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CHARACTERIZATION OF THE ANTICANCER PROPERTIES OF RUTHENIUM-DERIVED COMPOUNDS: MODE OF ACTION, OPTIMIZATION AND DEVELOPMENT OF EXPERIMENTAL TOOLS

Direttore della Scuola: **Ch.mo Prof. Pietro Giusti**
Coordinatore dell'indirizzo: **Ch.mo Prof. Pietro Palatini**
Supervisore: **Ch.mo Prof. Gianni Sava**

Directrice de l'Ecole: **Prof. Chaterine Florentz**
Directeur de thèse: **Dr. Christian Gaiddon**

Dottoranda/ Doctorante: **Vania Vidimar**

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Abstract

Platinum-based compounds are widely used anticancer drugs, despite severe side effects and drug-resistance phenomena. Over the past few years, a new class of platinum-free metal-based compounds, called RDCs (ruthenium-derived compounds), has been introduced with the aim of overcoming these issues. RDCs, in particular RDC11, have shown interesting and peculiar biological properties: a good anticancer activity *in vivo*, a reduced toxicity on healthy tissues and the ability to induce apoptosis through the induction of the CHOP/DDIT3 protein via a DNA-independent mechanism. Indeed, in contrast to platinum-based drugs, it has been suggested that ruthenium derivatives could exert their cytotoxicity independently from DNA interaction and through direct modulation of the activity of redox enzymes. In our study, we have compared the relative activity of platinum (cisplatin) and ruthenium (RDC11) derivatives on the HIF-1 and mTOR pathways, two pathways that are sensitive to the cellular metabolism. We showed that, unlike cisplatin, RDC11 was able to decrease HIF-1α and HIF-1β protein levels in normoxic and hypoxic conditions, leading to the reduction of the expression of HIF-1 target genes, such as *VEGF* and *GLUT1*. We have demonstrated that HIF-1α protein levels downregulation involves a complex mechanism associating changes in HIF-1α protein stability, *HIF1α* mRNA translation and synthesis. As mTOR controls HIF-1α translation, we analyzed the regulation of this pathway. We showed that, in contrast to cisplatin, RDC11 reduced the phosphorylation of the ribosomal protein S6 and Akt on specific sites that are markers for the activity of the mTORC1 and the mTORC2 complexes of mTOR. This observation correlates with a reduction in mRNA levels of RICTOR and RAPTOR, two components of mTOR. Finally, we showed that the inhibitory effect of RDC11 on the HIF-1 and mTOR pathways is consistent with its ability to reduce angiogenesis and potentiate the antitumor activity of the mTOR inhibitor rapamycin *in vivo*. Altogether, our results showed that ruthenium-derived compounds strongly impact metabolic pathways.

In parallel with the identification of RDCs direct targets, a structure/activity analysis to ameliorate the chemical and pharmacological features of RDCs, has been started. We have demonstrated that by changing ligands around the ruthenium center, it is possible to modulate several parameters, such as the redox value and the lipophilic/hydrophilic status, which might influence the ability of RDCs to enter the cells, to interact with intracellular targets and to alter their functions, as well as to modify their pharmacokinetic and distribution properties into the tissues. We have shown that the optimized RDCs reduce tumor growth in different mouse models and that they are more potent inducers of cancer cell death through the production of reactive oxygen species and activation of caspase 8, while retaining their ability to induce CHOP/DDIT3.

The fact that RDC11 was able to target molecular pathways (such as HIF-1α and Akt) directly

involved in the development of the metastatic process, led us to wonder whether it could exert an antimetastatic effect. The study of the antimetastatic effects of RDCs prompted us to undertake a further and separate study aimed to set up a model suitable for *in vitro* tests on chemicals endowed with the capacity to selectively target tumor metastases than being un-selective cytotoxics. We have set up the cell culture conditions suitable to recreate *in vitro* the metastatization of colorectal cancer cells towards the liver, the preferential site of metastatic colorectal carcinoma, in a model of bioreactor called "plastic mouse". We have demonstrated that the three different cell lines selected for our study can growth in the same environment, without undergoing modifications in viability and morphology, thus representing a good model for our purpose.

In conclusion, the results obtained during my PhD thesis have allowed us to identify a novel mechanism of action of RDC11, which is different from that of classical metal-based drugs, pointing out that platinum and ruthenium-based molecules can act differently, even if the latter was initially designed to mimic cisplatin. The second study has demonstrated the importance that the modifications of the ligands around the ruthenium center play in modulating the cytotoxicity and selectivity of the new generated RDCs towards different cancer types. This can be explained by their ability to interfere with different pathways crucial for cancer metabolism. Finally, we have made a breakthrough in developing an experimental tool to study the metastatic process *in vitro*. The plastic mouse will be useful in the future to screen potential antimetastatic molecules.

Riassunto

I derivati del platino sono ampiamente utilizzati come farmaci antitumorali, nonostante i gravi effetti collaterali e i fenomeni di farmaco-resistenza. Nel corso degli ultimi anni, una nuova classe di composti contenenti un metallo diverso dal platino, chiamati RDCs (ruthenium-derived compounds), è stata introdotta con lo scopo di superare queste limitazioni. Gli RDCs, e in particolare RDC11, hanno mostrato interessanti e peculiari proprietà biologiche: una buona attività antitumorale *in vivo*, una ridotta tossicità sui tessuti sani e la capacità di indurre l'apoptosi attraverso un meccanismo DNA-indipendente che implica l'induzione della via di segnalazione di CHOP/DDIT3. Contrariamente ai farmaci a base di platino, è stato suggerito che i derivati di rutenio possano esercitare la loro citotossicità indipendentemente dall'interazione con il DNA e attraverso la modulazione diretta dell'attività di enzimi ossido-riduttivi. In questo studio, abbiamo confrontato l'attività dei derivati del platino (cisplatino) e rutenio (RDC11) sulle vie di segnalazione di HIF-1 e mTOR, due vie sensibili al metabolismo cellulare. Abbiamo dimostrato come, a differenza del cisplatino, RDC11 sia in grado di diminuire i livelli di espressione delle proteine HIF-1α e HIF-1β in condizioni di normossia e ipossia, portando alla riduzione dell'espressione dei geni bersaglio di HIF-1α, come *VEGF* e *GLUT1*. Abbiamo dimostrato che la down-regulation dei livelli proteici di HIF-1α implica un complesso meccanismo che associa le variazioni della stabilità proteica di HIF-1α con la traduzione dell'mRNA di HIF-1α e la sua sintesi. Dal momento che mTOR controlla la traduzione di HIF-1α, abbiamo analizzato la regolazione di questa via da parte di RDC11. Abbiamo dimostrato che, diversamente dal cisplatino, RDC11 riduce la fosforilazione della proteina ribosomiale S6 e Akt su siti specifici che sono i marcatori per l'attività dei complessi mTORC1 e mTORC2 di mTOR. Questa osservazione è correlata con una riduzione dei livelli di mRNA di RICTOR e RAPTOR, due componenti di mTOR. Infine, abbiamo dimostrato come l'effetto inibitorio di RDC11 sulle vie di HIF-1 e mTOR sia coerente con la sua capacità di ridurre l'angiogenesi e di potenziare l'attività antitumorale della rapamicina, inibitore di mTOR, *in vivo*. Complessivamente, i nostri risultati hanno dimostrato che i derivati del rutenio hanno un forte impatto su diverse vie metaboliche.

In parallelo all'individuazione dei target diretti degli RDCs, è stato avviata un'analisi struttura/attività con lo scopo di migliorare le caratteristiche chimiche e farmacologiche di questi composti. Abbiamo dimostrato come, variando i ligandi attorno all'atomo di rutenio, sia possibile modulare diversi parametri, come il valore redox e lo stato di lipofilicità/idrofilicità, si possa influenzare la capacità degli RDCs di entrare nelle cellule, di interagire con i bersagli intracellulari e di alterare le loro funzioni, così come di modificare le loro proprietà farmacocinetiche e la distribuzione nei tessuti. Abbiamo dimostrato che gli RDCs ottimizzati riducono la crescita

tumorale in diversi modelli murini e che sono più potenti induttori del processo apoptotico nelle cellule tumorali attraverso la produzione di specie reattive dell'ossigeno e l'attivazione della caspasi 8, pur mantenendo la loro capacità di indurre la via di CHOP/DDIT3.

Il fatto che RDC11 agisca su vie di segnalazione cellulare (come quella di HIF-1α e Akt) direttamente coinvolte nella regolazione del processo metastatico, ci ha indotti a pensare ad un suo possibile effetto su tale processo. Per tale ragione, abbiamo intrapreso uno studio separato al fine di definire un modello cellulare *in vitro* per testare nuove molecole potenzialmente capaci di bersagliare in maniera selettiva le metastasi tumorali, piuttosto che essere dei composti citotossici non selettivi. Abbiamo creato le condizioni di coltura cellulare ideali per riprodurre *in vitro* la metastatizzazione di cellule tumorali colorettali verso il fegato, il sito preferenziale del carcinoma colorettale metastatico, in un prototipo di bioreattore chiamato "plastic mouse". Abbiamo dimostrato che le tre diverse linee cellulari selezionate per il nostro studio sono in grado di crescere nel medesimo ambiente, senza subire modifiche della vitalità e morfologia, rappresentando così un buon modello per il nostro obiettivo.

In conclusione, i risultati ottenuti durante la mia tesi di dottorato ci hanno permesso di identificare un nuovo meccanismo d'azione di RDC11, diverso da quello dei classici farmaci contenenti un metallo, sottolineando che i derivati del platino e del rutenio possano agire diversamente, anche se questi ultimi sono stati inizialmente progettati per imitare il cisplatino. Il secondo studio ha dimostrato l'importanza che le modificazioni dei ligandi attorno all’atomo di rutenio svolgono nel modulare la citotossicità e la selettività dei nuovi RDCs verso diversi tipi di tumori. Questo può essere spiegato grazie alla loro capacità di interferire con diverse vie di segnalazione, cruciali per il metabolismo delle cellule tumorali. Infine, abbiamo compiuto un passo avanti nello sviluppo di uno prototipo sperimentale per studiare il processo metastatico *in vitro*. Il plastic mouse risulterà utile in futuro per lo screening di potenziali farmaci antimetastatici.

Résumé

Les composés à base du platine sont largement utilisés pour le traitement du cancer, malgré les effets secondaires graves et les phénomènes de résistance. Au cours des dernières années, une nouvelle classe de composés contenants un métal différent du platine, appelés RDCs (ruthenium-derived compounds), a été développé pour éliminer ces limitations. Les RDCs, en particulier le RDC11, ont montré des propriétés biologiques intéressantes et particulières: une bonne activité anticancéreuse *in vivo*, une toxicité réduite sur les tissus sains et la capacité d'induire l'apoptose par l'induction de la voie CHOP/DDIT3 à travers un mécanisme ADN-indépendant. Contrairement aux médicaments à base de platine, il a été suggéré que les dérivés du ruthénium pourraient exercer leur cytotoxicité indépendamment de l'interaction avec l'ADN et par la modulation directe de l'activité des enzymes redox. Dans notre étude, nous avons comparé l'activité relative des dérivés du platine (cisplatine) et du ruthénium (RDC11) sur les voies de HIF-1 et de la protéine mTOR, deux voies qui sont sensibles au métabolisme cellulaire. Nous avons montré que, contrairement au cisplatine, le RDC11 était capable de diminuer les niveaux des protéines HIF-1α et HIF-1β dans les conditions de normoxie et hypoxie, en conduisant à la réduction de l'expression des gènes cibles de HIF-1, comme les gènes *VEGF* et *GLUT1*. Nous avons démontré que la baisse des niveaux de la protéine HIF-1α implique un mécanisme complexe associant des changements dans la stabilité de la protéine, la traduction de l'ARNm de HIF-1α et de sa synthèse. Comme le complexe mTOR contrôle la traduction de HIF-1α, nous avons analysé la régulation de cette voie. Nous avons montré que, contrairement au cisplatine, le RDC11 réduit la phosphorylation de la protéine ribosomique S6 et Akt sur des sites spécifiques qui sont des marqueurs de l'activité des complexes mTORC1 et mTORC2 de mTOR. Cette observation est corrélée avec une réduction des niveaux d'ARNm de RICTOR et RAPTOR, deux composantes du complexe mTOR. Enfin, nous avons montré que l'effet inhibiteur de RDC11 sur les voies de HIF-1 et mTOR est compatible avec sa capacité à réduire l'angiogenèse et de potentialiser l'activité anticancéreuse de l'inhibiteur de mTOR, la rapamycine, *in vivo*. Ces résultats ont montré que le RDCs impactent fortement les voies métaboliques.

En parallèle avec l'identification des cibles directes des RDCs, une analyse structure/activité a été commencée pour améliorer les propriétés chimiques et les caractéristiques pharmacologiques des RDCs. Nous avons démontré que, en changeant les ligands autour du centre du ruthénium, il est possible de moduler plusieurs paramètres, tels que la valeur redox et l'état de lipophilicité/hydrophilicité, qui pourrait influencer la capacité des RDCs de pénétrer dans les cellules, d'interagir avec des cibles intracellulaires et de modifier leurs fonctions, ainsi que de modifier leurs propriétés pharmacocinétiques et la distribution dans les tissus. Nous avons

montré que les RDCs optimisés réduisent la croissance tumorale dans des modèles murins différents et qu'ils sont des inducteurs plus puissants de la mort des cellules cancéreuses à travers la production d'espèces réactives de l'oxygène et l'activation de la caspase 8, tout en conservant leur capacité à induire CHOP/DDIT3.

Le fait que le RDC11 s'est démontré capable de cibler directement des voies moléculaires (tels que HIF-1α et Akt) impliqués dans le développement du processus métastatique, nous a conduit à nous demander s'il pourrait exercer un effet anti-métastatique. L'étude des effets anti-métastatique des RDCs nous a incité à entreprendre une étude séparée pour mettre en place un modèle approprié pour tester *in vitro* des produits chimiques dotés de la capacité de cibler sélectivement les métastases tumorales plutôt que d'être des cytotoxiques non-sélectifs. Nous avons mis en place les conditions de culture cellulaire appropriée pour recréer *in vitro* la métastatisation des cellules cancéreuses du colon vers le foie, le site préférentiel de cancer colorectal métastatique, dans un modèle de bioréacteur appelé "plastic mouse". Nous avons démontré que les trois différentes lignées cellulaires choisies pour notre étude peuvent pousser dans le même environnement, sans subir des modifications de la viabilité et de la morphologie, en représentent un bon modèle pour notre étude.

En conclusion, les résultats obtenus au cours de ma thèse de doctorat nous ont permis d'identifier un nouveau mécanisme d'action du RDC11, différent de celui des médicaments classiques à base de platine, en soulignant que les molécules à base de platine et de ruthénium peut agir différemment, même si ces derniers ont été initialement dessinés pour imiter le cisplatine. La seconde étude a démontré l'importance que les modifications des ligands autour du centre du ruthénium des RDCs optimisés ont dans la modulation de la cytotoxicité et de la sélectivité vers différents types de cancers. Ceci peut être expliqué par leur capacité d'interférer avec les différentes voies cruciales pour le métabolisme du cancer. Enfin, nous avons fait une percée dans le développement d'un outil expérimental pour étudier le processus métastatique *in vitro*. Le plastic mouse sera outil dans l'avenir pour tester des molécules anti-métastatiques potentielles.

1 | Introduction

According to the World Health Organization, cancer is a leading cause of death in industrialized countries and accounted for 7.6 millions of deaths (around 13% of all deaths) in 2008. Long term prognoses made by Globocan 2008, an international cancer statistics database, estimates that the deaths from cancer are projected to reach over 11 millions in 2030 [http://globocan.iarc.fr].

To date, different approaches have been developed to battle cancer. Surgery is often the first approach used to treat tumors. It offers the greatest chance for cure, especially if cancer has not spread to other sites of the body. To make sure that any remaining cancer cells are killed, surgery is often associated with radiotherapy (if the tumor has grown in one place) and/or with chemotherapy, when the tumor has spread to other sites of the body [O'Connell MJ et al, 1994; Recht A et al, 1996].

Chemotherapy, in particular, is one of the most widely used treatments for cancers of all types. It consists of using pharmacologically active molecules with the property of reducing cell proliferation and/or to induce cell death. In contrast to radiation, that is focused on a single part of the body, chemotherapy, is intended to reach any part of the body. This means that if cancer has spread or if it is not completely removed after surgery, chemotherapy will still reach and hopefully kill it. Even when chemotherapy cannot cure the disease, it can show palliation and help people to live longer than expected for that cancer type.

1.1 | General classification of anticancer chemotherapeutic drugs

Classically, chemotherapeutic drugs are grouped into four main classes according to their mode of action, that is sex hormones, immunostimulants, cytotoxic agents and targeted therapy drugs. Each class is discussed briefly below.

Sex hormones: certain sex hormones, or hormone-like drugs, inhibit tumors whose growth and development are dependent on the presence or on the absence of hormones. In particular, they prevent a cell from using a hormone as a growth factor or the body from making a hormone. They are used primarily for treating cancers developed in hormone-regulated organs, such as breast, prostate, uterus and kidneys.

Immunostimulants: they act by stimulating the body's own immune system to destroy cancer cells, facilitating their removal and eliminating healthy cells damaged by treatments such as surgery, radiotherapy or chemotherapy.

Cytotoxic agents: they inhibit the proliferation of cancer cells, usually by interfering directly or indirectly with DNA replication. Among this class, platinum-based drugs are the most widely used chemotherapeutics and represent the reference for treating many cancers. Despite the obvious benefits given by these agents, they have two main drawbacks that should be considered: toxicity and drug-resistance. Cytotoxic agents cause several toxicities because they usually hit rapidly dividing cells of both cancer and healthy tissues, resulting in an indiscriminate and non-specific therapy. Damage to healthy cells leads to the common side effects of immune suppression, nausea, and hair loss. Moreover, cancer cells often develop resistance to cytotoxic agents, rendering ineffective their use. For this reason, it is a common practice to administer these drugs in combination with other molecules. Such combinations are generally more effective than single drugs.

Targeted therapy drugs: in recent years, much interest has been paid to targeted cancer therapies (such as monoclonal antibodies, inhibitors of specific enzymes or growth factors or their receptors), which promises a solution to the limitations mentioned above. These therapies aim to employ drugs able to impede tumor development and growth, destroying specific molecules that are identified as essential to a tumor, but not to healthy cells. However, also targeted therapies have some limitations, such as the cost of development, toxicity, a narrow spectrum of activity and the potential for cells to develop resistance to them.

In light of these evidences, it is therefore necessary to improve current therapeutic approaches in order to increase treatment effectiveness on therapy-resistant tumors and to reduce "off-target" cytotoxicity, thus ameliorating the quality of life of the patient.

Moreover, a distinction has to be made between primary tumors and metastases, which are the main cause of death in solid tumors. While the most available treatments target primary tumors, only few therapeutic protocols are effective in preventing secondary tumors formation and development. Therefore, many efforts are still required to find effective treatments for metastatic diseases.

1.2 | Metal-based compounds in chemotherapeutical medicine

1.2.1 | Platinum derivatives

Medicinal chemistry is showing growing interest in the development of metal-based drugs which offer a much more diverse chemistry and important therapeutic applications that cannot be realized by organic agents. [Ott I and Gust R, 2007; Hannon MJ, 2007]. The field of metal-based anticancer drugs has started with cisplatin *[cis-diamminedichloroplatinum(II)]*, one of the leading agents in clinical use. Indeed, since the accidental discovery of its biological properties over 40 years ago [Rosemberg B et al, 1967], cisplatin has especially proven its efficiency in the treatment of testicular cancer, which was almost uniformly fatal in the pre-cisplatin era [Einhorn LH and Donohue J, 1977; Ozols RF and Williams SD, 1989]. Furthermore, cisplatin is also a component of standard treatment regimens for ovarian, bladder, cervical, head and neck and small-cell (SCLC) and non-small cell lung cancers (NSCLC) [Loehrer PJ and Einhorn LH, 1984; Ozols RF, 1992].

The efficacy and importance of cisplatin is emphasized by the fact that there is a growing number of combination chemotherapy regimens that today contain cisplatin or another platinum-based anticancer drug. It is estimated that as many as 50–70% of cancer patients are treated with a platinum derivative [Dyson PJ and Sava G, 2006].

Beside cisplatin, three other structurally related platinum drugs have also entered clinical use: carboplatin *[cis-diammine(1,1-cyclobutanedicarboxylato)platinum(II)]*, nedaplatin *[cis-diammine(glycolato)platinum(II)]*, and oxaliplatin *[(1R,2R)-cyclohexane-1,2-diamine](ethanedioato-O,O')platinum(II)]* [Galanski M et al, 2005] (Figure 1.2).

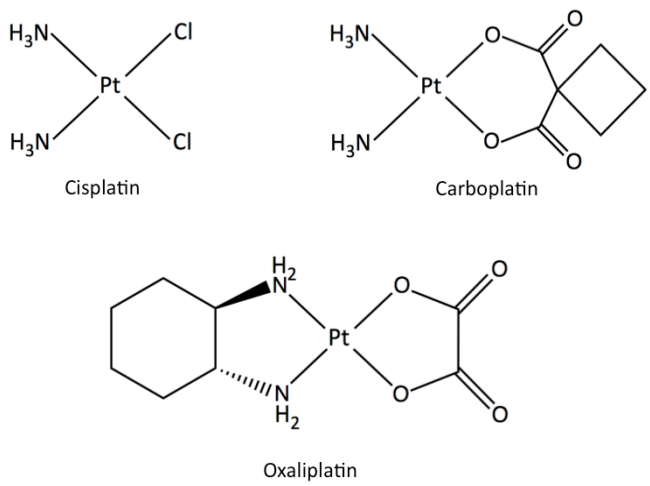


Figure 1.2 | **Structures of different platinum complexes**

In the past decade many researchers have studied the mode of action of cisplatin and it is now known that it acts by binding covalently to DNA, forming DNA adducts after intracellular activation by the aquation of the two chloride leaving groups. In particular, the principal adducts formed between cisplatin and double-stranded DNA are 1,2-intrastrand crosslinks of adjacent guanines with platinum binding to the N7 positions of the bases [Bernges F et Holler E, 1991; Burstyn JN et al, 2000]. These adducts cause distortions in DNA, leading to unwinding and bending, and activating various signal-transduction pathways, including those involving ATR, p53, p73, and MAPK, that lead to apoptotic cell death [Siddik ZH, 2003].

Although cisplatin and its derivatives are considered the most successful anticancer drugs, they are not without limitations, showing a high general toxicity to the kidneys and to the nervous, gastrointestinal and hematological system [Knox RJ, 1987]. Moreover, some tumors cannot be treated with platinum-based drugs. For example, glioblastoma and pancreatic cancers, two of the most aggressive tumors, are still refractory to cisplatin treatment [Wong E and Giandomenico CM, 1999]. Additionally, drug-resistance phenomena such as decreased drug accumulation, increased repair of platinum-DNA damage, alterations in proteins that recognize cisplatin-DNA damage and in pathways that determine sensitivity to apoptosis, lower the impact of these agents [Perez RP, 1998].

Similarly to all traditional anticancer drugs that target DNA, cisplatin effectiveness takes advantage of the rapid cell cycle of malignant cells. A drawback of this is that rapidly dividing healthy cells are affected as well, causing cisplatin severe side-effects such as nephrotoxicity and neurotoxicity [Bruijnincx PC and Sadler PJ, 2008]. A second drawback of cisplatin is represented by mechanisms of acquired or intrinsic resistance, which impair its intracellular concentration due to decreased drug uptake, increased efflux [Andrews PA and Howell SB, 1990; Parker RJ, 1991] or increased inactivation by sulfhydryl molecules such as glutathione [Ishikawa T and Ali-Osman F, 1993]. Moreover, altered expression of regulatory proteins, such as p53, involved in signal transduction pathways that control the apoptotic machinery can also affect sensitivity to the drug [Perego P et al, 1996]

These unresolved disadvantages have prompted the researchers to look for less toxic and more effective platinum-free metal-based anticancer therapeutics.

1.2.2 | Ruthenium derivatives

In the search for new metal-containing drugs with a reduced toxicity and a broader spectrum of activity than platinum compounds, ruthenium derivatives have raised great interest, since they display a reduced toxicity, a different mechanism of action, cisplatin non-cross-resistance and a different spectrum of activity [Aird RE et al, 2002].

From a chemical point of view, ruthenium anticancer drugs can be classified into two main groups: (a) ruthenium coordination complexes and (b) ruthenium organometallic complexes.

Nomenclature:

(a) In ruthenium coordination complexes the atom of ruthenium is bounded to the surrounding array of ligands via coordinate bonds. These bonds, which are generally much weaker than ordinary covalent bonds, allow ligand substitution reactions with components of the environment in which they are dissolved [Morris RE et al, 2001]. Indeed, these compounds can incur in hydrolytic processes during which the leaving groups are substituted by water molecules [Sava G et al, 2002; Bacac M et al, 2004]. The atom of Ru(III) of these complexes can be reduced to Ru(II) under physiological environments, according to the "activation by reduction" mechanism proposed by Clarke (see point (b) in the next paragraph, *Physico-chemical advantages*).

(b) In organometallic complexes, on the contrary, the central atom of ruthenium is bound covalently to carbon atoms of the surrounding ligands, avoiding ligand substitution reactions. Moreover, in these complexes, the atom of ruthenium does not undergo reduction from Ru(III) to Ru(II) because the atom of ruthenium is already at the oxidation state +2. Therefore, the activation by reduction mechanism does not occur for these compounds [Novakova O et al, 2003]. Chemically, both platinum and ruthenium are transition metals that share some similar physical and chemical properties. Nevertheless, some physico-chemical features make ruthenium derivatives a well suitable alternative to platinum drugs, justifying their further development [Brabec V and Novakova O, 2006].

Physico-chemical advantages:

(a) the availability of additional coordination sites

(b) the changes in the oxidation state

(c) the ability to mimic iron in binding with certain biological molecules

(a) In ruthenium derivatives, the availability of additional coordination sites allows designing different and original structures. In square-planar geometry Pt(II) compounds, the atom of Pt(II) can bind no more than four ligands, while in octahedral Ru(II) and Ru(III) derivatives, the atom of Ru(II) or Ru(III) can be surrounded by up to six different ligands. This difference in structural geometry, square-planar for Pt(II) derivatives and octahedral for Ru(II) and Ru(III) derivatives, is likely responsible for the different mechanism of action of ruthenium derivatives from Pt(II) compounds [Brabec V and Novakova O, 2006].

(b) Transition metal complexes are among the best-known materials involved in electron transfer processes, which represent one of the primary regulation mechanisms in biology, usually catalyzed by redox enzymes [Ryabov AD et al, 2005]. The redox potential of a complex can be modified by varying the ligands around the central metal and exploited to improve the effectiveness of drugs in the clinic. In ruthenium-based molecules, the oxidation states Ru(II) and Ru(III) are all accessible under physiological conditions. Based on this knowledge, Michael Clarke proposed the hypothesis of the "activation by reduction" mechanism, suggesting that Ru(III) complexes may serve as prodrugs that are activated by reduction *in vivo* to Ru(II) species in order to coordinate more rapidly to biomolecules [Clarke MJ, 2002]. According to this hypothesis, the activation of Ru(III) complexes in the corresponding reactive Ru(II) species may occur because of the intrinsic properties of cancer cells in solid tumors. These cells are characterized by a low oxygen content (hypoxia) and consequently they depend more on glycolysis than on oxidative phosphorylation for the energy supply [Okunieff P et al, 1994]. In these conditions, because of the generation of an excess of lactic acid, the pH inside these cells is lower [Wike-Hooley JL et al, 1984] favoring the production of Ru(II) species selectively in cancer cells and not in healthy tissues.

Actually, despite this fascinating and easy to understand hypothesis, there are no convincing data confirming that this mechanism of action is necessary for the activity of ruthenium compounds [Bergamo A and Sava G, 2011]. On the contrary, it is well established that some ruthenium derivatives, due to their redox potential, act as mediators of electron transfer to or from oxidized or reduced active sites of redox enzymes, such as glucose

oxidase, horseradish peroxidase [Ryabov AD et al, 2005] and thioredoxin reductase [Casini A et al, 2008], affecting their activity. This property could allow these compounds to target redox enzymes crucial for tumor metabolism and cancer energetic sustainment.

(c) Ruthenium belongs to the family of iron (it is placed in the same column as iron in Mendeleev's table). This chemical feature may explain the ability of ruthenium to mimic iron in binding to many biomolecules, including serum transferrin and albumin, exploiting the mechanisms that the cell uses for the detoxification of iron in healthy tissues [Messori L et al, 2000]. In mammals, these two proteins solubilize and transport iron, thereby preventing its toxicity. It was hypothesized that since rapidly dividing cells, such as cancer cells, require considerable amounts of iron, they increase the number of transferrin receptors on their surface, sequestering, in this manner, more of the circulating metal-loaded transferrin [Clarke MJ, 2002; Allardyce CS et al, 2002]. Actually, it has been demonstrated that some ruthenium derivatives bind to transferrin in place of iron [Messori L et al, 2000], but there are still no strong evidences in favor of transferrin-mediated ruthenium transportation into the cells [Bergamo A and Sava G, 2011].

On the whole, these favorable properties have stimulated the development of ruthenium-based molecules for treating cancer as alternative to platinum drugs. It is generally accepted that the first generation of ruthenium compounds, synthesized for anticancer purposes, consists of a series of complexes that mimic platinum drugs and target DNA, similarly to cisplatin.

1.3 | Ruthenium versus platinum-based drugs: how much the interaction with DNA is responsible for their anticancer activity?

The initial rationale for the synthesis of ruthenium drugs was to get compounds able to interact with DNA causing cell death through different mechanisms than those of cisplatin, especially avoiding cell chemoresistance, broadening the spectrum of activity and lowering the general toxicity. It is well established that, due to the different structural geometry, ruthenium compounds bind differently to DNA than cisplatin, forming predominately inter-strand crosslinks as opposed to the intra-strand crosslinks induced by cisplatin [Fruhauf S and Zeller WJ, 1991; Gallori E et al, 2000]. Hereafter, the general mechanism of action of the most important anticancer ruthenium derivatives (coordination complexes and organometallic

compounds) is reported, with the aim of highlighting how much it depends on DNA interaction.

1.3.1 | Ruthenium(III) coordination complexes

1.3.1.a | Amine-chlorido derivatives

One of the earliest types of anticancer ruthenium derivatives to be developed was that of the amine-chlorido complexes, clearly inspired by cisplatin (Figure 1.3.1.a). Those complexes, with general formula $[Ru(NH_3)_{6-x}Cl_x]^{Y+}$, were expected to bind to DNA similarly to cisplatin, and indeed the first experiments performed with some of these complexes, such as *cis*-$[Ru(III)(NH_3)_4Cl_2]^+$ (Figure 1.3.1.a), fulfilled this expectation [Clarke MJ et al, 1980].

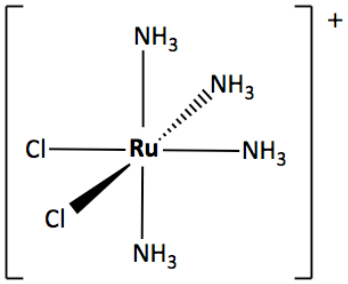


Figure 1.3.1.a | Structure of the amine- chlorido complex cis-$[Ru(III)(NH_3)_4Ru]Cl_2]^+$.

The amine-chlorido complexes are able to bind primarily to nitrogen sites on DNA bases, inhibiting DNA replication and inducing both mutagenic activity and the SOS repair mechanism [Yasbin RE et al, 1980]. They show a significant activity against leukemia models *in vivo* [Keppler BK et al, 1989]. However, because of their poor water solubility, they were not studied further on.

1.3.1.b | Dimethylsulfoxide Ru(III) complexes

Later, the next major class of compounds ideated and studied by Giovanni Mestroni and Gianni Sava at the University of Trieste and at the Callerio Foundation was that of the highly water-soluble Ru(III) dimethylsulfoxide complexes [Mestroni G et al, 1994]. It has been shown that NAMI-A [ImH][*trans*-$RuCl_4$(DMSO)Im] (Figure 1.3.1.b), the lead compound of this class, is able to form intrastrand adducts with DNA, but much fewer than cisplatin [Pluim D et al, 2004]. The condition that allows NAMI-A to bind DNA is probably the hydrolysis of the

chlorides and later of the DMSO ligand, which converts NAMI-A in a reactive aqua-complex, that subsequently coordinates to DNA [Bacac M et al, 2004]. Moreover, other studies have previously demonstrated that this compound formed very few DNA interstrand crosslinks [Novakova O et al, 2005]. These data suggested that NAMI-A binding to DNA was not the main reason of its biological activity.

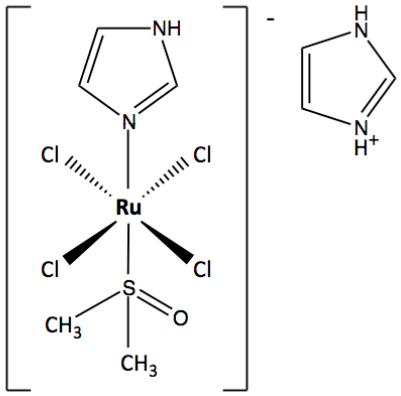
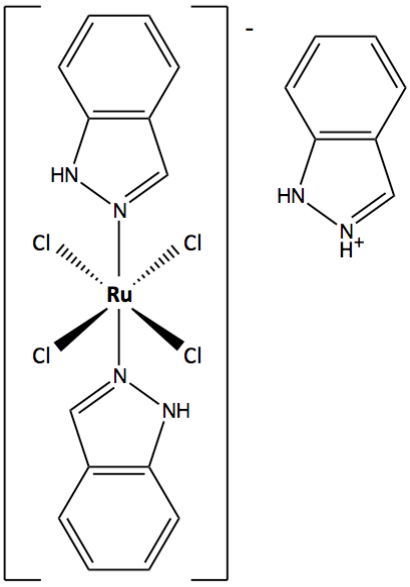


Figure 1.3.1.b │ Chemical structure of NAMI-A.

Generally, DNA damage correlates with high cytotoxicity (i.e. cisplatin). NAMI-A, in support of the previous data, does not modify *in vitro* the cell growth of different cancer cell lines when used in the micromolar range [Zorzet S et al, 2000]. Moreover, *in vivo* studies demonstrated that NAMI-A is free of direct effects on primary tumor growth [Bergamo A et al, 1999; Zorzet S et al, 2000] and it seems to stimulate fibrotic growth, with increased thickness of tumor capsule, cohesion among tumor cells and reduced vascular invasion of tumor mass [Sava G et al, 1998]. However, despite its lack of inhibition against primary tumor growth, NAMI-A is a potent antimetastatic agent [Sava G et Bergamo A, 2000; Sava G et al, 2003]. All these excellent results have made possible NAMI-A to enter clinical trials at the *Netherlands Cancer Institute* in Amsterdam where a group of adult patients with advanced malignancies received NAMI-A as an i.v. infusion. The drug was in general well tolerated. Main toxicities, possibly or probably related to study medication, were phlebitis, hypersensitivity reactions, and the formation of blisters. Renal toxicity was completely reversed few weeks after the end of drug administration [Rademaker-Lakhai JM et al, 2004]. At present, NAMI-A undergoes a clinical phase II trial on metastatic NSCLC. This is remarkably noteworthy because, although great improvements have been made in treating primary tumors (such as surgery, radiotherapy and also chemotherapy), secondary tumors (metastases) still represent a major challenge.

1.3.1.c | Chlorido-indazole Ru(III) complexes

Keppler and co-workers developed a Ru(III) chlorido-indazole complex, KP1019 *([IndH][trans-$RuCl_4(Ind)_2$])* (Figure 1.3.1.c) that showed a remarkable activity against cisplatin-resistant autochthonous colorectal tumors in rats, even if no pronounced antimetastatic activity was reported [Kapitza S et al, 2005].



Further studies demonstrated that the cytotoxic effect of KP1019 is mediated by oxidative stress. Indeed, KP1019 is not able to directly interact with DNA, but it causes DNA damage through the formation of reactive oxygen species, which react with DNA. The rate of induction of oxidative stress and DNA damage was found to be comparable to that of cisplatin. Moreover, KP1019 induces apoptosis through the mitochondrial pathway by downregulating bcl2, causing depolarization of mitochondria and activation of caspase 3 [Kapitza S et al, 2005; Heffeter P et al, 2010].

Figure 1.3.1.c | Chemical structure of KP1019.

KP1019 is also known to strongly bind to serum proteins, such as albumin and transferrin. This was considered to be important for the accumulation of the drugs into the tumor, likely through the transferrin pathway. This hypothesis was supported by the low toxicity of KP1019 observed in the clinical trial I concluded few years ago [Hartinger CG et al, 2006].

Moreover, the binding of KP1019 to serum proteins contrasts P-glycoprotein-mediated KP1019 efflux, making this ruthenium drug interesting for treatment of multidrug-resistant tumor types [Heffeter P et al, 2005].

KP1019, besides NAMI-A, is the second ruthenium derivate to undergo clinical I phase trials, together with its sodium salt KP1339 [Hartinger CG et al, 2006; Heffeter P et al, 2010].

If we take into account all the literature concerning NAMI-A and KP1019, which are the two reference compounds in the field of anticancer ruthenium-based drugs, it appears that these compounds seem to be active because of their interaction with molecular targets other than DNA.

On the one hand, NAMI-A modifies cell invasion and metastasis reducing tumor cell malignancy [Sava G et al, 2003], on the other hand KP1019 activates cellular signaling that leads to apoptosis through the mitochondrial pathway [Kapitza S et al, 2005].

1.3.2 | Ruthenium(II) organometallic compounds

The group of ruthenium-based anticancer drugs grew further on due to the development of organometallic complexes.

There are three main classes of well described ruthenium organometallic compounds in the literature: those developed by Peter Sadler [Dougan SJ et al 2006; Peacock AF and Sadler PJ, 2008], those by Paul Dyson [Ang WH et al, 2006; Vock CA et al, 2007] and more recently those by Michel Pfeffer [Gaiddon C et al, 2005; Meng X et al, 2009; Fetzer L et al, 2011]. One of the reasons for the development of these Ru(II) complexes has been the hypothesized mechanism of activation by reduction, which suggested that active and more stable Ru(II) species may be formed *in vivo* from Ru(III) precursors [Peacock AF and Sadler PJ, 2008].

The most numerous group of organometallic ruthenium compounds are the Ru(II) arene complexes, developed by Dyson, Sadler and co-workers. In these complexes, the presence of the arene unit stabilizes the metal at the +2 oxidation state. Ru(II) arene complexes show an half-sandwich, also-called “piano-stool” structure, in which the arene realizes the “piano” and the ligands (a monodentate leaving group and a chelating group) are the legs of the stool. This geometry, in particular the M-C bond and the ligands, allows the thermodynamic and kinetic reactivity of the metal to be controlled and also provide a scaffold for functionalization, optimizing in this manner the design of these compounds, both in term of biological activity and minimization of side-effects.

The presence of the arene plays a crucial role in the mechanism of action of these compounds because it works as an intercalator of DNA, allowing the distortion of the double strand [Liu HK et al, 2006]. Moreover, the type of leaving groups influences the rate of hydrolysis and cytotoxicity of the entire complex against tumor cells [Wang F et al, 2005 (a)]. Also the nature of the chelating ligand affects the reactivity of these complexes, influencing the rate of binding to DNA [Fernandez R et al, 2004].

1.3.2.a | Ru(II) arene ethylendiamine complexes

Among the organometallic Ru(II) arene synthetized by Sadler and co-workers, RM175 (Figure 1.3.2.a) *([η^6-biphenyl)Ru(II)-Cl(ethylendiamine)]PF_6)* showed interesting *in vitro* and *in vivo* activities. Special attention was attracted by the interaction of this compound with DNA nucleobases after aquation of the chloride.

Different studies, in fact, showed that it binds preferentially to N7 of guanine [Wang F et al, 2005 (b)]. RM175 interaction with DNA is confirmed also by other assays which show the ability of RM175 to interact with DNA in cell-free systems, allowing the formation of stable monofunctional adducts with a kinetic faster than that of cisplatin [Novakova O et al, 2003]. Interestingly, *in vivo* treatment with RM175 of both ovarian A2780 carcinoma and its cisplatin-resistant variant engrafted in nude mice showed a significant delay in cancer growth [Aird RE et al, 2002].

All the available data about RM175 suggest that, inside the cells, DNA may represent the favorite site of action.

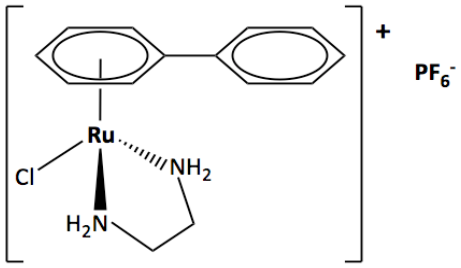


Figure 1.3.2.a | Chemical structure of RM175

1.3.2.b | Ru(II) arene PTA complexes

Later, on the basis of RM175 structure, Dyson and co-workers developed the family of RAPTAs compounds (Ruthenium Arene PTA, where PTA = 1,3,5-triaza-7-phosphoadamantane).

Differently from RM175, in RAPTA derivatives, the chelating ligand ethylendiamine is replaced with the PTA group in order to achieve a selective activation at low pH, a characteristic of hypoxic solid tumors. In this environment, in fact, the PTA unit undergoes protonation and the two chlorides are substituted with water molecules, thus allowing the compounds to interact with DNA [Scolaro C et al, 2005]. This process was demonstrated for

more than one RAPTA compound. It was found, in fact, that RAPTA-C and RAPTA-T exhibited pH-dependent DNA damage at low pH, whereas at the pH characteristic of healthy cells, little or no damage was observed [Allardyce CS et al, 2002; Scolaro C et al, 2005].

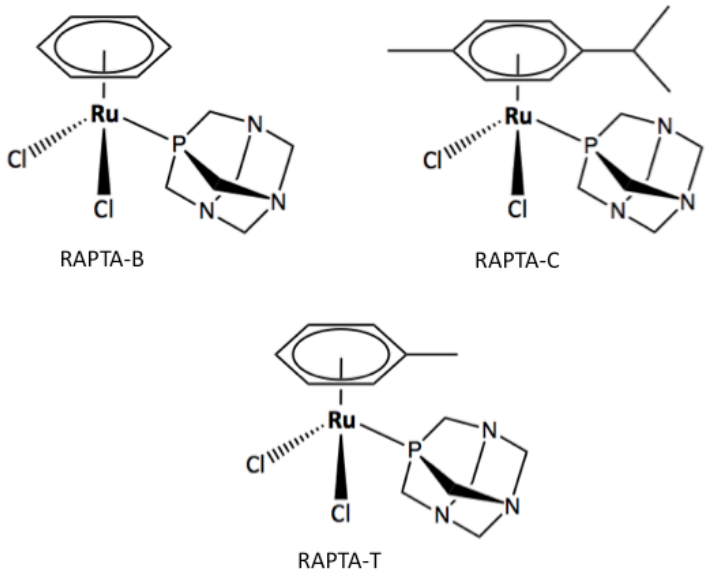


Figure 1.3.2.b | Chemical structure of RAPTA-B, RAPTA-C and RAPTA-T

In support of this, the protonation of the PTA ligand only at the pH typical of cancer cells, might explain the ability of RAPTA-T to be clearly more cytotoxic for the TS/A cancer cells than for the HBL-100 normal cells, suggesting that it may be selective toward cancer cells *in vivo*, possibly leading to low toxicity [Scolaro C et al, 2005]. Recently, Dyson and co-workers have also shown that RAPTA-T is able to interact selectively with transferrin in a cell-free system. This suggests a cooperative iron-mediated metal binding mechanism that may lead to the efficacy of the compound towards specific tumor types, reducing toxic side effects on healthy cells [Groessl M et al, 2010]. Moreover, in another recent study, RAPTA-T and RAPTA-C have showed interesting antiangiogenic activities, by inhibiting *in vitro* a number of endothelial cell (EC) functions essential in angiogenesis, such as proliferation, migration, and tube formation [Nowak-Sliwinska P et al, 2011].

The *in vivo* effects on tumor growth and metastases formation of RAPTAs were evaluated with RAPTA-B, RAPTA-C and RAPTA-T (Figure 1.3.2.b). RAPTA-B and -C are able to reduce lung metastases of MCa mammary carcinoma in CBA mice, mainly when administrated with fractionated treatment schedules without influencing the evolution of primary tumors [Scolaro C et al, 2005]. Also RAPTA-T showed similar effects in the same tumor model. These

results may be justified by the *in vitro* activity. It has been shown, in fact, that RAPTA-T is able to interfere with the detachment, adhesion and invasion of the highly invasive cells MDA-MB-231, without affecting these processes in the non-tumorigenic HBL-100 cells [Bergamo et al, 2008].

Although RM175 and analogs and RAPTAs share a similar structure and a similar mechanism to get the corresponding reactive species, they show opposite biological effects: while RM-complexes are quite good cytotoxics *in vitro*, mainly because of their interaction with DNA [Aird RE et al, 2002], RAPTAs are almost free of cytotoxicity. All these biological effects suggest that DNA is not the principle target responsible for RAPTAs activity.

1.3.3.c | Cycloruthenated compounds (Ruthenium(II)-derived compounds (RDCs)): a contribution of the laboratory in the field

Most of the anticancer ruthenium-containing compounds described in the literature have ligands that are relatively weakly bound to the metal via a heteroatom (N, O, S). When a ligand is bound to the metal via a coordination bond, it is very likely that, in an *in vivo* context, it can dissociate from the metal. In contrast to previous works, the group of Michel Pfeffer has recently synthesized several ruthenium-based complexes in which the ligand is bound to the metal via a strong covalent bond such as a C-M σ bond. This bond ensures the attachment of the ligand to the metal. Moreover, these stable ligands may additionally confer useful physical properties to the organometallic moiety (such as fluorescence), thus enabling the metal and ligand to be traced in the cells and *in vivo* [Leyva L et al, 2007].

In 2004, the laboratory of Michel Pfeffer (CNRS/UMR 7177) started a collaboration with Dr. Christian Gaiddon (INSERM U682) in order to characterize the anticancer potential of a pool of ruthenium-derived compounds (RDCs) with an innovative patented structure, mainly based on the C-M σ bond [Ruthenium Complexes For Treating Cancers, US 2008/0051370 A1, 2005]. This collaboration highlighted the cellular and molecular features of RDCs, which are the subject of this thesis work, providing interesting data that may contribute to improve future chemotherapeutic protocols.

Initially, some RDCs were synthesized in order to look like ruthenium compounds already known in the literature.

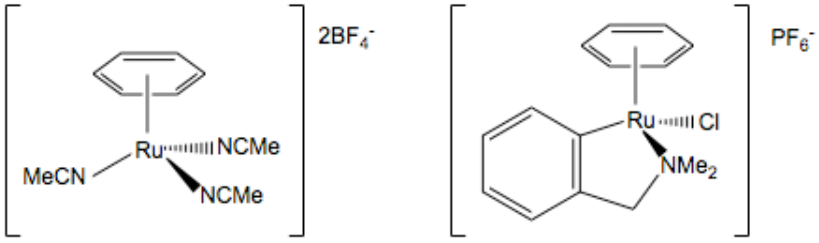


Figure 1.3.3.c (I) | Chemical structure of RDC2 (left) and RDC3 (right)

The ruthenium-derived compound RDC2 and RDC3, for example, were synthesized as a reference arene Ru(II) complexes (Figure 1.3.3.c (I)).

However, they did not display a remarkable cytotoxic activity. This lack of a success has led Pfeffer and co-workers to the idea that it was necessary to mimic, in some extend, cisplatin itself. Therefore, they reduced the number of coordination sites on the metal, obtaining compounds such as RDC9, RDC11 and RDC12. These compounds inhibited the proliferation of various cancer cell line (such as A172 glioblastoma cells, HCT116 colorectal adenocarcinoma cells, RDM4 linfoblastoma cells) with an IC_{50} (around 5 µM) ten times lower than that of RDC2 and RDC3. Moreover, RDC6, RDC9 and RDC11 showed the ability to arrest cells in G_1 phase and to induce apoptosis via the mitochondrial pathway through the activation of p53 and Bax. Interestingly, RDCs were found to be less sensitive to two resistant mechanisms developed by cancer cells. First, inactivation of p53 did not reduce the ability of RDCs to induce apoptosis. Second, overexpression of the ATP7B proteins, an event responsible for cisplatin resistance, scarcely affected RDCs biological activity [Gaiddon C et al, 2005].

Further studies have been focused on RDC11 (Figure 1.3.3.c (II)), one of the most active compounds of this new class of RDCs, with the aim to investigate the molecular mechanism involved in its biological activity.

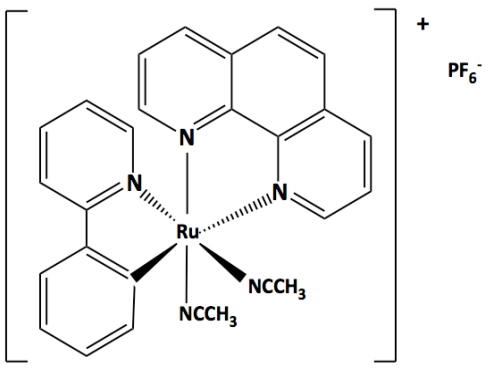


Figure 1.3.3.c (II) | Chemical structure of RDC11, the most active compound among RDCs

In vitro studies showed that RDC11 is able to reduce B16F10 mouse melanoma and U87 human glioblastoma cell number with an IC_{50} of 5 µM, similar to those of cisplatin [Meng X et al, 2009]. As previously shown for other RDCs [Gaiddon C et al, 2005], RDC11 favors the apparition of a sub-G1 fraction in cell cycle profiles, induction of nuclear condensation and activation of caspase-3, confirming the ability of RDC11 to induce apoptosis. Moreover, RDC11 is able to induce p53 protein levels and p53 target genes such as *p21*, *GADD45* and *PUMA* [Pietsch EC et al, 2008], even if it was shown that p53 induction was not necessary for RDC11 cytotoxicity. These controversial findings have prompted the researchers to verify whether RDC11 was able to interact with DNA inducing DNA damages.

Föster Resonance Energy Transfer (FRET) assays showed that the FRET efficiency (a decrease in FRET efficiency corresponds to a good ability of a compound to interact with oligonucleotides) decreases very rapidly with cisplatin (cisplatin/DNA ratio = 0.1/1), but not with RDC11 (RDC11/DNA ratio = 10/1). These data suggested that both cisplatin and RDC11 (used at the same concentration) induce a structural change of dsDNA, but the affinity of RDC11 for DNA is two orders of magnitude lower than that of cisplatin [Meng X et al, 2009].

Moreover, it has been shown that RDC11 binding to DNA is reversible and non covalent, with low association constants. The affinity constant and thermodynamic parameters obtained, led to the conclusion that RDC11 intercalates DNA base pairs, likely through the phenanthroline unit, which has the right dimension to be an intercalator group [Klajner M et al, 2010].

The weak affinity of RDC11 for DNA, together with the fact that p53 induction is not necessary for its cytotoxicity, suggested that other mechanisms might be involved. Indeed, Gaiddon and co-workers demonstrated that RDC11, unlike cisplatin, stimulate the activity of different components of the endoplasmic reticulum (ER) stress pathway. RDC11 induces the protein expression of CHOP (GADD153), which is a crucial mediator of the ER stress apoptosis, *CHOP* mRNA levels and also the mRNA of different *CHOP* target genes such as *Bip*, *XBP1* and *PDI*. In addition, it has been shown that the absence of p53 does not significantly affect the ability of RDC11 to induce CHOP, suggesting that the two mechanisms are activated in an independent manner. Silencing of CHOP significantly reduces the cytotoxicity of RDC11, while CHOP overexpression facilitates RDC11-induced cell death.

In vivo experiments with RDC11 were performed, providing interesting results. RDC11 is able to reduce the volume and weight of tumors in a model of syngenic B16F10 melanoma cells in C57BL/6 mice by 40% compared with the control. Its activity was better than cisplatin.

Similar results were obtained in a model of xenografted U87 glioblastoma cells in nude mice, after treatment with RDC11. This result was particularly encouraging and important, since that glioblastoma is still a disease with no available therapies. The *in vivo* assay provided important information about the toxicity of RDC11. After three weeks of treatment with RDC11 and cisplatin, the latter reduced body weight by about 25%, in contrast with RDC11, which did not show a significant effect. Moreover, the analysis of some markers of liver and kidney toxicity (such as uric acid, alpha-amilase, glucose, bicarbonate and iron) taken from the blood of the treated mice, showed that cisplatin, but not RDC11, is able to alter them. Electromyography was used to analyze the neurotoxicity of RDC11 on sensory nerves, recording their conduction in treated mice. While cisplatin significantly reduces the speed of conduction of sensory nerves, RDC11 affects it only partially [Meng X et al, 2009].

The lack of a strict correlation between RDC11 anticancer activity and its weak induction of DNA damage (if compared with cisplatin) suggested that other molecular targets, different from DNA, might be involved. These considerations also underlined that RDCs, and in particular RDC11, act with a mechanism of action different from that of cisplatin.

Moreover, a recent study by Pfeffer and co-workers demonstrated that almost all RDCs of second generation (RDCs of first generation are those described in the work of Leyva L et al, 2007) display good to very good cytotoxicities against HCT116 cells with IC_{50} in the nanomolar range, suggesting that the presence of a cycloruthenated unit confers to these compounds a good cytotoxic activity. Because it was not observed a direct structure-activity relationship, the redox potential and lipophilicities of RDCs were analyzed in order to establish a correlation with their *in vitro* activity. Results show that all RDCs having a low redox potential (in the range of 0.4-0.6V vs SCE: saturated calomel electrode, which is generally used as reference) and logP above 2.0 (logP measures how well a substance distributes between a lipid (oil) and water solvents; when logP>1 a substance is tendentially lipophilic) display the lowest IC_{50} values. On the contrary, the compounds having a hydrophilic behavior show the highest IC_{50} values (around 50 μM). Moreover, RDCs in which the C-M bond is absent were purposely synthesized to highlight the importance of the presence of this bond. Indeed, these compounds show only weak cytotoxicity. This study has demonstrated how the C-M bond, the lipophilicity and Ru III/II redox potential are essential in determining RDCs cytotoxicity [Fetzer L et al, 2011].

If no rational explanations were given about the exact role of the C-M bond in RDCs activity, a possible relation between redox potential and cytotoxicity of RDCs could be that

these compounds (as already shown for other cycloruthenated closely related to those described in Pfeffer's study [Ryabov AD et al, 2001]) alter somehow the behavior of certain redox enzymes (such as glucose oxidase and horseradish peroxidase) inside cells [Fetzer L et al, 2011].

This description of the mechanisms with which the main anticancer ruthenium compounds known in the literature act, provides enough information to answer to the initial question: *how much the interaction with DNA is responsible for their anticancer activity?*

Ruthenium compounds were thought to mimic platinum drugs and therefore to interact with DNA. Nevertheless, for neither RDCs nor the majority of the other ruthenium drugs described (with the exception of RM175) it is possible to establish a direct correlation between DNA interaction and their anticancer activity. Conversely, many of these compounds show mechanism of action that do not account for DNA damage (NAMI-A [Vacca A et al, 2002; Sava G et al, 2003], KP1019 [Kapitza S et al, 2005], RAPTA-T [Scolaro et al, 2005; Bergamo et al, 2008]) or only in a slight manner (RDC11 [Meng et al, 2009]).

Moreover, it is interesting to note that all these compounds have in common the central ruthenium core, but they do not share any similarity in terms of activity.

1.4 | No explicit targets different from DNA have been identified for ruthenium-based drugs yet: how to search for them?

Despite our increased knowledge on the mode of action of ruthenium drugs has notably increased, there are still no straightforward evidences of critical targets responsible for their anticancer activities. The main challenge, today, is to search for targets different from DNA, in order to get molecules more and more selective. In this respect, gene and protein expression microarray technologies has provided a fundamental contribution [Boccarelli A, Pannunzio Aa and Coluccia M, 2011; book].

The differential expression of genes, mRNAs and proteins between normal and malignant tissues allow to identify genes and pathways that are deregulated in a variety of human cancers [Michener CM et al, 2002]. Therefore, in the search for novel therapeutic targets for cancer treatment with ruthenium derivatives, genomics and proteomics can be taken into account. They might help researchers to obtain more information on gene interactions and protein interactions, in order to finally suggest new pathways to pursue and investigate [Slonim DK, 2002]. Protein synthesis and post-translational modifications are the most

important steps in the transition of a cell from a benign to a malignant phenotype [Michener CM et al, 2002], and therefore proteomics might provide more interesting results than genomics. Proteins might then represent an important target in order to develop novel and selective ruthenium-based drugs. The rationale is that, if these drugs are capable of interacting with a protein structure specifically owned by cancer cells, their selectivity will be strongly enhanced.

1.4.1 | Identification of RDC11 target genes

According to the literature, to date, no ruthenium-based drug has been submitted to microarray analysis for the purpose of investigating what genes are activated or inactivated in response to its presence. Actually, the first example of this technology applied to a ruthenium compound can be attributed to our lab in Strasbourg (article in preparation). RDC11 and cisplatin, in fact, were submitted to *Affimetrix®* microarray analysis which confirmed cisplatin-induced DNA damage through the induction of *p53*, *p21* and *GADD45* and RDC11-induced ER-stress by increasing the expression levels of *CHOP (GADD153)*, *TRB3* and *CHAC* genes. RDC11 was also able to affect the gene expression of several metalloproteases possibly involved in metastasis processes and solute carrier membrane transport proteins (SLC) that control nutrient import. Interestingly, these studies highlighted the ability of RDC11 to influence the expression of genes regulators of two relevant pathways for cancer development and growth: the hypoxia-inducible factor HIF-1 and the mammalian target of rapamycin mTOR pathways.

1.5 | Targeting the hypoxia-inducible factor HIF-1 for cancer therapy

The reduction of oxygen supply to a tissue below physiological levels is known as hypoxia. Hypoxia is a common feature of all solid tumors. In this condition, proliferating cancer cells are deprived of oxygen because of a limited blood supply from abnormal tumor microvasculature [Vaupel P and Mayer A, 2007].

Owing to an inappropriate accumulation of free radicals, O_2 deprivation makes hypoxic cells susceptible to genetic aberrations, DNA and protein damage which should limit cell growth leading to apoptosis. However, cancer cells display an array of genetic changes that improve survival making them able to adapt to hypoxic environments. As a result, they continue to proliferate acquiring additional mutations, leading to a more invasive and metastatic phenotype, and resistance to conventional treatments such as radio- and

chemotherapy [Hockel M and Vaupel P, 2001]. In particular, genetic alterations and intratumoral hypoxia can lead to HIF-1α overexpression, which has been associated with increased patient mortality in several cancer types [Semenza GL, 2003]. Therefore, HIF-1α represents an attractive therapeutic target.

1.5.1 | Hypoxia Inducible Factor 1 (HIF-1)

The adaptive response to changes in tissue oxygenation is mainly mediated by hypoxia-inducible factors (HIFs). HIF-1 is a heterodimeric transcription factor consisting of an O_2-responsive and highly regulated α subunit and a stable and constitutively expressed β subunit (also known as aryl hydrocarbon receptor nuclear translocator ARNT) [Semenza GL, 2003]. Mammals possess three isoforms of the α subunit, of which HIF-1α and HIF-2α are the most structurally similar and the best characterized [Majmundar AJ et al, 2010]. Little is known about HIF-3α, which seems to inhibit HIF-1α and HIF-2α activity in a dominant-negative way [Makino Y et al, 2001].

While HIF-1α is expressed ubiquitously in all cells and therefore responsible for a major component of HIF-1 activity in O_2-starved cells, HIF-2α and HIF-3α are selectively expressed in certain tissues, including vascular endothelial cells, type II pneumocytes, renal interstitial cells, liver parenchymal cells and cells of myeloid lineage [Bertout JA et al, 2008].

HIF-1β is not oxygen-responsive itself, but is necessary for the formation of the functional HIF-1 complex. In fact, only the HIF-1α/β heterodimer can bind to target genes on their hypoxic-response elements (HREs), a DNA binding motif in the promoter or enhancer region of the target gene. HIF-1 binding to HREs results in transcriptional upregulation of target genes that mediate multiple adaptations to hypoxia [Semenza GL and Wang GL, 1992].

On the whole, HIF-1 function is largely modulated by HIF-1α subunit stability based on oxygen availability. HIF-1α protein is composed of four functional domains: a bHLH domain, a PER-ARNT-SIM (PAS) domain (involved in dimerization and DNA binding), an oxygen-dependent degradation (ODD) domain (required for targeting to the proteasome and degradation) and two transactivation domains (N-TAD and C-TAD) required for transcriptional activation. HIF-1β contains bHLH, PAS and transactivation domains [Jiang BH et al, 1996]. As for any protein, the level of HIF-1α expression is determined by the rates of protein synthesis and protein degradation. HIF-1α protein synthesis is regulated via O_2-independent mechanisms, whereas degradation is regulated mainly via O_2-dependent mechanisms.

1.5.2 | O_2-dependent regulation of HIF-1α

In well-oxygenated environments, the oxygen-sensitive HIF-1α subunit is continuously expressed and degraded following a multi-step process. Firstly, HIF-1α is hydroxylated at the conserved proline residues Pro402 and Pro564. These modifications are mediated by PHDs (prolyl hydroxylase-domain proteins PHD1, PHD2 and PHD3) and required for interaction of HIF-1α with the VHL tumor-suppressor protein [Jaakkola P et al, 2001]. Because of their dependence on O_2 as a direct substrate, PHDs have been proposed to be "oxygen sensors" linking cellular O_2 concentration to HIF-1α molecular responses [Mole DR and Ratcliffe PJ, 2008] (Figure 1.5.2).

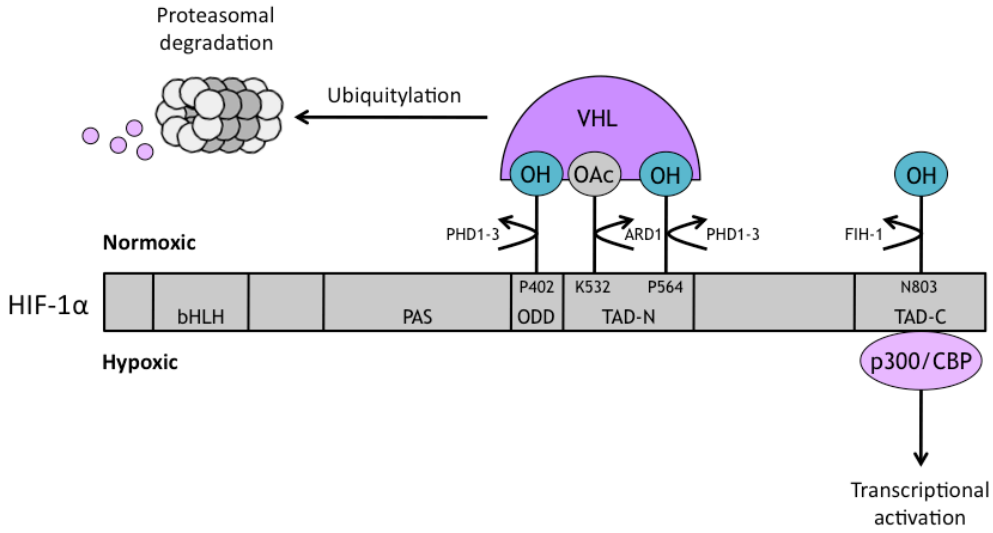


Figure 1.5.2 | **Functional domains of HIF-1α and its oxygen-dependent regulation activity.** HIF-1α consist of four functional domains: a bHLH domain and a PER-ARNT-SIM (PAS) domain (involved in dimerization and DNA binding), an oxygen-dependent degradation (ODD) domain (required for targeting to the proteasome and degradation) and two transactivation domains (N-TAD and C-TAD) required for transcriptional activation [Jiang BH et al, 1996]. In normoxic conditions, HIF-1α is hydroxylated, ubiquitylated and targeted for proteasomal degradation. Under hypoxic conditions, the mechanisms responsible for HIF-1α degradation are shutting down, resulting in a decreased rate of degradation of the protein. In these conditions, p300 and CBP can bind to HIF-1α allowing transcriptional activation of HIF-1α target genes. Adapted from Semenza GL, 2003.

PHDs belong to the α-ketoglutarate (2-oxoglutarate)-dependent dioxygenases superfamily of proteins which require oxygen and α-ketoglutarate as substrates and Fe(II) and ascorbate as cofactors to oxydate the two highly conserved proline residues of HIF-1α [Chan DA and

Giaccia AJ, 2010]. Of the identified HIF-1α prolyl hydroxylases, PHD2 is though to be the key oxygen sensor regulating HIF-1α. Silencing *PHD2* through RNA interference increased HIF-1α levels under normoxic conditions. This effect was not observed with either *PHD1* or *PHD3* [Berra E et al, 2003].

Secondly, HIF-1α hydroxylated is bound by the von Hippel-Liandau (VHL) tumor-suppressor protein which is the recognition component of an E3 ubiquitin-protein ligase that targets HIF-1α for degradation by the 26S proteasome [Cockman ME et al, 2000]. Acetylation of HIF-1α at lysine 532 (K532) by the ARD1 acetyltransferase enhances the interaction of VHL with HIF-1α, promoting its ubiquitylation and degradation. HIF-1α protein degradation is controlled by the ODD domain, indeed, deletion of the entire ODD region renders HIF-1α stable even in normoxic conditions [Jeong JW et al, 2002] .

O_2 also regulates the interaction of HIF-1α with transcriptional activators. The factor inhibiting HIF-1 (FIH-1), which is an α-ketoglutarate-dependent dioxygenases, mediates this effect through the hydroxylation of asparagine 803 residue (Asn 803), preventing the interaction of HIF-1α with co-activators p300 and CBP and therefore HIF-1α-mediated gene transcription [Hewitson KS et al, 2002].

In hypoxia, neither PHDs nor FIH-1 are functional, resulting in inhibition of prolyl hydroxylation within the ODD domain and prevention of the interaction of HIF-1α with VHL. As a result, HIF-1α ubiquitylation and degradation is blocked and consequently the level of HIF-1α increases, accumulates, translocates into the nucleus and dimerizes with HIF-1β via the bHLH and part of the PAS domain to form the HIF-1 complex. HIF-1 recruits the co-activators p300 and CBP and bind to the HRE within the promoter region of HIF-1-responsive target genes, thereby mediating their transcriptional activation [Semenza GL, 2003].

1.5.3 | O_2-independent regulation of HIF-1α

Growth factors, cytokines and other signaling molecules can lead to the induction of the synthesis and activation of HIF-1α [Richard DE et al, 2000; Gorlach A et al, 2001]. The increase in HIF-1α levels in response to growth-factor stimulation differs from the increase in HIF-1α levels in response to hypoxia in two important aspects. First, whereas hypoxia increases HIF-1α levels in all cell types, growth-factor stimulation induces HIF-1α expression in a cell-type-specific manner. Second, whereas hypoxia is associated with decreased degradation of HIF-1α, growth factors, cytokines and other signaling molecules stimulate HIF-1α synthesis via

activation of the PI3K or MAPK pathways [Semenza GL, 2003] (Figure 1.5.3). The main mechanism implicated in this HIF-1α non-hypoxic induction is an increase in HIF-1α protein translation. The increase in protein translation alone appears sufficient to shift the balance between synthesis and degradation towards an accumulation of normoxic HIF-1α [Déry MA et al, 2005].

Studies from several laboratories have identified the PI3K pathway and its downstream effectors, mTOR, AKT and p70S6 kinase (S6K), as mediating the increased HIF-1α translation. The activation of PI3K increases the rate of HIF-1α translation and this involves the activation of the ribosomal protein S6K by the PI3K/S6K/mTOR pathway. S6K regulates the translation of a group of mRNAs possessing a 5'-terminal oligopyrimidine tract (5'-TOP). The HIF-1α gene 5'-untranslated region (5'-UTR) contains these 5'-TOP tracts. Phosphorylation of the S6 protein of the 40S ribosomal unit by S6K increases the translation of the 5'-TOP mRNAs and therefore HIF-1α translation [Page EL et al, 2002].

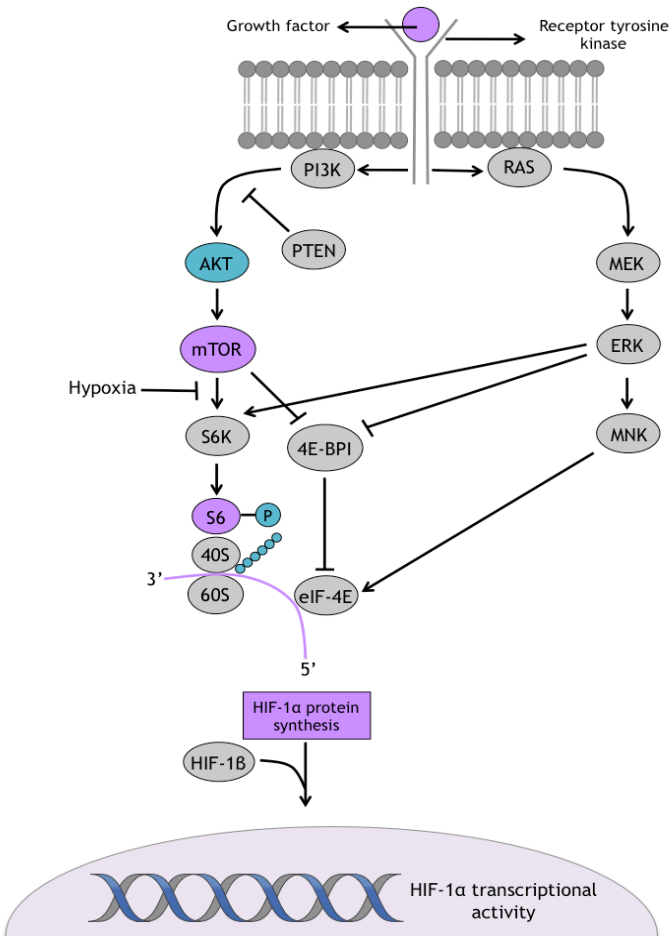


Figure 1.5.3 | **Oxygen-independent regulation of HIF-1α.** When a growth factor binds to its cognate receptor tyrosine kinase, it activates the phosphatidylinositol 3-kinase (PI3K) and mitogen-activated protein kinase (MAPK) pathways, resulting in an increase of translation of a subset of mRNAs (including HIF-1α mRNA) into protein. Adapted from Semenza GL, 2003.

In addition, activation of the RAF-MEK-ERK (MAPK) signaling pathway has also been shown to stimulate HIF-1α transactivation-domain function. This effect is due at least in part to the phosphorylation of the co-activator p300 by ERK, with which the transactivation domain TAD-C interacts [Sodhi A et al, 2000] .

1.5.4 | HIF-1α is regulated at post-translational level

HIF-1α mRNA levels are not affected by hypoxia neither in *in vitro* cell culture experiments nor in *in vivo* experiences by using hypoxic mouse tissues, despite marked increases in HIF-1α target genes mRNA can be observed [Wenger RH et al, 1997]. By contrast, HIF-1α protein levels change significantly according to oxygen concentration. This observation indicates that the prevalent mode of hypoxic induction of HIF-1α is the post-translational regulation and/or protein stability [Fandrey J and Gassmann M, 2009] (Figure 1.5.4).

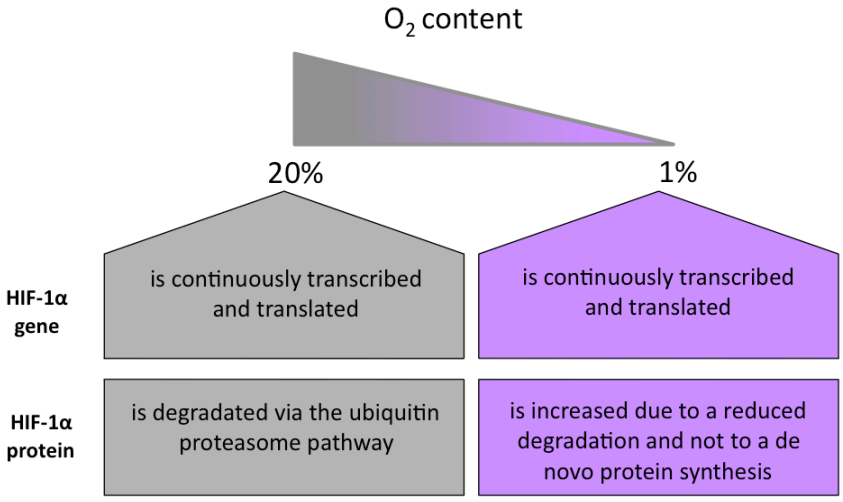


Figure 1.5.4 | **Regulation of HIF-1α gene and HIF-1α protein according to the oxygen content** (20%: normoxia; 1%: hypoxia).

1.5.5 | HIF-1α and cancer

Hypoxia is not the only condition responsible for HIF-1α overexpression in tumors. HIF-1α is overexpressed also as a result of genetic alterations, such as gain-of-function mutations in oncogenes (i.e. *ERBB2*) and loss-of-function mutations in tumor-suppressor genes (i.e. *VHL* and *PTEN*) [Semenza GL, 2003]. HIF-1α overexpression is associated with treatment failure and increased mortality [Zhong H et al, 1999; Talks KL et al, 2000].

A common approach for analyzing altered expression of proteins in human cancers is to perform immunohistochemistry on biopsy samples of patients. Immunohistochemical analysis using monoclonal antibodies revealed that HIF-1α is overexpressed in many human cancers. Significant associations between HIF-1α overexpression and patient mortality have been shown in cancers such as non-small-cell-lung carcinoma, colorectal cancer [Zhong H et al, 1999], pancreatic [Miyake K et al, 2008], head and neck [Koukourakis MI et al, 2002], renal [Klatte T et al, 2007], ovarian [Osada R et al, 2007], bladder, brain and prostate cancer [Talks KL et al, 2000]. Tumor hypoxia and HIF-1α overexpression is reported to correlate with an increased aggressiveness of tumor cell behavior, angiogenesis and metastasis and can be used as a marker to predict outcome in patients with metastatic disease [Koukourakis MI et al, 2002].

Surprisingly, not all tumors that exhibit HIF-1α overexpression are associated with decreased patient survival rates. For example, in early-stage esophageal cancer HIF-1α overexpression is associated with improved survival rates. This difference may be due to the dual role of HIF-1α in early carcinogenesis. Where on the one hand, HIF-1α promotes tumor angiogenesis and cell survival mediating an adaptive response, on the other hand, it cooperates with the apoptotic machinery via induction of apoptotic genes or crosstalk to p53 to mediate cancer cell death in response to cellular stress [Fillies T et al, 2005]. Therefore, the function of HIF-1α in tumor progression might depend on the cell type and cellular context as well as the stage of carcinogenesis.

1.5.6 | HIF-1α target genes

Given that cells and organs need to adapt to changes in oxygen supply, it is not surprising that more than 100 putative direct HIF-1α target genes are regulated in a tissue-specific manner [Ke Q and Costa M, 2006]. Four groups of direct HIF-1α target genes, particularly relevant for cancer, encode for proteins that are involved in angiogenesis, glucose metabolism, cell survival and invasion [Semenza GL, 2003].

1.5.6.a | Angiogenesis

Angiogenesis is a complex process that allows tumor to maintain its growth advantage facilitating metastatic spreading by establishing connections to the existing vasculature. A large number of genes involved in different steps of angiogenesis have been shown to be up-regulated in hypoxic environments [Semenza GL, 2002]. Among them, the vascular endothelial growth factor (*VEGF*) is the most potent endothelial-specific mitogen, which directly participates in angiogenesis by recruiting endothelial cells into hypoxic and avascular areas and stimulating their proliferation [Conway EM et al, 2001].

Therefore, the induction of *VEGF* and also other proangiogenic factors (LEP, leptin; LRP1, LDL-receptor-related protein 1; TGF-β3, transforming growth factor- β3) by HIF-1α leads to an increase in the vascular density and hence a decrease in the oxygen diffusion distance.

1.5.6.b | Glucose metabolism

In normoxic conditions, cells convert glucose into pyruvate which then enters the tricarboxylic acid cycle (TCA) and generates electrons which are essential for generating ATP by oxidative phosphorylation in the electron transport chain. When oxygen is low, cells switch to less efficient anaerobic glycolysis resulting in an increase in the conversion of glucose to lactate [Dang CV and Semenza GL, 1999].

With only 2 molecules of ATP from each glucose molecule produced by glycolysis (instead of 38 ATP provided by TCA cycle), hypoxic cells elevate their ability to generate ATP by increasing the glucose uptake. This is achieved by HIF-1α mediated up-regulating of the expression of glycolytic enzymes and glucose transporters. ENO-1, for example, is a glycolytic enzyme that catalyzes the conversion of 2-phosphoglycerate to phosphoenolpyruvate, the penultimate step of glycolysis [Zhang E et al, 1997]. Among the glucose transporters, GLUT-1

is the most responsible for glucose uptake. An increased rate of glucose transport is among the most characteristic biochemical markers of the transformed phenotype. Moreover, the glycolysis metabolic products, such as lactate and pyruvate, have been shown to cause HIF-1α accumulation under normoxia and regulate hypoxia-inducible gene expression, besides to decrease intracellular pH [Chen C et al, 2001].

1.5.6.c | Cell survival

Hypoxia and HIF-1α induce the expression of growth factors, such as insulin-like growth factor 2 (IGF2) and transforming growth factor alpha (TGF-α) [Feldser D et al, 1999]. When such growth factors bind to their corresponding receptor, signaling transduction pathways that lead to cell proliferation/survival and stimulation of HIF-1α expression are activated. Among these pathways, PI3K and MAPK contribute to HIF-1α activity, leading to increased HIF-1α transcriptional activation of target genes, thereby contributing to autocrine-signaling pathways, whose activation is crucial for cancer progression [Semenza GL, 2003].

1.5.6.d | Invasion

Hypoxia and HIF-1α cover a key role in the development of metastasis. HIF-1α is a major regulator of invasion, epithelial mesenchymal transition (EMT) and angiogenesis, all of which are needed for establishing a metastasis. As mentioned before, the normal adaptation to hypoxia is angiogenesis and cancer cells exploit this mechanism in a variety of ways. Endothelial cells, for example, remodel the extracellular matrix (ECM) in order to get new vessels in hypoxic tissues. Moreover, hypoxic cells secrete factors, such as urokinase-type plasminogen activator receptor (UPAR), matrix metalloproteinase-2 (MMP-2) and plasminogen-activator inhibitor 1 (PAI-1), which degrade the ECM allowing them to invade the basement membrane [Cassavaugh J and Lounsbury KM, 2011].

In addition to the ECM degradation, hypoxia is also able to increase the motility of cells in a paracrine manner through the secretion of factors such as TGF-α. In order for EMT to occur, cells must lose the expression of E-cadherins, which are the major proteins of the adherens junctions in epithelial cells. Hypoxia up-regulates transcriptional repressors of E-cadherin such as *SNAIL*, *TWIST* and *TCF*. The normal response to all these events is in favor of a highly aggressive, metastatic tumor cell [Finger E and Giaccia AJ, 2010].

1.5.7 | Agents that inhibit HIF-1α

A growing number of molecules have been shown to inhibit HIF-1α activity. Many of these act by reducing HIF-1α mRNA or protein levels, HIF-1α DNA-binding activity or HIF-1α-mediated transactivation of target genes and display anticancer activities.

Table 1.5.7 | List of the major anticancer drugs that inhibit HIF-1α activity

Target pathways/mechanism	Agents	References
Signaling		
Receptor tyrosine kinases	Genistein	Buchler P et al, 2004
	Bevacizumab (Avastin)	Calvani M et al, 2008
	Gefitinib (Iressa)	Pore N et al, 2006
	Erlotinib (Tarceva)	Pore N et al, 2006
	Cetuximab (C225)	Luwor RB et al, 2005
Ras-MAPK pathway	PD98059	Alam H et al, 2009
	BAY 43-9006 (sorafenib)	Kumar SM et al, 2007
PI3K-AKT pathway	LY294002	Sandau KB et al, 2001
	Wortmannin	Sandau KB et al, 2001
	Nelfinavir	Pore N et al, 2006
	Silibinin	Garcia-Maceira P and Mateo J, 2009
	NO-sulindac	Stewart GD et al, 2009
mTOR	Rapamycin	Pencreach E et al, 2009; Wang W et al, 2008
	Temsirolimus	Wan W et al, 2008
	Everolimus	Cejka D et al, 2008
HSP90	Geldanamycin	Alqawi O et al, 2006
	17-AAG	Kim WY et al, 2009
	Apigenin	Fang J et al, 2007
Soluble guanylyl cyclase (sGS)	YC-1 (sGS stimulator)	Li SH et al, 2008
COX-2	NS398	Huang SP et al, 2005
	Ibuprofen	Palayoor ST et al, 2003
Histone deacetylase	SAHA	Fath DM et al, 2006
	FK228	Mie Lee Y et al, 2003
	LAQ824	Qian DZ et al, 2004
	Trichostatin A	Yang QC et al, 2006
Microtubules		
Microtubule destabilisers	Curcumin	Thomas SL et al, 2008
	EF24	Thomas SL et al, 2008
	2-ME2	Mabjeesh NJ et al, 2003
	ENMD-1198	Lavalle TM et al, 2008
Microtubule stabilisers	Taxol	Kim HS et al, 2008
DNA binding/damage/cytotoxic		
DNA binding	Echinomycin	Kong D et al, 2005
	Polyamide	Olenyuk BZ et al, 2004
	DJ12	Jones DT and Harris AL, 2006
	Doxorubicin	Duyndam MC et al, 2007
	Cisplatin	Duyndam MC et al, 2007
p53		
p53-HDM2 interaction	Nutlins	Vassilev LT, 2004
	RITA	Yang J et al, 2009
Translation	PX-478	Koh MY et al, 2008
	Tunicamycin	Werno C et al, 2008
	UVC irradiation	Rapisarda A and Melillo G, 2007
Topoisomerase I/II	Topotecan	Rapisarda A et al, 2004
	NSC-644221	Creighton-Gutteridge M et al, 2007

DNA replication/transcription		
Topoisomerase I/II	Irinotecan	Rapisarda A et al, 2004
Mitochondria	Alkylimino-phenylacetate	Lin X et al, 2008
	Antimycin	Maeda M et al, 2006
	Rotenone	Hagen T et al, 2003
	Myxothiazol	Hagen T et al, 2003
Others		
Thioredoxin redox system	Pleurotin	Welsh SJ et al, 2003
RNA polymerase	TAS106	Yasui H et al, 2008
DNA synthesis/repair	TS-1	Zeng L et al, 2008
Multiple signaling pathways (Ras-MAPK; PI3K-AKT)	Resveratrol	Wu H et al, 2008
CDK	Flavopiridol	Newcomb EW et al, 2005

Agents that reduce HIF-1α protein levels do so by decreasing the rate of HIF-1α synthesis, increasing the rate of its degradation, or both. Moreover, since in many cancers the rate of HIF-1α protein synthesis is determined by mTOR activity, inhibitors of this pathway lead to loss of HIF-1α activity and biological consequences such as impaired tumor vascularization that may contribute to their therapeutic effect [Semenza GL, 2007; Poon E et al, 2009]. In table 1.5.7 the main anticancer agents that inhibit HIF-1α activity are listed by mechanism of action and target.

1.6 | HIF-1α meets the mTOR pathway

In addition to the activation of angiogenesis, hypoxic stress also leads to attenuation of protein synthesis through a mechanism that involves the mTOR pathway [Pouysségur J et al, 2006]. HIF-1α/hypoxia negatively regulates mTOR in two ways: first, by an increase in AMP levels which leads to the activation of AMPK (AMP-activated protein kinases), since during hypoxia mitochondrial respiration is impaired and second, by activating the tuberous sclerosis TSC1-TSC2 complex [Reiling JH and Hafen E, 2004] (Figure 1.6.a).

To avoid energy imbalance and death, cells quickly suppress biosynthetic programs during fasting, increase the recycling of aged proteins and organelles to provide an internal source of metabolites, and slow or stop proliferation. The mTOR pathway is at the interface between growth and starvation and it plays a major role in increasing regulation of protein synthesis, cell growth, metabolism and autophagy in many cancer types [Zoncu R et al, 2011]. Therefore, dysregulated mTOR signaling, caused mainly by mutations in tumor-suppressor

genes, fuels the destructive growth of cancers and thus it is actively pursued as a therapeutic target.

mTOR is a serine-threonine kinase and the catalytic subunit of two distinct complexes called mTOR complex 1 and mTOR complex 2 (mTORC1 and mTORC2). These complexes are defined by two accessory proteins: the regulatory-associated protein of mTOR (RAPTOR) and the rapamycin-insensitive companion of mTOR (RICTOR) which define mTORC1 and mTORC2 respectively [Kim DH et al, 2002]. RAPTOR and RICTOR function as scaffolds for assembling the complexes and for binding substrates and regulators [Sarbassov DD et al, 2004].

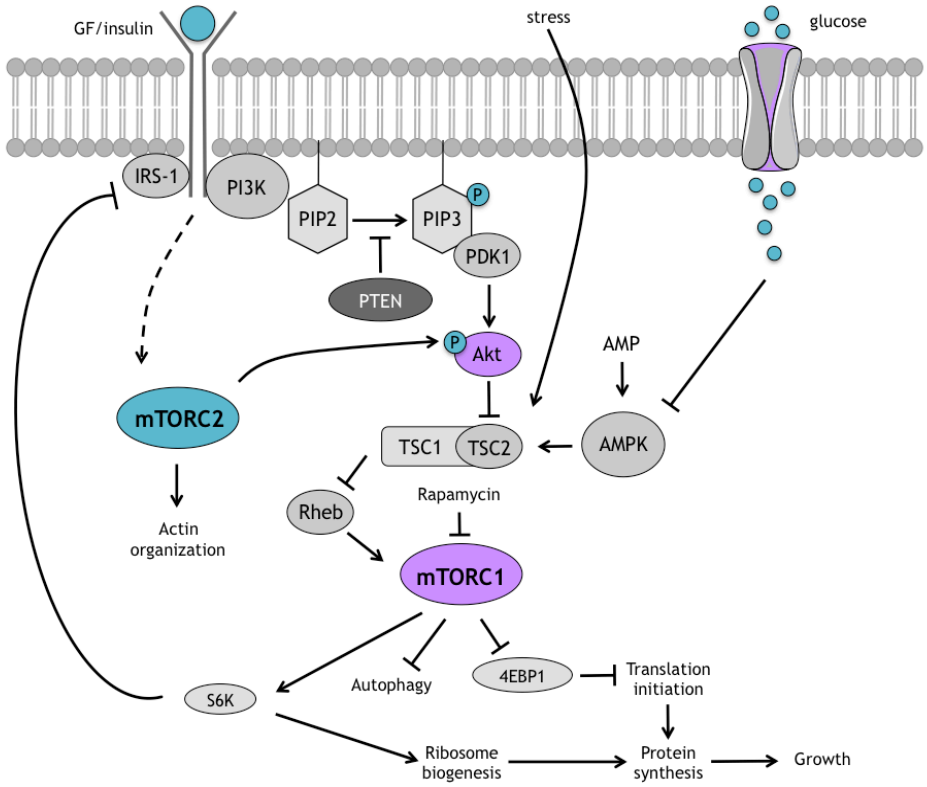


Figure 1.6.a | **The mammalian target of rapamycin (mTOR) signaling network.**
mTOR integrates input signals from growth factors, such as insulin, nutrients (glucose) and stress in order to regulate cell growth through different cellular processes. Adapted from Tsang CK et al, 2007.

Moreover, mTORC1 contains a unique component called PRAS40 (40kDa Pro-rich Akt substrate, a negative regulator), whereas mTORC2 contains PROTOR (protein observed with RICTOR) and mSIN1 (mammalian stress-activated map kinase-interacting protein 1) which help the complex to assemble and to target mTORC2 to membranes respectively [Frias MA et al, 2006; Yang Q et al, 2006].

mTORC1 and mTORC2 share mLST8 (mammalian lethal with SEC13 protein 8) and DEPTOR (DEP-domain-containing mTOR-interacting protein) which act as positive and negative regulators, respectively [Peterson TR et al, 2009] (Figure 1.6.b).

Rapamycin inhibits the ability of mTORC1, but not mTORC2, to phosphorylate its substrates. Rapamycin is a macrolide antifungal agent that binds the small 12kDa protein FK506-binding protein (FKBP12) and, in turn, rapamycin-FKBP12 avoids the binding of RAPTOR to mTOR in mTORC1 complex, thus preventing the access of mTOR to its substrates [Yip CK et al, 2010]. However, other studies have been shown that prolonged rapamycin treatment reduces mTORC2 levels in some cell lines [Sarbassov DD et al, 2004].

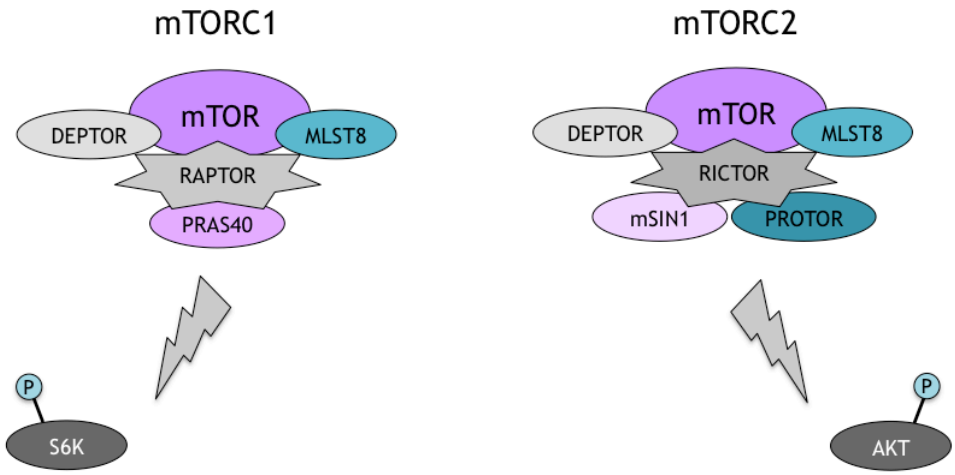


Figure 1.6.b | **Domain organization of mTORC1 and mTORC2.**
mTORC1 and mTORC2 share common components, such as mTOR, DEPTOR (DEP domain-containing mTOR-interacting protein) and MLST8 (mammalian lethal with SEC13 protein 8). Regulatory-associated protein of mTOR (RAPTOR) and 40 kDa Pro-rich Akt substrate (PRAS40) are unique to mTORC1; on the contrary, rapamycin-insensitive companion of mTOR (RICTOR), mammalian stress-activated map kinase-interacting protein 1 (mSIN1) and protein observed with RICTOR (PROTOR) are unique to mTORC2. The main substrate of mTORC1 is the kinase S6 (S6K), while Akt is the major substrate of action of mTORC2. Adapted from Zoncu R et al, 2011.

1.6.1 | Substrates and actions of mTORC1 and mTORC2

1.6.1.a | mTORC1

mTORC1 senses and integrates several environmental and intracellular signals (nutrients, growth factors, energy and stress) to regulate cellular processes involved in the promotion of cell survival and it is regulated mainly through the PI3K-Akt pathway [Facchinetti V et al, 2008]. mTORC1 regulates protein synthesis by directly phosphorylating the initiation factor 4E-binding protein 1 (4E-BP1) and also the ribosomal protein S6 kinase (S6K), leading to cell growth and G1 cell cycle progression [Guertin DA and Sabatini DM, 2007].

When phosphorylated by mTORC1, 4E-BP1 dissociates from the eukaryotic translation initiation factor 4E (eIF4E), allowing the latter to recruit the translation initiation factor eIF4G to the 5' end of most mRNAs [Hara K et al, 1997]. S6K, when phosphorylated by mTORC1, promotes mRNA translation by phosphorylating or binding multiple proteins, such as eIF4B, which collectively affect translation initiation and elongation [Wang X et al, 2001].

Moreover, mTORC1 actively suppress autophagy and, by contrast, inhibition of mTORC1 strongly induces autophagy. Autophagy is the controlled self-degradation of damaged, superfluous or even dangerous cellular components, providing substrates for energy production during periods of low extracellular nutrients [Mathew R et al, 2007]. Today, inhibition of autophagy is used or under investigation for cancer treatment, since suppression of protein degradation pathways leads to the accumulation of damaged or unwanted proteins, which if unresolved, is detrimental to cancer cells, leading to proteotoxicity [Pan JA et al, 2011].

mTORC1 also controls the activity of several transcription factors involved in lipid synthesis and mitochondrial metabolism. In many tumor types, constitutive mTORC1 activation may indirectly favor tumorigenesis by inhibiting autophagy. Moreover, it promotes angiogenesis by regulating HIF-1α. mTORC1 is therefore able to induce the growth of tumors by providing them nutrients and oxygen, thus validating it as a good therapeutic target [Zoncu R et al, 2011].

1.6.1.b | mTORC2

mTORC2 was initially identified as a mediator of actin cytoskeletal organization and cell polarization [Jacinto E et al, 2004]. More interesting, further evidences showed that mTORC2 phosphorylates and activates Akt, thus controlling cell survival, metabolism, proliferation and metastatic progression [Guertin DA and Sabatini DM, 2007]. Akt is a key regulator of the pathogenesis of cancer and its activation represents the most frequent alteration in human cancers [Bellacosa A et al, 2005].

It has been found that, to be fully activated and stabilized, Akt requires phosphorylation at both Ser473 and Ser308 by mTORC2 and PDK1, respectively [Bhaskar PT and Hay N, 2007].

Moreover, mTORC2 phosphorylates its substrates FOXO-1 and FOXO-3 (forkhead box protein O1 and O3), preventing them from translocating into the nucleus and activating gene expression programs that promote apoptosis. In this manner, mTORC2 may favor cell survival through inhibition of FOXO1 and FOXO3 by Akt. If, on the one hand, much more is known about mTORC1 regulation, which acts as a signal integrator for nutrients, growth factors, energy and stress, little is know about mTORC2 regulation. However, the available evidences suggest that, in contrast to mTORC1, only growth factors directly regulate mTORC2 [Zoncu R et al, 2011].

Today, phosphorylation of Akt at Ser473 is the most used marker of mTORC2 activation. Similarly, also mTORC1 phosphorylation of S6K and the ribosomal protein S6 still remains the preferential tool used to investigate its activation [Mavrommati I and Maffucci T, 2011].

Since rapamycin and its analogues partially inhibit mTOR through allosteric binding to mTORC1 but not mTORC2, the latter represents an emerging player in cancer [Yu K et al, 2010]. Thus, targeting mTORC1 and mTORC2 may become especially useful for treating cancers whose survival and progression depend on the activities of these complexes and their effectors.

1.6.2 | mTOR inhibitors: rapamycin and its derivatives

Rapamycin (commercially known as Sirolimus®) is a natural antibiotic produced by the bacterium *Streptomyces hygroscopicus* and isolated in 1975 from a soil sample collected on Easter Island (Rapa Nui), from where the name rapamycin is derived [Vézina C et al, 1975]. The antifungal and immunosuppressive properties of rapamycin were identified immediately [Martel RR et al, 1977]. For over twenty years rapamycin was abandoned, but since the

isolation of the *TOR* gene, a renewed interest in this molecule has grown. To be biologically active, rapamycin must interact with the protein FKBP12 (12-kDa FK506-binding protein) and subsequently, this complex binds directly to mTOR and inhibits the function of mTOR and the mTOR-mediated signaling network. The Development Therapeutic Branch of the National Cancer Institute (NCI) found that rapamycin had a broad antitumor activity in both *in vitro* and *in vivo* models. However, as Sirolimus® was poorly soluble in water, pharmaceutical companies began to develop rapamycin analogs, collectively known as "rapalogs", with improved pharmacokinetic properties [Tsang CK et al, 2007]. Generally, rapalogs display similar therapeutic effects to rapamycin but with improved hydrophilicity and are suitable for both oral and intravenous administration.

1.6.2.a | Resistance to rapamycin and rapalogs

Although clinical studies with rapamycin and rapalogs have validated mTOR as a cancer target, the effectiveness of these agents may be limited because of hyperactivation of PI3K/Akt pathway and resistance of mTORC2 signaling. The mTOR-target S6K phosphorylates IRS-1 (Insulin Receptor Substrate 1) in correspondence to serine residues, leading to its dissociation from tyrosine kinase receptors and inhibition of the PI3K signaling [Haruta T et al, 2000]. Rapamycin and its analogues by inhibiting mTOR decrease S6K serine phosphorylation, avoiding the inhibitory feedback loop to IRS-1, resulting in increased AKT phosphorylation [Sun SY et al, 2005].

Moreover, other mechanisms of resistance to rapamycin and rapalogs could derive from mutations that affect the binding of the complex rapamycin/FKBP12 to mTOR and to reduced levels of 4EBP1 [Kurmasheva RT et al, 2006].

Therefore, co-treatment with an agent able to overcome rapamycin/rapalogs resistance by decreasing rapamycin-induced-Akt phosphorylation, could improve the antiproliferative effect of rapamycin/rapalogs treatment [Cerovac V et al, 2010]. Lately, considering the limited antitumor activity of mTORC1 inhibitors and the fact that dysregulated mTORC2 contributes to the malignant phenotype, many efforts have been made to get mTOR-selective inhibitors able to induce a deeper or broader suppression of both mTORC1 and mTORC2 activities in tumor cells [Roulin D et al, 2010; Sini P et al, 2010].

WYE-132, for example, is a highly potent ATP-competitive and specific mTOR kinase inhibitor, which inhibits both mTORC1 and mTORC2 in different cancer models *in vitro* and *in*

vivo. It has been shown that WYE-132 selectively attenuates Akt function and inhibited proliferation of diverse cancer cell lines, in addition to a strong G_1 arrest [Yu K et al, 2010].

Another ATP-competitive mTOR kinase inhibitor, called AZD8055, was shown to inhibit both mTORC1 and mTORC2, decreasing the phosphorylation of their effectors, S6K and Akt respectively. These data showed that the ability of AZD8055 to completely inhibit mTORC1 and mTORC2 accountes for a greater inhibition of cell proliferation and induction of autophagy compared to rapamycin [Sini P et al, 2010].

1.6.3 | HIF-1α, mTOR and angiogenesis in cancer

Hypoxia activates and stabilizes HIF-1α which induces the expression of VEGF, VEGF receptor and other factors (LEP, LRP1, TGF-β3), thus stimulating neovascularization and proliferation [Shweiki D et al, 1995].

The PI3K/Akt/mTOR pathway is involved in the hypoxic response induced by HIF-1α in neoplastic cells. In particular, HIF-1α was reported to be up-regulated downstream by mTORC1, therefore promoting angiogenesis by transcribing VEGF [Skinner HD et al, 2004]. In this manner, mTORC1 and HIF-1α signaling induce angiogenesis in order to promote the growth of tumors by supplying them with nutrients and oxygen [Zoncu R et al, 2011].

Both *in vitro* and *in vivo* studies have shown the ability of mTOR inhibitors to prevent angiogenesis through inhibition of the HIF-1α/VEGF axis [Mayerhofer M et al, 2005; Del Bufalo D et al, 2006], which represents today an attractive target to treat tumors whose survival depends on HIF-1α and mTOR signaling pathways.

1.7 | A bioreactor for studying potential antimetastatic drugs

One of the aims of this thesis work was also to assess the antimetastatic potential of ruthenium-based molecules, such as RDC11 and its derivatives. Actually, screening a compound for its antimetastatic activity is not so a simple task. Two main limitations occur: the first concerns the use of too simplistic *in vitro* models that do not reflect what really happens in *in vivo* physio-pathological conditions, the second bears on ethical issues of animal testing.

Indeed, preclinical anticancer drug screening involves the use of static tissue/cell cultures to evaluate parameters such as cytotoxicity. These models provide an important and easy-to-do method to estimate drug concentrations that can be tested afterwards in animals and

clinical studies. However, cytotoxic tests simulate only constant concentrations of the drug overtime [Kirstein MN et al, 2006] and they cannot provide, for example, a likely idea of the complex process of metastatization and the possibility to discriminate the different sensitivity of primary tumor and metastasis to pharmacological treatment.

On the other hand, animal models are used to assess drug regimens, since cancers in mice mimic the microenvironment of human tumors. These models provide more information concerning, for example, the metabolic activation or detoxification of drugs, determination of the minimum effective exposures required for antitumor activity and drug availability in the tissues [Suggitt M and Bibby MC, 2005]. However, also animal models are not trouble-free; cost of housing and management of animals, the reproducibility in humans and ethical issues are the main problems that characterized them [Fitts DA, 2011]. Moreover, with these models it is not always possible to control variables such as maximum and steady state concentrations or to obtain multiple sequential blood samples in single animals. The understanding of the relationship between these variables and the antitumor activity is very important.

In the last fifteen years bioengineering has supported biology in order to design new tools for improving our knowledge in cell function and analyze the effects of different stimuli on it. These new tools, called bioreactors, aim to recreate better physiological conditions to those typically present during classic cell culture experiments. Moreover, bioreactors intend to replace or reduce the use of animals for drug screening, allowing the study of physiological and pathological behavior of human metabolism at cellular level [Vozzi F et al, 2009]. With respect to classical cell culture tools, bioreactors are characterized by an important physical feature, that is hydrodynamics, which occurs naturally in living tissues. Based on this property, many bioreactors and perfusion flow systems have been designed for tissue culture applications in order to mimic, for example, oxygenation and nutrient mass transfer for prolonged cell culture sustainability [Martin Y and Vermette P, 2005; Thouas GA et al, 2007].

Douglas Yee and co-workers developed a 3-dimensional system of cell growth in a bioreactor containing hollow fibers and a flow path driven by a pump to evaluate antineoplastic treatments, such as gemcitabine [Kirstein MN et al, 2006]. In 2004, Bibby MC's group set up the *in vivo* hollow fiber assay, a routine screening method for drug anticancer activity which allowed more to 50 cell lines to be inserted into small fibers that are subsequently implanted in mice [Suggitt M et al, 2004].

Later, Vozzi and co-workers developed a Multi-Compartmental Bioreactor (MCB) through which they were able to perform high-throughput experiments in an *in vivo*-like simulated environment for a long time (more than one week). This bioreactor was controlled by a software that enabled the temperature, pH, pressure and flow to be tightly regulated to simulate physiological or pathological conditions [Mazzei D et al, 2008]. They simulated a typical parallel experiment with four "animals". For each "animal", a bioreactor was installed in which cell were seeded under a constant flow of nutrient and gas.

Another example was that of Hourigan and collaborators, who developed a novel stirred bioreactor for culture of a transformed cell line under defined hydrodynamic condition *in vitro*, a suitable alternative method to study mechanisms of tumor progression and invasiveness *in vitro* [Thouas GA et al, 2007].

1.7.1 | *Contribution of the laboratory to the field*

Recently, the Callerio Foundation Onlus and the Department of Material Engineering of the University of Trieste have started a collaboration with the aim to develop an experimental tool in order to simulate *in vitro* the metastatization of cancer cells on healthy tissues and overcome the limitations of the commonly used *in vitro* and *in vivo* methodologies.

This project was encouraged by the National Centre for the Replacement, Refinement and Reduction of Animals in Research (NC3Rs) which published some guidelines to prompt researchers to improve animal testing [http://www.nc3rs.org.uk/page.asp?id=3].

Our experimental tool, called "Plastic mouse", would simulate *in vitro* complex biological processes that normally take place *in vivo*, such as metastasis, by reproducing biochemical and biophysical conditions to mimic those of the real biological systems. Therefore, the plastic mouse might be a reliable alternative to animal testing in validating new pharmacological therapies.

1.8 | Colorectal cancer (CRC)

According to the National Cancer Institute, every year almost fifty thousand people die from colorectal cancer in the United States [http://www.cancer.gov/cancertopics/colon-and-rectal], making it the second leading cause of death from cancer, after lung carcinoma [Markowitz SD and Bertagnolli MM, 2009]. CRC usually develops locally in the lower part of the descending colon or rectum, but frequently it may spread to other sites of the body, most commonly the liver, leading to negative outcomes for patients. Despite recent improvements, colorectal cancer is often resistant to chemotherapy, highlighting the need to develop novel therapeutic approaches.

1.8.1 | Current treatment of CRC

5-fluorouracyl (5-FU) has been the backbone of treatment regimens for CRC and metastatic CRC (mCRC) for over 40 years, mainly in association with folinic acid (leucovorin) [Machover D et al, 1986]. In 1996, irinotecan (CPT-11, Camptosar®, Pharmacia Corp.; Peapack, NJ) was approved by FDA for the treatment of CRC as both a single agent and also in combination with 5-FU and leucovorin [Kohne CH et al, 2005]. Also oxaliplatin plus infusional 5-FU/leucovorin obtained US FDA approval in this setting [Andre T et al, 2009].

Although cytotoxic agents are the most commonly used therapies, both in the adjuvant and metastatic setting, other strategies have been developed to manage this disease: the inibition of epidermal growth factor receptor (EGFR) with monoclonal antibodies such as cetuximab and panitumumab and blockade of angiogenesis with antibodies against VEGF receptor (VEGFR) such as bevacizumab, are currently used in the clinical practice [Waldner MJ and Neurath MF, 2010].

However, these strategies are not trouble free and their benefits are often short-lived and limited to small groups of patients, demonstrating the need to develop alternative therapies.

1.8.2 | Alternative strategies for the treatments of CRC

It has been shown that CRC is characterized by a dysregulation of the PI3K/Akt/mTOR pathway and often by an overexpression of HIF-1α. As already described above, PI3K/Akt/mTOR pathway is responsible for cell proliferation, survival, metabolism and motility of cancer cells and thus for negative outcomes for patients [Zhong H et al, 1999].

DNA microarray analysis showed that RDC11 was able to influence the expression of several genes involved in the regulation of the HIF-1α and mTOR pathways, making them attractive targets for a selective cytotoxicity against cancer cells. Inhibition of these two pathways, which are frequently up-regulated in CRC, could thus represents a promising therapeutic strategy for this tumor type.

Altogether, the following reasons have led us to select CRC as a suitable model for the present study:

- it is the second-leading cause of cancer-related deaths worldwide, still difficult to manage
- it is characterized by the up-regulation of the HIF-1α and mTOR pathways

because

- *Affimetrix* microarray analysis has showed that RDC11 is able to regulate the expression of several genes of the HIF-1α and mTOR pathways

an finally because

- the final negative outcome of CRC mainly depends on the metastatic spread to the liver.

2 | Aim of the study

Ruthenium-derived compounds (RDCs) are a relatively new class of organometallic ruthenium(II)-based molecules endowed with a significant ability to reduce tumor growth in mice, including glioblastoma, the most aggressive brain tumor for which there is still no cure, and to overcome resistance in cisplatin-insensitive cancer cell lines. RDC11, one of the most promising RDCs, was shown to induce apoptosis through a mechanism mostly independent of p53/DNA damages, but involving the endoplasmic reticulum stress/CHOP pathway. Although *in vitro* and *in vivo* properties of some RDCs have already been characterized, their exact mechanism of action is still unknown and further investigations are needed. *Affimetrix* microarray analysis revealed that RDC11 is able to alter the expression profile of genes involved in the regulation of two metabolic pathways essential for cancer development and growth: the pathway of the Hypoxia-inducible factor 1 (HIF-1) and the pathway of the mammalian target of rapamycin (mTOR). These observations, together with the fact that RDC11 affinity for DNA is 2 orders of magnitude lower than that of cisplatin, have prompted us to search for RDC11 DNA-independent molecular targets. Therefore, we started investigating in detail the effect of RDC11 on the HIF-1 and the mTOR pathways. We hypothesized that the inhibition of these two pathways by RDC11 could explain large part of its antitumor activity, thus adding new information in understanding its mode of action.

In parallel, we developed RDC11-like molecules in order to ameliorate the chemical and pharmacological features of ruthenium-derived compounds. In particular, we modified the nature of the ligands around the ruthenium center of RDC11 and analyzed how these modifications can modulate the cytotoxicity and selectivity of the new generated RDCs against cancer cells, both *in vitro* and *in vivo*. Since RDC11 had already been shown to regulate the endoplasmic reticulum stress pathway by inducing CHOP, we investigated the ability of the new RDCs to modulate this pathway potentially responsible for their cytotoxic activity.

A third direction explored concerns the possible antimetastatic role of RDCs. Since other ruthenium-based drugs (notably NAMI-A) have shown an important antimetastatic activity, and also considering that RDCs, in particular RDC11, inhibit pathways that are known to be involved in the regulation of tumor progression, we planned to evaluate their antimetastatic potential. Unfortunately, the available *in vitro* and *in vivo* methodologies for screening molecules in this respect present many limitations. For these reasons, we developed an alternative experimental tool, called “the plastic mouse”, with the goal of simulating the

metastatic process *in vitro* and efficiently screening potential antimetastatic molecules, such as RDCs.

Altogether, by analyzing the main molecular pathways targeted by RDC11 in cells, by studying the link between specific modifications of its molecular structure and the resulting antitumor activity and by setting up an *in vitro* device capable of testing its putative antimetastatic properties, I have analyzed in a comprehensive way the response of biological systems to this ruthenium-containing molecule, thus contributing to the pharmacological development of the class of Ruthenium-Derived Compounds.

3 | Materials and Methods

For materials and methods make reference to the two manuscripts in the results section. Hereafter, only the materials and methods relative to the chapter "Development of an experimental tool to recreate the metastatic process *in vitro*" are reported.

Trypan blue exclusion test

This test was performed seeding a different number of HT-29 cells in their own medium and in IHHs medium in six-well plates. After 72 hours cells were detached and counted using the Trypan blue exclusion test (Cook JA and Mitchell JB, 1989).

Sulforhodamine B cell viability test

Sulforhodamine-B (SRB) assay was performed to assess cell survival. SRB is a water-soluble dye that binds to the basic amino acids of the cellular proteins. Thus, colorimetric measurement of the bound dye provides an estimate of the total protein mass that is related to the cell number (Vichai V et al, 2006).
HT-29 and HCEC cells were seeded in 96-well plates in their own medium and in that of IHHs and after 96 hours, cells were fixed and stained with SRB 0.4% w/v in 1% acetic acid (50 µL/well) (Sigma-Aldrich). The dye was then dissolved in 10 mM TrisBase pH 10.5 and finally the absorbance measured by a spectrophotometer (SpectraCountTM, Packard) at the wavelength of 570 nm.

Morphological analysis using light microscopy

To perform this test, HT-29, HCEC and IHH cells were seeded in multi-well slides (Labtek, Nalge Nunc International), individually or in co-culture, for evaluating any morphological variation that might occur when cells are placed in contact with other cell types. Cells were finally observed by light microscopy.

4 | Results

4.1 | Differential regulation of the HIF-1 and the mTOR pathways by platinum and ruthenium anticancer organometallic drugs.

Ruthenium-derived compounds (RDCs) might represent a valid alternative to platinum-based chemotherapy. RDC11, one of the most active RDCs, has already shown to inhibit the growth of different tumors implanted in mice, more efficiently than cisplatin, displaying only a weak toxicity at hepatic, renal and neurological level [Meng X et al, 2009]. *In vitro*, RDC11 showed only a reduced ability to bind DNA and to induce DNA damage compared to cisplatin, suggesting the involvement of transduction pathways different from those of this platinum metal-based drug. Indeed, it has been found that RDC11 induce the activation of the transcription factor CHOP and several of its target genes, leading to apoptosis through the endoplasmic reticulum stress pathway. In support of a DNA-damage independent mechanism, *Affimetrix* microarray analysis showed that RDC11 was able to alter the expression of genes whose protein products are known to be the most relevant regulators of cancer cell metabolism, that is HIF-1α and mTOR [Wouters BG and Koritzinsky M, 2008]. We therefore decided to investigate whether RDC11 could affect HIF-1α and mTOR signaling, knowing that both are upregulated in the majority of solid and metastatic tumors. We thought that the validation of HIF-1α and mTOR as RDC11 putative targets could help us to add new information on the molecular basis of its anticancer activity.
Results are discussed in the following manuscript which is under evaluation:

Vidimar V, Licona C, Jenny M, Mellitzer G, Guenot D, Freund JN, Sava G, Pfeffer M, Gaiddon C. **Differential regulation of the HIF-1 and the mTOR pathways by platinum and ruthenium anticancer organometallic drugs.**

Differential regulation of the HIF-1 and the mTOR pathways by platinum and ruthenium anticancer organometallic drugs

Vania Vidimar[1,2,4], Cynthia Licona[1,2], Marjorie Jenny[1,2], Georg Mellitzer[1,2], Dominique Bagnard[1,2], Dominique Guenot[2,5], Jean-Noel Freund[1,2], Gianni Sava[4], Michel Pfeffer[2,3], Christian Gaiddon[1, 2*]

[1]UMRS682 INSERM, Strasbourg, France
[2]Université de Strasbourg, Strasbourg, France
[3]UMR 7177 CNRS, Institut de Chimie, Strasbourg, France
[4]University of Trieste, Department of Life Sciences, and Callerio Foundation Onlus, Trieste, Italy
[5] EA4438, Strasbourg, France

[*]Corresponding Author: Christian Gaiddon, U682 INSERM - Université de Strasbourg, 3 avenue Molière, Strasbourg 67200, France. Phone: +33 03 88 27 77 27**,** Fax: +33 03 88 26 35 38, Email Address: gaiddon@unistra.fr

Abstract

In recent years, ruthenium-based compounds were developed to overcome the severe side effects and drug-resistance phenomena of the widely used platinum-based anticancer drugs. In contrast to platinum-derived drugs, it was suggested that ruthenium compounds could exert their cytotoxicity in part independently from DNA and through direct modulation of redox enzymes. Therefore, we have compared the relative activity of platinum (cisplatin) and ruthenium (RDC11) derived compounds on the HIF-1 and the mTOR pathways, two pathways that are sensitive to the cellular metabolism. We showed that, in contrast to cisplatin, RDC11 was able to decrease HIF-1α and HIF-1β protein levels in normoxic and hypoxic conditions, which leads to decreased expression of HIF-1 target genes, such as *VEGF* and *Glut1*. We demonstrated that HIF-1α protein levels downregulation involved a complex mechanism associating changes in HIF-1α protein stability, *HIF1*α mRNA translation and *HIF1*α mRNA synthesis. As mTOR controls HIF-1α translation, we analyzed the regulation of this pathway. We showed that, in contrast to cisplatin, RDC11 reduced the phosphorylation of the ribosomal protein S6 and AKT on specific sites that are markers for the activity of the mTORC1 and the mTORC2 complexes of mTOR. This observation correlates with a reduction in mRNA levels of RICTOR and RAPTOR, two components of mTOR. Finally, we showed that the inhibitory effect of RDC11 on the HIF-1 and mTOR pathways is consistent with its ability to reduce angiogenesis and potentiate the antitumor activity of the mTOR inhibitor rapamycin *in vivo*. Altogether, our results show that ruthenium-derived compounds strongly impact metabolic pathways.

Introduction

Since the discovery of cisplatin, the study of metal-based drugs has turned out to be one of the most promising fields of antitumor chemotherapy research. Cisplatin and its derivatives (oxaliplatin and carboplatin) are widely used as anticancer agents worldwide and are frequently part of combinatory regiments associating targeted therapy. Platinum derivatives exhibit their cytotoxic properties through the interaction with DNA, forming DNA adducts which activate several signaling transduction pathways leading to cell growth arrest or cell death [1] [2] [3]. By affecting the DNA integrity, these platinum-compounds target rapidly dividing cells, including tumor cells as well as healthy tissues. This poor selectivity is the main cause of platinum compounds severe side effects such as neurotoxicity [4, 5]. Furthermore, due to mutation in key signaling pathways causing resistance, only a limited number of tumors can be effectively treated with platinum derivatives [6] [7].

These limitations have prompted the search for less toxic and more effective platinum-free metal-based antitumor drugs. Several metal compounds, as derivatives of iron, gold, titanium, gallium and ruthenium, have been designed on the basis of cisplatin chemical structure in order to ameliorate activity and reduce host toxicity [8] [9] [10] [11] [12] [13]. *In vitro* screening of all these molecules revealed ruthenium-based drugs as the least toxic. It has been proposed that this lower toxicity could be linked to the ability of ruthenium to mimic iron in binding to biomolecules, such as transferrin and albumin, exploiting the mechanisms that the cell is using for the detoxification of iron in healthy tissues [14] [15]. Beside this characteristic, ruthenium compounds present other interesting chemical properties for the development of anticancer compounds: (a) the availability of 6 coordination sites leading to multiple combinations of ligands, (b) a slow rate of ligand exchange, compatible with a reasonable stability in a biological context and (c) several possible oxidation states allowing a potential interference with biological macromolecules.

Therefore, various structures of ruthenium derivatives have been tested *in vivo* and they seem to offer promising anticancer activities, notably against cisplatin-resistant tumors [16], lower general toxicities compared to cisplatin [14] [15] [17] and even antimetastatic activity [18] [19]. To date, two ruthenium complexes have entered clinical trials, namely NAMI-A and KP1019 [20]. NAMI-A is an anti-metastatic compound, currently in phase II clinical trial, able to stabilize the progression of non-small cell lung carcinoma in pre-treated patients [21]. KP1019, which at present undergoes phase I clinical trials under the form of its salt derivate KP1339, is well tolerated and patients with advanced solid tumors experienced disease stabilization for more than two months [16] [22].

Although already tested in clinic and largely studied in pre-clinic, the mode of action of ruthenium derived compounds remains unclear. Since ruthenium derivatives have been often designed to mimic platinum compounds, it was expected that they showed a mechanism of action similar to that of cisplatin, especially for targeting DNA. It has been indeed

demonstrated that ruthenium compounds bind DNA, although quite differently from cisplatin [23-25] [26-29]. This may explain the dissimilar anticancer effects of ruthenium derivatives compared to cisplatin and their ability to exert cytotoxic effects on cisplatin-resistant cancer cells [30] [31] [26]. Since no strict correlation between cytotoxicity and DNA interaction has been found for ruthenium compounds, DNA-independent modes of action have been proposed.

We have developed a class of anticancer ruthenium compounds (RDC, ruthenium derived compounds) that present the particularity of having a covalent bond between the ruthenium atom and a carbon atom [26]. The presence of this covalent bond confers a specific range of redox potentials that correlates with the cytotoxicity of the compounds and that allow them to be efficient as mediators of electron transfer to or from oxidized or reduced active sites of redox enzymes, affecting their activity [32] [33]. This ability of ruthenium compounds to affect the activity of cellular redox enzymes is also supported by Sadler and co-workers that have shown that ruthenium derivatives interact with biological nucleophiles such as GSH [34] leading to GSH depletion and becoming more sensitive to reactive oxygen species (ROS) [35]. In addition, we have shown that the cytotoxicity of one of the RDC, RDC11, is dependent on the activity of the transcription factor CHOP, which is a critical effector of the endoplasmic reticulum stress signaling pathway [17]. This pathway, besides being activated by the accumulation of unfolded proteins, has been proposed to play a role in tumorigenesis via its induction by high glucose, ROS and hypoxia [36]. This observation further supports the idea that ruthenium derived compounds might regulate the activity of redox enzymes affecting the cellular metabolisms.

Therefore, we hypothesized that RDC molecules might regulate other intracellular signaling pathways that control, or are sensitive to, the cellular metabolisms. In this study, we analyzed the regulation of two of these pathways, the HIF-1 and the mTOR pathways, that have been strongly connected to the adaptive metabolic response of cancer cells to the hypoxia developed in poorly vascularized tumors [37]. HIF-1 (Hypoxia Inducible Factor 1) is a transcription factor that consists of two subunits: a constitutively expressed β subunit (Hif-1β) and a highly regulated α subunit (HIF-1α) [38]. HIF-1 is usually inactive under basal conditions but active in many hypoxic solid tumors, leading to resistance to chemotherapy and aggressive tumor progression [39]. HIF-1 regulates the transcription of genes involved in crucial aspects of cancer biology, such as angiogenesis and glucose metabolism. Angiogenesis is one of the main responses that cancer cells carry out in hypoxic conditions and involves the increase of VEGF production, while glucose metabolism represents the major source of energy in low-oxygen environments. The mammalian target of rapamycin (mTOR) is the catalytic subunit of two distinct protein complexes, namely mTORC1 and mTORC2 [40]. mTORC1, by phosphorylating the S6 kinase (S6K), which in turn phosphorylates the ribosomal protein S6, controls cell growth through the regulation of

protein translation, metabolism and autophagy. mTORC2 controls cell survival and cytoskeletal organization, mainly by phosphorylating AKT.

Both HIF-1α and mTOR are frequently deregulated and overexpressed in cancer and this is often associated with poor prognosis and negative outcome for patients [41] [42]. Therefore, alterations of the HIF-1 and mTOR pathways in tumors make them attractive targets for anticancer therapy. Based on these observations and the fact that ruthenium-derived compounds might regulate the cellular metabolisms, we compared the regulation of the HIF-1 and the mTOR pathways by both platinum- and ruthenium-derived compounds.

Materials and Methods

Chemicals. Ruthenium derived compounds were synthesized as previously described [26, 33]. Cisplatin was purchased from Mylan Pharmaceuticals, Deferoxamine Mesylate from Sigma-Aldrich®; MG132 and Lactacystin from Calbiochem. Rapamycin was obtained from Tocris Bioscience, such as ALLM, ALLN and Z-VAD-FMK.

Cell culture and MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) test. Human colorectal adenocarcinoma HCT116 and SW480 cells were maintained at 37°C in normoxic (20% O_2, 5% CO_2) or hypoxic (94% N_2, 5% CO_2, 1% O_2, Tri-Gas Incubator, Sanyo) conditions in DMEM 1 g/L glucose (Dulbecco's modified Eagle's medium; Life Technology) supplemented with 10% heat-inactivated fetal calf serum (Life Technology), penicillin/streptomycin (100UI/ml – 100 µg/ml) and gentamycin (50mg/ml). Cells were trypsinyzed for maintenance and experimentation plates as previously described [43]. HUVEC (Human Umbilical Vein Endothelial Cells) were grown in Endothelial Cell Basal Media from Cambrex supplemented with L-glutamine and 10% FBS. MTT test was performed using 96-well culture plates (Costar) as previously described [44].

Western Blot. Cells were grown in six-well plates, and for each condition, three wells were treated. Cells were lysed in 120 µl of Laemmli Sample Buffer 1X (125 mM Tris–HCl, pH 6.7, containing 3.3% SDS, 0.7 M 2-mercaptoethanol, 10% glycerol and 0.02% Bromophenol Blue), sonicated for 30 seconds and then boiled for 5 minutes before loading. Equal amounts of denaturated total-protein extracts were separated on a 12% SDS-polyacrylamide gel and then electro-transferred to nitrocellulose membranes (Bio-Rad Laboratories). Equal loading was ensured by using an antibody directed against actin (1/2000; Sigma). Immunoprobing was controlled with anti-HIF-1α (1/500; BD Transduction Labs), anti-HIF-1β (1/1000; Novus Biologicals), anti-S6 (1/1000; Cell Signaling Technology), anti-pS6 (S235, 236, 1/1000; Cell Signaling Technology), anti-PHD2 (1/1000, Abcam), anti-Akt (1/1000; Cell Signaling Technology) and anti-pAkt (1/200; Cell Signaling Technology). Nitrocellullose membranes were then probed with a secondary horseradish-peroxidase-conjugated antibody (anti-rabbit

or-mouse according to the first antibody, SantaCruz) diluted at 1/5000 and 1/1000 respectively. Antibody reaction was revealed with chemiluminescence detection procedures according to the manufacturer's recommendations (Immun-Star™ HRP Chemiluminescence Kits, Bio-Rad Laboratories) and by using the Molecular Imager® ChemiDoc™ XRS+ System (Bio-Rad Laboratories).

Quantitative reverse transcription-PCR (RT-qPCR). Expression of the different genes was quantitatively assessed by real-time PCR using *18S* as the normalizing gene. Cells were grown in six-well plates, and for each condition, three wells were treated. After treatment, cells were harvested and total RNA was isolated with TRIzol reagent (Invitrogen) according to the manufacturer's instructions. RNA was quantified using a Nanodrop 2000 Spectrophotometer (Thermo Scientific) and cDNA synthesized from 1µg total RNA using the iScript cDNA synthesis kit (Bio-Rad Laboratories). qPCR was performed in Bio-Rad iCycler thermal cycler using iQ SYBR Green supermix (Bio-Rad Laboratories). Specificity of the amplification was assessed by performing a melting curve analysis.
Nucleotide sequences of the primers were as follows: *VEGF* (forward 5'-ccttgctgctctacctccac-3'; reverse 5'-ccacttcgtgatgattctgc-3'); *GLUT1* (forward 5'-ggttgtgccatactcatgacc-3'; reverse 5'-cagataggacatccagggtagc-3'); *HIF1α* (forward 5'-tttttcaagcagtaggaattgga-3'; reverse 5'-gtgatgtagtagctgcatgatcg-3'); *HIF1β* (forward 5'-aggcttttctgcccaggt-3'; reverse 5'- ctgaaggaggatggagtctga-3'); ENO1 (forward 5'-tcccaacatcctggagaataa-3'; reverse 5'-atgccgatgaccaccttatc-3'); *Raptor* (forward 5'-aggaaaagaactacgccttgc-3'; reverse 5'- gcactggggtcaaactcc-3'); *Rictor* (forward 5'-agtgaatctgtgccatcgagt-3'; reverse 5'- agtagagctgctgccaaacc-3').

Determination of the NAD/NADH ratio following RDC11 treatment. Total NAD and NADH levels were quantified according to the protocol provided in the NAD/NADH Assay Kit (Abcam), using $2x10^5$ cells for each condition. Both NAD and NADH were calculated from a standard curve.

***In vitro* angiogenesis.** The anti-angiogenic potential of RDC11 was studied *in vitro* by looking at its ability to inhibit the generation of a capillary-like network by HUVECs. Briefly, 96-well plates were coated with ECMatrix™ (Millipore) which was allowed to polymerize at 37°C for 45 min. 5000 cells were then seeded in 200 µl of medium into each well. 1h later, cells were treated with 200 µl of medium containing cisplatin or RDC11. After 4h, cells were photographed and tube formation or intersections between cells were scored manually and expressed relative to controls.

***In vivo* angiogenesis.** The *in vivo* anti-angiogenic potential of RDC11, was assessed using the Matrigel plug assay [45]. C57BL/6 female mice (6 weeks old) were injected

subcutaneously with 600µl of cold liquid phenol-red free Matrigel (BD Bioscience) supplemented with VEGF (36ng/Matrigel plug; PeproTech), heparin (12U/Matrigel plug; Sigma), TNF-α (0.72ng/Matrigel plug; PeproTech), PBS (for controls) or RDC11 (5µM), near the abdominal midlines and at the base of the neck. The syringes containing Matrigel were kept on ice until the time of injection to prevent gelification. At 37°C (*in vivo*), Matrigel quickly polymerized to form a solid gel. After 4 days, mice were sacrificed and the Matrigel plugs explanted. Matrigel plugs were subsequently washed with PBS, photographed and then weighed. Their hemoglobin content was evaluated with the Drabkin's reagent kit according to the manufacturer's instructions (Sigma).

Intracellular reactive oxygen species (ROS) measurement

5-(and-6)-carboxy-2′,7′-dichlorodihydrofluorescein diacetate (carboxy-H_2DCFDA) (Molecular Probes) was used to detect intracellular ROS levels according to the manufacturer's instructions. For ROS quantification, cells were seeded in 96-well black plates (Greiner Bio-One) and treated with RDCs at the indicated concentrations and times. Afterwards, cells were washed with PBS and incubated with 10µM carboxy-H_2DCFDA in DPBS for 1h. Cells were then washed with PBS and fluorescence was measured by a plate reader (Perkin Elmer) with an excitation wavelength of 485 nm and an emission wavelength of 535 nm.

Results

RDC11 induces reactive oxygen species and NAD production

We have previously shown that, through their redox potential, ruthenium derived compounds are able to alter the activity of cellular redox enzymes involved in the metabolism, such as the glucose oxidase [32] [33]. To further explore a potential impact of ruthenium derived compounds on cellular metabolism we examined the effect of RDC11 on several markers of the metabolism in HCT116 human colon cancer cells. In these cells, RDC11 presents, similarly to cisplatin, an IC_{50} close to 5 µM (fig. 1A). We observed that treatment with RDC11 increased over time the amount of reactive oxygen species produced by the metabolism, starting already at 1h (fig. 1B). In addition, we checked the NAD/NADH ratio because of its importance in controlling the activity of cellular enzymes critical for cancer development, such as the PARP and the SIRT [46, 47]: as shown on Figure 1C, RDC11 produced a rapid increase in NAD production over NADH. Taken together, these data suggest that treatment with RDC11 indeed affects the cellular metabolism.

Platinum and ruthenium compounds differently impact on HIF-1 protein level and activity

We then monitored the activity of HIF-1, a key component of the signaling pathways that are sensitive to and control the cellular metabolism [37], upon RDC11 or cisplatin treatment. For

this, we followed the expression levels of its α and β subunits in colon cancer HCT116 and SW480 cells, in normoxic and hypoxic conditions. As expected, HIF-1α was not expressed or only expressed at low levels in the normoxic condition, due to its O_2-dependent prolyl hydroxylation with subsequent ubiquitylation and proteasomal degradation [38] (fig. 1D, E). RDC11, unlike cisplatin, was able to strongly reduce HIF-1α protein levels in hypoxia in both cell lines and also in normoxia in HCT116 cells after 6 and 24h of treatment. As described HIF-1β was constitutively expressed and RDC11 lowered its expression especially after 24h of treatment in normoxia and hypoxia in both HCT116 and SW480 cells.

We also checked if the drastic decrease of HIF-1 protein levels induced by RDC11 had consequences on the expression of different HIF-1 target genes [38], such as *VEGF* involved in angiogenesis or *Glut1* and *Eno1* involved in glucose metabolism. As expected, hypoxia induced the expression of these HIF-1 target genes (fig. 2). After 24h of treatment, RDC11 drastically reduced the mRNA level of all 3 genes, both in normoxia and hypoxia (fig. 2). This negative effect was already detected after 6h of treatment. Although cisplatin was able to reduce *VEGF* and *Glut1* levels in hypoxia, it was overall clearly less effective than RDC11. Altogether, these data indicated that RDC11 was more potent to reduce both HIF-1 protein levels and activity in colon cancer cells than cisplatin.

RDC11 reduces HIF-1α protein levels through multiple mechanisms

Since RDC11 drastically reduced HIF-1α protein levels, we wondered what mechanisms could be involved. Indeed, degradation of HIF-1α protein by the proteasome after hydroxylation by the iron-containing PHD2 enzyme is one of the important mechanisms regulating its cellular levels [38]. Thus, we assessed if RDC11 was still able to reduce HIF-1α levels in the presence of MG132, a potent and reversible proteasome inhibitor. As expected, in absence of RDC11, the MG132 treatment clearly stabilized HIF-1α proteins (fig. 3A,B). Strikingly, this accumulation of HIF-1α induced by MG132 was also detected but to a much lower extent in the presence of RDC11, both in normoxia and hypoxia. These results suggest that RDC11 indeed stimulates the degradation of HIF-1α proteins through the proteasome but also uses other mechanisms to drastically reduce HIF-1α proteins levels. Similar observations were made with another inhibitor of the proteasome pathway (ALLN) and inhibitors of calpaine (ALLM) and caspases (Z-VAD-FMK) were also not able to block the loss of HIF-1α proteins, suggesting that the activity of the ruthenium compound is only partially mediated by protein degradation.

However, we also noted that RDC11 tended to favor the hydroxylated form of HIF-1α (lower band) during hypoxia, in presence and in absence of MG132. To further understand the role of the hydroxylation in the RDC11-induced loss of HIF-1α, we used the iron chelator deferoxamine, which inhibits PHD enzymes (fig. 3C). As reported, deferoxamine induced HIF-1α stabilization through the accumulation of the non-hydroxylated form (upper band, fig.

3C). Upon PHD inhibition, RDC11 was still able to reduce the de-hydroxylated HIF-1α protein levels, although less efficiently. Moreover, MG132 reversed the effect of RDC11, suggesting that the loss of HIF-1α protein in this specific condition was predominantly dependent on the proteasome pathway. As these data suggested that RDC11 might affect HIF-1α hydroxylation status, we examined the expression of PHD2, the main regulator of HIF-1α, but RDC11 had no effect on PHD2 protein levels (fig. 3D).

Since RDC11 did not seem to reduce HIF-1α protein levels mainly by stimulating degradation, we also assessed its effect on mRNA levels (fig. 4). Indeed, RT-qPCR showed that RDC11, was able to strongly reduce *HIF1α* and *HIF-1β* mRNA in both normoxia and hypoxia after 24h of treatment in HCT116 cells, which is consistent with its drastic effect on HIF-1 proteins. However, the lower reduction of *HIF-1α* and *HIF-1β* mRNA observed 6h after RDC11 treatment suggests that other mechanisms also contribute to the rapid and dramatic loss of HIF-1α protein levels.

Ruthenium compounds and platinum compounds differently affect the mTOR pathway

Another important regulatory mechanism of HIF-1α protein levels acts on the control of its translation through the mTOR pathway. Indeed, the mTORC1 complex is able to activate the S6 kinase that in turn phosphorylates the ribosomal S6 protein controlling HIF-1α translation [48]. Therefore, we monitored the phosphorylation of S6. In contrast to cisplatin, RDC11 was able to strongly reduce S6 phosphorylation (S235, 236; pS6) in both normoxia and hypoxia, in HCT116 and SW480 cells, after 6 and especially 24h of treatment (fig. 5A-D). S6 protein level remained almost unchanged, suggesting a specific effect of RDC11 on the phosphorylation of S6 rather than on the total S6 protein level. This suggested a repressive effect or the ruthenium compound on the mTORC1 complex. Similar observation was done when cells were subjected to deferoxamine (fig. 5E, F). To assess the activity of the mTORC2 complex, we monitored the phosphorylation of AKT, which is a known target of this complex [48]. We observed that RDC11 reduced the phosphorylation of AKT levels after 6h of treatment in HCT116 cells (fig. 6A, B). This inhibitory effect was more pronounced when phosphorylation of AKT was enhanced using rapamycin, an inhibitor of the mTORC1 complex that stimulates the mTORC2 complex in a compensatory mechanism [49]. Importantly, RDC11 did not affect AKT levels.

To further understand how platinum and ruthenium compounds differently affect the mTOR pathway we followed the expression of some components of the mTOR protein complexes. The regulatory-associated protein of mTOR (RAPTOR) and the rapamycin-insensitive companion of mTOR (RICTOR) define mTORC1 and mTORC2 respectively and function as scaffolds for assembling the complexes and for binding substrates and regulators [50] [51]. RDC11 was able to reduce both *RAPTOR* and *RICTOR* mRNA already after 6h of treatment, while cisplatin was only efficient after 24h (fig. 6C, D).

RDC11 reduces angiogenesis both *in vitro* and *in vivo*

The ability of RDC11 to reduce mTOR, HIF-1α and *VEGF* mRNA levels led us to hypothesized that it could affect angiogenic processes. We first observed that, *in vitro,* RDC11 was able to reduce the formation of intersections between HUVECs 4h after treatment (fig. 7A) [52]. To assess the effect of RDC11 on angiogenesis *in vivo,* we used an assay in which angiogenesis was induced by different factors embedded in a Matrigel plug implanted subcutaneously in mice. 4 days after the injection, the plugs were extracted and the degree of vascularization was evaluated. The Matrigel plugs containing RDC11 were nearly uncolored, while the control plugs were redder, characteristic of the presence of small blood-filled channels (fig. 7B). Quantification of the hemoglobin content confirmed this difference. These results showed that RDC11 was able to exert an anti-angiogenic effect both *in vitro* and *in vivo*.

Combination of rapamycin and RDC11 inhibits the growth of Lewis lung carcinoma engrafted in mice

HIF-1α is frequently overexpressed in many solid tumors, included Lewis lung carcinoma, which is characterized by hypoxic regions with significant microvessels formation [53]. mTOR is the main upstream activator of HIF-1α in cancer cells and its inhibition by rapamycin [54] [55] leads to important antitumor effects which are mediated, in part, by the ability of rapamycin to prevent cellular responses to hypoxic stress [56]. Since RDC11 was able to affect both HIF-1α and mTOR *in vitro* and since rapamycin is a powerful mTOR inhibitor, we decided to verify whether the combination of RDC11 with rapamycin could increase the anticancer potential of RDC11 alone *in vivo* using the mouse lung tumor 3LL cells. We first tested the combinatory activity of RDC11 and rapamycin in vitro. A suboptimal dose of rapamycin that does not affect tumor cells growth or viability significantly increased RDC11 cytotoxicity (fig. 7C). Then, 3LL cells were implanted subcutaneously in C57BL/6 mice. After fifteen days, when tumors were about 80 mm^3, RDC11 was administered twice a week and rapamycin once a week both alone and in combination. At the end of the treatment, we observed that RDC11 reduced the volume of the tumors by nearly 40% (vs control, fig. 7D). Interestingly, the combination RDC11-rapamycin showed to be more effective in reducing the tumor volume by 56% compared to control. This result indicated that there was a synergistic effect between RDC11 and rapamycin, leading to an enhanced antitumor activity.

Discussion

Platinum-based drugs have greatly contributed to the treatment of cancer despite their severe side effects and drug-resistance phenomena. To overcome these drawbacks, platinum-free metal-based drugs have been thoroughly studied. Others and us have already identified some biological properties of different ruthenium-derived compounds, showing a lack of a strict correlation between DNA damage and their cytotoxicity [17]. In particular, we have previously shown that these compounds are able to alter the activity of redox enzymes that are involved in the cellular metabolism, which seems to correlate with their cytotoxicity [33] [57]. However, the physiological consequences of these alterations are still largely unknown. In this study, we have comparatively investigated the effect of ruthenium and platinum derived compounds on the Hypoxia-Inducible Factor 1 (HIF-1) and the mammalian Target of Rapamycin (mTOR) pathways that are two of the major signaling pathways that are sensitive and that control the cellular metabolism.

We first confirmed that the ruthenium compound RDC11 can alter the cellular metabolism, indicated by an increase in the ROS level (fig. 1, 8), as previously reported [34, 35]. This alteration of the cellular metabolism is further supported by our observation of an increase in the NAD/NADH ratio. This modification of the cellular metabolism might be a direct reflection of the uncontrolled reduction of cellular redox enzymes that have a redox potential slightly above the one of the ruthenium compounds [57]. As we have previously showed that the optimal redox potential for RDCs is between 0.3V and 0.6V, the redox enzymes or cofactors should have a redox potential above 0.3V to be reduced [33]. In addition, bioinformatic modeling studies indicated that the redox center of the ruthenium compound should be at no more that 3Å from the redox center of the enzyme to be targeted (AD Ryabov, personal communication).

This RDC11-induced perturbation of the cellular metabolism impacted on signaling pathways, namely on the HIF-1 pathway. Indeed, HIF-1α protein levels were dramatically reduced by RDC11 both in normoxia and hypoxia (fig. 1). Interestingly, cisplatin was less efficient. This points out that platinum- and ruthenium-based molecules can act differently, even if sharing similar chemical features. Moreover, RDC11 was able to reduce HIF-1β protein levels. This downregulation of both HIF-1 subunits correlated with a decrease in the expression of several HIF-1 target genes, linked to angiogenesis or glucose metabolism (fig. 2). On the contrary, cisplatin showed only a minor effect on HIF-1 target genes, suggesting again a different mode of action for the two types of drugs.

We investigated the mechanism of HIF-1α protein decrease by RDC11 and observed that the drug could act at multiple levels. Indeed, we found that RDC11-induced loss of HIF-1α proteins involved reduction of *HIF-1α* mRNA levels, increase of HIF-1α protein degradation and probably reduction of HIF-1α protein synthesis. RDC11 drastically decreased *HIF-1α* mRNA levels within 24h (fig. 4), suggesting repression of the *HIF-*

1α promoter or destabilization of the *HIF-1α* mRNA by a yet unknown mechanism. However, the progressive reduction of *HIF-1α* mRNA levels cannot account for the dramatic loss of HIF-1α proteins already observed 6h after RDC11 treatment, suggesting the involvement of other mechanisms, such as enhanced degradation by the proteasome pathway. This is consistent with the fact that HIF-1α is tightly regulated by this mechanism during the shift between normoxia and hypoxia [38]. However, the inhibition of the proteasome only slightly impacted on the ability of RDC11 to reduce HIF-1α protein levels, both in normoxia and hypoxia (fig. 3A), suggesting that enhanced degradation is not the prominent mechanism involved. Nevertheless, in the presence of the iron chelator deferoxamine, the effect of RDC11 was almost abolished after blocking the proteasome (fig. 3C), suggesting that, in some conditions, RDC11 acts through degradation. Deferoxamine has been used to mimic hypoxia, since it stabilizes HIF-1α proteins by blocking PHD2 enzyme activity. Interestingly, we observed that RDC11 favored the accumulation of the hydroxylated form of HIF-1α, without any effect on the protein level of PHD2 (fig. 3D). Since this enzyme contains an iron atom [38], it is tempting to imagine that the redox potential of RDC11 might interfere with this enzyme, maybe stimulating its activity somehow. Of note, degradation by other proteases (caspases, calpains; fig. 3B) does not seem to play a role in RDC11-dependent loss of HIF-1α.

Lastly, RDC11 might also inhibit HIF-1α translation. Indeed, we found that RDC11, in contrast to cisplatin, reduced the amount of the active form of the S6 protein (fig. 5) crucial for HIF-1α translation [38]. In the same line, we had shown previously that RDC11 induces the ER stress pathway, which is a negative regulator of mRNA translation [17].

In addition to HIF-1, RDC11 altered the activity of another, interrelated, crucial pathway for the cellular metabolism, i.e. the mTOR pathway. Indeed, we found that the activities of the mTORC1 and mTORC2 complexes, respectively tested by checking the phosphorylation of the S6 protein and AKT (fig. 5-6), was reduced upon RDC treatment. The repression of the mTOR complexes induced by RDC11 correlated with a diminution of *Raptor* and *Rictor* mRNA expression. This transcriptional regulation of the two components of the mTOR complexes might account for the reduced activity of the mTOR pathway. Alternatively, elevated NAD and sustained elevated ROS observed upon RDC11 treatment might enhance the activity of the AMPK, which can downregulate the activity of the mTORC1 complex [58-61].

The new information gathered on the mode of action of ruthenium compounds through this study is of interest for anticancer therapy. Indeed, we found that key actors of the tumorigenesis are affected by RDC11. The HIF-1 pathway plays a prominent role in the nutritional status of cancer cells and make them able to adapt to oxygen deprivation: consistently, we observed that RDC11 treatment reduced VEGF synthesis and angiogenesis (fig. 7). The downregulation of AKT phosphorylation, i.e. of its activity, is also an important

observation as AKT acts as a survival kinase in many cell types and is often constitutively activated in cancer cells [62]. The mTOR complexes themselves are generally considered as interesting targets for anticancer therapy due to their role in survival and cell growth. For example, rapamycin, an inhibitor of the mTORC1 complex, is tested for treating various cancers [63]. Interestingly, we observed that RDC11 acts synergistically with sub-optimal doses of rapamycin, likely because it inhibits both mTORC1 and mTORC2 (fig. 7C, D), which would represent an advantage for treating cancer.

When considering the platinum versus the ruthenium complexes, this study highlights a differential regulation of the mTOR and the HIF-1 pathways. This observation further supports our precedent findings that these two classes of organometallic compounds rely on different mechanisms of action. Platinum compounds act through DNA, while ruthenium compounds have a more complex mode of action, involving partly DNA, but also an alteration of the activity of redox enzymes involved in the cellular metabolism. In turn, alteration of the cellular metabolism impacts on the regulation of specific signaling pathways, such as the HIF-1 and mTOR pathways, which normally ensure a cellular homeostasis. The identification of the direct target of the ruthenium complexes represents an interesting and crucial challenge. Solving this challenge will pave the way for the design of more potent and more selective drugs that might target precise enzymatic activities essential for cancer cells survival, which remains the main obstacles of cancer chemotherapy today.

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Figure legends

Figure 1. RDC11 modulates ROS production, NAD/NADH ratio and represses Hif-1 protein levels.

A, HCT116 cells were treated for 48h at the indicated concentrations (μM) of cisplatin and RDC11. Cell viability was determined using the MTT test. Data are representatives of three independent experiments. Bars are means and asterisks indicate statistically significant difference (*=p<0.001) compared to control, as calculated by a One-Way ANOVA test followed by a Tukey post-test over the three independent experiments.

B. HCT116 cells grown on coverslips coated with polyornithine were treated with RDC (5μM) for the indicated time and labeled with carboxy-H2DCFDA. Fluorescence was quantified with a fluorimeter. Bars are mean and asterisks indicate statistically significant difference (p< 0.01) compared to control, as calculated by a one-way ANOVA test followed by a Tuckey post-test over the three independent experiments.

C. RDC11 increases the NAD/NADH ratio. $2x10^5$ HCT116 cells were treated with RDC11 (5μM) for the indicated time. NAD and NADH were subsequently quantified following the manufacturer's protocol. Both NAD and NADH were calculated from a standard curve (***=p<0.001; One-Way ANOVA + Tukey post-test).

D and **E**, Western blot analysis of HCT116 (D) and SW480 (E) colorectal cancer cells treated with 5μM of cisplatin or 5μM of RDC11 for the indicated time in normoxic (20% O_2) or hypoxic (1% O_2) conditions. Immunoblotting was performed with anti-HIF-1α, anti-HIF-1β and anti-actin antibodies.

Figure 2. RDC11 reduces mRNA levels of different *HIF1α* target genes. HCT116 cells were treated with 5 μM of cisplatin or 5 μM of RDC11 for the indicated time in normoxic (20% O_2) or hypoxic (1% O_2) conditions. RT-qPCR was performed using primers for *VEGF*, *GLUT1*, *ENO1*, and *18S* as housekeeping gene. Data represent relative change in the expression of the different genes in comparison with untreated cells (Ct) and were normalized with *18S*. Columns, means of triplicates; bars, SD. Asterisks indicate statistically significant difference (***=p<0.001; **=p<0.01; *=p<0.05) compared to control, as calculated by a One-Way ANOVA test followed by a Tukey post-test over the three independent experiments.

Figure 3. HIF-1α protein reduction involves only partly protease-dependent degradation

A, **B**, Western blot analysis of HCT116 colorectal cancer cells treated with 5 μmol/L of RDC11 for 6h in normoxic (A, 20% O_2) or hypoxic (B, 1% O_2) conditions, in absence or in presence of the indicated protease inhibitors (MG132, 10μM; ALLM, 10μM; ALLN, 10μM; Z-VAD-FMK, 20μM). Immunoblotting was performed with anti-HIF-1α and anti-actin antibodies.

Note the presence of two bands for HIF-1α, the upper band that correspond to the non-hydroxylated form and the lower band that is the hydroxylated form.

C. HCT116 cells were treated overnight with deferoxamine mesylate (DFO, 150μM) to chemically induce hypoxia and subsequently for six hours with RDC11 5μM together or not with MG132 10μM. Western blot was done using anti-HIF-1α and anti-actin antibodies.

D, HCT116 cells were treated overnight with 5μM of RDC11 in normoxia (20% O_2) or in chemically-induced hypoxia using deferoxamine mesylate (DFO, 150 μM). Western blot was performed using anti-PHD2 and anti-actin antibodies.

Figure 4. Differential effect of platinum and ruthenium derived compounds on HIF-1 mRNA levels.

A, **B,** expression of *HIF1α* (A) and *HIF1β* (B) mRNA in HCT116 cell treated with 5μM of cisplatin or 5μM of RDC11 at the indicated time in normoxic (20% O_2) or hypoxic (1% O_2) conditions, determined by RT-qPCR. Data represent relative change in the expression of the different genes in comparison with untreated cells (Ct) and were normalized to *18S* expression. Columns, means of triplicates; bars, SD. Asterisks indicate statistically significant difference (***=p<0.001; **=p<0.01; *=p<0.05) compared to control, as calculated by a One-Way ANOVA test followed by a Tukey post-test over the three independent experiments.

Figure 5. Differential effect of platinum and ruthenium derived compounds on the activity of the mTORC1 complex.

A, B, Western blot analysis of HCT116 (A) and SW480 (B) colorectal cancer cells treated with 5μM of cisplatin or 5μM of RDC11 at the indicated time in normoxic (20% O_2) or hypoxic (1% O_2) conditions. Immunoblotting was performed with anti-pS6, anti-S6 and anti-actin antibodies. **C**, **D,** Quantification of the image presented in A and B using Molecular Imager® ChemiDoc™ XRS+ System (Bio-Rad Laboratories). Bar indicate the intensity of the bands relative to the control.

E, F, HCT116 cells were treated overnight with 5μM of RDC11 in normoxia (20% O_2) or in chemically-induced hypoxia using deferoxamine mesylate (DFO) 150μM. F is the quantification of the image presented in respectively in E using Molecular Imager® ChemiDoc™ XRS+ System (Bio-Rad Laboratories). Bar indicate the intensity of the bands relative to the control.

Figure 6. Differential effect of platinum and ruthenium derived compounds on the activity of the mTORC2 complex

A, **B,** HCT116 cells were treated with 5μM of RDC11 or 100 nmol/L rapamycin (Rap) or both for 6h. Immunoblotting was done with an anti-pAKT and anti-AKT antibodies to distinguish phosphorylated AKT and total AKT. Anti-actin antibody was used as loading control. B is the

quantification of the image presented in respectively in A using Molecular Imager® ChemiDoc™ XRS+ System (Bio-Rad Laboratories). Bar indicate the intensity of the bands relative to the control.

C, D, HCT116 cell were treated with 5µM of cisplatin or 5µM of RDC11 for the indicated time in normoxic (20% O_2) or hypoxic (1% O_2) conditions. RT-qPCR was performed using primers for *RAPTOR* (C), *RICTOR* (D) and *18S* as housekeeping gene. Data represent relative change in the expression of the different genes in comparison with untreated cells (Ct) and were normalized to *18S* expression. Columns, means of triplicates; bars, SD. Asterisks indicate statistically significant difference (***=p<0.001; **=p<0.01; *=p<0.05) compared to control, as calculated by a One-Way ANOVA test followed by a Tukey post-test over the three independent experiments.

Figure 7. RDC11 inhibits angiogenesis both *in vitro* and *in vivo*.

A. HUVECs were seeded on ECMatrix™ and 1h later treated with cisplatin 5 µM and RDC11 2.5µM and 5µM. After 4 hours, cells were photographed and tube formation or intersections between cells were scored manually and expressed relative to controls. (**=p<0.01; *=p<0.05 vs Control; One-Way ANOVA + Tukey post-test)

B. C57BL/6 mice (6 week old) were injected s.c. three times with 600 µl of cold liquid Matrigel supplemented with VEGF (36ng/Matrigel plug), heparin (12U/Matrigel plug), TNF-α (0.72ng/Matrigel plug), PBS (for controls) or RDC11 (5µM). After 4 days, mice were killed and the Matrigel plugs explanted. Quantification of the hemoglobin content was performed with the Drabkin's reagent kit.

C. Suboptimal concentration of rapamycin favors RDC11 cytotoxicity. HCT116 cells were treated in 96-well plates for 48h at the indicated concentrations of RDC11, in absence or in presence of an inactive dose of rapamycin (10µM). Cell viability was determined using the MTT test. Data are representatives of three independent experiments. Bars are mean and asterisks indicate statistically significant difference (*=p<0.001) compared to control, as calculated by a One-Way ANOVA test followed by a Tukey post-test over the three independent experiments.

D. RDC11 and rapamycin inhibit the growth of Lewis lung carcinoma engrafted in mice better than RDC11 alone. C57BL/6 mice (8 week old) were injected s.c. with $6x10^5$ 3LL cells. When tumors were palpable (15 days post-injection), for RDC11 13µmol/kg and rapamycin 3,28 µmol/kg, alone or in combination, were administered to mice following this protocol: RDC11 twice a week and rapamycin once a week both alone and in combination. Bar represented the volumes of the tumors at the end of the treatment (day 30 post-injection). (n=10; *=p<0.01 vs Control; One-way ANOVA + Tukey post-test).

Figure 8. Schematic representation of the regulation of the cellular metabolic pathways by ruthenium derived compounds

Figure 1

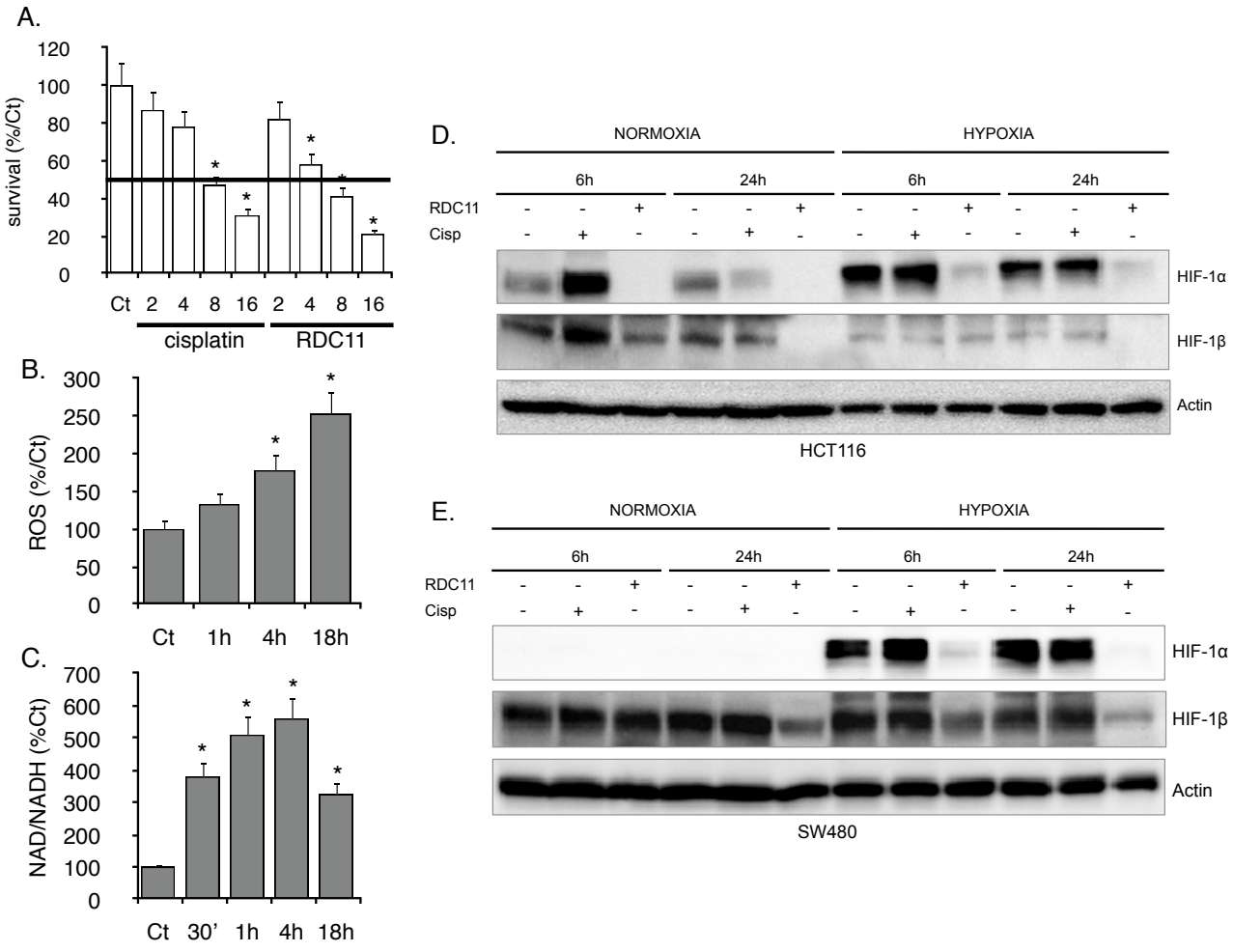
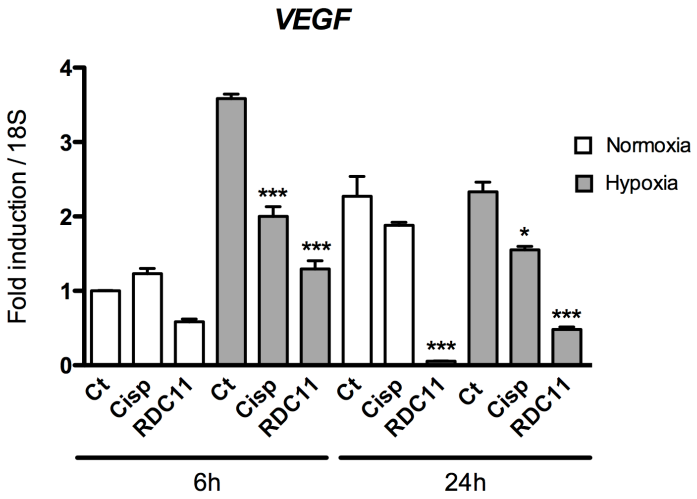
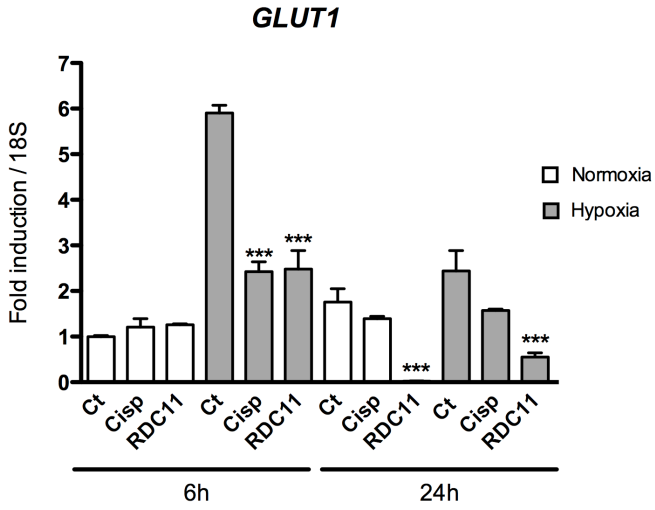

A.
survival (%/Ct)
120
100
80
60
40
20
0
Ct 2 4 8 16 2 4 8 16
cisplatin
RDC11
B.
ROS (%/Ct)
300
250
200
150
100
50
0
Ct 1h 4h 18h
C.
NAD/NADH (%Ct)
700
600
500
400
300
200
100
0
Ct 30' 1h 4h 18h
D.
NORMOXIA
HYPOXIA
6h
24h
RDC11
Cisp
HIF-1α
HIF-1β
Actin
HCT116
E.
NORMOXIA
HYPOXIA
6h
24h
RDC11
Cisp
HIF-1α
HIF-1β
Actin
SW480

Figure 2


VEGF
Fold induction / 18S
Normoxia
Hypoxia
Ct
Cisp
RDC11
6h
24h


GLUT1
Fold induction / 18S
Normoxia
Hypoxia
Ct
Cisp
RDC11
6h
24h

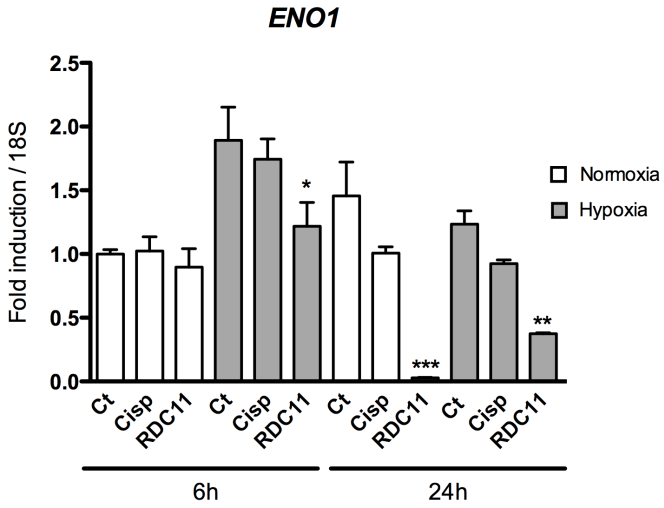

ENO1
Fold induction / 18S
Normoxia
Hypoxia
Ct
Cisp
RDC11
6h
24h

Figure 3

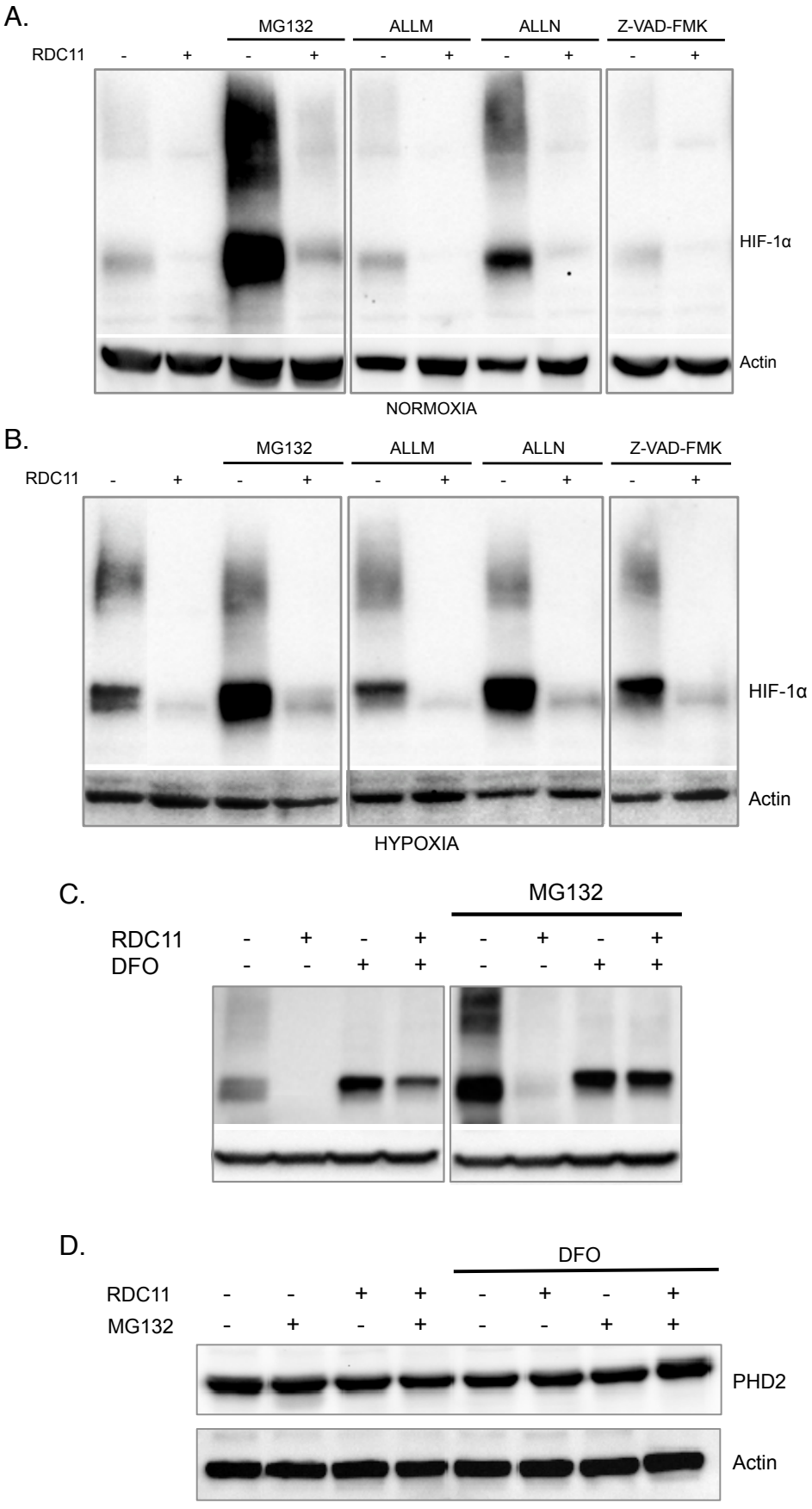
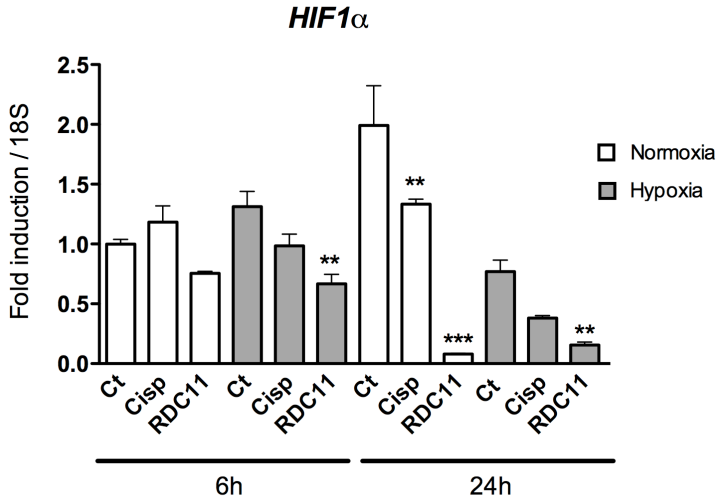

A.
MG132
ALLM
ALLN
Z-VAD-FMK
RDC11
- + - + - + - + - +
HIF-1α
Actin
NORMOXIA
B.
MG132
ALLM
ALLN
Z-VAD-FMK
RDC11
- + - + - + - + - +
HIF-1α
Actin
HYPOXIA
C.
MG132
RDC11 - + - + - + - +
DFO - - + + - - + +
D.
DFO
RDC11 - - + + - + - +
MG132 - + - + - - + +
PHD2
Actin

Figure 4


HIF1α
Fold induction / 18S
2.5
2.0
1.5
1.0
0.5
0.0
Normoxia
Hypoxia
**
**
***
**
Ct
Cisp
RDC11
Ct
Cisp
RDC11
Ct
Cisp
RDC11
Ct
Cisp
RDC11
6h
24h

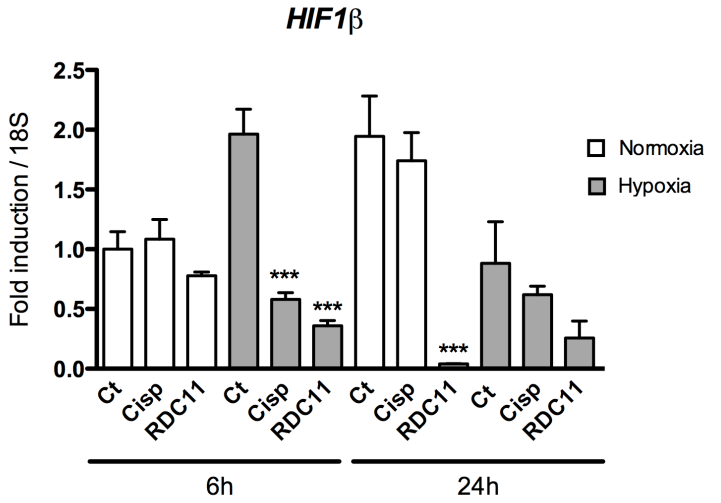

HIF1β
Fold induction / 18S
2.5
2.0
1.5
1.0
0.5
0.0
Normoxia
Hypoxia
***
***
***
Ct
Cisp
RDC11
Ct
Cisp
RDC11
Ct
Cisp
RDC11
Ct
Cisp
RDC11
6h
24h

Figure 5

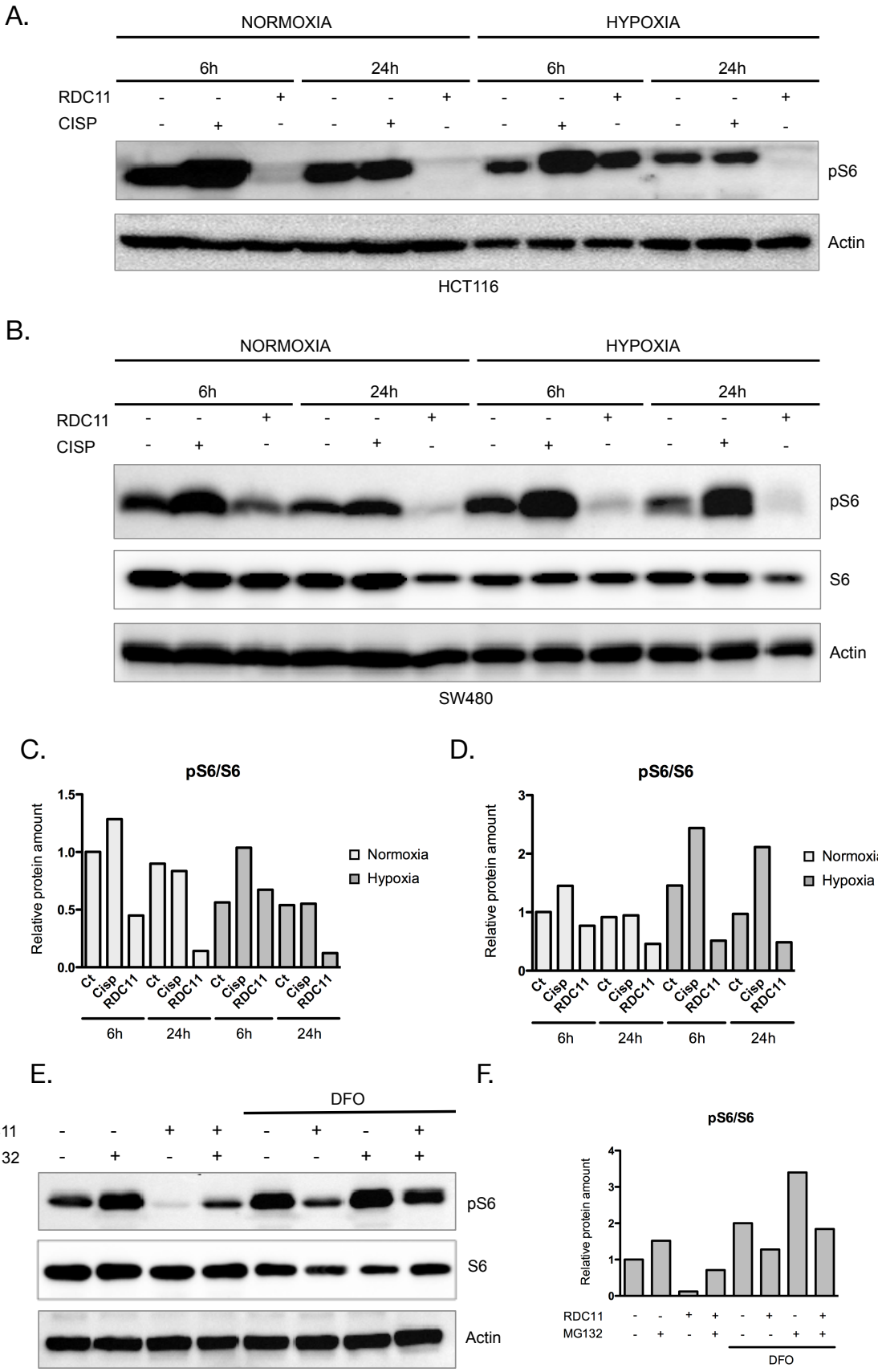

A.
NORMOXIA
HYPOXIA
6h
24h
6h
24h
RDC11
CISP
pS6
Actin
HCT116
B.
NORMOXIA
HYPOXIA
6h
24h
6h
24h
RDC11
CISP
pS6
S6
Actin
SW480
C.
pS6/S6
Relative protein amount
Normoxia
Hypoxia
Ct
Cisp
RDC11
6h
24h
D.
pS6/S6
Relative protein amount
Normoxia
Hypoxia
E.
DFO
RDC11
MG132
pS6
S6
Actin
F.
pS6/S6
Relative protein amount
RDC11
MG132
DFO

Figure 6

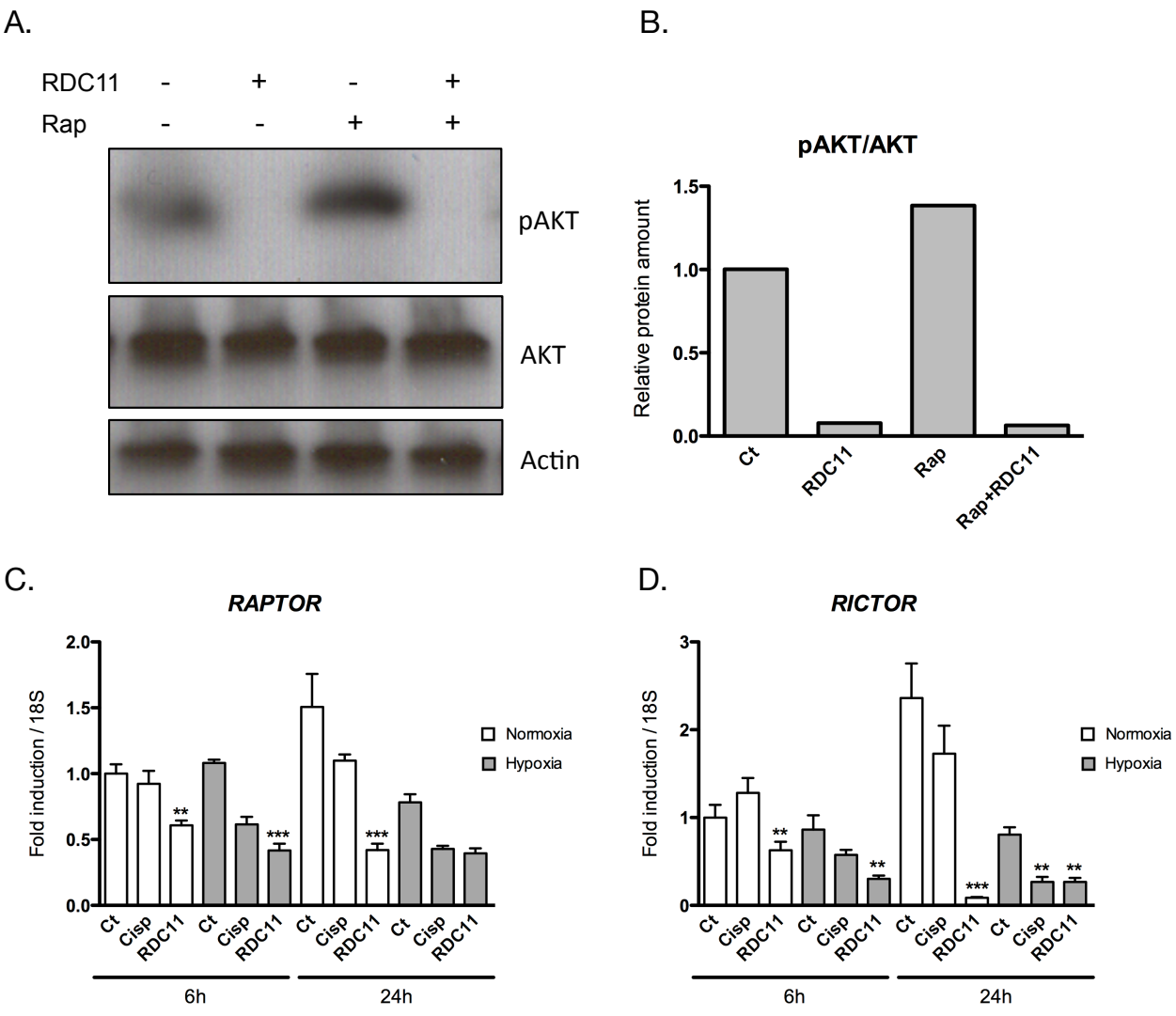

A.
RDC11 - + - +
Rap - - + +
pAKT
AKT
Actin
B.
pAKT/AKT
Relative protein amount
1.5
1.0
0.5
0.0
Ct
RDC11
Rap
Rap+RDC11
C.
RAPTOR
Fold induction / 18S
2.0
1.5
1.0
0.5
0.0
Normoxia
Hypoxia
**
***
***
Ct
Cisp
RDC11
6h
24h
D.
RICTOR
Fold induction / 18S
3
2
1
0
Normoxia
Hypoxia
**
**
***
**
**
Ct
Cisp
RDC11
6h
24h

Figure 7

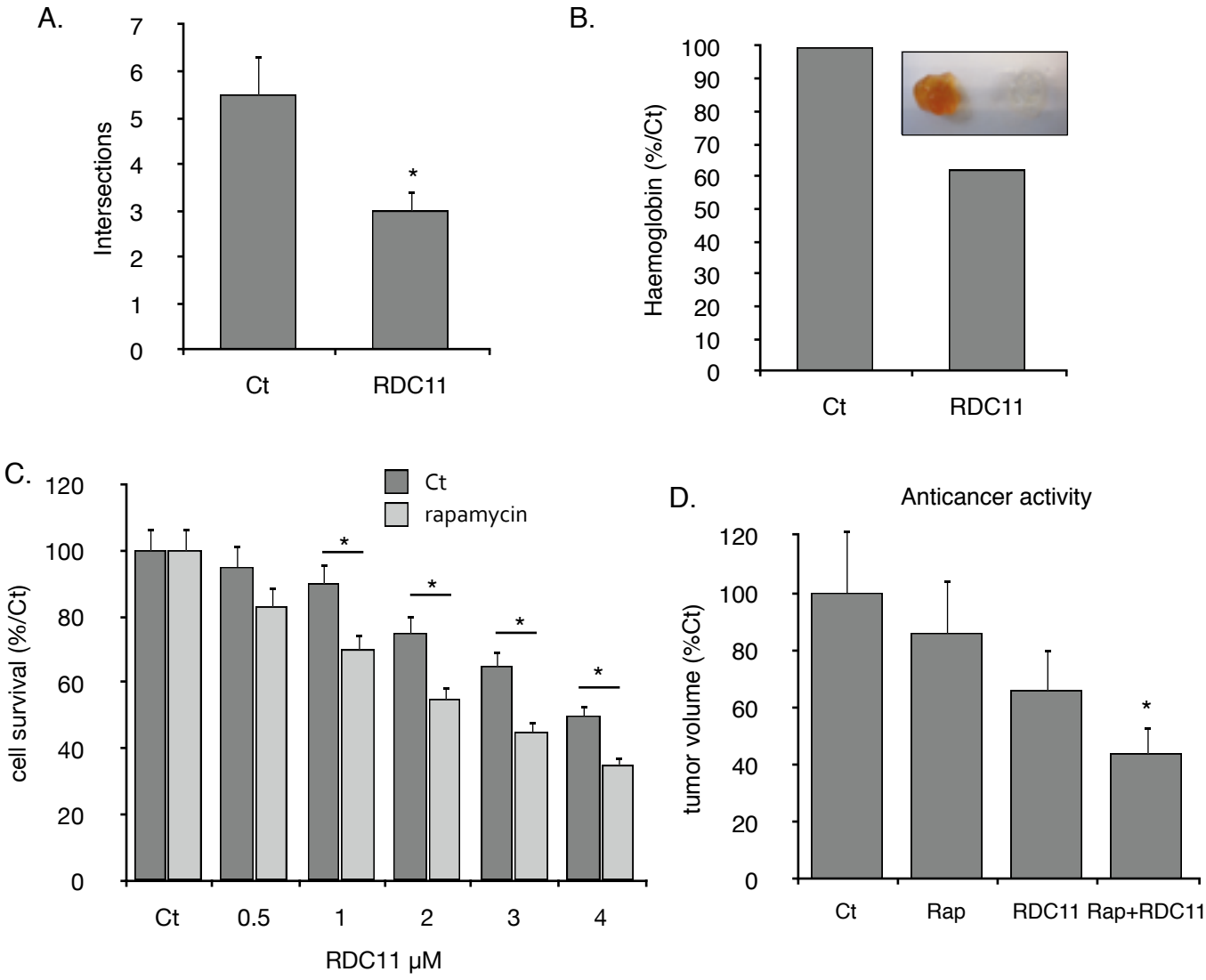
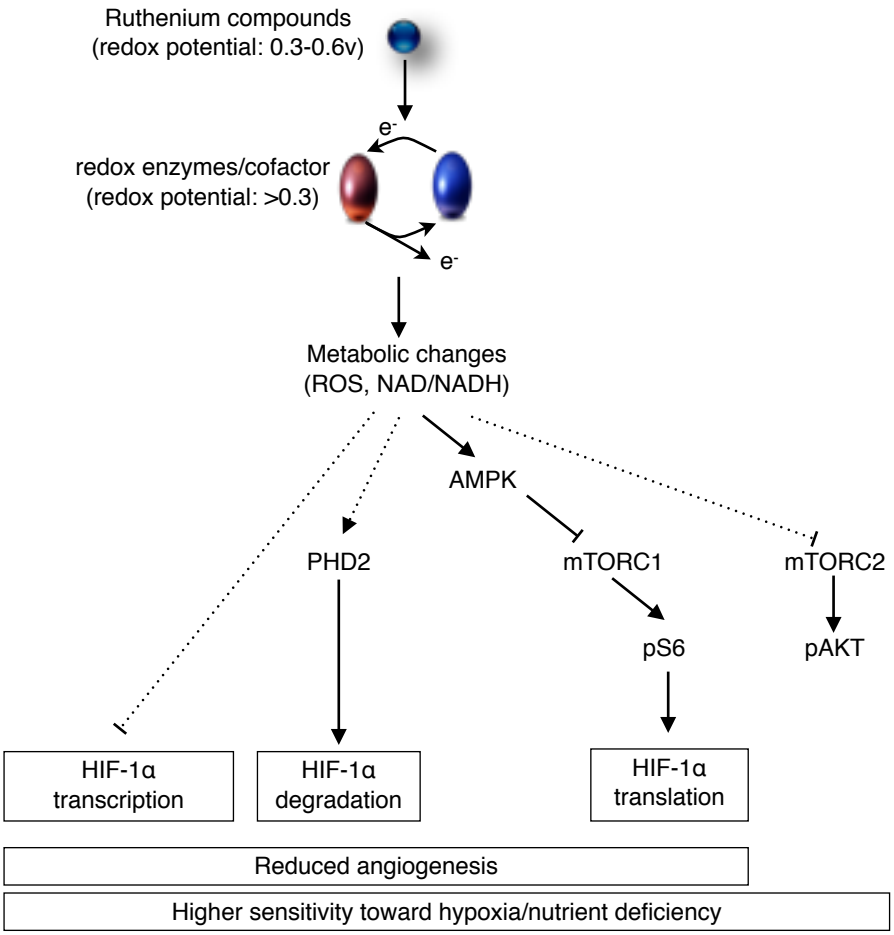

A.
Intersections
0
1
2
3
4
5
6
7
*
Ct
RDC11
B.
Haemoglobin (%/Ct)
0
10
20
30
40
50
60
70
80
90
100
Ct
RDC11
C.
cell survival (%/Ct)
0
20
40
60
80
100
120
Ct
rapamycin
*
*
*
*
Ct
0.5
1
2
3
4
RDC11 µM
D.
Anticancer activity
tumor volume (%Ct)
0
20
40
60
80
100
120
*
Ct
Rap
RDC11
Rap+RDC11

Figure 8


Ruthenium compounds
(redox potential: 0.3-0.6v)
e-
redox enzymes/cofactor
(redox potential: >0.3)
e-
Metabolic changes
(ROS, NAD/NADH)
AMPK
PHD2
mTORC1
mTORC2
pS6
pAKT
HIF-1α
transcription
HIF-1α
degradation
HIF-1α
translation
Reduced angiogenesis
Higher sensitivity toward hypoxia/nutrient deficiency

4.2 | New generation of anticancer ruthenium-derived compounds with improved solubility and cytotoxicity: role of radical oxidized species and caspase 8.

To date, two ruthenium derivates, namely NAMI-A and KP1019, have entered and passed early-phase clinical trials [Rademaker-Lakhai JM et al, 2004; Hartinger CG et al, 2006]. Following this success, no more ruthenium-based molecules have been submitted for clinical testing, basically because of some limitations, such as reduced water solubility, stability in aqueous solutions and not striking cytotoxicity.

Therefore, one of the principal aims to get better compounds for clinical use is to improve these parameters. Ruthenium-derived compounds (RDCs) have shown to be very stable molecules, thanks to the presence of a covalent bond between the atom of ruthenium and the atom of carbon of the ligand. The interesting *in vitro* and *in vivo* results accumulated with RDC11 in treating cancer have prompted us to develop other RDCs in order to ameliorate RDC11 solubility and cytotoxicity. Moreover, since it has already been shown that some RDCs, thanks to their redox potential, are able to affect the activity of specific redox enzymes involved in cell metabolism, we decided to investigate whether the $Ru^{III/II}$ redox potential could play a key role in defining RDCs cytotoxicity. The new generated RDCs have been studied both *in vitro* and *in vivo* in order to understand whether, by modifying ligands and thus parameters such as solubility and redox potential, it is possible to improve their effectiveness. In particular, since the DNA damage and endoplasmic reticulum stress pathways have already been shown to be regulated by RDCs, we decided to focus our attention on the effect of RDCs on these and also new pathways that could account for RDCs cytotoxicity. Results of this work were discussed in the following manuscript:

Vidimar V, Meng X, Mroczek M, Licona C, Fetzer L, Jenny M, Leyva M, Harlepp S, Hebraud P, Sirlin C, Mellitzer G, Loeffler JP, Sava G, Pfeffer M, Gaiddon C. **New generation of anticancer ruthenium-derived compounds with improved solubility and cytotoxicity: role of radical oxidized species and caspase 8.**

New generation of anticancer ruthenium-derived compounds with improved solubility and cytotoxicity: role of reactive oxygen species and caspase 8

Vania Vidimar[1,4,8#], Xiangjun Meng[5,7,8#], Marcelina Mroczek[3,8#], Cynthia Licona[1,8], Ludivine Fetzer[2,8], Marjorie Jenny[1,8], Mili Leyva[2,7,8], Sébastien Harlepp[3,8], Pascal Hébraud[3,8], Claude Sirlin[2,8], Georg Mellitzer[1,8], Gianni Sava[4], Michel Pfeffer[2,8], Christian Gaiddon[1, 8*]

[1]UMRS682 INSERM, Strasbourg, France
[2]UMR 7177 CNRS, Institut de Chimie, Strasbourg, France
[3]UMR 7504 CNRS, IPCMS, Strasbourg, France
[4]University of Trieste, Department of Life Sciences, and Callerio Foundation Onlus, Trieste, Italy
[5]Department of Gastroenterology, Shanghai First People's Hospital, School of Medicine, Shanghai Jiao Tong University
[6] IGBMC, Illkirch, France
[7] UMRS692 INSERM, Strasbourg, France
[8] Université de Strasbourg, Strasbourg, France

# Authors contributed equally to this work

Running title: Anticancer ruthenium compounds induce caspase 8

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*Corresponding Author: Gaiddon Christian, U682 INSERM - Université de Strasbourg, 3 avenue Molière, Strasbourg 67200, France. Phone: +33 03 88 27 77 27**,** Fax: +33 03 88 26 35 38, Email Address: gaiddon@unistra.fr

Abstract

Organometallic compounds which contain metals, such as ruthenium or gold, have been investigated as a replacement for platinum-derived anticancer drugs. They often show good antitumor effects, but the identification of their precise mode of action or their pharmacological optimization is frequently difficult. We have previously described a class of ruthenium(II) compounds with interesting anticancer properties. In comparison to cisplatin, these molecules have lower side effects, a reduced ability to interact with DNA, and they induce cell death in absence of p53 through CHOP/DDIT3. We have now optimized these molecules by improving their cytotoxicity and their water-solubility. We show that these optimized molecules reduce tumor growth in different mouse models and that they are more potent inducers of cancer cell death through the production of reactive oxygen species and activation of caspase 8, while retaining their ability to induce CHOP/DDIT3. Altogether our data suggest that water-soluble ruthenium(II)-derived compounds represent an interesting class of molecules that, depending on their structures, can target several pro-apoptotic signaling pathways.

Introduction

Cancer remains one of the first causes of death in industrialized countries, and the successes of its eradication results in many instances from improved diagnosis (early detection) and surgery rather than the treatment with drugs. The most frequent reasons that explain the failure of drug therapy are the existence of resistance mechanisms and the toxicity towards healthy tissues. Nevertheless, few examples of successful chemotherapy are still driving the search for new, more potent, more selective, less prone to resistance and better tolerated drugs. One of these examples is cisplatin, a drug showing a significant role in the management of a number of tumors [1-3]. However, cisplatin and more in general platinum-derived drugs display an often-severe toxicity (mainly neurotoxicity, nephrotoxicity) and a relatively frequent emergence of natural- or induced-resistance [4-6]. More recently, a significant interest was addressed to ruthenium-based drugs, because of some favorable properties that make them a suitable basis for the development of antitumor drugs, such as the oxidation state, the ligand exchange and the binding to biologically relevant proteins that make them suitable for the development of antitumor drugs. Compared to many organic compounds, ruthenium-based drugs also offer the advantage of a relatively low cost for their synthesis and purification.

Various ruthenium complexes were shown to present cytotoxicity against cancer cells, ligand-exchange abilities similar to those of platinum complexes, no cross-resistance with cisplatin and a reduced toxicity against healthy tissues at least in part explained by the selective transportation to cancer cells by the iron transport system [1-3, 7]. Following the pioneering work with ruthenium red [8] a number of ruthenium-based drugs were shown to

endow antitumor potential. Indeed, several teams have synthesized and characterized compounds containing ruthenium in oxidative state (II) or (III), showing their anticancer activity [9] [10] [11-16]. Two of these compounds, namely NAMI-A and KP1019, have successfully passed some initial phases of clinical trials [17] [18]. Beside these partial successes, the emergence of new ruthenium-based therapies have been slowed down by some limitations, such as a relatively poor level of solubility, and/or stability in aqueous solutions, a not impressive cytotoxicity (IC_{50} between 20 and 100 µM), and an uncertainty on the molecular mechanisms of action responsible for the antitumor effect.

The mechanism of action and the direct targets of ruthenium-based drugs are still a matter of debate. Indeed, depending on the drug, several modes of action have been proposed, such as interaction with DNA and activation of DNA damage pathways [19-21] [22-24], production of reactive oxygen species [25], inhibition of kinases [26], or of other enzymatic activities [27] [28], including extracellular metallo-proteases [29]. The differences observed may be due to variations in their structure. Even if most of the ruthenium-containing compounds have ligands that are relatively weakly bound to the metal via a heteroatom (N, O, S), there are differences in the types of ligands attached.

In order to improve the stability of ruthenium complexes and possibly to enhance their cytotoxicity and their pharmacokinetics, we have previously generated several ruthenium-based complexes in which the ligand is bound to the metal *via* strong covalent bonds such as a C-M σ bond [22, 30]. Beside the stability, these compounds present differences in their redox factor and a new variety of ligands. We have called these molecules RDCs (Ruthenium-Derived Compounds) and we have previously shown that several RDCs are cytotoxic *in vitro* for several cancer cell lines resistant to cisplatin [22]. One of them, RDC11, showed a good antitumor activity both *in vitro* and *in vivo* [31] with an IC_{50} often between 2 and 5 µM, and anticancer properties on models of ovarian cancer, melanomas and gliomas. Importantly, they showed, compared to cisplatin a reduced toxicity toward healthy tissues in vivo. We demonstrated that RDC11 exerts its antitumor effect via DNA-dependent and DNA-independent modes of action. We also identified one of the DNA-independent signaling pathways by showing an activation of the endoplasmic reticulum stress pathway, and in particular the transcription factor CHOP/DDIT3. However, the silencing of CHOP/DDIT3 by siRNA was not able to completely abolish RDC11 cytotoxicity, strongly suggesting that other signaling pathways are also involved.

In the present study, we developed RDC11 variants designed for improving their cytotoxicity and the solubility. The biological properties of these novel ruthenium-based organometallics are studied *in vitro* and *in vivo*, with a particular attention to the modulation of the pathways that RDC11 have been shown to regulate: the DNA damage pathways and induction of CHOP/DITT3. In addition, we also investigated novel regulations of pathways that could account for some of the cytotoxic activity of RDCs. For this, we focused on the production of reactive oxygen species and the induction of an extrinsic apoptotic pathway involving

caspase 8. Our results show that compared to RDC11, the novel RDC variants are more cytotoxic in vitro, with an IC_{50} in the nanomolar range, and that they induce strongly the production of ROS and caspase 8, which are required for their activity.

Results

Generating ruthenium compounds with an IC_{50} in the nanomolar range

On the basis of previously published structure function studies showing an improved cytotoxicity of RDCs when a phenanthroline was used as a ligand [22], we decided to substitute the two acetonitrile ligands of RDC11 by a second phenanthroline, naming the new molecule RDC34 (fig. 1A). An equivalent of RDC34 with another counter-ion (PF^{6-}) was also synthesized (RDC37). Previous works have established the ability of RDCs to modulate the activity of cellular enzymes through their redox potential [37]. We therefore modified the redox potential of RDC34 by adding on the phenylpyridine ligands a NO_2 (electron with drawing) or a NH_2 unit (electron releasing group), this leading to RDC40 and RDC41 respectively. Finally, in order to increase the water solubility of RDC34 (that slightly improved with the NH_2 group in RDC41), we added to the phenylpyridine ligand a spermine unit, this leading to RDC44, that proved indeed to be nicely water soluble (water solution of RDC44 of up to 25 mM were obtained) [38].

As colon cancers are one of the indications for platinum-derived treatments, the cytotoxicity of the RDC was first tested on a human colon cancer cell lines (HCT116). Cell survival was estimated using the MTT test after 48 hours of treatment. As previously observed, RDC11 has a cytotoxicity similar to cisplatin (IC_{50} between 4-8 µM) (fig. 1). Interestingly, RDC34 and RDC37, two derivatives that share the same structure around the ruthenium center but have a different counter-ion, and RDC40, characterized by the presence of the NO_2 group, showed an increased cytotoxicity (IC_{50} < 2 µM) (fig. 1). However, the addition of the NH_2 group decreased the cytotoxic activity (IC_{50} = 2-4 µM, RDC41) and that of the spermine moiety had an even worse effect with rising the IC_{50} to over 16 µM. Additional experiments gave IC_{50s} for RDC34, RDC40 and RDC41 of respectively 0.25 µM, 0.75 µM and 2-4 µM (supplementary data).

These results confirmed our hypothesis and put forward that with an additional phenanthroline the cytotoxicity of RDCs can strongly be improved, to an IC_{50s} in the nanomolar range. In addition, the redox potential and the solubility can further modulate RDC cytotoxicity for HCT116 cells *in vitro*.

Effects of the hydrophilic spermine substituent chain on cytotoxicity, cell uptake and DNA binding

The addition of the spermine chain to RDC34 (leading to RDC44) significantly decreased the cytotoxicity for HCT116 cells, indicating that improving the solubility in water does not necessarily lead to an increased cytotoxic activity (fig. 1). In order to understand the reason for this behaviour, we tested RDC34 and RDC44 cellular uptake. The presence of two phenanthrolines confers to RDC34 and RDC44 luminescent properties (with emission at 750nm) allowing us to follow their entry into living cells (fig. 2A, B). The molecular luminescence emission coefficients of RDC34 and RDC44 cannot be measured inside cells and the absolute concentration of RDC34 and RDC44 inside cells cannot be compared. The maximum of accumulation of RDC34 inside the cells was reached 1 hour after the compound was added. In contrast, cell uptake of RDC44 was much lower. In PBS, the emitted intensity of RDC44 is 20% less than the emission of RDC34, whereas in DMEM, RDC44 cell uptake is strongly diminished (fig. 2C). However, the increased cell uptake of RDC44 in PBS did not correspond to an increased cytotoxicity of this compound that showed IC_{50s} closed to those observed when cells were treated in complete medium (fig. 2D).

Even though there is no direct correlation between DNA binding and cytotoxicity that has been proven yet for RDCs, DNA is, in any case, one of the direct targets. We used the FRET approach based on the interaction between the RDCs and a double-stranded oligonucleotide labeled at each end by two fluorophores to test DNA binding. RDC44 showed a higher affinity for DNA compared to RDC34, as demonstrated by the drop in FRET transfer energy that occurred at the lowest concentration of RDC44 (fig. 2E). The affinity constants are obtained from the analysis of these data according to the McGhee and van Hippel model [39]: Ka=2.2 10^3 M^{-1} for RDC34 and 7.8 10^5 M^{-1} for RDC 44. The number of base pairs occupied by RDC44 along the DNA chain is equal to 3.9, larger than the occupation site size of RDC34 (2.3). It thus seems that the lack of significant cytotoxicity of RDC44 for HCT116 cells cannot be attributed nor to a default in cell entry and neither to the lack of interaction with DNA.

Modifications of RDC ligands modulate the selectivity between cancer cell lines

In order to further characterize the high cytotoxicity of RDC34, we tested its activity on cancer cells from various origins. RDC34 showed a significantly stronger cytotoxic activity against F10B16 (mouse melanoma), N2A (mouse neuroblastoma), A172 and U87 (human glioblastomas), HCT116 and SW380 (human colon cancer), and A2780 and OVCAR3 (human ovarian cancer) compared to cisplatin (fig. 3 and data not shown) with IC_{50s} mostly below 2 µM. To further characterize the cellular effect of RDC34, we performed FACS analysis on HCT116 colon cancer cells (fig. 4A). RDC34, better than cisplatin, induced the accumulation

of an elevated subG1 population at 24h and 48h, indicative of cell death. RDC34 induced also a G2/M arrest at 24h, while cisplatin blocked HCT116 cells in G1.

The cytotoxic potential of the new variants of RDCs, RDC11, RDC34 and RDC41 was submitted to the US National Cancer Institute (NCI) test on 60 cancer cell lines from various origins (fig. 4B). RDC34 showed a strong cytotoxic effect (indicated by the length of the bars on the left side) on almost all cell lines, except the NCI/ADR-Res cells. NCI/ADR-Res cells are resistant to Adriamycin and RDC34 provoked only a 50% cell cycle arrest on these cells. NCI/ADR-Res cells and kidney-derived cancer cell lines were mostly resistant toward RDC11 and RDC41. Globally taken together these data showed that RDC34, unlike RDC11 and RDC41, was endowed with a broader range of sensitive cell isotypes. NCI tests also showed the negative effect of NH_2 function on RDC34 (RDC41), leading to a significant decrease of cytotoxicity in almost all the 60 cancer cell lines. However, RDC41 showed an activity better than RDC11 on EKVX cells, MDA-MB-435, UACC-257 and HS57BT. It is also interesting to stress the significant activity of RDC11 on lung-derived cancer cells, on which RDC41 is only marginally effective (fig. 4B).

These data indicate the importance of the modifications of the ligands around the ruthenium atom for the antitumor activity and suggest the possibility to modulate the intensity of the cytotoxicity and also the specificity for selected tumor types.

Modifications of RDC ligands change the mode of action: role of caspase 8 and ROS

In order to understand how each modification of the ligands affects RDCs cytotoxicity and specificity towards cancer cells, we compared their DNA interactions. RDCs were incubated with double stranded supercoiled DNA for 14 hours and then samples were submitted to migration on an agarose gel. After migration, DNA was visualized with ethidium bromide. Increased molar ratios of RDCs versus nucleic acid bases were tested (fig. 5A). RDC34 showed an affinity for DNA a slight greater than that of RDC11, but inferior to RDC40 and RDC41. In particular, RDC40 formed a stable complex with DNA that remained in the loading pocket.

These data stress the lack of correlation between the DNA binding activity and the cytotoxicity. We therefore looked for another possible explanation for the higher cytotoxicity of RDC34 compared to RDC40 and RDC41. Previous observations indicated that redox potential allowed RDCs to modulate the cellular oxydo-reductase enzyme activity. We therefore hypothesized that such regulation might allow RDCs to induce the production of radical oxidized species (ROS) inside the cells. Incubation of HCT116 with RDCs and a fluorescent ROS probe (fig. 5B-G) showed that RDC34 was the most potent ROS inducer (fig. 5G, H), greater than RDC40 and RDC41, suggesting a correlation between the induction of ROS and cytotoxicity.

We therefore decided to investigate more in detail the mode of action of RDC34, comparing how this ruthenium compound was regulating the expression of p53 and CHOP/DDIT3, two

transcription factors previously shown be regulated by RDC11. We also followed the phosphorylation of H2AX at serine 137, which is a marker for DNA damages. RDC34 was as efficient as RDC11 to induce CHOP, but was more potent to induce p53 protein levels (fig. 6A). In these experimental conditions, cisplatin was unable to regulate CHOP, whereas both RDCs were less able to induce H2AX phosphorylation (marker for DNA damages)[31]. Besides the significant induction of p53 protein levels, RDC34 increased the expression of pro-apoptotic p53 target genes involved in either in the mitochondrial-dependent apoptotic pathway (noxa, bax, siva) or the mitochondria-independent pathway (fas, trail) were markedly induced by RDC34 (fig. 6B, C).

A fluorescent probe (JC-1) was used to further detect changes in the mitochondrial membrane potential, indicative of the involvement of the mitochondria in the apoptotic processes induced by RDC11 and RDC34 (fig. 6E, G). The involvement of a mitochondria-independent pathway was conversely tested by following caspase 8 activity. Compared to RDC11, RDC34 was more potent in inducing caspase 8 activity (fig. 6H). The concomitant use ROS and caspase 8 inhibitors reduced the cytotoxicity or RDC34, suggesting their importance for the antitumor activity of this drug (fig. 6I).

In vivo antitumor activity of optimized RDCs

To further examine the anticancer potential of the optimized compounds, we tested their activity on tumor growth. A preliminary experiment to evaluate host toxicity after single dose and after chronic treatment (3 weeks, 2 injections a week) showed that repeated doses of RDC34 higher than 4 µM/Kg were lethal (fig. 7A). Similar results were obtained with RDC44, a compound with significant lower cytotoxicity in vitro (table 1). RDC41 was the most tolerated compound and showed a DL_{50} of 30 µmol/Kg.

When tested at their maximum tolerated doses on the syngeneic model of 3LL cells implanted subcutaneously in B6 mice, RDC34 (4 µmol/Kg, 2X/week), RDC44 (4 µmol/Kg, 2X/week) and RDC41 (13 µmol/Kg, 2X/week) reduced tumor growth to 42% (RDC34), 38% (RDC44) and 36% (RDC41). The anticancer activity of RDC34 on human ovarian cancer cells (A2780) implanted into nude mice (fig. 7D) showed a significant reduction of tumor growth to approximately 36% of the controls.

Altogether these results indicated that optimized RDCs for cytotoxicity and solubility displayed anticancer properties in different tumor growth models.

Discussion and conclusion

The development of novel compounds to add or replace to the current anticancer therapies is a constant challenge for chemists and biologists. In the recent years, metal-based and organometallic compounds have been the focus of a particular interest based on the proven relative efficiency of platinum-derived compounds and their intrinsic physico-chemical properties of transition metals, such as ruthenium. Especially, the vast possibilities of combination with organic ligands and small ions make ruthenium-based molecules particularly suitable to be modeled and adapted to a specific need. In particular, the wide range of redox states and the hexahedral structure of this metal allow endless metal/ligand combinations with variations in redox state, lipophilic/hydrophilic status, ligand exchange properties, stability, and geometry.

Recently, a ruthenium (II)-based organometallic compound, called RDC11, was shown endowed with anticancer properties, such as the reduction of tumor growth in several *in vivo* models and a reduced chronic toxicity when compared to cisplatin. In order to optimize this compound, we have modified the ligands to gain in solubility and in cytotoxicity. Based on a previous structure/activity study, we chose to add a second phenanthroline that had proven to be important for RDC cytotoxicity, generating the compound RDC34. Furthermore, in order to improve the water solubility and to modify the redox potential of the compound, a NO_2, a NH_2 or a spermine group were added, generating three additional compounds, respectively RDC40, RDC41 and RDC44.

The variation in cytotoxicity between the compounds tested in the present study might involve several factors: 1) the redox potential, 2) the lipophilic status, and 3) the geometry. These factors might influence the ability of the compound to enter the cells, interact physically with intracellular targets and modify their functions. These factors also certainly affect the pharmacokinetic and the tissues distribution of the compounds in vivo and might explain the differences in toxicity.

Role of the phenantroline in anticancer activity

As we hypothesized, the addition of a second phenanthroline significantly increased the cytotoxicity of the new compound RDC34 in comparison to RDC11, for a number of cancer cell lines from different origins (fig. 1-4), with an IC_{50} often in the nanomolar range. Changing the counter-ion does not affect significantly the cytotoxicity, confirming the important role of the phenanthroline residue in the cytotoxic properties of RDC, independently of the counter-ion. Compared to RDC11, RDC34 is also more toxic when given to mice (fig. 7 and [31]). However, at the maximum doses free of host toxicity, RDC34 significantly reduces tumor growth either in a mouse model of Lewis lung carcinoma (3LL cells) and in xenograft of the A2780 human ovarian cancer cells. The increased toxicity of RDC34 could be related to its high lipophilicity [38], a property that might favor RDC34 distribution in the body after dosing.

In addition, the facilitation of cell uptake of the compound might also explain the greater cytotoxicity in vitro in cell cultures. Although the addition of a second phenanthroline significantly increased the cytotoxicity, the modification of the phenylpyridine by addition of a NO_2, NH_2 or spermine moiety modify also significantly the cytotoxicity of the RDCs (fig. 1-4), highlighting the functional interaction occurring between the ligands and their interdependence to produce a specific biological effect. Other aspects have also to be considered, such as the stability in blood or the sensitivity to liver enzymes, especially to understand the difference between the in vivo and the in vitro activities for RDC44.

Improving the water solubility

By adding the spermine chain (RDC44), the NH_2 function (RDC41), and in a lesser extend the NO_2 (RDC40) function (fig. 1), we purposely improve the water solubility of RDC34 as indicated by their LogP, which is 2.35 for RDC34, 2.05 for RDC40, 0.9 for RDC41 and inferior to 0 for RDC44. RDC44 is freely soluble in water up to a concentration of 25mM, simplifying its *in vivo* dosing of the experimental animals. However, as observed with RDC44 and in a lesser extent with RDC41 and RDC40, increasing the water solubility decreased the cytotoxicity (fig 1-4). Similar observation have been made previously for the ruthenium compound KP1019 and its water-soluble version KP1339 [40]. Although the low cytotoxicity of RDC44 was disappointing, the in vivo studies revealed interesting properties. Indeed, RDC44 displayed similar anticancer properties compared as RDC34 (fig. 7). A possible explanation could be that the spermine moiety confers a favorable pharmacokinetics of distribution, or alternatively that the spermine is somehow removed after its injection in mice, suggesting that RDC44 is a water-soluble pro-drug of RDC34.

The lower cytotoxicity of RDC44 could be due to a diminished cellular uptake through the lipophilic membrane barrier. Indeed, in standard medium condition RDC44 showed a diminished cellular accumulation. However, under PBS conditions both compounds (RDC34, RDC44) can enter the cells and the water-soluble compound RDC44 is still barely able to affect cell survival compared to the lipophilic RDC34 (fig. 2). Moreover, RDC44 is still able to interact with DNA with an affinity similar to RDC34 (fig. 2). This lack of correlation between the cytotoxicity and the ability to interact with DNA is also supported by the results obtained with RDC40 and RDC41, which display a better affinity to DNA than RDC34, although they show a weaker cytotoxicity (fig. 5). Therefore, our data suggest that the improvement of RDC water solubility does not affect significantly RDC uptake and RDC-DNA interaction. We can therefore hypothesize that besides DNA, RDCs recruit additional direct intracellular targets that can account for a variation in their biological function depending on RDCs lipophilicity.

Ligand variations and selectivity for different cancers

In favor of this hypothesis is the change of selectivity towards cancer cells of different origins observed in RDCs with variation in their ligands. Indeed, by modifying the ligand around the ruthenium center, we have observed that RDC34 displayed a strong cytotoxicity toward a broad range of cancer cell lines (fig. 4). RDC34 is particularly efficient on cancer cell lines of kidney origin that are poorly affected by RDC11. The introduction of the NH_2 function diminished the cytotoxicity of RDC41 at all levels comparable to that of RDC11. However few exceptions exist in which RDC41 is more active than RDC11, such as EKVX cells, MDA-MB-435, UACC-257 and HS57BT. It is likely that the ability of the various RDCs to interact with cellular DNA does not explain their selectivity for different cell lines but rather their ability to interact with different intracellular targets and to induce different stress signaling.

Role of ROS and caspase 8

As we have previously published for RDC11, the new generation of improved RDCs triggers at least two mechanisms: an interaction with DNA and an induction of CHOP/DDIT3 expression (fig. 5, 6). The interaction with DNA does not seem to play a major role in their cytotoxicity, as there is not a good correlation between the cytotoxicity and their ability to interact with DNA (fig. 1, 4). Interestingly RDC34 is a more potent inducer of the p53 protein levels and the p53 target genes compared to RDC11 even though its interaction with DNA is similar to RDC11 (fig. 5). This stronger effect might involve the higher ability of RDC34 to induce ROS production (fig. 6), as ROS can induce p53. The role of reactive oxygen species in the cytotoxicity of ruthenium compounds has been previously suggested [25, 28] and is supported by our present study as NAC significantly reduces RDC34 cytotoxicity (fig. 6). However, the correlation between production of ROS and RDC cytotoxicity is only partial as there is no difference in ROS production between RDC40 and RDC11 even though RDC40 is more cytotoxic than RDC11. Previous studies indicated that RDCs modulate the activity of oxido-reductase enzymes [41], suggesting that the production of ROS might be triggered by alterations of the activity of enzymes committed to produce or remove ROS, although these targeted enzymes remain to be identified yet.

Anyhow, RDC34 is a strong inducer of p53 and p53 target genes (e.g. Fas, Trail, fig. 5) connected to the extrinsic pro-apoptotic pathways involving caspase 8. The role of caspase 8 seems crucial to the pathway leading to cell death after treatment with RDC34 since its inhibition reduced these cytotoxic effects.

Overall this study indicates the crucial role of modifications of the ligand around the ruthenium center to optimize the molecules in order to improve the cytotoxicity and target cancers subtypes. In particular these modifications may help to understand the nature of the targets with which RDCs interact to determine their antitumor effect. Moreover, this study shows the possibility of producing a RDC prodrug that present a fair water-solubility without affecting significantly the in vivo anticancer activity of the pharmacophore.

Experimental section

Cell culture, MTT test, flow cytometry analysis

B16F10, U87, A172, 3LL, A2780, HCT116, SW480 cells were obtained from ATCC (Manassas, VA). Cells were maintained in DMEM with 10% FBS and incubated in presence of 5% CO2/95% air at 37°C. MTT tests were performed with cells cultured in 96-well culture dishes (Costar) as previously described [32]. Hypodiploid DNA was measured as described [33] using propidium iodide. The fluorescence of 10,000 cells was analyzed using a FACScan flow cytometer and CellQuest software (Becton Dickinson, San José, CA).

Western blot

Cells were treated in triplicates and Western blots were performed as previously described [34]. Equal loading was verified with an actin antibody (1/200; Dr. Aunis). Immunoprobing was performed with anti-p53 (1/250, Santa Cruz Biotechnology, Santa Cruz, CA), anti-phospho-H2AX antibody (1/3000, Millipore, Molsheim, France), anti-CHOP (1/1000, Santa Cruz Biotechnology), or anti-p53 (421, supernatant 1/3) antibodies. Membranes were probed with a secondary horseradish-peroxidase-conjugated antibody (anti-rabbit, -goat or –mouse) diluted at 1/2000, and then revealed with ECL (Pierce, France).

Quantitative Real-Time RT-PCR (RT-qPCR)

Total RNA was extracted using RNAII Nucleospin (Macherey-Nagel, Strasbourg, France). Reverse transcription was performed with 1µg RNA using Biorad iScript kit. qPCR was performed in Bio-Rad iCycler thermal cycler using iQ SYBR Green supermix (Bio-Rad Laboratories, Hercules, CA). Starting quantities of genes of interest were reported to those of housekeeping genes (TBP, 18s). Specificity of the amplification was controlled by a melting curve [35]. Primers sequences are shown in the supplementary material and methods. Probes: for *noxa* (PMAIP1), 5'-ggagatgcctgggaagaag-3'; 5'cctgagttgagtagcacactcg-3'; for *fas*, 5'-atggccaattctgccataag-3', 5'-tgactgtgcagtccctagctt-3'; *tbp*, 5'- cggctgtttaacttcgcttc-3', 5'-cacacgccaagaaacagtga-3'.

Evaluation of Mitochondrial Membrane Potential

The changes in ΔΨm were assessed using the lipophilic cationic membrane potential sensitive dye JC-1 (5,5',6,6'-tetrachloro-1,1',3,3' tetraethylbenzimidazolylcarbocyanine iodide), and FCCP (p-trifluoromethoxy carbonyl cyanide phenyl hydrazone) treatment was used positive control. Cells were firstly grown on the cover glasses (treated by 1/50 poly-lysine solution) and the staining procedure was the following: the DMEM was removed and 300µl of 10µg/ml JC-1 (Molecular Probes) solution (dissolved in PBS) was added to the cultures for 15 minutes [36]. The staining solution was removed and the cultures were rinsed with cold PBS for 2 times. Subsequently, cells were fixed in 4%PFA (glutaraldehyde) for 15

minutes and then coved with Vectashield mounting medium on glass slides for fluorescence. Intracellular distribution of the dye was assessed by confocal microscopy. Fluorescence present in the cell was measured at 488 nm excitation/510nm emission.

Assay for caspase 8 activity

Caspase 8 activity (1 unit is defined as the amount of enzyme required to cleave 1pmol of the substrate Ac-LEHD-pNA per minute at 30°C) was assayed by measuring the light intensity produced using a kit (Caspase-Glo®8 Assay, Promega) and a luminometer (Perkin Elmer HTS 7000, Bio Assay Reader). Briefly, Cells were cultured in 96-well plates in a final volume of 200µl. 3 wells per condition, then 50µl caspase-8 reaction buffer was added, incubated at room temperature for 1 hour before measurement.

FRET

FRET measurements are performed on a home build setup. The sample is enlightened at 488 nm with a laser, through a 63X oil-immersion objective. Emitted light is collected in the reflection geometry with the same objective. Reflected light at 488nm is stopped with a notch filter. Wavelengths smaller than 550nm are reflected by a dichroic mirror, and are filtered with a band pass filter in such a way that wavelengths between 500 and 550nm reach a Photomultiplier Hamamatsu H7421-40. On the other hand, larger wavelengths are filtered with a 520-650nm band pass filter before being collected with an avalanche photodiode. The signals are then sampled at 50Hz, and averaged during 300s. The maximum emission of Alexa 488 is centred at 520nm, and Alexa 568 at 610nm, thus independently collected the FRET efficiency is computed as: Q=I610/(I520+I610). Solid line: fit of the experimental data with a Hill function H(x) = base+A*1/(1+(xhalf/x)^rate). The critical transfer radius R0 of Alexa488/Alexa566 is 62Å. The end-to-end length of a 15 base pair long DNA strand is r=51Å.

Intracellular reactive oxygen species (ROS) measurement

5-(and-6)-carboxy-2′,7′-dichlorodihydrofluorescein diacetate (carboxy-H2DCFDA) (Molecular Probes) was used to detect intracellular ROS levels according to the manufacturer's instructions. For ROS quantification, cells were seeded in 96-well black plates (Greiner Bio-One) and treated with RDCs at the indicated concentrations and times. Afterwards, cells were washed with PBS and incubated with 10µM carboxy-H2DCFDA in DPBS for an hour. Cells were then washed with PBS and fluorescence was measured by a plate reader (Perkin Elmer) with an excitation wavelength of 485 nm and an emission wavelength of 535 nm.

RDC uptake and confocal microscopy

For quantification of RDC uptake, cells were grown on coverslips coated with poly-ornithine. Cells were treated with various concentration of RDC34 or RDC44 for the indicated time at 37°C in a cell culture incubator. Before observation under a home built confocal microscope and intensity measurements, cells were washed three times with PBS. The home build confocal microscope collects the fluorescence through an optical fiber coupled to a spectrometer, which splits the signal in the different wavelength. This signal is collected on a CCD camera that returns us the emission spectrum. This spectrum is then divided in ten different parts of wavelength width equal to approx. 20 nm. A series of spectral images of a given cell are then obtained. The images corresponding to the fluorescence emission of the studied labels (centered around 520 nm) and to the luminescence emission of the ruthenium compound (centered around 750 nm).

Chemical synthesis

Experiments were carried out under an argon atmosphere using a vacuum line. Diethyl ether and pentane were distilled over sodium/benzophenone, dichloromethane and acetonitrile over calcium hydride and methanol and ethanol over magnesium under argon immediately before use. The other starting materials were purchased from Sigma-Aldrich or Alfa Aesar and used as received without further purification.

All complexes were used with a purity >95%, as demonstrated by using several protocols. Indeed, all complexes were purified over chromatography columns carried out on Merk aluminum oxide 90 standardized. In order to verify the purity of the different complexes, several analyses were carried on. The NMR spectra were obtained at room temperature on Brucker spectrometers. 1H NMR spectra were recorded at 300.13 MHz (AC-300) or 400.13 MHz (AM-400), and referenced to SiMe4. 13C{1H} NMR spectra were recorded at 75.48 MHz (AC-300) or 100.62 MHz (AC-400) and referenced to SiMe4. The NMR assignments were supported by COSY spectra for 1H NMR. The infrared spectra were recorded on an alpha ATR spectrometer from Brucker Optics and analysed with OPUS software. The HPLC spectra were recorded on a Varian prostar 210 HPLC equipped with a Prostar 335 photodiode array detector and a Prostar 410 autosampler. The stationery phase was a 250mm x 4.6mm column packed with 10Å Kromasil C-8. The different compounds were dissolved into methanol (5.10-5 M) and then injected onto the column (5µL). ES-MS spectra and elemental analyses were carried out by the corresponding facilities at the Institut de chimie, Université de Strasbourg and at the Service Central d'Analyse du CNRS, Vernaison.

All compounds, RDC11 ([37], RDC34,37,40,41,44, were synthesized according to published procedures [38].

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Figure Legends

Figure 1: Optimized RDC for solubility and cytotoxicity reduce cell growth of HCT116 cells.

A, B. HCT116 human colon cancer cells were treated in 96-wells plates for 48 hours with the indicated concentration (μM) of cisplatin or RDC. Viability of the cells was evaluated using a MTT test. Insets: representations of RDC structures.

Data are representatives of three independent experiments. Bars are mean ±SD and asterisks indicate a statistically significant difference ($p < 0.01$) compared to control, as calculated by a one-way ANOVA test followed by a Tukey post-test over the three independent experiments.

Figure 2: Cell permeability and DNA interaction of optimized RDC.

A, B, represent HCT116 cells incubated with RDC44 and visualized with white light (A) and a confocal microscopy (at 750nm, B).

C. Quantification of RDC accumulation after 1 hour in HCT116. HCT116 were incubated for 1 hour with either RDC34 or RDC44 in either DMEM or PBS at the concentration of 5μM. After one hour, cells were washed three times, were observed under the confocal, and picture were taken before quantification.

D. HCT116 were treated at the indicated concentration (μM) for 2 hours either in DMEM or PBS, medium was replaced by DMEM with serum for 48 hours and MTT was performed. Data are representative of three independent experiments. Bars are mean and asterisks indicate statistically significant difference ($p < 0.01$) compared to control, as calculated by a one-way ANOVA test followed by a Tukey post-test over the three independent experiments.

E. Efficiency of the Förster Resonance Energy Transfer (FRET) between the extremities of a 14 base pairs-long double-stranded DNA labelled with Alexa-488 and Alexa-568. The measurements were performed at the equilibrium of complexation of DNA with the metal complex. Solid line is a guide to the eye. Analysis of these data leads to the affinity constants of RDC34 and RDC44 for DNA (see supplementary materials).

Figure 3: Cell growth inhibition by RDC34 on various cancer cells

RDC34 biological activity was tested on cancer cells from various origins (lung cancer, 3LL, A.; neuroblastoma, N2A, B.; glioblastomas, A172 and U87, C, D.; colon cancer SW480, E.; melanoma, F10B16, F.). Cells were treated in 96-wells plates for 48 hours with the indicated concentration (μM) of cisplatin or RDC. Viability of the cells was evaluated using a MTT test. Data are representatives of three independent experiments. Bars are mean±SD and asterisks indicate statistically significant difference ($p < 0.01$) compared to control, as calculated by a one-way ANOVA test followed by a Tukey post-test over the three independent experiments.

Figure 4: Optimized RDC induced cytotoxicity on cancer cells of various origins

A. Cell cycle profile analysis of HCT116 cells treated with cisplatin or RDC34 (2.5μM) for 24h or 48h. Cells were stained with propidium iodide and analyzed by FACS. Bars are mean±SD and asterisks indicate statistically significant difference ($p < 0.01$) compared to control, as calculated by a one-way ANOVA test followed by a Tukey post-test over the three independent experiments.

B. Graph indicating the cytostatic (bars on the right) and cytotoxic ((bars on the left) response of 60 cancer cells lines of the specified origins. The test was performed by the NCI with the indicated RDC at a concentration of 5μM.

Figure 5: Interaction of the optimized compounds with DNA and production of reactive oxygen species

A. Circular double-stranded DNA was incubated with RDCs at the indicated ratio (DNA base pairs/molecule of drugs). Complexes were run on a 1% agarose gel, and then stained with ethidium bromide to observe DNA relaxation.

B-G. HCT116 cells were grown on coverslips coated with polyornitine and were treated with RDC for overnight (16 hours) and labeled with carboxy-H2DCFDA for 1 hour. Cells were then observed under a microscope equipped for fluorescence. B. Control cells, C. cells treated with the positive control, menadione, D., cells treated with RDC11 (2.5μM), E., cells treated with RDC40 (2.5μM), F. cells treated with RDC41 (2.5μM), G., cells treated with RDC34 (2.5μM).

H. HCT116 cells were grown on black 96 well plates and were treated with the indicated RDC as described in B-G. After treatment, fluorescence was quantified with a fluorimeter. Bars are mean and asterisks indicate statistically significant difference ($p < 0.01$) compared to control, as calculated by a one-way ANOVA test followed by a Tukey post-test over the three independent experiments.

Figure 6: Optimized RDCs induce multiple signaling pathways, including ROS and caspase 8

A. Western blot analysis of HCT116 cells treated at the indicated concentrations of drugs for 24 hours. Immunoblotting were performed with anti-p53, anti-actin, anti-phospho-H2AX antibodies, and anti-CHOP.

B. C. HCT116 cells were treated with cisplatin, or RDC34 (5μM) with the indicated concentration (1, 2, 5μM) for 24h. RT-qPCR were performed using primers for noxa (B), and fas (C). Data are represented as fold inductions relative to untreated cells (Ct) and were normalized with both 18s and TBP levels.

D-G. HCT116 cells were incubated with the lipophilic cationic membrane potential sensitive dye JC-1 (5,5', 6, 6'-tetrachloro-1,1',3,3' tetraethylbenzimidazolylcarbocyanine iodide) to detect changes in mitochondrial membrane potential. We used FCCP (p-trifluoromethoxy

carbonyl cyanide phenyl hydrazone) as positive control (E). Cells were firstly grown on the cover glasses (treated by 1/50 polylysine solution before) and staining procedure was performed. The DMEM was removed and 300µl of 10µg/ml JC-1 (Molecular Probes) solution (dissolved in PBS) was added to the culture cells for 15 minutes. The staining solution was removed and the culture cells were rinsed twice with cold PBS. Cells were fixed in 4% PFA (glutaraldehyde) for 15 minutes and mounted on glass slides for fluorescence. Intracellular distribution of the dye was assessed by confocal microscopy. Fluorescence signal was measured at 488 nm excitation/510 nm emission.

H. HCT116 cells were incubated for the indicated time with the indicated concentration of drugs (in µM). Then caspase 8 activity was tested using a Promega Kit. Bars are mean and asterisks indicate a statistically significant difference ($p < 0.01$) compared to control, as calculated by a one-way ANOVA test followed by a Tukey post-test over the three independent experiments.

I. HCT116 were treated with the combination of drugs (NAC, 10µM; Caspase 8 inhibitor, C8inh 10µM) that included RDC34 at the indicated concentrations. Cell survival was tested 48 hours after using MTT tests. Bars are mean and asterisks indicate statistically significant difference ($p < 0.01$) compared to control, as calculated by a one-way ANOVA test followed by a Tuckey post-test over the three independent experiments.

Figure 7: Anticancer activity of optimized RDC

A. C57BL/6 mice (8-weeks old) were injected repeatedly over 30 days with RDC34 twice a week at the indicated concentration. Graph represents survival percentage from groups of 6 animals.

B. C. D. C57BL/6 mice (8-weeks old) were injected subcutaneously with $5X10^5$ 3LL cells. Injections of equivalent doses of RDC or cisplatin (13.3 µmol/Kg) started when tumors were palpable (10 days after injection, ± 80 mm3) and were performed twice a week. Graph shows tumor volumes. Data are representative of three independent experiments (n=8).

E. Nude mice (Swiss nu/nu, Charles River, 8-weeks old) were injected subcutaneously with $5X10^6$ A2780 cells. Injections of molar equivalent doses of RDC34 or cisplatin were performed twice a week during 12 days when tumors were palpable. Graph represents tumor volumes. Data are representative of two independent experiments (n=8). Asterisks indicate statistically significant difference ($p < 0.01$) compared to control, as calculated by a one-way ANOVA test followed by a Newman-Keuls test over three independent experiments.

Abbreviations

CHOP: C/EBP homologous protein, also known as growth arrest- and DNA damage-inducible gene 153 (GADD153)
DDIT3: DNA damage inducible transcript 3
FACS: Fluorescence Activated Cell Sorter
FRET: Förster resonance energy transfer
NAC: n-acetylcysteine

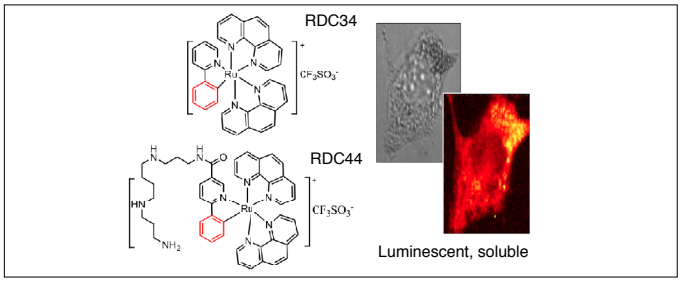

RDC34
RDC44
Luminescent, soluble

Figure 1

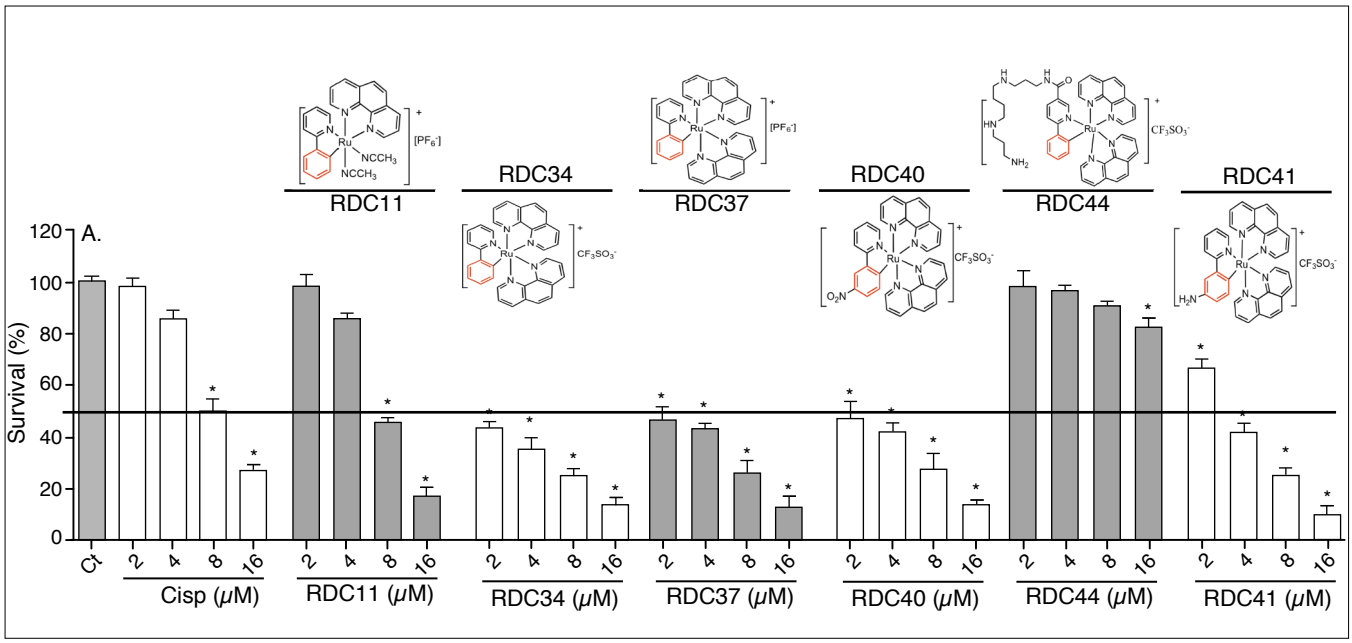

A.
Survival (%)
120
100
80
60
40
20
0
Ct
2
4
8
16
Cisp (μM)
RDC11
RDC11 (μM)
RDC34
RDC34 (μM)
RDC37
RDC37 (μM)
RDC40
RDC40 (μM)
RDC44
RDC44 (μM)
RDC41
RDC41 (μM)
[PF6-]
NCCH3
CF3SO3-
O2N
H2N
HN
NH2
Ru
*

Figure 2

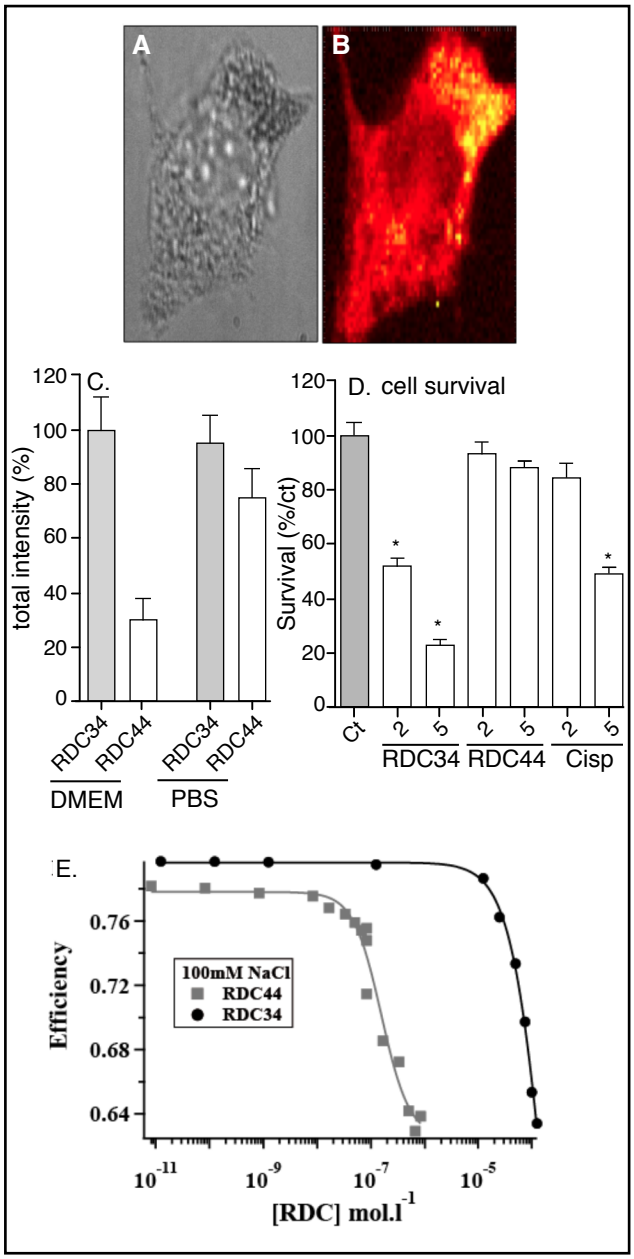

A
B
C.
total intensity (%)
120
100
80
60
40
20
0
RDC34
RDC44
RDC34
RDC44
DMEM
PBS
D. cell survival
Survival (%/ct)
Ct
2
5
2
5
2
5
RDC34
RDC44
Cisp
E.
Efficiency
0.76
0.72
0.68
0.64
100mM NaCl
RDC44
RDC34
10-11
10-9
10-7
10-5
[RDC] mol.l-1

Figure 3

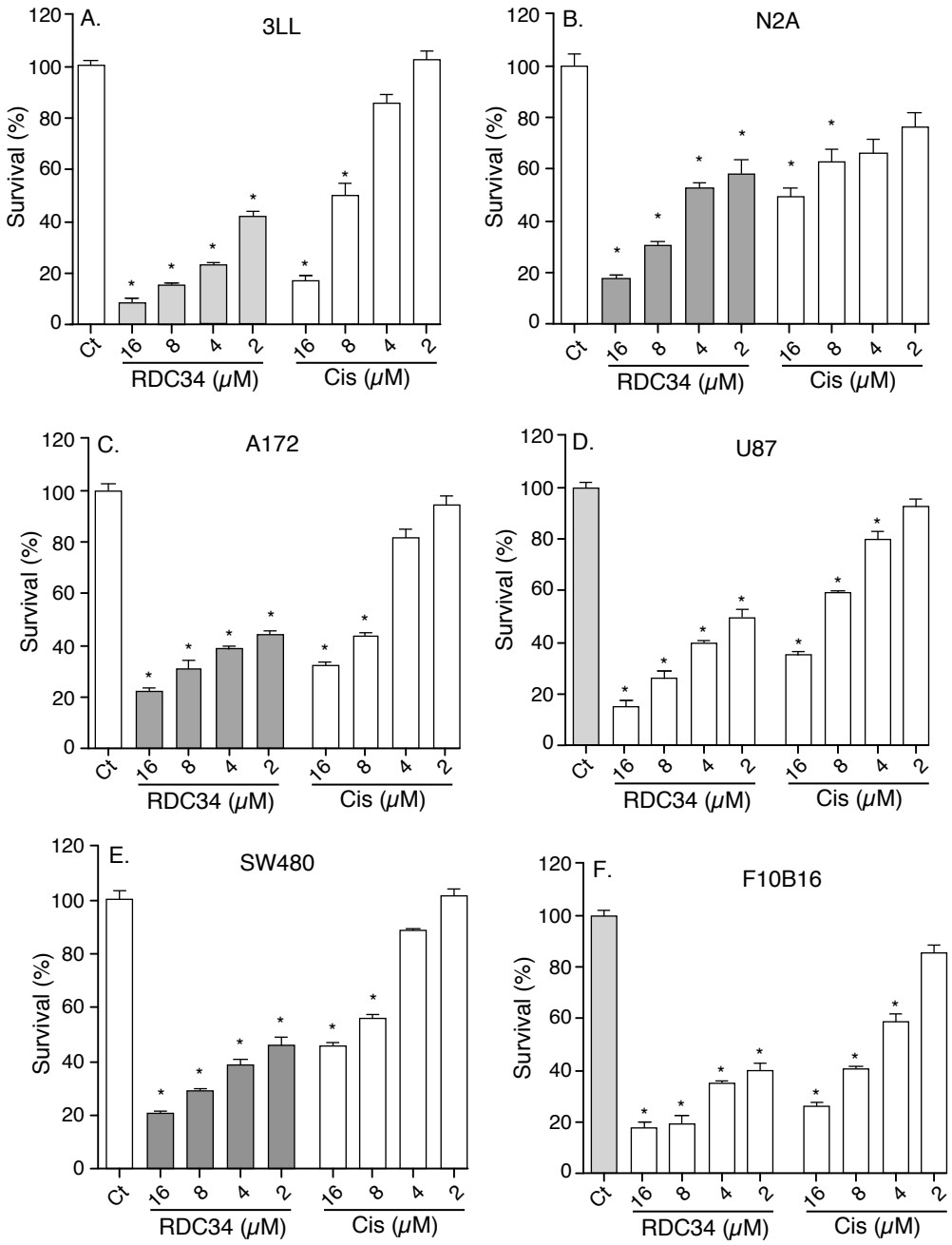

A.
3LL
B.
N2A
C.
A172
D.
U87
E.
SW480
F.
F10B16
Survival (%)
0
20
40
60
80
100
120
Ct
16
8
4
2
RDC34 (μM)
Cis (μM)

Figure 4

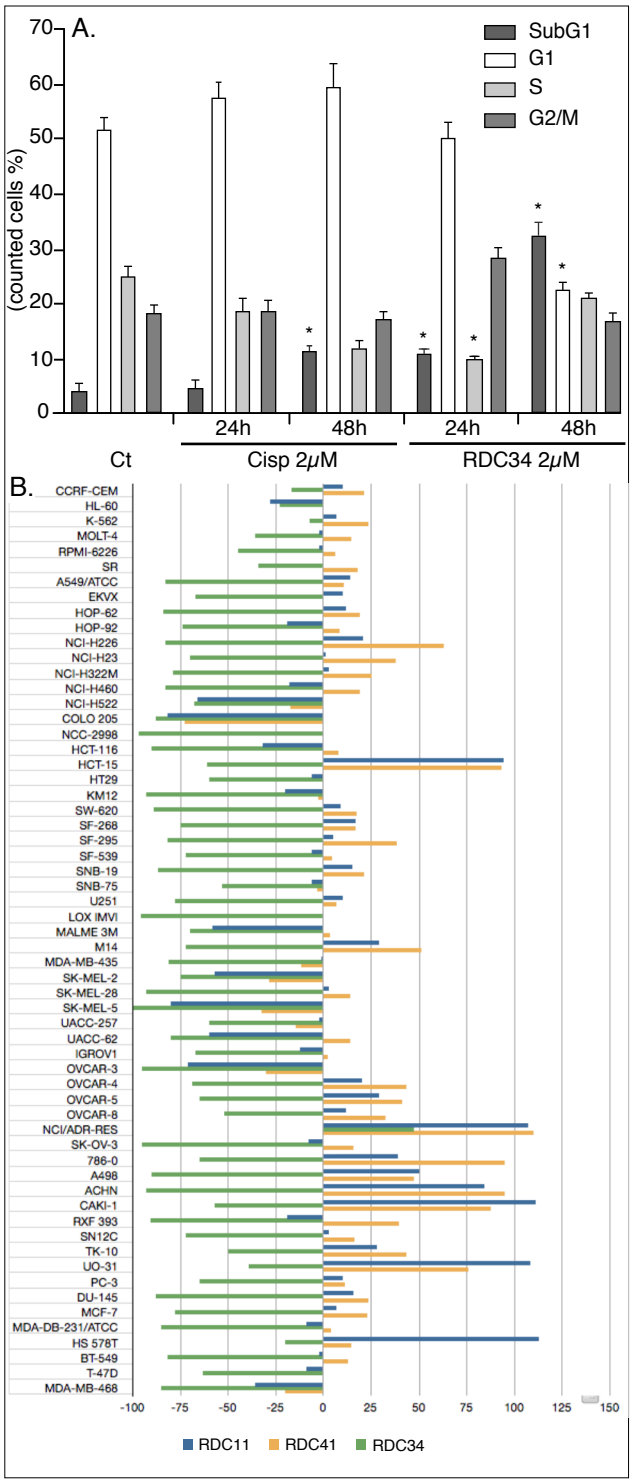

A.
(counted cells %)
70
60
50
40
30
20
10
0
SubG1
G1
S
G2/M
24h
48h
24h
48h
Ct
Cisp 2μM
RDC34 2μM
B.
CCRF-CEM
HL-60
K-562
MOLT-4
RPMI-6226
SR
A549/ATCC
EKVX
HOP-62
HOP-92
NCI-H226
NCI-H23
NCI-H322M
NCI-H460
NCI-H522
COLO 205
NCC-2998
HCT-116
HCT-15
HT29
KM12
SW-620
SF-268
SF-295
SF-539
SNB-19
SNB-75
U251
LOX IMVI
MALME 3M
M14
MDA-MB-435
SK-MEL-2
SK-MEL-28
SK-MEL-5
UACC-257
UACC-62
IGROV1
OVCAR-3
OVCAR-4
OVCAR-5
OVCAR-8
NCI/ADR-RES
SK-OV-3
786-0
A498
ACHN
CAKI-1
RXF 393
SN12C
TK-10
UO-31
PC-3
DU-145
MCF-7
MDA-DB-231/ATCC
HS 578T
BT-549
T-47D
MDA-MB-468
-100
-75
-50
-25
0
25
50
75
100
125
150
RDC11
RDC41
RDC34

Figure 5

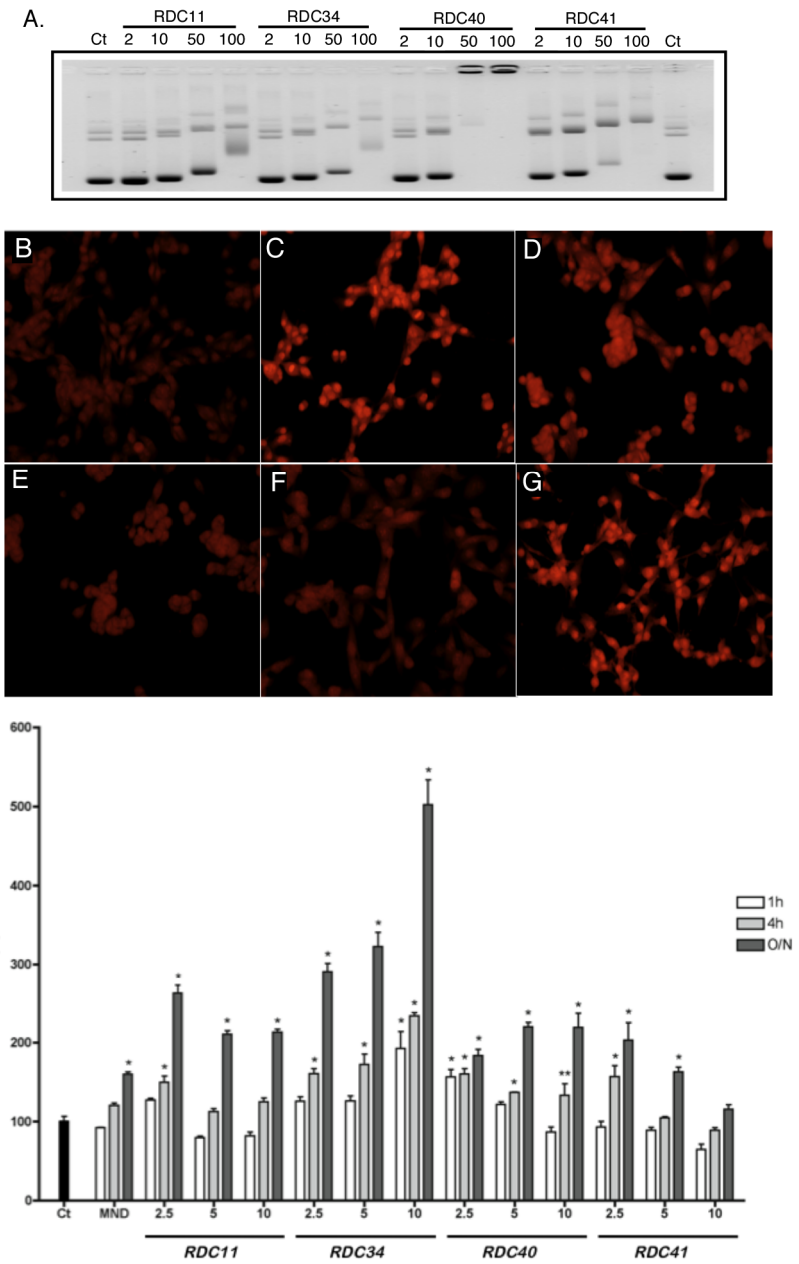

A.
RDC11
RDC34
RDC40
RDC41
Ct 2 10 50 100 2 10 50 100 2 10 50 100 2 10 50 100 Ct
B
C
D
E
F
G
RFU (%)
600
500
400
300
200
100
0
1h
4h
O/N
Ct MND 2.5 5 10 2.5 5 10 2.5 5 10 2.5 5 10
RDC11
RDC34
RDC40
RDC41

Figure 6

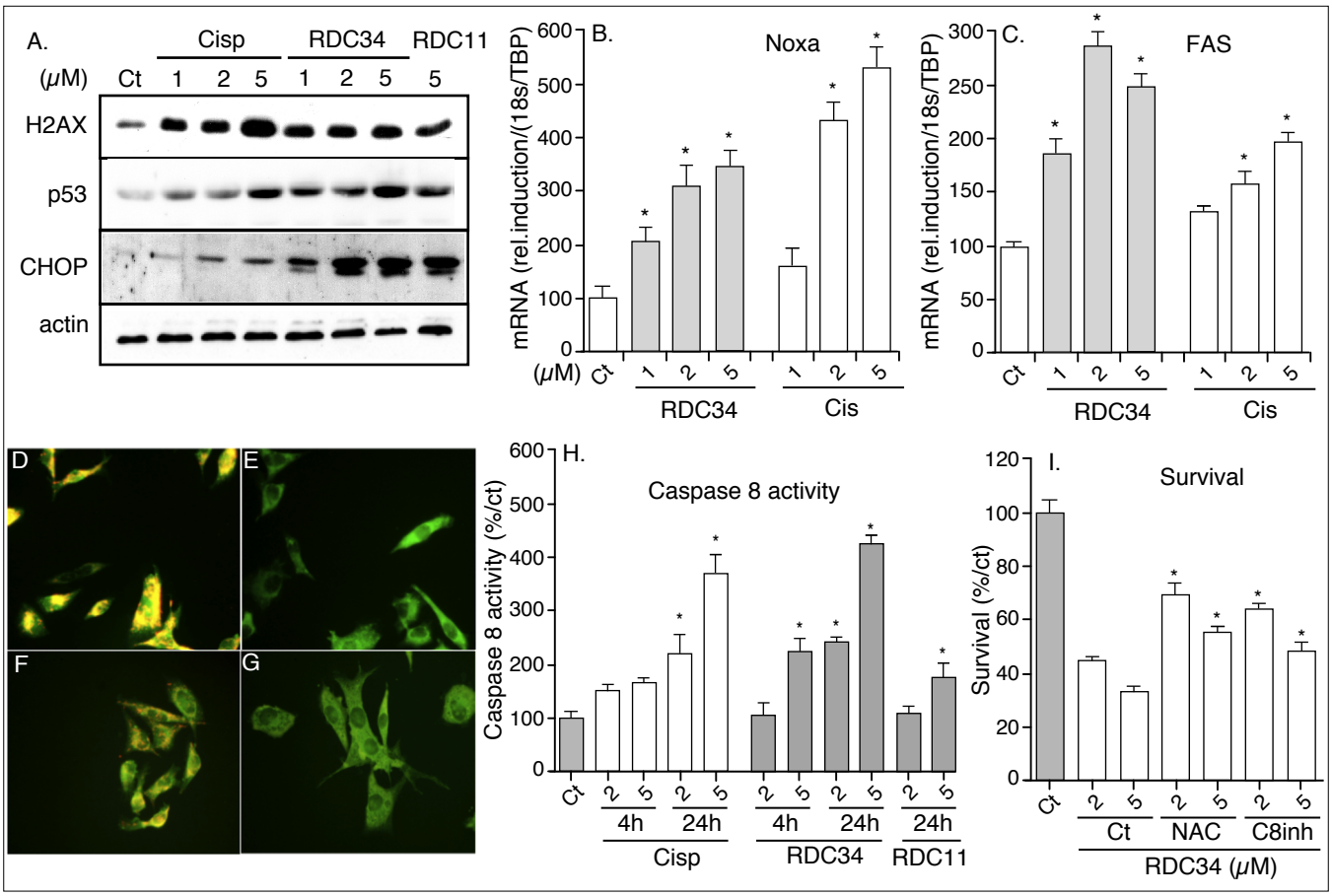
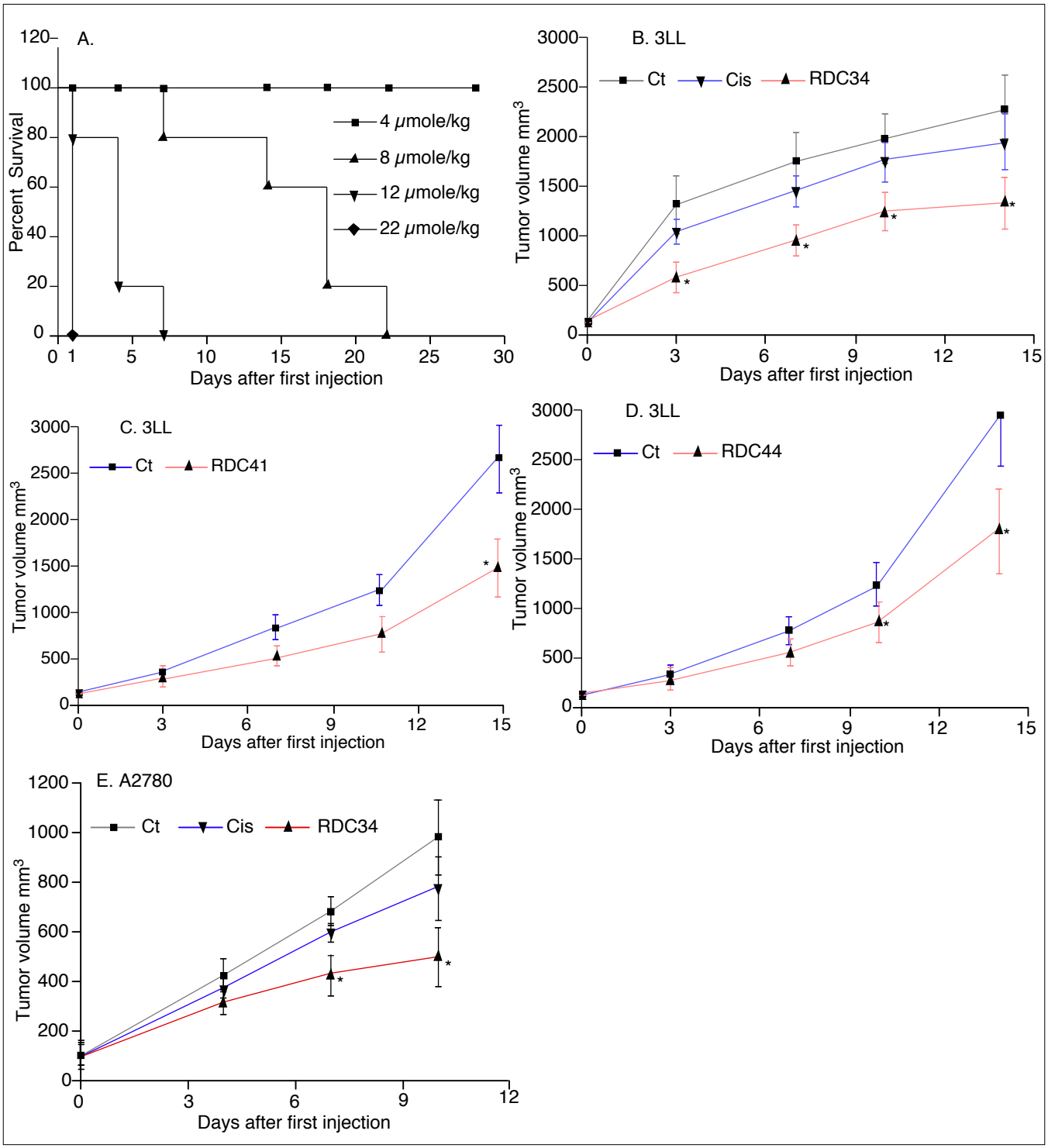

A.
Cisp
RDC34
RDC11
(μM)
Ct
1
2
5
1
2
5
5
H2AX
p53
CHOP
actin
B.
Noxa
mRNA (rel.induction/(18s/TBP)
(μM)
Ct
RDC34
Cis
C.
FAS
mRNA (rel.induction/18s/TBP)
RDC34
Cis
D
E
F
G
H.
Caspase 8 activity
Caspase 8 activity (%/ct)
4h
24h
Cisp
RDC34
RDC11
I.
Survival
Survival (%/ct)
Ct
NAC
C8inh
RDC34 (μM)

Figure 7


A.
Percent Survival
Days after first injection
4 μmole/kg
8 μmole/kg
12 μmole/kg
22 μmole/kg
B. 3LL
Ct
Cis
RDC34
Tumor volume mm3
Days after first injection
C. 3LL
Ct
RDC41
Tumor volume mm3
Days after first injection
D. 3LL
Ct
RDC44
Tumor volume mm3
Days after first injection
E. A2780
Ct
Cis
RDC34
Tumor volume mm3
Days after first injection

4.3 | Development of an experimental tool to recreate the metastatic process in vitro

The activity of potential anticancer agents is usually studied by using both *in vitro* and *in vivo* methodologies. These approaches, even if well-established, present some drawbacks: with the commonly used *in vitro* techniques is often difficult to reproduce the physio-pathological processes that characterize *in vivo* systems, especially those that take place in tumors; also animal experimentation (*in vivo* models) is not without limitations, since it does not completely reproduce what really happens in humans cancers, and is continuously matter of ethical-scientific debates.

In order to overcome these drawbacks, the National Center for the Replacement, Refinement and Reduction of Animals in Research (NC3Rs) published some guidelines to prompt the research in improving animal testing [http://www.nc3rs.org.uk/page.asp?id=3].

To find a solution to this purpose, the researchers of the Department of Material Engineering of the University of Trieste and the Callerio Foundation Onlus developed a bioreactor to mimic the complex metastatic process *in vitro.* This device, named "plastic mouse", will be useful to better characterize the metastatic cell behaviour, to test potential antimetastatic molecules and has the long-term aim to reduce animal consumption.

It is known from the literature that two ruthenium derivates, namely NAMI-A and RAPTA-T, affect some of the most important steps that characterize the metastatic process, such as the activity of membrane metalloproteases required for cell diffusion. Moreover, in this thesis work, the ability of RDC11 to act on two signaling pathways (HIF-1 and mTOR pathways) involved in the control of tumor progression was highlighted. These evidences led us to imagine a possible involvement of RDCs, in particular RDC11, in the control of the metastatic process and have further convinced us to exploit the above experimental tool for studying their antimetastatic potential.

We choose colorectal cancer as model for the study, since it represents the second-leading cause of cancer-related deaths worldwide, whose prognosis depends on the progression of the malady to the liver.

Structure of *the plastic mouse*

The plastic mouse (Fig. 4.1) consists of two compartments connected by a system of internal microcircuits. Each compartment should contain a population of cells of human

origin. In our model, the first compartment should contain HT-29 colorectal metastatic cells grown on a monolayer of healthy colon epithelial cells, which represent the site of development of the primary tumor. In the second compartment, healthy hepatocytes IHH should be seeded to reproduce the secondary site of invasion and adhesion of colon cancer cells. The internal system of microcircuits should simulate blood-like vessels that allow cancer cells and the medium to move from the first compartment (the colon) to the second (the liver). A peristaltic pump and a system of filtration are used to assure a constant flux of the medium and to discard dead cells from the system. Moreover, an external reservoir, controlled by the operator, will allow the replacement of the old medium with the fresh one in a quick and easy manner. The discarded medium would be used to analyze biochemical parameters, such as pH, or to quantify the content of extracellular proteins or growth factors likely modified by the contemporary presence of the three cell types. This system will allow to maintain three different cell lines in the same culture conditions in order to realize an *in vitro* colorectal cancer as much reliable as possible. The plastic mouse is made of a transparent, biocompatible material (polymethyl methacrylate) and has the same size as any 96-well culture plate. It is easy to handle and allows visual, as well as UV and optical absorbance monitoring.

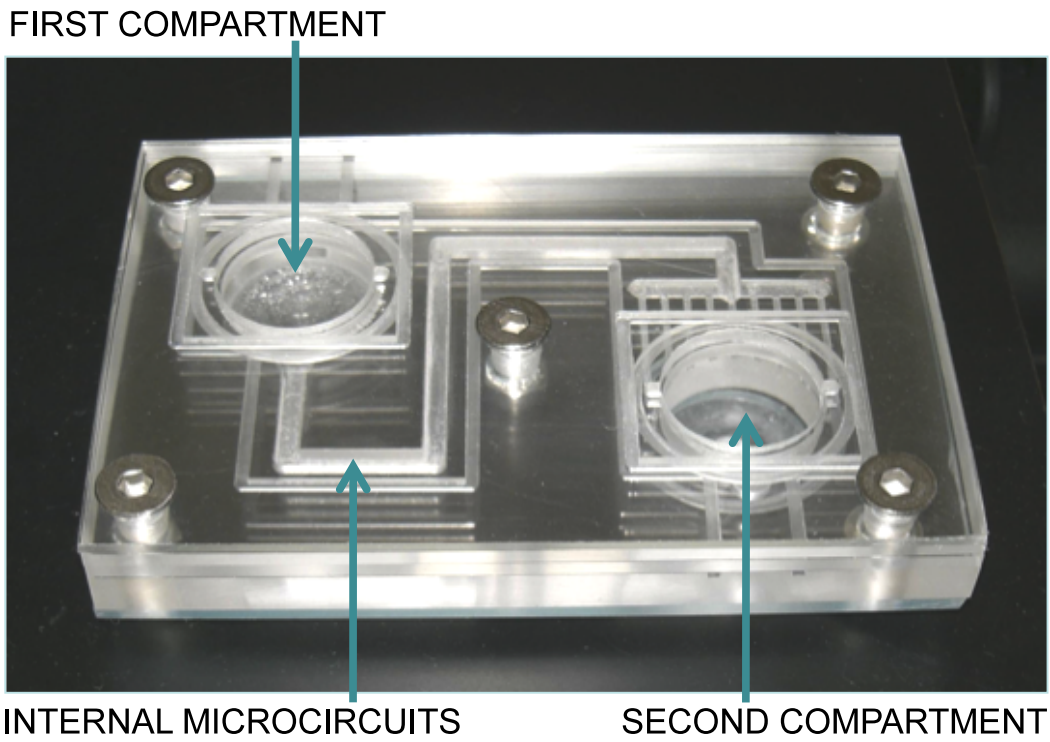


Fig 4.1 | Layout of the first prototype of the plastic mouse.

Adaptability of the three cell lines to the same cell medium

To set up the culture conditions, we performed some preliminary experiments in order to evaluate whether HT-29 and HCEC cells could grow in hepatocytes IHH medium. We chose this medium since IHHs require specific components, such as insulin and dexamethasone, for maintaining the differentiation state (Husson A et al, 1985).

We observed that, when seeded at low or medium density, HT-29 in their standard (DMEM, 10% serum) medium showed the same growth rate than that in IHH medium. On the contrary, when seeded at high density, we have seen a significant increase of the number of HT-29 cells in the medium of IHHs. Moreover, we performed the Sulforhodamine B assay (SRB) to assess cell survival. This test showed that both HT-29 and HCEC cells were able to grow in IHH medium (Fig. 4.2).

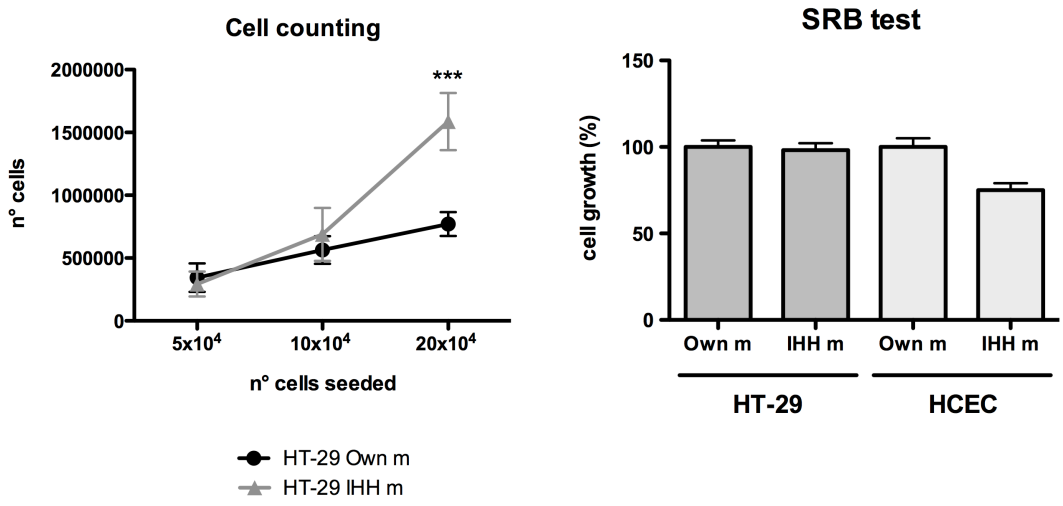


Figure 4.2 | Left: HT-29 cell counting in their standard medium and in IHH medium by using the trypan blue exclusion test. Right: HT-29 and HCEC cell viability in their own medium and in IHH medium measured by SRB test. ***=p<0.001 vs HT-29 Own m, One-Way ANOVA+Tukey post test.

In order to evaluate whether the morphology of HT-29 and HCEC cells underwent variations because of the presence of IHH, HCEC or the IHH medium, we started a co-culture of HT-29 with IHH or with HCEC cells in IHH medium and we monitored any shape variation using a light microscope.

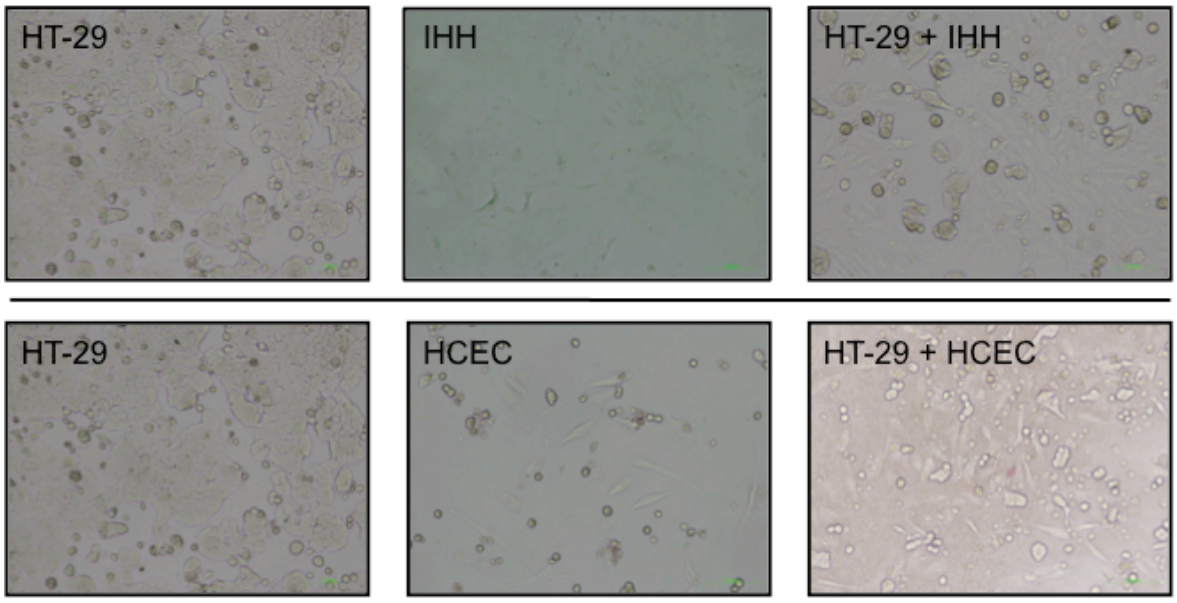


Figure 4.3 | Images of HT-29, HCEC and IHH cells grown alone or HT-29 in co-culture with IHH or HCEC in IHH medium, acquired using a light microscope (100X).

We observed that when HT-29 were grown together with IHH, there was a general maintenance of the morphological features typical of both cell lines grown alone: HT-29 arrange in the form of agglomerates, while IHH showed an elongated shape and appeared translucent. The only different is that, in co-culture conditions, HT-29 give rise to smaller aggregates.

Also for the co-culture of HT-29 with HCEC cells there is a general maintenance of the morphology of both cell lines, except that HCEC appeared more numerous in the presence of HT-29.

These preliminary tests demonstrated that the three cell lines we have selected represent a good choice concerning the setting-up a co-culture system, since they substantially maintain each own characteristics in the common medium.

5 | Discussion

During my PhD, I added new information on the mechanism of action of a relatively new class of ruthenium-based organometallic compounds, namely RDCs (Ruthenium-Derived Compounds). RDC11, the best known among RDCs, unlike cisplatin, has already been shown to poorly interact with DNA and to induce apoptosis through a mechanism independent of p53/DNA damage, unlike cisplatin [Meng X et al, 2009]. Therefore, we believed that other transduction pathways could be responsible for its anticancer activity. In support of this hypothesis, we have demonstrated that RDC11 is able to affect the HIF-1 and mTOR pathways, which are among the most important regulators of cancer cell metabolism [Wouters BG and Koritzinsky M, 2008]. We have shown that RDC11 strongly reduces HIF-1α protein levels in colorectal cancer cells. Also HIF-1β protein levels are also down-regulated by RDC11, suggesting that this dual protein level diminution might explain its effectiveness in targeting HIF-1, thus abolishing more efficiently its functions. In tumors, a consequence of the up-regulation of HIF-1α protein levels is the increase of HIF-1α target genes expression [Carroll VA and Ashcroft M, 2006]. As expected, RDC11 induces a down-regulation of the mRNA levels of the most important HIF-1α target genes (such as *VEGF* and *GLUT1*), whose protein products play a key role in angiogenesis and energy metabolism. Conversely, cisplatin shows only a weak effect on the same mRNAs. These results demonstrate that RDC11 is able to unsettle those processes that normally ensure the nutritional status of cancer cells, making them able to survive in low-oxygen environments and to grow out of the physiological control.

We have demonstrated that the reduction of HIF-1α protein levels by RDC11 can be explained by an effect at the transcriptional, rather than the post-translational level (degradation). Indeed, we have observed that RDC11 strongly downregulates *HIF-1α* mRNA, while only moderately affecting the degradation of the protein. Interestingly, RDC11 is able to stabilize, and even increase, the hydroxylated form of HIF-1α in hypoxia, a condition in which HIF-1α hydroxylation is normally inhibited. This evidence, together with the fact that RDC11 does not alter the protein expression of PHD2 (the prolyl hydroxylase responsible for HIF-1α hydroxylation) led us to hypothesize that it might activate PHD2, with consequent down-regulation of HIF-1α protein levels in hypoxia. However, the precise mechanism by which RDC11 likely could increase PHD2 activity remains to be determined. Surprisingly, we noticed that there was no perfect correlation between HIF-1α protein levels and *HIF-1α* mRNA after 6

hours of treatment with RDC11 in normoxia (indeed, the protein levels were already very low compared to those of mRNA). These results led us to think that another mechanism could be involved at early times: we hypothesized an effect at the translational level, which is consistent with the fact that RDC11 is able to induce the endoplasmic reticulum (ER) stress pathway. Recent evidences have shown that the cellular compartment where RDCs tend to localize is the ER, where RNA is more abundant than DNA. Since RDC11 displays only a weak affinity for DNA, it is reasonable to think that RNA could represent a potential and more attractive target for RDCs. In fact, accumulating evidences support the key role of RNA in the development of cancer. While DNA is (without some specific exceptions) the same in every cell of the body (encoding all the possible functions a cell might have), RNA is the working copy of genomic DNA, indicating those functions that have been currently or recently turned on. As a consequence, it is more interesting to identify what functions are operating in a cell, in order to select which ones have been gone astray in a cancer cell. Thus, targeting RNA represents a novel approach to selectively kill tumor cells. A future study in this respect will help us to establish whether RDCs may interact with the operating parts encoded in RNA, which seems more interesting than searching for interactions with entire DNA sequences.

One of the mechanisms that regulates HIF-1α protein expression is accountable to the mTOR pathway via the activation of the ribosomal protein S6, that allows HIF-1α translation. mTOR is the catalytic subunit of two functionally distinct complexes, namely mTORC1 and mTORC2. We have shown that RDC11 is able to repress both mTORC1 and mTORC2 activities, as confirmed by the reduction of the phosphorylation levels of their downstream effectors, S6 and AKT respectively. Interestingly, unlike RDC11, cisplatin increases phosphorylation of the ribosomal protein S6. Moreover, RDC11 down-regulates *Raptor* and *Rictor* mRNA, the two components that confer to mTORC1 and mTORC2 respectively, a different sensitivity to rapamycin and whose overexpression is associated with colorectal cancer progression and metastasis [Gulhati P et al, 2011]. These results are very interesting, since the majority of the mTOR inhibitors described in the literature, such as rapamycin and its analogs, inhibits only mTORC1. mTORC2 is resistant to these drugs, with consequent over-activation of AKT, which directly drives tumorigenesis promoting proliferation, survival and metastatic progression [Hsu PP and Sabatini DM, 2008]. Therefore, RDC11 may overcome rapamycin/rapalogs-resistance thanks to its ability to inhibit rapamycin-induced-AKT phosphorylation. This study has contributed to the discovery of a novel mTOR inhibitor, which is able to induce the suppression of both mTORC1 and mTORC2 activities in cancer cells *in vitro*. Moreover, the

combination RDC11-rapamycin has shown a better effect in reducing tumor growth *in vivo*. We think that this effect can be mediated by the ability of RDC11 to inhibit both mTORC1 and mTORC2, but we have hypothesized that it probably accounts also for another mechanism: the inhibition of angiogenesis. In support of this hypothesis, we have demonstrated that RDC11, besides down-regulating *VEGF* mRNA, prevents angiogenesis *in vivo* by reducing microvessel-like structures and the hemoglobin content in Matrigel plugs implanted in mice. Additional experiments using vascularized tumor model will have to be performed to confirm this observation.

In parallel with the identification of RDCs direct targets, we have started a structure/activity analysis to ameliorate the chemical and pharmacological features of RDCs. We have demonstrated that by changing ligands around the ruthenium center, it is possible to modulate several parameters, such as the redox value and the lipophilic/hydrophilic status, which might influence the ability of RDCs to enter the cells, to interact with intracellular targets and to alter their functions, as well as to modify their pharmacokinetic and distribution properties into the tissues. We have shown that RDC34, resulting from the replacement of the two acetonitrile groups of RDC11 with a second phenanthroline, is more cytotoxic than RDC11 *in vitro* and also when given to mice. We think that this increased cytotoxicity could be due to higher lipophiliticy of RDC34, that allows it to easily enter the cells *in vitro* and better distribute into the body *in vivo*. Also the addition of a NO_2 (RDC40), NH_2 (RDC41) or a spermine moiety (RDC44) to RDC34, in order to modify the redox potential and water solubility, modulates RDCs cytotoxicity, highlighting the key role that ligands play in producing a different and specific biological effect. We have generated more water-soluble RDCs than RDC34, even if we observed that an increase in water solubility is accompanied by a decrease in cytotoxicity, especially for RDC44. Surprisingly, RDC44 shows a similar anticancer activity compared to RDC34 *in vivo*. The lower cytotoxicity of RDC44 could be explained by a reduced cellular uptake through the lipophilic membranes. On the contrary, the good anticancer activity *in vivo* could be due either to the spermine moiety that confers a better pharmacokinetics of distribution or to the fact that it is somehow removed in the body: we indeed suggest that RDC44 is a water-soluble prodrug of RDC34. Moreover, we have observed a lack of correlation between the cytotoxicity of the new generated RDCs and DNA binding, suggesting that RDCs gain access to additional direct intracellular targets that could account for a variation in their biological function depending on their lipophilic/hydrophilic potential. In support to this hypothesis, we have demonstrated that RDCs endowed with

variations of their ligands, show a different selectivity for cancer cells of different origins. For instance, RDC34 proves to be cytotoxic towards the majority of the cell lines tested, with a particular inclination for kidney-derived cancer cells that are barely affected by RDC11. However, RDC11 still remains the most effective against lung-derived cancer cells. The addition of the NH_2 group reduces the cytotoxicity of RDC41 below that of RDC11, unless few exceptions. These results showed that the ability of some RDCs to interact with DNA (such as RDC40 and RDC41) does not explain their capacity to selectively act on cancer cell lines from different origins, underlining, once again, that they can interact with intracellular targets different from DNA. Despite RDC34 has been shown to interact *in vitro* with DNA as well as RDC11, it is a more potent inducer of p53 protein and of its target genes. However, it is also important to note that these results where mainly obtained *in vitro* (pure plasmidic DNA or double stranded oligonucleotides incubated with organometallic molecules) or using indirect markers, such as the phosphorylation of histone H2X and the induction of p53. It is therefore possible that in the chromatin context (DNA+proteins), RDCs might present a different affinity for the DNA/histone complex. Nevertheless, the enhanced induction of p53 by RDC34 can also be explained by the ability of RDC34 to strongly induce ROS, since ROS are known to induce p53. Previous studies have already suggested the role of ROS in determining the cytotoxicity of ruthenium compounds [Jakupec MA et al, 2005; Daugan SJ et al, 2008] and our work supports this evidence, since RDC34 loses its cytotoxicity once ROS are inhibited. However, the correlation between RDC34 cytotoxicity and the induction of ROS is not a common feature to the other RDCs that we have examined. For instance, there is no difference in ROS production between RDC11 and RDC40, even if the latter is more cytotoxic than RDC11. However, the production of ROS can be triggered also by other mechanisms, such as the alteration of the activity of redox enzymes. Indeed, before our work, it has already been demonstrated that some RDCs alter the activity of redox metabolic enzymes [Ryabov AD et al, 2001; Le Lagadec R et al, 2006], suggesting that this mechanism might account for the ability of a subset of our RDCs to induce ROS. Moreover, we have found that the induction of the pro-apoptotic factor caspase 8 by RDC34, which normally occurs after p53 induction, is crucial for its cytotoxicity, since its inhibition reduces RDC34 activity *in vitro*.

It is known from the literature that some ruthenium derivatives are able to affect different steps of the metastatic progression *in vitro* [Vacca A et al, 2002; Bergamo A et al, 2008] and also to act directly on metastasis *in vivo* [Sava G et al 2003]. These evidences, together with the fact that we have demonstrated that RDC11 is able to target molecular pathways (such as

HIF-1α and AKT) directly involved in the development of the metastatic process, led us to wonder whether it could exert an antimetastatic effect. The study of the antimetastatic effects of RDCs prompted us to undertake a further and separate study aimed to set up a model suitable for *in vitro* tests on chemicals endowed with the capacity to selectively target tumor metastases than being un-selective cytotoxics. During my PhD I have set up the cell culture conditions suitable to recreate *in vitro* the metastatization of colorectal cancer cells towards the liver, the preferential site of metastatic colorectal carcinoma, in a model called "plastic mouse". We have demonstrated that the three different cell lines selected for our study can growth in the same environment, without undergoing modifications in viability and morphology, thus representing a good model for our purpose. Once the plastic mouse will be technically operative, it will be used to study the antimetastatic potential of novel anticancer drugs, including RDCs

In conclusion, the results obtained during my PhD thesis have allowed us to identify a novel mechanism of action of RDC11, which is different from that of classical metal-based drugs, pointing out that platinum and ruthenium-based molecules can act differently, even if the latter were initially designed to mimic cisplatin. We have demonstrated that RDC11 is able to reduce HIF-1α at the transcriptional and translational level through a complex process that involves the downregulation of the mTOR pathway and the slowing down of the angiogenic process, emphasizing its anticancer activity. To our knowledge, these effects represent a unique characteristic for a ruthenium derivative, since in the literature there are no other examples of organometallic anticancer molecules able to affect these two cellular pathways. The second study has demonstrated the importance that the modifications of the ligands around the ruthenium center play in modulating the cytotoxicity and selectivity of the new generated RDCs towards different cancer types. This can be explained by their ability to interfere with different pathways crucial for cancer metabolism. Now we have to find out the direct targets of our compounds in order to explain how they can regulate these intracellular pathways at molecular level and to suggest what changes on the molecules can lead to more specific compounds. Finally, we have made a breakthrough in developing an experimental tool to study the metastatic process *in vitro*. The plastic mouse will be useful in the future to screen potential antimetastatic molecules. Many efforts are still be necessary to reach our final goal, that is to reduce animal consumption during preclinical trials. The fact that metastases are today the main cause of cancer-related deaths worldwide motivates us to

pursue our objective in order to contribute to get an appropriate tool for studying potential RDCs antimetastatic drugs.

6 | Perspectives

This work, by analyzing the mechanisms of action and the link between structure and function of RDC11, set the basis for further developing the class of Ruthenium-containing antineoplastic drugs. Our results confirmed that RDC11 cytotoxicity is not only linked to its DNA binding ability, but relies also on other mechanisms: besides the already established mechanisms (induction of reticulum stress/CHOP pathway), we characterized an inhibition of two molecular pathways, notably the HIF-1α and mTOR pathways. However, molecular basis underlying these inhibitions are not yet fully understood: experimental data revealed the involvement of an early translational block mechanism, followed by a late transcriptional/post-transcriptional regulation event, thus suggesting that RDC11 could act on specific RNAs, or more generally interfere with RNA metabolism (miRNAs, mRNA processing, or mRNA recruitment into specific intracellular compartments). It is already known that RNA in a cell is subject to many of the same insults as DNA. RNA damage, such as oxydation, can induce apoptosis and may be exploited for anti-cancer chemotherapy [Bellacosa A and Moss EG, 2003]. This novel field concerning interactions of RDCs with RNA molecules seems by now very promising and will require a deeper analysis. Moreover, understanding the precise way by which RDC11 blocks HIF-1 and mTOR pathways could shed light on druggable molecular mechanisms that could be targeted for blocking tumoral progression and that are still unknown.

Moreover, a targeted modification of the chemical structure of the drug, could eventually be applied for further enhancing specific activities or features of the latter, as already discussed concerning water solubility and/or redox potential of RDC11-like molecules.

A last consideration has to be done concerning the putative role of RDC11 as an antimetastatic drug. It has already been shown that some Ruthenium containing drugs revealed to be very potent metastasis inhibitors. The challenge of treating and preventing metastases is actually one of the most important themes in cancer pharmacology, since metastases represent the leading cause of death in cancer patients. An important follow up of this study would be the identification of the specific molecular pathways/mechanisms targeted by RDCs or Ruthenium containing drugs (like NAMI-A) and responsible for the antimetastatic effect. Assessing these mechanisms, together with the possibility of using a device like the plastic mouse, could boost the development of targeted drugs, which could be extremely effective in treating cancer, especially by preventing relapses.

7 | Bibliography

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8 | SINTESI DELLA TESI

Caratterizzazione delle proprietà antitumorali di composti derivati del rutenio: meccanismo d'azione, ottimizzazione e sviluppo di dispositivi sperimentali.

I derivati del platino hanno contribuito enormemente al trattamento del cancro, nonostante i gravi effetti collaterali (neurotossicità, nefrotossicità…) ed i fenomeni di farmaco-resistenza. Per superare queste limitazioni, derivati contenenti metalli diversi dal platino sono stati ampiamente studiati. Da qualche anno, il laboratorio studia dei derivati di rutenio (RDCs, ruthenium-derived compounds) che presentano proprietà biologiche interessanti e particolari: una buona attività antitumorale *in vivo*, una debole tossicità sui tessuti sani, un'attività su tumori resistenti al cisplatino e un meccanismo d'azione multiplo che implica, in parte, l'interazione con il DNA e l'alterazione dell'attività di enzimi ossido-riduttivi. L'alterazione dell'attività di enzimi ossido-riduttivi rappresenta un meccanismo d'azione originale che permetterebbe di bersagliare enzimi che partecipano alla regolazione del metabolismo delle cellule tumorali.

Durante il dottorato, mi sono occupata di studiare in dettaglio le proprietà biologiche degli RDCs in modo da poterli ottimizzare. Tre aspetti complementari sono stati sviluppati:

1) lo studio comparativo degli effetti del derivato di rutenio RDC11 e del cisplatino nel regolare le vie di segnalazione di HIF-1 e mTOR, entrambe coinvolte nella regolazione delle variazioni metaboliche proprie delle cellule tumorali.

2) l'ottimizzazione di RDC11 al fine di migliorarne la citotossicità e la solubilità

3) lo sviluppo di un dispositivo sperimentale per poter testare *in vitro* i potenziali effetti antimetastatici di composti organometallici, quali gli RDCs.

1) In questa prima parte, abbiamo voluto investigare il meccanismo d'azione di uno dei più promettenti RDCs, ovvero RDC11, su due vie di segnalazione fondamentali per il metabolismo delle cellule tumorali: la via di HIF-1 (hypoxia inducible factor 1) e la via di mTOR (mammalian target of rapamycin). Per tale ricerca, abbiamo scelto di utilizzare due linee cellulari di carcinoma colorettale: le HCT116 e le SW480. Il tumore al colon-retto, oltre ad essere la seconda causa mondiale di morte dovuta al cancro, è spesso caratterizzato dalla sovraespressione della proteina HIF-1α e, nonostante rientri tra i tumori trattabili con un derivato del platino (l'oxaliplatino), spesso risulta resistente a questo farmaco. Abbiamo

dimostrato che RDC11 riduce i livelli di espressione della proteina HIF-1α nelle due linee cellulari selezionate. Inoltre, riduce anche l'espressione della seconda subunità di HIF-1, ovvero HIF-1β, suggerendo che questa doppia inibizione possa essere ancora più efficace nel bersagliare la proteina HIF-1 e nel annullare quindi le sue funzioni. RDC11 diminuisce anche i livelli di mRNA di numerosi geni target di HIF-1α (come *VEGF*, *GLUT1*, *ENO1*, *Adenylate Kinase 3*) che svolgono ruoli importanti nell'angiogenesi e nel metabolismo energetico. In questo modo, RDC11 può ostacolare quei processi che assicurano lo stato nutrizionale delle cellule tumorali e che le rendono capaci di adattarsi alla mancanza di ossigeno.

Come per tutte le proteine, i livelli di espressione di HIF-1α dipendono dall'equilibrio tra la sintesi proteica (trascrizione e traduzione) e la degradazione. Abbiamo visto come RDC11 sia in grado di prevenire l'accumulo di HIF-1α (sia in condizioni di normossia che ipossia) in seguito all'inibizione della sua degradazione con l'inibitore del proteasoma MG132, suggerendo che il proteasoma non sia coinvolto nella degradazione della proteina in seguito a trattamento con RDC11. Simili risultati sono stati ottenuti con inibitori di calpaine (ALLM), catepsine (ALLN) e caspasi (Z-VAD-FMK), escludendo quindi un coinvolgimento di queste vie nel processo di degradazione di HIF-1α. Questi risultati dimostrano come la capacità di RDC11 di ridurre i livelli proteici di HIF-1α non sia correlata alla degradazione della proteina. Per questo motivo, abbiamo voluto indagare se RDC11 potesse agire a livello dell'mRNA di HIF-1α, ed infatti, abbiamo visto come RDC11 riduca significativamente i livelli di mRNA del gene *HIF-1α*, diversamente dal cisplatino. E' stato interessante notare come non vi fosse una perfetta correlazione tra i livelli proteici di HIF-1α e i suoi livelli di mRNA dopo 6 ore di trattamento con RDC11 in normossia (i livelli proteici erano infatti più bassi rispetto ai livelli di mRNA) e ciò ci ha indotti a pensare che un ulteriore meccanismo potesse essere implicato a tempi più precoci: ovvero un effetto di RDC11 a livello traduzionale. Tale risultato è in effetti compatibile con l'abilità di RDC11 di indurre lo stress del reticolo endoplasmatico.

Uno dei meccanismi che regola l'espressione di HIF-1α è riconducibile alla via di mTOR, attraverso l'attivazione della chinasi S6 che induce la traduzione della proteina HIF-1α. Ciò ci ha indotto a considerare mTOR come un potenziale bersaglio di RDC11. mTOR è la subunità catalitica di due complessi funzionalmente distinti, chiamati mTORC1 ed mTORC2. mTORC1 controlla la proliferazione cellulare attraverso la regolazione della sintesi proteica, lipidica e l'autofagia, fosforilando ed attivando il suo effettore a valle S6 chinasi (S6K) che a sua volta attiva la proteina ribosomale S6. Contrariamente, mTORC2 regola la crescita cellulare e l'organizzazione del citoscheletro attraverso l'attivazione della proteina Akt. La fosforilazione

di Akt è una delle più frequenti alterazioni nei tumori umani. I miei risultati mostrano come RDC11 sia capace di ridurre la fosforilazione della proteina ribosomale S6 e di Akt, senza modificare significativamente i livelli totali di espressione delle proteine, suggerendo la capacità di tale composto di reprimere l'attività di mTORC1 e mTORC2. Inoltre, ho dimostrato che RDC11 inibisce i livelli di mRNA di *Raptor* e *Rictor*, i due componenti che distinguono mTORC1 da mTORC2 rispettivamente, diversamente sensibili alla rapamicina. Questi risultati dimostrano come RDC11 sia capace di ridurre l'attività di mTOR attraverso l'inibizione di entrambi i suoi complessi. Ciò risulta particolarmente interessante, poiché la maggior parte degli inibitori di mTOR noti in letteratura (tra cui la rapamicina), inibisce solamente il complesso mTORC1, visto che mTORC2 è resistente alla rapamicina stessa.

Dal momento che la rapamicina inibisce solamente mTORC1 e che RDC11 reprime entrambi i complessi di mTOR, abbiamo voluto valutare se la combinazione di queste due molecole avesse un effetto ancora maggiore nell'inibire la crescita tumorale *in vivo*. Effettivamente, abbiamo osservato come la combinazione di RDC11 con la rapamicina fosse la più efficace nel ridurre il volume tumorale (carcinoma polmonare di Lewis 3LL impiantato in topi femmina C57BL/6) alla fine del trattamento. Questo risultato, assieme al fatto che RDC11 si è dimostrato abile nel ridurre l'mRNA del gene *VEGF*, ci ha indotti ad esaminare se il nostro derivato di rutenio esercitasse un effetto anti-angiogenico *in vivo*. L'analisi dei pellets di Matrigel (contenenti fattori di crescita pro-angiogenici e RDC11) prelevati dai topi dopo quattro giorni dall'impianto, hanno mostrato come il contenuto emoglobinico dei pellets dei topi trattati con RDC11 fosse inferiore di circa la metà rispetto a quello dei pellets dei topi di controllo. Questo esperimento preliminare suggerisce come RDC11 possa indurre un effetto anti-angiogenico.

2) Gli studi descritti in precedenza hanno come obiettivo principale quello di identificare i target intracellulari diretti degli RDCs, in modo da permettere la razionalizzazione del processo di ottimizzazione di tali composti. Nell'attesa di identificare i bersagli diretti, abbiamo intrapreso un'analisi struttura/attività per migliorare le proprietà chimiche e farmacologiche degli RDCs. Questo studio ha dimostrato che l'aggiunta di una seconda fenantrolina agli RDCs induce un aumento della citotossicità. In particolare, la sostituzione dei due gruppi aceto nitrile di RDC11 con una seconda fenantrolina, ci ha permesso di ottenere una nuova molecola, denominata RDC34. Inoltre, poiché in precedenza è stato dimostrato che gli RDCs modulano l'attività di enzimi ossido-riduttivi grazie al loro potenziale redox, abbiamo voluto modificare quest'ultimo parametro aggiungendo alla fenantrolina di RDC34

un gruppo elettron-attrattore NO_2 o un gruppo elettron-donatore NH_2, ottenendo rispettivamente le molecole RDC40 e RDC41. Infine, per ottimizzare la solubilità in acqua di RDC34, abbiamo aggiunto all'unità fenilpiridinica una spermina, ottenendo RDC44, che ha mostrato una buona solubilità in ambiente acquoso. La citotossicità di questi nuovi RDCs è stata valutata mediante test MTT sulle cellule HCT116. E' stato interessante notare come RDC34 ed RDC40 (quest'ultimo caratterizzato dall'aggiunta del gruppo NO_2) mostrassero un'aumentata citotossicità (IC_{50}<1 μM) in confronto a RDC11, mentre l'aggiunta di un gruppo NH_2 ha mostrato sempre una ridotta citotossicità (IC_{50} entro 2-4 μM), ma inferiore a quella di RDC34 e RDC40. Sorprendentemente, l'aggiunta dell'unità sperminica ha diminuito notevolmente la citotossicità (IC_{50} superiore a 16 μM). Questi risultati hanno confermato la nostra ipotesi iniziale, dimostrando che l'aggiunta di una seconda fenantrolina incrementa fortemente la citotossicità portando l'IC_{50} nel range del nano molare, e hanno dimostrato come l'incremento della solubilità in acqua non sia sempre indice di aumentata citotossicità. Quindi, potenziale redox e solubilità possono modulare la citotossicità degli RDCs per le cellule HCT116 *in vitro*.

Il potenziale citotossico di RDC11, RDC34 e RDC41 è stato sottoposto al National Cancer Institute (NCI) test su 60 linee cellulari tumorali di diversa origine. RDC34 si è rivelato essere il più citotossico nei confronti di quasi tutte le linee cellulari, ad eccezione di quelle adriamicina-resistenti. Mentre RDC34 si è rivelato il più efficace contro le linee tumorali di origine renale, RDC11 si è confermato il più attivo sulle linee tumorali di derivazione polmonare. Questi risultati sottolineano l'importanza che le modificazioni dei ligandi hanno nel modulare l'attività antitumorale, e suggeriscono come possano influenzare l'intensità della citotossicità e la selettività verso diversi tipi di tumori. Per meglio comprendere ciò, abbiamo confrontato l'abilità di legare il DNA e di indurre le ROS (un potenziale indicatore dell'alterazione dell'attività enzimatica redox) da parte dei nuovi RDCs. L'interazione con il DNA non sembra ricoprire un ruolo chiave nel definire la citotossicità di questi composti. A sostegno di ciò, abbiamo dimostrato come RDC34, il più citotossico tra gli RDCs, mostri un'abilità di legare il DNA leggermente minore a quella di RDC11. La citotossicità di RDC34 può essere spiegata con un altro meccanismo: abbiamo infatti osservato come RDC34 sia il più potente induttore di specie reattive dell'ossigeno (ROS) a livello intracellulare e come questo sia in correlazione con la sua abilità di aumentare i livelli della proteina p53 e dei suoi geni bersaglio. Tuttavia, questa spiegazione non può essere estesa ad RDC11, RDC40 e RDC41 in quanto non si sono osservate variazioni significative nella produzione di ROS. RDC34 è

risultato anche un potente induttore della caspasi 8, che riveste un ruolo cruciale nel processo apoptotico. Inoltre, RDC34 si è dimostrato essere il più efficace *in vivo* nel trattare diversi tipi di tumori, sia singenici che xenograft.

3) L'ultima parte di questo lavoro di tesi, invece, si è occupata dello sviluppo di un bioreattore per contribuire alla validazione *in vitro* di nuovi approcci terapeutici. L'idea di questo progetto è nata dalla necessità di trovare delle alternative alle tecniche *in vitro* ed *in vivo* comunemente utilizzate per lo studio dei farmaci, dal momento che entrambe presentano numerose limitazioni di natura etica e scientifica. Inoltre gli RDCs, ed in particolare RDC11, sono capaci di inibire due vie di segnalazione implicate nel controllo del processo metastatico (HIF-1 e mTOR), suggerendo una potenziale attività anti-metastatica. La novità di tale sistema risiede nella possibilità di ricreare *in vitro* un ambiente in cui possono aver luogo processi fisio-patologici simili a quelli riscontrati nei tumori, con l'ulteriore vantaggio di essere facilmente controllato. Tale prototipo, chiamato "plastic mouse", potrà risultare utile per studiare l'efficacia di potenziali molecole ad attività anti-metastatica. Attualmente il plastic mouse è in fase di ottimizzazione per la simulazione del processo metastatico, sia per quanto riguarda l'aspetto ingegneristico che biologico. Il tumore al colon-retto è stato scelto come modello per il nostro studio, dal momento che è una patologia di grande impatto sociale nei paesi occidentali. Per sviluppare il modello di carcinoma metastatico del tumore al colon-retto, le HT-29 sono state utilizzate come cellule tumorali e invasive, le cellule HCEC (cellule epiteliali sane di colon) per simulare il sito principale di crescita del tumore e le IHH (epatociti sani) per mimare il sito secondario di invasione e adesione delle cellule tumorali. Tale dispositivo permetterà di studiare il comportamento di cellule tumorali che sono libere di muoversi da un compartimento che rappresenta il colon, ad un altro che rappresenta il fegato, l'organo bersaglio delle metastasi del tumore colorettale. L'uso del plastic mouse consentirà di coltivare insieme tre linee cellulari che verranno adattate alle medesime condizioni di coltura, in modo da creare un modello *in vitro* di tumore al colon-retto più realistico possibile. Dopo aver testato l'efficacia del dispositivo e sviluppato un modello cellulare adeguato, il plastic mouse sarà utilizzato per lo screening di potenziali composti anti-metastatici. Il mio lavoro è consistito nell'attuare un complesso sistema di co-colture, riuscendo a dimostrare che le tre differenti linee cellulari scelte sono in grado di adattarsi allo stesso terreno di coltura, senza subire variazioni in termini di vitalità e morfologia.

In conclusione, la prima parte di questo studio ci ha permesso di identificare un nuovo meccanismo d'azione di RDC11, diverso dal cisplatino. RDC11 è in grado di inibire la proteina HIF-1α a livello trascrizionale e traduzionale, attraverso un meccanismo complesso che implica anche la diminuzione della via di mTOR, valorizzando così la sua attività antitumorale, caratterizzata anche da un rallentamento del processo angiogenetico. Questi effetti sembrano essere una caratteristica unica per un derivato a base di rutenio, dal momento che in letteratura non vi sono evidenze di altri organometallici capaci di agire su queste due vie di segnalazione. Il secondo studio, ci ha permesso di ottimizzare le proprietà chimiche dei nuovi RDCs, migliorandone la citotossicità e la selettività verso diversi tipi di tumori. Ciò può essere spiegato grazie alla loro capacità di interferire con diverse vie di segnalazione cellulare cruciali per il metabolismo dei tumori. Ora dobbiamo identificare i bersagli diretti di tali composti in modo da spiegare come regolino l'attività di queste vie intracellulari a livello molecolare. La terza parte di questa tesi, nonostante sia alle prime fasi di sviluppo, ci ha permesso di selezionare il mezzo di coltura comune alle tre linee cellulari, dimostrando come esse possano adattarsi ad un ambiente diverso dal proprio. Molto resta ancora da fare per poter raggiungere il nostro obiettivo finale, ovvero quello di ottenere un dispositivo utile a ridurre il consumo di animali nella sperimentazione pre-clinica.

9 | SYNTHÈSE DE LA THÈSE

Caractérisation des propriétés anticancéreuses des composés dérivés du ruthénium: mode d'action, optimisation et développement d'outils expérimentaux.

Les dérivés du platine ont contribué énormément au traitement du cancer, malgré les effets collatéraux graves (neurotoxicité, toxicité rhénale…) et les phénomènes de résistance. Pour dépasser ces limitations, des dérivés contenants des métaux (ruthénium, or, galium, iridium…) différents du platine sont largement étudiés. Le laboratoire étudie depuis quelques années des dérivés du ruthénium (RDCs, ruthenium derived compounds) qui présentent de propriétés biologiques intéressantes et particulières: une activité anticancéreuse *in vivo*, une faible toxicité vis-à-vis des tissus sains, une activité sur des tumeurs résistantes, un mode d'action multiple impliquant, en partie, une interaction avec l'ADN et la dérégulation de l'activité d'enzyme de type redox. Cette dérégulation de l'activité d'enzyme redox représente un mode d'action originale qui permet d'espérer de cibler des enzymes participant au métabolisme spécifique à la cellule cancéreuse.

Mon travail de thèse a consisté à caractériser plus en détail les propriétés biologiques de ces composés à base de ruthénium de manière à pouvoir les optimiser. Trois aspects complémentaires ont été développés:

1) l'étude comparative des effets d'un composé à base de ruthénium (le RDC11) et du cisplatin sur la régulation de voies de signalisation HIF-1 et mTOR qui sont impliquées dans les changements métaboliques propres à une cellule cancéreuse.

2) l'optimisation du RDC11 afin d'améliorer la cytotoxicité et la solubilité du composé

3) le développement d'une approche expérimentale permettant de tester simplement les effets antimétastatiques des composés organométalliques

1) Dans cette première partie, nous avons voulu rechercher le mécanisme d'action d'un des plus prometteurs RDCs, c'est-à-dire RDC11, sur deux voies de signalisation très importantes pour le métabolisme des cellules cancéreuses: la voie de HIF-1, (hypoxia inducible factor 1) et la voie de mTOR (mammalian target of rapamycin). Pour ce travail nous avons choisi deux lignées de cancer du colon : HCT116 et SW480. Les tumeurs au côlon, au-delà d'être la seconde cause mondial de mort due au cancer, sont caractérisées souvent par le surexpression de la protéine HIF-1α et, malgré qu'elles soient une des tumeurs traitables

avec un dérivé du platine (l'oxaliplatin), elles deviennent souvent résistantes à ce médicament. Nous avons montré que le RDC11, et non le cisplatin, réduit les niveaux d'expression de la protéine HIF-1α dans deux lignes du cancer du côlon. Le RDC11 réduit aussi les niveaux d'expression de la deuxième sous-unité de HIF-1, c'est-à-dire HIF-1β, en suggérant que cette double inhibition puisse être encore plus efficace en ciblant la protéine HIF-1 et en annulant ses fonctions. Le RDC11 diminue les niveaux de mARN de nombreux génes cibles de HIF-1α (comme *VEGF*, *GLUT1*, *ENO1*, *Adenylate Kinase 3*) qui ont des rôles importants dans l'angiogenèse et dans le métabolisme énergétique. De cette manière, le RDC11 peut contrarier ces processus qui assurent le statu nutritionnel des cellules cancéreuses et les rendent capables de s'adapter au manque d'oxygène.

Comme pour toutes les protéines, les niveaux d'expression de HIF-1α dépendent de l'équilibre entre la synthèse protéique (transcription et traduction) et la dégradation. Nous avons montré que l'inhibition de la voie du protéasome par le MG132 ne reverse que partiellement la perte de HIF-1α induite par le RDC11. Des résultats semblables ont été obtenus avec des inhibiteurs de calpaines (ALLM), cathepsines (ALLN) et caspases (Z-VAD-FMK), en excluant donc une implication de ces voies dans le procès de dégradation de HIF-1α. Ces résultats montrent que la capacité de RDC11 de réduire les niveaux protéiques de HIF-1 et la dégradation de la protéine ne sont pas corrélées. Pour ce motif, nous avons recherché si le RDC11 pouvait agir au niveau de l'ARNm de HIF-1α. En effet, nous avons vu que le RDC11 réduisait significativement les niveaux d'ARNm du gène HIF-1α. Cependant, il a été intéressant de remarquer qu'il n'y a pas une corrélation parfaite entre les niveaux protéiques de HIF-1α et ses niveaux de mARN après 6 heures de traitement avec RDC11 en normoxie (les niveaux protéiques étaient en effet plus bas vis-à-vis des niveaux d'ARNm). Ceci nous a conduit à penser qu'un autre mécanisme pouvait être impliqué à un temps plus précoces: c'est-à-dire un effet de RDC11 au niveau traductionnel. Ceci étant par ailleurs compatible avec l'idée que le RDC11 induit un stress du réticulum endoplasmique.

Un des mécanismes régulant l'expression de HIF-1 fait appel à la voie mTOR via l'activation de la S6 Kinase qui favorise la traduction de HIF-1. Ceci nous a conduit à considérer la voie de mTOR comme un cible du RDC11. mTOR est la sous-unité catalytique de deux complexes fonctionnellement différentes, nommés mTORC1 et mTORC2. mTORC1 contrôle la prolifération cellulaire à travers la régulation de la synthèse protéique, lipidique et l'autophagie, en phosphorylant et activant son effector S6 kinase (S6K), qu'il active la protéine à son tour la protein ribosomal S6. mTORC2 règle la croissance cellulaire et l'organisation du

cytosquelette, à travers l'activation de la protéine AKT. La phosphorylation d'AKT est une des altérations les plus fréquentes dans les tumeurs humaines.

Mes résultats montrent que le RDC11 est capable de réduire la phosphorylation de la protéine S6 et d'AKT sans changer significativement les niveaux totales d'expression des protéinés, suggérant que le RDC11 peut réprimer l'activité de mTORC1 et de mTORC2. Par ailleurs, j'ai montré que le RDC11 réduisant les niveaux d'ARNm de *Raptor* et *Rictor*, les deux composants qu'ils distinguent mTORC1 de mTORC2 respectivement et qui sont différemment sensibles à la rapamycin. Ces résultats montrent comme RDC11 est capable de réduire l'activité de mTOR à travers l'inhibition des deux ses complexes. Ce résultat est particulièrement intéressant, car le plus grande partie des inhibiteurs de mTOR connus en littérature, tel que la rapamicyn, inhibe le complexe mTORC1 uniquement. Par exemple, le complexe mTORC2 est résistant à la rapamycin.

Etant donné que la rapamicyn inhibe uniquement le complexe mTORC1 et que le RDC11 inhibe les deux complexes de mTOR, nous avons évalué si la combinaison de ces deux molécules avait un meilleur effet en réduisant la croissance des tumeurs *in vivo*. Effectivement, nous avons observé que la combinaison de RDC11 avec le rapamicyn était la plus efficace en réduisant le volume de la tumeur (carcinome pulmonaire de Lewis 3LL implanté en souris femelles C57BL/6), à la fin du traitement. Ce résultat, avec le fait qui RDC11 s'est montré capable de réduire l'ARNm du gène *VEGF*, nous a induit à examiner si notre dérivé de ruthénium exerçait un effet anti-angiogenique *in vivo*. J'ai donc développé une approche consistant à implanté chez des souris des pellets de Matrigel (contenants facteurs de croissance pro-angiogeniques et du RDC11) et à analyser après 4 jours le contenu en hémoglobinique. L'hémoglobine des pellets des souris traitées avec du RDC11 était inférieure de moitié vis-à-vis des pellets des souris contrôles. Cet essai préliminaire suggère que le RDC11 puisse induire un effet anti-angiogénique.

2) Les études décrites ci-dessus ont pour objectif d'identifier les cibles intracellulaires directes des composés dérivés du ruthénium, ce qui permettrait de rationaliser le processus d'optimisation de ces composés. En attendant l'identification de ces cibles directes, nous avons utilisé des analyses structure/activité afin d'améliorer les propriétés chimiques et pharmacologiques de nos composés. Ces travaux ont montré que l'addition d'une phenantroline aux RDCs apportait une augmentation de la cytotoxicité. Ainsi, nous avons décidé de remplacer les deux groupes acetonitrile du RDC11 par une deuxième phenantroline, en dénommant la nouvelle molécule RDC34.

En outre, puisqu'il a été démontré que les RDCs modulent l'activité d'enzymes de type redox grâce à leur potentiel redox, nous avons également modifié ce dernier en ajoutant à la phenantroline de RDC34 un groupe électro-attracteur NO_2 ou un groupe électro-donneur NH_2 en obtenant respectivement le RDC40 et le RDC41. Finalement, pour augmenter la solubilité dans l'eau du RDC34 nous avons rajouté une spermine au groupe phenylpiridine, en obtenant le RDC44, qu'il a montré une bonne solubilité en milieu aqueux. La cytotoxicité de ces nouveaux RDCs a été évaluée par le test MTT sur les cellules HCT116. Il a été intéressant de remarquer que le RDC34 et le RDC40, ce dernier caractérisé par l'addition du groupe NO_2, présentait une cytotoxicité augmentée (IC_{50}<1 µM) comparativement au RDC11, alors que l'addition dans un groupe NH_2 a toujours montré une cytotoxicité réduite (IC_{50} parmi 2-4 µM), mais inférieur à celle du RDC34 et du RDC40.

De façon surprenante, l'addition d'une unité sperminique a diminué considérablement la cytotoxicité (IC_{50} supérieur à 16 µM). Ces résultats ont confirmé notre hypothèse initiale, en montrant que l'addition d'une deuxième phenantroline augmente fortement la cytotoxicité en portant l'IC_{50} dans le range du nanomolaire, et ils ont aussi montré que l'accroissement de la solubilité dans l'eau n'est pas forcément un index pour augmenter l'activité biologique. Le potentiel redox et la solubilité peuvent, ensuite, moduler la cytotoxicité des RDCs pour les cellules *in vitro*.

Les composés RDC11, RDC34 et RDC41 ont été soumis au National Cancer Institute (NCI) afin de les tester sur 60 lignes cellulaires cancéreuses de différentes origines. Le RDC34 s'est révélé être le plus cytotoxique sur presque toutes les lignes cellulaires, à l'exception de celles résistantes à l'adriamycine. Ces résultats soulignent l'importance que les modifications des ligands ont en modulant l'activité anticancéreuse, et ils suggèrent comme ils peuvent influencer l'intensité de la cytotoxicité et la sélectivité vers les différents types de tumeurs. Pour mieux comprendre ceci, nous avons comparé la capacité des nouveaux RDCs de lier le ADN et à produire des ROS, un indicateur potentiel de la perturbation de l'activité d'enzyme redox.

L'interaction avec l'ADN ne semble pas avoir un rôle clé pour définir la cytotoxicité de ces composés. En effet, nous avons montré que le RDC34, le plus cytotoxique parmi les RDCs, a montré une habilité de lier l'ADN légèrement plus faible que celle du RDC11. La cytotoxicité du RDC34 peut être expliquée avec autres mécanismes: nous avons en effet observé que le RDC34 favorise plus la formation d'espèces réactives de l'oxygène (ROS) au niveau intracellulaire. De plus, le RDC34 induit plus fortement la caspase 8, qui est impliquée dans

l'apoptose d'origine extrinsèque. Par ailleurs, le RDC34 s'est a aussi montré efficace *in vivo* pour traiter différents types de tumeurs en singénique ou en xénogreffe.

3) La dernière partie de cette thèse a porté sur le développement d'un bioréacteur pour contribuer à la validation *in vitro* de nouvelles approches thérapeutiques pour le traitement du cancer métastatique. L'idée de ce projet est née de la nécessité de trouver des alternatives aux techniques *in vitro* et *in vivo* couramment utilisés pour l'étude des médicaments antimétastatiques, puisque les deux présentent de nombreuses limitations de nature éthique et scientifique. En outre, les RDCs, et in particulier RDC11, sont capables d'inhiber deux voies de signalisation impliquées dans le contrôle de la progression tumorale (HIF-1 et mTOR), suggérant une activité antimétastatique possible. La nouveauté de ce système, le « plastic mouse », réside dans la possibilité de recréer *in vitro* un environnement dans lequel il est possible de reproduire des processus physio-pathologiques similaires à ceux trouvés dans les tumeurs, avec l'avantage supplémentaire d'être facilement contrôlé. Actuellement, le «plastic mouse» est en phase d'optimisation pour la simulation du processus métastatique. Le cancer métastatique du colon a été choisi comme modèle pour notre étude, vu l'important impact social qu'il a dans les pays occidentaux.

Pour mettre en place le modèle du cancer métastatique du colon *in vitro*, les HT-29 ont été utilisées comme cellules tumorales invasives, les cellules HCEC (ligne cellulaire dérivée par l'épithélium intestinal) pour simuler le site principal de la croissance tumorale et les IHH (hépatocytes) pour représenter le site secondaire de l'invasion et d'adhésion des cellules cancéreuses. Le dispositif permettra d'étudier le comportement des cellules cancéreuses qui sont libres de se déplacer d'un puits/chambre représentant le côlon à un puits/chambre qui simule le foie, l'organe cible des métastases du cancer au colon. L'utilisation du « plastic mouse » permettra de garder ensemble trois types de cellules différentes qui seront adaptés aux mêmes conditions de culture, afin de créer un modèle *in vitro* du cancer du colon le plus réaliste possible. Après avoir testé le dispositif et développé un modèle de cancer métastatique du colon, le « plastic mouse » sera utilisée pour l'étude des composés anti-métastatiques.

Mon travail a consisté à mettre en place ce système complexe de co-culture. Après avoir réaliser certaines améliorations, j'ai réussi à montrer que les différentes lignées cellulaires peuvent s'adapter à un même milieu de culture, sans modifier leurs caractéristiques.

En conclusion, la première partie de cette étude nous a permis d'identifier un nouveau mécanisme d'action d'un composé organométallique à base de ruthénium, le RDC11,

différent du cisplatine. Le RDC11 est apte à réprimer la protéine HIF-1α au niveau transcriptionnel et traductionnel, via un mécanisme complexe mettant en jeu une diminution de l'activité de la voie mTOR, favorisant ainsi son activité anticancéreuse, aussi caractérisée par un ralentissement du processus angiogénique. Ces effets semblent être une caractéristique unique pour un dérivé à base de ruthénium, puisque dans la littérature il n'y a pas d'autres exemples de composés organometalliques capables d'agir sur ces deux voies de signalisation cellulaire. La seconde étude nous a permis d'optimiser les propriétés chimiques des nouveaux RDCs en améliorant la cytotoxicité et la sélectivité vers les différents types de tumeurs. Ceci peut être expliqué grâce à leur capacité d'interférer avec différentes voies de signalisation cellulaire cruciales pour le métabolisme des tumeurs. Il nous reste à identifier les cibles directes des ces composés afin d'expliquer au niveau moléculaire comment ils régulent l'activité de ces voies intracellulaires. La troisième partie de cette thèse, malgré qu'elle soit encore aux premiers stades d'optimisation, nous a permis de sélectionner le milieu de culture commun aux trois lignées cellulaires, en démontrant qu'elles peuvent s'adapter à un environnement autre que le leur. Beaucoup d'efforts seront nécessaires pour atteindre notre objectif principal, c'est-à-dire d'obtenir un dispositif utile pour réduire la consommation d'animaux dans les essais précliniques.