF-2 is responsible for many of the pathologies described in ccRCC and this feature could be explained by the fact that HIF-2 is less sensitive to FIH1 target than HIF-1 thereby compensating its degradation effects. Recently, Schonenberger 2015 provided evidence linking HIF-2-signaling to peroxisome homeostasis.

As previously discussed, in VHL^{-/-} ccRCC pVHL is not the only one to be mutated, suggesting that additional biological and genetic events are necessary for malignant transformation. This notion is reinforced by studies showing that thousands of p-VHL null related lesions can be found in the kidney of patients with VHL disease, but not all will progress to induce kidney cancer ([Walther 1995](#)).

Through the literature HIF-1 α in the context of clear cell carcinoma is presented mainly as a tumor suppressor contrary to HIF-2 α that is taken as a driver oftumorigenesis. HIF-1 α resides on chromosome 14q, which is frequently deleted in clear cell renal carcinomas ([Shen 2011](#)). Shen and colleagues showed that many ccRCC cell lines harbour focal, homozygous, HIF-1 α deletions or aberrant HIF-1 α isoforms due to alternative mRNA splicing. They observed as well that cell proliferation was suppressed when they reintroduced the wild type form of HIF-1 α in both in vitro and in vivo models.

In addition, [Adam 2011](#) and colleagues, found that inactivation of HIF-1 α , but not HIF-2 α , exacerbates renal cyst development following Fumarate hydratase (FH) inactivation in a mouse model, suggesting an antiproliferative role for HIF-1 α and not for HIF-2 α .

Interestingly, they couldn't find any evidence that inactivation of Hif-1 α or Hif-2 α , either alone, or in combination was sufficient to initiate cyst formation or to disrupt the renal tubule architecture.

The notion that HIF-1 α is a tumor suppressor is at the centre of several debates. Through the generation of the TRACK mouse model (transgenic cancer of the kidney), ([Fu 2011](#)) where HIF1 α is constitutively expressed in the tubular cells, it has been observed that its presence activates the transcription of genes that cause increased glucose uptake, glycolysis, and lactate production, mediating a metabolic switch to aerobic glycolysis (the Warburg effect). These changes resemble those found by [Minton 2015](#) through the analysis of the transcriptome and metabolome in human clear cell renal cell carcinomas.

Moreover, no increased tumorigenesis was found in transgenic mice that constitutively express HIF-2 α , suggesting that HIF-2 α is not sufficient to cause neoplastic transformation of proximal tubules, arguing against the idea that HIF2 α activation is critical for ccRCC tumorigenesis. Of note transcriptome analysis of the cell of these transgenic mice did not resemble human ccRCC ([Fu 2013](#); [Nanus 2015](#))

Finally, as described by [Salama 2015](#) and colleagues, exhaustive ChIP-Sequencing to analyse HIF DNA binding and associated gene expression in response to HIF-1 α and HIF-2 α expression in cell lines and human renal cell carcinoma tissues, showed an overall association between HIF-1 α binding and genes associated with good prognosis and between specific HIF-2 α binding and genes that are associated with poor prognosis. Interestingly, individual gene analyses revealed that both HIF-1 α and HIF-2 α associated gene-predictors included genes associated with both good and poor prognoses. In summary, it is possible that each HIF α isoform activates a complex mix of pro-tumorigenic and anti-tumorigenic effects.

2.3 HIF isoforms as prognosis factors

The importance of the different isoforms of HIFs during ccRCC initiation and progression could suggest that HIF is a key predictive prognosis factor, however, the clinical outcome has shown heterogeneous results.

No relationship in patient survival was found between high expression of both HIF-1 α and HIF-2 α in the studies of 168 ccRCC patients ([Biswas 2012](#)).

HIF-2 α expression correlated with increased tumor volume in two studies, depending on the status of c-myc ([Biswas 2012](#); [Gordan 2008](#)).

Targeted therapies using inhibitors of both the vascular endothelial growth factor (VEGF) pathway and the mammalian target of rapamycin (mTOR) pathway are part of the current treatment of ccRCC. Inhibition of each pathway interferes with the HIF signalling pathway and it could be hypothesized that the level of HIF expression would predict the response to these treatments. Unfortunately, Choueri 2013 and colleagues found no correlation between HIF expression (HIF-1 α or HIF-2 α) and either the overall response rate or progression-free survival with the VEGF/PDGF inhibitor Pazopanib.

It is also important to mention that previous studies didn't show any effect on the response rate related to VHL inactivation, in patients treated with Sunitinib, Sorafenib, Axinitib, or Bevacizumab. ([Choueri 2008](#)).

CHAPTER 3: NOVELS TARGETS AGAINST ccRCC

1. ATAXIA TELANGESTASIA MUTATED KINASE (ATM)

As it will be later discuss, two important kinases that where never described before as potentials therapeutic targets against ccRCC, came out from our screening. In the next chapter I will describe the main characteristic and properties of each kinase. As well, the inhibitors that have been develop to inhibit these kinases will be discussed.

1.1 ATM structure

The ATM protein is a very large protein of 370 KDa that is encoded in the human chromosome 44q22-23 and shares high homology with the mouse protein (84%). Homologs of this protein have been found in all eukaryotes.

The structure of this protein is briefly described in the figure N°11.

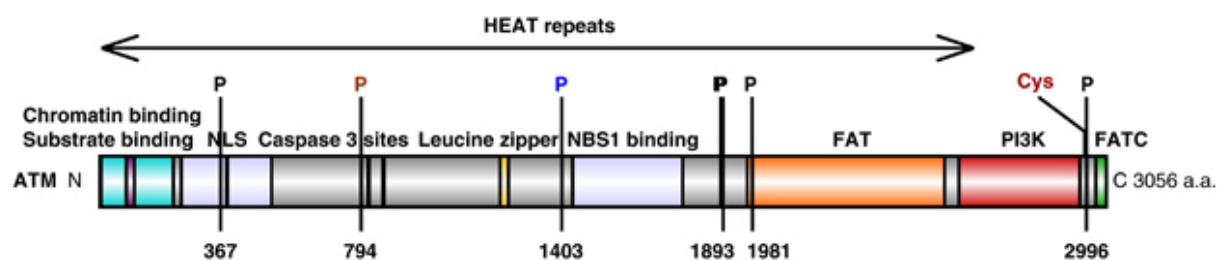


Figure 11: Domain structure of human ATM.

ATM shares homology with others phosphoinositide-3 kinase (PI3K)-like kinases through a conserved domain named FAT (named after the FRAP, ATR, and TRRAP proteins), a PI3K-like serine/threonine kinase domain and a FAT C-terminal domain (FATC). This region can interact with several proteins such as ATR, mTOR and DNA-PKCs.

The N-terminal end has a chromatin-interaction domain, a nuclear localization sequence (NLS), two caspase 3 cleavage-sites, and a putative leucine zipper region. This region can also interact with several substrates such as NBS1, p53, BRCA1, LKB1 and BLM. ([Fernandez 2005](#), [Young 2005](#)) Picture taken from ([Cremona 2014](#)).

ATM contains several auto-phosphorylation sites: serine 367, 1893, 1981, 2996 whose phosphorylation is induced after ionizing radiation (IR) and serine 1403 whose phosphorylation is induced in mitosis ([Kozlov 2011](#)).

ATM was first found as a gene mutated in the rare human disease called ataxia-telangiectasia, where patients are prone to thymic lymphoma and other cancers. T cells show radio-resistant DNA synthesis and failure to stop or slow the cell cycle progression after DNA damage induced-radiation, therefore, contributing to cancer predisposition ([Savitsky 1995](#)).

1.1. ATM cell function

A) ATM role in DNA damage response activity.

Double strand breaks (DSBs) can be a direct consequence of DNA damage induced by X-rays, ionising radiation (IR), or radio-mimetic chemicals or can be produced indirectly, for example, by reactive oxygen species (ROS) that are often generated as by-products of normal cellular metabolic processes.

DSBs can also be generated during replication fork collapse, which occurs after prolonged replication fork stalling or when the replication machinery encounters certain lesions such as DNA single strand breaks (SSBs) or an abortive topoisomerase complex (reviewed in [Shiloh 2003](#)).

Upon the formation of a DSB, the first factor to sense the damage is the MRE11/RAD50/NBS1 (MRN) complex stabilizing the free DNA ends ([Williams 2007](#)). Once bound to a DSB, the MRN complex triggers the activation of ATM via its association with the C-terminus of NBS1 ([Falck 2005](#)).

In the absence of DNA damage, ATM exists as an inactive dimer. Following break recognition by the MRN complex the inactive ATM dimers dissociate and form active monomers. This damage-induced event occurs by autophosphorylation of ATM in trans on serine (S) 1981, S367, S1893 and S2996, as well as acetylation on lysine 3016 by the histone acetyltransferase Tip60 ([Bakkenist 2003](#), [Kozlov 2011](#)).

One of the first targets of the activated ATM is the histone H2A variant (γ H2AX), which becomes phosphorylated on S139 ([Rogakou 1998](#)).

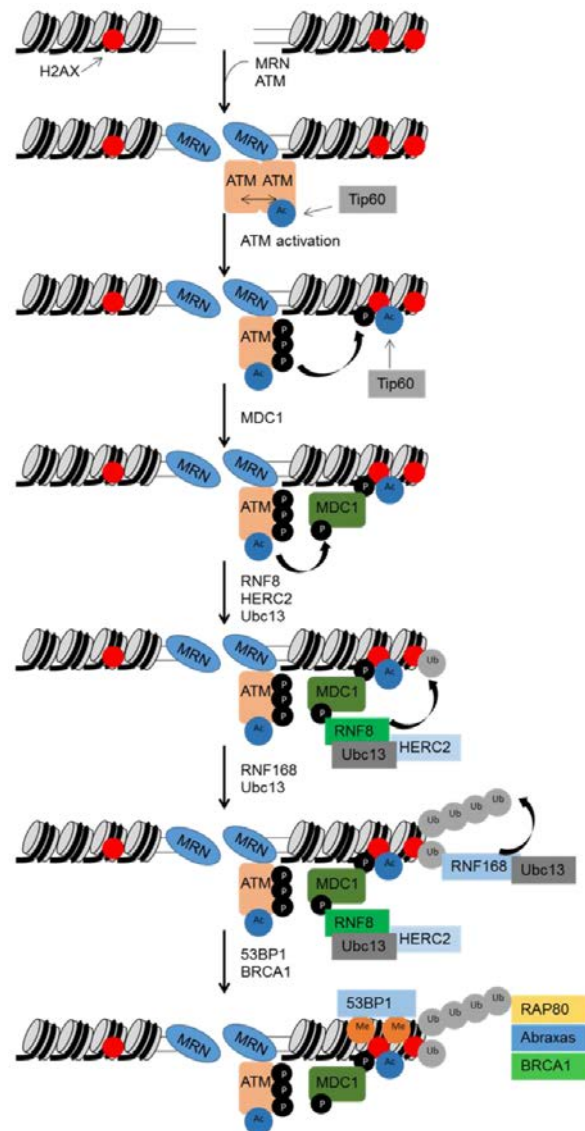
The phosphorylation and dephosphorylation of γ H2AX leads to the recruitment of the mediator protein called mediator of DNA damage checkpoint 1 (MDC1), which interacts directly with the γ H2AX, via its BRCA1 C-terminal (BRCT) domain ([Lee 2005](#)).

The binding of γ H2AX to MDC1 stimulates the recruitment of additional MRN complexes to the site of DNA damage, which serves to recruit other activated ATM therefore spreading the γ H2AX signal along the chromatin in either side of the lesion. Using this positive ATM-dependent feedback loop, this process results in amplification of the intracellular signal generated by the DNA damage ([Chapman 2008](#), [Melander 2008](#), [Spycher 2008](#)).

MDC1 has also the function to recruit others DNA damage response (DDR) proteins to the site of damage. Through its interaction with ATM, the N-terminus of MDC1 becomes phosphorylated, creating a binding platform recognized by the forkhead-associated (FHA) domain of the E3 ubiquitin ligase, RNF8 ([Huen 2007](#), [Kolas 2007](#)). RNF8 catalyses the ubiquitylation of H2A and γ H2AX, surrounding the break. The ubiquitylation of H2A-type histones signals the recruitment of another E3-ubiquitin ligase, RNF168.

RNF168, together with the E2 conjugating enzyme UBC13, catalyses the lysine 63 (K63) - linked poly-ubiquitylation of H2A/ γ H2AX, which is thought to facilitate chromatin relaxation proximal to the break and also create an interaction platform for the recruitment of downstream DNA repair/checkpoint proteins, such as BRCA1 and 53BP1 ([Doil 2009](#), [Stewart 2009](#)).

Poly-ubiquitylated H2A and γ H2AX are both recognized by the tandem ubiquitin-interacting motifs (UIMs) of RAP80 (receptor associated protein 80) an adaptor protein that is involved



in facilitating the recruitment of the BRCA1-A complex to sites of DNA damage via its association with another protein, Abraxas ([Kim 2007](#), [Sobhian 2007](#)).

Figure 12: ATM activation in response to DSBs

The MRN complex rapidly migrates to sites of DSBs and, along with the acetyltransferase Tip60, contributes to activation of ATM kinase activity. Phosphorylation of histone H2AX by ATM results in binding of MDC1 which subsequently mediates recruitment of factors, such as 53BP1 and BRCA1, which participate in DSB repair and regulation of cell cycle checkpoints. Picture taken from ([Hollingworth and Grand 2015](#)).

B) ATM signaling in cancer cells.

Several studies have shown that ATM main function is to be a tumor suppressor through the induction of cell cycle arrest and apoptosis.

In the figure N°13 we can observe the large group of substrates that ATM orchestrates after DSB to induce beside DNA repair, several anti-tumorigenic responses as for example apoptosis, cell-cycle arrest. But cancer cells can escape from activated ATM by inducing its downregulation. The miR18 has been shown to be upregulated in breast cancer and to downregulated ATM activation. As another example, [Le Guezennec 2010](#) and colleagues have shown that the WIP1 phosphatase can decrease ATM activity through desphosphorylation of ATM and its substrates as p53.

The ATM activated protein is often present in cancer cells that have already evaded the cell cycle arrest and apoptosis. This augmentation can be induced by enhancers of the ATM signalling (as for example pancreatic cancer cells that overexpress the transcription factor CUX1 which increases ATM expression) or due to precancerous lesions that caused replication stress, activating the DNA damage response, but stayed active once it was uncoupled from cell-cycle and apoptosis.

As reviewed by ([Carmona 2014](#)), the benefits of ATM signalling for cancer cells may include chemoresistance, radioresistance, metastasis and cell survival.



C) ATM Dependent Modulation of Signalling Pathways Outside DDR Implicated in Cancer.

ATM has been shown to be a key player in the maintenance of the genome stability and DNA damage response and that dysregulation of these pathways is strongly linked to cancer initiation and progression. Interestingly, ATM has also been found to have extended functions in signalling networks that sustain tumorigenesis, including oxidative stress, hypoxia, receptor tyrosine kinase and AKT serine-threonine kinase activation (Reviewed by [Stagni 2014](#)).

Alternative functions for ATM were found from a global proteome analysis performed by ([Matsuoka 2007](#)), who identified about 900 ATM targets in response to ionizing radiation (IR), among which several are proteins that exert important functions outside of the DNA damage response. Importantly, albeit ATM was originally identified to be mostly present in the nucleus, several reports have demonstrated also its cytoplasmic localization ([Li 2009](#)) and more recently to the mitochondria ([Valentin-Vega 2012](#)) and to participate to selective autophagy and to the peroxisomes homeostasis ([Tripathi 2016](#)), pointing the possibility that ATM participates in several signalling cascades others than DNA damage response. Some of these functions are summarized in the next table (Table N°7).

References		
ATM and Insulin resistance	<p>Insulin can activate ATM kinase activity in the cytosol modulating AKT phosphorylation and, together with the PI3K pathway, upregulating the translocation of GLUT4 to the cell surface, inducing increase of glucose uptake.</p> <p>In the A-T disease, ATM deficiency causes insulin-resistance due to a decrease of AKT ser473 phosphorylation and activity.</p>	(Halaby 2008)
ATM and AKT	<p>Various studies indicate ATM as a major determinant of full AKT activation, through AKT phosphorylation on serine 473. Even more, ATM inhibitors have shown to inhibit the pro-survival signals induced by AKT in cancer cells.</p>	(Golding 2009) (Golding 2011) (Li 2010)
ATM and MET	<p>It has been shown that ATM kinase activity maintains upregulated the level of the MET oncogene expression in response to IR through the transcription factor NF-κB, which in turn leads to overexpression of MET at the cell surface.</p>	(Bacco 2011)
ATM and Oxidative Stress	<p>There is increasing evidence that ATM deficiency leads to increased oxidative stress due to ROS accumulation. The oxidative stress regulated by ATM has cellular functions as including:</p> <ul style="list-style-type: none"> • Haematopoietic and neuronal stem cell self-renewal (NSCs): Loss of ATM induces upregulation of the oxidative stress p38 MAPK signalling pathway and NSCs becomes defective for proliferation. • Novel angiogenesis due to ROS accumulation: ATM in mice was shown to be activated specifically in immature vessels in response to the accumulation of ROS. ATM deficiency either by ATM defective cells or by ATM inhibitors decreases tumor angiogenesis and enhances the antiangiogenic action of agents that block VEGF, suggesting that ATM activation in response to ROS collaborates with tumor growth through induction of neoangiogenesis. 	(Ito 2004) (Ito 2006) (Guo 2010) (Okuno 2012)
	<p>One of the most important responses to Hypoxia inside the tumors to sustain their growth is angiogenesis. It has been shown that cells that lack ATM are incapable to upregulate HIF-1α and this could be due to impaired sensing of oxygen</p>	

ATM in Oxygen sensing and Cell metabolism	<p>variations. HIF-1 expression correlates with hypoxia-induced angiogenesis as a result of the induction of a major HIF-1 target gene, the vascular endothelial cell growth factor (VEGF).</p> <p>Under condition of hypoxia, ATM induces phosphorylation of HIF-1α at Ser 696. This way, through HIF, the cell adapts its metabolism by inhibiting mTORC1 and in consequence cell metabolism and growth. Downregulation of ATM levels, a frequent event in cancer, shows that when HIF-1α is no longer phosphorylated, it cannot control mTOR repression, allowing tumor growth. When activated by ROS, ATM localizes to peroxisomes to repress mTORC1 signaling targeting peroxisomes for selective autophagy (pexophagy).</p>	<p>(Ditch 2013)</p> <p>(Cam 2010)</p> <p>(Hanahan2011)</p> <p>(Tripathi 2016)</p>
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Table 7: Role of ATM in independent-DNA damage pathways.

1.2. Modulation of ATM Activity in Cancer Therapy

ATM kinase activation and therefore DNA damage response can enable tumors to resist classical treatments as IR or other chemotherapeutic drugs. This response is beneficial for the tumor cells only when the tumor suppressor activity of DDR has been disabled, for example, by deactivating p53 through mutation or loss of expression ([Halazonetis 2008](#)).

As previously described, A-T-patients have increased radiosensitivity to IR. This observation raised the question of how to sensitize tumors to IR or other chemotherapeutic agents through the inhibition of ATM to overcome resistance ([Jiang 2009](#), [Bouwman 2012](#)).

So far, four ATM kinase inhibitors have been produced: (1) KU-55933 ([Hickson 2004](#)); (2) CP466722 ([Rainey 2008](#)) ; (3) KU-59403([Batey 2013](#)) and (4) KU-60019 ([Golding 2009](#)). All these compound structures are represented in the figure N°14:

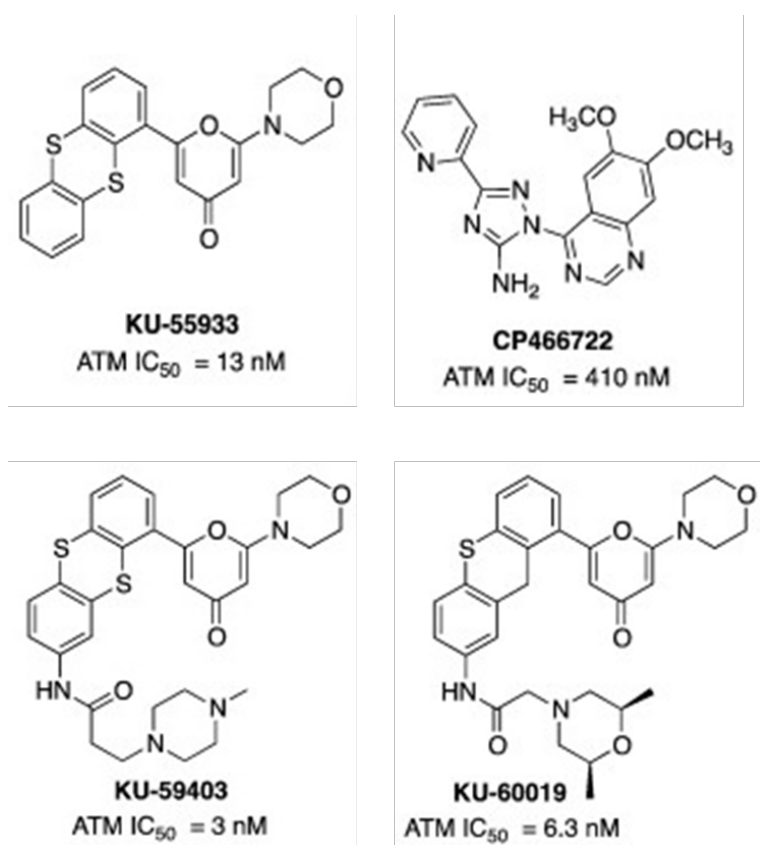


Figure 14: Chemical structures of ATM inhibitors.

IC₅₀ in vitro activities are indicated below each figure. Adapted from ([Gavande 2016](#)).

ATM inhibitor KU-59403

KU59403 is a novel ATM inhibitor developed from LY294002 (PI3K inhibitor), which is more potent against ATM than the previous lead KU55933 (IC₅₀ 3 nM vs 13 nM), and has at least 1000 times greater specificity for ATM over other members of the PI3K family tested.

This inhibitor was tested, *in vitro*, in human colon, osteosarcoma and breast cancer cell lines, showing to be more effective in combination with etoposide or doxorubicin than alone, even if this enhancement was independent of p53 status. *In vivo*, tumor mice studies demonstrated increased responsiveness to chemotherapeutic treatment with etoposide and irinotecan, as well as improved pharmacokinetics and bioavailability as compared to other ATM inhibitors ([Batey 2013](#))

ATM inhibitor KU-60019: This inhibitor has been raised as an improvement of the inhibitor KU-55933. [Golding 2012](#) and colleagues have shown that KU-60019 is ten times more potent on radio-sensitizing human glioma cells and inhibiting migration, invasion and growth. The mechanistic underlying these effects seems to rely in the ATM regulation over signalling pathways as AKT and MEK/ERK. In another study, mice carrying orthotopic glioblastoma (GBM) were 2 to 3 times more sensitive to IR after treatment with KU-60019. This effect was even stronger in mice that carried GBM with p53 mutations than genetically matched wild type glioma ([Biddlestone-Thorpe 2013](#)).

The compound CP466722 showed a distinct radio-sensitizing effect but no further *in vivo* data of this compound has been reported.

All these recent developments are encouraging and support the future clinical development of ATM inhibitors; however, at this time no clinical trials are underway.

The modulation of ATM in cancer therapy can be applied in several contexts of the tumor physiology. A possible exploitation may be a synthetically lethal combination with the inhibition of other components of the DNA damage response (DDR). For example, since ATM is involved in the homologous recombination, its inhibition may be convenient to sensitize cells to PARP-inhibitors ([Williamson 2012](#) , [Gilardini-Montani 2013](#)).

Outside the DDR context, another very interesting example of the use of ATM inhibitors as a therapeutic tool is its capacity to sensitize cancer cells to the TNF-related apoptosis-inducing ligand (TRAIL).

In the case of HCC, the resistance to TRAIL-induced apoptosis is due to high expression of FLICE-inhibitory proteins (cFLIP) and can be overpassed through FLIP inhibitors ([Safa 2011](#)). Interestingly, in HCC, ATM activation induces (c-FLIP) proteins downregulation ([Stagni 2010](#)) suggesting that ATM modulation can enable the DDR-dependent enhancement of TRAIL sensitivity at least in some context.

2. PROTEIN KINASE CK2

2.1 CK2 Structure

The protein kinase CK2, wrongly called casein kinase 2, was discovered almost 60 years ago from studies using casein as substrate (Burnett and Kennedy, 1954). It has been extensively studied showing that CK2 is a multifunctional, ubiquitously expressed and constitutively active, protein kinase that preferentially phosphorylates serine, threonine, and tyrosine residues within clusters of acidic residues ([Pinna 1990](#); [Basnet 2014](#)).

The human enzyme structure mostly appears as a heterotetrameric protein complex with two catalytic subunits CK2 α (42 kDa) and/or α' (38 kDa) and one dimer of a regulatory subunit CK2 β (28 kDa) ([Allende 1995](#)) ([Guerra 1999](#)) ([Litchfield 2001](#)). The α and α' subunits exhibit 90% of homology in their catalytic domains as well they show similar action in their enzymatic activity in vitro (Figure N°15) ([Bodenbach 1994](#), [Litchfield 1990](#)).

CK2 β represents the regulatory subunit, being at the centre of the tetrameric complex as a dimer, and with the two catalytic subunits attaching separately to this dimer ([Boldyreff 1996](#), [Chantalat 1999](#), [Gietz 1995](#), [Graham and Litchfield, 2000](#)).

Although this subunit is not required for the activity of the catalytic subunits, CK2 β binding regulates a large range of substrates that usually are not phosphorylated or only weakly phosphorylated. In opposite, only a few substrates are phosphorylated by the catalytic subunit alone and not by the holoenzyme ([Meggio 1992](#), [Pinna 2002](#)).

In addition, it has been reported that close association between CK2 and some of its substrates is often bridged by the CK2 β dimer ([Guerra 1999](#), [Filhol 1992](#), [Golden 2015](#), [Deshiere 2013](#), [Escarqueil 2000](#), [Maizel 2002](#), [Huillard 2010](#)). This means that any change in CK2 β expression might lead to a shift in the balance of phosphorylated CK2 α - and holoenzyme-specific substrates.

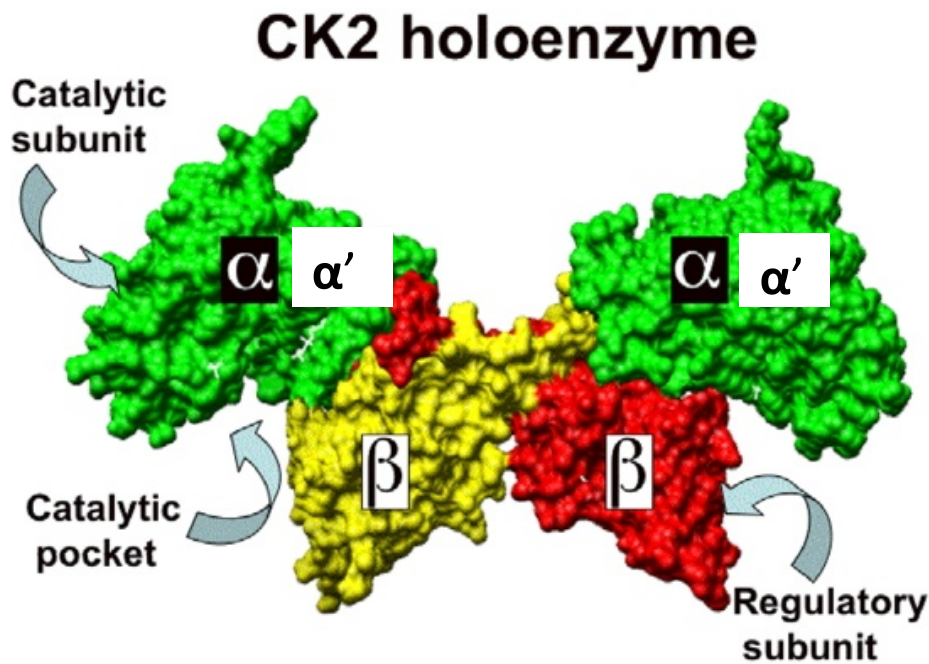


Figure 15: Tetrameric representation of CK2.

CK2 is formed by a complex that can be a combination of catalytic subunits (CK2 α / α , CK2 α / α' or CK2 α' / α') alone or bound to a dimer of regulatory subunits β . Picture taken from Cochet's Lab.

The extent of the influence that this enzyme can have over the cellular process can be glimpsed looking at the recent analysis of 10,899 phosphorylation sites present in the cellular proteome, where 2275 of them matched to a typical CK2 phosphorylation consensus motif (more than 20%) ([Salvi 2009](#)).

2.2 Cellular functions in Cancer Disease

Interestingly, fluctuations in CK2 levels have been noted in several human diseases, and more specifically increased levels of CK2 has been reported in a number of cancers ([Niefind 2009](#)). As reviewed by ([Ruzzene 2010](#)) high CK2 activity creates an environment that gives selective advantages for tumor development. Some of these advantages are due to the activity of CK2 in: regulating oncogenes ([Channavajhala 2002](#), [Seldin 1995](#), [Duncan 2007](#)); stabilizing most of the onco-kinome through CK2 phosphorylation of the co-chaperon CDC37 which is critical to preserve many onco-kinases (for example Raf1, AKT, Aurora A between others) in their active conformation ([Miyata 2004](#)); counteracting the efficacy of anti-tumor

drugs, notably of imatinib ([Mishra 2007](#)), it contributes to the multi-drug resistance phenotype ([Maira 2007](#)) and it supports neo-vascularization ([Ljubimov 2004](#)).

In 2015, [Filhol 2015](#) has made a detailed analysis of how this enzyme is implicated in tumorigenesis by participating in the regulation of several processes that have been previously described as “Hallmarks of cancer” ([Hanahan and Weinberg 2011](#)). The next figure N°16 represents most of the different proteins that are under the influence of CK2 in cancer. The wide spectrum of different cellular processes that are implicated ranges from: regulation of the expression of proteins essential for proliferation, evading growth suppressors, avoiding immune destruction, enabling replicative immortality, tumor-promoting inflammation, invasion, and metastasis, inducing angiogenesis, regulating genome instability and mutation, resisting cell death, and deregulating cellular energetics, all relevant for cancer progression.

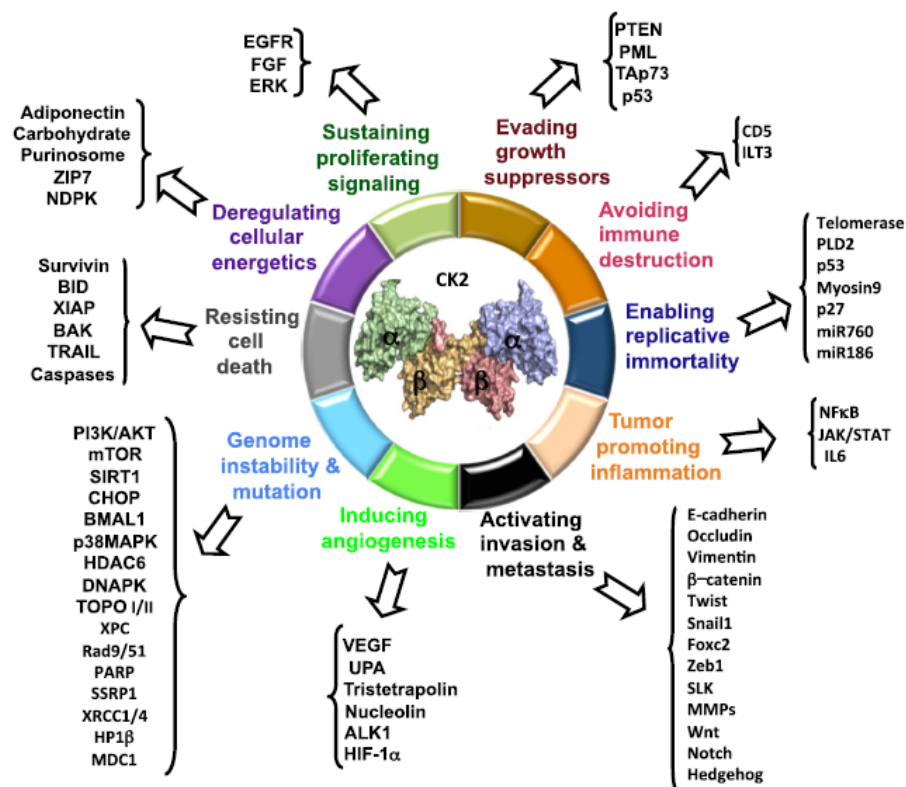


Figure 16:CK2 contributes to the “Hallmarks of Cancer”. Dysregulated levels of CK2 activity may reinforce the progression of cancer disease through the promotion of hallmarks. Figure taken from ([Filhol 2015](#))

Finally, elevated CK2 expression has been found in a number of cancers as described in table 8, this high expression is correlated with aggressive phenotype and in some cases poor prognosis.

Type of cancer	Subtype of cancer	CK2 subunits mRNA expression			References
		CK2 α	CK2 α'	CK2 β	
Non-Small cell lung cancer (NSCLC)	Squamous Cell Carcinoma	UP	UP		Bhattacharjee 2001 Hou 2010 Garber 2001 Okayama 2012 Beer 2002
	AdenoCarcinoma	UP	UP	UP	
	Large cell carcinoma (LCLC)	UP		UP	
Small Cell Lung Carcinoma (SCLC)		UP			
Lung Carcinoid				DOWN	
Breast Cancer	Ductal Breast Carcinoma	UP	DOWN		Sorlie 2001 Sorlie 2003 Zhang 2011 Curtis 2012 Zhao 2004 Finak 2008 Richardson 2006
	Invasive Ductal Breast Carcinoma	UP	DOWN	UP	
	Lobular Breast Carcinoma		DOWN		
	Invasive Lobular Breast Carcinoma		DOWN	UP	
	Mucinous Breast Carcinoma	UP			
	Invasive Mucinos Breast Carcinoma			UP	
	Breast Carcinoma		DOWN	UP	
	Invasive Breast Carcinoma	UP	DOWN		
Ovarian Carcinoma	Invasive Breast Carcinoma Stroma	DOWN	DOWN	DOWN	Zhang 2011 Yoshikara 2009 Lu 2004 Adib 2004 Bonome 2008
	Invasive Medullary Carcinoma			UP	
	Ovarian Serous Adenocarcinoma	UP	DOWN	UP	
	Ovarian Carcinoma	UP		UP	
	Ovarian Serous Cystadenocarcinoma	UP			
Colon Cancer	Ovarian Endometroid Adenocarcinoma			UP	KI 2007 Skrzypczak 2010 Hong 2010
	Ovarian Mucinous Adenocarcinoma		DOWN		
	Colon Carcinoma	UP	UP	UP	
Prostate Cancer	Rectal Adenocarcinoma	UP	UP	UP	Tomlins 2007 Lapointe 2004 Singh 2010
	Cecum Adenocarcinoma	UP	UP	UP	
	Prostatic Intraepithelia Neoplasia	UP	UP		
Pancreatic Cancer	Prostate Adenocarcinoma	UP			Iacobuzio 2003, Logsdon 2003 Pilarsky 2004, Buchholz 2005
	Prostate Carcinoma	UP		UP	
	Pancreatic Adenocarcinoma	UP	DOWN		
	Pancreatic Ductal Adenocarcinoma	UP	DOWN		

Table 8: Dysregulated CK2 mRNA expression levels in 6 types of cancer. Table adapted from [Ortega 2014](#).

2.3 CK2 Protein expression in Renal Cancer

Dysregulation of the different subunits of CK2 was first described by [Stalter 1994](#). Their study was the first to show a dysregulation of the expression of the different subunits when comparing the ratio of CK2 α and CK2 β in normal and tumour tissues of patients with renal clear cell carcinoma. They observed an increased expression of the CK2 β subunit that could be related to excess of the enzymatically active CK2 α subunit but as well due to increased expression of the CK2 β subunit.

Although this study showed the presence of overexpressed CK2 in ccRCC, there were no further studies showing that CK2 was a good target to treat this disease. Our team, since a long time has some experience in the development of CK2 inhibitors and in the understating of how this inhibition affects this multipleiotropic kinase ([Prudent 2009](#)). There were no further studies showing the effect of dysregulated CK2 subunits in clear cell renal cell cancer. The next book chapter “Dysregulated Expression of Protein CK2 in Renal Cancer” published by our team in 2015 aims to not only detect the levels of CK2 subunits in human kidney cancer samples but as well to determine *in vitro* the effects of inhibiting this key enzyme in a model of aggressive clear cell carcinoma.

2.4 CK2 and the HIF axis

As reviewed by [Hascoet 2014](#), there is evidence showing that CK2 is at the origin of HIF accumulation in ccRCC. The induction of HIF by CK2 can be VHL and non –VHL dependent. In the first case two pathways have been described: a) under conditions of hypoxia, CK2 is activated and the catalytic subunits translocate to the nucleus to phosphorylate the Histone deacetylases 1 and 2 (HDAC 1 and HDAC 2) ([Pluemsampant 2008](#)). These active HDACs will then downregulate the expression of VHL and in consequence will stabilize HIF-1 α . HDAC regulates also p53 stabilization ([Kim 2001](#)). Or b) CK2 can directly phosphorylate VHL on at least 3 different amino acid residues, reducing VHL activity and stability as well as decreasing transcriptional activity of p53, impeding HIF degradation ([Ampofo 2010](#)).

In the case of VHL independent HIF accumulation, Cdc37 is phosphorylated by CK2 at Ser13 and this phosphorylation is essential for the binding of Cdc37 to various HSP90 client protein kinases. As well, inhibition of HSP90 has been shown to induce HIF-1 α ubiquitination and degradation through an alternative E3 ubiquitin ligase that is not part of the VHL complex ([Issacs 2002](#)). In conclusion, blockage of CK2 disrupts the activity of the Cdc37/HSP90 complex and by so the degradation of several client proteins such as HIF-1 α ([Guerra 2015](#)).

2.5 CK2 is a druggable target.

It is known that only one tenth of the human genome is sensitive to be targeted by drug, but even so only half of them are pertinent targets ([Hopkins 2002](#)). Inside this “druggable genome”, kinases take a key role due to their capacity to regulate almost all signaling pathways inside the cell by promoting or repressing changes in gene expression through the phosphorylation of target proteins at specific residues. As mentioned before, dysregulation of these signaling pathways by protein kinases can have dramatic effects on the control of cell growth, proliferation and apoptosis, resulting in a variety of human diseases, including cancer ([Johnson 2005](#)) ([Hannahan 2000](#)).

A) CK2 inhibitors

Even if CK2 has not been proven as a direct cause of tumorigenesis, the huge number of substrates that are under the influence of CK2 phosphorylation has turned it as an interesting target for the design of drug inhibitors.

In the next figure N°16 some of these inhibitors are represented but I will only describe those that have had more success in being used for research in cancer.

The main problem that CK2 inhibitors confront is that most of them inhibit other proteins as well. The next table N°11 summarizes the efficacy and specificity of these so-called “specific inhibitors”.

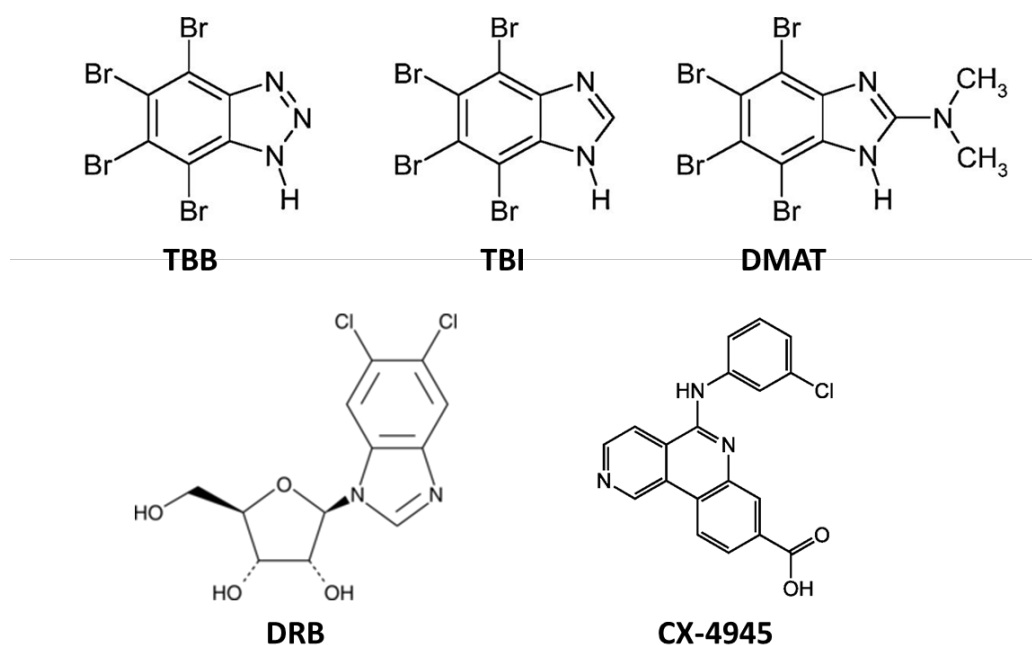


Figure 17: Chemical structures of CK2 inhibitors. Pictures taken from [Pagano 2008](#), Cozza 2015

Name	Target kinases and cancer experimental use	Reference
DRB (5,6-di-chloro-1-(b-D-ribofuranosyl)-benzimidazole)	CK2 (IC ₅₀ =4-10 µM), Cdk7 (IC ₅₀ = ~20 µM), Cdk8 (IC ₅₀ = ~20 µM), and Cdk9 (IC ₅₀ = 3 µM). DRB inhibits an elongation step during RNA polymerase II transcription of the carboxyl-terminal domain of CTD kinases. This can trigger p53-dependent apoptosis of human colon adenocarcinoma cells without inducing genotoxic stress to healthy cells.	Zandomeni 1986 Raaf 2008 Rickert 1999 Yamaguchi 1998 Poele 1999
TBI/TBBz (4,5,6,7-tetrabromo-1H-benzimidazole)	CK2 IC ₅₀ = 0.60 µM, PIM1 IC ₅₀ = 0.115 µM, PIM2 IC ₅₀ = 0.18 µM, PIM3 IC ₅₀ = 0.07 µM, HIPK2 IC ₅₀ = 0.7 µM, HIPK3 IC ₅₀ = 1.22 µM, DYRK1a IC ₅₀ = 2.1 µM, DYRK2 IC ₅₀ = 0.34 µM, DYRK3 IC ₅₀ =3.7 µM, PKD1 IC ₅₀ = 0.34 µM, CDK2 IC ₅₀ = 0.58 µM. This inhibitor was tested against glioblastoma, and other transformed cells as Jurkat and showing to be more efficient than TBB decreasing cell viability and inducing apoptosis and necrosis in transformed human cell lines as HeLa, Jurkat and HL-60.	Pagano 2008 Cozza 2015 Kaminska 2009 Zien 2005
DMAT(2-dimethylamino-4,5,6,7-tetrabromo-1H-benzimidazole)	CK2 IC ₅₀ = 0.13 µM, PIM1 IC ₅₀ = 0.148 µM, PIM2 IC ₅₀ = 1.6 µM, PIM3 IC ₅₀ = 0.097 µM, HIPK2 IC ₅₀ = 0.37 µM, HIPK3 IC ₅₀ = 0.59 µM, DYRK1a IC ₅₀ = 0.41 µM, DYRK2 IC ₅₀ =0.35 µM, DYRK IC ₅₀ =3 1.7 µM, PKD1 IC ₅₀ = 0.18 µM CDK2 IC ₅₀ = 0.64 µM. DMAT has been shown to be efficient against glioblastoma, breast cancer and human adrenocortical cell lines. Moreover, mouse models showed the efficacy of DMAT to inhibit hepatocellular and prostate carcinoma growth.	Pagano 2008 Cozza 2015 Kaminska 2009 Lawnicka 2010 Yde 2007 Trembley 2014 Sass 2011
	CK2 IC ₅₀ = 0.15 µM, PIM1 IC ₅₀ = 1.04 µM PIM2	Pagano 2008

TBB (4,5,6,7-tetrabromobenzotriazole)	IC ₅₀ = 4.3 µM, PIM3 IC ₅₀ = 0.86 µM, HIPK2 IC ₅₀ = 5.3 µM, HIPK3 IC ₅₀ = 4.9 µM, DYRK1a IC ₅₀ = 4.36 µM, DYRK2 IC ₅₀ = 0.99 µM, DYRK3 IC ₅₀ =5.3 µM, PKD1 IC ₅₀ = 5.9 µM, CDK2 IC ₅₀ = 14.0 µM. Used in the study of leukemia, breast cancer, glioblastoma and prostate cancer cell lines.	Cozza 2015 Ruzzene 2002 Gray 2014 Kaminska 2009 Orzechowska 2012
Quinalizarin (1,2,5,8-tetrahydroxyanthraquinone)	CK2α2β2 IC ₅₀ =0.15 ± 0.02 µM CK2α IC ₅₀ =1.35 ± 0.15 µM. This inhibitor is an anthraquinone derivate that has high selectivity for CK2 compared to a panel of 75 kinases. It has been tested against prostate and Jurkat cancer cells. It has been shown to have anti-angiogenic effects in the treatment of endometriosis.	Cozza 2015 Cozza 2009 Schneider 2011 Feng 2012

Table 9: Most representative ATP-competitive inhibitors of CK2. As observed through their IC₅₀, DMAT and TBI/TBBz are less specific towards CK2 by affecting other proteins, principally PIM 1, 2 and 3. Table adapted from [Cozza 2015](#) and [Pagano 2008](#).

Other inhibitors of CK2 that belong to the family of flavonoids (apigenin and quercetin) have been shown to be more potent than DRB but were proven to be poorly specific towards CK2. Importantly none of these inhibitors has reached the clinical phase trial.

2.6 The CX-4945 inhibitor

A) Specificity and cell biology function.

Although the compounds described above have shown to have certain efficacy in the inhibition of cancer cells, none of them has reached the clinical trial phase. In 2010, Cylene Pharmaceuticals, released a novel ATP-competitive inhibitor of both CK2α and CK2α' catalytic subunits, the so called CX-4945 (5-(3-chlorophenylamino)benzo[c][2,6]naphthyridine-8-carboxylic acid) or Silmitasertib). ([Siddiqui-Jain 2010](#); [Pierre 2011](#); [Chon 2015](#))

In their study, CX-4945 was found to be a very selective inhibitor for recombinant human CK2 (CK2α IC₅₀= 1nmol/L and K_i =0.38 nmol/L, CK2α' IC₅₀=1 nmol/L). Moreover, to assess the selectivity of this compound they confronted it to a panel of 238 kinases using a

concentration that was 500-fold greater than the IC₅₀ of CK2. As a result, only 7 of the 238 kinases tested were inhibited by more than 90% and those 7 were further subjected to IC₅₀ determination where the levels were at least 17 nmol/L for the closest kinase (DAPK3 a kinase involved in regulation of apoptosis, autophagy, transcription, translation and actin cytoskeleton reorganization). As well, when profiled against breast, prostate, colon, pancreas, ovarian, leukaemia, lung, melanoma and myeloma cancer cell lines, most of these cell lines showed to be sensitive to the antiproliferative effects of this drug.

Xenograft models of breast and pancreatic cancer, showed not only good tolerance to the gavage with CX-4945 but also decreased up to 50% the size of the initial tumor.

Finally, this inhibitor was proved to be antiangiogenic when tested over HUVEC cells as well to affect HIF-1 α activation in hypoxia conditions ([Siddiqui-Jain 2010](#)).

B) Clinical Phase trials

As mention before, from all the CK2 inhibitors, CX-4945 is the only inhibitor that is currently in Phase I and II clinical trials (ClinicalTrials.gov Identifier: NCT02128282).

This Phase I ([ClinicalTrials.gov Identifier:NCT00891280](#)) trial was performed over patients with advanced solid tumor cancers (Lung cancer, Breast cancer, Renal Cancer, Inflammatory breast cancer, Head and neck cancer, Prostate cancer, Colorectal cancer), Castleman's disease (multi-centric disease and Multiple myelomato test the safety, tolerability and highest safe dose level of this CK2 inhibitor).

Phases I of ([ClinicalTrials.gov Identifier: NCT02128282](#)) measured the safety and tolerability of increasing doses of CX-4945 in combination with gemcitabine plus cisplatin. The subsequent Phase II trial is a randomized study of antitumor activity in cholangiocarcinoma patients. The objective of this phase II, is to compare the standard-of-care protocol of gemcitabine plus cisplatin versus a treatment with CX-4945 in combination with gemcitabine plus cisplatin at the maximum tolerated dosis (MTD) obtained in the first phase. The estimated completion date is December 2016.

C) CX-4945 in combinational therapy

Soon after the appearance of CX-4945, several reports showed that this inhibitor could be used to overcome resistance in human T lymphoblastoid (CEM) cells that were resistant to vinblastin. Moreover, a pretreatment with CX-4945 was able to increase doxorubicin accumulation in multidrug resistant (MDR) cells ([Zanin 2010](#)).

Later, the same team demonstrated that the combination of CX-4945 plus imatinib has an effect to overcome resistance in Chronic Myeloid Leukemia imatinib resistant cells ([Borgo 2013](#)). [Manni 2013](#) reported that bortezomib, a proteasome inhibitor, had anti-tumor activity in Multiple Myeloma and Mantle cell lymphoma cells by stabilization of I κ B α in the NF- κ B signaling pathway. Since CK2 stimulates cell survival in these cells through regulation of STAT3 and NF- κ B, the authors demonstrated that CK2 inhibitors cooperate with bortezomib in causing Multiple Myeloma and Myeloid Cell Leukemia cell apoptosis by down modulating these signalling cascades and by potentiating the proteotoxic effects due to proteasome blockage.

In conclusion, ccRCC represents a disease which treatment has improved over the last years but has still some limitations in inducing the long-term response.

The objective of my PhD was to find a novel combinational therapy to target ccRCC. The procedure used to detect potential pathways implicated in kidney cancer is described in the next section. As well validation and characterization of the hit were also described.

RESULTS (i)

Search for novel therapeutic targets against ccRCC by High-Throughput screening.

1.1 Set up of conditions for High-throughput Screening (HTS)

Finding novel therapeutic targets has been the objective of my PhD. In order to do that, we decided to perform a dual chemical and genetic HTS.

Increasing clinical evidence shows that cytotoxic drugs that are given in combination can lead to synergistic effect in killing cancer cells due to targeting different molecular mechanisms between the cell heterogeneous tumor, at the same time blocking possible drug resistance ([Chabner 2005](#); [Al-Lazikani 2010](#)).

For a long time the discovery of combinational therapy was obtained through empirical tests in clinical trial-and-error way. Shortly it became evident that this mechanism was not efficient to detect clinically important drug combinations due to the enormous amount of novel target agents in oncology and other areas of medicine ([Kummar 2010](#)). HTS approach has arisen as a rapid, selective and reliable assay to compare the interactions between drugs or sensitizing agents over cancer cells in early phase of drug discovery ([Zhang 2012](#); [Macarron 2011](#); [Kenny 2014](#)).

The correct design is a key process for a successful HTS. In the following points I will describe the different parameters that were set up before performing our screening.

A) 786-O as cell line model

As it has been previously described, from all types of different Kidney Cancer, 75% are Clear Cell Renal Carcinoma type. The 786-O cell line derived from this disease, lacks the tumor suppressor VHL, is radio- and chemotherapy resistant, has increased mobility and is highly metastatic ([Kondo 2002](#); [Kim 2004](#); [Roberst 2009](#); [Pan 2015](#)). This cell line has been used in several and different type of screenings as for example to find diagnostic markers for

therapeutic targeting and RCC patient monitoring ([Boysen 2012](#)), or to select inhibitors against gene expression enhanced by hypoxia-inducible factor 2 ([Woldemichael 2006](#)).

B) Kinases as targets

Protein Kinases are key regulators of cell function that constitute one of the largest and most functionally diverse gene families. By adding phosphate groups to substrate proteins, they direct the activity, localization and overall function of many proteins ([Khuory 2011](#); [Lahiry 2010](#)).

As reviewed by [Gross 2015](#) and others, the abnormal activation of protein phosphorylation is frequently either a driver or a direct consequence of cancer disease. Figure N°1 represents the different cellular pathways in which dysregulated kinases induce tumorigenesis. After a deep search in literature, we chose for the screening, the kinase that have been previously shown to be implicated in kidney cancer, as well to other key players in other type of cancers. We then initiated a screening to identify the pair of genes that, when lost or dysregulated together, affects the cell viability of kidney cancer cells.

To inhibit these kinases we used two methods:

Kinases drug inhibitors: Inhibitors have been shown to have an effect over cells ([Muellner 2011](#)). The inhibitors to inhibit these kinases were part of a library that was selected for being cell permeable, have low toxicities and that have been already tested in other types of cancers.

Table N°1 represents the list of 80 drug inhibitors that we used to chemically inhibit 45 different kinases and 4 key cellular pathways (microtubules, DNA, topoisomerase and autophagy) that have been implicated in renal carcinoma development.

shRNA lentivirus: Each one of the selected target genes was silenced by a transduction with a MISSION® pLKO.1-puro-CMV-TurboGFP™ Target Plasmid DNA (Sigma-Aldrich). This shRNA Lentivirus is under the control of U6 and CMV promoters and endowed with a Puromycin resistance for its selection and GFP immunofluorescence co-expression to confirm by fluorescence its transfection. Table N°2 and Figure N°2 represent the list of genes that were included in the library and the vector used.

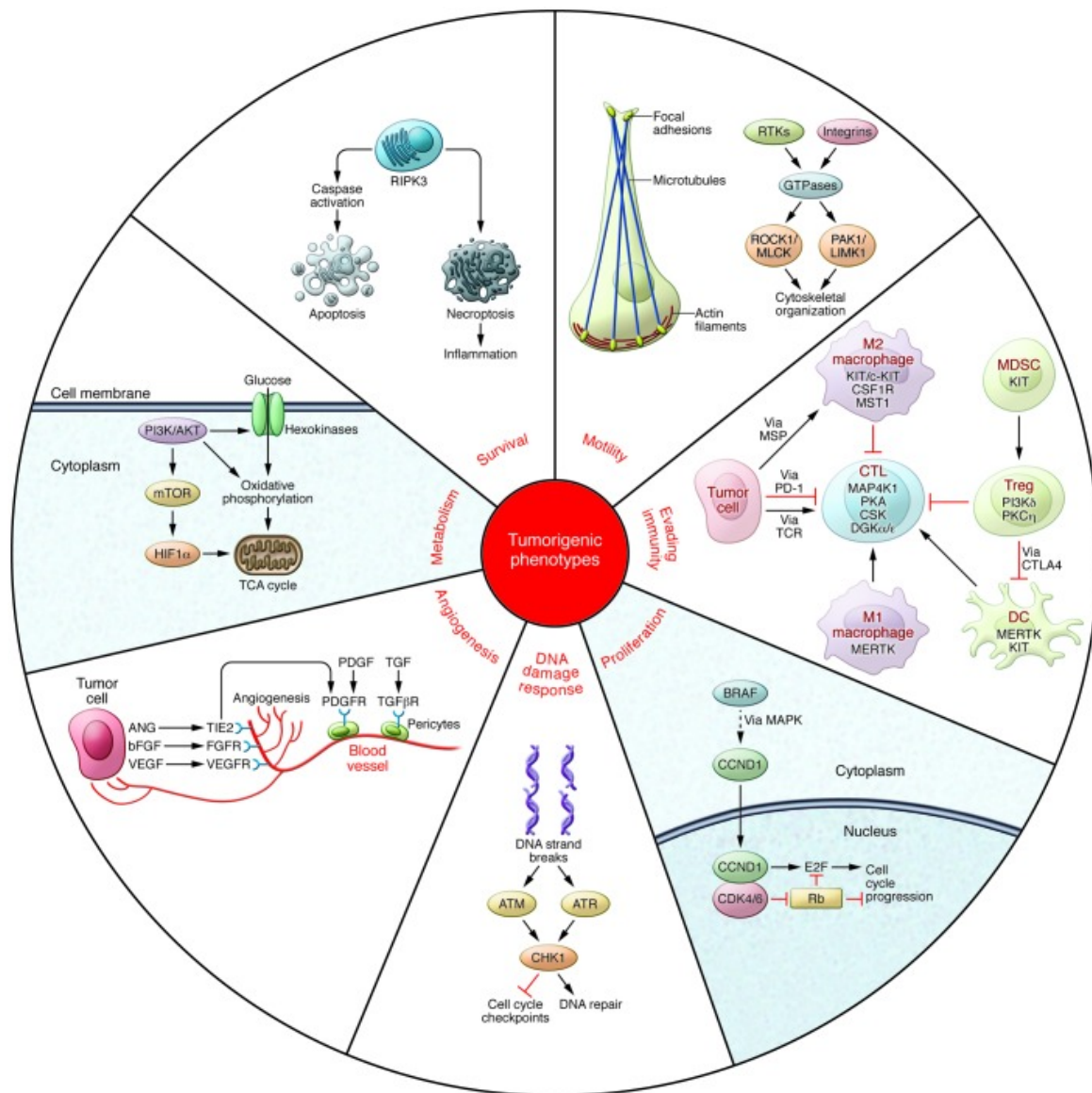


Figure 1: Kinases in Cancer disease. Mechanism in which kinases have been shown to be regulated by activation of kinases includes Survival: Modulation of apoptosis regulators and necroptosis. Motility: Cytoskeletal dynamics are mediated by RTKs and integrin clustering to promote the activation of FAK and GTPases. Immunity evasion: MAP4K1, PI3K δ , MST1R, MERTK, and PKC η , represent some of the potential targets for reversing the myriad mechanisms involved in tumor-mediated immunosuppression of

the tumor microenvironment. Proliferation: MAPK and CCND1 pathway. DNA damage checkpoints: After DSB ATM and ATR phosphorylate CHK1 and induce DNA repair mechanisms. Angiogenesis: Tumor cells modulate the microenvironment by secreting angiogenic factors (ligands for VEGFR, FGFRs, and TIE2) for neovascularization. Metabolism: PI3K/AKT pathway activity is directly correlated with increased glucose metabolism in cancer cells. Picture taken from [Gross 2015](#).

Inhibitor	Target	Inhibitor	Target
AG-370	<i>PDGFR</i>	PERIFOSINE	<i>AKT</i>
AG-490	<i>JAK-STAT</i>	SU-4312	<i>PDGFR/VEGFR</i>
APATINIB	<i>VEGFR-2</i>	RAPAMYCIN	<i>m -TOR</i>
AT-9283	<i>AURORAA/B</i>	ZM-336372	<i>c-RAF</i>
BML257	<i>AKT1</i>	AURO1 INHIBITOR	<i>AURORA1</i>
CYT 387	<i>JAK1/JAK2</i>	DASATINIB	<i>BCR/ABL SRC c-KIT</i>
DOXORUBICIN	<i>Topoisimerase</i>	OSI-930	<i>c-KIT</i>
ENZASTAURIN	<i>PKCβ</i>	AZ-960	<i>JAK2</i>
FASETIN	<i>GLUT1</i>	IMATINIB	<i>BCR/ABL</i>
GDC-0941	<i>PI3K</i>	LAPATINIB	<i>EGFR/HER2</i>
GEFITINIB	<i>EGFR</i>	TYRPHOSTIN 25	<i>EGFR</i>
H-89	<i>PKA</i>	STF-62247	autophagy inducer
HYPERICIN	<i>DNA fragmentation</i>	SARACATINIB	<i>SRC</i>
INDIRUBIN	<i>GSK-3β</i>	KU-55933	<i>ATM</i>
INIPARIB	<i>PARP</i>	PAZOPANIB	<i>VEGFR/PDGFR/c-KIT</i>
KEMPOLLONE	<i>GSK-3β</i>	BARASERTIB	<i>AURORA B</i>
KN62	<i>Ca⁺⁺/CaM kinase II</i>	TEMSILORIMUS	<i>m-TOR</i>
LAVENDUSTIN A	<i>EGFR</i>	CRENOLANIB	<i>PDGFRα</i>
LY294002	<i>PI3K</i>	TGFR INHIBITOR	<i>TGF- β</i>
MK-177	<i>WEE1</i>	GW-5074	<i>c-RAF</i>
NVP-ADW742	<i>IGF-1R</i>	AG-126	<i>ERK1/ERK2</i>
PF-2341066	<i>c-Met</i>	PICEATANNOL	<i>SYK</i>
PF-562271	<i>FAK</i>	BOSUTINIB	<i>SRC</i>
PHA665762	<i>c-Met</i>	PP1	<i>SRC</i>
RG-1462	<i>EGFR</i>	CP-690550	<i>JAK1-3</i>
RUXOLITINIB	<i>JAK/STAT</i>	TSU-68	<i>PDGFRβ&FGFR1</i>
SB203580	<i>P38 MAPK</i>	GSK1838705	<i>IGFR</i>
SP600	<i>JNK</i>	CHIR99021	<i>GSK3β</i>
SU11274	<i>c-Met</i>	AG-490	<i>JAK2</i>
SUNITINIB	<i>VEGFR/PDGFR</i>	ML-7	<i>MYLK</i>
SGX523	<i>c-Met</i>	AZD-0530	<i>SRC</i>
TIE-2	<i>ANG2/Tie-2</i>	PACLITAXEL	<i>MICROTUBULES</i>
TOSASERTIB	<i>AURORA</i>	CX-4945	<i>CK2</i>
TYRPHOSTIN 1	<i>EGFR</i>	KX2-391	<i>SRC</i>
U0126	<i>MEK1/MEK2</i>	KRN633	<i>VEGFR</i>
VEMURAFENIB	<i>MEK</i>	AXITINIB	<i>VEGFR&PDGFR</i>
WP1130	<i>BCR/ABL</i>	GSK1120212	<i>MEK1/2</i>
Y-27632	<i>ROCK</i>	INCB018424	<i>JAK/STAT</i>
YM155	<i>Survivin</i>	5FU	<i>DNA</i>
SORAFENIB	<i>RAF/MEK/ERK VEGFR/PDGFR</i>	ROSCOVITINE	<i>CDC2/CYCLIN B/A/E CDK5/p35</i>

Table 1: List of chemical inhibitors used during the High Throughput screening.

shRNA gene
ATM
ATR
AURKB
AUROKA
BCL2A1
CA9
CDK6
CDSF1R
CHK1
CHK2
CK2
FGFR1
HCK
KIT
LCK
MAP14
MAPK1
MELK
MET
NEK6
PAX2
PCTK3
PDGFR1
PDK1
PIM2
PLK2
PNCK
PRKCD
PTK2
PXK
SRC
TP53
TRIB3

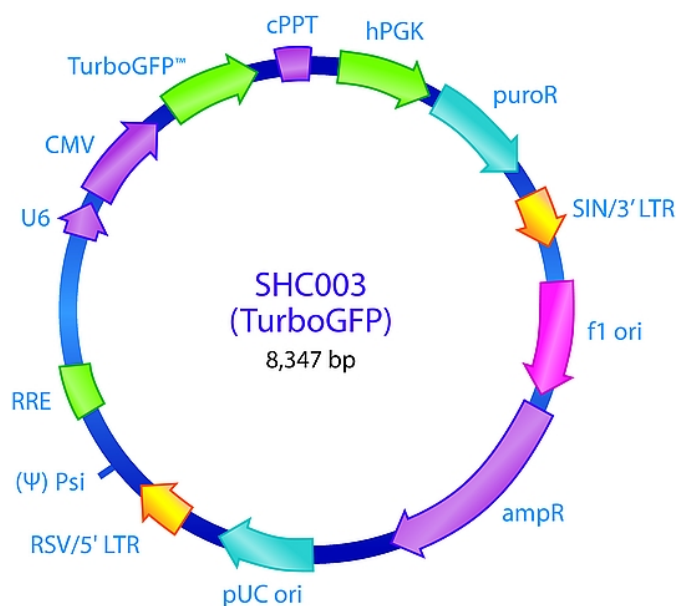


Table 2 (Left Panel): List of genes included in the shRNA gene library.

Figure 2 (Right Panel): Schematic representation of the pLKO1-puro-CMV-tGFP plasmid vector used for the gene library. The shRNA coding sequence was introduced downstream of U6 promoter.

C) Cells seeding and Cell Viability Markers

Based on previous experiments, drug treatments were performed to last 48h. As cell proliferation rates often vary between cell lines, cell seeding per well in 96 well plates was optimized by treatment with 2 different drugs with known effect (Staurosporine and CX-4945). In order to measure the effect of our combinations we took advantage of the TECAN Fluorescence Reader Platform (TECAN's Infinite M1000 microplate reader), provided by the CMBA to measure several cell parameters such as GFP fluorescence, vital Hoechst and cell viability. To evaluate the cell viability several reagents were tested to obtain a robust signal after treatment. Prestoblu[®] (Life technologies) which is quickly reduced by metabolically active cells provided a quantitative measure of viability and cytotoxicity with higher sensitivity than other classical cell viability markers (MTT or WST-1). A cell density of 5000 cells per well was found to be the best experimental condition for accurate drug sensitivity evaluation.

D) Determination of Z'Score Factor

In high-throughput screens, experimenters often compare a large number (hundreds of thousands to ten millions) of single measurements of unknown samples to positive and negative control samples. The particular choice of experimental conditions and measurements is called an assay. Large screens are expensive in time and resources. Therefore, prior to starting a large screen, smaller test (or pilot) screens are used to assess the quality of an assay, in an attempt to predict if it would be useful in a high-throughput setting. ([Zhang 1999](#) ; [Macarron 2011](#)).

In order to obtain these values we tested several bio-active molecules as positive controls versus non-bioactive molecules as negative controls. Finally, correct Z'Factor values were reached using 20 μ M of CX-4945 as bioactive positive control and 0.5% DMSO as non-active control. Figure N° 3 represents the mathematical equation to obtain the values of Z' factor: It is the ratio between the average of the stronger signals over the weaker signals and it gives an idea of separation between the signal of the bio active control and the signals given by the bio inactive control.

Z' Values, that determine that the experiments performed in HTS are robust and reliable, are between 0.5 and 1. Lower values (0-0.5) would indicate marginal assay showing that the distance between the bioactive control and bio inactive control are too close. In a preliminary manual screening of compounds over cells, we found a Z'-factor of $\leq 0.5 \geq 1$.

$$Z\text{-factor} = 1 - \frac{3(\sigma_p + \sigma_n)}{|\mu_p - \mu_n|}.$$

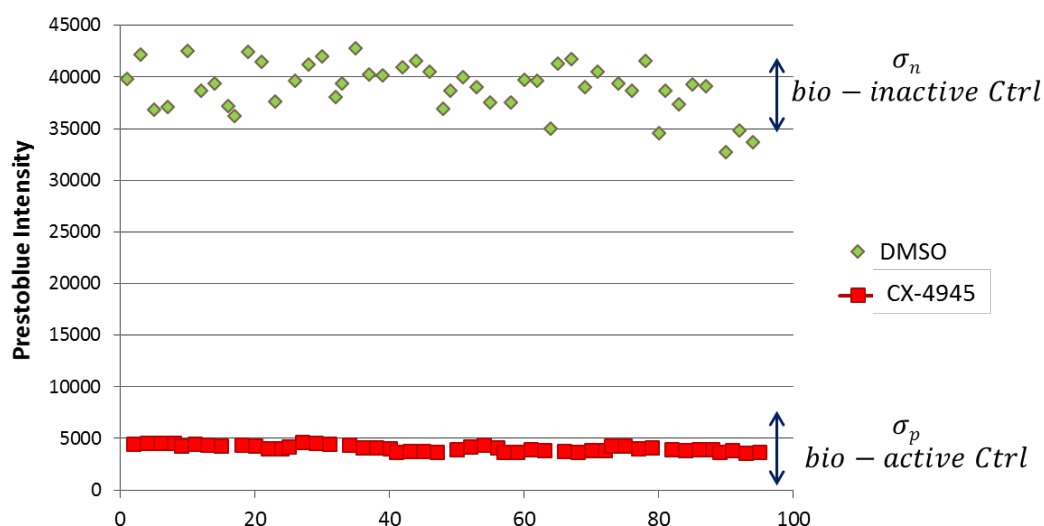


Figure 3: Representation of Z' factor equation and example of a test run for our HTS. DMSO: bio-inactive control. CX σ_p : Standard deviation of bio-active control; σ_n : Standard deviation of bioactive control. μ_p : Means of bio active control μ_n : Means of bio inactive control.

1.2 The Screening

To perform the screening we decided to run each time 4 shRNA target genes plus one shCtrl (non-target). shRNA transductions were started 3 weeks before adding the drugs. Transductions with each Mission® lentiviral Transduction particles (10^6 TU/ml) were made using polybrene (final concentration $8\mu\text{g/ml}$). The detailed planning is represented in Figure N° 4. After transduction, selection and propagation, cells were seeded at 5000 cells per well making 3 96-well plates for each transduced cell line in order to add the drugs at 3 concentrations ($1\mu\text{M}$, $5\mu\text{M}$ and $20\mu\text{M}$). This gave a total of 15 microplates to be run each time and 13 runs in total. Cell viability was monitored using PrestoBlue® and Hoechst. The negative control, showing no cell viability inhibition, thereafter designed “bio-inactive

control” was the solvent DMSO at 0.5%. The positive control, mimicking the desired inhibitory activity, was the CK2 inhibitor CX-4945 (Calbiochem) at 20 μ M final concentration.

Dilution, distribution and screening of chemicals were operated in a fully automated manner on 96-well microplates, on the robotic platform whose core instrument is TECAN’s Freedom EVO workstation using the TECAN’s LiHa MCA 96-channel pipetting head and the TECAN’s Infinite M1000 microplate reader. For primary screening, the compound library (80 compounds, dispatched in the 10 central columns of eight 96-well microplates) was first defrost, then 3 μ L of pure DMSO or of 1mM CX-4945 were added in the 16 wells left empty in each compounds microplate (8 wells per control), for bio-inactive and bioactive control data points, respectively. Then, the automated protocol was as follows:

1. The compound library (4 μ L compound/well, at 5mM) was diluted by adding 96 μ L/well of RPMI Medium, as described above. From this 200 μ M solution containing 4% DMSO, a serial dilution in RPMI Medium containing 4% DMSO was performed to obtain 50 μ M and 10 μ M solutions.
2. Then 10 μ L/well of 200 μ M, 50 μ M or 10 μ M solution were added in each well of black, V-bottomed polystyrene 96-well assay microplates (Greiner #651209).
4. Assay microplates containing 5000 transduced cells per well seeded the day before in 90 μ L plus 10 μ L of compounds were cultured at 37°C in 5% CO₂ incubator.
5. 48h later, 10 μ L of Prestoblu[®] were dispensed in each well of every assay plate, and the fluorescence emission at 590nm was quantified at 30 minutes after excitation at 560nm, to measure cell viability. Then the assay plates were washed and cells were stained with Hoechst for 15 minutes, before quantification of fluorescence emission at 530nm (GFP) and 483nm (Hoechst), after excitation at 485nm and 343nm respectively.

Data analysis was done using the in-house TAMIS software: raw data from each compound-containing well was normalized using the mean values of bioactive and bioinactive controls (100% and 0% inhibiting activity, respectively) to obtain a percentage of inhibiting activity for each compound replicate. Chemicals inducing more than 20% inhibition of cell viability were considered as primary hits. The percentage of inhibition was calculated as follows: $((\text{Sample raw value} - \text{DMSO plate mean value}) * 100) / (\text{CX-4945 plate mean value} - \text{DMSO plate mean value})$.

A schematic representation of different steps during the High-Throughput screening are represented in Figure N°5.

The secondary screen of hits and analogs was performed at the average chemical concentrations of 1μM, 5μM or 10μM, each in triplicates in the same experimental conditions as the primary screening.

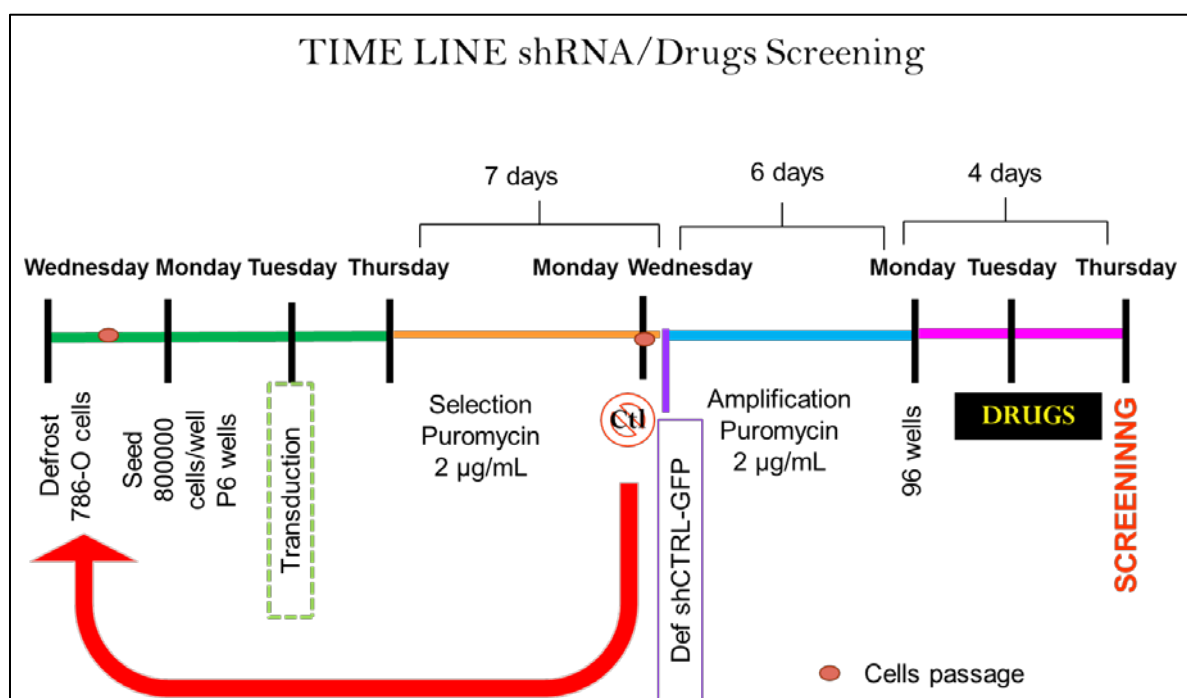


Figure 4: High Throughput Screening Timeline. Before to begin with the robotic part of the screening, several steps had to be done: To start from the same number of cell passage, vials of 786-O were defrost 5 days before seeding to be transduced. Puromycin selection was added during seven days until all CTL (non-transduced cells) died. Then amplification of the novel shTarget cell line was done in puromycin conditions. Finally, after 6 days of amplification cells were seeded in 96 wells plates and the robot next day added drugs. Screening started after 48h of treatment. For the next series of shTargets, new cells were defrosted once the CTL cells of the ongoing group died (red arrow).

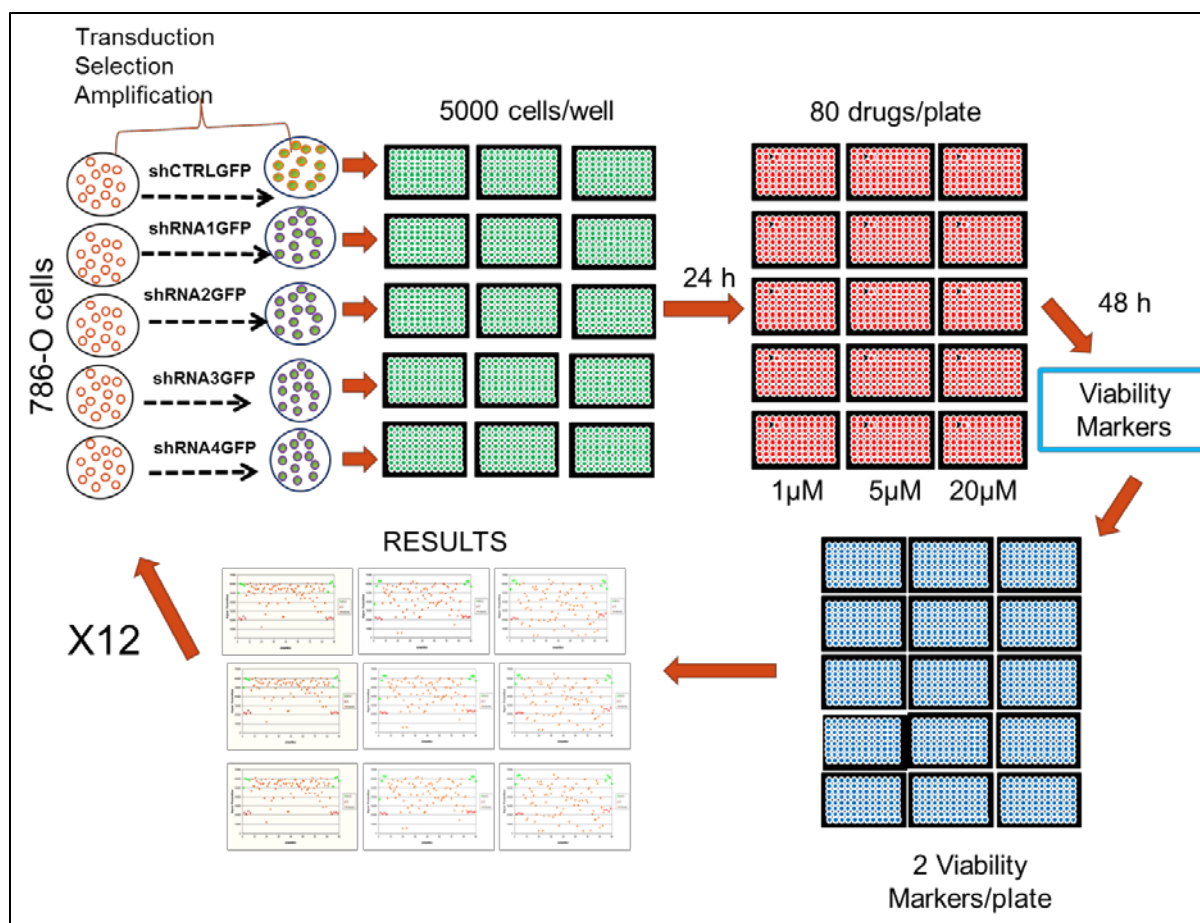


Figure 5: High Throughput Screening steps. Once the shRNA cells are ready, 96 wells are seeded at concentration of 5000 cells per well. After 24h drugs are added in 3 concentrations (1 μ M, 5 μ M, and 20 μ M). After 48h Prestobluue and Hoechst were added sequentially and processed by TECAN Robot to quantify cell viability after relevant fluorescence reading. Clouds of data were analysed by the platform expert by mathematical software. In total 13 runs were performed. shCTL (non-target) was added as control.

The selection of the hits of interest was made based in 3 required points that the combined inhibition had to have:

Sensitivity: We looked for combination where there was a good cell growth inhibition at low drug concentrations (compare to sh-CTL).

Specificity: The drugs that induce differential effect did not had to have the same effect in several other shRNA cell lines.

Strong or Mild effect: The combination of both inhibited pathways had to have increased cell growth inhibition effect at low and medium concentration (compared to shCTL).

1.3 Validation of hits through molecular biology studies

The intensive works done in the design and the setting up of conditions for the screening gave place to the obtention of several hits that were selected for a second round of validation. This validation was done using the Prestoblué viability assay (again using the robotic platform). This led to the confirmation of at least 7 novel potential combinations that are described in the table N°1.

shRNA	Drug inhibitor (Principal Target)
CK2 α	ATM inhibitor (ATM)
CHK-2	Kenpaullone (GSK-3 β)
BCL-2	TGF- β inhibitor (TGF- β)
CHK-1	SXG523 (MET)
ATM	PF2341066 (MET)
MAPK1	KN62 (CamK)
PDK1	Apatinib (VEGFR)

Table 1: List of hits obtained after validation of the screening.

Finally, we focused our work on two hits: Kenpaullone with shCHK2 and ATM inhibitor with shCK2. This last combination is presented in the patent named: “Novel therapeutic drug combination against Kidney Cancer”. V/Ref: AD16453 N/Ref.: VMA/sf – F0263S00627/EP/OEB. However, some work has been done on kempaullone and CHK2.

1.4 Kenpaullone and CHK2

CHK2 is a Serine/threonine-protein kinase that regulates cell cycle arrest at checkpoint level through phosphorylation of CDC25A, CDC25B and CDC25C, inhibiting their activity and by so inhibiting the phosphorylation of CDK-cyclin complex and cell cycle progression. In addition CHK2 activates DNA repair and apoptosis in response to the presence of DSB ([Zannini 2014](#)).

The fact that CHK2 is also implicated in DNA damage response raised the interest as a possible target in cancers that had been pre-treated with a DSB inducer.

Kenpaullone (ENZO Life Sciences) is an inhibitor of the serine/Threonine kinase glycogen synthase kinase-3 beta (GSK-3 β) (IC₅₀=23nM) but it also inhibits CDK1/cyclin B (IC₅₀=400nM), CDK2/cyclin A (IC₅₀=680nM) and CDK5 (IC₅₀=850nM).

This inhibitor has been shown to increase neurogenesis of human neural progenitor cells through stimulation of Wnt/ β -catenin signaling pathway ([Lange 2011](#)) and to induce the expression of microRNA-182 (miR-182) and therefore reducing ischemia/reperfusion (I/R)-induced cardiac cell death through down-regulation of BNIP3 ([See-Yeon 2016](#)). As reviewed by [McCubrey 2014](#), GSK-3 β is implicated in cancer as a tumor promoter being overexpressed in various tumor types including colon, liver, ovarian and pancreatic tumors or as a tumor suppressor by regulating the Wnt/ β -catenin pathway and inducing β -catenin ubiquitin/proteasome-dependent degradation. As well, this kinase interacts with the PI3K/PTEN/Akt/mTORC1 pathway, often dysregulated in kidney cancer ([Porta 2014](#)).

To obtain robust results we validated manually the data obtained by the robot. Briefly, shRNA against CHK1 protein (shCHK1), CHK2 protein (shCHK2) and non-Target protein (CTRL) were transduced as described before. 5000 cells were seeded in 96-well plates and after 24h Kenpaullone was added at concentrations of 1 μ M, 5 μ M, 10 μ M and 20 μ M in triplicate. After 48h of treatment in normoxia (25% O₂), 10 μ L of Prestoblue was added and fluorescence was read in FLUOstar Optima microplate reader at excitation/emission of 560/590nm. DMSO at a final concentration of 0.5% was added as negative control. Results show that there is a dose dependent increase in cell viability inhibition when shCHK2 and Kenpaullone are in combination (Figure N°6).

shCHK1 cell line was also compared as a second control. There is large evidence that CHK1 and CHK2 have different roles. After DSBs, the first is activated to control the replication checkpoint that monitors S-phase replication and mitosis entry. In contrast, CHK2 arrests the cell cycle at G1/S and G2/M impeding the activation of cyclin-dependent kinase 2(Cdk2) through degradation of Cdc25A, therefore blocking for G1/S and S phase progression ([Falck 2001](#)). Another mechanism involves the phosphorylation and activation of p53, that in consequence upregulates the expression of p21 leading to G1/S transition arrest ([McNeely 2014](#)).

Western blot analysis proved that the shRNA CHK1 and CHK2 as well as Kenpaullone were efficient in inhibiting cell viability (Figure N°7).

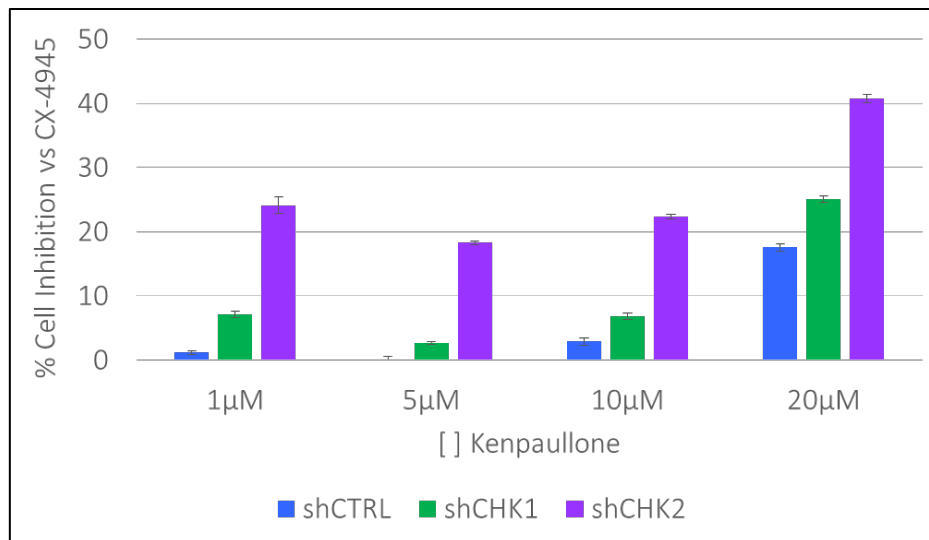


Figure 6: Validation of the hit shCHK2 and Kenpauillone through cell viability test. Non-target shRNA (blue bar), shCHK1 (green bar) and shCHK2 (purple bar) were combined with increasing doses of Kenpauillone. Inhibitory values were compared to the effect of 20 μM of CX-4945 on the same cells.

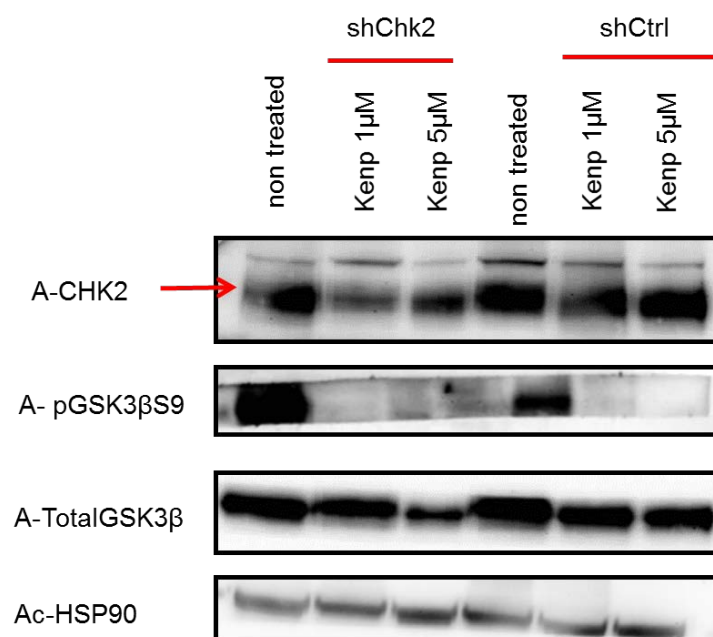


Figure 7: Validation of the hit “shCHK2 and Kenpauillone”. Western Blot showing the efficiency of shRNA against CHK2 and the inhibition of the GSK-3β serine 9-phosphorylation by Kenpauillone. HSP90 was used as loading control.

Since the transduction and selection to obtain the knockdown cell lines is a time consuming process and the use of shRNA strategy as a target therapy is still not optimal for use in the clinic, we decided to replace the shRNA by using a drug that would inhibit the same shRNA

target. For this purpose, the selective CHK2 Inhibitor II (Calbiochem) (IC₅₀= 15nM) was chosen to replace the shCHK2-mediated silencing. An IC₅₀ was performed as described in ([Arienti 2005](#)). Briefly, 5000 cells of 786-O were seeded in a 96 well plate. After 24h increasing doses of Kenpaullone alone or in combination with CHK2 Inhibitor II were added and left during 48h in normoxia conditions (21%O₂). Prestobblue metabolic test was used to measure the changes in cell viability. As shown in figure N°8, at 10μM a synergetic effect is given by the drug combination compared to the drugs alone indicating that inhibition of both GSK-3β and CHK2 inside the ccRCC significantly affect their survival.

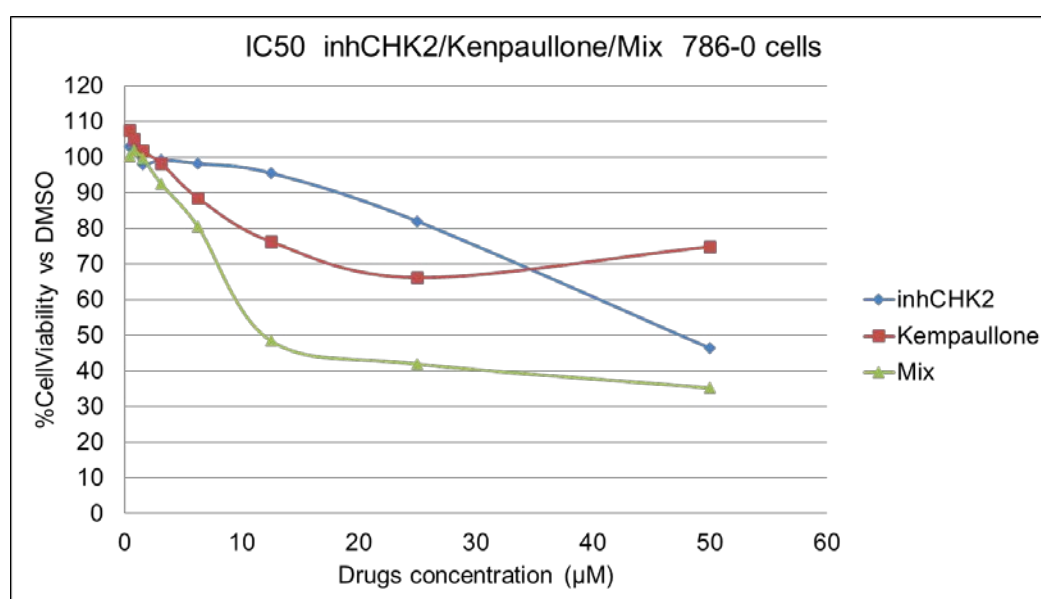


Figure 8: Combination of drug inhibitors against CHK2 and GSK-3β increases toxicity in 786-O cells. IC₅₀ was determined using increasing concentrations of drugs alone or in combination. Prestobblue was used to assay the cell viability.

As previously described, the main characteristic of the ccRCC is the lack of functional VHL tumor suppressor protein. Since normal cells express VHL, our goal was to find out a drug combination that would mostly kill VHL-deficient renal cancer cells whereas having little effect in their normal counterpart. To this purpose, we have re-introduced this VHL protein in the 786-O VHL null cells by lentiviral transduction. We obtained two cell lines, 786-O pbabe-VHL⁻ (empty vector) and 786-O pbabe-VHL⁺ cell lines. Then we compared the effect of our drug combinations in these two different cell lines.

ccRCC are highly vascularized tumors, in part due to increased levels of angiogenic factors associated with these tumors, such as the vascular endothelial growth factor (VEGF) ([Rioux-Leclercq 2007](#)). These angiogenic factors are mostly regulated by the HIF transcription

factors ([Calzada 2007](#)). Diminished oxygen availability is a common feature of malignant tumors and in fact, it has been shown to enhance proliferation and metastasis. Moreover, as reviewed by [Giullano and Pagès 2013](#) patients carrying highly vascularized tumor that are treated with angiogenic inhibitors will develop resistance to treatment through the activation of alternative pathways that can be stimulated in hypoxic conditions. These mechanisms of resistance include the activation of alternative pathways as, development of aggressive cells, appearance of Cancer stem cells, Autophagy, Dormancy and recruitment of vascular progenitors. Therefore, there is a need to find alternative therapies to either enhance the effectiveness of anti-angiogenic inhibitors or to overcome their resistance.

To determine the effect of the different environments that are present in a tumor, we evaluated the effect of our drugs on the viability of 786-O VHL⁺ and VHL⁻ cells in normoxic (21% O₂) or hypoxic (1.5% O₂) conditions.

Surprisingly, in normoxia, we found an additive effect (at lower concentration) of the mix of the drugs on the 786-O VHL⁻ cells whereas this effect was not observed in 786-O VHL⁺ cells. Moreover, a synergetic-like effect was found in conditions of Hypoxia (1.5%O₂) but at very high concentration, which might be explained by the inhibition of other pathways aswell (Figure 9).

786-OVHL NULL

786-OVHL +

NORMOXIA

% synergy vs DMSO		Kenpaullone			
		0 μM	5μM	10μM	15μM
inhChk2	0 μM				
	10μM		12	0	0
	15μM		12	0	0
	20μM		15	0	0

HYPOXIA

% synergy vs DMSO		Kenpaullone			
		0μM	5μM	10μM	15μM
inhChk2	0 μM				
	10μM		7	0	0
	15μM		17	17	38
	20μM		17	0	4

% synergy vs DMSO		Kenpaullone			
		0μM	5μM	10μM	15μM
inhChk2	0 μM				
	10μM		14	18	17
	15μM		15	0	1
	20μM		33	0	15

Figure 9: Kenpaullone and CHK2 II inhibitors combination matrix. Strong effect: red squares. Mild effect effect: Orange square and No-effect or low effect in yellow. White is indicated when drugs alone had higher effect that the combination of both.

Discussion

Here we report, as a second hit of this screening, a potential combinational therapy by using CHK2 and GSK-3 β kinases.

[Kang 2008](#) and colleagues showed that there is a strong correlation between GSK-3 β inactivation and Cdc25A overproduction in human cancer. In the opposite side, after DBS CHK2 phosphorylates Cdc25A and induces its degradation ([Sancar 2004](#)). As well, thymidylate synthase inhibitors 5-fluoro-2'-deoxyuridine (FdUrd) associated accumulation of Cdc25A, showed to induce premature mitotic entry and clonogenic death in colon cells ([Parsels 2004](#)).

We showed that, in conditions of normoxia and at low concentration, combination of the inhibitors CHK2 and Kenpaullone decreases cell viability in VHL null cells.

Nevertheless, Kenpaullone inhibits also other pathways as CdK1, more experiments are needed to better understand the mechanisms underlying the efficacy of this combination.

Conclusions

Today, HTS is one of the most used tools in pharmaceutical research for the discovery of novel drugs against several diseases. It provides not only fast, quality and novel data but also opens the door for the deciphering of key question as for example what are the genes that can compensate drug inhibition and induce resistance.

Our studies provide experimental and technical data for the design, set up of conditions and validation of hits for the discovery of novel therapeutic targets against clear Cell Renal cell carcinoma.

The co-inhibition of the CHK-2 and the GSK-3 β pathways seems to be a potential therapy, although the toxicity does not discriminate between cells carrying the VHL protein. Thus, it could be interesting to further investigate its applicability in certain cases as for example as part of the cycle of treatments applied in mRCC.

RESULTS (ii)

PATENT: “Novel therapeutic drug combination against Kidney Cancer ». (V/Réf. : AD16453 N/Réf. : VMA/sf – F0263S00627/EP/OE)

Sofia Giacosa, Catherine Pillet, Caroline Barette, Emmanuelle Soleilhac, Claude Cochet, Odile Filhol

Introduction

Renal cell carcinoma (RCC) is the eighth leading malignancy in the world, accounting for 4% of all cancers. Approximately 30% of RCC patients present with metastatic disease at the time of diagnosis and nearly half of the remainder will subsequently develop metastasis ([Chung 2012](#), [Negrier 1998](#))

Clear Cell Renal Cell Carcinoma (ccRCCs) is the most common form of kidney cancer, accounting for 75% of all cases ([Lopez-Beltran 2006](#), [Moch 2000](#), [Amin 2002](#)).

Inactivating mutations or silencing of a crucial gene: The Von Hippel Lindau (VHL) Tumor Suppressor Gene represents ccRCC fingerprints.

It has been shown that, under normoxic conditions, the oxygen-sensitive HIF α subunit is degraded by VHL-mediated ubiquitination. However, under hypoxic conditions, HIF α degradation is suppressed, leading to enhanced nuclear localization of HIF α and transcription of various target genes, including the angiogenic gene factor VEGF ([Kaelin 2008](#)) and other genes involved in tumor progression and metastasis.

RCC is notably highly resistant to chemotherapy. Moreover, treatment of mRCC is difficult because it shows no or limited responsiveness to conventional anticancer therapies, such as radiation and chemotherapy, or cytokine therapy ([Figlin 2012](#))

However, several available kinase-targeted agents have shown beneficial effects for the treatment of patients with metastatic renal cell carcinoma (mRCC), including Tyrosine kinase inhibitors (TKIs) sunitinib, sorafenib, pazopanib, axitinib, cabozantinib and the VEGF-targeted antibody bevacizumab and the mammalian target of rapamycin (mTOR) inhibitors temsirolimus and everolimus ([Maj-Hes 2013](#), [Hutson 2007](#)). Although these targeted agents

demonstrate antitumor activity and prolonged progression-free survival (PFS) in patients with mRCC, eventually all patients relapse.

Thus, the lack of long-term efficacy of current treatments reveals the urgent need to find other therapies focusing on the different pathways involved in this devastating disease.

Along this goal, we have taken advantage of a new approach called Synthetic Lethality. This is a combination of mutations or inactivation of two or more genes that leads to cell death, whereas a mutation in only one of these genes does not, and by itself is said to be viable. A synthetic lethal approach to cancer therapy is currently being explored as a means to develop targeted therapies to reduce off-target effects in chemotherapies and chemopreventive drugs ([McLornan 2014](#)).

Genetic or pharmacological interference are among major mechanisms to achieve synthetic lethality in cancer. New drug combinations can be identified from high-throughput screens using small interfering RNA (siRNA), short hairpin RNA (shRNA), RNA interference (RNAi) or small chemical inhibitors ([Muellner 2015](#)).

To achieve we use highly aggressive 786-O VHL null ccRCC human cell line, we have combined 36 shRNAs to silence different genes that have been shown to be involved in different types of cancer with 80 different drugs (FDA and non-FDA approved). Cell viability markers were used to evaluate the effects of each combination in each cell line versus the control.

Materials and Methods

Chemicals

All compounds were dissolved in DMSO at a concentration of 10mM. CX-4945 was synthesized at the Plateau Synthèse Organique, Département de Chimie moléculaire, UJF, Grenoble, according to the method described by [Pierre 2010](#). The chemical library was composed of 2 commercial libraries, one from ENZO (screen-well kinases inhibitors) and the other from Selleckchem (Tyrosine kinase inhibitors) that were complemented with other inhibitors (see Table 1).

Lentiviral particles were provided by Sigma-Aldrich coming from the PLKO1 vector Hpgk-puro-cMV-tGFP containing different shRNA sequences targeting different genes (see Table 2).

Cell culture

ccRCC cell lines 786-O, RCC4 and ACHN as well as normal renal proximal tubular epithelial cell line RPTEC were obtained from ATCC and Evercyte (Germany) respectively. Cells were grown in 10-cm-diameter plates in a humidified incubator (37°C, 5% CO₂) with RPMI 1640 medium (Gibco) containing 10% of fetal bovine calf serum, penicillin [100U/mL], streptomycin [100µg/mL] concerning 786-O and ACHN. RPTEC were cultured in ProXup (Evercyte). DMEN medium Glutamax 4,5mg/mL (Gibco) containing 10% of fetal bovine calf serum, penicillin [100U/mL], streptomycin [100µg/mL] was used for the RCC4 cells and McCoy's medium containing 10% of fetal bovine calf serum, penicillin [100U/mL], streptomycin [100µg/mL] was used for the Caki-2 cell line. 786-O and ACHN cells were passed every 2-3 days and only once a week for RPTEC.

The parental VHL null cell line was used to generate its derivative lines containing either the empty expression vector HA-pBABE or a functional VHL construct HA-VHL (VHL⁺ cells). Stable transfectants were maintained in medium supplemented with 2µg/ml puromycin.

Transduction of shRNA in 786-O cells

For infection, 786-O cells were plated into 6 well-plates (8×10^5 in 2 mL of serum-supplemented RPMI 1640 medium). The day after, adherent cells were incubated with lentiviral particles (1-5 MOI) diluted in 1000 µl of serum-supplemented medium containing 8µg/µL polybrene. After 4h, 1 mL of medium were added to cultures and transduction was maintained for 16 hours before to wash cells and change the medium. For stable transduction, puromycin selection started 36h post-infection (at the concentration of 2µg/mL) and was maintained during all cell culture.

Spheroids culture

For 3-D culture (spheroids), the spheroids were prepared in 96-wells U-bottom tissue culture plate with low evaporation Lid (Microtest™, Becton Dickinson Labware, San Jose, CA) coated with 20mg/mL of poly-HEMA per well to allow the formation of spheroids. Renal cancer cells 786-O WT, 786-O pBABE and 786-O VHL (taken from exponentially growing cultures) were seeded at a density of 1×10^3 cells/ml in RPMI supplemented with 10% FCS. 90 μ L (1×10^3 cells) of this cell suspension were deposited in each well then tissue culture plates were incubated at 37°C and 5% CO₂ for 3 days before drug treatment for further 48h.

Viability assay

Cytotoxicity was measured using PrestoBlue® assay (Invitrogen, Carlsbad, CA). Cell lines were seeded in a 96-well microtiter plate at a concentration of 5×10^4 cells/mL. Cells were allowed to attach for 24 h at 37°C and 5% CO₂. The cells were exposed to the negative control DMSO or positive drug CX-4945 at 20 μ M for 48h or the other molecules at indicated concentrations.

Western Blot analysis

Cells were lysed in RIPA buffer containing a cocktail of protease and phosphatase inhibitors (Tris HCl pH 7.4 10 mM, NaCl 150 mM, SDS 0.1%, Na Deoxycholate 0.5%, EDTA 1 mM, Triton X100 1%, containing a protease inhibitor cocktail (Sigma P8340), phosphatase inhibitor cocktails 1 and 2 (Sigma P2850, P5726), 15 min and clarified at 16,000g for 15 min. Homogenates were quantified using BCA protein Assay kit (Thermo Scientific). SDS-PAGE was performed using pre-cast 4-12% gradient gel (Bio-Rad) and electrophoresed in NuPAGE buffer at 150 volts for 75 minutes. Separated proteins at 20 μ g/lane were transferred to PVDF membranes (60 minutes at 100 Volts). Blotted membranes were blocked during 1h at room temperature with saturation buffer (1% BSA in TBST), and then incubated with primary antibody diluted in saturation buffer, during 1h30. Secondary antibodies were added for 1 hour. Detection was achieved by using Luminata Forte Western HRP substrate (Millipore) and Fusion FX acquisition systems. Anti- β -actin (Abcam), GAPDH (Ambion) or HSP90 (Cell Signaling) were used to control for equal protein loading.

Fully automated, High-Throughput screening assay

In 96-well plates, 90µl of either Ctrl- or targeted- shRNA expressing 786-O cell suspension (55.5×10^3 cells/ml) were seeded and plates incubated overnight in a 5% CO₂, 37°C, humidified atmosphere. Then, 10 µl of each compound was added at the indicated concentrations (1, 5 or 20 µM final concentration) using the CMBA's robotic platform. DMSO was used as a negative control and 20 µM CX-4945 as a positive control. Plates were further cultured for 48 hrs. Cells were labeled with vital Hoechst (33342) together with Prestoblue and the fluorescence reading was made through the Tecan's Infinite M100 reader. See Flow chart in Figure 1.

Data Processing and Hit selection

Primary and secondary High Throughput Screening assays were evaluated and validated using a simple statistical parameter, the Z'-factor ([Zhang 1999](#)).

Raw data were first imported into the database of the homemade analysis software TAMIS, used for analysis. For each test plate and assay type (PrestoBlue or vital Hoechst), mean of the eight DMSO negative control values was considered as 0% activity and mean of the eight CX-4945 positive control values was considered as 100% activity. Then, each compound X-related raw value was translated in activity percent, compared to DMSO (0%) and CX-4945 (100%). Finally, for each compound X/targeted-shRNA Y pair and assay, the activity percentage obtained with the compound X/Ctrl-shRNA was taken from the compound X /targeted-shRNA Y activity percentage in order to identify compound X/ targeted-shRNA Y pair showing additive, or even synergistic, effect compared to compound X/Ctrl-shRNA pair

Statistical analysis

The statistical significance of differences between the means of two groups was evaluated by using GraphPad version 6. Tests are indicated in below each legend.

3D assay

Three days pre-formed spheroids were treated with the different drugs at indicated concentrations during 48h. After that, Hoechst (200ng/mL) and Propidium Iodide (1µg/mL) were added as markers of cell nuclei and cell death respectively.

Images of IP and Hoechst fluorescence were acquired at the CMBA platform using the automated microscope ArrayScanVTI (ThermoScientific). ArrayScanVTI is a fluorescent microscope which allows the rapid and fully automated acquisition of images of fixed or live cells in 96-well microplates at different magnifications. Dedicated softwares are used to automatically perform High Content image Analysis like spheroid area measurement, fluorescent intensity measurement of each staining and counting of the number of dead cells.

3D cell migration assay

Cell migration assay was performed as described in [Gunther 2003](#). Briefly, 3 days-old round bottom plate pre-formed spheroids were transferred to flat bottom 96 well plate and treated with either DMSO, KU-60019 (10 μ M), CX-4945 (7.5 μ M) or mix of both for 48h. Upon adherence to the solid support, spheroids disassembled and released cells migrated away radially from their initial position. Cell spreading was followed during 7 days. Markers, Hoechst 33342 (blue) and Propidium Iodide (red), were added to visualize cell spreading and dead cells respectively. Quantification was made using Image J software.

Fresh Tissue Sectioning

A Vibratome VT1200 (Leica Microsystems) was used to cut thin (300–500 μ m) slices from fresh tissue. Samples were soaked in ice-cold sterile balanced salt solution (HBSS), orientated, mounted, and immobilized using cyanoacrylate glue. Slicing speed was optimized according to tissue density and type; in general, slower slicing speed was used on the softer tissues and vice versa (0.03–0.08mm/neoplastic tissue; 0.01–0.08 mm/s normal tissue). Vibration amplitude was set at 2.95–3.0 mm.

Organotypic Tissue Cultures

Tissue slices were cultured on organotypic inserts for up to 120 h (one slice per insert; Millipore). Organotypic inserts are Teflon membranes with 0.4- μ m pores that allow preservation of 3D tissue structure in culture. Tissue culture was performed at 37 °C in a 5% CO₂ humidified incubator using 2 ml of DMEM media supplemented with 20% inactivated FBS (GIBCO), 100 U/mL penicillin (Invitrogen) and place in a rotor agitator to allow gas and

fluids exchanges with the medium. The tissue slices were harvested at baseline time (T0) and thereafter, at 24h intervals; the slices were incubated with the drugs at the indicated concentrations and after 24h and 48h, medium-containing Luciferin was added and imaged using IVIS. ROI levels allowed quantifying the efficacy of the drug treatment.

Results

Dual genetic/drug High-throughput screening identifies the combinatory synthetic lethal effect of CK2 and ATM kinase inhibition in a CCRCC cell line.

As a first step to identify potential novel therapeutic targets in ccRCC, we applied our screening platform to interrogate drug-gene interactions using the 786-O VHL⁻ cell line. Cancer-relevant genetic aberrations were selected using literature and database search. This yielded a list of 36 genes that have been linked to several cancers including ccRCC (Table 2). To assess the effect of inhibition of these genes in cancer, we manipulated their expression using RNA interference (RNAi). The chemical library mainly consisted of clinically relevant kinase inhibitors (including a series of FDA-approved therapeutics) that altogether comprised 80 small molecules (Table 1). Next, to search for drug-gene interactions we have established a multiplexed assay to analyze the cellular fitness of the shRNA-engineered cell lines in response to our chemical library. This method enabled us to query a 36 x 80 drug-gene matrix, which allowed the interrogation of almost 2,880 drug-gene pairs. The library was screened at three concentrations, yielding over 8,640 data points (Figure 1A). As mentioned before cell viability markers were used to measure the effects of each combination in each cell line versus the control.

Data analysis revealed several gene-drug interactions including synthetic-lethal interactions between components of signaling pathways and specific kinase inhibitors. Among them, we found that the shRNA-mediated knockdown of CK2 α expression enhanced the sensitivity of 786-O VHL⁻ cells to an inhibitor of Ataxia Telangiectasia Mutated (ATM) kinase (Calbiochem) (Figure 2A). To confirm the specificity of the targeted-kinases we used another ATM inhibitor KU-60019 (from Selleckchem) and Ellipticine another inhibitor of CK2 (Figure S2A-C). As KU-60019 showed to be significantly more efficient, we used this ATM inhibitor in the follow up experiments. Moreover, the same enhanced effect was also observed when the cells were treated with a combination of KU-60019 plus CX-4945, a protein kinase CK2 inhibitor, suggesting a synthetic-lethal interaction (Figure 2B).

One of the roles of VHL is to promote the degradation of HIF factors under normoxic conditions. Indeed, pVHL complex tags HIF-1 α with ubiquitin and thereby marks it for

degradation by the 26S proteasome. It is admitted that in tumor, the cell environment is hypoxic. Consequently, it has been previously shown that there are differences in drug effects due to different VHL status in hypoxic conditions ([Hazzouri 2006](#)). We then stably reintroduced the tumor suppressor VHL in 786-O cells and measured the sensitivity of these cells to the CK2 and ATM inhibitors (Supplemental figure). Thus, we looked at the effect of the CK2 and ATM inhibitors in low oxygen (1.5 %). As illustrated in Figure 2C, 786-O VHL⁺ cultured and treated under hypoxic conditions are less sensitive to each inhibitor alone. However, the most striking observation was the strong synergetic effect of the combination in the VHL⁻ cells as compared to the VHL⁺ cells. The calculated theoretical additive cell death effect of both inhibitors was ~10% whereas the observed effect was 37%, clearly indicating a synthetic lethality process. This difference was less marked in the normoxia conditions (Figure S2D)

Caki2 cell line was isolated from primary ccRCC and has a loss-of-function mutation in the von Hippel-Lindau (VHL) tumor-suppressor protein and are known to form tumors in immunocompromised mice ([Miyake 2012](#), [MSSCC website](#)). In VHL mutated form of ccRCC cells like Caki2, this synergetic effect was also present in hypoxia conditions (Figure 2D). As comparison, RPTEC that are described as “normal” renal epithelial cells, are as sensitive to these inhibitors as 786-O VHL⁺ cells (Figure 2E) in normoxic conditions and some more sensitive in hypoxia conditions (Figure S2E) suggesting a role of HIF in this response.

Drug combination induces cell cycle arrest and cell stress in monolayer cell culture.

Western blot analysis was performed to visualize the inhibition in hypoxia and normoxia conditions, of ATM and CK2 in 786-O cell lines by Ku60019 and CX-4945 respectively (Figure 3A-B). ATM was clearly inhibited by Ku60019 in all conditions (VHL⁺/-, normoxic or hypoxic conditions) as evidenced by reduction in the canonical phosphorylation of P-ATM Ser1981, as CK2 in 786-O VHL⁻ using either P-AKT Ser129 or P- α -Catenin Ser641 but CK2 inhibition (α -catenin is decreased in hypoxia) in VHL⁺ both under normoxic and hypoxic conditions. Surprisingly, substrates phosphorylation was less affected in the mix both in normoxia and hypoxia.

To understand how cell viability might be affected by the treatments, we looked for apoptosis markers. Since neither PARP cleavage nor caspase activation were detected (data

not shown), we dyed the cells with the KIT Live and Dead to measure the number of dead/lived cells after 48h of treatment. Although there was a notably decrease in the total number of lived cells that remained after treatment with the mix, no significant difference was found between the number of dead cell in all conditions, indicating that the inhibitors did not induce apoptosis (Figure 3C). Cell cycle analysis was performed by FACS using Propidium Iodide labeling. Figure 3E shows that the G1 phase was extended in cells when ATM was inhibited whereas CX4945 was without effect. Moreover, the combination of ATM and CK2 inhibitors increased the amount of G1-arrested cells (79%), while decreasing cells in S and G2/M phases (4 and 17% respectively) as compared to DMSO-treated cells. These data were confirmed by western blot analysis of proliferation-related markers (p38 MAPK and p42/p44 MAPK) as well as cell cycle arrest markers (P53 and BAX) (Figure 3D).

Multicellular Tumour Spheroids show cell death.

In order to be closer to the tumor environment, we performed the drug treatments in Multicellular Tumor Spheroids, which are known to mimic micro-tumors more closely than cancer cell line monolayers. Indeed, tumor spheroids represent quite realistically the 3-D growth and organization of solid avascular tumors and consequently, simulate more precisely the cell-cell interactions and microenvironmental conditions found in tumors, especially nutrient and oxygen gradients. Several studies indicated that drug sensivity testing performed on tumor spheroids can effectively predict the efficiency of new antitumor compounds in patients and has proven in numerous studies to be of tremendous interest in characterizing the effects of chemotherapeutics ([Dubessy 2000](#)). In that context, Tumor spheroid models are particularly interesting as they reproduce the multicellular resistance (MCR) associated to cell-cell interaction, low drug penetration, resistance of quiescent cells located in the deepest and hypoxic regions ([Desoize 2000](#), [Hirschhaeuser 2010](#)). Tumor spheroids generated from the 786-O shCK2 α and shATM cells were treated for 48h with ATM or CK2 inhibitors, respectively. As shown in Figure 4A and B, 5 μ M ATM inhibitor and 5 μ M CX-4945 were the optimal concentrations that show a synergetic effect on the viability compared to the shCTL as visualized by propidium iodide staining. These results confirm in a 3D cell model, the one obtained during the screening. To validate that increased PI staining was due to apoptosis, we treated 786-O null cells with ZVAD, and apoptosis inhibitor. Results showed a clear decrease in the efficacy of drugs to kill the cells in the presence of ZVAD

(Figure 3C). Moreover, another ccRCC patient derived cell line with mutation in VHL gene, R305, cultured in 3D and treated , with the combination of KU+CX also induce cell death as shown in Figure 3D.

Co-inhibition of CK2 and ATM decreases cell migration and proliferation in 3D *in vitro* and *in vivo* models.

Migration and invasion of drug resistant cells is one of the first mechanisms that the primary tumor uses to escape in order to metastasize in another organ ([Friedl 2003](#)). To determine the capacity to migrate of 786-O cells we performed a test described in Figure S4A. Briefly, 786-O spheroids were treated with the drugs alone or in combination and then placed in flat bottom plates. During 7 days cells spreading was measured as well as their capacity to survive. As observed in Figure 4E, after 5 days of culture in flat bottom wells there is significant difference in the migration of cell treated with KU+CX compared to the drugs alone. Moreover, cell viability test indicates that even after 7 days a single dose of KU+CX has inhibited cell proliferation (Figure 4F). This result indicates that in an environment that is closer to the one inside the tumor, the inhibition of CK2 α and ATM together induces a synthetic lethal effect.

Co-inhibition of CK2 α and ATM induces cell death *in vivo* and *ex vivo* models.

The majority of preclinical kidney cancer research is based on established cell lines. However, these cell lines frequently have undergone multiple changes influencing their biological behavior and therefore no longer reflect the primary tumor of origin. Freshly isolated primary tumor cells, in contrast, may be more closely related to the malignant cells of the tumor ([Goodspeed 2016](#)). But it is most likely that separated tumor cells will behave differently *in vitro*, as both cell-cell and cell-matrix interactions are highly different compared to the *in vivo* situation. Therefore, to investigate tumor cell behavior *ex vivo* it is necessary to maintain or reconstitute an environment closely resembling the tumor tissue ([Griffith 2006](#)).

To achieve this, the inventors took advantage of their expertise in cutting pre-induced xenografts of kidney tumors ([Vaira 2010](#)). Briefly, 786-O Luciferase cells were injected under the kidney capsule of nude mice and tumor growth was monitored through Luciferase

intensity using an IVIS Imaging reader. After 6 weeks of growth, mice were euthanized and tissue slices were generated from the tumors using a Vibratome (Leica). Tissue slices were placed in special supports that allow oxygen-gas exchange and were treated for 48h at different drug concentrations. Read-out of the drug effect on tumor slices was performed *in vivo* using either Luciferase activity measurement with IVIS Imaging reader or immunofluorescence (cell live and dead kit). Quantification of Luciferase (Figure 5A Right Panel) activity indicates a decrease in the luminescent signal when compared to the tissue slice culture treated with only the vehicle. Figure 5B confirms the induction of apoptosis visualized by the presence of PI labeling. Quantification of the toxicity given by the drugs in tumor (Figure 5B1) and normal (Figure 5B1) tissue indicates that the inhibitors less affect normal cells.

Claims

1. A combination of a first agent inhibiting a protein kinase CK2 (CK2) and a second agent inhibiting an Ataxia Telangiectasia Mutated (ATM) kinase, for use as a medicament.
2. The combination of claim 1, for the use of claim 1, wherein said first agent is selected from the group consisting of chemical molecules inhibiting the kinase activity of CK2, siRNAs targeting a gene encoding CK2 α and/or α' and shRNAs targeting a gene encoding CK2 α and/or α' , and said second agent is selected from the group consisting of chemical molecules inhibiting the kinase activity of ATM kinase, siRNAs targeting a gene encoding ATM kinase and shRNAs targeting a gene encoding ATM kinase.
3. The combination of claim 1 or claim 2, for the use of claim 1, wherein said first agent is selected from the group consisting of 5-(3-chlorophenylamino)benzo[c][2,6]naphthyridine-8-carboxylic acid, 5,11-Dimethyl-6H-pyrido[4,3-b]carbazole, benzo[g]-pyridoindole and derivatives thereof.
4. The combination of any of claims 1 to 3, for the use of claim 1, wherein said second agent is selected from the group consisting of 2-((2S,6R)-2,6-dimethylmorpholino)-N-(5-(6-morpholino-4-oxo-4H-pyran-2-yl)-9H-thioxanthen-2-yl)acetamide, 2-Morpholin-4-yl-6-thianthren-1-yl-pyran-4-one and derivatives thereof.

5. The combination of any of claims 1 to 4, for the use of claim 1, wherein said first agent is 5-(3-chlorophenylamino)benzo[c][2,6]naphthyridine-8-carboxylic acid and said second agent is benzo[g]-pyridoindole.

6. The combination of any of claims 1 to 5, for the use of claim 1, wherein said first and second agents are comprised in a composition or in a kit of parts.

7. The combination of any of claims 1 to 6, for use as a medicament for treating a solid tumor that harbors a VHL inactivation.

8. The combination of any of claims 1 to 6, for use as a medicament for treating a renal clear cell carcinoma (ccRCC).

9. The combination of any of claims 1 to 6, for the use of claim 8, wherein said RCC is a metastatic renal cell carcinoma (mRCC).

10. ATM kinase inhibitor, for use as a medicament for potentiating the effect of a CK2 inhibitor in the treatment of a cancer that harbors a VHL inactivation.

11. The ATM kinase inhibitor of claim 10, for the use of claim 10, which is selected from the group consisting of 2-((2S,6R)-2,6-dimethylmorpholino)-N-(5-(6-morpholino-4-oxo-4H-pyran-2-yl)-9H-thioxanthen-2-yl)acetamide, 2-Morpholin-4-yl-6-thianthren-1-yl-pyran-4-one and derivatives thereof.

12. The ATM kinase inhibitor of claim 10 or claim 11, for the use of claim 10, for potentiating the effect of a CK2 inhibitor elected from the group consisting of 5-(3-chlorophenylamino)benzo[c][2,6]naphthyridine-8-carboxylic acid, 5,11-Dimethyl-6H-pyrido[4,3-b]carbazole, benzo[g]-pyridoindole and derivatives thereof.

13. The ATM kinase inhibitor of claim 10 or claim 11, for the use of claim 10 or claim 12, for potentiating the effect of a CK2 inhibitor in the treatment of a cancer selected amongst RCC, ccRCC and mRCC.

14. A CK2 inhibitor, for use as a medicament for potentiating the effect of an ATM kinase inhibitor in the treatment of a cancer that harbors a VHL inactivation.

15. The CK2 inhibitor of claim 14, for the use of claim 14, which is selected from the group consisting of 5-(3-chlorophenylamino)benzo[c][2,6]naphthyridine-8-carboxylic acid, 5,11-Dimethyl-6H-pyrido[4,3-b]carbazole, benzo[g]-pyridoindole and derivatives thereof.

Tables and Figures

Table 1 Inhibitors and their targets

Inhibitor	Target	Inhibitor	Target
AG-370	<i>PDGFR</i>	PERIFOSINE	<i>AKT</i>
AG-490	<i>JAK-STAT</i>	SU-4312	<i>PDGFR/VEGFR</i>
APATINIB	<i>VEGFR-2</i>	RAPAMYCIN	<i>m -TOR</i>
AT-9283	<i>AURORAA/B</i>	ZM-336372	<i>c-RAF</i>
BML257	<i>AKT1</i>	AURO1 INHIBITOR	<i>AURORA1</i>
CYT 387	<i>JAK1/JAK2</i>	DASATINIB	<i>BCR/ABL SRC c-KIT</i>
DOXORUBICIN	<i>Topoisimerase</i>	OSI-930	<i>c-KIT</i>
ENZASTAURIN	<i>PKCβ</i>	AZ-960	<i>JAK2</i>
FASETIN	<i>GLUT1</i>	IMATINIB	<i>BCR/ABL</i>
GDC-0941	<i>PI3K</i>	LAPATINIB	<i>EGFR/HER2</i>
GEFITINIB	<i>EGFR</i>	TYRPHOSTIN 25	<i>EGFR</i>
H-89	<i>PKA</i>	STF-62247	autophagy inducer
HYPERICIN	<i>DNA fragmentation</i>	SARACATINIB	<i>SRC</i>
INDIRUBIN	<i>GSK-3β</i>	KU-55933	<i>ATM</i>
INIPARIB	<i>PARP</i>	PAZOPANIB	<i>VEGFR/PDGFR/c-KIT</i>
KEMPOLLONE	<i>GSK-3β</i>	BARASERTIB	<i>AURORA B</i>
KN62	<i>Ca⁺⁺/CaM kinase II</i>	TEMSILORIMUS	<i>m-TOR</i>
LAVENDUSTIN A	<i>EGFR</i>	CRENOLANIB	<i>PDGFRα</i>
LY294002	<i>PI3K</i>	TGFR INHIBITOR	<i>TGF-β</i>
MK-177	<i>WEE1</i>	GW-5074	<i>c-RAF</i>
NVP-ADW742	<i>IGF-1R</i>	AG-126	<i>ERK1/ERK2</i>
PF-2341066	<i>c-Met</i>	PICEATANNOL	<i>SYK</i>
PF-562271	<i>FAK</i>	BOSUTINIB	<i>SRC</i>
PHA665762	<i>c-Met</i>	PP1	<i>SRC</i>
RG-1462	<i>EGFR</i>	CP-690550	<i>JAK1-3</i>
RUXOLITINIB	<i>JAK/STAT</i>	TSU-68	<i>PDGFRβ&FGFR1</i>
SB203580	<i>P38 MAPK</i>	GSK1838705	<i>IGFR</i>
SP600	<i>JNK</i>	CHIR99021	<i>GSK3β</i>
SU11274	<i>c-Met</i>	AG-490	<i>JAK2</i>
SUNITINIB	<i>VEGFR/PDGFR</i>	ML-7	<i>MYLK</i>
SGX523	<i>c-Met</i>	AZD-0530	<i>SRC</i>
TIE-2	<i>ANG2/Tie-2</i>	PACLITAXEL	<i>MICROTUBULES</i>
TOZASERTIB	<i>AURORA</i>	CX-4945	<i>CK2</i>
TYRPHOSTIN 1	<i>EGFR</i>	KX2-391	<i>SRC</i>
U0126	<i>MEK1/MEK2</i>	KRN633	<i>VEGFR</i>
VEMURAFENIB	<i>MEK</i>	AXITINIB	<i>VEGFR&PDGFR</i>
WP1130	<i>BCR/ABL</i>	GSK1120212	<i>MEK1/2</i>
Y-27632	<i>ROCK</i>	INCB018424	<i>JAK/STAT</i>
YM155	<i>Survivin</i>	5FU	<i>DNA</i>
SORAFENIB	<i>RAF/MEK/ERK</i>	ROSCOVITINE	<i>CDC2/CYCLIN B/A/E</i>
	<i>VEGFR/PDGFR</i>		<i>CDK5/p35</i>

Table 2

Gene	Accession Number	Gene	Accession Number
PDK1	NM_002610	BCL2A1	NM_004049
PLK2	NM_006622	TP53	NM_000546
PNCK	NM_198452	ATM	NM_000051
HCK	NM_002110	ATR	NM_001184
NEK6	NM_014397	CHEK1	NM_001274
TRIB3	NM_021158	CHEK2	NM_007194
PCTK3 / CDK18	NM_002596	MAPK14	NM_001315
MET	NM_000245	CDK6	NM_001259
MELK	NM_014791	PTK2	NM_005607
AURKB	NM_004217	SRC	NM_198291
KIT	NM_000222	PAX2	NM_000278
PRKCD	NM_006254	CSF1R	NM_005211
PXK	NM_017771	FGFR1	NM_015850
PDGFRL	NM_006207	CA9	NM_001216
LCK	NM_005356	AURKA	NM_003600
PIM2	NM_006875	FLT1	NM_002019
MAPK1	NM_138957	CSNK2A1	NM_001895
AURKB	NM_004217	CSNK2B	NM_001320

Figure 1

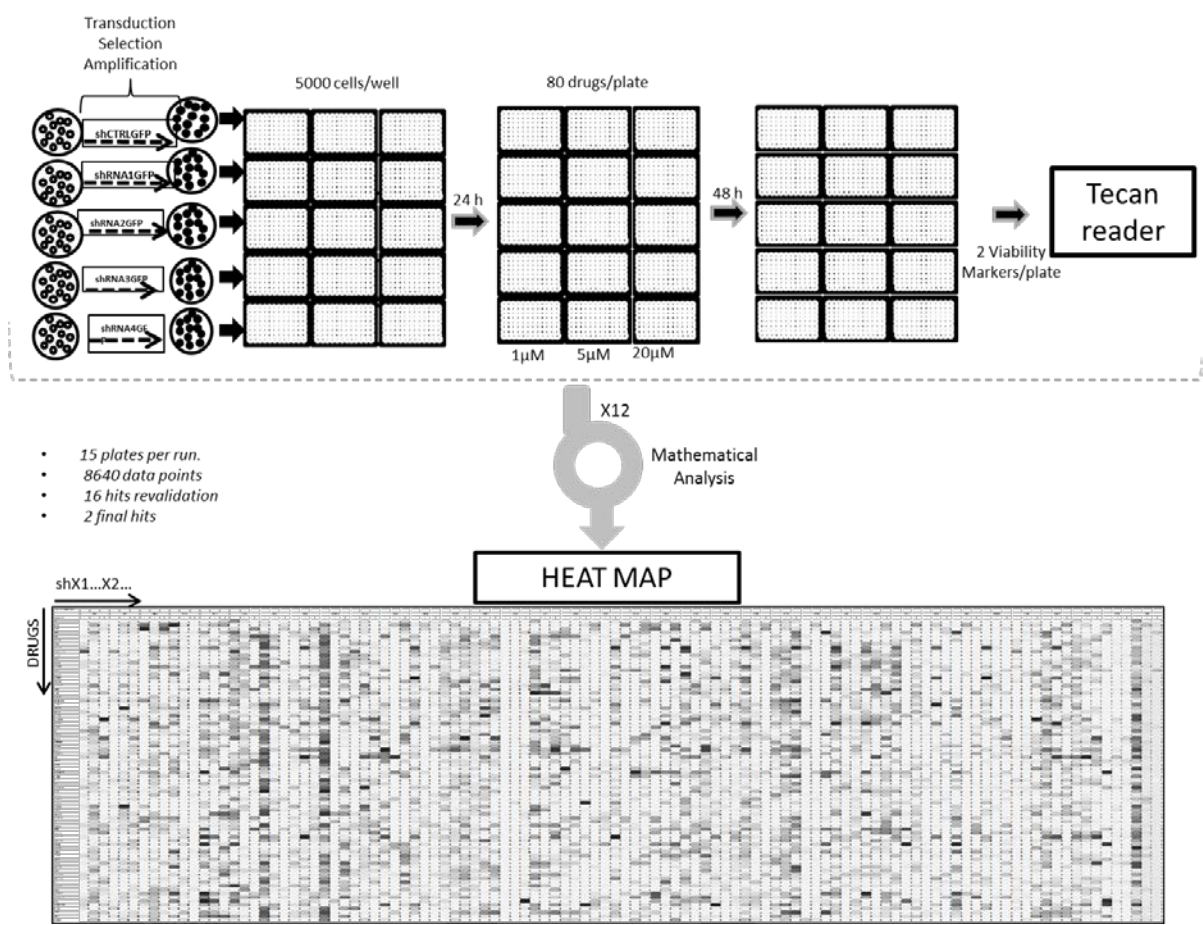


Figure 2

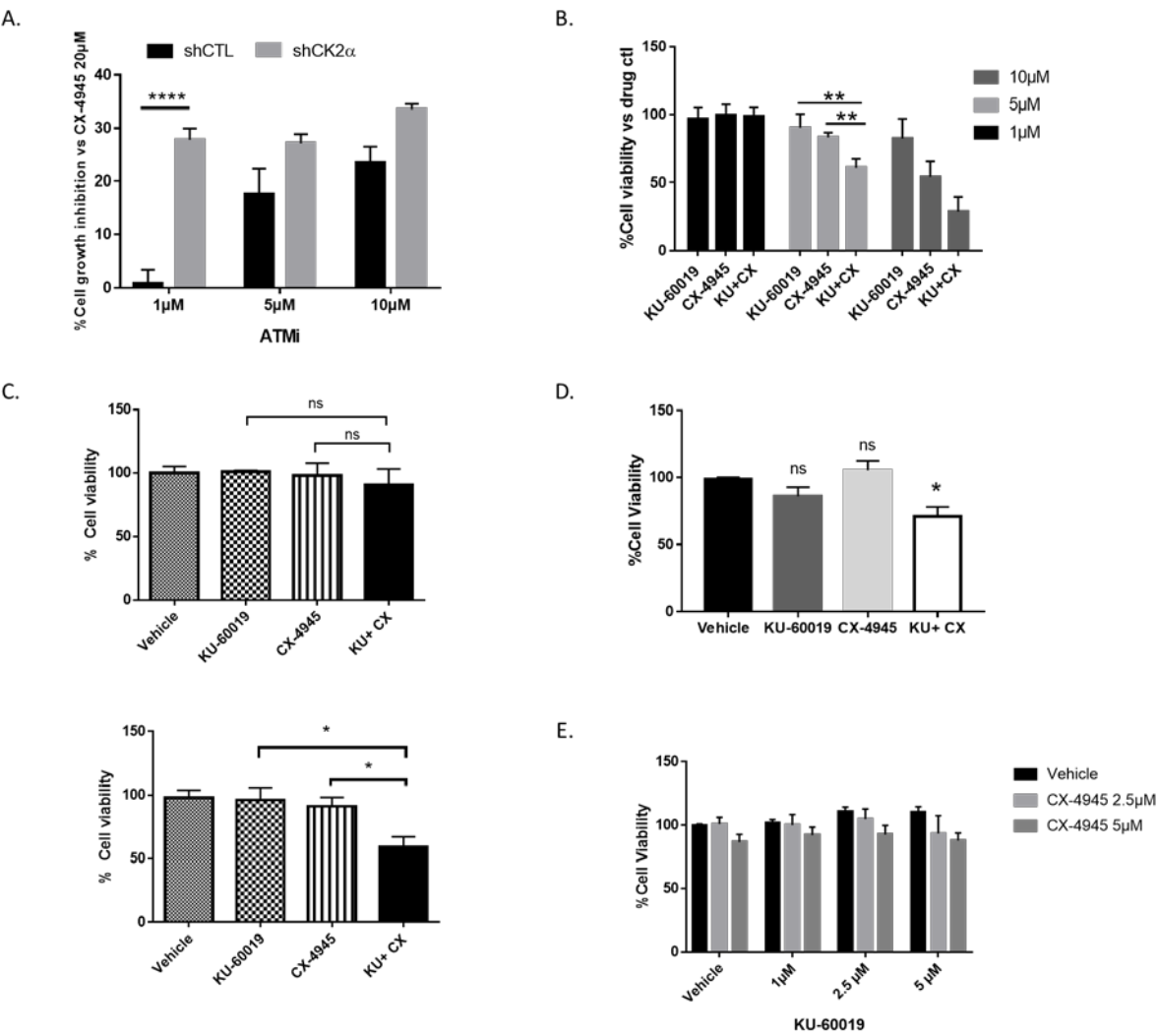


Figure 3

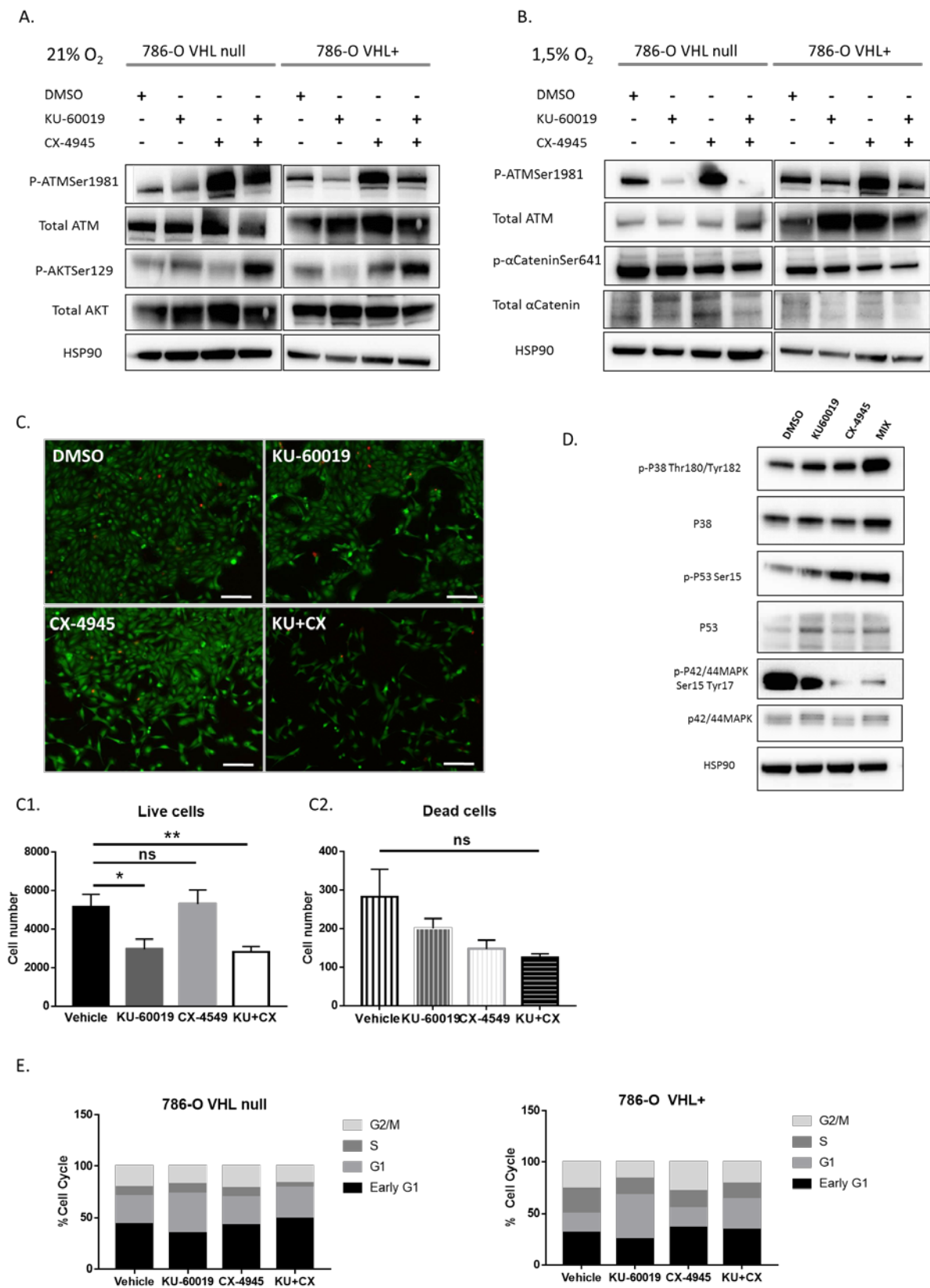


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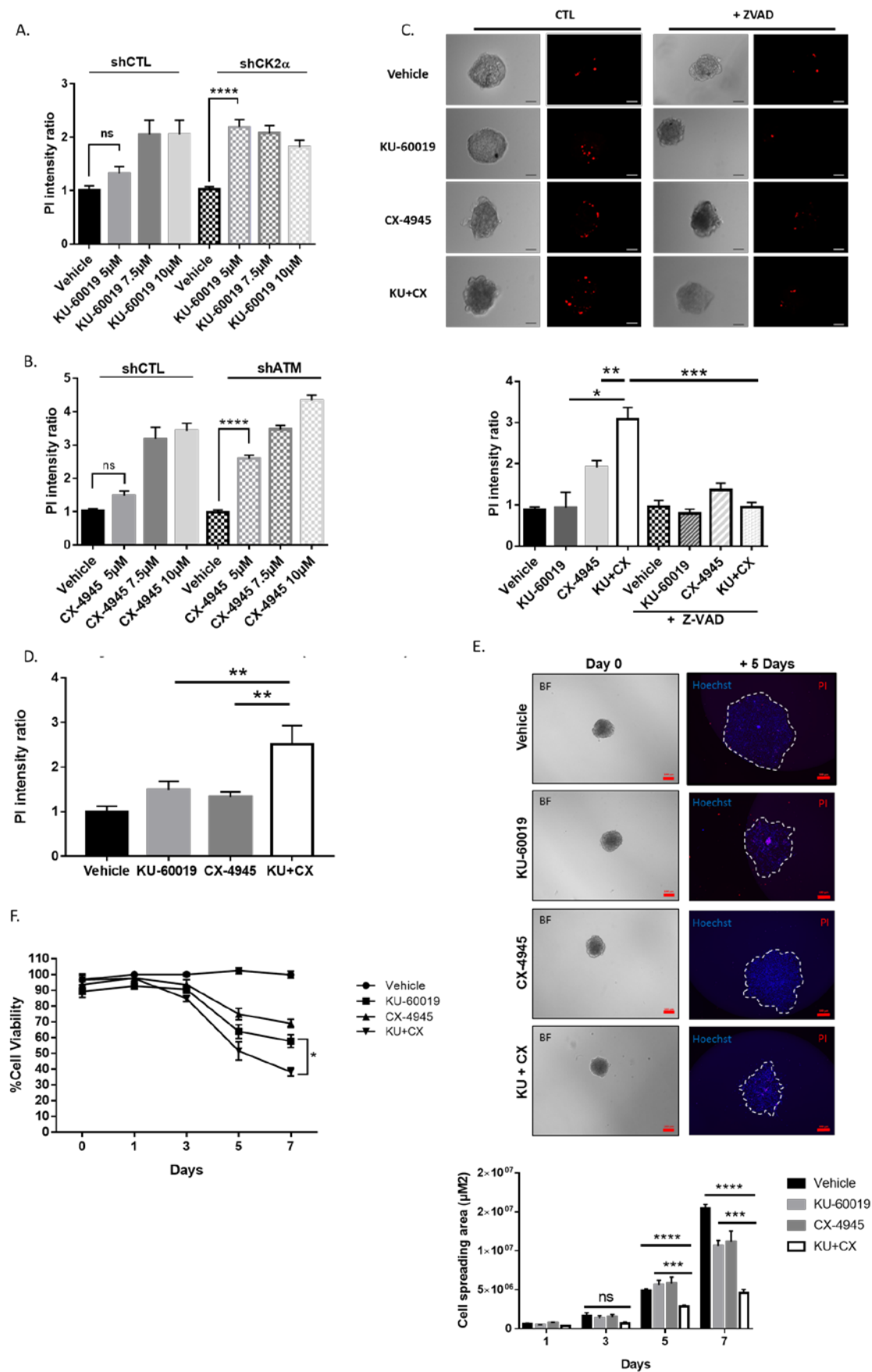
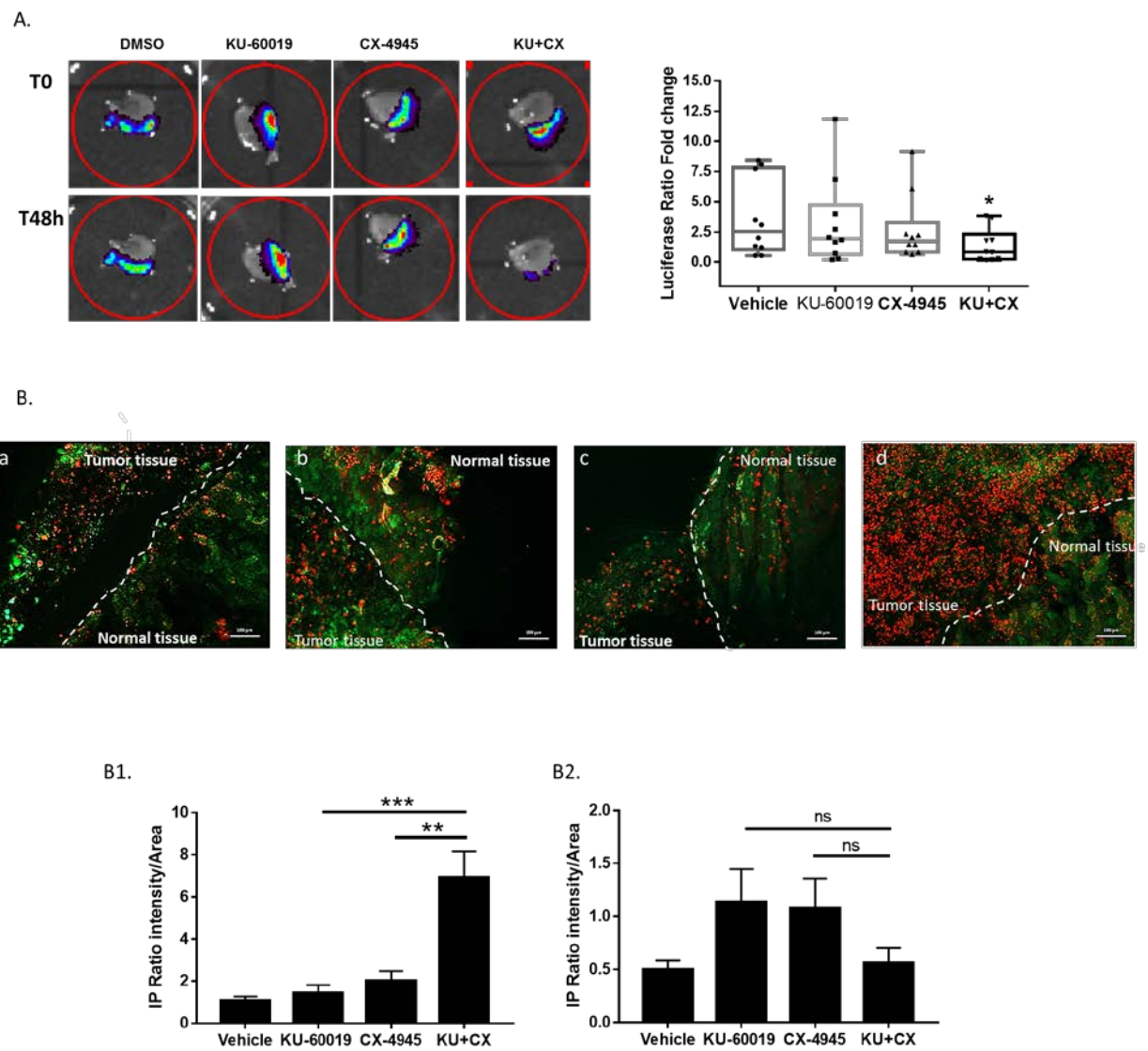
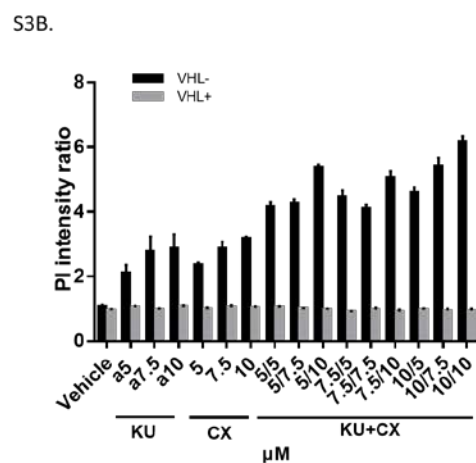
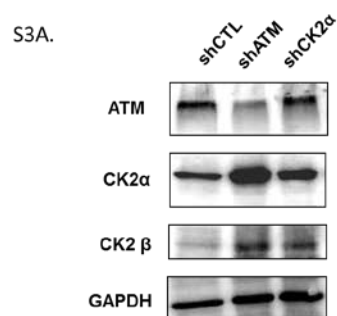
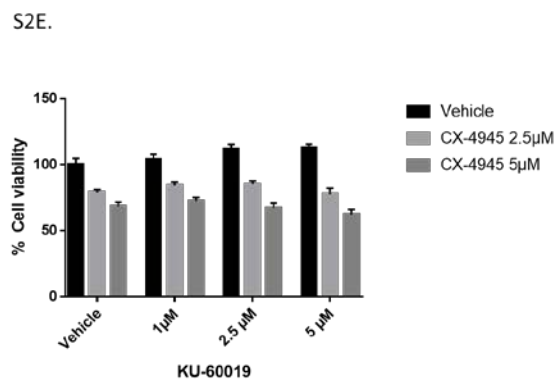
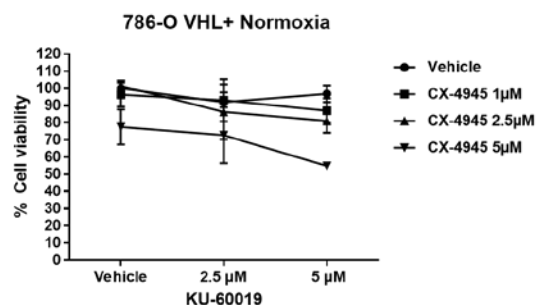
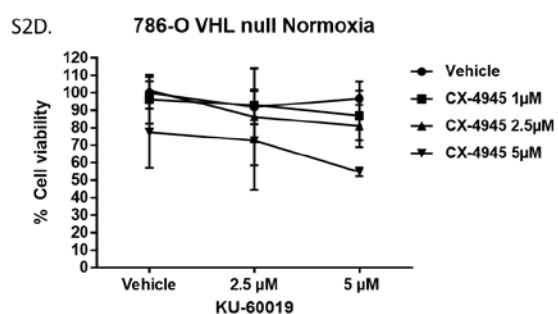
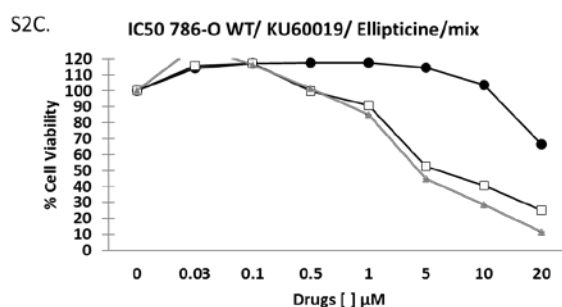
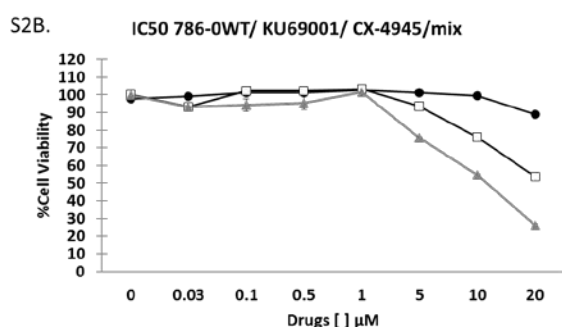
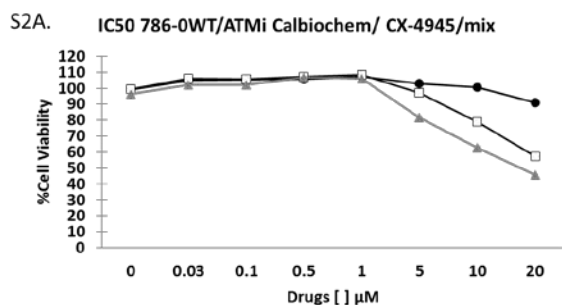


Figure 5

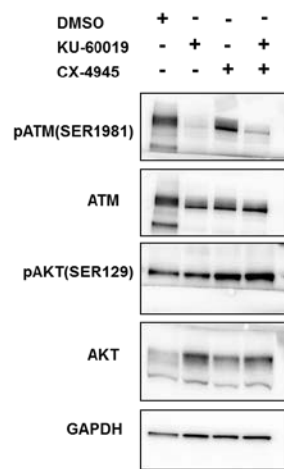


Supplementary Figures:

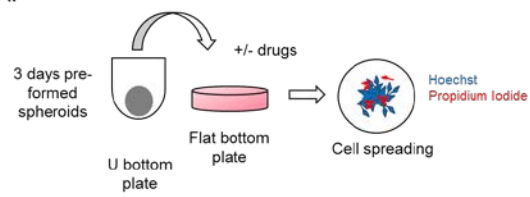


786-O VHL-		
Vs Vehicle	Mann-Whitney	p value
A5	*	0.152
A7.5	*	0.0217
A10	**	0.056
CX5	**	0.0022
CX7.5	**	0.0023
CX10	**	0.0012
A5+CX5	***	0.0007
A5+CX7.5	**	0.0013
A5+CX10	***	0.0007
A7.5+CX5	**	0.0012
A7.5+CX7.5	**	0.0023
A7.5+CX10	**	0.0012
A10+CX5	**	0.0022
A10+CX7.5	**	0.0012
A10+CX10	**	0.0022

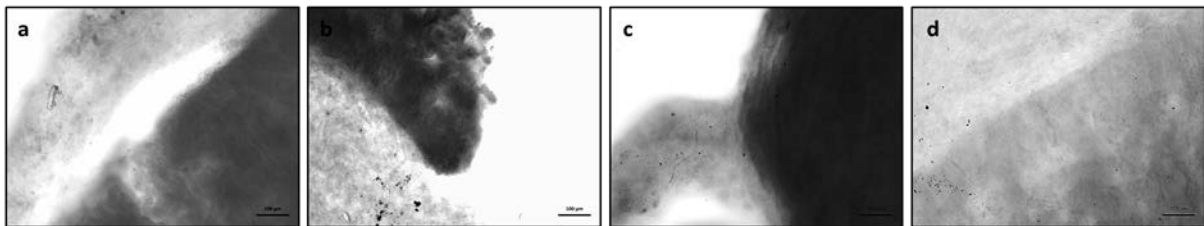
S3C.



S4A.



S5A.



Figures legends

Figure 1: Flow chart of the screening. a. 786-O cell line was transduced with either shRNA non-target (shCTL) or shRNA against different gene kinase targets (shLIBRARY). After selection, cells were seeded in 96 well plates and treated against 80 different kinases inhibitors. Prestoblu[®] cell viability marker kit was added and the read out was performed through CMBA robot platform. Conditions where the cell viability was higher in the shCTL cell line comparing to a shRNA gene target cell line in presence of a certain inhibitor (represented as shA, shZ, etc) were confirmed. Validation was made through the same process indicated above. The shRNA gene targets that had lower cell viability ratio than its control in the presence of the same drug were considered as Hits.

Figure 2: Co-Inhibition of CK2 α and ATM induces enhanced cell toxicity in ccRCC cells. A) shCTL and shCK2 α 786-O cells were treated with indicated concentrations of ATM inhibitor for 48h and -cell viability was measured using Prestoblu[®]. **** represents $p < 0.0001$ (Two way ANOVA analysis) as the difference between % cell growth inhibitions of ATMi in shCTL vs shCK2 α . B) Relative sensitivity of 786-O cells to a dual drug inhibition of CK2 α (CX-4945 inhibitor) and ATM (KU-60019 inhibitor) kinases at different concentrations (1 μ M in black; 5 μ M in light grey and 10 μ M in dark grey). (** $P \leq 0.001$ Mann Whitney comparing CX-4945 5 μ M vs KU (5 μ M) +CX (5 μ M) and KU-60019 5 μ M vs KU (5 μ M) +CX(5 μ M). C) Relative sensitivity of 786-O cells with re-introduced VHL (Upper panel) or without (Lower panel) to drugs alone (KU-60019 or CX-4945) or combined, in Hypoxia conditions (1.5% O₂). (* $P < 0.05$ Mann Whitney comparing CX-4945 2.5 μ M alone or KU-60019 5 μ M vs MIX KU (5 μ M) +CX (2.5 μ M). D-E) Relative sensitivity of CAKI-2 ccRCC cell line or RPTEC normal cells to drugs alone (KU-60019, CX-4945) or combined in Hypoxia conditions (1.5% O₂) compared to the Vehicle treatment (DMSO). (* $P < 0.05$ Mann Whitney when comparing MIX (KU (5 μ M) +CX (5 μ M)) to Vehicle). No significant difference was found in the RPTEC cells compared to the control in normoxia conditions.

Figure 3: Cell stress and no apoptosis in monolayer culture. 786-O cells (VHL+ and VHL-) cultured under A) normoxia (21% O₂, left panel) or B) hypoxia (1.5 % O₂, right panel) were treated for 48h with DMSO (Ctrl), 5 μ M KU60019 (ATMi), 2.5 μ M CX-4945 or both (mix).

Expression (total) and phosphorylation (P-) of ATM, AKT and/or α catenin were assessed by western Blot. HSP90 was used as a loading control. C) 786-O cell images showing lived cells (green cells) relative to dead cells (red cells) in response to Vehicle (DMSO), KU-60019, 5 μ M CX-4945 2.5 μ M or KU 5 μ M+CX 2.5 μ M, after 48h of treatment in Hypoxia conditions (1.5% O₂) (Live/Dead Kit). Bar scale: 200 μ m. C1): Cell number quantification of live cells (plain bars) and C2) dead cells (motif bars) when treated with either drugs alone or in combination as indicated above. (Significant difference between CX-4945 2.5 μ M vs MIX KU (5 μ M) + CX (2.5 μ M) is represented * $P < 0.05$ Mann Whitney test). NS: no significant difference. D) Stress pathways of 786-O VHL null cells after treatment. Expression (total) and phosphorylation (P-) of p38, MAPK, p53 and BAX were assessed by western Blot. HSP90 was used as a loading control. E) FACS analysis of 786-O VHL null (left panel) and 786-O VHL+ (right panel) cells expressing the FUCCI cell cycle probes, treated with drugs alone or in combination (KU 5 μ M, CX 2.5 μ M) for 48H in hypoxia conditions (1.5% O₂). Increased G1 phase and shorter S phase when 786-O VHL null cells are compared to 786-O VHL cells.

Figure 4: Cell death is induced in tumor environment conditions. A) Multicellular Tumor Spheroids (MTCS) were pre-formed for 3 days with indicated 786-O sh-transduced cell lines before treatment for 48h with vehicle or increasing concentrations of KU-60019 or CX-4945 (5, 7.5 and 10 μ M). Significant difference (**** $P \leq 0.0001$) was observed when comparing the treatment of either shCK2 α MCTS to Vehicle (DMSO) with 5 μ M KU-60019 (A) or shATM MCTS to vehicle with 5 μ M CX-4945 (B) (Kruskal-Wallis non-parametric test). Cell death was monitored by Propidium Iodide quantification using ArrayScan® VTI HCS Reader. C) 786-O VHL null MCTS where treated with either vehicle (DMSO), drugs alone (KU-60019 10 μ M; CX-4945 7.5 μ M) or in combination (KU 10 μ M/CX 7.5 μ M) during 48H, in presence or absence of 15 μ M Z-VAD (apoptosis inhibitor). Upper panel: Pictures representative of MCTS treated as indicated above in presence or absence of Z-VAD apoptosis inhibitor. Red marker: Propidium Iodide. Bar scale: 50 μ m Pictures were taken with AxioObserver z1 Zeiss microscope. Lower panel: Quantification of Propidium iodide intensity. Significant difference was found between KU-60019 treatment (* $p < 0.05$), CX-4945 (** $p \leq 0.001$) and the combinational treatment. As well, *** $p = 0.0001$ represents the difference of KU+CX in absence of Z-VAD

compared to the effect of either Vehicle + Z-VAD, CX-4945+Z-VAD, KU-60019+ Z-VAD or MIX+Z-VAD. (Mann-Whitney test for all the describe conditions).

D) ER305 MCTS cells were treated with either vehicle (DMSO), drugs alone (KU-60019 10 μ M; CX-4945 7.5 μ M) or in combination (KU 10 μ M/CX7.5 μ M) for 48H before cell death quantification. Significant differences (** $p \leq 0.001$) when comparing KU or CX alone vs MIX.

E) Upper panel: pictures of spheroids at day 0 and day 5 Bar scale at day 0: 100 μ m. Bar scale at 5 days 500 μ m. Lower panel: Kinetic analysis of cell spreading. The area of cell spreading was measured over time from MCTS treated with vehicle (black), KU (light grey) CX (dark grey) or both (white). After 5 and 7 days of treatment significant differences were observed between MCTS treated with CTL (**** $p < 0.0001$) and KU-60019 or CX-4945 (*** $p = 0.0001$) versus KU+CX. (Kruskal-Wallis test for all described conditions). F) Cell viability kinetics was also measure after 0, 3, 5 and 5 days of cell spreading in presence of treatments described above. At day 7, * represents $p < 0.05$ between cell spreading of MCTS treated with KU-60019 versus the mix. (Kruskal-Wallis test).

Figure 5: *Ex vivo* models as pre-clinical tests for drug combination efficacy. A) Tissue slices were imaged for Luciferase activity before treatment and 48h after incubation with indicated molecules (dots: vehicle, square: 10 μ M ATM triangle: 10 μ M CX-4945, inverse triangle: 10 μ M ATM+ 10 μ M CX-4945). Right panel: Luciferase ratio fold changes on each condition compared to changes in the Control after 48 hours of treatments (* $p < 0.05$). Mann-Whitney test. B) Tissue slices cultures carrying tumour and normal kidney tissue (indicated in the pictures) were marked with Live and Dead Kit after 48h of treatments with a. Vehicle; b. KU-60019 10 μ M c. CX-4945 10 μ M and d. KU 10 μ M+CX10 μ M. Intensity of Propidium iodide (Red marker =dead cells) was measured on both normal and tumour areas. B1) Significant difference was observed between KU-60019 (*** $p = 0.0005$), CX-4945 (** $p = 0.007$) alone versus KU+CX. Right Panel: PI intensity in normal tissue shows no significant difference when comparing each condition alone versus the mix of drugs. Bar scale 100 μ m (Mann-Whitney test for all describe conditions).

Supplementary figures legends

Figure 2: Alternative inhibition of hit pathways. S2A-B) IC₅₀ of 2 different ATM inhibitors (ATMi (Calbiochem) and KU-60019 (ENZO) in combination with CX-4945 to test their efficacy on 786_O cell viability ●: ATM inhibitor, □: CX-4945, Δ: both). Cell viability was measured and represented as percentage of viability compared to cells treated with the vehicle (DMSO) taken as 100%. S2C) The same test was used to determine IC₅₀ of CK2 inhibitor Ellipticine. Figure S2D: 786-O cells (VHL- lower panel and VHL+ lower panel) were culture under 20 % O₂ (normoxia) and treated with either vehicle, 2.5μM or 5μM of KU-60019 in combination with either vehicle or increasing concentrations of CX-4945, Vehicle (plain lines with circles), 1 μM (plain line with squares), 2.5μM (plain line with triangles) or 5μM (plain line with inverse triangles). Cell viability was measured and represented as a percentage compared to DMSO taken as 100%. No significant difference was found when compared drugs alone versus combination (2way ANOVA test). SDE: RPTEC cells were treated with indicated concentrations of KU-60019 in the absence or in the presence of Vehicle, 2.5 or 5μM (black, light grey and dark grey bars respectively) of CX-4945 for 48h. Cell viability was measured as described before. No significant differences were found between CX-4945 alone versus combined with KU. *** represents $p < 0.0001$ as the difference between KU-60019 at 2.5μM or combined with CX-4945 1μM (2 Way ANOVA test).

Figure 3: S3A) Western Blot analysis of stable shATM and shCK2α cell lines showing decreased protein expression levels of either ATM or CK2α after shRNA transduction and selection. CK2β levels do not change, showing specificity of shRNA against CK2α. Loading was normalized using GAPDH. S3B) 786-O VHL null and 786-O VHL+ MCTS where treated with either vehicle (DMSO), increase concentration of KU-60019 or CX-4945 alone or in combination during 48H. Propidium Iodide intensity was quantified using ThermoFisher Cellomics' Arrayscan machine. Lower panel: p values for each condition. In VHL+ cells no significant difference was observed for all conditions (Mann-Whitney test). S3C) Western Blot Analysis of drug targeted proteins in MCTS 786-O VHL-213 cells after treatment with Vehicle, KU-60019 10 μM, CX-4945 7.5 μM or combination of both for 48H. Represented proteins (pATM(Ser1981); ATM; pAKT(Ser129); AKT. GAPDH was used as loading control.

Figure S4A: MCTS cell spreading experiment schema: MCTS were pre-formed during 3 days in a low-binding U bottom plate, moved to a flat bottom plate and immediately treated for 48h with vehicle, drugs alone or in combination. Cell spreading was followed by addition of Hoechst marker and measure using Image J software. Dead cells were visualized using Propidium Iodide marker.

Figure S5A: Bright field pictures of tissue slices cultures treated with a. Vehicle; b. KU-60019 10 μ M c. CX-4945 10 μ M and d. KU 10 μ M+CX10 μ M. The tumour tissue (or infiltrated tissue) is often seen as more “transparent” and disorganized than the normal tissue. Bar scale 100 μ m

Discussion

After surgery, targeted therapy has been the main front line treatment in ccRCC. Although it has shown promising results in a first time, soon it became evident that tumors were not only developing resistance but also becoming more aggressive and inducing metastasis, decreasing patient survival up to a 50% ([Husillos–Alonso 2015](#), [Penticuff 2015](#)).

So the question became: How do cells become resistant to these so “effective” target therapies. The first answer was that cancer cells could change site of the inhibitor’s interaction. Although recent reports about the development of second and third class of same targeted therapies tends to show an alternative, this seems to be only a short time alternative until the cells adapt and resist again.

As well, resistance has been shown to be induced by upregulation of an alternative pathway ([Buckzec 2014](#)). To counter this mechanism, we have searched for novel combinational therapies using an approach of dual genetic and chemical screening. A striking observation has come out from the data analysis. We found that in our cell line, dual inhibition of the Ataxia telangiectasia mutated (ATM) kinase and the protein kinase CK2 is more effective to decrease cell viability than either single inhibition. Moreover, validation of this combined inhibition of CK2 and ATM kinases led us not only to propose an alternative treatment against ccRCC but also to demonstrate the importance of mimicking the tumor environments, either by 3D MCTS or tissue slice culture, in novel drugs validation.

Although ATM has been shown to induce synthetic lethality together with MET after p53 activation ([Sullivan 2012](#)) and CK2 inhibition combined with both conventional chemotherapeutics and novel agents, similar to heat-shock protein 90, proteasome and tyrosine kinases inhibitors ([Mandato 2016](#)) to our knowledge until today, these two kinases together have never been considered before as partners to induce synthetic lethality.

Protein kinase CK2 is a multifunctional and pleiotropic serine/threonine kinase that plays major roles in cell cycle progression, apoptosis, cell differentiation and transcription processes ([Litchfield, 2003](#)). As a signaling protein, CK2 could be targeted to different cellular compartments in response to various stresses such as hypoxia, heat shock, DNA damage ([Filhol 2009](#)). In addition, CK2 α is overexpressed and its activity is enhanced in multiple forms of human cancers ([Ruzzene 2009](#)) including prostate ([Laramas 2007](#)) and glioblastoma ([Zheng 2013](#)). Thus, CK2 is now regarded as a potential target for specific therapy in human malignancies and a first highly selective CK2 inhibitor, called CX-4945 (Silmitasertib) has been developed and entered into Phase I/II clinical trials for the treatment of solid tumors and myeloma patients ([Siddiqui-Jain, 2010](#), [Pierre 2010](#)). We have previously report that inhibition of CK2 in clear renal cell carcinoma induces cell death but regardless of the VHL status of the cell ([Roelants 2015](#)).

Our studies provide a novel application for the use of this potent anticancer drug through its combination with another key player kinase, ATM.

Ataxia Telangiectasia Mutated protein (ATM) is a large serine/threonine protein kinase, that belongs to the phosphoinositide 3-kinase-related protein kinase (PIKK) family (Cremona 2014). ATM plays a central role in DNA repair response: activated by DNA damage, ATM phosphorylates itself and downstream effectors that arrest cell cycle allowing DNA repair or, should DNA damage be too severe and not retrievable, inducing apoptosis. ATM is a worth-investigating target for tumor radio- and chemo-sensitization ([Shiloh 2013](#)). During last years, pharmaceutical industries and research laboratories have developed a series of small molecules, capable to inhibit ATM with increasing specificity. Although small-molecule ATM kinase inhibitors, such as CP466722 and KU-60019 were identified using in vitro kinase assays, new strategies are developed to find novel drugs ([Guo 2014](#)). Furthermore, several

preclinical studies have demonstrated that these inhibitors alone or in association with other treatments may improve therapeutic outcomes.

MultiCellular Tumor Spheroid (MCTS) is a 3D model where the organization of a microtumor is reproduced, recapitulating cell-cell and cell-microenvironment interactions ([Dubessy 2000](#), [Nath 2016](#)). MCTS accurately reproduce the 3D architecture of solid tumors, filling the gap between monolayer cultured cells and animal models, and their use as evaluation models for new anti-cancer strategies have been increasing in the last years ([Hirschhaeuser 2010](#), [Desoize 2000](#)). With respect to modeling in vivo tissue functionality, numerous studies have shown that cell responses to drugs in 3D culture are improved compared to those evaluated in 2D ([Dufau 2015](#)). Interestingly, we found that in monolayer culture, the impaired viability induced by our drug combination was enhanced when we used hypoxia conditions but we did not observed apoptosis related cell death. Instead, ccRCC spheroids clearly showed apoptosis cell signal.

Cell migration is often a mechanism of resistance that the tumor develops in response to the drug. In this study, we determined that even after 7 days after the first and only drug dose, ccRCC cell lost their capacity to migrate. Since loss of cohesion inside the tumor allows the tumor cells to escape and induce metastasis. It would be interesting to study how cell aggregation and the cell-to-cell adhesion are affected by this novel therapy inside the 3D spheroid ([Saia 2015](#)).

These results strongly implicate that the microenvironment inside the tumor, either by decreased levels of oxygen, ATP, cell cycle or nutrients, sensitizes these cancer cells to the inhibition of CK2 and ATM.

The von Hippel Lindau tumor suppressor is either lost or non-functional in the majority of kidney cancer patients. This characteristic inside the tumor has been the focus of several researchs to find inhibitors that will only affect pVHL null cells. For example, [Turcotte 2008](#) and others have described an inhibitor that induces autophagic cell death only in cancer cells that lack VHL. We consistently found that either cells with re-introduced VHL or normal cells

as RPTEC, or even more, normal tissue surrounding the tumor was almost not affected by the inhibition of these pathways. More studies are needed to better understand the mechanisms underlying this combination.

Considering that the CK2 inhibitor CX-4945 is currently evaluated for the treatment of several types of cancers, our findings reveal a strategy to potentially improve the efficacy of CK2 inhibition in RCC by co-treatment with ATM inhibitors. There are still many questions to be answered as well as several experiments to be performed to understand the mechanism, but we strongly believe that our combination of CK2 and ATM inhibitors can offer a preclinical rationale to treat advanced stage RCC, with immediate implications for clinical evaluation.

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Conclusion

High-Throughput screening has arisen as a potent tool to the discovery of novel drugs and targets against cancer disease, and it has been shown to be useful to test potential combination between already discovered drugs and to take advantage of the genome heterogeneity in cancer cells while searching for targets.

In this study we aimed to search novel therapeutic targets against ccRCC using a dual screening that combined genetic and chemical approach.

To assure the quality and reproducibility of a HTS, we set up several conditions before to start the screening.

Although clear cell renal cell carcinoma was first discovered in patients carrying the von Hippel Lindau disease, in most cases, ccRCC arises due to somatic bi-allelic mutations in VHL gene localized the short arm of chromosome 3 (Foster 1994).

Because in the majority of patients with kidney cancer the disease is ccRCC, we decided to perform the screening using a patient derived cell line that has been already used as a model of this disease. 786-O cell line has been shown to be VHL null but to express HIF-2 α subunit (Hu 2003).

The different type of mutations inside the gene (deletion, inactivation, missense, frameshift, etc) can lead to different expression levels of HIF-1 α and HIF-2 α affecting different pathways implicated in metabolism, cell survival and disease progression (Gordan 2008). This indicates that it is important to have in account the genetic background of the cell line chosen for the screening since it is often than in the same disease, there are different gene mutations or levels of expression of the same protein. This difference can have a lead to different drug response.

Targeted therapies against ccRCC showed promising results at the beginning but later it became evident that the cancer cell could find an alternative pathway to resist the drug effect. In our approach, we seek to identify the molecular pathways that are linked to the drug response. shRNAs were used as an alternative to limit the inhibition of the off-target pathways that are potentially targeted by chemical compounds. Sh/siRNA therapy is a promising technique that can be used therapeutically to block the synthesis of disease-causing proteins. However, the main obstacle to their use as therapeutic drugs is the difficulty of their delivery into the cell cytosol, their potential off-target effects associated with high cell genotoxicity. Today various pathway-based mechanisms have been studied to overcome these barriers using chemical inhibitors instead of siRNA (Wittrup 2015).

Thus, as a second step, we used chemical inhibitors to replace the shRNA target genes. For this purpose we looked for inhibitors that were described to have high specificity for our genes of interest.

SUPPLEMENTARY RESULTS (i)

Study of the phenotype given by drug inhibitors

The main objective of drug discovery is to kill malignant cells and a minimal effect over normal cells. The fact that cancer is often caused by dysregulation in the activity or in the expression of molecular targets that differ from normal cells facilitates the goal of killing these cancer cells.

Sequential screening has been the main driving force to find novel therapeutic targets in different types of cancer. Through a High Throughput screening, our objective was to find a novel combinational therapy that would inhibit only clear cell renal carcinoma cells. (see patent) .

Although this objective was achieved, several questions remained unanswered. Between them, were how the drugs affect the structure of our cells, and why our drug combination would not affect cells that have the von Hippel Lindau protein?

1.1 Intracellular vacuolar structures

Together with decreased cell death in re-introduced pVHL cells, we observed that when we confronted them to the inhibitor of KU-60019, or with the combination of CX-4945 and KU-60019, 786-O cells showed increased number of “Holes or Vacuoles” compared to the control treated cells (DMSO). Moreover, the 786-O VHL+ cells showed bigger vacuoles when treated with the inhibitor of ATM than the VHL null cells. In appearance, the size of vacuoles in both cell lines were smaller when they were treated with the combination of CX-4945 and KU-60019 than with the inhibitor of ATM alone (Figure N°1A). Vacuoles were persistent after 48h of treatment in both hypoxia (Figure N°1A) and normoxia conditions (data not shown).

In collaboration with the Institute of Structural Biology - Grenoble, cells were included in resin (EPON) after PFA fixation and cut into slices that were analysed through Electronic

Microscopy. This microscope has the power to observed structures with magnification up to 10.000.000X obtaining a higher resolution power than a classic microscope.

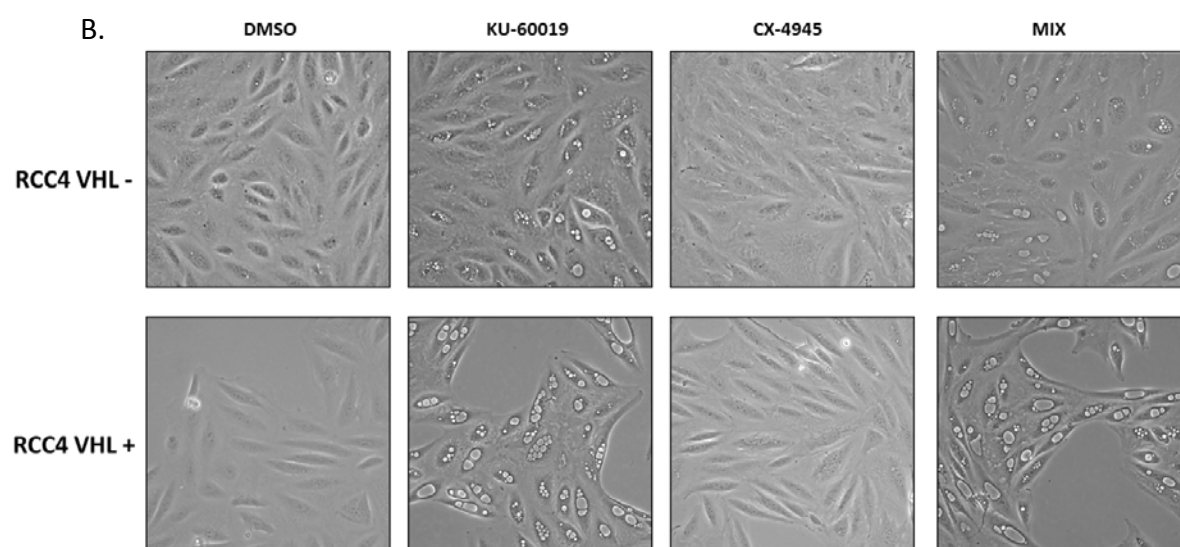
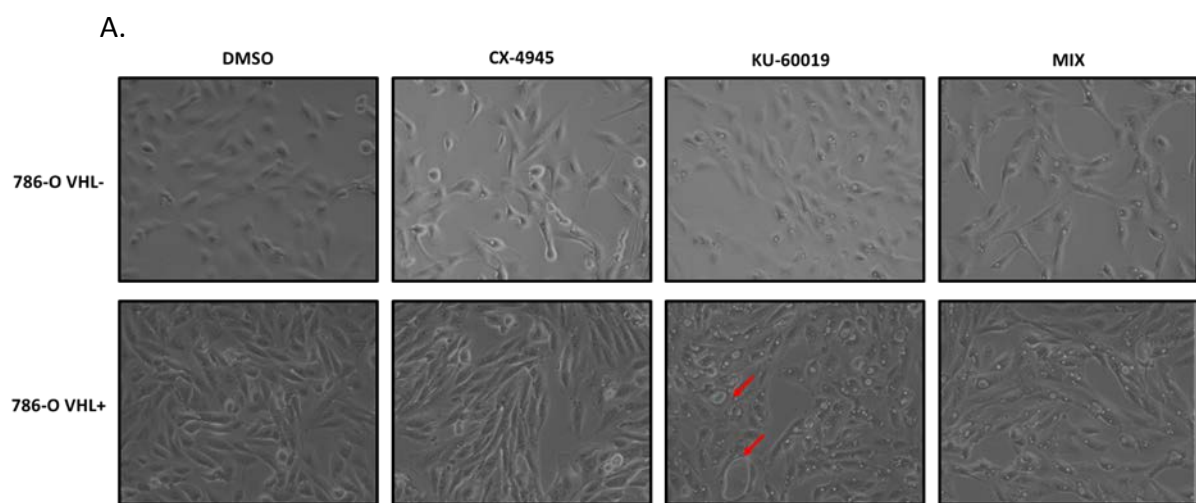
In presence of 5 μ M of KU-60019 the cell morphology is modified due to the appearance of very big vacuoles or vesicles in the cytoplasm. These vacuoles seems to fuse between them and to become bigger by doing so. Finally, these structures seem to be empty as no debris or organelles is visible even in the highest magnification (X4800) observed. Strikingly, there is no visible membrane surrounding these vacuoles. Figure N°2A

In presence of 2.5 μ M CX-4945, both cell lines present very small vacuoles and cell morphology resembles to the CTL where there are no vacuoles. Figure N°2A, 2C.

Treatment with the combination of ATM and CK2 inhibitors shows a mix of small and big vacuoles that fuse between them in the VHL+ cells (Figure N°2A and 2B). In the VHL null cell there are mainly small vacuoles and the fusion is less frequent. Figure N°2C

In all cases the ultrastructure of the cells looks normal (beside the presence of the vacuoles), with organized ribosomes and mitochondria showing normal morphology.

Video microscope showed as well that these vacuoles fuse between each other. Interestingly this fusion does not impair the movement neither the division of the cells. Video N°1-8



C.

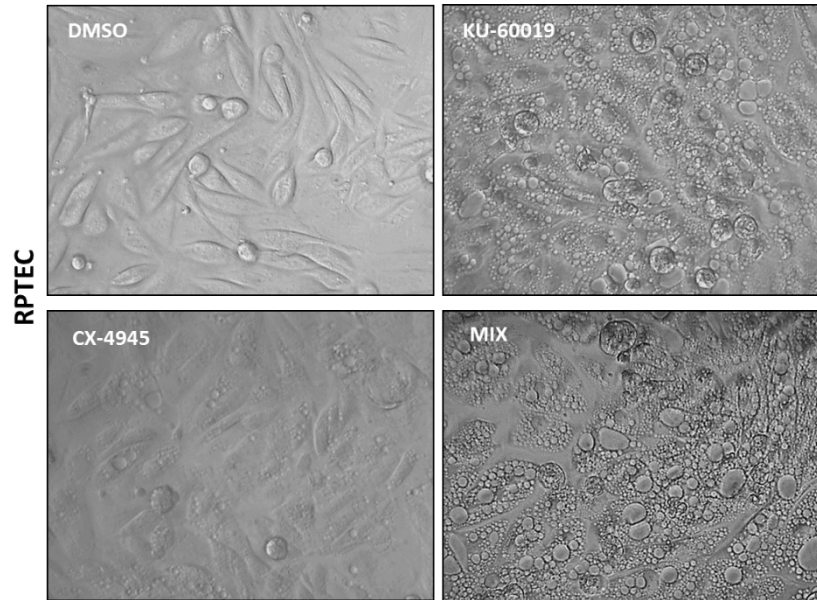
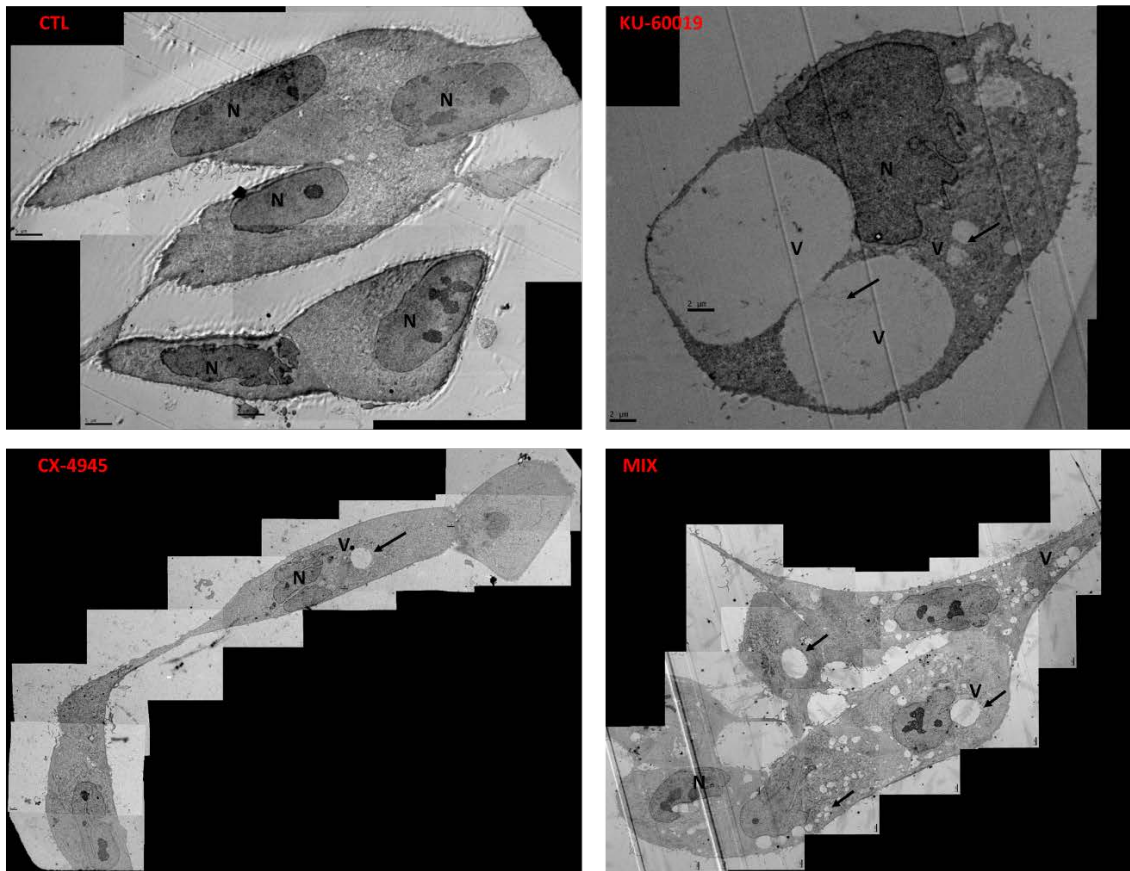
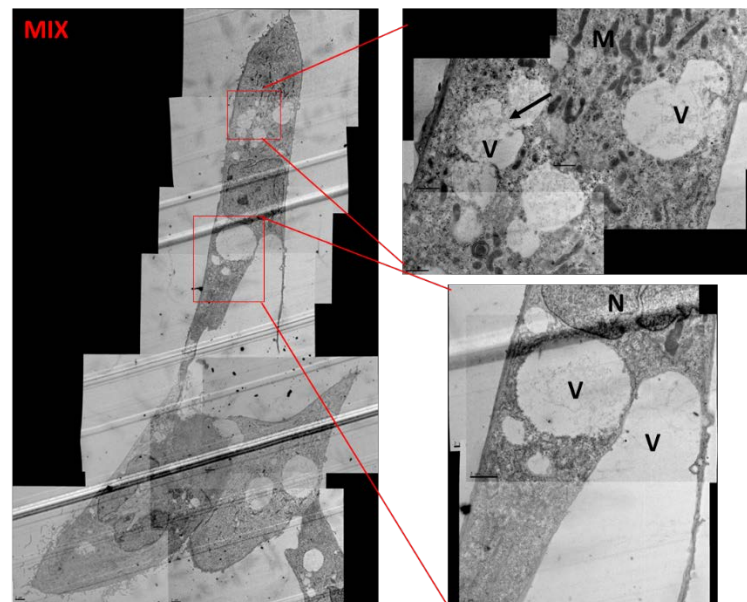


Figure 1: Inhibition of ATM induces morphological changes with giant vacuolization in VHL+ cells. ccRCC cell lines 786-O WT A and 786-O VHL+ cells B; RCC4- and RCC4+ cells and C; primary Renal Proximal Tubule Epithelial cell line (RPTEC) where treated with either DMSO (CTL), 5 μ M KU-60019, 2.5 μ M CX-4945 or the MIX of both during 48h in hypoxia conditions (A;B) and in normoxia conditions (C). Bright field pictures where taken with Axioplan Zeiss microscope. 10X magnification.

A.



B.



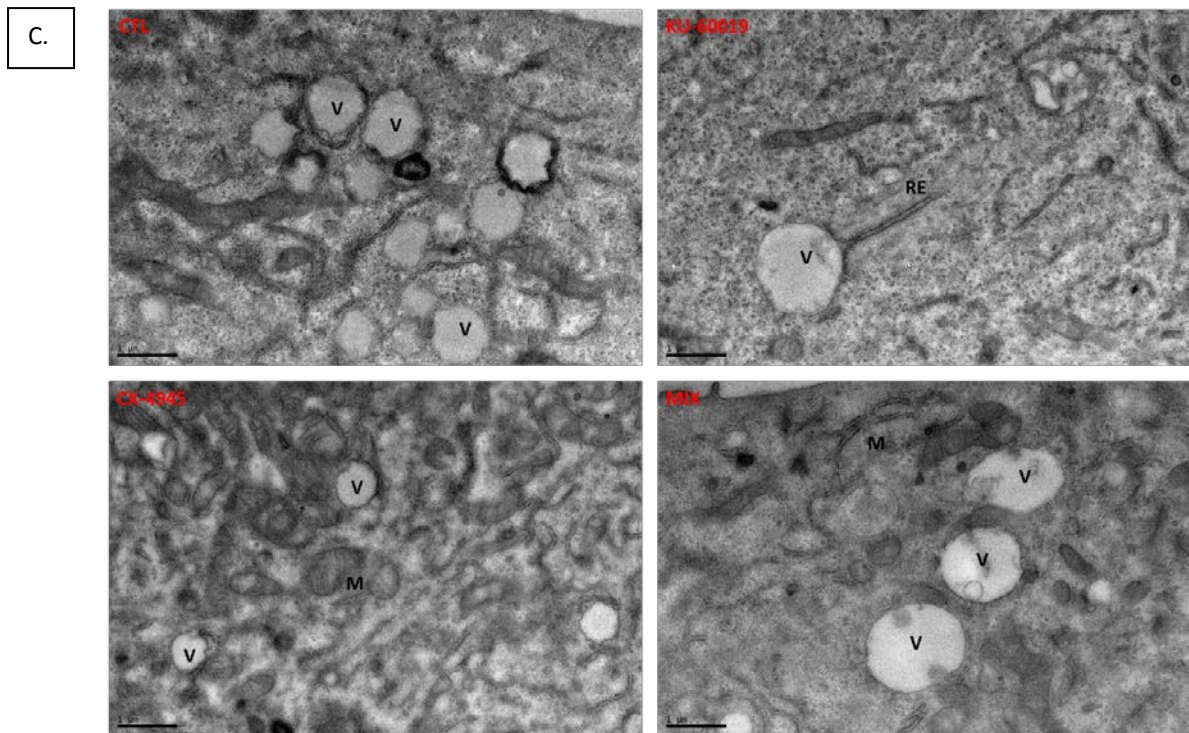


Figure 2: ATM inhibitors induce formation of empty structures and their size is pVHL dependent. A-C 786-O VHL + and VHL null cells were treated with either DMSO (CTL), 5 μ M KU-60019, 2.5 μ M CX-4945 or the MIX of both during 24h in hypoxia conditions and transmission electron microscopic study was conducted as described. A; cells in magnification of 440X for DMSO and MIX and 690X for KU and CX treatment. B; VHL+ cells picture with magnification of 4800X (right panels) showing fusion of vacuoles in presence of the ATM inhibitor. C; 786-O WT cell pictures with 2900X magnification showing detailed vacuole structures. Nuclei (N), Vacuoles (V) and arrows, Mitochondria (M), Endoplasmic reticulum (RE).

7 Origin of vacuoles

A) *Endocytosis*

Endocytosis is the mechanism used by the cells to transport macromolecules and particles inside their cytoplasm. The components are engulfed by the cell where the plasma membrane surrounds them to later internalize them into vesicles inside the cell, through an energy-using process. Usually, the cell uses this process to trap important chemical substance that cannot pass through the hydrophobic plasma by passive means (i.e polar molecules). Once the molecules travels inside the cytoplasm through a vesicle they can follow two different pathways. A) The vesicle can fuse with early endosomes and then with acidic late endosomes that will meet lysosomes and induce the degradation of the materials. B) It can be recycled and sent again to the cell surface. ([Cooper 2000](#)).

During endocytosis, molecules are delivered from the plasma membrane to early endosomes in endocytic vesicles. There are two main pathways of endocytosis: **Pinocytosis**, in which there is internalization of small macromolecules and extracellular fluids and **Phagocytosis**, where there is the engulfment of big particles as microorganisms and cellular debris. Only the first one will be briefly discussed.

Pinocytosis can be separated into different groups: ([Soldati 2006](#); Cooper 2000; [Mayor 2007](#); [Damm 2005](#), [Doherty 2009](#)) and will be represented in figure N°18.

- 1) Clathrin-dependent endocytosis: Several macromolecules bind to their specific receptors on the cell surface. These receptors are localised in clathrin-coated regions of the membrane called Clathrin-coated pits. The macromolecules are internalized in small vesicles to then fuse with early endosomes for degradation or recycling.
- 2) Macropinocytosis: Is a regulated non-selective uptake of solute and molecules from the extracellular medium. The surface of the membrane ruffles through an actin-dependent process that gives place to large endocytic vacuoles called macropinosomes.
- 3) Caveolae pinocytosis: Caveolae are small invaginations that are stabilized by dimeric proteins called caveolin to form the flask-shape structure of the caveolae. Caveolin proteins

insert in the plasma membrane and self-associate, forming a caveolin coat on the surface of the membrane. Finally, caveolar cargoes are diverse, ranging from lipids, proteins and lipid-anchored proteins to pathogens.

4) Clathrin and caveolae independent pathways are regulated by proteins as Cdc42, Arf1, RhoA, Rac1, Arf6 and are in charge of transport of cargos as fluid phase markers, CTxB, GPI-linked proteins, IL2R β , FC ϵ RI, Kir3.4, γ c-cytokine receptor, MHC class I proteins, CD59, carboxypeptidase E, proteoglycans, etc.

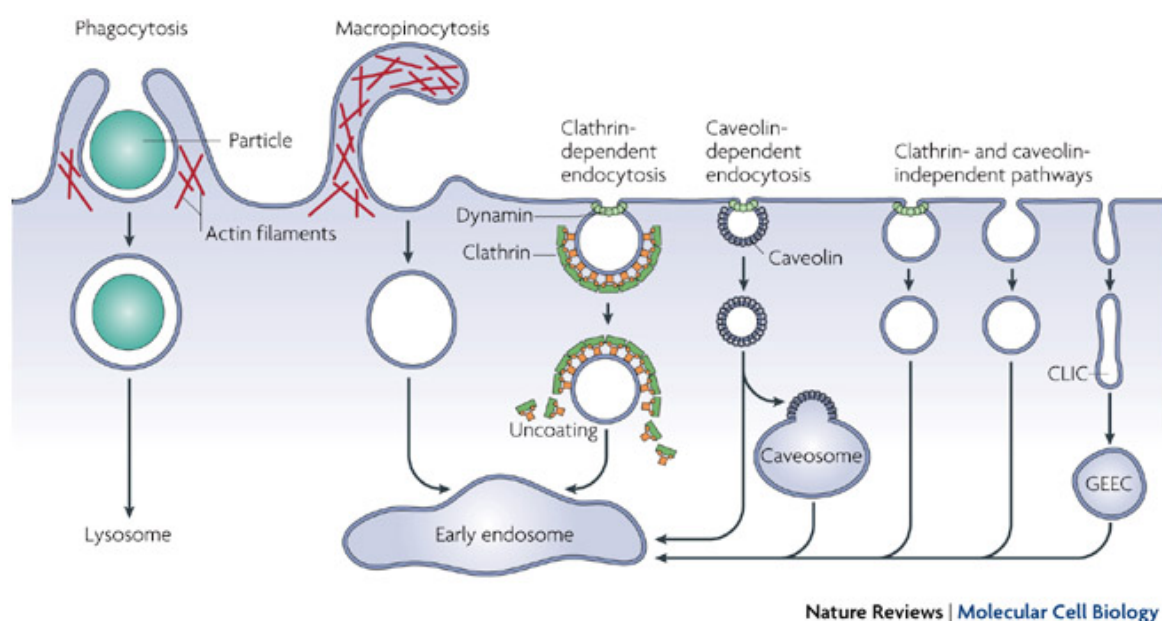


Figure 18: Putative endocytic portals

Clathrin-dependent and independent pathways are represented together with the intermediate structures before the fusion to the early endosome or lysosome in the case of phagocytosis. Picture taken from [Pagano 2008](#).

To determine if these vacuoles were a consequence of accumulation of extracellular material inside the cells we took advantage of the impermeable fluorescent dye, Lucifer yellow. This marker for fluid phase endocytosis has been already used in mammalian cells and in *Saccharomyces cerevisiae* ([Johnson 2011](#)). The accumulation of Lucifer yellow within KU-60019 induced vacuoles (Figure 3) revealed their endosomal origin.

To better understand these structures we co-treated both cell lines with inhibitors of the different pinocytic pathways (data not shown). Only treatment with Amilorid (EIPA), an inhibitor of Macropinocytosis, induced a decrease in the number of vacuoles in both KU-60019 and KU+CX conditions. Moreover this was true in hypoxia (Figure N° 4) and normoxia conditions (data no shown). The evaluation of cells containing vacuoles in each cell line upon inhibitor treatment confirmed our hypothesis, indicating that these vacuoles could be a result of increased macropinocytosis, induced mostly by the inhibitor KU-60019 (Figure N°4). Moreover, we observed differences in the size and number of vacuoles after treatment with EIPA. In both cell lines the number of vacuoles which area was $200\mu\text{m}^2$ disappear and the number of small vacuoles went up to 70% in the case of the KU-60019 treatment. This could be due to a deficiency in the fusion of the remaining vacuoles upon EIPA inhibition (Figure N°5).

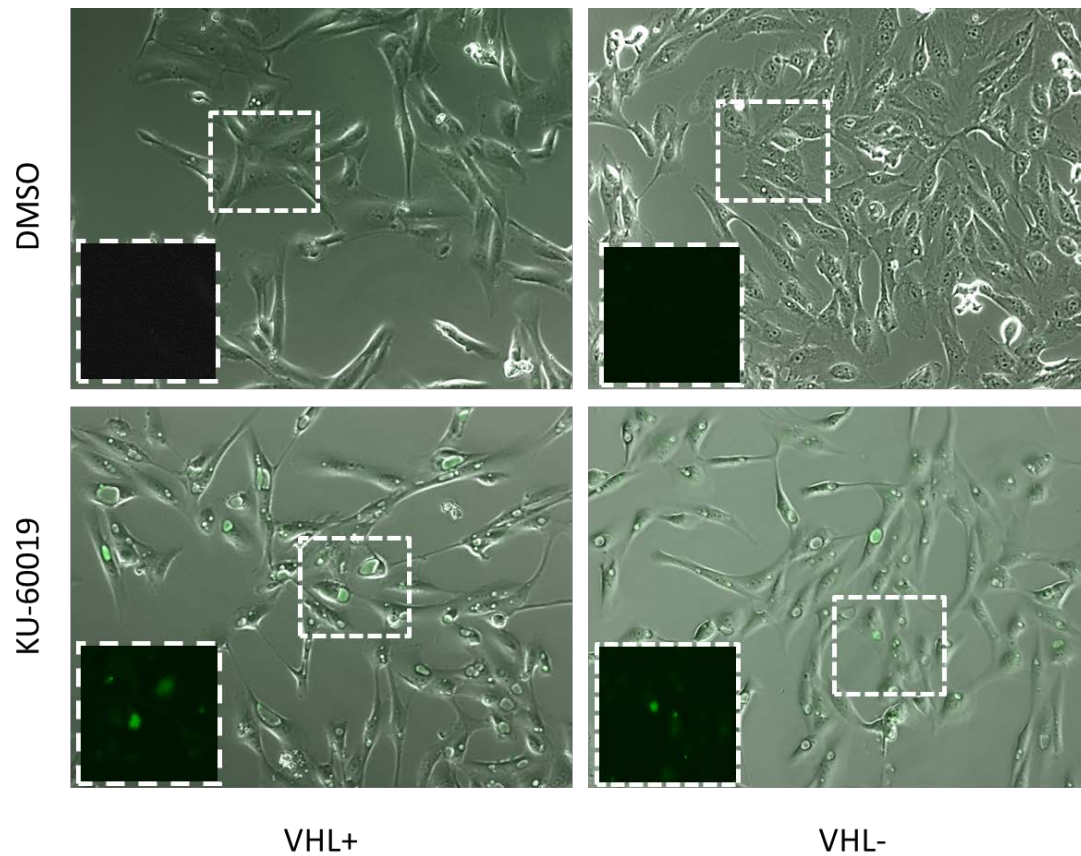


Figure 3: Localization of Lucifer yellow in KU-60019 induced vacuolated 786-O VHL null and VHL re-introduced cells. 786-O cells were treated with 5 μ M KU-60019 or DMSO in the presence of 0.5 mg/ml Lucifer yellow in DMEM medium for 24 h at 37°C. Cells were visualized by visible light and fluorescence microscopy. (Small square) Green fluorescence; and (Big square) Merged image

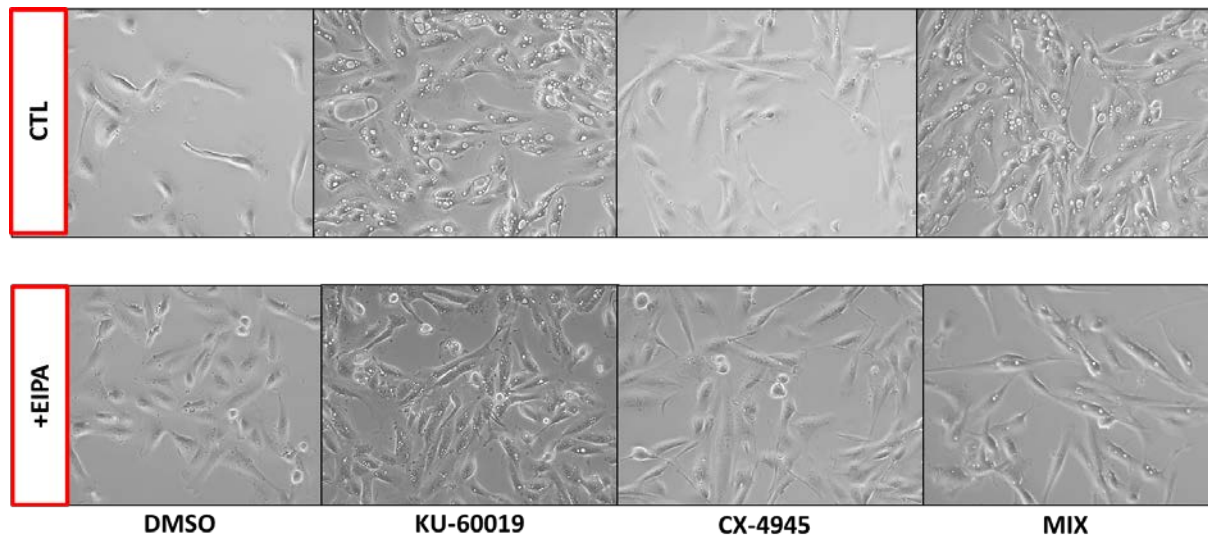


Figure 4: Macropinocytosis as mechanism of extracellular engulfment. 786-O WT and VHL+ were treated with 5 μ M KU-60019, 2.5 μ M CX-4945 and the combination of both during 48h in hypoxia conditions (1.5% O₂). After 24h of treatment, EIPA was added in presence of CK2 and ATM inhibitors.

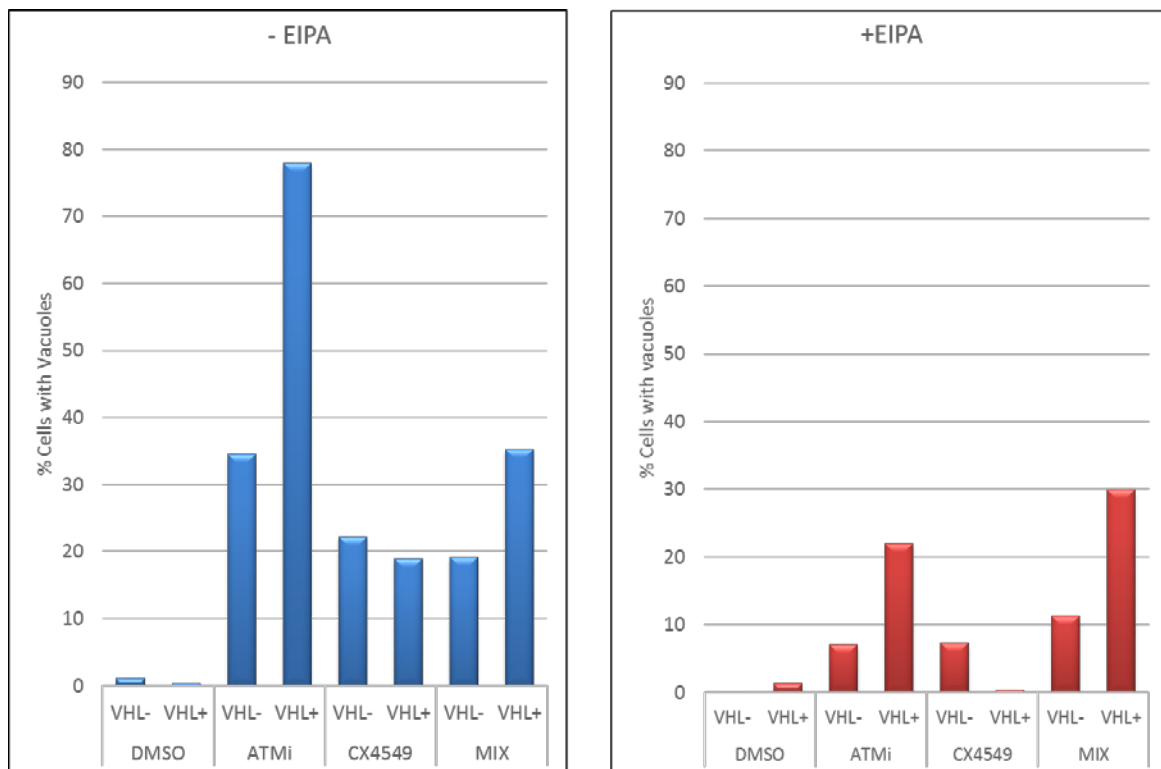


Figure 5: Quantification of cells with vacuoles in 786-O WT and VHL+ treated cells before (left panel) and after (right panel) co-treatment with the macropinocytosis inhibitor Amilorid (EIPA).

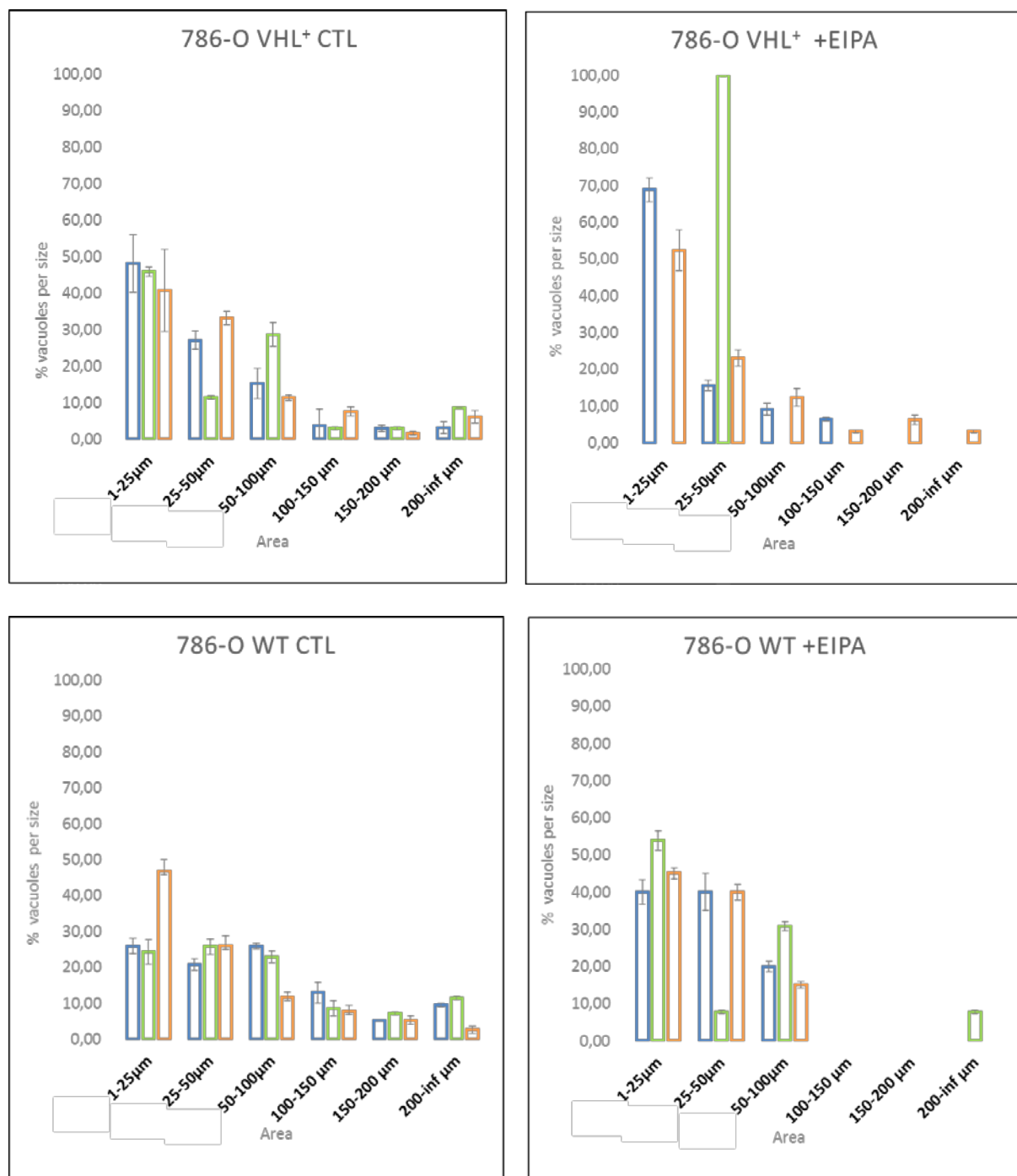


Figure 6: Quantification of vacuoles per size in each treatment condition. 786-O WT and VHL⁺ treated cells before (left panel) and after (right panel) co-treatment with Amiloride (EIPA) during 24h. Vacuole size was measured using Image J software. Blue Bar: KU-60019; Green Bar: CX-4945; Orange Bar: KU+CX.

B) Early and Late endosomes

There is a large literature that demonstrates that cancer cells can acquire MDR by increasing the expression of transporters on endosomal membranes. Drugs like Sunitinib or Doxorubicin that enter into the cell are protonated inside acidic compartments as late endosomes and lysosomes and becomes impermeable. This sequestration decreased to efficacy of cancer drugs to reach their target. ([Lee 2006](#))

Immunofluorescent staining with antibodies against Early Endosomes Antigen 1 (EEA1) and Lysosome Associated Membrane Protein 1 (LAMP1) a marker of a major integral membrane glycoprotein of late endosomes and lysosomes, showed that large vacuoles are most likely to have a lysosomal/late endosomes origin whereas small vacuoles gave signal with the early endosome antibody (Figure N°7 and N°8). Western Blot analysis using the early endosome marker rabaptin-5 showed small changes in its level indicating that in this phenotype there is an increase number of early endosomes formation (Figure N°9).

Taken together, these results showing the specific localization of LAMP1 marker inside the bigger vacuoles and the presence of early endosomes could indicate that after only 24h of treatment with an ATM inhibitor there is an increased maturation of early endosomes into late endosomes that will later fuse with the lysosomes.

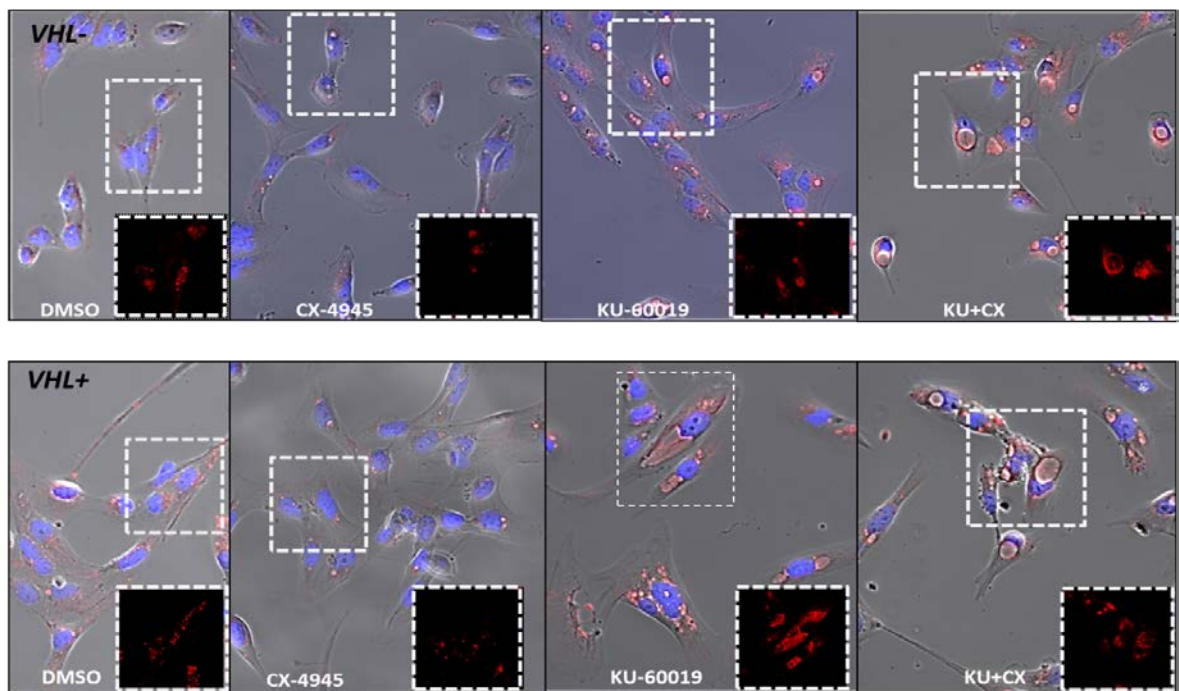


Figure 7: Vacuolization is due to late endosomes formation. 786-O WT and VHL+ were treated with 5 μ M KU-60019, 2.5 μ M CX-4945 and the combination of both during 48h in hypoxia conditions (1.5% O₂). Then cells were fixed and labeled with LAMP1 antibody (red). Nuclei were stained with Hoechst (blue).

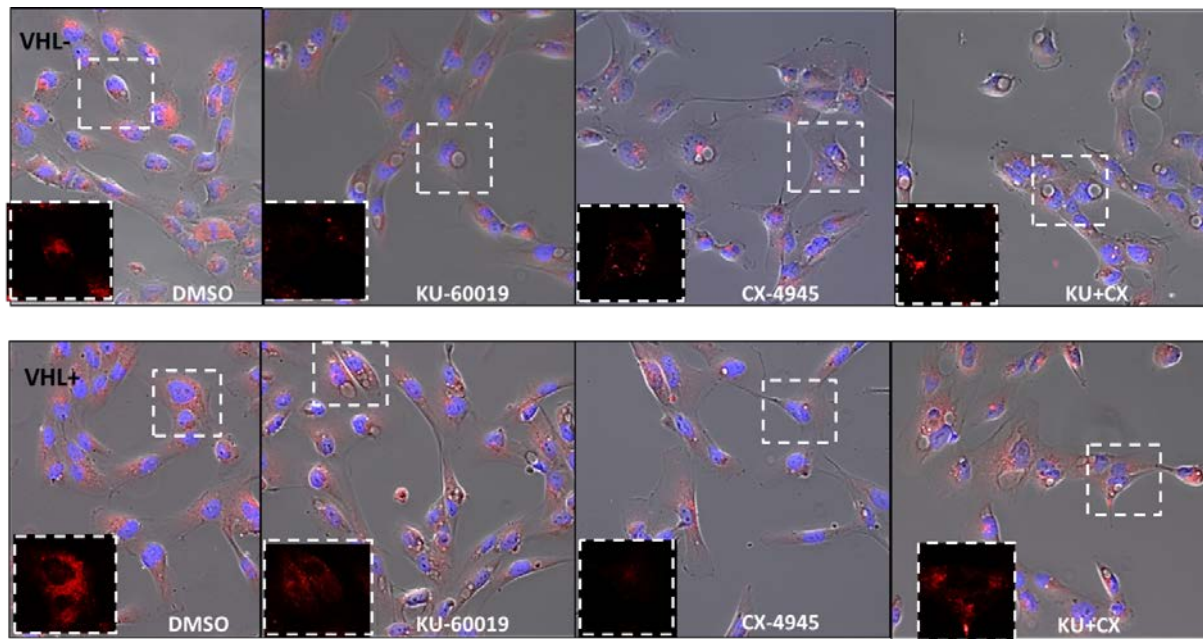


Figure 8: EEA1 labels distinct endosomal populations outside vacuoles. 786-O WT and VHL+ cells were treated with 5 μ M KU-60019, 2.5 μ M CX-4945 and the combination of both during 48h in hypoxia conditions (1.5% O₂). Then cells were fixed and label with EEA1 antibody (red). Nuclei were stained with Hoechst (blue).

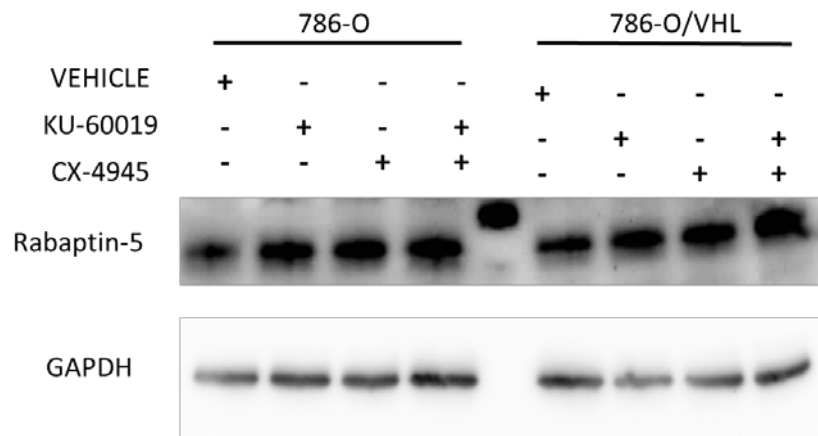


Figure 9: Early endosome accumulation. Western blot analysis using antibodies against rabaptin-5 GAPDH was used as loading control.

8 Mechanism of resistance

The major factor in the failure in the treatment of cancer is due to the capacity of cancer cells to acquire resistance to chemotherapy. This mechanism is known as “Multidrug resistance”. This resistance can be intrinsic due to mutations in the drug receptor or acquired. In the last context, at the beginning chemotherapy kills drug sensitive cells but several mechanisms to survive are activated in some part of the population. As the tumor grows again, chemotherapy is no longer efficient over these cells that are now modified and no longer affected by the treatment. ([Holohan 2013](#); [Szakacs 2006](#)).

So far, the mechanism of MDR that has been described, includes: reduced cellular drug uptake, ATP-driven drug efflux from the cell, quantitative and qualitative alterations in target proteins, drug compartmentalization (lysosomal sequestration), autophagy, and evasion of apoptosis ([Gillet 2010](#); [Zhitomirsky 2015](#); [Pisco 2014](#); [Luqmani 2005](#); [Sun 2015](#))

D) Lysosomal sequestration

Doxorubicin and Mitoxantrone, are two basic drugs to be known that can be concentrated in acidic endosomes of cells. This process is called “lysosomal sequestration”.

Inhibition of these lysosomes with Bafilomycin A or chloroquine inside breast cancer cells has been shown to increase cytotoxicity and cell death of these multidrug resistant cancer ([Lee 2006](#)). In addition, Azijli and colleagues demonstrated that several cancers (like colon and renal cancers) that had Sunitinib and others tyrosine kinases inhibitors were more resistant to these treatments due to lysosomal sequestration of these drugs (impeding them to reach their targets) ([Azijli 2015](#), [Giuliano 2015](#)).

Next, we sought to determine if a decrease in the number of vacuoles inside the cells would have an incidence in cell viability of the two cell lines. However, there was no significant effect on the efficiency of our drugs to kill VHL null cells when we added EIPA during the last 24H of treatment. In addition, VHL+ cells did not show any significant difference in capacity to proliferate (Figure N°10).

Video microscopy showed that VHL+ cells are capable of moving and divide in presence of these vacuoles.

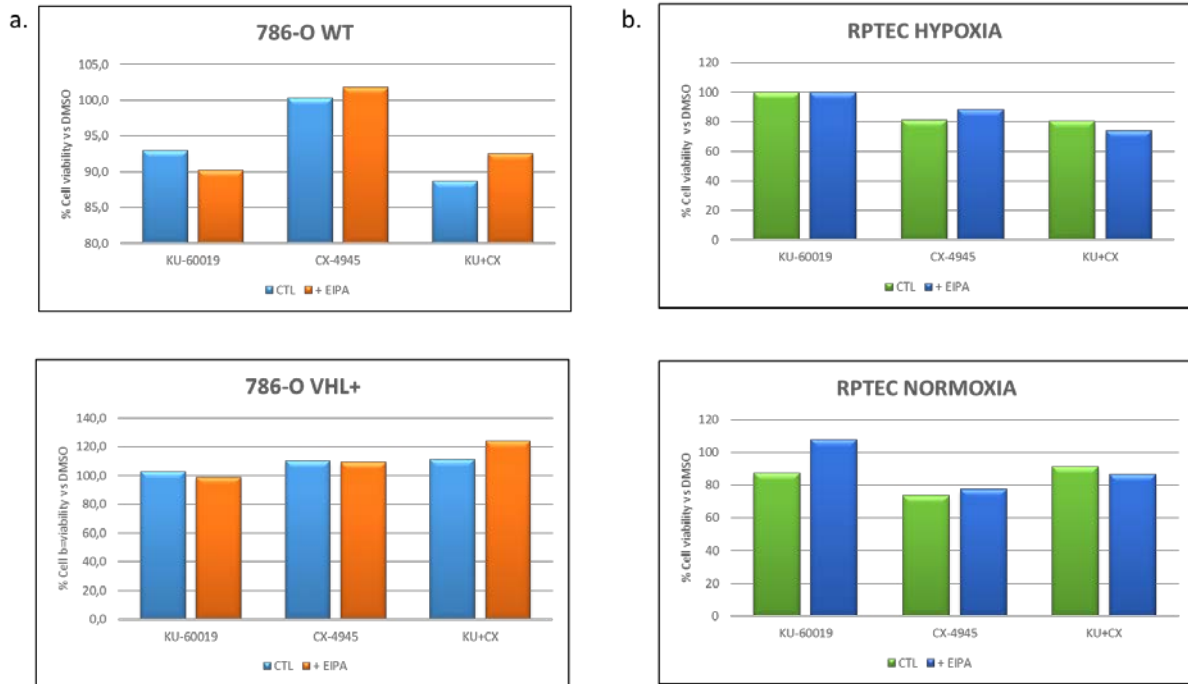


Figure 10: Macropinocytosis does not influence cell viability. A. 786-O VHL null cells (upper panel) and 786-O VHL+ cells (lower panel) were treated during 48h with drugs (DMSO, KU-60019, CX-4945, MIX) in presence (orange column) and absence (blue column) of EIPA inhibitor during the last 24h. RPTEC cells were treated during 48h with drugs (DMSO, KU-60019, CX-4945, MIX) in presence (blue column) or absence (green column) of EIPA inhibitor during the last 24h in presence of hypoxia (upper panel) (1.5% O₂) or normoxia (25% O₂). Cell viability was measured using Prestobblue and compared to the CTL (DMSO).

E) Autophagy

Autophagy is a cellular process where protein-aggregates and damaged-organelles goes under catabolic lysis via the lysosomal pathway ([Kumar 2012](#)). As review by [Wang 2014](#) , autophagy is present in the human kidney playing a role in normal and pathological conditions. In the case of ccRCC, one role that it has been found is to induce autophagic cell death. In VHL deficient RCC cells, the compound STF-62247 activated LC3-II and the formation of autophagic vacuoles, increasing apoptosis. Consequent inhibition of these vacuoles by an autophagy inhibitor led to deficiency in the cytotoxic effect of STF-62247 in VHL-deficient cells compared to VHL positive RCC cells, suggesting that the autophagy pathway is critical for STF-62247 induced cell death in VHL-deficient RCC cells ([Turcotte 2008](#)). But, another study showed that the NVP-BEZ235, inhibitor of the PI3K/mTOR pathway, can induce apoptosis in 786-O RCC at the same time that would induce autophagy to protect the cells. Even more, the authors confirm that a combination of NVP and autophagy inhibitors increased cell death ([Li 2013](#)).

Western Blot analysis showed accumulation of the protein p62 in both cell lines when treated with KU-60019 alone and in less proportion when combined with the CK2 inhibitor (Figure N°11). Contrarily, LC3-II levels did not seems to change compared to their LC3-I (Figure N°11). This would indicate a blockage in the early autophagy process before the fusion of the autophagosome to the lysosome.

In a recent study, Giuliano 2015 and colleagues demonstrated that autophagy and lysosomal sequestration are linked in mRCC that are resistant to Sunitinib treatment. In their study they showed that small doses of this drugs can be retained by the acidic lysosomes. This leads to decrease levels of Cathepsin B (CTSB) and by so increase levels of autophagosome that will not arrive to full maturation.

Because we also observed accumulation of SQSTM1/p62, we thought that this could indicate an accumulation as well of autophagosomes inside the cell. To check this possibility we inhibited the early autophagosome elongation by using 3-MA (3-methyladenine) inhibitor. No differences were observed in the cytotoxic effects of our inhibitors in presence or absence of 3-MA in the VHL null cells (Figure N° 12) neither in the pVHL reintroduced cell

lines (data not shown). Finally, no autophagic bodies were observed in the electronic microscopy study (Figure N°2). All these results suggest that even in presence of autophagy involved-proteins, this mechanism is not responsible for the resistance to our inhibitors.

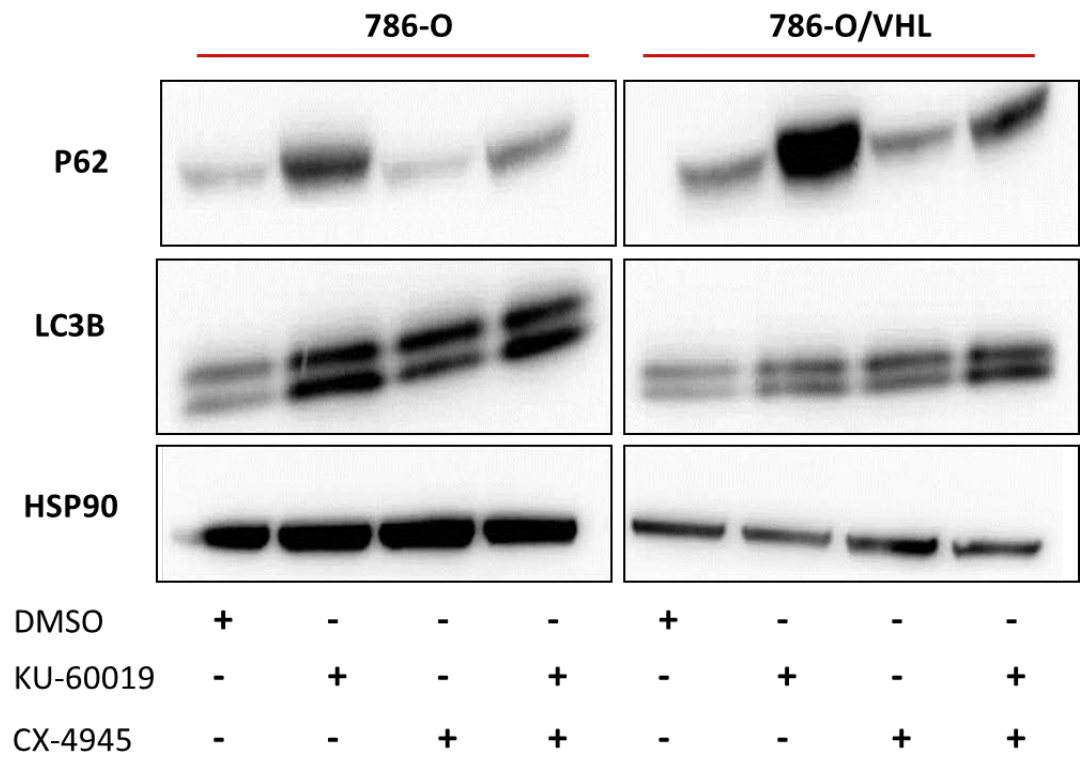


Figure 11: p62 accumulates in presence of ATM inhibitor. Western blot analysis using antibodies against p62 and LC3-II (LC3B). HSP90 was used as loading control.

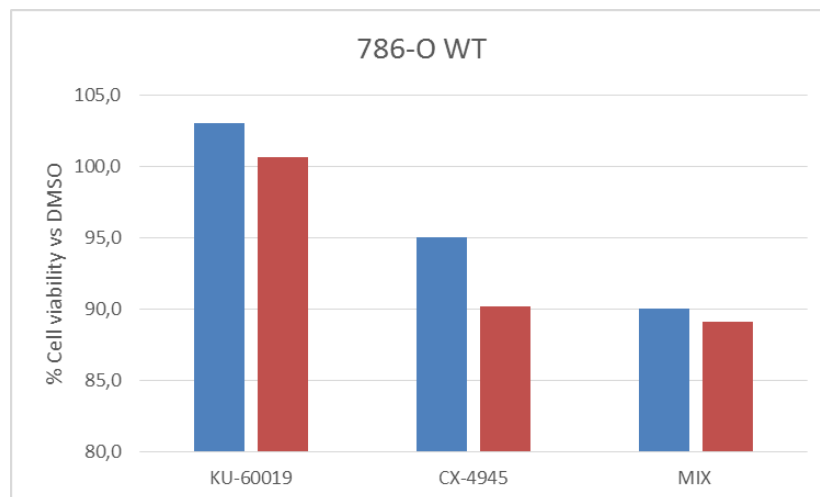


Figure 12: Early autophagosome inhibition does not affect cell toxicity of KU+CX drug combination. 786-O cells were treated with drugs as described before in absence (blue) or presence (red) of 3-MA inhibitor. Cell viability was measured using Prestoblue and compared to CTL (DMSO).

Discussion

The high toxicity in normal cells and the induction of resistance to treatment is one of the main obstacles in the several steps that are necessary to pass before the approval of new drugs to be used in patients with cancer. As an alternative to overcome these problems, new strategies arise based on differences in the activity or expression of the molecular targets, driving cancer-specific cellular phenotypes in cancer cells versus normal cells.

In our study, the resistance to the inhibitor of ATM by the kidney normal cells (RPTEC) that carried pVHL, came together with the formation of giant vacuoles structures inside the cells. Moreover when we re-introduced pVHL in ccRCC cell lines we obtain a significant decrease in the efficacy of KU-60019 or CX-4945 in single or combined fashion compared to the effect in VHL null cells (See patent Figure N°2 and Figure N°2E supplementary results).

Vacuolization was previously described in kidney cancer cells but in an opposite way to our results since it was present in RCC4 cells that lack VHL. In the study of ([Turcotte 2008](#)) the inhibitor STF-62247 induced autophagy increasing cell death. Indicating that autophagy is an important target in ccRCC. However, morphology of these vacuoles was not similar to the ones found in our study.

In our study, the autophagy marker p62 was upregulated after treatment of KU-60019. Interestingly LC3-II did not show any increase (Figure N°12). This could indicate a dysregulation at the level of early autophagosomes formation. Autophagy deficiency in the liver and p62 accumulation is associated with enhanced ROS production and activation of Nuclear Factor (erythroid-derived 2)-like 2 (NRF2), a transcription factor that controls the expression of key ROS scavengers ([Taguchi 2011](#)).

To test this possibility in our kidney model, we used the inhibitor of early autophagosome formation 3-MA in our cells. No decrease in cell viability was observed (Figure N° 13). Moreover, the absence of autophagic bodies in E.M. (Figure N°2) together with the continuous presence of these vacuoles after treatment with bafilomicyn A1 (data not shown) indicate that they did not have an autophagic origin. Interestingly, [Singha 2013](#) and colleagues reported that Manumycin A, an inhibitor of farnesyl protein transferase, reduces

cancer cell viability through induction of non-apoptotic, non-autophagic cytoplasmic vacuolation death in Triple negative Breast cancer cells with increased levels of p62 and LC3.

Our electronic microscopy analysis revealed that these giant vacuoles were empty structures, with no cellular debris or organelles (Figure N°2). In addition, through videomicroscopy, fusion of these structures was observed at higher magnification in 786-O VHL+ cells treated with KU-60019 or CX-4945 alone or in combination. These observations suggest that this vacuolization may participate to the cellular response to our inhibitors.

Cell induced vacuolization (independent from autophagy) has been already described in the case of *Mycoplasma pneumoniae* CARDS Toxin ([Johnson 2011](#)) where it was observed that this toxin induces large structures derived from the endocytic pathways in HeLa cells. Using a Lucifer yellow test, we demonstrated that these vacuoles have a fluid phase content and by blocking the endocytic mechanism of macropinocytosis we revealed these vacuoles ontogeny.

We established that vacuoles formation occurs in both VHL-deficient and VHL + cells mainly after KU-60019 treatment. Although vacuoles were also observed after treatment with CX-4945 or the combination of both drugs, the percentage of vacuolized cells was higher in the presence of VHL indicating that this tumor suppressor participates to their formation.

Given the roles of VHL in modulating several pathways (hypoxia response, metabolism ,etc) this enlarged vacuolization could be a mechanism whereby VHL is protecting normal cells from our drug inhibitors. For example, the GLUT-1 inhibitor STF-31 was shown to affect only VHL deficient cells where GLUT-1 is upregulated through HIF dysregulation. Normal kidney cells with functional VHL express GLUT2 and very low levels of GLUT1 ([Chan 2011](#)). Lysosomal sequestration has been described as a mechanism of resistance to Sunitib in ccRCC ([Azijili 2015](#)), where weak base drugs cross the membrane and are capted inside the acidic lysosomes where they become protonated and impermeable, decreasing their capacity to reach their targets.

In our study we observed an increased expression of the lysosomal-associated protein LAMP1 inside the large vacuoles in response to KU-60019. (Figure N°9) Although LAMP1 proteins are mainly associated with lysosomes, they also shuttle between endosomes and

the plasma membrane ([Rohrer 1996](#)). The early endosome antigen EEA1 was also present but its staining was outside the bigger vacuoles so it could be possible that inhibition of ATM accelerates early endosome maturation and fusion towards the lysosome.

Conclusion

Molecule targeting specifically renal cell carcinomas with low toxicity in normal cells has become a reality since the advances that had been made in understanding the significant role of the von Hippel Lindau tumor suppressor proteins.

Selective VHL null drug induce autophagic cell death, as well as metabolic inhibition of GLUT1 protein are some of the recent examples of how the difference of having or not VHL can affect drug response.

In this study we sought to understand if the presence of giant vesicles organelles inside our VHL positive cell could have an influence in the drug response. Preliminary results indicate that inhibition of macropinocytosis did not influence the toxicity of our drugs in both VHL- and VHL+ cells, and therefore cannot explain the difference in the observed vesicle formation.

After several efforts, we came to the conclusion that these vacuoles have an endocytic origin with uptake of extracellular fluid and with increased number of lysosomal associated proteins inside. Our results suggest that VHL-deficient cells are altered either in the endocytosis fluid phase uptake or in the lysosomal sequestration step thereby facilitating the drugs to reach their targets. Obviously, further studies need to be done to understand which signalling pathways are linked to the formation of these structures and what roles they play in response to our drug combination.

SUPPLEMENTARY RESULTS (ii)

Preliminary results on the mechanistic of CK2/ATM drug combination

1.1 ATM/CK2 dysregulation in ccRCC human samples

As described before, co-inhibition of ATM and CK2 kinases has been shown to induce cell death in Multicellular Tumor Spheroids (MCTS) and in ex-vivo tissue slice culture models of ccRCC. This combined drug treatment induced a caspase-dependent apoptosis mainly in malignant cell region. The main difference between normal and cancer cells is that normal cells express the von Hippel Lindau tumor suppressor. In VHL-deficient ccRCC several signalling pathways, are dysregulated such as genes involved in histone modification ([Dalgliesh 2010](#)), metabolic and catabolic processes ([The Cancer Genome Research Atlas Network 2013](#)), excretion ([Gerlinger 2012](#)), oxidation reduction, ion transport and response to chemical stimulus, while simultaneously upregulated genes were associated with immune and inflammatory responses, response to hypoxia, stress, wounding, and vasculature development.

We have previously shown that CK2 expression levels can be dysregulated in tumor samples from ccRCC patients ([Roelants 2015](#)). Using the same approach, we looked at ATM by checking both its expression level and its activation in normal and tumor tissue from 6 patient samples. Surprisingly, 4 of 6 of the tumor samples had a decreased expression of ATM protein as compared to normal corresponding tissue. Lack of ATM expression has been shown to be related to sporadic breast cancer ([Kovalev 2000](#)) and in childhood tumors facilitating active mTORC1 signaling under hypoxic conditions ([Cam 2010](#)). Interestingly, this ATM dysregulation in ccRCC tumors has never been described before.

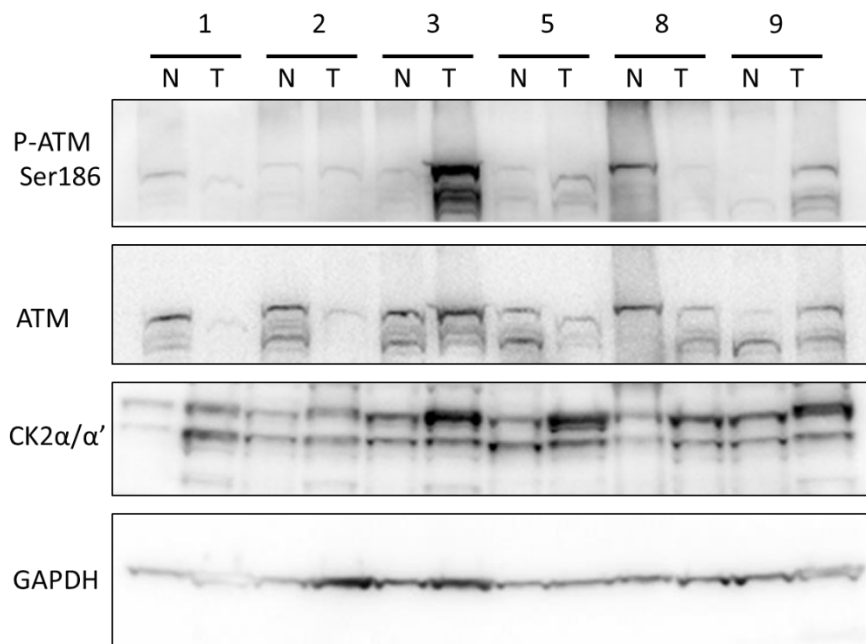


Figure 1: Differential levels of CK2, ATM in normal kidney and ccRCC tumor samples of patients. Western blot analysis against protein levels of ATM, Phospho-Ser186-ATM, CK2α, CK2β, GAPDH as loading control.

9 HIF-2α: The Redemption

Although clear cell renal carcinomas can overexpress HIF-1α and/ or only HIF-2α subunits, the last one has been described as the main tumor driver in clear cell carcinoma ([Kondo 2002](#), [Kondo 2003](#), [Kaelin 2009](#)). Thus we aimed to determine the expression levels of HIF-2α in the different cells lines used in our studies (Figure N°2). RPTEC cells are normal kidney cells that, as expected, expressed low levels of HIF-2α. This was also true for cancer cells that had re-introduced VHL expression. RCC4 cell line expresses not only HIF-2α but also HIF-1α. In the case of RCC4+ the presence of HIF-2α could be explained as the conditions of growth may induce overexpression of HIF-1α and therefore the VHL pathways is saturated, leading to stabilization of HIF subunits ([Raval 2005](#)).

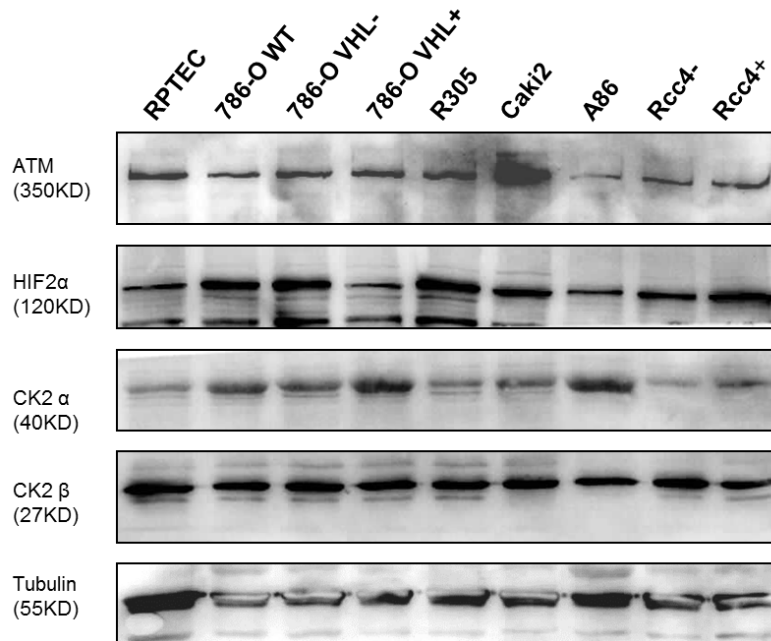


Figure 2: Protein expression levels of CK2 subunits, ATM and HIF-2α in RPTEC (kidney normal cell line) and ccRCC established cell lines (786-O WT, 786-O VHL-, 786-O VHL+, R305, CAKI2, A86, RCC4-, RCC4+, A86) were analysed by Western blot. Tubulin was used as loading control.

In addition to the basal levels of HIF-2α in the ccRCC cell lines, we also observed an increase of HIF-2α after 48h of combined drug treatments (Figure N°4). It is worth noting that apoptosis seemed to appear only at this time point (Figure 4C Patent) and only in cells lacking VHL. This led us to hypothesize that the presence of HIF-2α was necessary for the effect of our drug combination on cancer cells. To verify this idea, 786-O cells were transfected with CRISPR/CAS9 plasmid to generate HIF-2α (EPAS1) knock out cell lines (sgHIF-2α-1 and sgHIF-2α-2). MCTS generated with these cell lines were treated with CX-4945 and KU-60019 alone or in combination and cell death was quantified by PI measurement. Strikingly, as shown in figure N°3, the absence of HIF-2α impaired significantly the capacity of KU-60019 to induce cell death when added alone or together with CX-4945. Moreover, Western blot analysis showed that the drugs alone or in combination induced an accumulation of HIF-2α in MCTS from 786-O VHL null cells together with an increase of p53 phosphorylated on Ser15 (Figure N°4).

[Chesnel and colleagues](#) have shown that there is a third isoform of VHL expressed in ccRCC, the pVHL172. 786-O cells that were forced to express this isoform pVHL 172 do not target HIF-2 α for degradation (Hascoet et al 2016 *in preparation*). Thus, thanks to the generous gift of Dr Yannick Arlot, we took these cells named A86 to prepare MCTS and to perform the same experiment described before. Interestingly, the effect of co-inhibition of CK2 and ATM in these cells that express stable levels of HIF-2 despite the presence of VHL(172), was the same than the one obtained in VHL null cells. Again, in response to the combined drug treatment, p53(ser15) was also activated in A86 cells (Figure N°5 and Figure N°6). These results indicate, that the presence of HIF-2 α was necessary to activate the signalling to induce cell apoptosis.

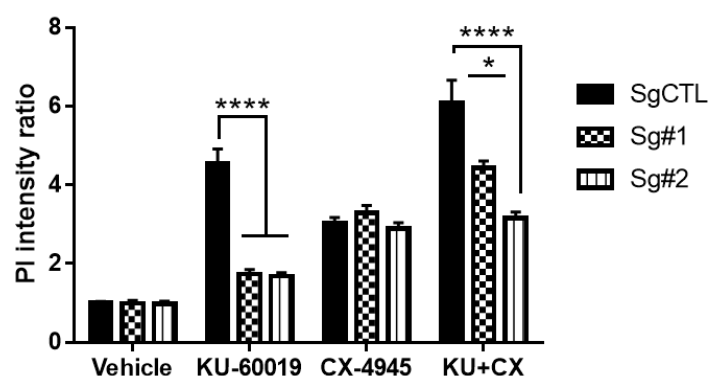


Figure 3: 786-O HIF2 α CRISPR/cas9 (sg1 and sg2) and 786-O CTL CRISPR/cas9 (sgCTL) MTS were treated either with vehicle (DMSO) or KU-60019 (10 μ M), CX-4945 (7.5 μ M) alone or in combination for 48h. Then, cell death was quantified by PI measurement. Significant difference was found in the KU-60019 condition (**** $p \leq 0.00001$) and in the mix condition when compared to sgCTL versus sg1 and sg2. (**** $p \leq 0.00001$; * $p < 0.05$). Kruskal- Wallis test for all the conditions.

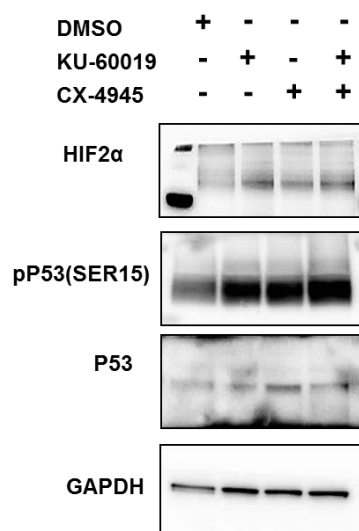


Figure 4: 786-O VHL null MTS Western blot analysis showing protein expression levels of HIF2 α , pP53(SER15), P53 and GAPDH as loading control.

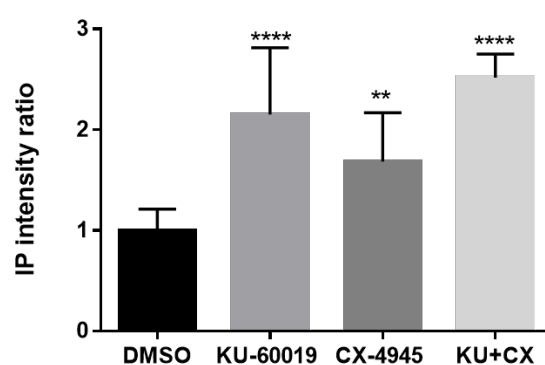


Figure 5: MTS of the ccRCC A86 cell line were treated either with vehicle (DMSO) or KU-60019 (10 μ M), CX-4945 (7.5 μ M) alone or in combination for 48h. Then, cell death was quantified by PI measurement. Significant differences were found between drug treatments and DMSO. (Kruskal- Wallis test for all the conditions. (** $p \leq 0.001$; **** $p \leq 0.00001$)).

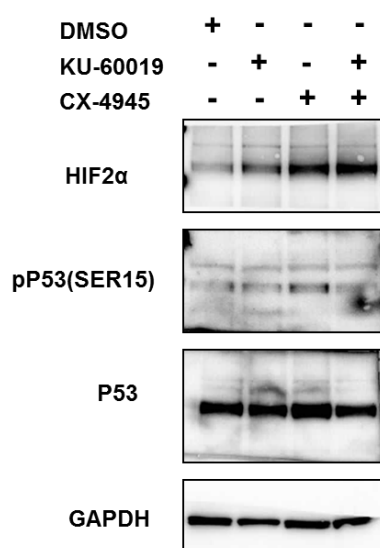


Figure 6: A86 MTS Western blot analysis showing protein expression levels of HIF2 α , pP53 (SER15), P53, GAPDH as loading control.

These unexpected results pointed out to the possibility that HIF-2 α is necessary to create the proper environment for the effect of combined CK2 and ATM inhibition. This raises the question whether there is a functional link between HIF-2 α and the CK2/ATM kinases.

10 HIF2 α is a substrate for CK2.

The fact that HIF-2 α was necessary to increase drug efficacy in ccRCC has been described in a study where HIF-2 α was identified as a predictor of better response to sunitinib and longer survival time ([Garcia-Donas 2013](#)). ATM was shown to phosphorylate HIF-1 α on Serine 696 mediating downregulation of mTORC1 signaling ([Cam 2010](#)). Although HIF-1 α and HIF-2 α share extensive sequence homology and overlapping functional similarity, we could not find any potential ATM consensus phosphorylation sites on HIF-2 α .

It was shown that CK2-mediated phosphorylation of HIF-1 α affects its transcriptional activity even though the mechanism of this posttranslational regulation is not known ([Kietzman 2016](#), [Mottet 2005](#)). Alternatively, an indirect CK2-mediated regulation of HIF-1 α was reported in which CK2 phosphorylates VHL, reducing its destabilizing effect on HIF-1 α , thereby increasing HIF-1 α levels and contributing to tumorigenesis ([Ampofo 2010](#)).

Interestingly, we showed in HIF-2 α -overexpressing HEK293T cells, that HIF-2 α immunoprecipitates contained a CX-4945-sensitive kinase that phosphorylates HIF-2 α *in vitro* (Figure N°7A). Similarly, a specific CX-4945-sensitive CK2 activity could be detected in HIF-2 α immunoprecipitates (Figure N°7B). Finally, recombinant HIF-2 α was phosphorylated by recombinant CK2 holoenzyme (Figure N°7C).

Whether CK2-mediated HIF-2 α phosphorylation affects its stability, localization, and transcriptional activity remains to be explored.

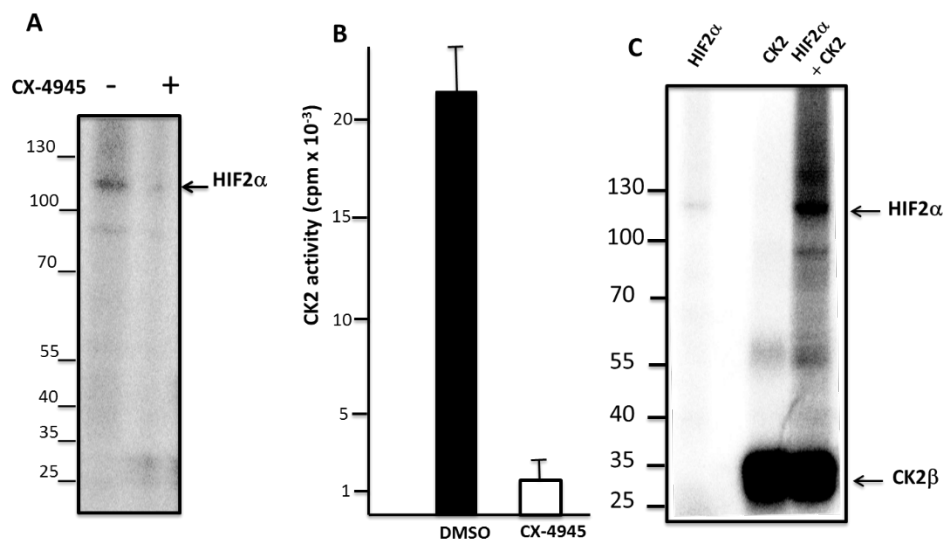


Figure 7:A. HIF2α was immunoprecipitated from transiently transfected HEK293T cells. Beads were then resuspended in 20 μl of kinase buffer in the absence or presence of CX-4945. Kinase reactions were initiated by addition of 10 μCi [γ-32P]-ATP for 10 min at 20°C. Phosphorylated proteins were analysed by SDS-PAGE and autoradiography.

B. CK2 activity was assayed in HIF2α immunoprecipitates in the absence or presence of CX-4945, using the specific CK2 peptide substrate RRREDEESDDEE.

C. Beads containing HIF2α were resuspended in kinase buffer in the absence or presence of recombinant CK2 (20nM) and the kinase reactions were initiated as in A. Phosphorylated proteins were analysed by SDS-PAGE and autoradiography.

Although CK2 has been shown to be upregulated in ccRCC, its role in the absence of VHL and with high basal levels of HIF-2α, is so far not known. In this context, our results suggest that CK2 is a major player in the activity of HIF-2α. Overexpression of HIF-2α enhanced the susceptibility to our drug combination whereas CRISPR/CAS9-mediated knockout of HIF-2α inhibited it. This suggests that HIF-2α acts as a cooperative mediator that synergizes CK2 and ATM inhibition to induce cell death of VHL-deficient renal carcinoma cells.

Figure N°8 represents a proposed model for the mechanistic of CX-4945/KU-60019 combination in ccRCC. In basal levels CK2 phosphorylates and stabilizes HIF-2α that will go to the nucleus to induce transcription of pro-tumorigenic genes. CX-4945 inhibits CK2 thereby



Discussion

Although CK2 and ATM had been separately described as interesting targets to treat diverse types of cancers such as Glioblastoma, Breast Cancer, Colon cancer, between others, the combination of these two targets has never been considered before in the treatment of kidney cancer, more specifically in the treatment of Clear Cell Renal cell carcinoma.

The dysregulation in the protein expression of certain proteins makes them potential targets ([Alxesen 2007](#)). Looking at the levels of p-ATMser1981 in tumor and normal samples, we found decreased levels of this form that is often activated in case of DNA damage. Deficiency in the expression of DNA repair proteins has been associated with deficient DNA repair and tumorigenesis ([Kovalev 2010](#)). Moreover, absence of ATM in lung small cell cancer has been proposed to favour the synthetic lethality of inhibition of CHK1 and Irinotecan ([Al-Almahaide 2014](#)).

In the case of CK2, it has been largely demonstrated that dysregulation of its different subunits can affect several signalling pathways inside the cells. In addition, upregulation of the expression of this multi-subunit kinase was found in several cancers including kidney cancer.

The collaboration of CK2/ATM kinases has been previously described in the literature. It has been shown that down-regulation of CK2 results in reduction of ATM autophosphorylation ([Guerra 2014](#)) or γ -H2AX ATM dependent phosphorylation (Reviewed by [Cochet 2009](#)). The main difference between these studies and ours is that CK2 was required after induction of double strand breaks. In our case, we did not induce DNA damage indicating that the activation of pATM(ser1981) was a consequence of the CK2inhibition by CX-4945 (Figure 2 of the patent).

As previously discussed, HIF-2 appears to be necessary for this two pathways to act in a synthetic lethal manner. Accumulation of HIF-2 has been described to induce cell death in colon cancer cells ([Sun 2013](#)). Moreover, HIF-1 α is phosphorylated by ATM to control mTOR signaling ([Cam 2010](#)). Since HIF-2 α is mostly expressed in ccRCC, we aimed to determine if ATM could stabilize HIF-2 α . We did not find any ATM phosphorylation of HIF-2 α . In contrast,

we observed that HIF-2 α is phosphorylated by CK2 *in vitro*. Although CK2 has been shown to regulate HIF-1 α ([Mottet 2005](#)), HIF-2 α phosphorylation was not described in the literature before.

Our hypothesis (Figure N°8), does not explain why after 48h of treatment, both HIF-2 α expression and cell death increase. One explanation could be that HIF-2 α is phosphorylated by other kinases but the process of cell death is already underway. So HIF-2 α accumulation does not act to induce cell survival but activates genes implicated in cell death.

In conclusion, there are still several questions about how the co-inhibition of CK2 and ATM can kill cancer cells lacking VHL. The presence of HIF-2 α as a key player in this synthetic lethality could be a door that opens to its redemption, as it has been shown with other “evil” players in cancer signalling before.

FINAL CONCLUSION AND PERSPECTIVES

Today, High Throughput Screening is one of the most used tools in pharmaceutical research for the discovery of novel drugs against several diseases. It provides fast, quality and novel data but also opens the door for the deciphering of key question as for example what are the genes that can compensate drug inhibition and induce resistance.

Importantly, a recent report showed in a randomized phase 2 study, the positive effect of a synergistic combination of VEGFR inhibitor tyrosine kinase inhibitors and mTOR inhibitors for the treatment of metastatic renal cell carcinoma, validating the combination strategy of molecularly targeted drugs in this cancer ([Motzer 2015](#)).

The main objective of this PhD was to find a novel combinatory therapy against the clear cell renal cell carcinoma disease. To this end, an HTS was designed to target part of the kinome using an approach that combined two different strategies for protein/gene inactivation. In one hand, si/shRNA silencing was performed generating multiple stable cell lines. On the other hand, drug inhibitors, mostly commercially available, were used to target different kinases and non-kinases proteins associated with cancer signalling pathways.

The setting up of experimental conditions to obtain the most reproducible and robustness screening process was a key step in the accurate discovery of exploitable hits. Although the validation of our hit was later on performed in 3D cell culture, our initial screening was carried out using 2D monolayer cultures allowing a fast and efficient hit identification due to appropriated determination of the Z' factor.

This strategy led us to the discovery a potent drug combination that strongly affects the viability of renal cancer cells. Data from the screening revealed that the co-inhibition by KU-60019, a specific ATM chemical inhibitor in CK2-silenced 786-O cells induces synthetic lethality. Moreover, this effect was reproduced using CX-4945, a CK2 chemical inhibitor that is now in clinical phase studies for other types of cancer.

The absence of pVHL is the main characteristic of ccRCC and can differentiate them from normal renal cells. Importantly our results showed that our drug combination was more toxic for cancer cells than for normal cells.

Notably, the effect of our drug combination was associated with the presence of giant vacuoles in VHL-expressing cells. This observation suggests a potential role of these vacuoles

in conferring any advantage for cell to survive the treatment. The phenotypic characterization of these vacuoles showed that they were connected to the macropynocytosis process. However, their absence did not compromise the cell survival. On the other hand, upon drug treatment, these vacuoles showed an increased number of lysosomal associated proteins and may have an endocytic origin. Further studies will be required to clearly characterize these “puzzling” intracellular structures.

The development of many new anticancer drugs relies on the knowledge that cancers arise from specific aberrations in regulatory pathways in which protein kinases play crucial roles.

Our data show that CK2 and ATM are relevant targets for molecularly targeted drugs in renal cancer cells. The activity of CK2 is associated with the maintenance of cell proliferation in cancer cells ([Ruzzene 2010](#)). This multifunctional kinase promotes antiapoptotic, prosurvival mechanisms and inhibition of caspase-mediated apoptosis ([Olsten 2004](#); [Ruzzene 2010](#)). CK2 activity is also key to the development of multidrug resistance phenotypes and plays a role in “nononcogene addiction” in several cancers ([Ruzzene 2010](#)). CK2 has also been involved in the cellular DNA damage response and repair pathways ([Rabalski 2016](#)).

CK2 inhibition has already been shown to be effective in combination with EGFR inhibition and to prevent DNA repair after treatment with chemotoxic agents ([Siddiqui-Jain 2012](#); [Bliesath 2012](#)). Similarly, cotargeting BRAFV600E and CK2 synergistically reduced proliferation in patient-derived melanomas ([Parker 2014](#)).

Evidence has accumulated showing that ATM is part of many signalling networks, including cell metabolism and growth, apoptosis, oxidative stress and chromatin remodelling, all of which can affect cancer progression. Its high number of targets as well as its many potential ways for modulation place ATM as a signalling hub implicated on one side on the DNA repair machinery and on the other side on the response to several cellular stress ([Cremona 2014](#)).

Interestingly, ATM is also activated under hypoxic conditions by a mechanisms that is independent of the presence of DNA breaks ([Bencokova 2009](#)). Thus ATM inhibitors have been tested for anticancer activity. Among them KU60019 is a potent radiosensitizer in vitro inhibiting AKT pro-survival signalling and reducing cell motility and invasion ([Golding 2009](#)). Recent research has also tested the effects of ATM inhibitors in combination with other

treatments raising the possibility of a synthetic lethal approach to therapy ([Sullivan 2012](#); [Li 2010](#); [Golding 2012](#)). Thus, ATM inhibitors could become useful in combination with other drugs. However, at present, there is no report of ATM inhibitors in use in clinical trials, though this situation is likely to change.

Regarding the mechanism of action behind this combined drug treatment, we observed that HIF-2 α levels were increased after treatment. Therefore, using CRISPR/CAS9 technology to delete the HIF-2 α gene we could observe that the absence of HIF-2 α expression significantly impeded the synthetic lethal effect of co-inhibiting CK2 and ATM. Thus, we aimed to determine whether HIF-2 α was a substrate of one of these kinases. In addition, using a cell line that has an isoform of pVHL that do not target HIF-2 for degradation we observed the same effect. All these results seem to indicate that the presence of activated HIF-2 is necessary. Preliminary results showed that CK2 is found associated with and can phosphorylate HIF-2 α but further studies are required to confirm this point. One approach could be to mutate amino acids in the phosphorylation site of HIF-2 α by CK2 using gene editing (CRISPR/CAS9 technology).

The microenvironment inside a tumor can strongly affect the behavior of malignant cells. Hypoxia is frequently observed and it has been strongly associated with tumor propagation, malignant progression, and resistance to therapy. To mimic the environment inside tumor, we took advantage of the multicellular tumor spheroid model to test our drug combination. Whereas in the 2D cell model, our drug combination led to a decrease in cell viability associated with high levels of stress but with no signs of apoptosis (even in conditions of hypoxia), in the multicellular tumor spheroid model the same regimen induced a strong caspase-dependent apoptotic cell death.. Migration and invasion of drug resistant cells is one of the first mechanisms that the primary tumor uses to escape in order to metastasize in another organ ([Friedl 2003](#)). Interestingly, we could observe that survival as well as cell spreading out of the spheroids were strongly impaired in response to our drug combination.

To understand the pharmacodynamics and pharmacokinetics underlying these combined therapy treatment inside the spheroids, the next step would be to determine in future experiments, the levels of hypoxia, the presence of reactive oxygen species, the metabolic

changes and the way how signaling pathways are activated in response to CK2 and ATM inhibition .

In addition, since renal cancer has been described as a highly vascularized tumor, co-culture as spheroids of these cells with endothelial cells to observe drug response could help to improve the treatment. The main limitation for these 3D studies relies not only on the difficulty to obtain reproducible and similar spheroids but also on that most protocols are adapted for 2D cell culture. Both the design and the read-out of the experiments are much more complex.

To be even closer to a model that preserve the native tissue together with its architecture and proper environment, we took advantage of the “*ex vivo*” ‘tissue slice culture’ (TSC) model. With this preclinical model we could determine the effects of the co-inhibition of CK2 and ATM both in the malignant and normal parts of the kidney tissue, where the cellular heterogeneity and cell-cell interactions are maintained. Importantly, the low levels of cell death in normal tissue compared to the massive apoptotic signal observed in the malignant cells in presence of CX-4945 and KU-60019 make this novel combination a promising treatment against ccRCC.

In addition, these observations illustrate the power of working with tissue slice culture models in the sense that we have the ability to test a number of different drug combinations. Not only can we vary the drug combinations we can also vary the order or what's called the sequencing of the drugs to minimize the resistance.

In the aim to achieve the objective of “from the bench to the patient’s bed”, Patient derived xenografts (PDX) are ideal models to predict patient drug response by comparing the drug response in the patient to their mouse model. Along this line, it would be interesting to use ccRCC derived PDX not only to confirm the results obtained with the tissue slice culture generated from the 786-O cell line, but also to determine the potential response to this combined therapy in each patient genetic background. This would allow the classification of non-responders and responders patients.

In conclusion, in this work, a novel combinational therapy that kills selectively VHL deficient renal cancer cells and has little effect over normal cells, has been identified, validated and

patented. The signalling mechanism underpinning this toxic drug interaction is complex and requires further investigations.

Beside, we also expect to validate in preclinical drug assays the other hits that were obtained during our screening campaign.

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APPENDIX