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Original Articles

A redox ruthenium compound directly targets PHD2 and inhibits the HIF1 pathway to reduce tumor angiogenesis independently of p53

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ABSTRACT

Targeting specific tumor metabolic needs represents an actively investigated therapeutic strategy to bypass tumor resistance mechanisms. In this study, we describe an original approach to impact the cancer metabolism by exploiting the redox properties of a ruthenium organometallic compound. This organometallic complex induced p53-independent cytotoxicity and reduced size and vascularization of patients-derived tumor explants that are resistant to platinum drugs. At the molecular level, the ruthenium complex altered redox enzyme activities and the intracellular redox state by increasing the NAD + /NADH ratio and ROS levels. Pathway analysis pointed to HIF-1 as a top deregulated metabolite pathway. Unlike cisplatin, treatment with the ruthenium complex decreased HIF1A protein levels and expression of HIF1A target genes. The rapid downregulation of HIF1A protein levels involved a direct interaction of the ruthenium compound with the redox enzyme PHD2, a HIF1A master regulator. HIF1A inhibition led to decreased angiogenesis in patient-derived xenografted using fragments of primary human colon tumors. Altogether, our results show that a ruthenium compound impacts metabolic pathways acting as anticancer agents in colon cancer via an original mechanism of action that affects redox enzymes differently than platinum-based drugs.

1. Introduction

The tumor microenvironment is one of the most important determinants of cancer progression together with the accumulation of intrinsic molecular alterations, such as mutations in the p53 tumor suppressor gene [1]. In recent years, multiple studies have highlighted that cancer cells have different metabolic profiles due to the poorly irrigated tumor by blood vessels and reduced oxygenation. To survive, cancer cells shift from oxidative toward glycolytic metabolism [1]. Several molecular mechanisms ensure this shift, such as the HIF1/VEGF and mTOR pathways that respond to reduction in oxygen and other nutrients, and reprogram the cancer cell metabolism by impacting the activity of metabolic redox enzymes [2]. Thus, small molecules (e.g. rapamycin), or monoclonal antibodies (e.g. anti-VEGF) have been developed to target these pathways. Although these therapies have achieved improvements, clinical data indicate drawbacks mostly linked to their ability to target a single molecule. This limits their use only to a subset of cancers presenting a particular molecular signature, thus favoring the development of resistance by clonogenic expansion [3]. As a consequence, scientists remain challenged to develop new specific drugs directed against novel targets. A complementary and attractive strategy would be to identify anticancer candidates that simultaneously target several key components of the cancer metabolism in order to slow down the development of resistance mechanisms in cells deprived of the drug's single target.

Since the discovery of cisplatin, metal-based drugs turned out to be

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one of the most active fields in antitumor chemotherapy research. Platinum derivatives exert their cytotoxic properties through the formation of DNA adducts, which activate several signaling transduction pathways leading to cell growth arrest or cell death [4]. This poor selectivity is mostly responsible for the side effects of platinum drugs, such as neurotoxicity [5,6]. Furthermore, tumor cell resistances due to mutations in key signaling pathways, such as in p53, limit their use [7].

Ruthenium complexes have been intensively investigated as potential alternative because of the interesting chemical properties of ruthenium: (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) a broad range of redox potentials allowing a potential interference with biological macromolecules [8,9]. Therefore, various structures of ruthenium derivatives have been tested *in vivo* and they seem to offer promising anticancer activities, especially against cisplatin-resistant tumors or even anti-metastatic activity, while presenting a general lower toxicity on healthy tissues compared to cisplatin [8–17]. To date, two ruthenium complexes, namely NAMI-A and KP1019, have entered clinical trials [18].

Even though ruthenium-based compounds have already been tested in humans and largely studied in pre-clinical *in vitro* models, their mode of action remains unclear and most likely involves multiple cellular targets. Indeed, some ruthenium compounds bind DNA, although quite differently from cisplatin [19,20] [21-26]. However, the presence of ruthenium confers also a specific range of redox potentials to the compounds that correlates with their cytotoxicity, and that allows them to be efficient mediators of electron transfer to or from oxidized or reduced active sites of redox enzymes thereby affecting their activity [27–29]. As such, ruthenium derivatives can interact with glutathione (GSH) [30], leading to GSH depletion and increased sensitivity to reactive oxygen species (ROS) [31].

To gain a better understanding of the molecular pathways impacted by ruthenium complexes, we investigated further the mode of action of one of these molecules, RDC11 (ruthenium derived compound 11), which is an organoruthenium compound characterized by a covalent bond between the ruthenium atom and a carbon of a phenylpyridine. RDC11 previously showed interesting anticancer activity *in vivo* in syngeneic models [25,26]. In addition, RDC11 interacts with DNA less efficiently than cisplatin while having a higher cytotoxicity, suggesting that target(s) other than DNA are involved in the ruthenium complexes' cytotoxicity against tumor cells [25,26]. To further understand the signaling pathways involved we performed a transcriptomic approach showing that multiple mechanisms were affected differently than with cisplatin, in particular epigenetic modulators [32]. However, our unsupervised pathway analyses pointed towards additional pathways deregulated by RDC11 treatment, in particular metabolic pathways, suggesting a multimodal mechanism of action. Therefore, we hypothesized that the ability of redox ruthenium-based molecules to interfere with redox enzymes might affect intracellular signaling pathways that are sensitive to the cell metabolism. Hence, we decided to focus on the regulation of one of these pathways, the HIF1 (Hypoxia Inducible Factor 1) pathway, which is strongly connected to the adaptive metabolic response of cancer cells to the hypoxia developing in poorly vascularized tumors [2]. In the current study, we show that alterations of the HIF1 pathway by an organoruthenium compound is an effective strategy to disrupt cancer metabolism and a novel way to bypass mechanisms responsible for platinum drugs' resistance.

2. Materials and methods

2.1. Chemicals

Ruthenium derived compounds were synthesized as previously described [21,28]. Cisplatin was purchased from Mylan Pharmaceuticals, Deferoxamine Mesylate from Sigma-Aldrich® and MG132 from Calbiochem. ALLM, ALLN and Z-VAD-FMK were obtained from Tocris Bioscience. Glucose oxidase from *Aspergillus niger* was obtained from Sigma (211 U mg^{-1}), and D-glucose was obtained from Acros.

2.2. Cell culture, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) test and NCI IG_{50}

Human colorectal adenocarcinoma HCT116 and SW480 cells, obtained from ATCC, 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% fetal calf serum (Life Technology), penicillin/streptomycin (100 UI/mL – 100 µg/ml) and gentamycin (50 mg/mL). HUVEC (Human Umbilical Vein Endothelial Cells, ATTC) were grown in Endothelial Cell Basal Media from Cambrex, supplemented with L-glutamine and 10% FBS. Cell lines are every 6 months tested for mycoplasmas (MycoTOOL, Roche) and are not maintained more than 20 passages. MTT test was performed using 96-well culture plates (Costar) [33]. NCI renamed the IC_{50} into the GI_{50}, the concentration that causes 50% growth inhibition, to emphasize the correction for the cell count at time zero; thus, GI_{50} is the concentration of test drug where $100 \times (T-T0)/(C-T0) = 50$ (dtp.cancer.gov/databases_tools/docs/compare/compare_methodology.htm#specon).

2.3. Ruthenium complex-matrix affinity precipitation

Cells were grown in 10 cm plates. Precipitations were performed as previously described [6]. Cells were lysed in 1 mL of NP40 lysis Buffer 1X (125 mM Tris–HCl, pH 6.7, containing 0.1% NP40, 10% glycerol, 150 mM NaCl). Equal amounts of total-protein extracts (3 mg) were incubated for 5 h with 100 µL of 50% slurry RDC11-matrix beads. After 4 washes in NP40 lysis buffer, complexes were either boiled or eluted using RDC11 (5 µM) or cisplatin (5 µM). PHD2 was then detected by Western blot. Matrix-RDC11 was synthesized attaching RDC11 onto a Hypogel 400-COOH (Sigma). Successful synthesis was assessed by spectroscopy.

2.4. Quantitative reverse transcription-PCR (RT-qPCR)

Gene expression was assessed by qPCR using *18S* as the normalizing gene [5]. Total RNA was isolated with TRIzol reagent (Invitrogen). RNA was quantified using a Nanodrop 2000 Spectrophotometer (Thermo Scientific) and cDNA synthesized from 1 µg 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 are indicated in supplementary material and methods.

2.5. 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 and as previously described [34]. For ROS quantification, cells were seeded in 96-well black plates (Greiner Bio-One) and treated with RDC. Afterwards, cells were washed with PBS and incubated with 10 µM carboxy-H2DCFDA in DPBS for 1 h. 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.

2.6. Clonogenic assay

Cells were transfected by control siRNA or HIF1A-directed siRNA

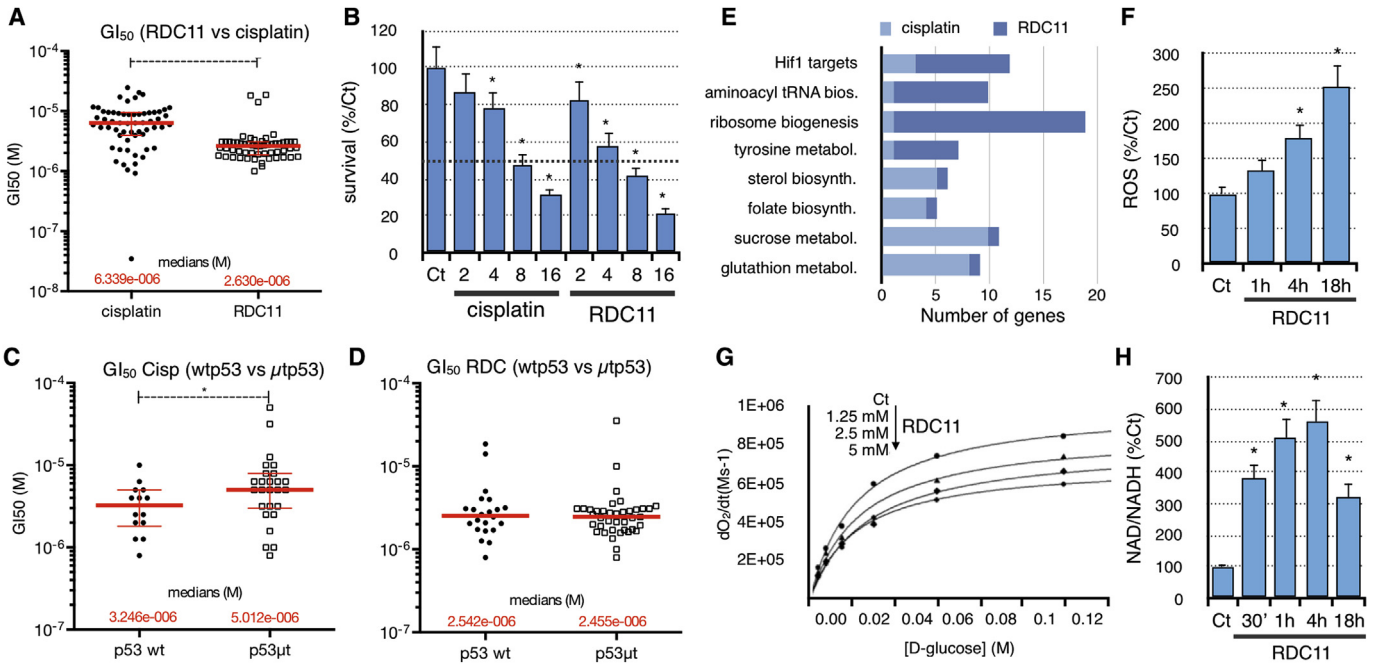

Fig. 1. An organoruthenium compound induces cytotoxicity and regulates cellular metabolism independently of p53.

A, Growth inhibition (GI) caused by the organoruthenium compound and cisplatin on the 60-cancer cell line panel of the NCI. Median GI_{50} (dose for growth inhibition of 50%) for all NCI cell lines is indicated. * indicate $p < 0.05$ as calculated by *t*-test.

B, Survival dose response of HCT116 colon cancer cells upon of RDC11 and cisplatin treatment. Cells were treated for 48 h at the indicated concentrations (µM) of cisplatin and RDC11 at confluence. Cell viability was determined using the MTT test. 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.

C, D, Growth inhibition (GI) caused by the organoruthenium compound and cisplatin on the 60-cancer cell line panel of the NCI classified by expression of wild-type (p53 wt) or mutated (p53 µt) p53 protein. * indicate $p < 0.05$ as calculated by *t*-test.

E, Graphs represents number of genes in the indicated pathways that are regulated by RDC11 and cisplatin at 24 h. Microarray data were analyzed using AltAnalysis and R bioinformatics tools to identify in KEGG, Gene Ontology, miRNA, transcription factors databanks, the signaling pathways and mechanisms corresponding to the mis-regulated genes.

F, Production of radical oxygen species (ROS) in HCT116 cells treated with RDC11. HCT116 cells grown on coverslips coated with polyornithine were treated with RDC11 (RDC, 5 µM) for the indicated time and labelled 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.

G, RDC11 increases the NAD+/NADH ratio. 2×10^5 HCT116 cells were treated with RDC11 (5 µM) for the indicated time. NAD+ and NADH were subsequently quantified following the manufacturer's protocol **(Supplementary materials and methods).** Both NAD+ and NADH were calculated from a standard curve (*** = $p < 0.001$; One-Way ANOVA + Tukey post-test).

H, Measure of O_2 consumption by glucose oxidase in presence of RDC11. Purified glucose oxidase was incubated with increased concentration of glucose and RDC11 (1.25, 2.5 and 5 mM). O_2 was measured using a Clarke electrode and represented as dO_2/dt(Ms-1).

(50 nM, Qiagen™, USA) for 48 h using RNAiMax (Invitrogen™, USA). Cells were then harvested and diluted after treatment, and either 100 or 200 cells were seeded in 6-well microplates. They were then cultured for 12 days, and clones were stained with a methylene blue solution. Surviving positive clones, defined as clones composed of > 50 cells were counted. The plating efficiency (PE) was determined from the number of positive clones obtained when cells were not treated. The percentage of surviving clones in other experimental conditions was calculated by normalizing the number of positive clones with respect to the PE.

2.7. Measure of purified PHD2 activity

Biotinylated peptides derived from the HIF1A Oxygen dependent degradation domain (ODDD: Biotin-DLDLEALAPYIPADDDFQL) were immobilized on NeutrAvidin-coated 96-well plated. 50 ng of purified PHD2 enzyme (Recombinant Human EGLN1/PHD2 Protein; H00054583-P01, Novus Biological) was then incubated at 30 °C for 1 h in a reaction buffer containing 40 mmol/L Tris–HCl, pH 7.4, 4 mmol/L 2-oxoglutarate, 1.5 mmol/L FeSO4, 10 mmol/L KCl, and 3 mmol/L MgCl2 in absence or presence of the ruthenium complexes, deferoxoamine or cisplatin [35]. Peptide hydroxylation was detected using a polyclonal rabbit antibody raised against a hydroxylated HIF1A (D43B5, Cell Signaling), followed by addition of a goat anti-rabbit HRP-conjugated secondary antibody.

2.8. In vitro angiogenesis

The anti-angiogenic activity was studied by looking at 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. 1 h later, cells were treated with 200 µL of medium containing cisplatin or RDC11. After 4 h, cells were photographed and tube formation or intersections between cells were scored manually and expressed relative to controls.

2.9. In vivo angiogenesis plug assay

C57BL/6 female mice (6 weeks old) were injected subcutaneously with 600 µl of cold liquid phenol-red free Matrigel (BD Bioscience) supplemented with VEGF (36ng/Matrigel plug; PeproTech), heparin (12 U/Matrigel plug; Sigma), TNF-α (0.72 ng/Matrigel plug; PeproTech), PBS (for controls) or RDC11 (5 µM), near the abdominal

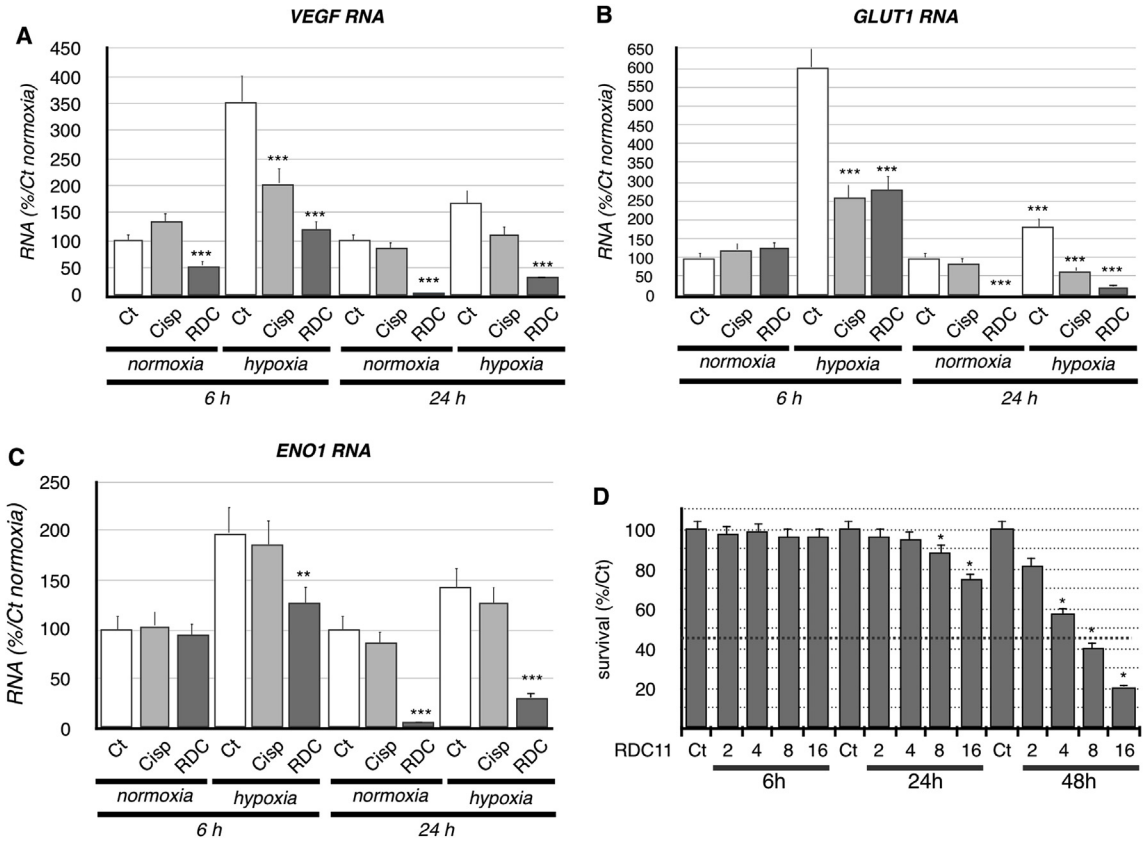

Fig. 2. RDC11 reduces expression of HIF1A target genes.
A-C, HCT116 cells were treated with 5 μM of cisplatin or 5 μM of RDC11 for the indicated time in normoxic (20% O_2) or hypoxic (1% O_2) conditions. RT-qPCR was performed using primers for *VEGF* (A), *GLUT1* (B), *ENO1* (C), and *18S* as housekeeping 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.
D. Survival dose response of HCT116 colon cancer cells upon of RDC11 treatment. Cells were treated for the indicated time at the indicated concentrations (μM) of RDC11. Cell viability was determined using the MTT test. 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.

midlines and at the base of the neck. 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).

2.10. In vivo angiogenesis in human xenografted colon tumors

Fragment of a surgical piece of a human colon tumor was subcutaneously implanted and amplified in nude mice. Pre-amplified tumors were extracted and homogenized prior to implantation into novel nude mice. Two homogenous tumor samples (100 μL) were implanted subcutaneously at each side of mice back. Treatments were started once tumors reached 100 mm^3 of volume. Tumor volumes and mouse weights were monitored twice a week. Vascularization was monitored by the hemoglobin content measurement by a colorimetric assay and the expression of HIF1 target genes by RT-qPCR. Experiments were conducted in compliance with the French Animals Committee guidelines for the welfare of animals in experimental procedures and approved by a regional ethical review committee.

3. Results

3.1. The organoruthenium compound RDC11 alters redox enzyme activity and metabolic pathways independently of p53

We assessed the activity of the organoruthenium compounds RDC11 on the NCI-60 human tumor cell lines panel (Fig. 1A, Supplementary Table S1). GI_{50}, which corresponds to the concentration that inhibit 50% of cell growth as measured by the NCI was used to compared the activity between compounds. We found that the activity of RDC11 (median GI_{50} = 2.63 μM) is higher than that of cisplatin (median GI_{50} = 6.339 μM). A typical difference of the response between cisplatin and RDC11 is shown in the colon cancer cell line HCT116 in Fig. 1B. Importantly, unlike cisplatin, the activity of RDC11 was not affected by the presence of p53 mutations (Fig. 1C and D). This result was confirmed using siRNA against p53 and HCT116 cells in which p53 was deleted [21], which did not alter RDC11 cytotoxic activity (Supplementary Figs. S1C and D). In addition, RDC11 displayed anticancer activity in several different colon cancer cell lines (Supplementary Table S2), while presenting a reduced cytotoxicity on healthy cells (Supplementary Figs. S1A and B).

To identify signaling pathway deregulated by RDC11, we performed an unbiased wide transcriptomic experiment followed by bioinformatics pathway analysis [32]. Amongst the deregulated pathways, several cellular metabolic pathways were present (Fig. 1E). Interestingly, RDC11 and cisplatin displayed different alterations of these

pathways. Notably, RDC11 inhibited the expression of genes regulated by the HIF1 pathway unlike cisplatin (Supplementary Tables S3–5). Interestingly, on HCT116 human colon cancer cells, RDC11 increased the levels of reactive oxygen species (ROS) (Fig. 1F). Use of ROS chelators (NAC: N-acetyl-cysteine) reduced slightly the cytotoxicity of RDC11 (Supplementary Fig. S2C). RDC11 also produced a rapid increase in NAD + levels over NADH (Fig. 1H). Similar results were obtained in SW480 colon cancer cells (Supplementary Figs. S2A and B). The ratio NAD/NADH is of importance in controlling the activity of cellular enzymes critical for cancer development, such as PARP and SIRT [36]. These changes in metabolic markers, suggested that RDC11 has the ability to impact on redox enzymes, as previously described for other complexes [29]. Therefore, we used Clarke electrodes to follow O_2 consumption by the redox enzyme glucose oxidase in presence of increased concentration of glucose and RDC11 (Fig. 1G, Supplementary Fig. S1E). We found that increasing quantity of RDC11 reduced the activity of the glucose oxidase redox enzyme. Taken together, these data suggested that treatment with RDC11 affected the cellular metabolism and the HIF1 pathway.

3.2. Platinum and ruthenium compounds differently impact the HIF1 pathway

HIF1 is a transcription factor composed of two subunits: the constitutively expressed β subunit (HIF1B) and the highly regulated α subunit (HIF1A) [37]. The unsupervised pathway analysis identified the HIF1 pathway as deregulated based on change in the expression of several of its targets genes: *VEGFA*, *GLUT1* (*SLC2A1*), *PSD3*, *KLF6*, *NRN1*, *P4HA2*, *EGLN3*, ENO1, *TSC22D* (Fig. 1E; Supplementary Table S3). The unsupervised pathway analysis did not identify the HIF1A pathway as deregulated under the cisplatin condition. However, 3 of these genes were also downregulated by cisplatin but with a lower intensity - *P4HA2*, *VEGFA*, *GLUT1*. Because *VEGF,* involved in angiogenesis, and *GLUT1* and *ENO1,* involved in glucose metabolism are hallmarks of an HIF1A response [38] and resulted in their downregulation by RDC11 in the transcriptomic analysis, we further analyzed their expression. As expected, hypoxia increased the expression of these HIF1 target genes (Fig. 2A–C). After 24 h of treatment, the RDC11 drastically reduced the mRNA level of all 3 genes, both in normoxia and hypoxia. This negative effect was already detected after 6 h. Although cisplatin was able to reduce *VEGF* and *GLUT1* levels in hypoxia, it was clearly less effective than RDC11. Similar results were obtained with additional HIF1A target genes at the mRNA level and the protein level (Supplementary Figs. S3A and B). Importantly, the inhibition of the HIF1 target genes by RDC11 was also observed in the SW480 cells that harbor a mutated p53 protein (p53R273H/P309S). To note, the loss of gene expression was not due to cellular loss/cytotoxicity as we normalized our experiments toward total RNA and reference genes. Furthermore, at 6 h and 24 h there was no significant cytotoxicity detected (Fig. 2D). Altogether, these data indicated that RDC11 was more potent than cisplatin to reduce the activity of HIF1 target gene in colon cancer cells via a p53 independent pathway.

We then monitored the protein levels of HIF1A and HIF1B subunits in HCT116 and SW480 colon cancer cells under the same conditions. As expected HIF1A was not expressed (SW480 cells) or only expressed at low levels (HCT116 cells) in the normoxia condition, due to its O_2-dependent prolyl-hydroxylation with subsequent ubiquitination and proteasomal degradation (Fig. 3A and B). RDC11, unlike cisplatin, strongly reduced HIF1A protein levels in hypoxia in both cell lines and also in normoxia in HCT116 cells, after 6 and 24 h of treatment. HIF1B was constitutively expressed and RDC11 lowered its expression especially after 24 h of treatment in normoxia and hypoxia in both HCT116 and SW480 cells. This inhibition of HIF1A protein level might explain at least in part the cytotoxic activity of the ruthenium compound as the silencing of HIF1A decreased cell survival (Fig. 3C). Inversely, overexpression of HIF1A reduced RDC11 cytotoxicity (Supplementary Fig. S2D).

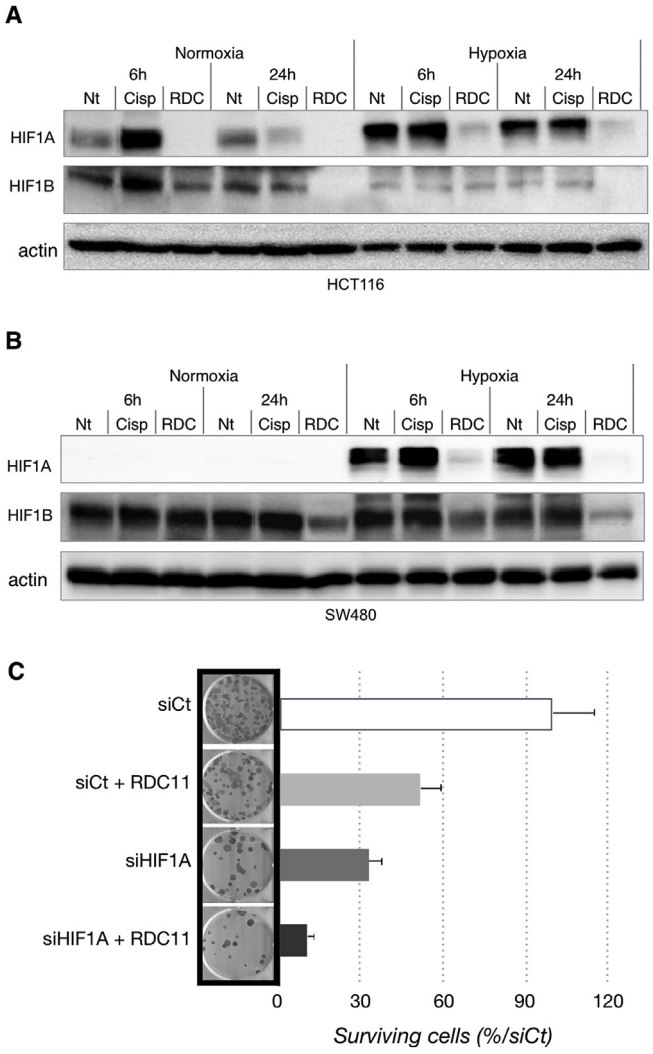

Fig. 3. RDC11 reduces protein levels of HIF1A.
A, B, Western blot analysis of HCT116 (A) and SW480 (B) colorectal cancer cells treated with 5 μM of cisplatin or 5 μM of RDC11 for the indicated time in normoxic (20% O_2) or hypoxic (1% O_2) conditions. Immunoblotting was performed with anti-HIF1A, anti-HIF1B and anti-actin antibodies **(Supplementary materials and methods)**. **C**, HIF1A silencing reduces cell survival and co-operates with RDC11 cytotoxicity. Graphical representation of a clonogenic survival assay of cancer cells that were treated with 0.5 μM RDC11, with an anti-HIF1A siRNA, or with a combination of both molecules. Non-treated cells, as well as cells transfected with a non-specific scramble siRNA were used as negative controls. The percentage of surviving clones in other experimental conditions was calculated by normalizing the number of positive clones with respect to the PE. Pictures showing representative examples of clonogenic survival assays corresponding to each condition are shown left to the graph.

Additionally, RT-qPCR analyses showed that RDC11 was able to strongly reduce *HIF1A* and *HIF1B* mRNA after 24 h of treatment in HCT116 cells, which is consistent with its drastic effect on HIF1 protein levels (Supplementary Fig. S3C). However, the lower reduction of *HIF1A* and *HIF1B* mRNA observed 6 h after RDC11 treatment suggests the existence of other mechanisms (*i.e.* independent of mRNA synthesis and degradation) that can contribute to the rapid and dramatic loss of HIF1A protein levels.

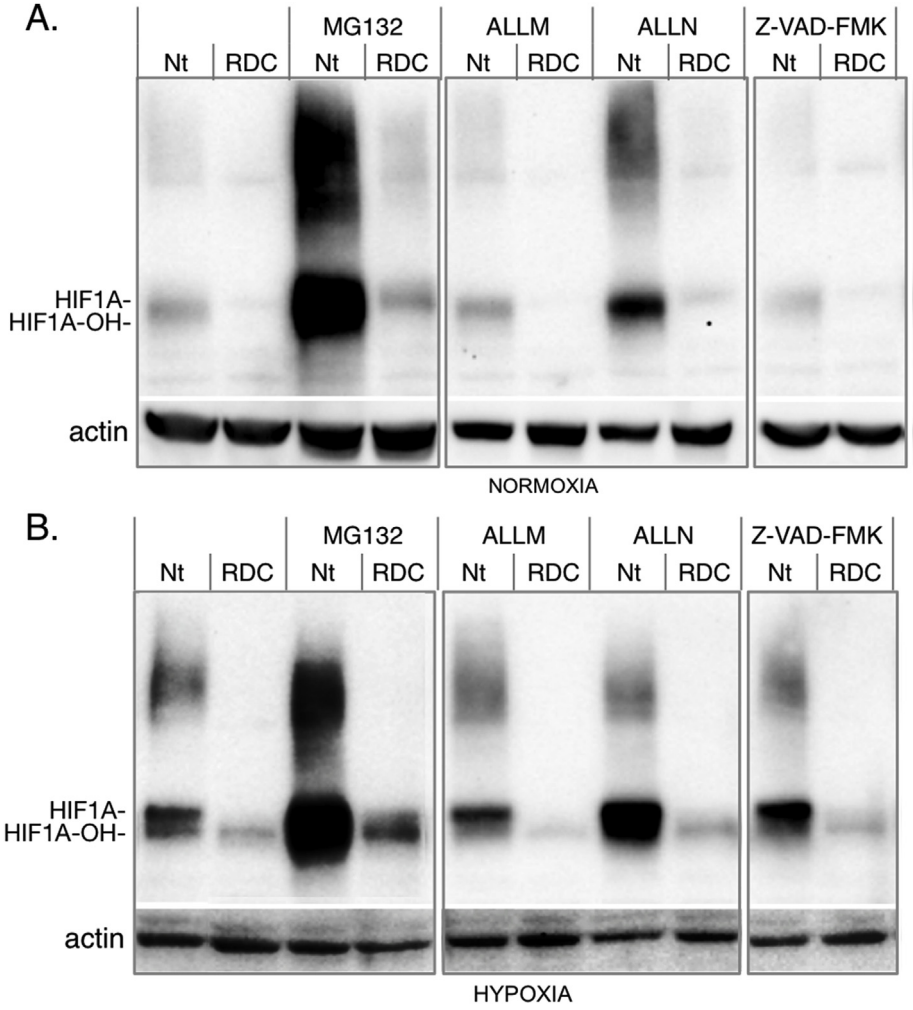

Fig. 4. HIF1A protein reduction involves only partly protease-dependent degradation.
A, **B**, Western blot analysis of HCT116 colorectal cancer cells treated with 5 μM of RDC11 for 6 h in normoxic (A, 20% O_2) or hypoxic (B, 1% O_2) conditions, in absence or in presence of the indicated protease inhibitors (MG132, 10 μM; ALLM, 10 μM; ALLN, 10 μM; Z-VAD-FMK, 20 μM). Immunoblotting was performed with anti-HIF1A and anti-actin antibodies. Note the presence of two bands for HIF1A, the upper band that correspond to the non-hydroxylated form and the lower band that is the hydroxylated form.

3.3. The ruthenium complex reduces HIF1A protein levels through multiple mechanisms

Degradation of HIF1A by the proteasome after hydroxylation by the iron-containing PHD2 enzyme is one of the important mechanisms regulating its cellular levels [37]. Thus, we assessed whether RDC11 was still able to reduce HIF1A protein levels in the presence of MG132, a potent and reversible proteasome inhibitor. As expected, in absence of RDC11, the MG132 treatment clearly stabilized HIF1A proteins (Fig. 4A and B). Strikingly, the accumulation of HIF1A induced by MG132 was also detected, although to a much lower extent, in the presence of RDC11, both in normoxia and hypoxia. In addition, RDC11 favored the accumulation of hydroxylated HIF1A in hypoxia conditions, suggesting that RDC11 promoted the activity of the hydroxylases such as PHD2. Indeed, *in vitro* treatment of cell extracts with RDC11 stimulated hydroxylation of an HIF1A peptide encompassing the oxygen-dependent degradation domain [39] (Supplementary Fig. S5A). In this assay, deferoxamine (DFO) and inhibitor of PHD2 (N-oxal) reduced the RDC11-dependent hydroxylation of the HIF1A peptide, while cisplatin had no impact. Similar results were obtained with PHD2 siRNA (Supplementary Fig. 4). Altogether, these results indicated that RDC11 stimulated the degradation of HIF1A proteins through the proteasome, but it cannot completely account for the drastic reduction of HIF1A protein levels, suggesting the involvement of other mechanisms.

3.4. The ruthenium complex interacts with the hydroxylase PHD2

RDC11 promoted the appearance of the hydroxylated form of HIF1A (lower band) during hypoxia (Fig. 4B), suggesting a possible involvement of the PHD2 hydroxylase. To investigate the direct interaction between PHD2 and RDC11, covalently attached RDC11 molecules onto a matrix (Hypogel 400) (RDC11-matrix) were incubated with cellular protein extracts. After several washes, proteins were detached by boiling or by competition with free RDC11 or cisplatin and separated on SDS-PAGE before immunoblotting for PHD2 detection (Fig. 5A). A naked matrix (Matrix-Ø) was used to assess the specificity of the interaction. Incubation of the cellular extracts with the RDC11-matrix pulled PHD2 down. The specificity of the interaction between RDC11 and PHD2 was confirmed by the absence of bands when the naked matrix was used or when proteins were eluted with cisplatin. This represents the first demonstration of a direct interaction between a ruthenium compound and a redox enzyme in cancer cells.

The iron chelator deferoxamine (DFO), which inhibits PHD enzymes, was used to further investigate the role of PHD2 in RDC11 regulation of HIF1A. As expected, DFO induced HIF1A stabilization through the accumulation of its non-hydroxylated form (upper band,

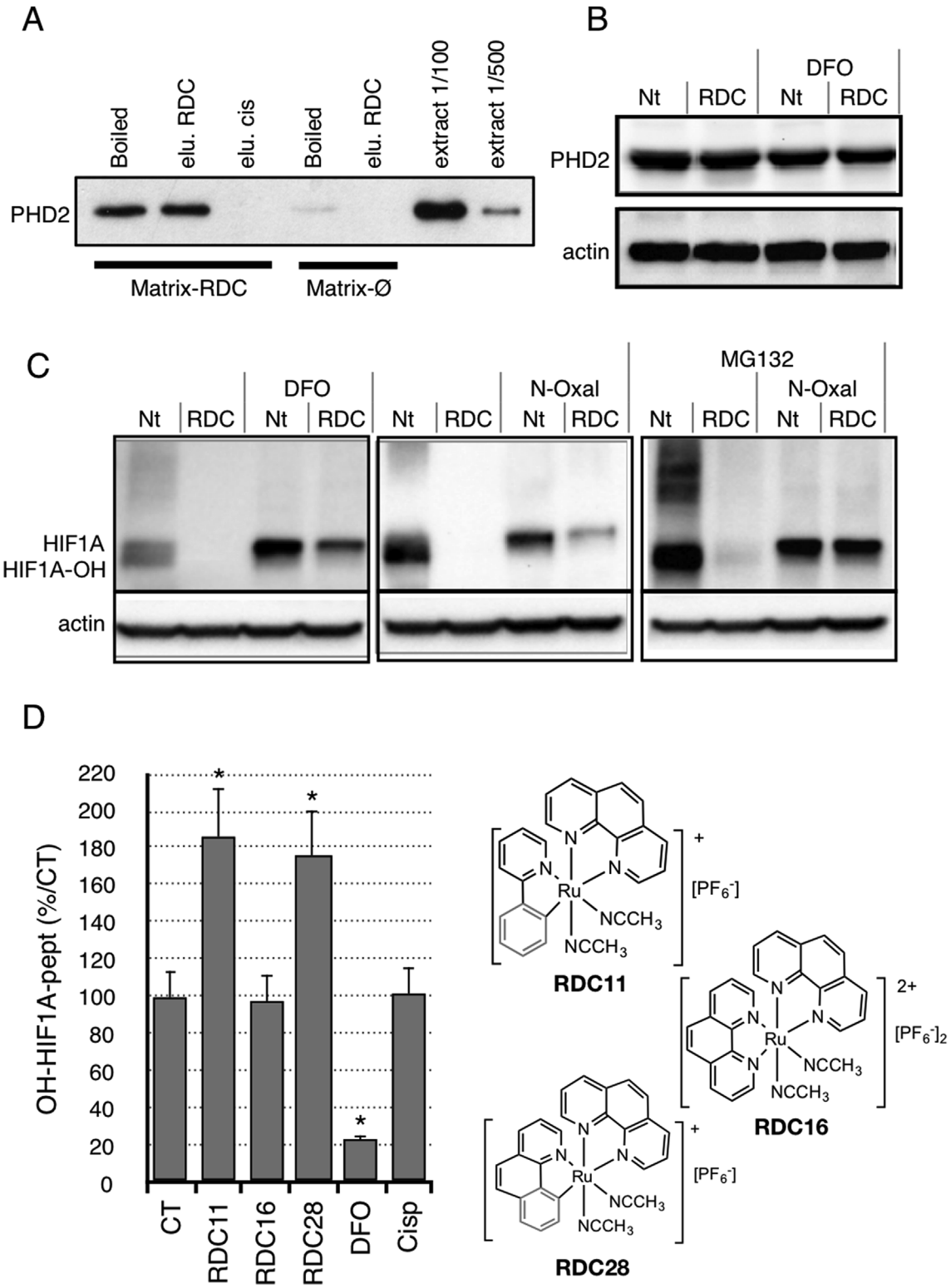

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Fig. 5C). Upon PHD2 inhibition, RDC11 did not induce the appearance of the lower and hydroxylated band of HIF1A. Similarly, inhibition of PHD2 activity with its selective inhibitor N-oxalilglycine stabilized HIF1A with an accumulation of its non-hydroxylated form. In addition, RDC11 inhibited significantly less HIF1A protein level in both cases, indicating the partial dependence of RDC11 activity on PHD2 function. Although these data suggested that RDC11 might affect HIF1A hydroxylation, RDC11 had no effect on PHD2 protein levels (Fig. 5B). In addition, the activity of purified PHD2 was increased by RDC11 treatment unlike cisplatin (Fig. 5D). Interestingly, another ruthenium complex with a similar structure (RDC28 [28]) also enhanced PHD2 activity, in contrast to a close structural variant lacking the carbon-ruthenium bond (RDC16 [40]). Hence, these results indicate that RDC11 directly interacts with PHD2 and that this function is important

Fig. 5. RDC11 interact with PHD2 and its activity is dependent upon PHD2 function.
A, HCT116 cells were lysed and 3 mg of protein extract was incubated with RDC11-matrix or empty matrix (Matrix-O) for each indicated condition. After 4 washes, protein complexes were detached by boiling (boiled) or competition with RDC11 (5 μM, elu. RDC11) or cisplatin (5 μM, elu cis). Proteins were then separated on SDS PAGE and immunoblotted with PHD2 antibody.
B, HCT116 cells were treated overnight with deferoxamine mesylate (DFO, 150 μM) to chemically induce hypoxia and subsequently for six hours with RDC11 5 μM. Western blot was done using anti-HIF1A and anti-actin antibodies.
C, HCT116 cells were treated overnight with 5 μM of RDC11 in normoxia (20% O_2) or in chemically-induced hypoxia using deferoxamine mesylate (DFO, 150 μM), or with MG132 10 μM). N-Oxalilglycine (8 μM) was used to inhibit PHD2. Western blot was performed using anti-HIF1A and anti-actin antibodies.
D, Purified PHD2 (50 ng) were incubated with Biotinylated peptides derived from the HIF1A Oxygen dependent degradation domain (ODDD: Biotin-DLDLEALAPYIPADDDFQL) immobilized on NeutrAvidin-coated 96-well plated. Reaction was performed in presence of the indicated compounds (5 μM, RDC11, RDC16, RDC28, Cisp; 150 μM: DFO) for 1 h. Peptide hydroxylation was detected using a polyclonal rabbit antibody raised against a hydroxylated HIF1A followed by addition of a goat anti-rabbit HRP-conjugated secondary antibody. Columns represent average of triplicates with error bars and * indicate $p < 0.001$ compared to control, as calculated by a One-Way ANOVA test followed by a Tukey post-test.

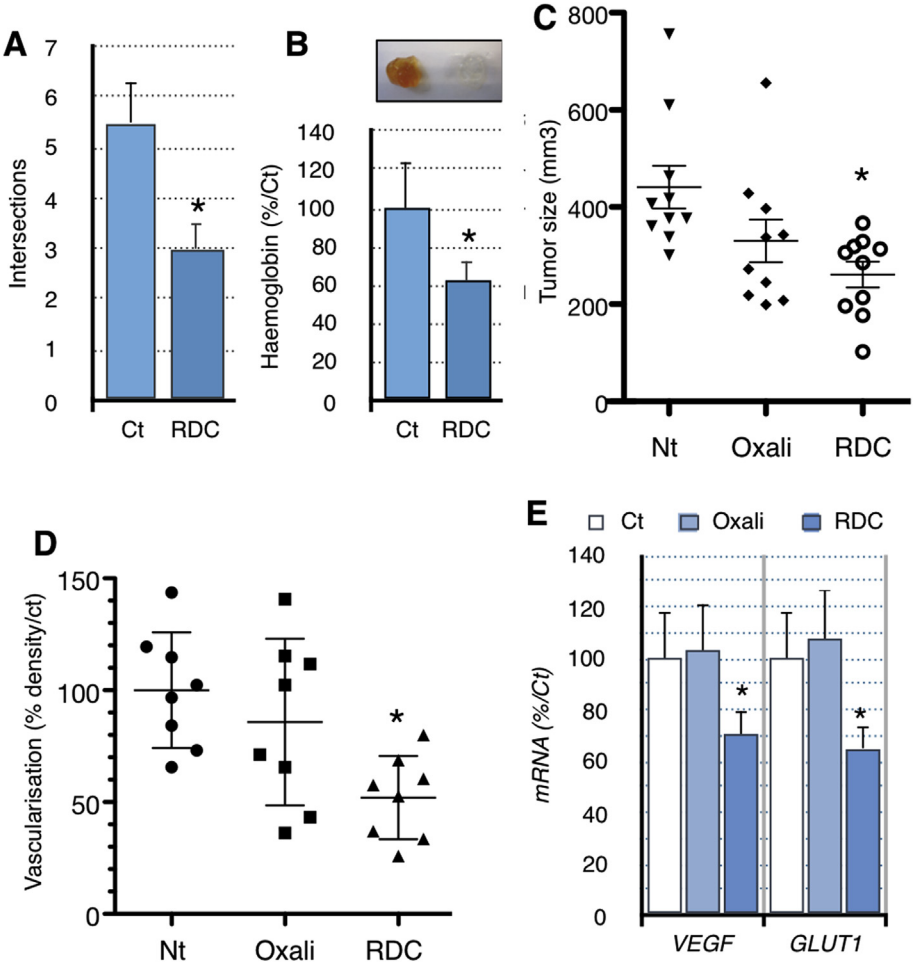

Fig. 6. RDC11 inhibits angiogenesis both *in vitro* and *in vivo*.
A, HUVECs were seeded on ECMatrix™ and 1 h later treated with RDC11 5 μM. After 4 h, cells were photographed and tube formation or intersections between cells were scored manually and expressed relative to controls. (** = $p < 0.01$; * = $p < 0.05$ vs Control; One-Way ANOVA + Tukey post-test).
B, C57BL/6 mice (6-week-old) were injected subcutaneously 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 (5 μM). 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, D, E, pre-amplified homogenized fragments of human colon tumors were subcutaneously implanted in nude mice. Mice were treated with oxaliplatin (11mole/Kg) or RDC11 (11 μmol/kg) twice a week once the tumor reached 100 mm^3. The dose 11μM/Kg corresponds to the MTD in nude mice. Tumor size was followed (D). Vascularization was estimated by colorimetric assay detection of hemoglobin (C) and expression of HIF-1 target genes (*vegf* and *glut1*) by RT-qPCR 21 days after the first treatment. Bars represented means ± standard deviations (n = 8; * = $p < 0.05$ vs Control; One-way ANOVA + Tukey post-test).

for RDC11-dependent downregulation of HIF1 protein levels.

3.5. The ruthenium complex reduces angiogenesis both in vitro and in vivo

HIF1A has a critical role in controlling angiogenesis [41]. *In vitro* tests showed that RDC11 was able to reduce the formation of intersections between HUVEC cells 4 h after treatment (Fig. 6A). To evaluate the effect of RDC11 on angiogenesis *in vivo,* we used an assay where angiogenesis was induced by different factors embedded in a Matrigel plug subcutaneously implanted in mice. Four days after implantation, the plugs were extracted and the degree of vascularization was evaluated by assessing the hemoglobin content. The Matrigel plugs containing RDC11 were nearly uncolored, while the control plugs were redder, sign of the presence of small blood-filled channels (Fig. 6B). 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*.

RDC11 was active against the growth of primary human colon tumor fragments from a patient tissue xenografted in nude mice. The xenografted mice were treated with RDC11 (11 μmol/kg) or Oxaliplatin (11 μmol/kg). Tumors were analyzed 21 days after the first treatment to ensure the structural integrity of the tumors. RDC11 significantly reduced tumor size (± 45%) as compared to Oxaliplatin (Fig. 6C). We then analyzed the tumors for hemoglobin content and expression of HIF1A target genes (*VEGF* and *GLUT1*). Treatment of the tumors with RDC11 reduced both the vascularization and the expression of HIF1A target genes (Fig. 6D and E). The reduced vascularization was further confirmed by analyzing CD31 staining, a marker for angiogenesis (Supplementary Figs. S5B and C).

4. Discussion

The redox properties of ruthenium complexes have made them promising anticancer candidates and potential alternatives to platinum compounds. Indeed, instead of solely targeting DNA like platinum derivatives, ruthenium drugs have been shown to affect the intracellular redox state and activity of metabolic enzymes, which seems to correlate with their cytotoxicity [28] [42] [30] [31] [29]. However, the physiological relevance for their anticancer activity of the functional interaction between redox enzymes and ruthenium drugs is still largely unknown.

This is the first comprehensive study showing a direct link between alteration of redox enzymes by a ruthenium complex (RDC11) and the consequent impact on cancer cell metabolism and tumor growth *in vivo*. Here we demonstrate that the organoruthenium compound RDC11 alters the activity of two redox enzymes involved in the cellular metabolism, namely glucose oxidase and PHD2. Importantly, we report a physical interaction between PHD2 and RDC11 and how this alters PHD2 function, thus affecting the HIF1 pathway, a master regulator of cancer cell survival and tumor angiogenesis. The alteration of these redox enzymes correlates with an increase in ROS and NAD + metabolites. However, it is very likely that the cytotoxicity of RDC11 is not mediated solely by a specific impact on PHD2 and glucose oxidase, but rather on a variety of redox enzymes as well as other types of targets.

The alteration of the metabolism induced by RDC11 might be a direct reflection of the alteration of cellular redox enzymes that have a redox potential in the range of redox potential of the ruthenium compounds [42] [28]. Indeed, PHD2 activity was altered with ruthenium complexes containing a carbon-ruthenium bond and not with a structural equivalent lacking it. These complexes differ only by their redox potential. This multi-targeting property of RDC11 represents an advantage to avoid the development of resistance mechanisms as often seen for selective inhibitors. For instance, RDC11 retains its cytotoxicity on cancer cells that have mutated p53, suggesting that this activity does not depend on the p53 status, which has been recently shown to control cancer cell metabolism [43]. By avoiding the need of the p53 pathway, RDC11 retains its activity on cancer cells with mutated p53 proteins, which represent more than 50% of the tumors.

The RDC11-induced perturbation of the redox enzymes and cellular metabolism affected several metabolic signaling pathways, particularly the HIF1 pathway. Indeed, HIF1A protein levels were dramatically reduced by RDC11 both in normoxia and hypoxia. Interestingly, this effect of RDC11 was much more pronounced than that of cisplatin, pointing out that platinum- and ruthenium-based molecules act differently. The downregulation of both HIF1 subunits correlated with a decrease in the expression of several HIF1 target genes (i.e. *VEGF* and *GLUT1*) linked to angiogenesis or glucose metabolism. Interestingly, multiple studies highlight the role of HIF1 in cancer aggressiveness and platinum resistance [44]. Indeed, silencing of HIF1A decrease cell survival. Hence, the downregulation of HIF1 activity induced by the ruthenium compound might help to bypass tumor resistance and have a double impact on tumor growth by decreasing cancer cell survival and reducing angiogenesis. It is to note that despite inhibiting angiogenesis, RDC11 did not reduce metastasis formation when cancer cells were implanted in the tail's veins of mice (C. Gaiddon; unpublished observations). However, the downregulation of the HIF1 might provide some insights about how ruthenium compounds induce the ER stress pathway [25,45]. Indeed, hypoxia has been shown to induce ER stress [46]. Furthermore, glucose deprivation is a known inducing condition for ER stress activation [47]. Therefore, by acting on the HIF1 pathway, in particular by reducing the expression of glucose transporter GLUT1, ruthenium complexes might favor ER stress.

We identified a PHD2-mediated protein degradation of HIF1A as one of the mechanisms involved in the quick loss of HIF1A proteins observed as early as 6 h after RDC11 treatment. Indeed, RDC11 elevated the level of HIF1A hydroxylation in cells, and blocking the activity of PHD2 with a selective inhibitor affected the ability of RDC11 to reduce HIF1A protein levels. In addition, RDC11 interacted directly with PHD2, an enzyme that controls HIF1A protein levels [37]. Finally, RDC11 induces the activity of purified PHD2 *in vitro*, unlike a non-cytotoxic ruthenium complex. Therefore, we believe that PHD2 is a direct target of RDC11 and that RDC11 might stimulate the activity of PHD2 by a mechanism yet to be discovered but that likely involves the redox potential of the ruthenium complex. However, our results clearly show that mechanisms other than interaction with PHD2 are involved as proteasome inhibitors do not reverse the level of HIF1A protein levels.

For instance, in addition to affecting HIF1A protein degradation, RDC11 drastically decreased *HIF1A* mRNA levels within 24 h, suggesting repression of the *HIF1A* promoter or destabilization of the *HIF1A* mRNA. The exact contribution of these mechanisms in RDC11-induced HIF1A protein loss and its nature remains to be investigated. Destabilization of *HIF1A* mRNA might involve change in expression of miRNA targeting HIF1A. In this respect, our bioinformatic analysis also showed that the aminoacyl tRNA biosynthesis and ribosome biogenesis are affected by RDC11. Both these pathways are known to be deregulated in cancer and linked to mTOR signaling, which is deployed by cancer cells to foster cell survival and proliferation via upregulation of protein biosynthesis [48]. Because RDC11 induces the ER stress pathway [25], which negatively regulates mRNA translation and crosstalk with mTOR [49], we believe that aminoacyl tRNA biosynthesis and ribosome biogenesis could also account for RDC11 anticancer activity. Preliminary results indicate that indeed, RDC11 inhibits the activity of mTOR (V. Vidimar and C. Gaiddon; unpublished observation). However, further investigation is needed to elucidate this.

Nevertheless, by impacting the activity of redox enzymes, the ruthenium compound RDC11 represents an innovative tool to manipulate the cellular metabolism and target cancer cells. Similar observations of inhibition of HIF1A protein levels and activity were done with variants of RDC11, such as RDC34 (C. Licona and C. Gaiddon; unpublished observations) also containing a Ru-C bond and the phenylpyridine and phenanthrolin ligands. This new information on the mode of action of ruthenium compounds is of high interest for anticancer therapy since key actors of tumorigenesis are affected by ruthenium complexes. The HIF1 pathway plays a prominent role in the nutritional status of cancer cells and makes them able to adapt to oxygen deprivation. Our observation that RDC11 reduced VEGF levels and angiogenesis may represent one of the aspects in support of its value for the control of cancer growth. In this respect, the identification of PHD2 as a one of the direct targets of a ruthenium compound alleviate a recurrent unresolved issue in our understanding of the mode of action of these compounds and critical bottleneck in designing more potent and more selective drugs. Interestingly, the regulation of PHD2 seems to be unique to ruthenium complexes with Ru-C bond, as previously shown with other enzymes [42]. Importantly, our results also clearly show that ruthenium complexes do not have a single target. PHD2 and glucose oxidase are not sufficient to explain all the cytotoxicity of RDC11 neither the loss of HIF1A expression. However, this multimodal intracellular action will help to bypass the development of resistances in cancer cells. As PHD2 and other redox enzymes are often ubiquitously present in cancer cells, it is expected that the effects observed in colon cancer cells might also happen in other cancer cell types, although this might have to be confirmed by additional studies.

Finally, when considering ruthenium complexes as a therapeutic alternative, this study highlights two characteristics that can been seen as critical advantages. The first is that platinum and ruthenium complexes clearly display a differential regulation of the HIF1 pathway, which shows that their mode of action differ significantly. Moreover, ruthenium compounds have a more complex mode of action, involving the alteration of the activity of redox enzymes responsible for the cellular metabolism. The fact that ruthenium-based drugs, unlike platinum drugs, target the tumor cells by affecting their metabolic needs, also

provides molecular basis to explain how these drugs can bypass cancer cell resistance mechanisms [21,25,26]. The second important feature is that ruthenium compounds are likely to target simultaneously multiple redox enzymes via their redox potential. This represent an advantage by reducing the ability of the tumors to easily develop resistance mechanisms, as seen when a unique target is aimed. These two properties, independence from p53/DNA damage and simultaneous redox targets, are competitive advantages that point to ruthenium compounds as anticancer drugs.

Conflicts of interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.canlet.2018.09.029.

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