Development of new drugs to overcome resistance to anti-angiogenic therapy in renal cell carcinoma

Final Thesis

Bachelor's Degree of Biotechnology

UNIVERSITAT ROVIRA i VIRGILI

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Index

Centre Information

This project has been based on the results obtained during a three-month internship in the Bellvitge Biomedical Research Institute (IDIBELL), Barcelona.

IDIBELL is a biomedicine research centre founded in 2004 made up of Hospital Universitari de Bellvitge, Hospital de Viladecans, Institut Català d'Oncologia and Universitat de Barcelona. The centre is a certified health research institute by Instituto de Salud Carlos III, it is a member of CERCA institution from Generalitat de Catalunya and it has received the "HR Excellence in Research" from the European Commission.

The institute has a wide variety of research areas, emphasizing on those subjects of clinical application, such as oncology, neuroscience and translational medicine, in order to improve life quality of patients.

My internship was performed in the oncology area, which focuses on the causes of cancer and its prevention, early diagnosis and the evaluation and improvement of quality in cancer care. Specifically, I was part of the Program Against Cancer Therapeutic Resistance (ProCURE), a newly launched research platform that aims to address the existence of therapeutic resistance in cancer.

ProCURE is constituted by multidisciplinary teams in order to successfully address cancer resistance. One of those teams focuses its research on angiogenesis and tumor malignization, highlighting the mechanisms of tumor adaptation to anti-angiogenic therapies. Moreover, they are studying why anti-angiogenics lead to invasion and metastasis in renal and neuroendocrine tumors.

Summary

Angiogenesis is a fundamental mechanism for tumor progression that induces blood vessel formation. In turn, it increases levels of oxygen and nutrients in the growing tumor mass. Targeted therapies inhibiting angiogenesis are currently used to treat several cancer types, such as renal cell carcinoma. However, these treatments fail to produce long-term effectiveness in most patients, due to tumor adaptation and subsequent resistance to therapy. Furthermore, there are no guidelines to follow after anti-angiogenic first-line treatment.

Transcriptome characterisation pointed out several altered pathways in resistant tumors, highlighting an overexpression of the resistant factor 1 (RF-1). Several studies have already validated RF-1 as a good anti-tumor target. Considering its emerging role in cancer progression and angiogenesis, different RF-1 inhibitors have been synthesized.

IRF-1 is an anti-RF-1 drug used together with chemotherapy. This small molecule inhibitor was tested in an *in vivo* model of resistant renal cancer and failed to significantly reduce tumor progression, besides presenting bad stability and pharmacokinetics. Consequently, efforts have been focused on the design of more effective RF-1 inhibitors.

Virtual screening of RF-1 revealed different structures that were most likely to bind to the target and inhibit its enzymatic activity. Three candidates, HIT101, HIT102 and HIT103, showed the strongest affinity with RF-1 and were further evaluated in two genetically modified renal cancer cell lines. HIT103 was the most active compound against RF-1 in proliferation assays, although HITs effect on cell migration was not clearly elucidated. In consequence, further experiments are required to reveal how the novel HITs allow to overcome resistance of renal tumors to anti-angiogenic therapies.

Keywords: Angiogenesis, anti-angiogenic therapy, tumor resistance, resistance factor 1, HIT compounds.

Introduction

Cancer cells are structures that have lost their capacity to grow under controlled conditions. In consequence, new cells are continuously formed avoiding contact inhibition, which causes their accumulation and further generation of a tumor cell mass (Hanahan and Weinberg, 2011).

Hanahan et al. proposed distinctive hallmarks of cancer, in order to provide a coherent organisation and understanding of the disease complexity. They included common cancer features, such as sustained proliferative signalling, avoidance of growth suppressors, resistance to cell death, replicative immortality, activation of invasion and metastasis, reprogramming energy metabolism, overpassing immune destruction and the induction of angiogenesis (Hanahan and Weinberg, 2011).

The thread of this thesis is the angiogenesis hallmark, which has been established as an important target to design new drugs in order to prevent tumor mass development in certain types of cancer (Folkman, 2007).

Tumor angiogenesis

Angiogenesis is the process where new blood vessels are formed from existing vasculature. It is a natural phenomenon during embryonic development and organism growth. During adulthood, it occurs at specific times and in a non-continuous process, for example, in wound healing or during the female reproductive cycle. However, it is also a fundamental mechanism for tumor growth (Hanahan and Weinberg, 2011).

The tumor mass usually grows until it reaches 1-2 mm^3. However, its unlimited proliferation capacity requires an extension of the vessel network to ensure an adequate oxygen and nutrient supply to the developing tumor. These elements are sustained by new blood vessels, which are induced by the neoplastic aggregate through increased expression of pro-angiogenic growth factors (Jiménez-Valerio and Casanovas, 2013).

The principal pro-angiogenesis factor is the vascular endothelial growth factor (VEGF), which plays a central role in blood vessel formation. There are other angiogenesis-triggering growth factors, such as fibroblast growth factor (FGF), platelet-derived growth factor (PDGF), transforming growth factor α (TGF-α) and placental growth factor (PlGF). In addition, angiopoietins, ephrins and some integrins are also involved in tumor angiogenesis (Folkman, 2007).

To enhance the pro-angiogenic effect, tumor cells are able to decrease expression levels of endogenous anti-angiogenic factors, contributing to a sustained imbalance that leads to the formation of a weak and dysfunctional vascular system (Folkman, 2007). Therefore, the vascular tree of the tumor becomes a disorganised network with dead-end vessel branches and areas of intermittent blood flow that cause lower perfusion and hypoxia (Jiménez-Valerio and Casanovas, 2013).

Hypoxic microenvironment

The deprivation of oxygen supply in tumors can trigger its proliferation due to the ability of cancer cells to activate a pro-survival signalling pathway. Hypoxia leads to the activation of the heterodimer hypoxia-inducible factor 1 (HIF-1) that, in turn, increases the expression of hypoxia-dependent genes, which are involved in cell survival, angiogenesis and glucose transport. HIF-1 is a central regulator of cell adaptation to the lack of oxygen, which may play an important role in hypoxic cancer cells survival (Rapisarda and Melillo, 2009).

Alpha subunit of the HIF-1 factor binds to the von Hippel-Lindau protein (pVHL), a protein involved in HIF-1α ubiquitination and further degradation by the proteasome. Under normoxic conditions, HIF-1 is not assembled and it does not promote the expression of pro-angiogenic and other survival molecules. Otherwise, under hypoxic conditions, HIF-1α cannot bind to pVHL, avoiding its degradation and, therefore, activating HIF-1, which will be translocated to the nucleus and further activate the hypoxia responsive element (Fig. 1). Genes modulated by HIF-1 are involved in glucose mobilisation, angiogenesis, proliferation, oxygen consumption, migration and invasion, and their expression is associated with poor prognosis and treatment failure (Rapisarda and Melillo, 2009).

The study of families with renal cell carcinoma, in the setting of von Hippel-Lindau (VHL) syndrome, a disorder characterised by the development of multiple tumors, has allowed recognizing genetic alterations involved in renal cancer. The *VHL* gene encodes for pVHL, the tumor suppressor protein that prevents the uncontrolled growth of cells. More than 50% of individuals with renal cancer have mutations in *VHL* gene, producing a defective pVHL. It has been demonstrated that, even under normoxic conditions, there is an accumulation of HIF-1, since HIF-1α cannot be degraded. In this context, the transcription of pro-survival and pro-angiogenic factors is increased (Kaelin, 2004; Rapisarda and Melillo, 2009; Jiménez-Valerio and Casanovas, 2013).

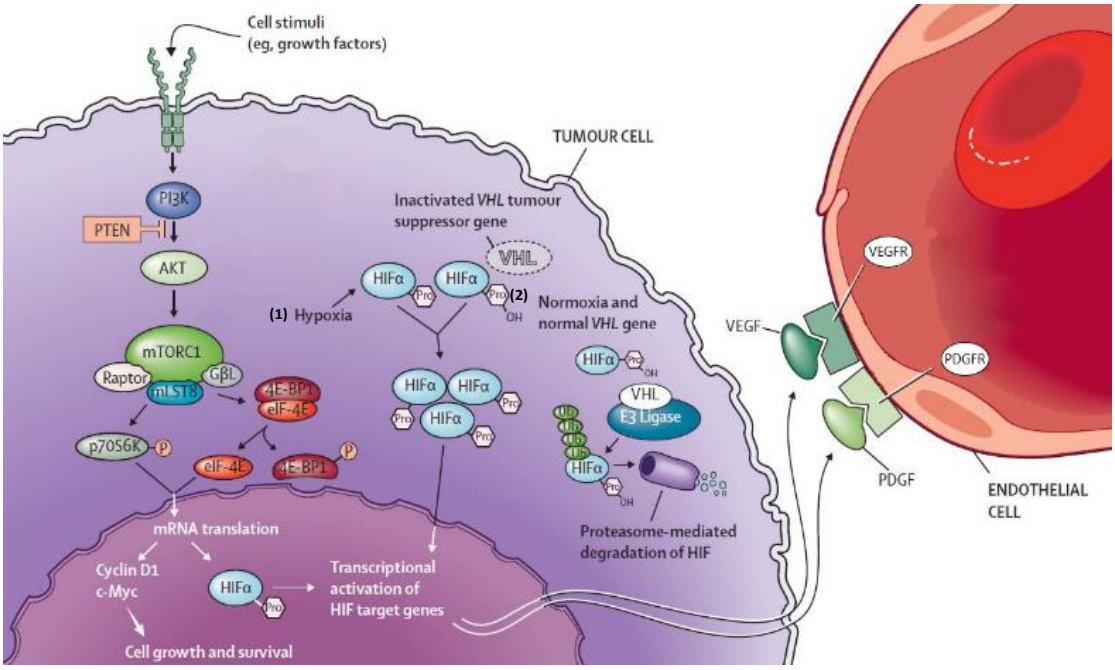

Figure 1. Molecular mechanisms in renal cell carcinoma (1) under hypoxia conditions, when von Hippel-Lindau (VHL) gene is inactivated. Accumulation of hypoxia-inducible factor-alpha (HIF-α) occurs and translocates into the nucleus, leading to transcription of hypoxia-inducible genes, including vascular endothelial growth factor (VEGF) and platelet-derived growth factor (PDGF). (2) Under normoxia conditions and active VHL gene, VHL protein targets the hypoxia-inducible factor-alpha (HIF-α) for ubiquitination and further degradation by the proteasome (Adapted from Rini, B. I. et al., 2009).

Angiogenesis as a therapeutic target for renal cell carcinoma

Kidney cancer is the sixth most common cancer in men and the eighth most common cancer in women (Cancer.Net Editorial Board, 2019). Clear cell renal cell carcinoma (ccRCC) is the most prevalent type of renal cancer and it represents 70% of all renal cell carcinoma (RCC) cases (Shingarev and Jaimes, 2017).

The use of angiogenesis inhibitors as a first-line treatment for metastatic RCC (mRCC) is a highly expanded strategy used to stop continuous tumor growth. It became a new approach since anti-angiogenic therapy did not affect cancer cells themselves, but endothelial cells supporting tumor development.

There are different strategies followed by anti-angiogenic treatment, including the inhibition of angiogenic protein synthesis by tumor cells, the use of selective inhibitors or monoclonal antibodies to neutralise angiogenic proteins, the inhibition of endothelial cell receptors or direct induction of endothelial cell apoptosis. These strategies are able to specifically target angiogenic growth factors and its receptors (Wu, Huang and Chang, 2008).

The main target for anti-angiogenic therapy is VEGF, which promotes endothelial cell development and migration, and it is expressed by up to 60% of human tumors (Folkman, 2007). Bevacizumab was the first humanized monoclonal antibody against VEGF, approved in 2009 for treating RCC. Furthermore, small-molecule tyrosine kinase inhibitors, such as sunitinib and axitinib, blocked VEGF and PDGF receptors in endothelial cells preventing coupling with its ligand (Shingarev and Jaimes, 2017). It also should be mentioned that other drugs can target different molecules such as FGF, FGF receptor, Ang and semaphorins (SEMAs) (Folkman, 2007; Jiménez-Valerio and Casanovas, 2013) (Fig. 2).

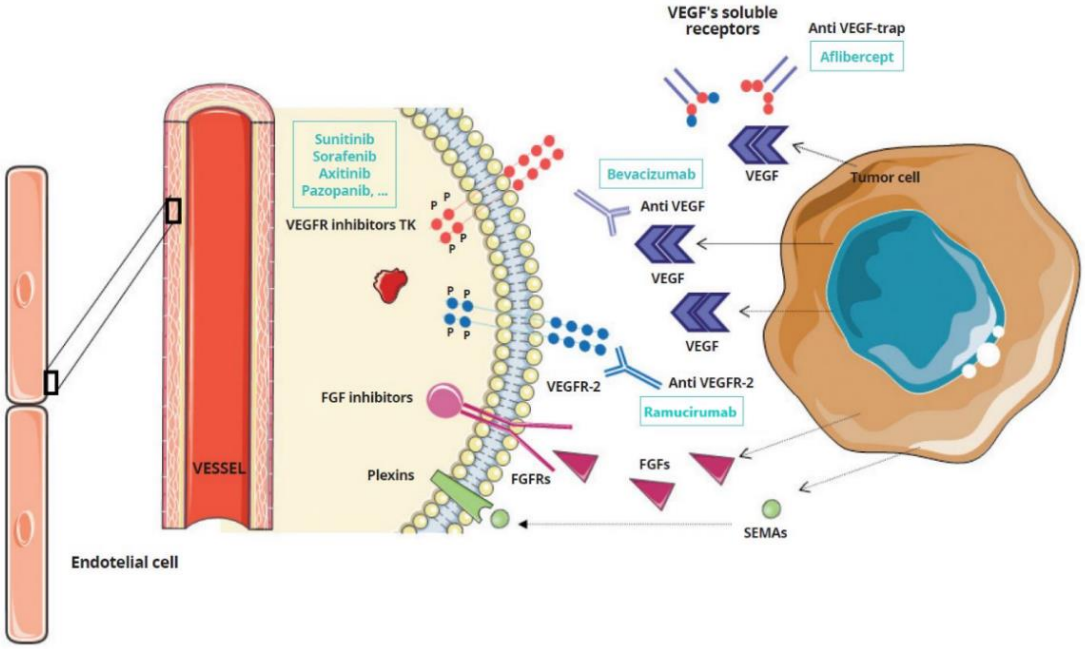

Figure 2. Direct anti-angiogenic therapeutic drugs. Sunitinib, sorafenib, axitinib and pazopanib are small-molecule tyrosine kinase inhibitors that block vascular endothelial growth factor receptors (VEGFR) in endothelial cells. Aflibercept acts as a soluble receptor targeting VEGF isoforms with higher affinity than its natural receptors. Monoclonal antibodies can also be used against pro-angiogenic molecules, such as bevacizumab, blocking VEGF antigen and ramucirumab, targeting VEGFR-2. Other therapies can target fibroblast growth factor (FGF) and semaphorins (SEMAs) (Adapted from Jiménez-Valerio and Casanovas, 2013).

In addition to direct anti-VEGF therapies, there are also indirect drugs that target tumor cell signalling pathways involved in the synthesis of proteins that activate angiogenesis. An example would be everolimus, which acts against mammalian target of rapamycin (mTOR) in the phosphatidylinositol 3-kinase/protein kinase B (PI3K/AKT) pathway. It blocks the translation of proteins involved in cell cycle regulation, glycolysis and hypoxia adaptation, therefore, inhibiting tumor growth and expression of hypoxia-inducible factors that lead to angiogenesis (Folkman, 2007).

Resistance to anti-angiogenesis treatment

The specificity given by anti-angiogenic therapy let to an initial presumption that it would evade drug resistance, as non-tumor cells are genetically more stable and less likely to accumulate mutations than cancer cells (Jiménez-Valerio and Casanovas, 2013). Anti-angiogenic therapies became a good alternative to chemotherapy in certain types of cancer, as they could avoid side effects produced by the lack of specificity of chemotherapeutic agents.

However, anti-angiogenic drugs are not able to eliminate tumor mass, enabling growth relapse. Tumors may develop resistance to anti-angiogenic therapy, specifically if it only targets one single protein. This effect occurs by the ability of tumors to express between five to eight different angiogenic proteins, so that a compensatory effect is produced by those that have not been targeted, increasing their expression (Folkman, 2007). While VEGF-targeted therapies have demonstrated efficacy in extending the progression-free survival of mRCC patients, their benefits are transient and fail to produce durable responses for the majority of patients. Moreover, mRCC patients treated with tyrosine kinase inhibitors develop resistance within a median of 6–15 months (Rini and Atkins, 2009; Jimenez-Valerio *et al.*, 2016).

Two different types of tumor resistance to anti-angiogenic therapy have been described depending on tumor response, the intrinsic and the acquired resistance (Fig. 3).

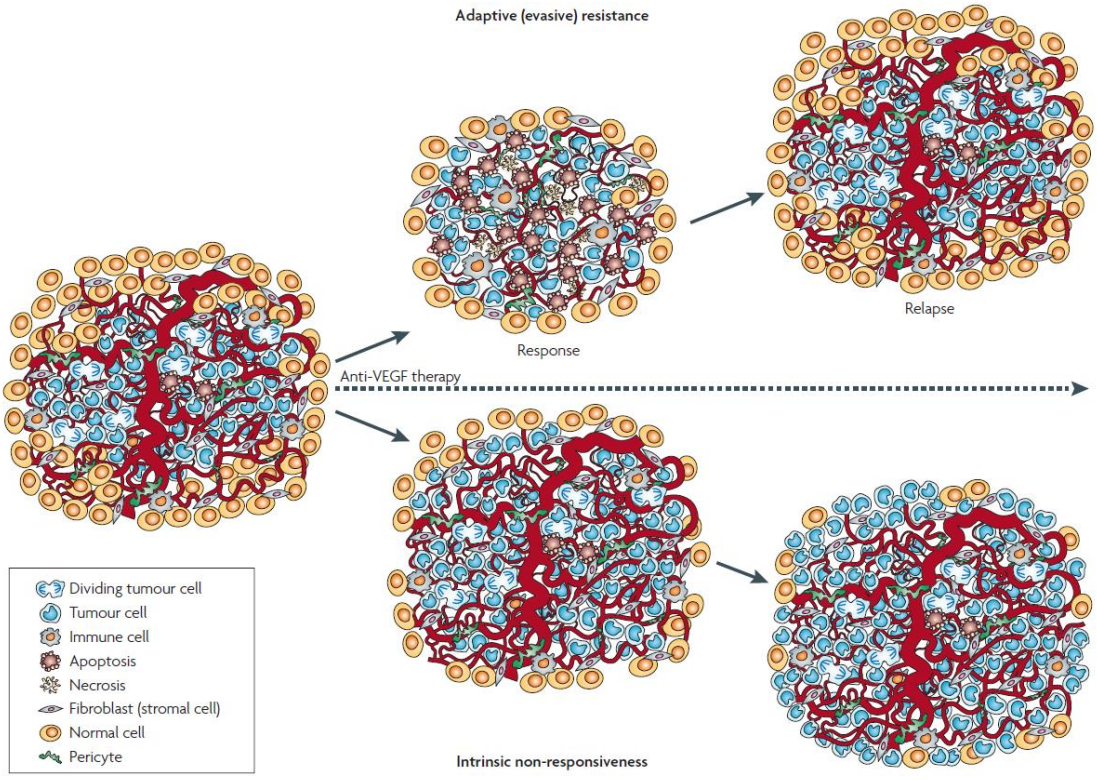

Figure 3. Types of tumor resistance in response to anti-angiogenic therapy. Anti-angiogenic resistance refers to the ability of tumors to evade the therapeutic blockade by inducing mechanisms that enable neovascularization or reduce the dependence of new blood vessels, leading to renewed tumor growth and progression. However, intrinsic non-responsiveness is a pre-existing condition characterized by the absence of beneficial effect of an anti-angiogenic therapy, growing and progressing unabated during the course of the anti-angiogenic therapy (Bergers and Hanahan, 2008).

Intrinsic resistance

In the context of intrinsic resistance, anti-angiogenic therapy has no effect on the tumor. It has been demonstrated that tumors are able to keep growing since the beginning of the treatment. The ability to express multiple pro-angiogenic genes (*FGF1, FGF2, EFNA1, EFNA2 and ANGPT1*) is thought to be one of the mechanisms involved in this type of resistance, limiting the efficacy of anti-VEGF therapy. Another condition involved in this process is the de-regulation of the HIF pathway. Renal tumors express high levels of angiogenic molecules controlled by this pathway, thereby reducing the effect of anti-angiogenic therapy. Furthermore, using the normal vasculature contiguous to the tumor may be another potential mechanism supporting tumor development, as well as the mobilization of latent vessels (Folkman, 2007).

Acquired resistance

Otherwise, the treatment has an initial effect in acquired resistance, decreasing tumor development and stopping and/or reducing its size. Resistance appears when pro-angiogenic factors are again overexpressed allowing tumor revascularisation regardless of therapy. This relapse can also be due to an increased expression of alternative pro-angiogenic factors other than VEGF, the recruitment of pro-angiogenic cells from stroma, the coating of vessels by pericytes, or vascular mimicry, which is the ability of tumor cells to reproduce host blood vessels network to nourish itself. However, clinical evidence demonstrated reversibility of acquired resistance, suggesting that this type of resistance is due to physiological changes in the microenvironment and adaptations to therapy, instead of the accumulation of gene mutations (Rini and Atkins, 2009).

In order to overcome resistance, various strategies have been explored, such as increasing treatment dose, using non-cross-resistant drugs, switching to another VEGF inhibitor and doing a strategic treatment interruption. Results obtained from these approaches so far have been modest, suggesting a clear need for the development of novel agents to combat resistance, which may involve exploring new targets and new mechanisms of treating RCC (Vasudev and Reynolds, 2014; Oudard *et al.*, 2016).

Novel strategies to overcome anti-angiogenic resistance

a. Metabolic symbiosis

Recently, this research group has described an alternative mechanism of tumor resistance to anti-angiogenics that does not require tumor revascularisation. Metabolic symbiosis is a model of acquired resistance that consists in metabolic tumor adaptation to the hypoxic environment after anti-angiogenic therapy (Fig. 4). This new concept has been developed in studies with renal cell carcinoma orthoxenograft mouse models and also in human clinical samples (Jimenez-Valerio *et al.*, 2016).

After an efficient response to anti-angiogenic first-line treatment with sunitinib, tumor re-growth is observed in animal models, as well as in patients. Vascular failure in the tumor followed by hypoxia induces the differentiation of two different metabolic regions within the tumor, based on their proximity to the remaining vasculature (Jimenez-Valerio *et al.*, 2016).

The hypoxic area, located far from the remaining blood vessels, triggers HIF-1α transcription factor that, in turn, induces expression of transporters that stimulate glucose import (glucose transporter 1, GLUT1) and lactate export (monocarboxylate transporter 4, MCT4). Thus, activating anaerobic glycolysis and leading to high levels of lactate secretion. In consequence, extracellular accumulation of lactate induces the expression of lactate importers (monocarboxylate transporter 1, MCT1) in normoxic cancer cells, proximal to lasting vascular tissue, leading to the catabolization of lactate by oxidative phosphorylation. Normoxic cells do not uptake glucose from near remnant blood vessels because they have not upregulated glucose transporters in contrast to hypoxic cells, making glucose available for hypoxic cancer cells. This situation triggers mTOR signalling that promotes metabolic symbiosis and tumor survival (Jimenez-Valerio *et al.*, 2016).

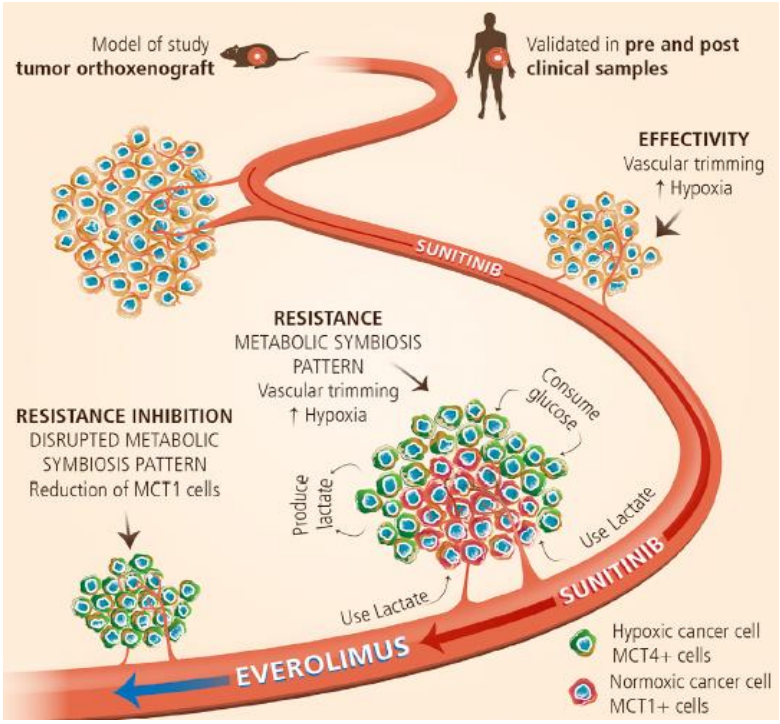

Figure 4. Metabolic symbiosis pattern. After an effective response to the first-line treatment with sunitinib, tumor cells develop a mechanism of adaptation to hypoxia. Hypoxic cancer cells (green), located far from the remaining vasculature, increase the expression of glucose transporter 1 (GLUT1) and monocarboxylate transporter 4 (MCT4), which promote glucose uptake and lactate secretion. However, normoxic tumor cells (red), which are proximal to remaining blood vessels, are induced by the extracellular accumulation of lactate to express lactate importers (monocarboxylate transporter 1, MCT1), therefore, developing a resistance mechanism to the anti-angiogenic therapy. Second-line treatment with everolimus (mTOR inhibitor) disrupted metabolic symbiosis eliminating the MCT1+ subpopulation and leading to toxic accumulation of lactate and increased necrosis by oxygen and nutrient reduction (Adapted from Jimenez-Valerio et al., 2016).

Blocking mTOR signalling switches MCT1+ subpopulation from lactate metabolization to glucose consumption by aerobic glycolysis. In consequence, the hypoxic tumor cells die due to the lack of glucose and the toxic accumulation of lactate, underlying the disruption of the metabolic symbiosis pattern. This situation results in a decrease of tumor viability, but with limited survival benefits, suggesting that mTOR inhibitors, such as everolimus, may not be the appropriate drugs for treating tumor adaptive resistance (Elizabeth *et al.*, 2016; Pisarsky *et al.*, 2016).

Highly selective drugs targeting MCT1 and MCT4, and predictive biomarkers are a promising alternative to treat anti-angiogenic resistance and to detect pre-treatment responses (Jiménez Valerio, 2013). Several small molecule MCT1 inhibitors have already been reported, with favourable results inhibiting tumor growth in diffuse large B-cell lymphoma, non-Hodgkin lymphoma and Burkitt's lymphoma (Curtis *et al.*, 2017).

b. Metabolic escape by nitrogenous bases catabolism

Transcriptome analysis of different genes involved in angiogenesis revealed an increased expression of the resistance factor 1 (RF-1) in anti-angiogenic treatment-resistant tumors (Jiménez Valerio, 2013).

RF-1 is an enzyme involved in purine and pyrimidine metabolism and it is considered a pro-angiogenic factor, which promotes endothelial cell growth and major tumor aggressiveness, related with poor prognosis. Therefore, the development of new therapies inhibiting RF-1 are considered an attractive approach for stopping tumor growth (Jiménez Valerio, 2013).

This group performed an RF-1 genetic knockdown in a ccRCC cell line and its effect was compared to an RF-1 pharmacologic inhibition in a tumor orthoxenograft mice model. Results clearly demonstrate that inhibiting RF-1 pathway is an effective alternative to treat anti-angiogenic resistant tumors (Fig. 5). The chemical inhibitor was only used as a proof of concept (PoC) drug for validation hypothesis purposes, and now, new compounds designed with related RF-1 structure should be tested to maximize effectiveness and minimize toxicity for patients (Jiménez Valerio, 2013).

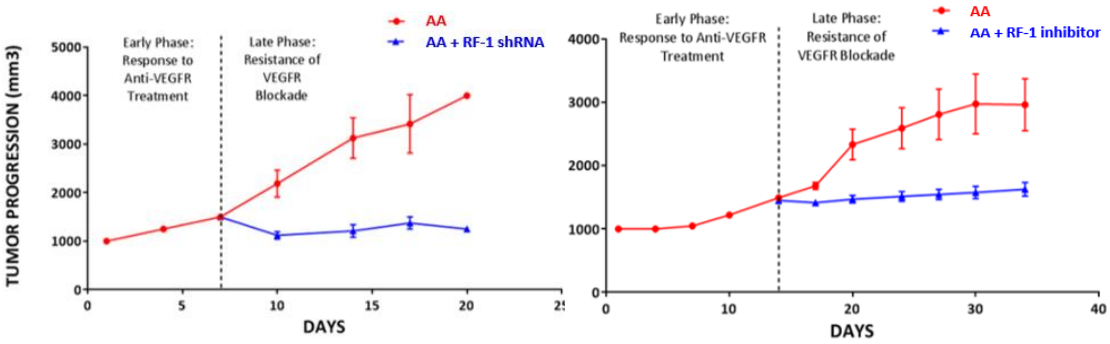

Figure 5. Tumor progression in 786-O (left) and REN28 (right) renal cell carcinoma (RCC) orthoxenograft tumors. Animals were treated with anti-angiogenics (AA) until tumors reached resistance, and then randomized in 2 groups: AA continues treatment (red), and combination of AA and genetic or pharmacologic inhibition of RF-1 (blue) is given (unpublished data from Jimenez-Valerio, G.)

Current RF-1 inhibitors have been developed in academic studies and have been published in scientific journals. The chemical RF-1 inhibitor, IRF-1 (inhibitor of resistance factor 1), blocks RF-1 enzymatic activity and it is currently used in patients. However, it was approved as a preventing drug to avoid degeneration of a chemotherapeutic agent, but not because of its therapeutic effect (Committee for Medicinal Products for Human Use, 2016).

In addition, IRF-1 presents bad stability and pharmacokinetics due to poor absorption and rapid degradation in the organism (Committee for Medicinal Products for Human Use, 2016). Nevertheless, the use of IRF-1 in patients indicated that RF-1 inhibition was not toxic for humans, thus benefiting the development of new and better therapeutic options against RF-1.

Opportunity remains for drugs that both overcome resistance and have better safety profiles compared to tyrosine kinase inhibitors. Additionally, current therapies are used in a non-targeted manner due to the absence of predictive biomarkers. A more personalized approach would allow patients to be specifically matched with available and emerging therapies (Global Data, 2016).

Previous work

The present group performed a target validation and a computational drug discovery of RF-1 in order to develop better prolife inhibitors compared to standard anti-RF-1 drugs. Analysis of the new therapeutic target resulted in several relevant scaffolds or structural parts of the molecule involved in the binding interaction. Two active sites were revealed, suggesting that their inhibition could allow a greater blockage of RF-1 activity. A virtual screening exposed forty-eight molecules as potential inhibitors of RF-1. Three of these structures were selected according to greater affinity with the target, as they interacted with both catalytic and allosteric binding sites. These structures became HIT101, HIT102 and HIT103 candidate drugs, which could possibly block resistance to anti-angiogenics through RF-1 optimal inhibition.

In addition, a proof of concept drug (PoC) was also evaluated to test anti-angiogenic and anti-tumor activity of the target.

During this project, *in vitro* analysis of HIT101, HIT102 and HIT103 function were performed by proliferation and migration assays in modified RCC cell lines, 786-O sh91 and SN12C sh91.

Hypothesis

New HIT compounds, HIT101, HIT102 and HIT103, exert a greater inhibition of RF-1 compared to standard anti-RF-1 drugs, such as inhibitor of resistance factor 1 (IRF-1) and proof of concept (PoC) drug, thus decreasing renal tumor resistance after anti-angiogenic therapy.

Objectives

The first objective is to investigate the effect *in vivo* of the commercial RF-1 inhibitor, IRF-1, after anti-angiogenic therapy in ccRCC tumor progression.

The second aim is the validation *in vitro* of RF-1 inhibitors as new therapeutic factors in order to overcome resistance to anti-angiogenic treatments. Specifically, to elucidate the effect of the novel HIT candidates, HIT101, HIT102 and HIT103, on proliferation and migration rate of modified renal cell lines 786-O and SN12C.

Materials and methods

In vitro experiments

– Cell cultures

The human ccRCC cell lines 786-O and SN12C were kindly provided by Dr. Jesús del Pozo from Universidad Autónoma de Madrid (Spain) and Dr. Manel Esteller from IDIBELL (Spain), respectively. These renal cell lines were genetically modified by a short hairpin RNA 91 (shRNA 91) expression vectors in order to inhibit RF-1 expression with doxycycline (DOX) treatment, known as *tet on* system. Non-silencing (shNS) expression vectors were used as control. Both cell lines were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 5% non-essential amino acids, 50U/ml penicillin, 50µg/ml streptomycin, 2mM glutamine, 1mM HEPES pH 7.5 and 2mM sodium pyruvate (all Gibco, Life Technologies, USA). All cells were maintained at 37°C in humidified conditions with 5% CO_2.

– Cell proliferation assay

The modified 786-O sh91, 786-O shNS, SN12C sh91 and SN12C shNS cells were seeded into 12-well plates and incubated at 37°C in complete RPMI 1640 medium until they reached 50-60% of confluence. After that, RF-1 inhibitors (Table 1) and DOX (5µg/ml) were added in duplicate to the medium and incubated at 37°C. After 24h, proliferating cells were labelled with EdU (5-ethynyl-2´-deoxyuridine) using the Click-It EdU Imaging Kit (Thermo Fischer Scientific™, USA), while total cells were marked with 4',6-diamidino-2-phenylindole (DAPI). EdU detection was performed following the manufacture instructions. For each condition, 12 photos were taken at 10X magnification using Zeiss Apotome.2 (Carl Zeiss, Germany) microscope. Cell proliferation was determined using ImageJ (NIH, USA) software.

Table 1. List of RF-1 inhibitors used for cell proliferation assay of 786-O sh91, 786-O shNS, SN12C sh91 and SN12C shNS cells with their respective tested concentrations and the company from which they were purchased.

RF-1 inhibitors	Concentration (μM)	Company
Inhibitor of resistance factor 1 (IRF-1)	100	Sigma-Aldrich, USA
Proof of concept (PoC)	100	Tractus Company Limited, England
HIT101	100	ChemDiv, USA
HIT102	100	Life Chemicals Inc., Europe
HIT103	100	Life Chemicals Inc., Europe

– Cell migration assay

Migration analysis of modified human ccRCC (786-O sh91, 786-O shNS, SN12C sh91 and SN12C shNS) was performed through wound healing assay. The amount of 20000 cells per section (40000 in total) of a silicone insert (Applied BioPhysics Inc., USA) (Fig. 6) were seeded on a 24-well plate in RPMI 1640 medium for 24h, until cells reached confluency. Wounding of the cellular monolayer was performed by detaching the insert from the plate with a quick and precise movement upwards in order to create a straight line at the bottom of the well. At this time (t=0h), three photos per well were taken at 10X magnification using inverted microscope Leica DMi1 (Leica Microsystems, Germany).

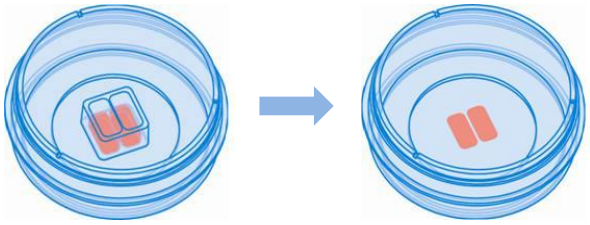

Figure 6. Wounding of cell monolayer after removing the silicon insert used to perform wound healing assay (Applied BioPhysics Inc., 2019).

After that, RF-1 inhibitors (Table 2) and DOX (5μg/ml) were added to the medium. To determine the wound healing course, 786-O cells were incubated at 37°C and serial images were taken every 3h for the following 9h, while SN12C cells were imaged for 24h. Cell migration was quantified as the change in wound width over time. Wound width was calculated by tracing the cell-free area in captured images using ImageJ software (NIH, USA).

Table 2. List of RF-1 inhibitors used for the wound healing assay of modified human ccRCC (786-O sh91, 786-O shNS, SN12C sh91 and SN12C shNS) with their respective tested concentrations and the company from which they were purchased.

RF-1 inhibitors	Concentration (µM)	Company
Inhibitor of resistance factor 1 (IRF-1)	100	Sigma-Aldrich, USA
Proof of concept (PoC)	100	Tractus Company Limited, England
HIT101	1 and 10	ChemDiv, USA
HIT102	1 and 10	Life Chemicals Inc., Europe
HIT103	0.1 and 1	Life Chemicals Inc., Europe

In vivo experiments

– Animal model and conditions

Animals used in this study were male athymic nu/nu mice (Harlan Laboratories Inc., USA) maintained in individually ventilated cages at 20-22^{o}C in sterile conditions. Animals were under an artificial circadian 12h light/dark cycle and received *ad libitum* standard diet and water. Experiments were performed inside a vertical laminar flow cabinet. All animal experiments were developed according to IDIBELL's Ethical Committee of Animal Experimentation and following Spanish laws and European directives on ethical usage of rodents for animal research.

– 786-O-derived orthoxenograft mice model

Human cancer cells from 786-O cell line were directly injected in the kidney of 4-5 weeks old mice. After injection, tumor cells were able to colonize the kidney and develop a palpable tumor mass in approximately 20-30 days, then perpetuated throughout successive passages to obtain an experimental cohort.

– Anti-angiogenic treatment

Mice were treated with two anti-angiogenics: I) DC101, a monoclonal antibody against mouse VEGFR-2 obtained from hybridoma culture from ATCC (ATCC, USA) and II) IRF-1, an RF-1 inhibitor that is currently used in combination with chemotherapy, which was administered to mice as a second-line treatment (Sigma-Aldrich, USA).

Once tumors started to be palpable (1000mm^3, approximately), mice were randomized in two groups, control (treated with 1X PBS by intraperitoneal injection) (n = 4) and DC101 (n = 14). Treatment was administered twice a week by intraperitoneal injection dosed at 1mg/kg mice. Tumor growth was followed by palpation twice a week, and treatment was considered ineffective (tumor resistance) when primary tumors grew 50% more of their initial volume (1500mm^3, approximately).

After 10-15 days since first DC101 administration, tumors developed resistance and DC101-treated animals were randomized into three groups, according to second-line treatment: DC101 (n = 5), IRF-1 (n= 4) and DOX (n = 5). IRF-1 was administered by oral gavage at a dose of 100mg/kg mice and 2mg/ml of DOX were fed with water + 3% sucrose to increase palatability. Animals were sacrificed after 14 days of treatment and tumor tissue was weighted and processed for further histological and molecular analysis.

– Quantification of tumor volume and weight

Mice were placed in supine position held by head and tail with one hand, exposing the kidney area to the operator. Tumor palpation of the right kidney was performed with the thumb and forefinger from the other hand. An estimated tumor volume was established according to measurements in Fig. 7.

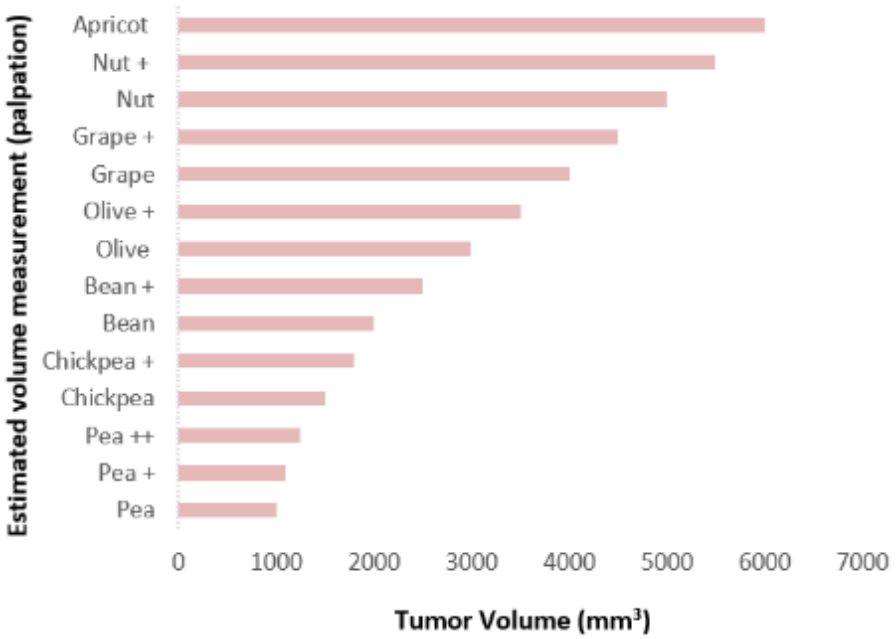

Figure 7. Equivalence between estimated tumor volume determined by palpation and real tumor volume (mm³) (adapted from Jiménez Valerio, G. A., 2013).

To calculate the equivalence between palpation and real tumor volume, Dr. Jimenez Valerio performed an initial experiment sacrificing the animals when they reached the estimated measures (pea, chickpea, bean…) and then, quantifying the volume displaced by the tumor when it was inserted in a container with a known volume of physiological serum (Fig. 8).

Tumor weight was determined directly on a highly sensitive scale, FX-300i (A&D Weighing, Japan).

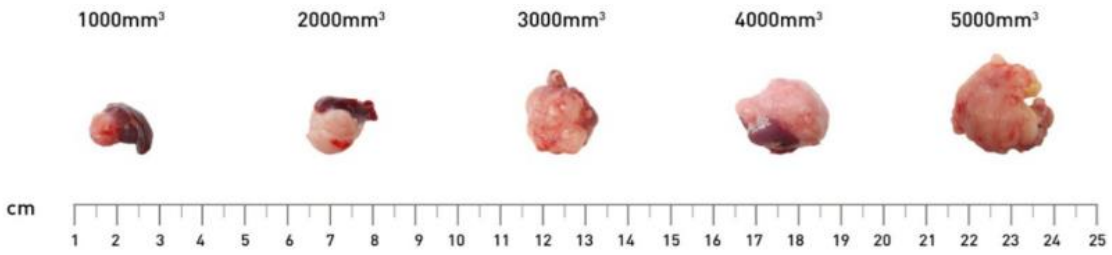

Figure 8. Volume of renal tumors (Jiménez Valerio, G. A., 2013).

– Obtaining and processing of samples

Once the tumor volume, weight and measurements were obtained, different tumor fragments were collected for further histological and molecular studies. One piece of tumor with kidney was fixed in formaldehyde 4% to be included in a cassette for paraffin embedding. Another piece was included in Tissue-Tek O.C.T. (Sakura Finetek, Japan) and kept at -80^{o}C for further analysis of frozen tissue. Finally, other fragments were directly frozen in cryotubes and maintained at -80^{o}C for protein and RNA extraction. Additionally, lungs and liver were also fixed in formaldehyde 4% to be included in paraffin.

Statistical analysis

Data analysis and graph generation was performed using GraphPad Prism 6.01 software (GraphPad Inc., USA). Results are presented as mean ± standard deviation (SD). A non-parametric test, Mann-Whitney, was used due to small sample size and lack of normal distribution. Results were considered statistically significant when $p < 0.05$ (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001).

Results

Activity of IRF-1 in ccRCC tumors

Due to increased RF-1 expression in resistant tumors, an evaluation of a commercial RF-1 inhibitor was performed in a long-term tumor progression experiment (Fig. 9). IRF-1 effect was analysed after anti-angiogenic therapy in comparison to genetic inhibition of RF-1. During early phase of the *in vivo* study, mice were treated with VEGFR-2 inhibitor, DC101. After seven days, tumor volume regrew and resistant phenotype was developed. A group of mice continued to be treated with DC101, which showed a lineal increase in tumor volume (3000 mm^3). Another group was treated with IRF-1, which reduced tumor growth (2500 mm^3), compared to standard treatment (DC101) and control group. Finally, treatment with DOX induced genetic inhibition of RF-1 and kept tumor volume constant over time (1600mm^3), confirming that there is a comparable effectiveness between RF-1 inhibitors and anti-angiogenic therapies.

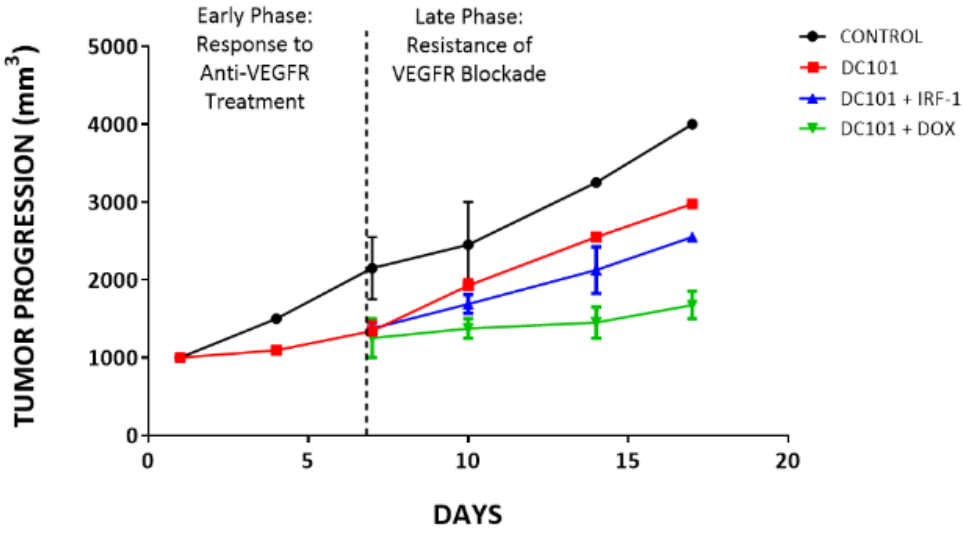

Figure 9. Effect in 786-O-derived mice tumors volume progression (mm^3) of anti-angiogenic drug, IRF-1, compared to standard treatment, DC101, and DOX-induced RF-1 genetic inhibition, after 17 days since tumor was palpable.

At the end of the experiment, weight and tumor volume were evaluated (Fig. 10). Inhibition of RF-1 with the chemical inhibitor IRF-1 after continuous administration of DC101 did not show a significant decrease in both tumor weight and volume (2.4g and 2500mm^3) compared to DC101 therapy alone (2.7g and 3000 mm^3). However, DOX-induced genetic silencing of RF-1 did significantly reduce tumor weight and volume (1.5g and 1600mm^3) after anti-VEGFR-2 therapy. Consequently, new drugs were necessary to design in order to stop resistance to anti-angiogenics.

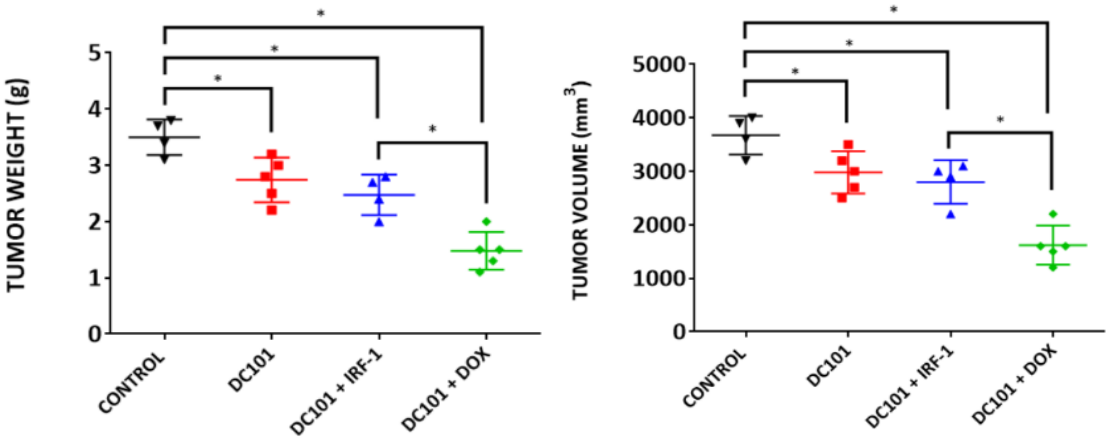

*Figure 10. Effect of anti-angiogenic treatment with DC101 (n = 5), DC101 + IRF-1 (n = 4), and DOX-induced genetic RF-1 inhibition (n = 5) on tumor weight (g) and volume (mm^3), compared to control (n = 4) in 786-O-derived mice tumors. Data are presented in scatter plot with mean and SD values represented in error bars. Data were analysed by Mann-Whitney non-parametric test and statistically significant differences were observed between control group and treatments, and between DC101 + IRF-1 and DC101 + DOX (*p<0.05).*

The novel HITs identified through the virtual screening of RF-1 were validated in an *in vitro* setting.

Effect of novel HITs in 786-O and SN12C tumor cell proliferation

Proliferation of 786-O and SN12C sh91 cells was studied in seven different conditions, including control, DOX, previously tested anti-angiogenic drugs such as, PoC (100µM) and IRF-1 (100µM), and the three new HIT compounds, HIT101 (100µM), HIT102 (100µM) and HIT103 (100µM). Initially, experiments were performed at higher doses in order to further study the dose response and settle the appropriated drug concentration.

First, cellular response of 786-O sh91 to new HIT compounds was observed with fluorescence microscope. Fig. 11 shows total cells (blue) and proliferating cells (green) stained with DAPI and EdU, respectively. Images depict a greater reduction of 786-O sh91 proliferation when cells were treated with HIT101 (Fig. 11E), HIT102 (Fig. 11F) and HIT103 (Fig. 11G) drugs, compared to PoC (Fig. 11C) and IRF-1 (Fig. 11D). The greatest effect was observed with HIT103 treatment, where proliferating cells were reduced and similar to DOX treatment (Fig. 11B), which genetically inhibited RF-1 activity.

786-O sh91

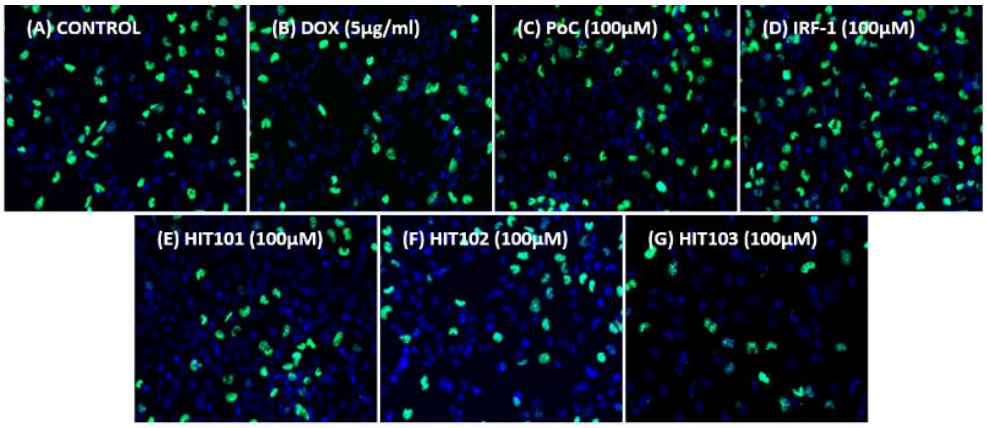

Figure 11. Representative images of 786-O sh91 cells taken at 10X with Zeiss Apotome.2 microscope (Carl Zeiss, Germany). Dividing cells were labelled with EdU (green), and nuclei of total cells were stained with DAPI (blue). Cells were treated with different conditions, including (A) control, (B) 5µg/ml doxycycline (DOX), (C) 100µM proof of concept drug (PoC), (D) 100µM inhibitor of resistance factor-1 (IRF-1), (E) 100µM HIT101, (F) 100µM HIT102 and (G) 100µM HIT103.

To verify the correct inhibition of RF-1 by the vector, a random shRNA was induced, targeting a non-related RF-1 gene (shNS). When 786-O shNS cells were treated with DOX, RF-1 inhibition was not induced and proliferation was similar to control group, confirming that RF-1 silencing was the responsible for stopping renal tumor cell growth (Fig. 12).

786-O shNS

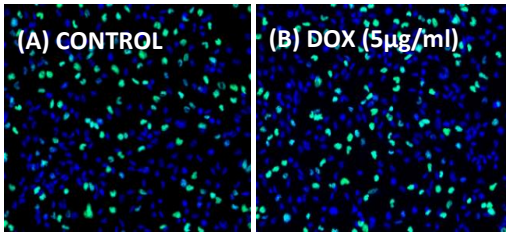

Figure 12. Representative images of 786-O shNS cells taken at 10X with Zeiss Apotome.2 microscope (Carl Zeiss, Germany). Dividing cells were labelled with EdU (green), and nuclei of total cells were stained with DAPI (blue). Cells were divided in, (A) control and (B) treated with 5µg/ml doxycycline (DOX) in order to induce sh non-silencing (NS) expression.

According to SN12C sh91 cells, images from the microscope show a significant reduction of SN12C sh91 with HIT103 condition (Fig. 13G), where proliferating cells were decreased compared to control and to other anti-angiogenic treatments. In addition, HIT103 effect was better than DOX-induced RF-1 genetic inhibition (Fig. 13B). At first glance, other HITs seemed to be similar to DOX effect in cell proliferation.

SN12C sh91

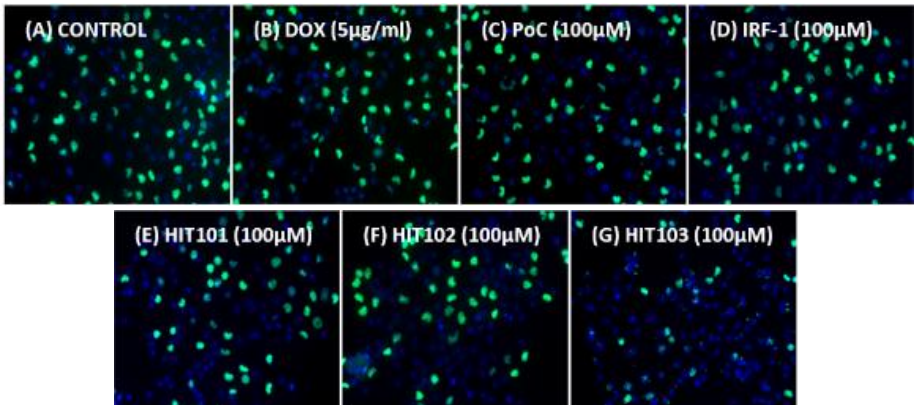

Figure 13. Representative images of SN12C sh91 cells taken at 10X with Zeiss Apotome.2 microscope (Carl Zeiss, Germany). Dividing cells were labelled with EdU (green), and nuclei of total cells were stained with DAPI (blue). Cells were treated with different conditions, including (A) control, (B) 5µg/ml doxycycline (DOX), (C) 100µM proof of concept drug (PoC), (D) 100µM inhibitor of resistance factor-1 (IRF-1), (E) 100µM HIT101, (F) 100µM HIT102 and (G) 100µM HIT103.

Likewise, when the genetic expression of RF-1 was not inhibited, proliferation of SN12C cells treated with DOX was similar to control (Fig. 14).

SN12C shNS

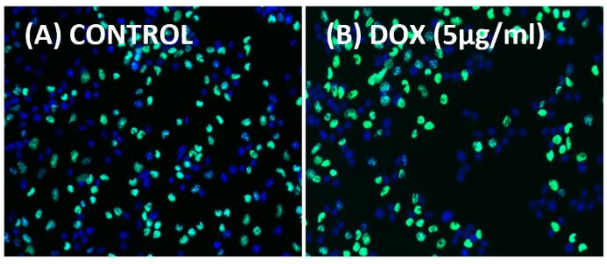

Figure 14. Representative images of SN12C shNS cells taken at 10X with Zeiss Apotome.2 microscope (Carl Zeiss, Germany). Dividing cells were labelled with EdU (green), and nuclei of total cells were stained with DAPI (blue). Cells were divided in, (A) control and (B) treated with 5µg/ml doxycycline (DOX) in order to induce sh non-silencing (NS) expression.

Quantification of HITs effect *in vitro* in 786-O and SN12C tumor cell proliferation

A quantitative analysis of 786-O and SN12C sh91 proliferation was performed. The percentage of proliferation in 786-O sh91 cells (Fig. 15A) significantly decreased with DOX (45.9 ± 18.8%) compared to control group (54.7 ± 15.3%), proving that knockdown of RF-1 gene reduced tumor cell growth. Experiments with PoC also revealed a significant decrease in cellular proliferation by 8% compared to control group. Remarkably, IRF-1, which is already a commercial drug, did not reduce cell proliferation (57.0 ± 15.9%), confirming that it was not an optimal RF-1 inhibitor. RCC cells significantly decreased when they were treated with HIT101 (43.7 ± 20.7%), HIT102 (45.9 ± 16.3%) and HIT103 (20.8 ± 6.7%) compared to control group. However, HIT103 was the most effective drug decreasing 34% of cell growth and presented statistically greater difference compared to control, corresponding with the effect seen in the microscope (Fig. 11G).

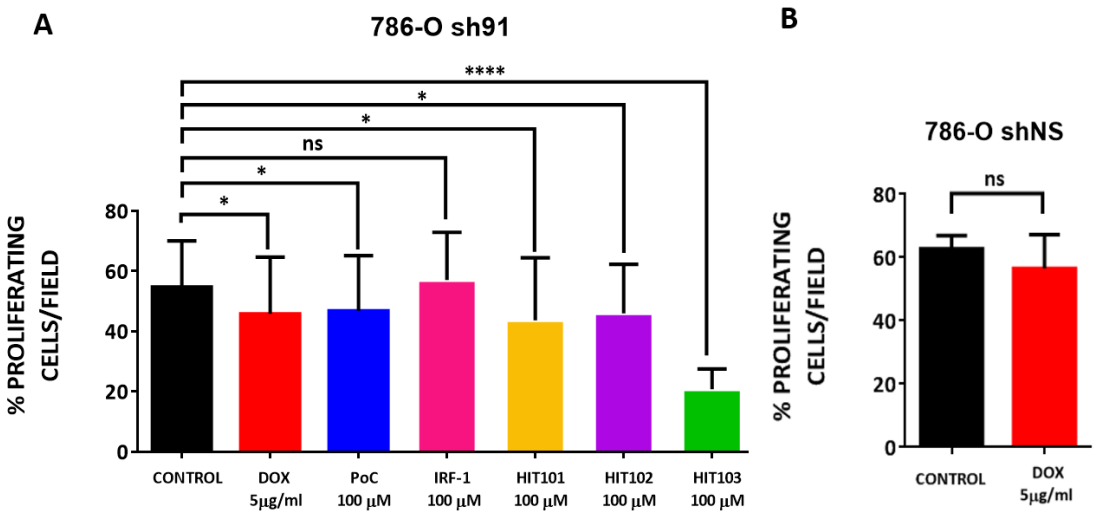

*Figure 15. Proliferation of (A) 786-O sh91 cells under the effect of 5µg/ml doxycycline (DOX) and 100µM RF-1 inhibitors (PoC, IRF-1, HIT101, HIT102 and HIT103) and (B), 786-O sh non-silencing (NS) cells induced with 5µg/ml doxycycline (DOX) and not induced (CONTROL). Data are presented as mean ± standard deviation (SD) represented in error bars. Data were analysed by Mann-Whitney non-parametric test and results for (A) 786-O sh91 were statistically significant for DOX, PoC, HIT101, HIT102 (*p<0.05) and HIT103 (****p<0.0001) and no significance (ns) was observed for IRF-1 (n = 3). Results for (B) 786-O shNS showed no significance (ns) (n = 3).*

Results from 786-O shNS proliferation assay showed no significant differences between control and DOX conditions (Fig. 15B), proving that RF-1 genetic inhibition reduced renal tumor growth. However, a slightly decrease in cell proliferation was observed, due to the antibiotic nature of doxycycline.

According to SN12C sh91, cells followed the same trend as in 786-O sh91. Quantitatively, DOX treatment was able to significantly reduce 15.4% of cell proliferation (Fig. 16A), which was 1.75 times higher than DOX effect in 786-O cells. The effect of PoC remained minor (46.3 ± 8.1%) compared to HIT compounds. Treatment with IRF-1 (49.2 ± 6.2%) did not show any significant differences in comparison to control group (50.5 ± 6.6%) demonstrating that it did not optimally inhibit RF-1 in SN12C cells either. The major decrease in cell growth was observed with HIT103, which was able to significantly reduce 26.8% of tumor cell proliferation compared to control, being the most inhibitory compound.

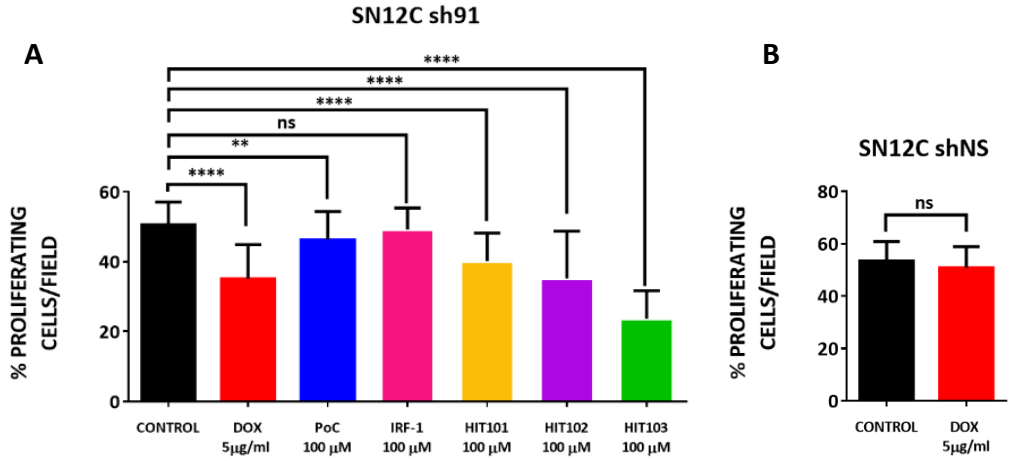

*Figure 16. Proliferation of (A) SN21C sh91 cells under the effect of 5µg/ml doxycycline (DOX) and 100µM RF-1 inhibitors (PoC, IRF-1, HIT101, HIT102 and HIT103) and (B), SN12C sh non-silencing (NS) cells induced with 5µg/ml doxycycline (DOX) and not induced (CONTROL). Data are presented as mean ± standard deviation (SD) represented in error bars. Data were analysed by Mann-Whitney non-parametric test and results for (A) SN12C sh91 were statistically significant for PoC (**p<0.01), DOX, HIT101, HIT102 and HIT103 (****p<0.0001) and no significance (ns) was observed for IRF-1 (n = 4). Results for (B) SN12C shNS showed no significance (ns) (n = 4).*

To ensure there were no other factors involved in cell proliferation, a shNS was also induced in SN12C cells. Proliferation assay resulted in no differences between control and DOX-treated group (Fig. 16B), verifying that RF-1 was one of the factors involved in SN12C tumor cell growth.

Evaluation of HITs doses to inhibit ccRCC cell growth

The three new compounds HIT101, HIT102 and HIT103 were tested in 786-O and SN12C sh91 cells at different concentrations (100µM, 50µM, 10µM, 1µM, 0.1µM, 0.01µM, 0.001µM, 0.0001µM and 0µM), in order to define the lowest drug dose that induced an anti-proliferative effect on tumor cells.

Best dose response was determined by qualitative observation of cells in the 96-well plate with Leica DMi1 (Leica Microsystems, Germany) microscope. The best anti-proliferative effect was observed at 1µM and 10µM for HIT101 and HIT102, whereas 0.1µM and 1µM were the best doses for inhibiting cell proliferation with HIT103 drug (Fig. 17). The strongest effect was produced by HIT103, which showed a similar behaviour compared to genetic inhibition of RF-1 (Fig. 17 DOX).

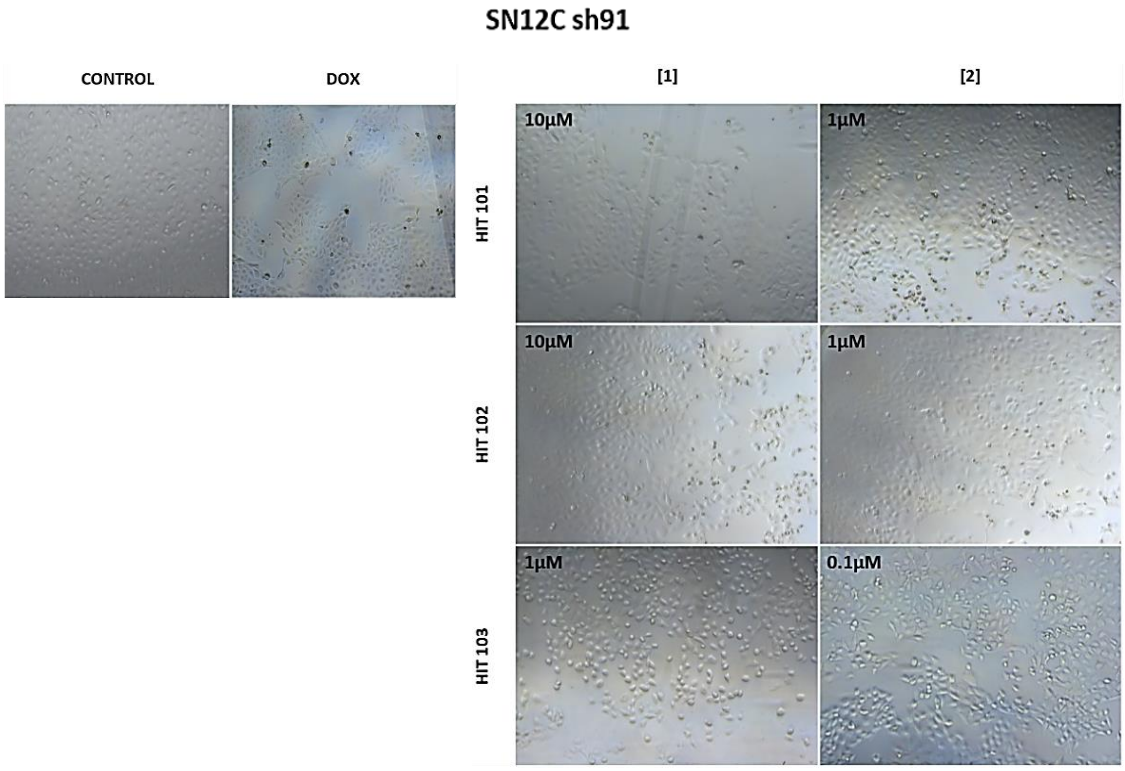

Figure 17. Representative images of dose response in SN12C sh91 cells with different drug concentrations (HIT101 and HIT102: 10µM and 1µM, HIT103: 1µM and 0.1µM) compared to control and doxycycline (DOX) treatment. Images were taken at 10X using Leica DMi1 inverted microscope (Leica Microsystems, Germany).

Effect of novel HITs in 786-O and SN12C tumor cell migration

Migration of modified 786-O and SN21C cells was studied in order to evaluate the effectiveness of new therapeutic compounds (HIT101, HIT102 and HIT103) compared to PoC and IRF-1, previously used. Wound healing assay was performed in the same seven conditions as described in the proliferation assay (control, DOX, PoC 100µM and IRF-1 100µM), but this time testing the dose of HIT101, HIT102 and HIT103 obtained in the dose response experiment (Table 3).

Table 3. Summary of HITs concentrations that demonstrated better inhibitory activity in 786-O and SN12C cell growth.

RF-1 inhibitor	**Concentration (µM)**
HIT101	1 and 10
HIT102	1 and 10
HIT103	0.1 and 1

Untreated 786-O sh91 cells showed a total closure of the wound after 9h (Fig. 18), while induction of RF-1 genetic inhibition with DOX stopped cell migration, so the wound was not completely closed after the same time. Treatment with HIT101 1µM and HIT103 1µM slowed cell migration compared to HIT102 1µM. Cells treated with standard RF-1 inhibitors, IRF-1 100µM and PoC 100µM, avoided wound closure after 9h, although its width was narrower than treatment with HITs (Annexed Fig. 1). In addition, HIT101 and HIT103 demonstrated better effects at 1µM than at 10µM, while HIT102 presented a similar effect at both concentrations (Annexed Fig. 1). Moreover, wound width was wider under HIT101 and HIT103 1µM treatment than RF-1 genetic inhibition induced by DOX.

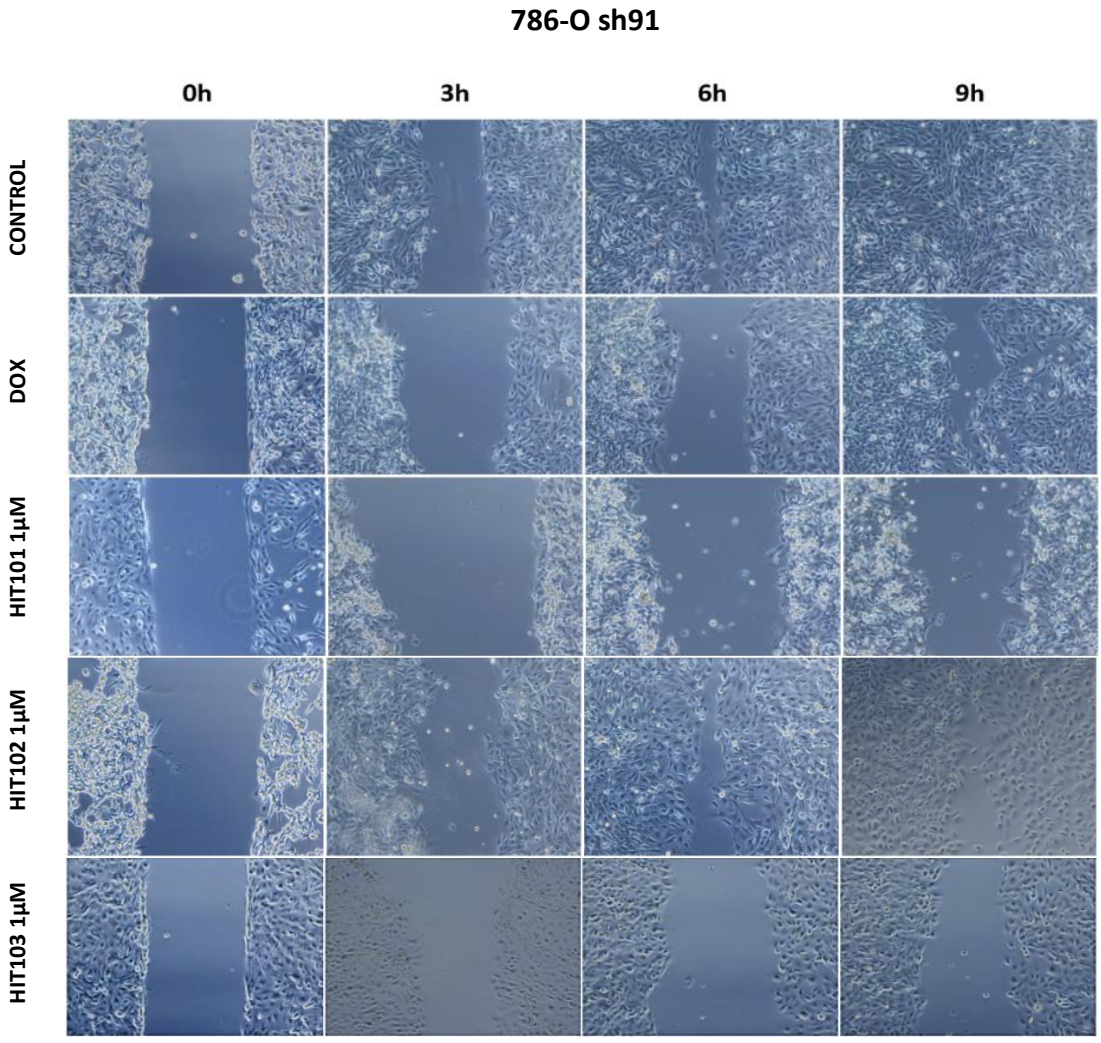

Figure 18. Representative images of 786-O sh91 migration assay of control, doxycycline (DOX), HIT101 1µM, HIT102 1µM and HIT103 1µM conditions. Images were taken at 10X every 3h, for a total of 9h with Leica DMi1 inverted microscope (Leica Microsystems, Germany).

According to SN12C sh91 cells, the wound could still be observed in control condition after 9h. However, it had to be considered that SN12C cells proliferate slower than 786-O, making it difficult to differentiate whether the closure of the wound was due to the effect of the drug or that cells took longer to grow. For this reason, photos of SN12C migration assay were taken up to 24h after treatment.

Remnants of the wound could still be observed in control condition after 24h (Fig. 19). However, untreated cells migrated faster than cells treated with HIT101 1µM and HIT102 1µM. SN12C sh91 treated with standard RF-1 inhibitors, IRF-1 100µM and PoC 100µM, migrated faster than HIT-treated cells, although they also avoided wound closure after 24h (Annexed Fig. 2). Differences between 1µM and 10µM were not clear, even though in all cases there was no closure of the wound (Annexed Fig. 2). In addition, HIT101 and HIT102 demonstrated a greater reduction of migration compared to genetic silencing of RF-1 induced by DOX.

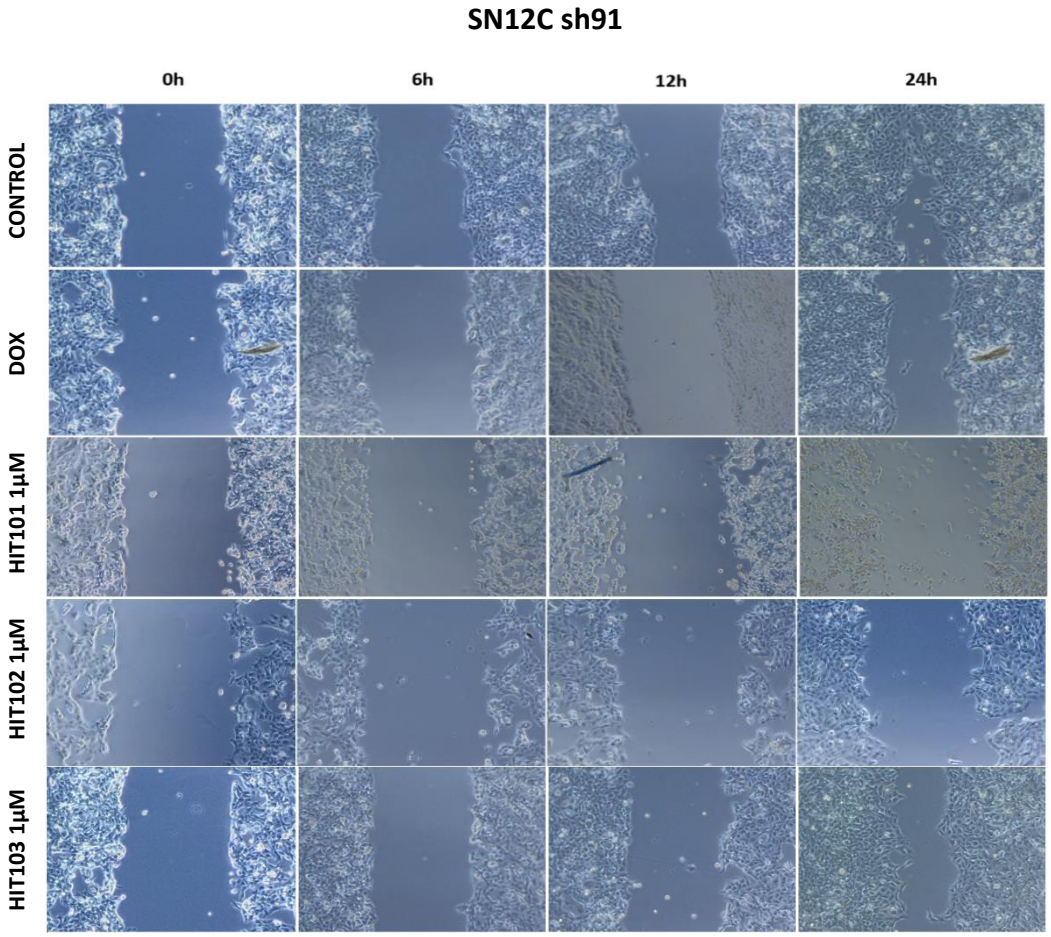

Figure 19. Representative images of SN12C sh91 migration assay of control, doxycycline (DOX), HIT101 1µM, HIT102 1µM and HIT103 1µM conditions. Images were taken at 10X every 6h, for a total of 24h with Leica DMi1 inverted microscope (Leica Microsystems, Germany).

Quantification of HITs effect *in vitro* in 786-O and SN12C tumor cell migration rate

Quantification of cell movement showed that the chemical inhibitors, PoC and IRF-1 had a similar effect in both RCC cell lines, decreasing migration velocity compared to control and equalling the effect of RF-1 genetic inhibition with DOX (Fig. 20 and 21). However, new HIT compounds showed different effects depending on drug dose and cell type.

The greatest reduction in 786-O migration velocity occurred with HIT101 and HIT103 treatment at 1µM (Fig. 20). The effect of these drugs reduced by 76% the rate at which cells migrate (0.24 ± 0.04nm/h) compared to control group (1 ± 0nm/h) and decreased velocity more than RF-1 genetic inhibition with DOX (0.76 ± 0.19nm/h). Although a positive effect of all new HIT compounds was observed in proliferation assays, the same cannot be concluded for cell migration. The HIT102 component did not demonstrate a good inhibition of RF-1, since the migration rate only reduced up to 0.77 ± 0.22nm/h and 0.74 ± 0.03nm/h for 10µM and 1µM, respectively, compared to control (1 ± 0nm/h). Remarkably, higher doses of HIT101 did not demonstrate a greater inhibitory effect on cell migration. More experiments are needed in order to increase the consistency of these results.

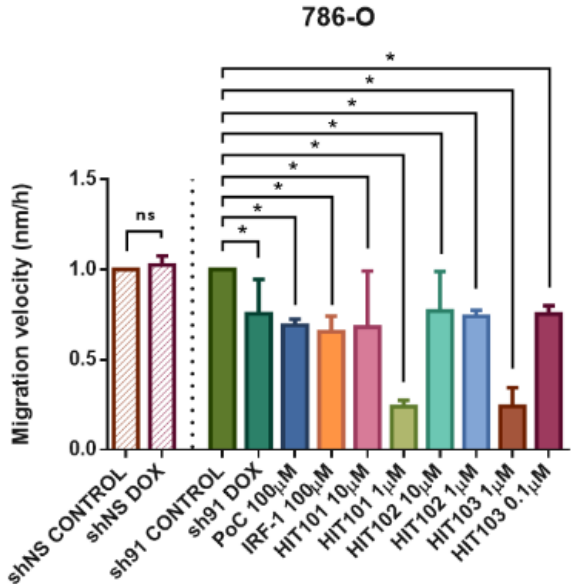

*Figure 20. Quantification of migration velocity (nm/h) of 786-O sh91 and shNS cells after treatment with DOX, PoC 100µM, IRF-1 100µM, HIT101 10µM, HIT101 1µM, HIT102 10µM, HIT102 1µM, HIT103 1µM and HIT103 0.1µM. Data are presented as mean ± standard deviation (SD) represented in error bars. Data were analysed by Mann-Whitney non-parametric test and results for 786-O shNS showed no significance (ns), whereas for 786-O sh91 were statistically significant in all conditions (*p<0.05) (n = 3).*

Migration velocity of SN12C sh91 was significantly decreased by HIT102 1µM (0.41 ± 0.14nm/h) compared to control (1 ± 0nm/h) (Fig. 21). However, the same drug at a higher concentration (10µM), only reduced the migration rate by 7% (0.93 ± 0.04nm/h) compared to control. In addition, HIT101 1µM and HIT103 1µM, with the greatest effect on 786-O cells, did not produce the same result on SN12C. On one hand, HIT101 1µM could not be evaluated since cells were detached from the plate and it was not possible to determine their migration rate. On the other hand, HIT103 1µM reduced 38% of migration rate compared to control, whereas in 786-O cells it was able to decrease 76% of migration velocity.

In 786-O and SN12C shNS modified cells, no significant differences were observed between control and DOX treatment, proving that variations in cell migration were due to the inhibition of RF-1 (Fig. 20 and 21).

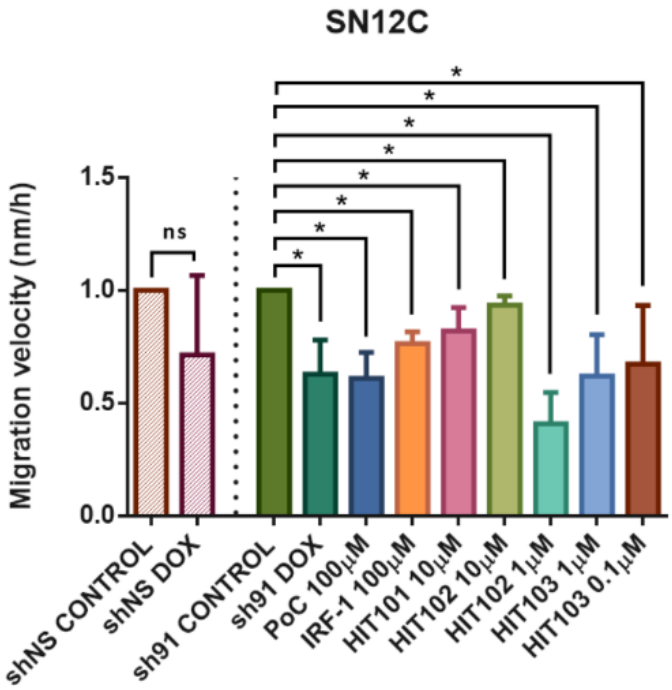

*Figure 21. Quantification of migration velocity (nm/h) of SN12C sh91 and shNS cells, after treatment with DOX, PoC 100µM, IRF-1 100µM, HIT101 10µM, HIT102 10µM, HIT102 1µM, HIT103 1µM and HIT103 0.1µM. Data are presented as mean ± standard deviation (SD) represented in error bars. Data were analysed by Mann-Whitney non-parametric test and results for SN12C shNS showed no significance (ns), whereas for SN12C sh91 were statistically significant in all conditions (*p<0.05) (n = 3).*

In vitro proliferation assays placed HIT103 as the best candidate for RF-1 inhibition, although some variations were observed in the migration rate. More experiments are required for consistent data in order to perform future *in vivo* studies.

Discussion

Vascular network plays a fundamental role in supplying oxygen and nutrients to the organism and removing catabolic waste and circulating cells for immune surveillance (De Falco, 2014). Consequently, angiogenesis alterations, such as inadequate vessel maintenance, disproportionate growth and abnormal remodelling are related to many diseases, including cancer (De Falco, 2014).

Inhibition of blood vessel growth became successful and led to the approval of anti-angiogenic therapies alone or in combination with other drugs for hepatocellular carcinoma and renal cell carcinoma, among other cancer types (Beijnum *et al.*, 2015). These therapies demonstrated clinical benefits by lengthening lifetime of patients with lung, renal, pancreatic neuroendocrine and ovarian cancer (Al-Husein *et al.*, 2012). However, anti-angiogenics failed to produce enduring clinical responses in most patients resulting in transitory improvements, as tumors were able to overcame anti-angiogenic therapy and start to regrew (Rini and Atkins, 2009; Jimenez-Valerio *et al.*, 2016).

Most of the resistance mechanisms to anti-angiogenic therapy are not genetic, which could explain why resistance can be reversible and transient. Up to five mechanisms have been described in order to escape anti-angiogenic treatment, which include growth factor redundancy, recruitment of bone marrow-derived cells, local stroma cells, vessel co-option and vascular mimicry (Rini and Atkins, 2009). In addition, a new mechanism of adaptive resistance involving a functional compartmentalization of energy metabolism in the tumor has been described (Jimenez-Valerio *et al.*, 2016).

In previous analysis from this group, an increased expression of pro-angiogenic factors from the VEGF-pathway was observed in response to anti-angiogenic therapy, as well as certain alternative molecules involved in different processes. One of the upregulated VEGF-alternative factors in resistant tumors was RF-1. Increased RNA and protein expression levels were observed compared to tumors that responded to therapy, pointing it out as a new alternative pro-angiogenic factor (Jiménez Valerio, 2013; Bassani, 2017).

RF-1 is described as an enzyme involved in nucleic acid homeostasis that has received increasing attention due to its overexpression in tumors and its participation in angiogenesis mechanisms, evasion from apoptosis, tumor invasion and metastasis (Jiménez Valerio, 2013). RF-1 also plays an important role in the development of tumor resistance to anti-angiogenic therapy. Anti-angiogenic resistant patients with ccRCC presented increased RF-1 expression levels after treatment (Jiménez Valerio, 2013). Indeed, patients with pancreatic, colon, gastric and renal cell cancer with high RF-1 expression levels had poorer prognoses than those without high RF-1 expression, pointing it out as a new therapeutic target for cancer research (Jiménez Valerio, 2013; Bassani, 2017).

The angiogenic activity of RF-1 has been confirmed with several assays and its inhibition with small molecules blocked blood vessel formation mechanisms (Jiménez Valerio, 2013). Thus, it has been described as a promising VEGFR-alternative target for treating tumor anti-angiogenic resistance.

The use of RF-1 chemical inhibitors as a second-line treatment induced the same effect as the standard anti-angiogenic therapies, such as DC101 or bevacizumab, decreasing tumor weight, volume and vascular density, and increasing tumor necrosis (Jiménez Valerio, 2013; Bassani, 2017). The effect of IRF-1 supressed the growth of RF-1-expressing tumors and inhibited metastasis of RF-1-expressing cancer cells *in vivo* (Jiménez Valerio, 2013; Bassani, 2017). However, the results obtained during the *in vivo* experiment in this project demonstrated a poor effect of IRF-1, as tumor progression did not significantly decreased after resistance. These facts were consistent with bad stability and rapid degradation of IRF-1 in the system described in the literature (Committee for Medicinal Products for Human Use, 2016).

In previous *in vitro* experiments from this group, IRF-1 did not show any effects in the proliferating rate of the renal carcinoma cell line, SN12C, compared to RF-1 genetic silencing and PoC drug (data not published). In consequence, a computational drug discovery analysis was performed in order to design a more effective and selective drug against the new therapeutic target. This group revealed that RF-1 presents two active sites, and that both should be inhibited in order to achieve greater affinity with the target, which translates into better inhibitory capacity.

A virtual screening of RF-1 was performed to study its binding interaction with different molecules. Results showed forty-eight structures that could be active against the target, and three of these HITs (HIT101, HIT102 and HIT103) were selected according to the binding affinity. HIT validation *in vitro* was performed through analysis of RCC tumor cell proliferation and migration. Their effect was compared to genetic inhibition of RF-1 through DOX-induced silencing RNA and to chemical RF-1 inhibitors previously tested in other studies, such as IRF-1 and PoC drug (Lu, Klein and Schwartz, 2009; Jiménez Valerio, 2013).

Evaluation of tumor response to RF-1 inhibition *in vivo* has demonstrated that the anti-VEGFR-2 antibody, DC101, has a limited therapeutic capacity (Fig. 9), confirming what has been reported in different publications (Loges, Schmidt and Carmeliet, 2010; Falcon *et al.*, 2016; Bassani, 2017). Second-line therapy with IRF-1 demonstrated a decrease in tumor progression, although major tumor stabilisation was observed with DOX-induced RF-1 genetic silencing.

Proliferation experiments of 786-O and SN12C ccRCC models *in vitro* (Fig. 15 and 16, respectively) showed a significant decrease with HIT compounds, demonstrating a better inhibitory capacity than small molecule RF-1 inhibitors, such as IRF-1 and PoC, and RF-1 genetic silencing with DOX treatment. It was recently reported that novel compounds also demonstrated a better RF-1 inhibitory effect compared to IRF-1 (Zhao *et al.*, 2018). In addition, other publications showed that PoC drug had modest effect on lung and pancreatic tumor cell growth *in vitro*, inhibiting 10% of proliferation at the highest concentration tested (100µM). However, in combination with other anti-angiogenic therapies, PoC was able to inhibit 80% of tumor growth (Lu, Klein and Schwartz, 2009). In this project, the novel compound HIT103 yielded the best results, decreasing 34% and 26.8% of 786-O and SN12C cell proliferation, respectively, hence it was interesting to study its effect through other experiments.

Migration assays have shown that 786-O and SN12C ccRCC cell models were affected differently by the studied treatments (Fig. 20 and 21, respectively). Migration rate of 786-O and SN12C cells was decreased discreetly with PoC drug compared to control, and it was similar to the genetic inhibition of RF-1 induced with DOX. The velocity of 786-O cells decreased with IRF-1 treatment, compared to PoC drug, while it increased in SN12C cells. Novel compounds demonstrated the greatest effectiveness on decreasing tumor cell migration. 786-O cells migrated slower with HIT101 1µM and HIT103 1µM treatment (Fig. 20), while SN12C cells showed a decreased migration rate with HIT102 1µM (Fig. 21). Differences could be due to diverse proliferating rates between cell lines, although more experiments are required to make data consistent.

Considering the *in vitro* proliferation experiments, HIT103 was the optimal candidate to treat tumor re-growth after anti-angiogenic therapies and to delay resistance acquisition in combination with VEGF-inhibiting drugs.

An *in vivo* validation of the results would be necessary to postulate that inhibitory capacity of HIT103 is stronger than RF-1 genetic silencing and other chemical inhibitors. In addition, an *in vivo* testing of HIT103 effects in tumor growth, metastasis and invasion should also be performed.

Looking ahead, this research group has also studied the role of RF-1 as a biomarker to select anti-angiogenic resistant patients. The determination of RF-1 protein levels in tissue or plasma could be associated to a better or worse response to anti-angiogenic treatment and could allow the selection of subgroups of patients to benefit the most from that treatment. In fact, this research group owns a patent in national phases of RF-1 as a patient biomarker. This approach would allow extending the use of new treatments in different tumor types.

Conclusions

Currently, more research groups are studying from different perspectives the way to treat cancer, since focusing on one approach is an overly simplistic view. RF-1 is emerging as one of the potential targets involved in the development of tumor resistance to anti-angiogenic therapies.

This study corroborated RF-1 inhibition as a good mechanism for stopping tumor regrowth after anti-angiogenic therapy in ccRCC-derived orthoxenograft mice model. However, it also evidenced the need to develop better inhibitors rather than IRF-1.

The novel therapeutic compounds, HIT101, HIT102 and HIT103 were presented as an alternative way to block tumor resistance to anti-angiogenic therapies through inhibition of RF-1.

Proliferation and migration of modified renal cell lines 786-O and SN12C were reduced when RF-1 was inhibited. Specifically, HIT103 yielded the best results in proliferation assays for both cell types, decreasing the percentage of cell growth. In migration assays, 786-O sh91 migrated slower with HIT101 1µM and HIT103 1µM treatment, while velocity of SN12C cells decreased the most with HIT102 1µM.

To sum up, HIT candidates inhibited more effectively RF-1 rather than standard anti-RF-1 drugs, such as IRF-1 or PoC, specially HIT103 in proliferation assays. However, further experiments are needed in order to obtain major number of data and confirm the effects of novel HIT compounds *in vivo* in tumor regression for renal cancer treatment.

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I would like to express my gratitude to Dr. Oriol Casanovas for his generosity and for allowing me to do an internship in his research group from IDIBELL. I would like to thank my supervisor Gabriela Jiménez for her patience and the confidence she has placed in me since the first day. I would also like to thank my academic tutor Maria Montserrat Poblet for her guidance during this project. Finally yet importantly, thank you to my parents and my brother for all their support and esteem, especially during the last five years of work.

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Self-evaluation

The realization of my bachelor final thesis at IDIBELL has given me the possibility to put into practice all the techniques and knowledge acquired during my degree.

Literature research and weekly seminars have allowed me to expand and understand more about the field of oncology, especially regarding anti-angiogenic therapies applied in renal cancer. I have also learned to use other informatic resources and softwares for data analysis and bibliography management tools.

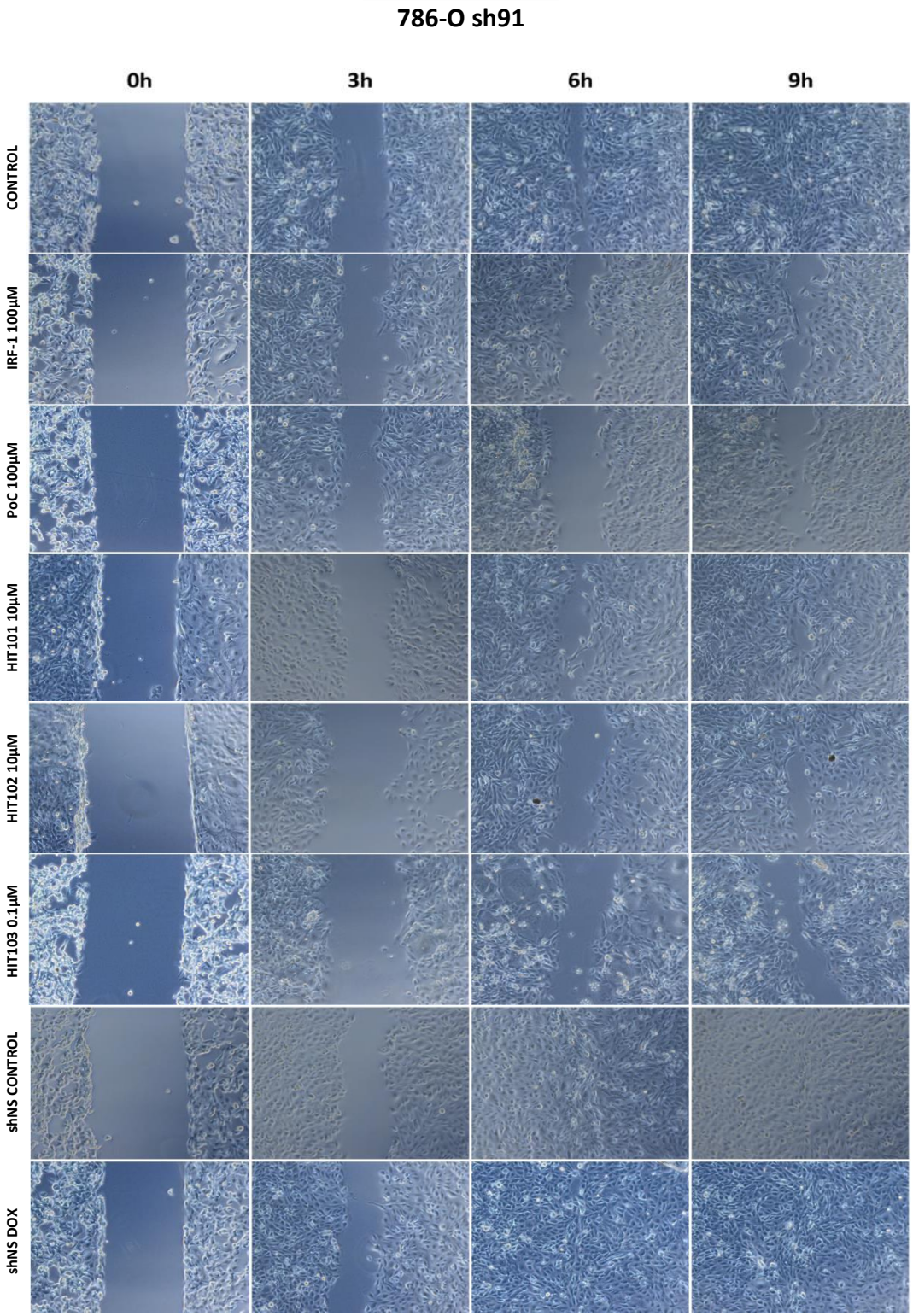
The daily work at the laboratory taught me how to plan and structure my experiments and to develop my technical skills in cell culture, proliferation and migration assays, as well as working with an *in vivo* mouse model. The 786-O-derived orthoxenograft mice has allowed me to see how tumor cells respond to the treatment in the organism of a mammal, which has made me realise the importance of animal models, especially when working with patient samples. I have also been acquainted with the scientific methodology and learned how to overcome obstacles when executing the experiments, such as problems with diluting new drugs or unexpected results.

Moreover, the writing of the thesis developed my critical thinking, as I was able to analyse and evaluate affirmations that were taken for granted and may not be reliable, such as the effect of the already commercialised IRF-1 in the *in vitro* analyses.

Finally, I would like to put in value all the learning received from the group leader, Dr. Oriol Casanovas and my professional tutor, Dr. Gabriela Alejandra Jiménez, who have advised me and gave me orientation during my stay in the laboratory, not only scientifically but also making my day-to-day much easier.

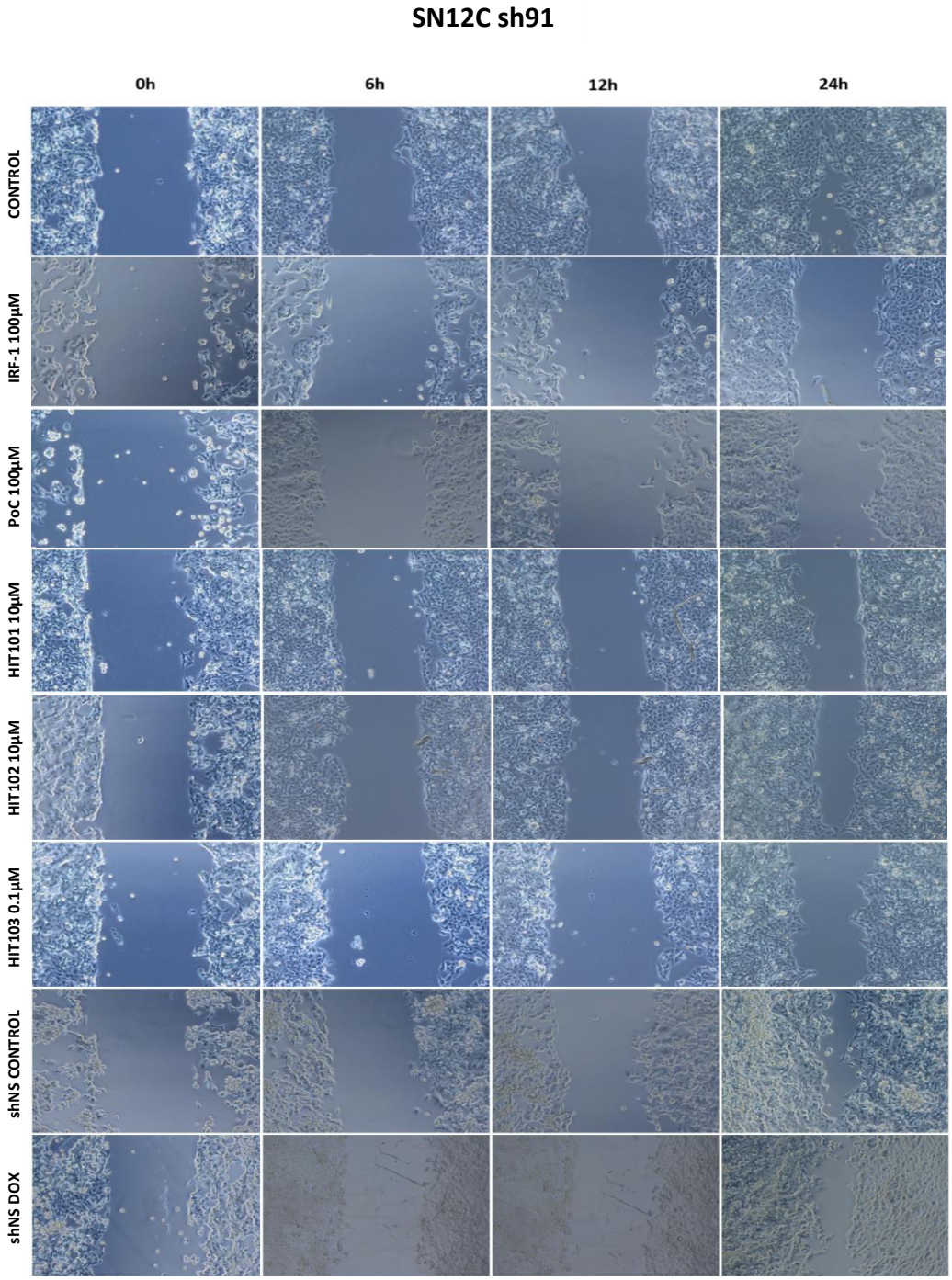
Annexes

Annex 1. 786-O migration assay



Annexed Figure 1. Images of 786-O sh91 migration assay of control, inhibitor of resistance factor 1 (IRF-1) 100µM, proof of concept drug (PoC) 100µM, HIT101 10µM, HIT102 10µM, HIT103 0.1µM, non-silencing sh control and non-silencing sh DOX conditions. Images were taken at 10X every 3h, for a total of 9h with Leica DMi1 inverted microscope (Leica Microsystems, Germany).

Annex 2. SN12C migration assay



Annexed Figure 2. Images of SN12C sh91 migration assay of control, inhibitor of resistance factor 1 (IRF-1) 100µM, proof of concept drug (PoC) 100µM, HIT101 10µM, HIT102 10µM, HIT103 0.1µM, non-silencing sh control and non-silencing sh DOX conditions. Images were taken at 10X every 6h, for a total of 24h with Leica DMi1 inverted microscope (Leica Microsystems, Germany).