**Universidade do Minho**
Escola de Ciências

Ana Rita Pinto Brás

# Unraveling the molecular targets of new ruthenium-based anticancer drugs

Fevereiro de 2018

UMinho | 2018 Ana Rita Pinto Brás **Unraveling the molecular targets of new ruthenium-based anticancer drugs**

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# Unraveling the molecular targets of new ruthenium-based anticancer drugs

Tese de Mestrado

Mestrado em Genética Molecular

Trabalho efetuado sob orientação de

**Doutora Ana Preto (UMinho)**

**Doutora Andreia Valente (ULisboa)**

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# Declaração

**Nome:** Ana Rita Pinto Brás

**Endereço eletrónico:** ritabras_2_vsc@hotmail.com

**Telefone:** 910617976

**Número do Bilhete de Identidade:** 14649348 6 ZY2

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**Orientadores:** Doutora Ana Preto (Universidade do Minho)
Doutora Andreia Valente (Universidade de Lisboa)

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# Unraveling the molecular targets of new ruthenium-based anticancer drugs


## Abstract

Colorectal cancer (CRC) is one of the most commonly diagnosed cancers worldwide. Nowadays, there are limited chemotherapeutic agents available for the treatment of CRC, which is frequently accompanied by severe side effects and acquisition of resistance. Moreover, CRC that harbor mutations in *KRAS* or *BRAF* and/or *PIK3CA* associated with EGFR overexpression do not respond to EGFR inhibitors available. This constitutes a clinical relevant problem that needs to be overcome.

Ruthenium (Ru) drugs had arisen as one of the most promising metallodrugs with characteristics that increase their specificity and selectivity toward cancer cells. For these reasons, three new multifunctional polymer-metal conjugates of ruthenium (RuPMC) were synthesized, one taking advantage of Ru anticancer properties (PMC79) and two resulting from Ru functionalization to improve the targeting approach (PMC78 and PMC85).

Here, we aimed to assess the effect of the newly synthesized Ru compounds in CRC cells with different genetic background and unravel their mechanisms of action and molecular targets. For that matter, we studied the effect of Ru compounds on cell proliferation, cell cycle and cell death. Further, we evaluated the effect of Ru compounds on the expression of proteins associated to different signaling pathways and of GLUT1. We also analyzed their effect on actin filaments of the cytoskeleton.

The results showed that our compounds induce apoptosis but do not interfere with cell cycle. Moreover, the Ru compounds influence differently the expression of AKT and ERK. The most interesting result was observed with PMC79 in SW480 cells, which decreased the expression levels of p-AKT and p-ERK proteins. We also observed that the RuPMCs affect the actin cytoskeleton and $\beta$-actin expression. Additionally, we could observe that PMC79 upregulated the expression of GLUT1 in SW480 cells, and the combination of PMC79 with STF-31 results in a synergistic effect which potentiate the Ru compound effect.

Our compounds seem to affect differently the two cell lines, being SW480 the most sensitive cell line, mainly to the compound PMC79, however, PMC78 and PMC85 also showed very promising results. In this work, the first steps were taken toward the discovery of the molecular targets and mechanism of action of new Ru compounds in CRC cells, that might be promising agents for CRC therapy.



**Keywords:** Colorectal cancer; ruthenium; apoptosis; *KRAS* mutations; actin cytoskeleton; GLUT1.

# Descobrir os alvos moleculares de novos agentes anticancerígenos derivados do ruténio


## Sumário

O cancro colorretal (CCR) é um dos cancros mais diagnosticados em todo o mundo. Atualmente, existem poucas drogas disponíveis para o tratamento de CCR, que é frequentemente acompanhado de efeitos secundários e aquisição de resistência. CCR com mutações em *KRAS* ou *BRAF* e/ou *PIK3CA* associado à sobre expressão de EGFR não respondem aos inibidores de EGFR disponíveis. O que constitui um grave problema clínico que precisa de ser resolvido. As drogas de ruténio (Ru) surgiram como opções promissoras, com características que aumentam a sua especificidade e seletividade para as células cancerígenas. Por estas razões, três novos conjugados multifuncionais polímero-ruténio foram sintetizados, um juntando as propriedades anticancerígenas do Ru (PMC79), e dois resultantes da funcionalização do Ru, a fim de melhorar a abordagem ao alvo (PMC78 e PMC85).

Neste trabalho pretendemos avaliar o efeito de compostos de Ru recentemente sintetizados em células de CCR com diferentes características genéticas e descobrir os seus mecanismos de ação e alvos moleculares. Para isso, foi estudado o efeito dos compostos na proliferação celular, ciclo celular e morte celular. Também avaliamos o efeito dos compostos na expressão de proteínas associadas a diferentes vias de sinalização e do GLUT1, e o seu efeito nos filamentos de actina do citoesqueleto.

Os resultados mostraram que os compostos induzem apoptose, mas não interferem com o ciclo celular. Para além disso, influenciam de forma diferente a expressão de AKT e ERK. O resultado mais interessante foi observado com o composto PMC79 na linha celular SW480, que diminuiu os níveis de expressão de AKT e ERK. Observamos também que os compostos afetam o citoesqueleto e a expressão de $\beta$-actina. Adicionalmente, o composto PMC79 induziu um aumento da expressão de GLUT1 na linha celular SW480 e a combinação de PMC79 com STF-31 resultou num efeito sinergístico que potenciou o efeito do composto de Ru.

Os nossos compostos parecem afetar as duas linhas celulares de forma diferente, sendo a linha celular SW480 mais sensível, principalmente ao composto PMC79, no entanto os compostos PMC78 e PMC85 também mostraram resultados muito promissores. Neste trabalho foram dados os primeiros passos em direção à descoberta dos alvos moleculares e mecanismo de ação destes novos compostos de Ru em células de CCR, que podem vir a ser drogas promissores para a terapia de CCR.



**Palavras-chave:** cancro colorretal; ruténio; apoptose; mutações em *KRAS*; citoesqueleto de actina; GLUT1.

# Scientific output

## Articles

Teixeira, R. G., **Brás, A. R.**, Côrte-Real, L., Tatikonda, R., Sanches, A., Robalo, M. P., Avecilla, F., Moreira, T., Garcia, M. H., Haukkac, M., Preto, A., Valente, A. (2018). "Novel ruthenium methylcyclopentadienyl complex bearing a bipyridine perfluorinated ligand showing strong activity towards colorectal cancer cell lines". *European Journal of Medicinal Chemistry*, 503–514. https://doi.org/10.1016/j.ejmech.2017.11.059

**Brás, A. R.**, Moreira, T., Francisco, R., Silva, S., Preto, A., Valente, A. "Anticancer properties of new ruthenium-targeted drugs toward colorectal cancer cells". (*in preparation*)

## Abstracts in proceeding

A. Valente, T. Moreira, **A. R. Brás**, A. Preto, M. H. Garcia, "Cancer targeted therapy: the next generation of metallodrugs". *J Biol Inorg Chem* (2017) 22 (Suppl 1): S1–S278, DOI: 10.1007/s00775-017-1475-y

## Oral communications

Ricardo G. Teixeira, **Ana R. Brás**, Leonor Côrte-Real, Rajendhraprasad Tatikonda, M. Helena Garcia, Matti Haukka, Ana Preto, Andreia Valente. "Novel ruthenium cyclopentadienyl complexes bearing bipyridine based (macro)ligands with strong activity towards cancer cells". 4th European Conference on Smart Inorganic Polymers, March 01-03, **2018**, Zagreb, Croatia.

## Posters

Andreia Valente, Tiago Moreira, **Ana R. Brás**, Ana Preto, M. Helena Garcia, "Cancer targeted therapy: the next generation of metallodrugs". ICBIC18 – International Conference on Biological Inorganic Chemistry July, 31st – August, 4th – 2017 - Florianópolis – Brazil.

# Table of contents

# List of abbreviations and acronyms

| | |
|---|---|
| **2-DE** | Two-dimensional gel electrophoresis |
| **5-FU** | 5-Fluorouracil |
| **AKT** | Protein kinase B |
| **APC** | Adenomatous polyposis coli |
| **APS** | Ammonium persulfate |
| **ATCC** | American Type Cultured Collection |
| **ATG5** | Autophagy protein 5 |
| **Bad** | Bcl-2-associated death promoter |
| **Bcl-2** | B-cell lymphoma 2 |
| **Bim** | Bcl-2-like protein 11 |
| **BRAF** | B-Raf proto-oncogene |
| **BSA** | Bovine serum albumin |
| **Caspase-9** | Cysteine-aspartic acid protease 9 |
| **CIMP** | CpG island methylator phenotype |
| **CIN** | Chromosomal instability |
| **CRC** | Colorectal cancer |
| **CREB** | cAMP response element-binding protein |
| **CTC** | Computed tomographic colonography |
| **DACH** | 1,2-diaminocyclohexane |

| | |
|---|---|
| **DAPI** | 4',6-diamidino-2-phenylindole |
| **DCBE** | Double-contrast barium enema |
| **DMEM** | Dulbecco's Modified Eagle's Medium |
| **DMSO** | Dimethyl sulfoxide |
| **DNA** | Deoxyribonucleic acid |
| **dUTP** | Deoxyuridine triphosphate |
| **EDTA** | Ethylendiamine tetraacetic acid |
| **EGFR** | Epidermal growth factor receptor |
| **EPR** | Enhanced permeation and retention |
| **F-actin** | Filamentous actin |
| **FAP** | Familial adenomatous polyposis |
| **FBS** | Fetal bovine serum |
| **FdUMP** | Fluorodeoxyuridine monophosphate |
| **FdUTP** | Fluorodeoxyuridine triphosphate |
| **FIT** | Fecal immunochemical test |
| **FOBT** | High-sensitivity guaiac-based fecal occult blood test |
| **FOLFIRI** | Folinic acid/5-FU/irinotecan |
| **FOLFOX** | Folinic acid/5-FU/oxaliplatin |
| **FOXO** | Forkhead box O |
| **FUTP** | Fluorouridine triphosphate |

| | |
|---|---|
| **GLUT** | Glucose transporter |
| **HCl** | Hydrochloric acid |
| **HIF** | Hypoxia-inducible factor |
| **HK2** | Hexokinase 2 |
| **HNPCC** | Hereditary nonpolyposis colorectal cancer |
| **$IC_{50}$** | Half-maximal inhibitory concentration |
| **IgG1** | Immunoglobulin G1 |
| **JNK** | Jun N-terminal kinase |
| **KRAS** | Kirsten RAS |
| **LC3II/I** | Microtubule-associated proteins 1A/1B light chain 3A |
| **LOH** | Loss-of-heterozygosity |
| **MAPK/ERK** | Mitogen-activated protein kinase |
| **Mcl-1** | Myeloid cell leukemia 1 |
| **MEK** | Mitogen-activated protein kinase kinase |
| **MLH1** | MutL homolog 1 |
| **MMR** | Mismatch repair genes |
| **MSH2** | MutS homolog 2 |
| **MSI** | Microsatellite instability |
| **MSI-H** | Microsatellite instability-high |
| **MSI-L** | Microsatellite instability-low |

| | |
|---|---|
| **$Na_3VO_4$** | Sodium orthovanadate |
| **NaCl** | Sodium chloride |
| **NaF** | Sodium fluoride |
| **$NH_4Cl$** | Ammonium chloride |
| **PAGE** | Polyacrylamide gel electrophoresis |
| **PARP** | Poly (ADP-ribose) polymerase |
| **PBS** | Phosphate buffered saline |
| **PBS-T** | Phosphate buffered saline with 0.05% Tween 20 |
| **PCR** | Polymerase chain reaction |
| **PI** | Propidium iodide |
| **PIK3CA** | Phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha |
| **PIP2** | Phosphatidylinositol-2-phosphate |
| **PIP3** | Phosphatidylinositol-3-phosphate |
| **PKM2** | Pyruvate kinase isozymes 2 |
| **PMC** | Polymer-metal conjugate |
| **PMS2** | Postmeiotic segregation increased 2 |
| **PMSF** | Phenylmethylsulfonyl fluoride |
| **$PPh_3$** | Triphenylphosphine group |
| **PVDF** | Polyvinylidene fluoride |
| **p-AKT** | Phosphorylated-AKT |

| | |
|---|---|
| **p-ERK** | Phosphorylated-ERK |
| **ROS** | Reactive oxygen species |
| **RPMI** | Roswell Park Memorial Institute |
| **RT** | Room temperature |
| **RuCp** | Ruthenium-cyclopentadienyl |
| **RuPMC** | Polymer-metal conjugate of ruthenium |
| **SCFA** | Short-chain fatty acid |
| **SD** | Standard deviation |
| **SDS** | Sodium dodecylsulfate |
| **SMAD4** | Sma- and Mad-related protein 4 |
| **SRB** | Sulphorhodamine B |
| **STF-31** | 4-[[[[4-(1,1-dimethylethyl)phenyl]sulfonyl]amino]methyl]-N-3-pyridinyl-benzamide |
| **tAKT** | Total AKT |
| **TEMED** | Tetramethylethylenediamine |
| **tERK** | Total ERK |
| **TfR** | Transferrin receptor |
| **TP53** | Tumor protein p53 |
| **Tris** | Tris(hydroxymethyl)aminomethane |
| **TS** | Thymidylate synthase |
| **TUNEL** | Terminal transferase dUTP nick end labeling |

**VEGFR** Vascular endothelial growth factor receptor

**XELIRI** Capecitabine/irinotecan

**XELOX** Capecitabine/oxaliplatin

# List of figures

# List of tables

# 1. Introduction

## 1.1. Cancer: an overview

Cancer is a generic term for a large group of diseases, characterized by the rapid and uncontrolled growth of abnormal cells. It can be classified as benign, if restricted to a region of the body, or malignant, if it spreads to adjacent parts of the body and, consequently, to other organs, creating thus metastases, which are the leading cause of cancer death.

Nowadays, more than 100 different types of cancer are known, and this disease is one of the leading causes of death worldwide, affecting people of all age groups and social conditions. A recent study revealed that in 2012, there were 14.1 million new cancer cases and, in 2015, cancer was responsible for 8.8 million cancer deaths worldwide. The most common types of cancer diagnosed were breast, prostate, lung, colorectal and cervix uteri (Torre et al., 2015).

The continued growth and ageing of the world population significantly affect the incidence of cancer, as well as, exposure to common risk factors, such as tobacco, alcohol consumption, unhealthy diet with high sugar and fat content, physical inactivity, overweight and stress (Anand et al., 2008; Torre et al., 2015). Genetic predisposition, certain types of infections and environmental exposure to different sort of chemicals and radiation are also other causes of this illness (Irigaray et al., 2007).

Although the increase in cancer cases is a consequence of modern life and increased longevity, it is known that it is not a disease exclusive of modern times, since there are archaeological records that prove their existence in ancient times (Binder, Roberts, Spencer, Antoine, & Cartwright, 2014).

Cancer can be prevented by modifying or avoiding key risk factors mentioned above. However, cancer mortality can also be reduced if cases are detected and treated early. There are several treatment options known such as surgery, chemotherapy, radiotherapy, immunotherapy and palliative care, which may be used alone or in combination depending on the type of cancer, its localization and its grade of progression (World Health Organization, 2015).

The transformation of a normal cell into a cancer cell requires few molecular, biochemical and cellular changes, despite all potential causes of cancer and carcinogenic pathways (Argyle & Blacking, 2008). In general, these changes occur due to the accumulation of genetic mutations in normal cells affecting their proliferation, differentiation, and development (Knowles & Selby, 2005).

There are two broad classes of genes that play a key role in inducing cancer, oncogenes and tumor suppressor genes. These genes are involved in processes of cell growth, survival and proliferation. Oncogenes are genes that undergo gain of function mutations which are advantageous for tumor progression. These mutations are acquired and permanently activate the oncogenic protein, leading to uncontrolled cell growth (Lodish, Berk, Zipursky, & et al, 2000). Oncogenes usually give rise to proteins involved in the process of cell proliferation and apoptosis, acting as transcription factors, growth factors, growth factors receptors, chromatin remodelers, signal transducers or apoptosis regulators (Argyle & Blacking, 2008; Croce & D. M., 1995). Tumor suppressor genes are genes that, in their normal state, encode proteins involved in the regulation of cell proliferation, repair deoxyribonucleic acid (DNA) mistakes or trigger apoptosis. In these genes, mutations lead to their inactivation and consequently to a loss of function (Argyle & Blacking, 2008; Lodish et al., 2000).

Carcinogenesis (the process of cancer development) is a multistage process (Knowles & Selby, 2005). Each stage reflects genetic changes in the cell with a selective advantage that drives the progression towards a highly malignant cell (Argyle & Blacking, 2008). During carcinogenesis, cells acquire the capability to sustain growth signals, evade growth suppressors, resist to cell death (apoptosis), replicate limitless and be immortal through the reactivation of telomerase, attract and sustain angiogenesis for nutrient supply, invade other tissues and create metastasis, avoid the destruction by the immune system and promote tumor inflammation (**Figure 1**). In addition, tumor cells have high genomic instability and also a deregulation of cellular metabolism (Argyle & Blacking, 2008; Hanahan & Weinberg, 2000, 2011). Together, these characteristics are known as the "Hallmarks of Cancer" and are shared by all cancers (Hanahan & Weinberg, 2000).

The tumor microenvironment also plays a key role in tumor progression. It provides favorable conditions for the survival and growth of cancer cells. The environment of solid tumors is an organ-like structure that is heterogeneous and structurally complex (Trédan, Galmarini, Patel, & Tannock, 2007). It is constituted by cancer cells and cancer stem cells, but also by normal cells like fibroblasts, inflammatory cells and cells of the immune system that are embedded in an extracellular matrix and nourished by a vascular network (Hanahan & Weinberg, 2011; Tomida & Tsuruo, 1999; Trédan et al., 2007).

The microenvironment of cancer cells is conditioned by insufficient and aberrant vascularization leading to a low concentration of oxygen (hypoxia), low pH and decreased supply

of nutrients such as glucose and essential amino acids (Tomida & Tsuruo, 1999; Trédan et al., 2007). These are very particular characteristics that distinguish tumor microenvironment from the environment of normal cells. It is known that the tumor microenvironment might be associated with drug resistance by solid tumors (Trédan et al., 2007).

Considerable efforts have been made in cancer research areas, in order to address the high incidence and mortality levels and, thus, better understand this disease, discover more effective forms of diagnosis and therapy.

Recently, polymer-metal complexes of ruthenium arise as promising candidates for the drug-delivery application and cancer treatment. In the following sections, a review of the literature on colorectal cancer and therapies used today, focusing on its advantages and disadvantages will be carried out. Finally, the features which make these novel ruthenium-based compounds promising candidates for cancer therapy will be also referred.

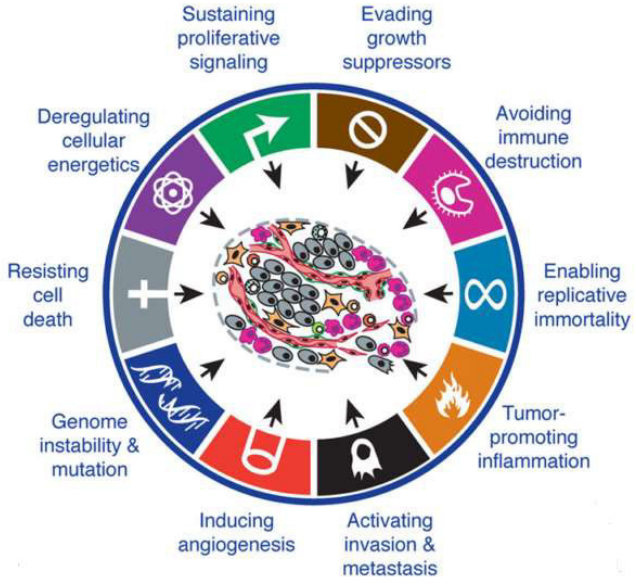



**Figure 1. The ten hallmarks of cancer**: acquired capabilities of cancer cells during carcinogenesis. Adapted from: Hanahan and Weinberg, 2011.

## 1.2. Colorectal cancer

### 1.2.1. From epidemiology to risk factors

Colorectal cancer (CRC) is an important cause of global morbidity and mortality (**Figure 2**), being the third most commonly diagnosed cancer in males and the second in females. In 2012, were estimated 1.4 million cases and 693.900 deaths worldwide (Torre et al., 2015), with almost 55% of the cases occurring in more developed countries (Ferlay, Soerjomataram, *et al.*, 2013).

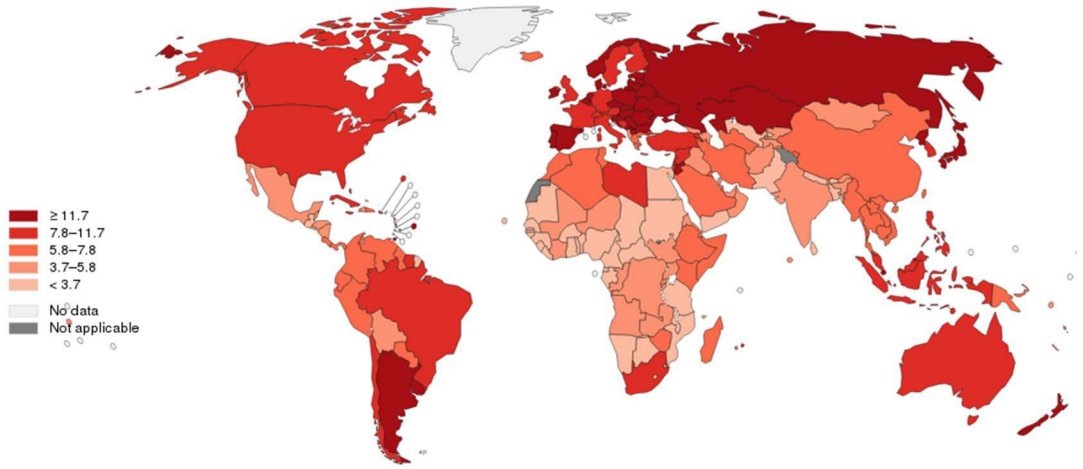



**Figure 2. Worldwide distribution of colorectal cancer mortality in both sexes,** according to the age-standardized rate per 100.000. Source: GLOBOCAN 2012 (International Agency for Research on Cancer 2017). Available from: http://gco.iarc.fr/today, accessed on 21/08/2017.

In Portugal, CRC is also a frequent disease being the second type of cancer most diagnosed as well as the second leading cause of cancer death (J. Ferlay, Steliarova-Foucher, et al., 2013).

Nowadays several risk factors associated with CRC are known. A diet rich in meat and fat, and poor in fiber, folate, and calcium, and tobacco and alcohol consumption are some of the environmental factors associated with this disease (Potter, 1999). Additionally, there are other conditions associated with a sedentary lifestyle, namely, obesity and diabetes mellitus, which are other factors related to CRC development (Weitz et al., 2005).

Although several studies suggest that the male sex, with older age, is the most propitious to develop this type of cancer, it is known that CRC affects equally the opposite sex. In women,

the onset of this cancer is closely related to hormonal factors, such as nulliparity, late-age at first pregnancy and early menopause (Weitz et al., 2005).

The best way to prevent this disease is always to avoid risk factors and maintain a healthy diet by ingesting more vegetables, fruits, micronutrients and foods rich in fiber and calcium, and avoiding the consumption of processed and red meat, alcohol and tobacco (Huxley et al., 2009). Furthermore, maintaining a healthy body weight and regular physical activity are critical to the proper functioning of the body.

Screening can also prevent cancer through the detection and removal of precancerous growths, as well as detect cancer at an early stage, when treatment is more successful. Some screening methods used today are colonoscopy, flexible sigmoidoscopy, double-contrast barium enema (DCBE), computed tomographic colonography (CTC), high-sensitivity guaiac-based fecal occult blood test (FOBT), fecal immunochemical test (FIT) and stool DNA test (American Cancer Society, 2014).

Depending on the stage of cancer, as well as other factors, different types of treatment may be used combined and at the same time or used one after another. There are several types of treatment for colorectal cancer, such as surgery (the type of surgery depends on the location of cancer, in the colon or rectum), radiation therapy, chemotherapy, and targeted therapy that will be further discussed (American Cancer Society, 2014).

### 1.2.2. Anatomy and histophysiology of the colon and rectum

In order to better understand the carcinogenic mechanisms of CRC, in this section, a little of the anatomy, histology, and physiology of the major component of the large intestine will be addressed.

With variable length, which can go from 90 cm to 150 cm, the colon is constituted by ascending colon, transverse colon, descending colon, sigmoid colon and rectum (**Figure 3**). Its main function is the absorption of water, electrolytes, and minerals by the mucosa, and the storage and secretion of residues resulting from digestion (feces) (Kararli, 1995; Levine & Haggitt, 1989).

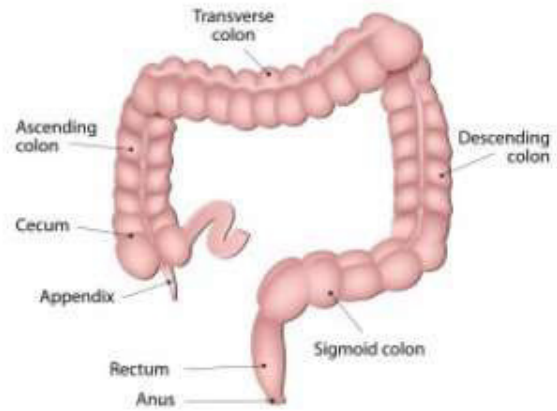



**Figure 3. Anatomy of the large intestine.** Source: Medical News Today. Available from: https://www.medicalnewstoday.com/articles/155598.php, accessed on 05/01/2018.

In this portion of the digestive system are located between 500 to 1000 different microorganisms that metabolize carbohydrates and dietary fiber with the production of short-chain fatty acids (SCFA), flatus and water. The SCFA produced can then be absorbed by the mucosal epithelial cells and serve as a source of dietary calories (Levine & Haggitt, 1989; Sherwood, 2010). Several authors have already proposed the potential of SCFA in the prevention and therapy of colorectal cancer (Ferro et al., 2016; Marques et al., 2013; C. S. F. Oliveira et al., 2015).

Despite the metabolism of carbohydrates and dietary fibers by microorganisms, in the colon, there is no digestion by enzymes (Sherwood, 2010). This function is restricted to the small intestine.

Regarding the histology of the colon and rectum, these are composed of four layers: mucosa, submucosa, muscular and serosa. In the colon, the mucosa (innermost layer) is composed by epithelial cells, lamina propria (rich in lymphocytes) and muscular mucosa (with lymph nodes) (Levine & Haggitt, 1989; Sherwood, 2010). Almost all CRC derive from the epithelial cells of the colon and rectum. In contrast to the small intestine, the colon does not have villi, however, it has the Lieberkühn Crypt which is longer than those in the small intestine, and is constituted by goblet cells, endocrine cells, Paneth cells (important in defense against pathogens), and stem cells (Clevers, 2013). The mucosa layer of the rectum is characterized by a large number of goblet cells. These cells are responsible for the production of mucus that helps to protect the mucosa during the secretion of feces (Levine & Haggitt, 1989).

After this brief overview of the anatomy and histophysiology of the final part of the digestive system, in the next section some of the genetic aspects of colorectal cancer will be addressed.

### 1.2.3. The genetics of colorectal cancer

Colorectal cancer is divided into sporadic, and familial or hereditary syndromes (Móran et al., 2010). CRCs occur sporadically in most of the cases (75%–80%) and are only related to hereditary factors in 10%–30% of the total incidence (Cruz-Bustillo Clarens, 2004; Móran et al., 2010). However, up to 25% of patients have a family history of CRC, suggesting a specific contribution by genes that are yet to be identified (Nosho et al., 2008).

Familial or hereditary CRC syndromes result from germline mutations in genes involved in DNA replication and repair mechanisms (Al-Sohaily, Biankin, Leong, Kohonen-Corish, & Warusavitarne, 2012). They can be divided into adenomatous polyps, which include Lynch syndrome (also called hereditary nonpolyposis colorectal cancer (HNPCC)), familial adenomatous polyposis (FAP), attenuated FAP, and MUTYH-associated polyposis. Hamartomatous polyps are the second type of hereditary CRC and include Peutz-Jeghers syndrome and juvenile polyposis syndrome. The third type is the hyperplastic polyposis, an unusual condition that has a substantial cancer risk and must be distinguished from the other conditions (Jasperson, Tuohy, Neklason, & Burt, 2010).

The most common hereditary CRCs disorders are Lynch syndrome and FAP, which are inherited, autosomal-dominant syndromes (Al-Sohaily et al., 2012; Jasperson et al., 2010; Van Wezel, Middeldorp, Wijnen, & Morreau, 2012). The Lynch syndrome belongs to the microsatellite instability (MSI) pathway, which is known to be related with germ-line mutations in mismatch repair genes (MMR), such as MutL homolog 1 (*MLH1*) (32% of cases), MutS homolog 2 (*MSH2*) (39%), postmeiotic segregation increased 2 (*PMS2*) (15%), and *MSH6* (14%) (Palomaki, McClain, Melillo, Hampel, & Thibodeau, 2009). However, MSI pathway is also involved in sporadic CRC as it will be explained later. Patients with Lynch syndrome present an increased risk of developing other cancers, including ovary, stomach, pancreas, and brain, and normally they arise earlier in life than in the general population (Al-Sukhni, Aronson, & Gallinger, 2008).

Familial adenomatous polyposis is characterized by over a hundred colonic adenomas, and a high penetrance of colorectal cancer with an average age of cancer presentation of 39 years (Clevers, 2006). FAP is caused by mutation of the adenomatous polyposis coli (*APC*), tumor suppressor gene which activates the Wnt pathway. In normal cells, APC regulates $\beta$-catenin intracellular levels, targeting it for ubiquitination (Rao & Kuhl, 2010). These proteins are involved in the Wnt signaling pathway and regulate processes such as cell communication,

growth, and controlled destruction. However, mutations in APC inhibit its binding to β-catenin, leading to the accumulation of that protein within the cell. Increased β-catenin levels can initiate transcriptional activation of c-myc, which lead to uncontrolled proliferation (Kaldis & Pagano, 2009). *APC* mutations also have an important role in the tumorigenesis of sporadic CRCs.

Sporadic CRC is defined as occurring in an individual with no known familial history of a hereditary CRC syndrome and no personal history of a condition associated with an increased CRC risk, namely inflammatory bowel disease (Arvelo, Sojo, & Cotte, 2015; Rosa et al., 2015). Genetically, sporadic CRCs develop by the accumulation of a series of abnormalities in tumor suppressor genes and oncogenes and are divided into three phenotypes (sometimes shared with hereditary CRC), as it will be discussed below. Several authors have suggested the theory of adenoma-carcinoma sequence as the tumorigenic model of sporadic CRC (Fearon & Vogelstein, 1990; Polakis, 2000). The adenoma-carcinoma sequence states that at least seven different events are required for CRC pathogenesis and is initiated by mutation of the *APC* tumor suppressor gene, followed by successive mutations in other genes, as Kirsten RAS (*KRAS*), loss of 18q, Sma- and Mad-related protein 4 (*SMAD4*), and tumor protein p53 (*TP53*) (**Figure 4**). However, other investigators have described another route to colorectal carcinogenesis through serrated polyps (Bettington et al., 2013).

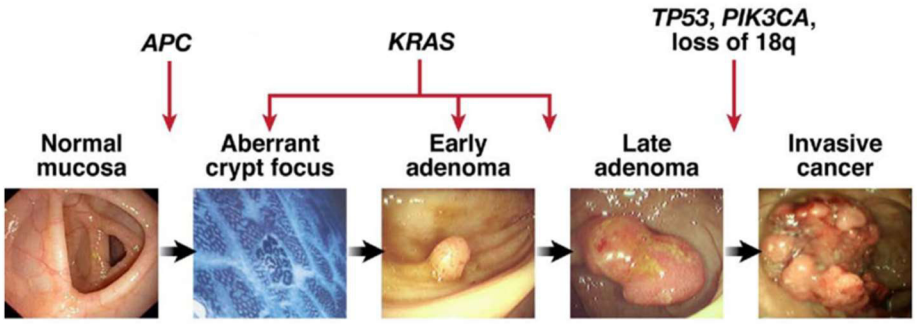



**Figure 4. Multistep genetic model of colorectal carcinogenesis.** The initial step of colorectal tumorigenesis is the mutation in *APC*, which leads to the activation of the Wnt signaling pathway. Progression to larger adenomas and early carcinomas requires activating mutations of the proto-oncogene *KRAS*, mutations in *TP53*, and loss of heterozygosity at chromosome 18q. Mutational activation of *PIK3CA* occurs late in the adenoma–carcinoma sequence in a small proportion of colorectal cancers. Adapted from: Pino & Chung, 2010.

The chromosomal instability (CIN) pathway is the most common phenotype to sporadic CRC (The Cancer Genome Atlas Network, 2012; Yamagishi, Kuroda, Imai, & Hiraishi, 2016). It accounts for 65–70% of sporadic CRC (Al-sohaily, Biankin, Leong, Kohonen-corish, &

Warusavitarne, 2012; Yamagishi et al., 2016). These tumors are characterized by the accumulation of numerical or structural chromosomal abnormalities, resulting in aneuploidy karyotype, frequent loss-of-heterozygosity (LOH) at tumor suppressor gene loci, and chromosomal rearrangements (Grady & Carethers, 2008; Pino & Chung, 2010). Moreover, CIN tumors are distinguished by the accumulation of mutations in specific oncogenes and tumor suppressor genes (e.g., *APC*, *KRAS*, phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit alpha (*PIK3CA*), B-RAF proto-oncogene serine/threonine kinase (*BRAF*), *SMAD4*, and *TP53*), thereby activating pathways critical for carcinogenesis (Grady & Carethers, 2008).

CpG Island Methylator Phenotype (CIMP) pathway is described as an epigenetic instability. It refers to the presence of concomitant hypermethylation of multiple genes (DNA methylation occurs commonly at the 5′-CG-3′ (CpG) dinucleotide) (Al-sohaily et al., 2012; Samowitz et al., 2005). Methylation of gene promoter region results in gene silencing, hence providing an alternative mechanism for loss of function of tumor suppressor genes. Genes involved in colorectal carcinogenesis that are silenced by DNA hypermethylation include *APC*, *MCC, MLH1, MGMT*, and several others genes (J. J. L. Wong, Hawkins, & Ward, 2007). CIMP is commonly associated with *BRAF* mutations (found in approximately 80% of tumors with epigenetics abnormalities) (Nosho et al., 2008).

Microsatellite instability phenotype is caused by DNA MMR deficiency and is characterized by frequent mutations in simple nucleotide repeat sequences (Horvat & Stabuc, 2011; Rosa et al., 2015). MSI tumors lose MMR function early in the cancer progression sequence. Sporadic tumors almost uniformly lose MMR function due to hypermethylation of the promoter of *hMLH1*. In the milieu of MMR absence, a hypermutable phenotype develops in which multiple mutations occur in DNA. Although most mutations occur in noncoding sequences such as intronic DNA microsatellites, certain genes such as *TGFBR2*, *ACVR2*, *BAX*, *hMSH3*, *hMSH6*, and others that have coding microsatellite sequences become frameshifted in the absence of DNA MMR. These mutations help drive the progression of the tumor (Grady & Carethers, 2008). *BRAF* mutations are principally found in sporadic tumors with MSI (Thiel & Ristimäki, 2013). This phenotype accounts for approximately 15% of sporadic CRCs and is divided in MSI-high (MSI-H) and MSI-low (MSI-L) depending on the number of microsatellite panel markers modified (Yamagishi et al., 2016).

### 1.2.4. Colorectal cancer: main mutations and signaling pathways involved

Although often viewed as a single disease, CRC more accurately represents a group of heterogeneous subtypes that result from different combinations of genetic events and epigenetic alterations, and lead to different therapeutics outcomes (Samadder et al., 2013). As reported above, several tumor suppressor genes and oncogenes are involved in this pathology and the most frequent mutations are observed in the *APC*, *KRAS*, *BRAF*, *PIK3CA*, *TP53*, *SMAD4,* phosphatase and tensin homolog *(PTEN)*, MMR genes (*MLH1*, *MSH2*, *MSH6*, *PMS2*), *TGFBR2* and *MUTYH*.

Despite the occurrence of these mutations in different subtypes of colon cancer and at different stages of carcinogenesis, those that are important effectors in key signaling pathways involved in the progression of colorectal cancer stand out, namely *KRAS*, *BRAF*, *PIK3CA,* and *PTEN*.

Concerning *KRAS* (40%) and *BRAF* (15%) mutations in sporadic CRC, they occur alternatively in CRC (Laurent-Puig et al., 2009). These mutations, activate the RAS/RAF/MAPK pathway and are the most frequent oncogenic events in sporadic CRC (C. Oliveira et al., 2003; Rajagopalan et al., 2002; Yuen et al., 2002). Mitogen-activated protein kinase (MAPK) pathway is responsible for a variety of fundamental cellular processes such as proliferation, differentiation, motility, stress response, apoptosis, and survival. This pathway is activated by an extracellular signal that binds to epidermal growth factor receptor (EGFR) (tyrosine kinase receptor). Then the downstream effectors of this pathway (RAS, RAF, mitogen-activated protein kinase kinase (MEK) and mitogen-activated protein kinase (MAPK but hereafter referred as ERK)) are sequentially activated by phosphorylation (**Figure 5**).

The high frequency of activating mutations in *KRAS* and *BRAF* suggests that they are the regulatory hotspot of the MAPK pathway (Orton et al., 2005). This may indicate that RAF is a major effector pathway of RAS in human carcinogenesis (Dhillon, Hagan, Rath, & Kolch, 2007). Another hypothesis may be that *KRAS* and *BRAF* mutations may be synthetic lethal (Orton et al., 2005). Previous studies have analyzed *KRAS* mutations distribution which indicated that G12D, G12V, and G13D, G13C are the most frequent mutations subtype in codons 12 and 13, respectively (Neumann, Zeindl-Eberhart, Kirchner, & Jung, 2009; Shen et al., 2013). In *BRAF*, the V600E mutation, in exon 15, is the most frequent subtype followed by V600M mutation, and

R461K and G465E are the most common mutations, in exon 11 (Shen et al., 2013). *BRAF* mutations are associated with an extremely aggressive phenotype and poor prognosis. Currently, patients with *BRAF* mutation in metastatic CRCs do not respond to any current chemotherapy (Popovici et al., 2012). Little is known about this CRC subtype since the number of patients with tumors with mutated *BRAF* decreases in later lines of therapy, and the median overall survival of this patients is less than 1 year (E Van Cutsem et al., 2010; Eric Van Cutsem et al., 2011).

Beyond the role of MAPK pathway, the PI3K pathway also plays an essential role in colorectal carcinogenesis. PI3K signaling pathway regulates a broad spectrum of cellular mechanisms including survival, proliferation, growth, metabolism, angiogenesis, and metastasis (Fruman & Rommel, 2014). This pathway is also activated by a tyrosine kinase receptor which phosphorylates PI3K protein, which in turn activates the protein kinase B (PKB) (hereafter referred as AKT) (**Figure 5**). Activated AKT leads to the activation of mTOR. The PI3K pathway could be antagonized by a tumor suppressor gene, *PTEN* (Wyatt, Filbin, & Keirstead, 2014). In CRC, 20% of patients carry a *PIK3CA* mutation (Laurent-Puig et al., 2009).

The MAPK and PI3K signaling cascades are pathways that co-regulate each other. AKT directly phosphorylates and inactivates RAF, KRAS activates PI3K through direct interaction with its catalytic subunit, while MEK suppresses PI3K signaling by promoting membrane localization of PTEN (S. K. Hong, Jeong, Chan, & Park, 2013).

Therapies for this type of cancer remain limited and colorectal patients that harbor mutations in *KRAS* or *BRAF* and/or *PIK3CA* do not respond to available treatments, thus this disease is considered a subset equivalent to the triple negative form of breast cancer (Berg & Soreide, 2012; S. Hong et al., 2016; Janku et al., 2011).

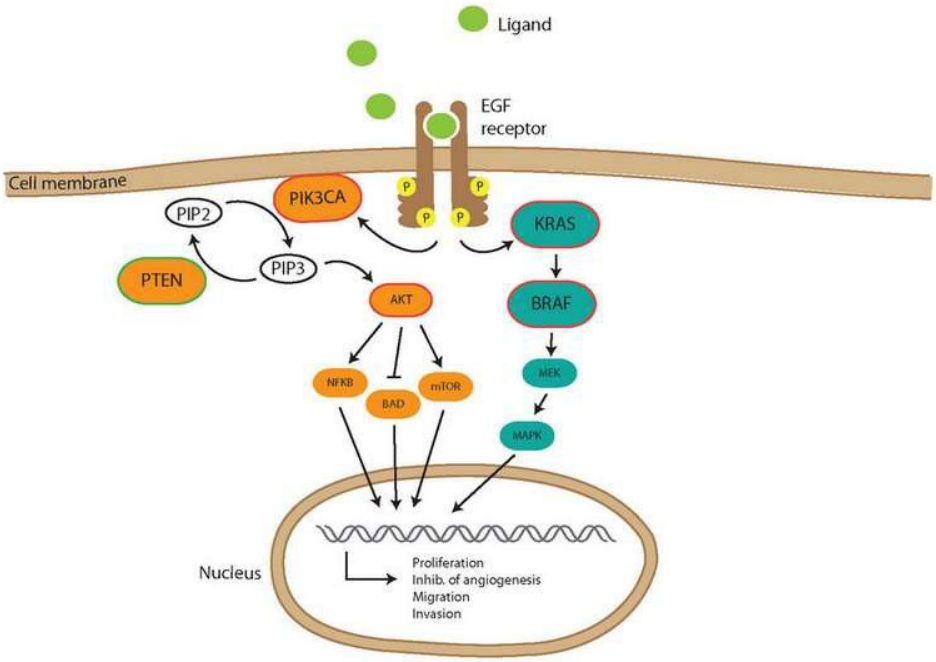



**Figure 5. MAPK and PI3K signaling pathways.** Ligand binds to the extracellular domain of the receptor and results in receptor dimerization and phosphorylation of the intracellular domains. Activated EGFR leads to activation of the KRAS, which in turn activates the BRAF protein, MEK, and ERK (MAPK), and leads to the expression of growth-promoting genes. In addition to activation of KRAS, EGFR activates the PI3K which phosphorylates phosphatidylinositol-2-phosphate (PIP2) to phosphatidylinositol-3-phosphate (PIP3), which in turn activates AKT and several downstream effectors, leading to protein synthesis, cell growth and survival, proliferation, migration, and angiogenesis. Adapted from: Berg & Soreide, 2012.

## 1.3. Chemotherapy in colorectal cancer treatment

The choice of treatment method for CRC is very important because each tumor responds to different methods differentially. It is selected according to many factors including tumor type, stage of the disease, patient's age, patient's level of health, and attitude toward life (Gulbake, Jain, Jain, Jain, & Jain, 2016; Millan et al., 2015).

Currently, four approaches are used in the treatment of CRC (surgery, chemotherapy, radiotherapy and targeted therapies) being surgery the mainstay. In early-stage disease (stage I or II), surgical excision can be used without the need for further treatment options. In stage III of the disease, surgery followed by adjuvant chemotherapy is commonly used. At this stage, the CRC is already metastasized (Marshall, 2008). Patients with stage IV disease require

chemotherapy or targeted therapies combined with surgery (Yasuhiro Matsumura, 2008; Schmoll et al., 2012).

The most widely used chemotherapy agent in the world to treat CRC is fluoropyrimidine, 5-fluorouracil (5-FU). However, 5-FU possesses success rates as low as 10%–15% because of its severe side effects and resistance (Pardini et al., 2011; Provenzale et al., 2015; Eric Van Cutsem et al., 2016). 5-FU is an anti-metabolic drug, similar to uracil (with a fluorine atom at the C-5 position instead of hydrogen), which inhibits essential biosynthetic processes and is incorporated into DNA and RNA, inhibiting their normal function. 5-FU was developed in the 1950s and enters the cell by the same transport mechanism as uracil (Longley, Harkin, & Johnston, 2003). Nowadays, it is known that its mechanism of action varies according to dosage, route, and method of administration.

When 5-FU enters the cells, it is transformed into fluorodeoxyuridine monophosphate (FdUMP). This nucleotide metabolite reacts with thymidylate synthase (TS) enzyme inhibiting its action. Fluorodeoxyuridine triphosphate (FdUTP) appears then, after several enzymatic steps of FdUMP phosphorylation and is incorporated into DNA leading to blockade of DNA synthesis and function. Due to the inhibition of the TS enzyme, an accumulation of deoxyuridine triphosphate (dUTP) occurs, which can then be incorporated into the DNA, resulting in single and double DNA strand breaks (Longley et al., 2003; Rose, Farrell, & Schmitz, 2002). Damage to DNA that occurs during this process leads to cell death by apoptosis (Nita et al., 1998).

Beyond this pathway of action, sometimes 5-FU has a cytotoxic effect directed to RNA, which result in the production of fluorouridine triphosphate (FUTP) metabolite. In these cases, FUTP is incorporated into RNA, causing problems in critical steps of RNA processing and mRNA translation (Aschele, Sobrero, Faderan, & Bertino, 1992; Sobrero, Aschele, & Bertino, 1997).

5-FU has been the backbone of systemic combination chemotherapy for the treatment of CRC and other gastrointestinal cancers, breast cancer, and head and neck cancer (Lee, Beumer, & Chu, 2016; Wilhelm et al., 2016). This drug is normally used in combination with other chemotherapeutic agents, such as irinotecan, oxaliplatin and leucovorin (folinic acid), in CRC, either to maximize their therapeutic effects or to mitigate adverse effects. The most well-known combinations of these drugs are folinic acid/5-FU/oxaliplatin (FOLFOX), capecitabine/oxaliplatin (XELOX), folinic acid/5-FU/irinotecan (FOLFIRI), and capecitabine/irinotecan (XELIRI). More recently, tas-102 and the combination of trifluridine and tipiracil were approved as new chemotherapeutic agents for the treatment of CRC (Lee & Sun, 2016; Zaniboni, 2015).

As mentioned above, there are other chemotherapeutic agents which are used in combination with 5-FU in the treatment of CRC. One of them is irinotecan, a chemotherapeutic drug found in the 1990s that was synthesized from camptothecin. Its mechanism of action involves the inhibition of topoisomerase I activity through the formation of a ternary complex between irinotecan, DNA and topoisomerase I. This complex block DNA uncoiling and cause breaks in the double-strand DNA, leading to DNA damage and cell death (Goldberg et al., 2004; Hsiang, Hertzberg, Hecht, & Liu, 1985). The combination of 5-FU with either irinotecan or oxaliplatin has been widely accepted as standard cytotoxic chemotherapy for metastatic CRC (Colucci et al., 2005; Goldberg et al., 2004).

Leucovorin is another drug belonging to the set of compounds used to treat CRC. Known to potentiate the effect of 5-FU, this drug is metabolized within the cell to the reduced folate 5,10-methylenetetrahydrofolate and forms a ternary complex with FdUMP metabolite and TS, maintaining the enzyme in an inhibited state (Buyse et al., 2000; Rose et al., 2002). The combination of 5-FU/Leucovorin was considered a standard therapy for patients with advanced CRC for many years (Buyse et al., 2000). Although the combination with 5-FU is the most classical in the CRC treatment, leucovorin is also combined with oxaliplatin and irinotecan (De Gramont et al., 1997; Goldberg et al., 2004).

Another drug involved in this process is oxaliplatin. This compound belongs to the group of one of the great success stories in the field of medicinal inorganic chemistry. Platinum anticancer agents are approved worldwide, for over 30 years, to treat several types of cancer in humans. Included in this group are cisplatin, carboplatin, and oxaliplatin, which have DNA as their main target.

Cisplatin was the first to be discovered and is the most commonly used agent of the set. In its mechanism of action, the drug interacts with DNA to form inter-/intra-strand cross-links, as well as DNA-protein cross-links (Kelland, 2007). These adducts cause DNA distortions, including unwinding and flexing, which lead to the activation of DNA repair mechanisms, G2 cell cycle arrest, and apoptotic cell death. It also inhibits DNA replication, RNA transcription and is implicated in signaling pathways that control growth, differentiation and stress responses (Chaney et al., 2004; Kelland, 2007; Siddik, 2003).

Despite the clinical success of cisplatin, it has many disadvantages associated, as the resistance which can be inherent or acquired. In CRC case, the resistance to cisplatin is associated with mechanisms of DNA repair and removal of DNA adducts in the nucleus, and

nonspecific inactivation and efflux at the cytoplasmic level (Raymond, Faivre, Chaney, Woynarowski, & Cvitkovic, 2002). Thus, the second and third generation of platinum-drugs was created to overcome these limitations of cisplatin.

Carboplatin and oxaliplatin entail less pronounced side effects and show significant activity in cisplatin-resistant cancer types (E. Wong & Giandomenico, 1999). These platinum (II and III) complexes kill cancer cells by modification of DNA and subsequent induction of apoptosis (Johnstone, Wilson, & Lippard, 2013). Carboplatin is used for the same spectrum of cancers as cisplatin, but with the advantage of fewer side effects, whereas oxaliplatin responds to a different spectrum of cancers (usually resistant to cisplatin and carboplatin) and has found a major applicability in CRC (Kelland, 2007). The mechanism of action is the same of cisplatin however, oxaliplatin forms a greater number of intra-strand adducts which, if not repaired, block the DNA replication and transcription (Seetharam, Sood, & Goel, 2009).

Oxaliplatin belongs to 1,2-diaminocyclohexane (DACH) carrier ligand family. This type of ligands on the DNA adducts differs from cisplatin and carboplatin (which have cis-diamine ligands) and are responsible for the activity of oxaliplatin in colon cancer cells, since the DNA adducts formed by it cannot be recognized by mismatch repair proteins, DNA damage-recognition proteins and trans-lesion DNA polymerases, which in turn recognize the DNA adducts ligands formed by cisplatin and carboplatin and eliminate the drug, repairing the DNA (Chaney, Campbell, Bassett, & Wu, 2005; Xu et al., 2009).

Several studies have demonstrated the efficacy of this drug in the therapy, as a front-line agent, and also combined with 5-FU, leucovorin, and irinotecan in the treatment of advanced colorectal cancer (Giacchetti et al., 2006; Goldberg et al., 2004; Rothenberg et al., 2003). However, chemotherapy with platinum-based compounds is frequently accompanied by severe side effects and their activity is limited to a small spectrum of tumors due to acquired and intrinsic resistance to treatments (A. Bergamo, Gaiddon, Schellens, Beijnen, & Sava, 2012; Morais, Valente, Tomaz, Marques, & Garcia, 2016).

In addition to the aforementioned drugs, other metal-based drugs are used, such as gold, iron, ruthenium, copper, palladium and silver (Antunovic et al., 2015; Milacic, Fregona, & Dou, 2008; Morais et al., 2016; Samie et al., 2016; Tan, Yan, Lee, & Lim, 2010).

Beyond these chemotherapeutic agents and the different possible combinations between them, sometimes they are also used in conjunction with targeted therapies such as bevacizumab, cetuximab, and panitumumab (Zaniboni, 2015). The use of these therapies targets the two major

growth factor pathways in CRC, the vascular endothelial growth factor receptor (VEGFR) pathway and the EGFR pathway (Lee & Sun, 2016). Cetuximab and panitumumab are monoclonal antibodies that target EGF receptors. Cetuximab was approved in 2004 and is a chimeric immunoglobulin G1 (IgG1) monoclonal antibody which targets the external cell surface domain of EGFR (Lee & Sun, 2016). The receptor-antibody complex is internalized in the cell and degraded, which results in a down-regulation of the EGFR expression by the cells (Dequanter, Shahla, Paulos, & Lothaire, 2010). Panitumumab is a fully human anti-EGFR antibody and was approved in 2006. This IgG2 monoclonal antibody binds to the extracellular domain of the EGFR with high affinity and inhibits downstream signaling (Ciardiello & Tortora, 2008). Although, recent works have demonstrated that tumors with mutations in key downstream effectors of EGFR-signaling pathways, as *KRAS*, *BRAF*, *PIK3CA*, do not correspond to EGFR antibodies (S. Hong et al., 2016; Temraz, Mukherji, & Shamseddine, 2015). This is a clinical relevant problem that needs to be overcome. In this way, novel targeted therapies are being extensively tested with the purpose to target downstream effectors of these important pathways (Roock, Vriendt, Normanno, Ciardiello, & Tejpar, 2011). Bevacizumab is another monoclonal antibody used in targeted therapy, approved in 2004. It is a humanized IgG1 antibody that blocks the action of VEGF (Kelland, 2007). This antibody targets specifically VEGF-A isoform and prevents its binding to similar receptors, inhibiting its activity (Ferrara, Hillan, & Novotny, 2005).

Despite recent progress in cancer therapy, much remains to be done with regard to available drugs and their side effects. In this way, the first goal of researchers working in this area is to try to discover new anticancer drugs with higher efficacy, reduced toxicity, lack of cross-resistance or improved pharmacological characteristics as compared with the parent compound. With this objective in mind, in the following section, some characteristics of ruthenium complexes and the advantages of polymer-metal complexes of ruthenium comparing with the free drug will be deepened.

## 1.4. New metal-drugs under development

Because of the severe side effects of the available therapies and acquisition of resistance of some tumors, research has been directed toward the development of compounds based on other metals. Among the several metal complexes explored so far, ruthenium (hereafter also referred by its chemical symbol - Ru) compounds appear as some of the most promising metallodrugs. The low toxicity, the different mechanisms of action, the spectrums of activity and the potential to overcome platinum-resistance are some of the characteristics that will be deepened in the next section and make these drugs so attractive in future therapies (Antonarakis & Emadi, 2010; A. Bergamo et al., 2012).

### 1.4.1. Ruthenium-drugs

Ruthenium is now a clear candidate for the search for new chemotherapeutics, since complexes bearing this metal core present several properties that make them attractive within this area. Ruthenium is a transition metal belonging to the group 8 of the periodic table with important differences from platinum-drugs. Firstly, Ru(III) drugs may accumulate preferentially in cancer cells compared to normal tissues, possibly by using transferrin to enter in the tumors (Guo et al., 2013). Transferrin is an iron-binding blood plasma glycoprotein, which transports iron and delivery it into the cell through transferrin receptor 1. This receptor is reported to be overexpressed in cancer cells because of the role of iron in several enzymatic reactions critical for DNA replication and cellular respiration (Daniels et al., 2012; Jr., 2007). Ru(III) might mimic iron binding to serum protein, thereby reducing the concentration of free plasma ruthenium and increasing the concentration that reaches the cancer cells compared to healthy cells (Allardyce & Dyson, 2016). Transferrin receptors in the cell-surface bind to ruthenium-loaded transferrin and the complexes are endocytosed and transferred to acidic non-lysosomal compartments where ruthenium is released. This creates an indirect drug targeting mechanism as the ruthenium drugs are selectively accumulated in cancerous cells (Kostova, 2006). Beyond transferrin-dependent transport mechanism, ruthenium complexes can also enter cells by passive diffusion (Webb & Walsby, 2015). The high affinity of ruthenium to transferrin makes these compounds one of the least toxic metallic complexes (Allardyce & Dyson, 2016).

Secondly, ruthenium drugs possess multiple oxidation states (II, III, IV) accessible under physiological conditions. Ru compounds may remain in its relatively inactive Ru(III) oxidation state until reaches the tumor (**Figure 6**). The characteristics of the tumor microenvironment, as low $O_2$ concentrations and acidic pH, leads to the reduction of Ru(III) to Ru(II), in the bloodstream or within the cells, by a mechanism of "activation-by-reduction" (Gianni Sava & Bergamo, 2000). This feature, in some way, makes the complexes more selective to target cancer cells.

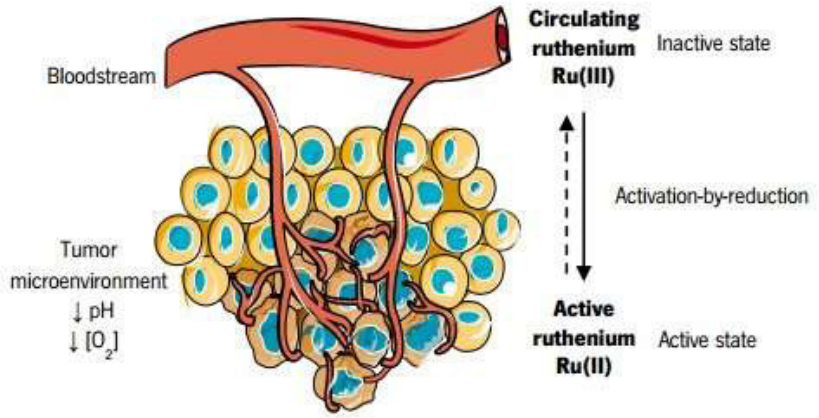



**Figure 6. "Activation-by-reduction" mechanism of ruthenium(III) complexes.** Ruthenium complexes circulate in the bloodstream in their inactive state until reach the reducing environment of the tumors. In the tumor microenvironment, due to the lower pH and the lower concentration of oxygen, Ru agents are reduced and become in its active state. This characteristic allows ruthenium drugs to be more specific in targeting cancer cells. Adapted from: Antonarakis & Emadi, 2010.

Another property of Ru complexes is the favorable ligand-exchange kinetics with low toxicity. This characteristic is shared with cisplatin and is correlated with the capacity of changing some ligands by other molecules found under physiological conditions, activating the compound (Antonarakis & Emadi, 2010). Both drugs have slow metal-ligand exchange rates, comparable to those of cell division processes, which give them high kinetic stability, minimizing side reactions, and make them highly active in killing cancer cells (Wang & Lippard, 2005). This property allows the Ru compounds to remain intact until they reach the cancer cells and gives more control over the stability of the complexes modifying the variation of the ligands (Bruijnincx & Sadler, 2009).

Taking into account the particular characteristics stated above, currently, three Ru(III) complexes went into clinical trials. The first one, NAMI-A is an antimetastatic drug with anti-angiogenic and anti-invasive properties (**Figure 7A**) (Alberta Bergamo & Sava, 2011; Dyson &

Sava, 2006). It is the most stable Ru drug remaining in its inactive state at physiological pH of 7.4 until reaches the reducing microenvironment of the tumor (Amin & Buratovich, 2009; Antonarakis & Emadi, 2010). It enters the cells by passive diffusion and active transportation, through human serum albumin and transferrin (Cetinbas, Webb, Dubland, & Walsby, 2010). NAMI-A cytotoxicity induces G2/M cell cycle arrest when the compound enters the nucleus, however, its mechanism of action involves other targets because this drug preferentially accumulates in the cell walls (Wang & Lippard, 2005; Webb & Walsby, 2013). This complex also interacts with the cytoskeleton and actin-dependent adhesion (G Sava et al., 2004). *In vivo* studies, showed that NAMI-A distributes to the whole body and concentrates on collagen-rich tissues and in kidney and liver (G Sava et al., 2004). Recently, NAMI-A entered in phase I/II clinical trials, where the Ru compound was applied together with gemcitabine in patients with non-small cell lung cancer (Leijen et al., 2015). Although the combination of the two drugs was only moderately tolerated. The results also showed that NAMI-A was less active than gemcitabine alone, and also have more side effects. Unfortunately, NAMI-A is not designed to be applied in this type of cancer and the set of results obtained call into question a possible resumption of clinical trials with this complex (Allardyce & Dyson, 2016).

The second Ru(III) agent entering clinical trials was KP1019 (**Figure 7B**). This drug is active against primary tumors and is endowed with interesting properties against CRC (A. Bergamo et al., 2012; Alberta Bergamo & Sava, 2011; G Sava et al., 2004). KP1019 was developed to treat solid tumors (Alberta Bergamo & Sava, 2011). Concerning its mode of action, KP1019 binds to transferrin and enters the cell by endocytosis of transferrin receptors, where it becomes active (Pongratz et al., 2004). KP1019 also induces apoptosis via the mitochondrial pathway and lead to reactive oxygen species (ROS) overproduction (Hartinger et al., 2006; Scolaro, Hartinger, Allardyce, Keppler, & Dyson, 2008). This drug also accumulates preferentially in the cytoplasm which suggests that its main targets are in the cytosolic fraction (Heffeter et al., 2010; Webb & Walsby, 2013). KP1019 entered in phase I clinical trials in which five out of six evaluated patients experienced disease stabilization with no severe side effects (Hartinger et al., 2008). Although, higher doses of this compound are insoluble in the bloodstream, which impairs the progress of clinical trials. In this way, NKP-1339, a more soluble compound derived from KP1019, was developed (**Figure 7B**) (Hartinger et al., 2006). This compound entered in clinical trials and showed promising results in several types of cancer, with no severe side effects (Trondl et al., 2014).

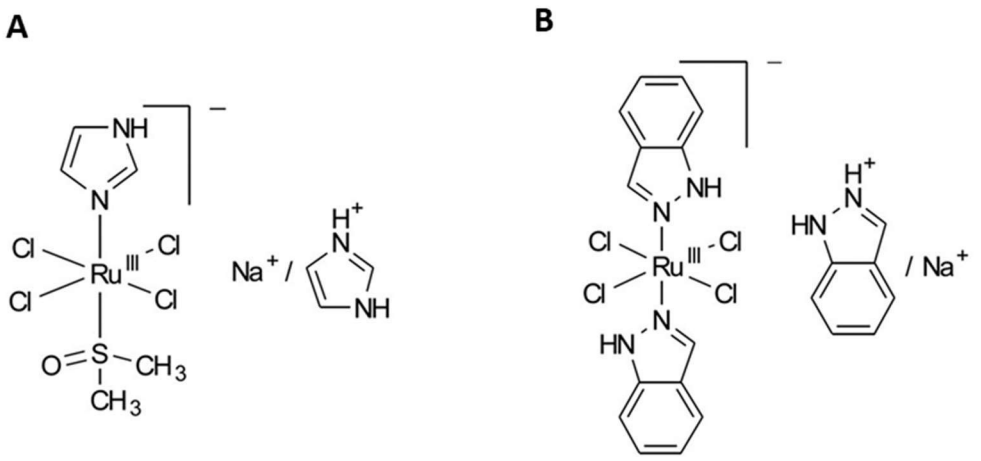



**Figure 7. Structures of NAMI-A (A) and KP1019/NKP-1339 (B).** Adapted from: Trondl et al.,2014.

## 1.4.2. Polymer-ruthenium conjugates in cancer treatment

The principal disadvantages of chemotherapy are related to the fact that it has strong side effects and lack of selectivity. One way of overcoming this issue would be the development of a drug that would act only in tumor cells without triggering responses in healthy cells. To this end, the controlled delivery of the drug would be a more effective way of treating cancer.

Polymer-metal conjugates (PMCs) arise as a polymeric controlled drug delivery that constitutes a promising alternative to the conventional drug delivery approaches in cancer therapy (H. Maeda, Bharate, & Daruwalla, 2009; Park et al., 2008; Parveen, Misra, & Sahoo, 2012). PMCs are generated from the combination of the chemical coordination with a controlled polymerization (Andres & Schubert, 2004; Fraser, C & Smith, 2000). These macromolecules have high molecular weight and can more readily accumulate in cancer cells by the "enhanced permeation and retention (EPR) effect" (**Figure 8**) (Hiroshi Maeda, 2001; Y Matsumura & Maeda, 1986). EPR effect states that macromolecules selectively accumulate in tumors relative to healthy tissues, due to their defective vessel vascular structure, as an irregular shape with random and large fenestration, and decrease lymphatic drainage. This passive targeting results, thus, in the passive accumulation of macromolecules in solid tumors, increasing the therapeutic index, while preventing the undesirable side effects generated by free drugs (Kopecek, Kopecková, Minko, & Lu, 2000). This phenomenon can increase the drug concentration in tumor compared to that of the blood, as high as 10–100 times, and is recognized to overcome the multi-drug resistance (Antonarakis & Emadi, 2010; Süss-Fink, 2010).

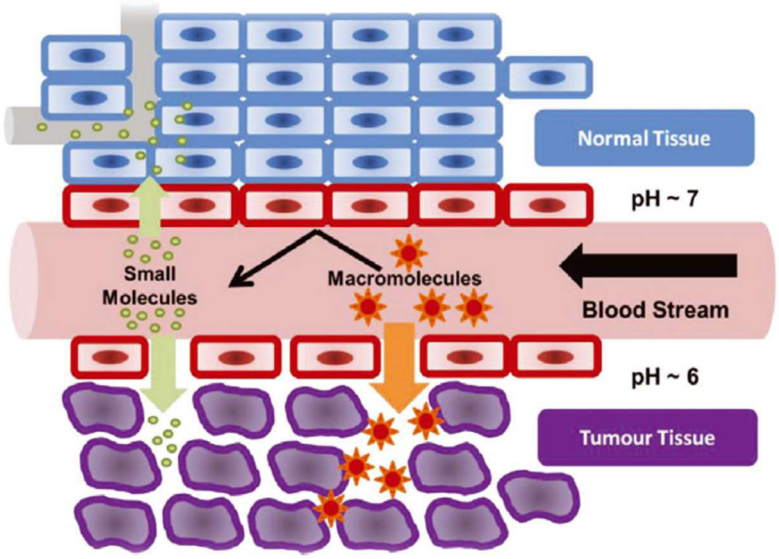



**Figure 8. Enhanced permeation and retention effect.** Macromolecules are more likely to accumulate and subsequently be retained in tumor tissue, because of increased permeability and lack of a lymphatic drainage system. Due to this, polymer-metal conjugates are retained in solid tumors, increasing de efficacy of the drug and decreasing the side effects of the treatment. Adapted from: Blunden & Stenzel, 2015.

Polymer-drug conjugates present some advantages compared to the parent free drugs like passive tumor targeting by the EPR effect, decreased toxicity, capability of solubilization in biological fluids of insoluble low molecular weight compounds, ability to overpass some mechanisms of drug resistance, ability to elicit immunostimulatory effects and stabilization, and prolongation of the plasma half-life of the low molecular weight drugs or proteins (Greco & Vicent, 2009). In these complexes, the drug is covalently bound to a polymeric carrier, usually through a biodegradable linker, which allows the release of the drug under specific conditions (acidic pH in lysosomal/endosomal compartments).

As noted above, Ru agents have emerged as a very effective alternative to platinum-based complexes to possess characteristics that make them very versatile and attractive compounds. In addition to the ruthenium (III) agents, new organometallic complexes of ruthenium (II) begun undergoing preclinical evaluation, especially those with "piano stool" geometry (A. Bergamo et al., 2012; Peacock & Sadler, 2008). This appears to be somewhat contradictory since the complex is in its active state, which may result in increased systemic toxicity. However, some studies reported that the coordination of an arene ligand to the Ru(II) afforded to the complex greater stability and allowed the compound to be modified in a rational functionalization way (**Figure 9**). Beyond this important feature, the arene ligand also increases the degree of selectivity of the compound to biomolecular targets, while its geometry allows the exchange of

ligands (in the legs of the stool) in order to modulate the pharmacological properties of ruthenium(II)-arene compounds (Nazarov, Hartinger, & Dyson, 2014).

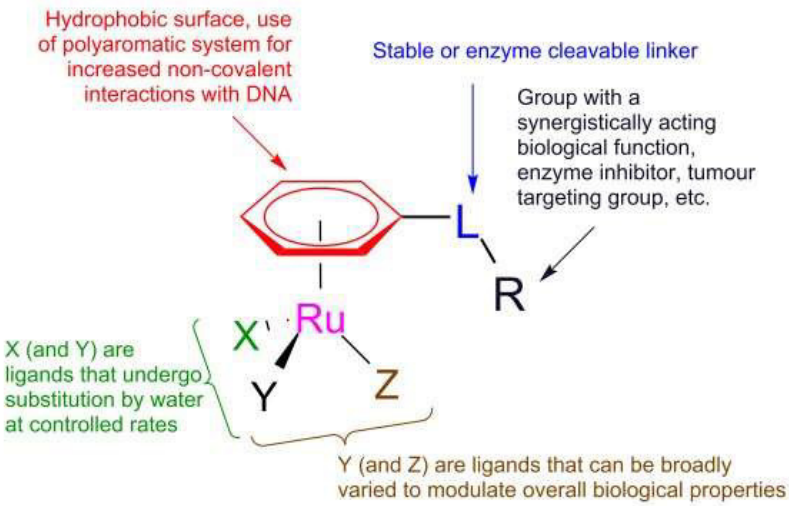
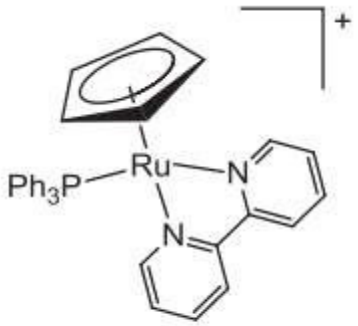



**Figure 9. Ruthenium(II)-arene structure.** Piano stool geometry of a complex and the different ways in which the central structural motif can be altered in order to increase the pharmacological properties of the compound. Adapted from: Nazarov, Hartinger, & Dyson, 2014.

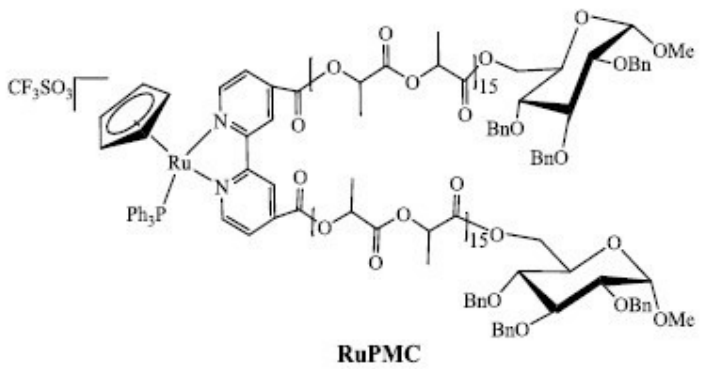
Over the last few years, our group has been dedicated to a family of "piano stool" compounds, Ru(II)-cyclopentadienyl complexes (RuCp), and its functionalization, as potential anticancer drugs for chemotherapy (Morais et al., 2016). The structure of the first RuCp complex (also designated as TM34) synthesized by our group comprises the central core of Ru(II) and the different ligands to which it is attached, specifically the cyclopentadienyl group ($\eta^5$-$C_5H_5$) (arene), the triphenylphosphine group ($PPh_3$) and the bidentate N,N'-heteroaromatic ligands, and which form the "piano stool" structure (**Figure 10**) (Moreno et al., 2011; Tomaz et al., 2012).




**TM34**

**Figure 10. TM34 structure.** Adapted from: Valente et al., 2013.

Based on the characteristics of TM34, such as adequate solubility and exceptional stability, high cytotoxicity against several types of cancer cell lines with half-maximal inhibitory concentration ($IC_{50}$) values in the sub-micromolar range, apoptosis as the dominant cell death mechanism and the fact that this complex surpasses cisplatin in efficiency and spectrum of action/range of activity, the second generation of RuCp compounds was designed (Valente et al., 2013). This new family was structurally based on TM34, with an appended macromolecule in the bipyridine ligand (herein referred as 'bipyridine macroligand'), thus leading to polymer–metal conjugates of ruthenium, or RuPMCs. In addition, the polymer was further functionalized to incorporate a moiety suitable for active targeting, the bipyridine macroligand that comprises a polylactide chain end capped with a glucose derivative as the tumor-targeting moiety (**Figure 11**) (Valente et al., 2013).



RuPMC

**Figure 11. D-Glucose end-capped polylactide ruthenium-cyclopentadienyl (RuPMC).** Adapted from: Valente et al., 2013.

RuPMC exhibits a pH-dependent hydrolysis which is important for drug delivery, since the measured pH in most solid tumors range from 5.7–7.2, while in blood it remains well-buffered and constant at pH 7.4 (Tannock & Rotin, 1989). This characteristic discards the need for a biodegradable linker and provides the opportunity for site-specific drug delivery, mainly within endosomal/lysosomal compartments, where the pH approaches 4.5–6.0 (Ruth Duncan, 2003). This conjugate should also have a different mechanism of action from TM34 due to the high molecular weight introduced by the polymeric chains (Côrte-Real et al., 2013; Valente et al., 2013). Regarding for cytotoxicity, comparing the $IC_{50}$ values between TM34 and RuPMC reveals a slight decrease in the cytotoxicity, it might be expected that the prolonged plasma half-life of the RuPMC can considerably improve the chemotherapeutic efficacy, and thus, decrease the

necessary dose for treatment, allowing a positive final outcome, as it has been described for many platinum-related compounds (Uchino et al. 2005; Nishiyama & Kataoka 2001; Cabral et al. 2005; Kim et al. 2008). The RuPMC also triggers a mechanism of cell death by apoptosis verified by mitochondrial changes and accumulation of ROS in cancer cells (Matos et al., 2018). Importantly, this conjugate is also more active than other reported polymer-metal complexes of cisplatin and ruthenium. All these characteristics together with the EPR effect (which allow a better internalization), make RuPMC a good candidate for the drug-delivery application. Taking into account all the mentioned, a *rationale* for the synthesis of the three new compounds studied in this work was created.

The first compound was PMC79, also designated as the parental compound, and is endowed with promising characteristics for cancer therapy (**Figure 12**). PMC79 showed a great stability in dimethyl sulfoxide (DMSO): Dulbecco's Modified Eagle's Medium (DMEM), even after being about 24 hours in solution, and in relation to its cytotoxicity, it had low $IC_{50}$ values in several cell lines, when compared to cisplatin (unpublished data). In view of its structure, it has also been noted that the presence of the $PPh_3$ ligand influences the cytotoxicity of the compound since when substituted by other molecules (DMSO and carbon monoxide), the cytotoxicity of the compound decreases (Côrte-Real et al., 2015).

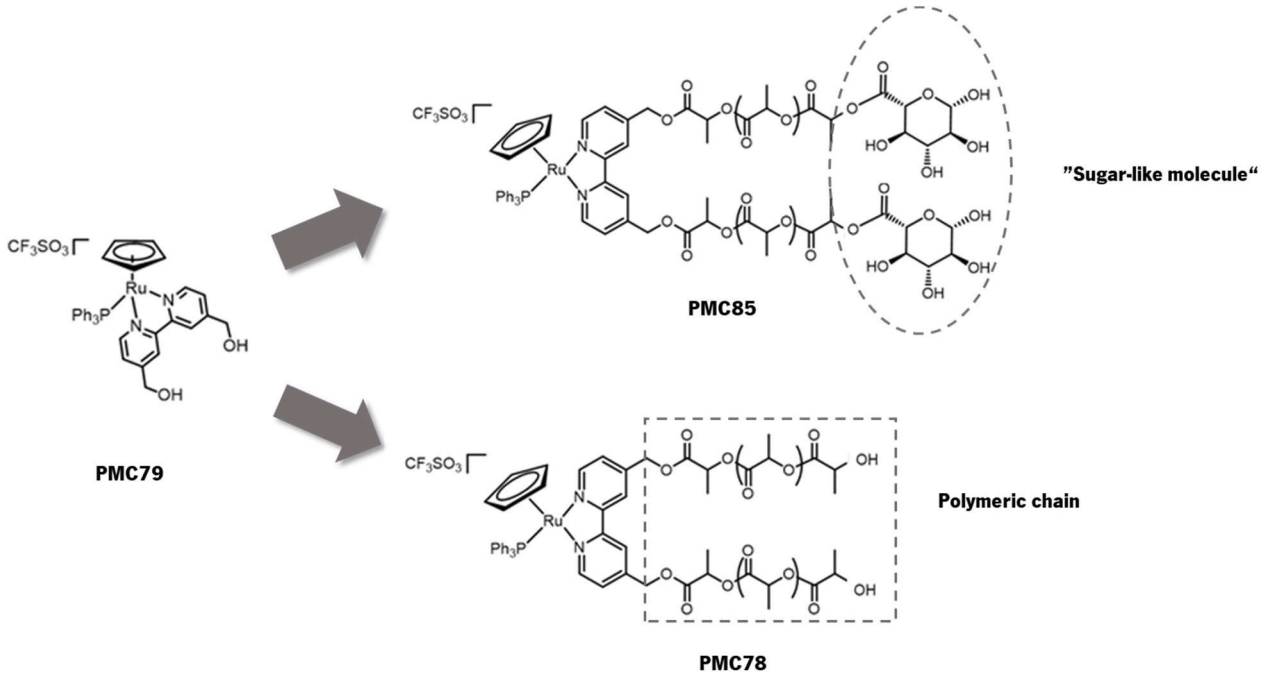



**Figure 12. Polymer-metal complexes of ruthenium.** The structure of our compounds, evidencing the typical piano stool geometry in PMC79, the addition of the polylactide polymer in PMC78, and the addition of the polylactide polymer with the sugar-derivative in PMC85.

The second compound, PMC78, was designed based on the knowledge of the existence of a biological phenomenon associated with cancer, the EPR effect, which leads to the selective accumulation of the macromolecules in the malignant tissues. This compound was designed from the parent compound, PMC79, considering its promising features. In this way, to the bipyridine ligand of PMC79, a polylactide polymer was added to increase its molecular weight (**Figure 12**).

The *rationale* for the last compound, also known as PMC85, was thought to further increase its target approach. The glucose transporters (GLUTs) are known to be overexpressed in cancer cells, in particular, GLUT1, which is the principal glucose transporter in several types of cancer (Amann et al., 2009; Yang et al., 2016). In this way, a glucose derivative was included to the end-chain of the polylactide polymer, so that it might be recognized by the glucose receptors present on the cell surface thus allowing specific targeting (**Figure 12**). Overall, our compounds bring together several characteristics that make them very promising, taking advantage of ruthenium's anticancer properties (PMC79) as well as its functionalization in order to improve the targeting approach (PMC78 and PMC85).

Previous results of our group have already shown some effects of this three new Ru compounds in different cell lines. The study began with breast cancer cell model MCF7 (Garcia, Valente, Morais, & Tomaz, 2016; unpublished data). The results showed that the compounds induced changes in the cytoskeleton, interfering with filamentous actin (F-actin) polymerization. In addition, the type of cell death induced by our compounds was proven to be apoptosis (Garcia, Valente, Morais, & Tomaz, 2016; unpublished data). More recently, the evaluation of the anticancer properties of our compounds started in a triple negative breast cancer cell model MDA-MB-231 (Moreira, 2016). In this cell line, cytoskeleton alterations were again observed. The results also showed the three Ru compounds affected the clonogenic ability of the cells and in the case of the PMC85 compound, it also affected the migration of the cells in a manner similar to cisplatin, which was used as a comparison model. PMC85 demonstrated to be the most efficient compound in this cell line.

The effects of the three compounds were evaluated in colorectal cancer cells. The work of Tiago Moreira showed the $IC_{50}$ values, at 48 h, were in the micromolar range, for RKO and SW480 cell lines (Moreira, 2016). The results also demonstrated that the compounds affect the cell lines in different ways. Regarding the effect in the clonogenic ability, in RKO cell line, the compounds decreased the ability to form colonies and the more pronounced effect was observed with the

PMC85 compound. In the case of SW480 cells, the compounds did not affect the clonogenic ability. The effect of the different compounds in the migration of cells was also assessed however, the results showed the compounds did not affect the migration in both cell lines.

The exact mechanism of action and targets of Ru complexes are still not well understood. In addition, the type of cell death and its mechanism remain unknown, as well as other anticancer properties of these compounds. Thus, all conditions are fulfilled for the continuation of this work, using as a model of colorectal cancer the RKO and SW480 cell lines, as well as, using cisplatin as a comparison model in order to find out if our compounds are more appropriate than this platinum drug for CRC therapy.

# 2. *Rationale* and aims

CRC is an important cause of global morbidity and mortality, being in Portugal the second leading reason of cancer death (J. Ferlay, Steliarova-Foucher, et al., 2013; Torre et al., 2015). The risks associated with this type of cancer are mainly due to lifestyle, however, it could also have hereditary causes (Potter, 1999; Weitz et al., 2005). Several genetic alterations have been associated with colorectal carcinogenesis, being *KRAS* and *BRAF* genes mutations the most frequent related to sporadic CRC (Ahmed et al., 2013; Móran et al., 2010).

There are few chemotherapeutic agents available for the treatment of CRC, being 5-FU the chemotherapy agent more frequently used in CRC treatment, however 5-FU possesses low success rates due to undesirable side effects and resistance (Pardini et al., 2011; Provenzale et al., 2015; Eric Van Cutsem et al., 2016). Cisplatin is a classical drug used in the clinics, although it is not used in CRC therapy due to severe side effects and to the fact that the majority of CRC are resistant to cisplatin treatment (Morais et al., 2016). Despite the chemotherapeutic drugs available, colorectal patients harboring mutations in *KRAS* or *BRAF* and/or *PIK3CA* do not respond to EGFR antibodies, which creates a relevant clinical problem that needs to be overcome (S. Hong et al., 2016; Temraz et al., 2015).

Because of the lack of specific anticancer agents in colorectal cancer therapy, there is an increased need to find new drugs that could rise the efficacy and specificity of treatment, overcoming the resistance mechanisms of the agents currently used, and reducing the range of side effects. In this way, a new family of multifunctional polymer-ruthenium conjugates has been synthesized and tested as anticancer agents (Garcia et al., 2016; Valente et al., 2013). These conjugates comprise a RuCp molecule, a biodegradable polymer, and a sugar-like molecule. The conjugation of these characteristics allows the accumulation of the drug by EPR effect and make these conjugates more specific, efficient and selective to target cancer cells. Thus, RuPMCs arise as a promising candidate for the drug-delivery application and cancer treatment.

**Our general aim** is to determine the potential use of the new ruthenium agents (RuPMCs) as novel anticancer drugs to use in colorectal cancer therapy.

**Specifically, we aim to answer the following questions:**

1. What are the effects of ruthenium-cyclopentadienyl derived compounds in colorectal cancer cells survival?
2. What are the mechanisms of action of these ruthenium compounds?
3. What are the possible molecular targets of these ruthenium compounds?

# 3. Materials and methods

## 3.1. Cell lines and culture conditions

The cell lines used in this work SW480 and RKO are colorectal cancer-derived cell lines obtained from American Type Culture Collection (ATCC). SW480 cells harbor mutations in $KRAS^{G12V}$ and $TP53^{R273H/P309S}$ and RKO cells harbor $BRAF^{V600E}$ and $PIK3CA^{H1047R}$ mutations.

Initially, one vial of frozen cells from each cell line, stored either in liquid nitrogen or at -80 °C, was thawed and the content, cells blended with freezing mixture (DMSO and fetal bovine serum (FBS), 1:4 (v/v), respectively), was transferred to a 15 ml falcon tube where it was, carefully, resuspended in 9 ml of complete medium. The cell suspension was then centrifuged at 1200 rpm for 5 minutes and the pellet resuspended in 5 ml of fresh complete medium, which was transferred into a sterile culture plate.

All cell lines were maintained at 37 °C under a humidified atmosphere containing 5% $CO_2$. SW480 cells grew in Roswell Park Memorial Institute (RPMI) 1640 medium (Biowest®) and RKO cells in Dulbecco's Modified Eagle's Medium (DMEM) High Glucose (Biowest®). Both supplemented with 10% FBS (v/v) (Biowest®) and 1% penicillin/streptomycin (v/v) (Biowest®).

Cells were subcultured once a week when at least 80% of confluence was reached at a dilution of 1:10 and 1:20 for SW480 and RKO cell lines, respectively.

In order to maintain the stock of the cell lines used, during the first subcultures some of the unused cell suspension was centrifuged at 2000 rpm for 10 minutes. The pellet was resuspended in 1 ml of freezing mixture and transferred to a cryotube which was then stored in liquid nitrogen or at -80 °C.

## 3.2. Mycoplasma detection

Contamination with *Mycoplasma* is one of the main contaminations found in cell culture, essentially due to improper handling. It is difficult to detect and causes changes in several cellular characteristics that may interfere with the results.

In order to test for mycoplasma contamination, 200 µl of cell supernatant was collected from culture plates with almost 100% confluence for a 1.5 ml microtube, which was heated in a dry bath at 95 °C for 10 minutes. After that the samples were stored at -20 °C.

The test was performed by polymerase chain reaction (PCR) using Venor™GeM Mycoplasma Detection Kit (Sigma-Aldrich®). Cells that showed positive results for *Mycoplasma* contamination were discarded.

## 3.3. RuPMC compounds and cisplatin: dilution, storage and $IC_{50}$

The ruthenium compounds used throughout this work were synthesized by Doctor Andreia Valente and her team, in the Department of Chemistry and Biochemistry of the Faculty of Sciences of the University of Lisbon.

The RuPMC compounds were dissolved in DMSO and the aliquots stored at -20 °C, protected from light, and discharged after one month, at which time new samples were prepared. Each aliquot could only be thawed once.

Cisplatin was dissolved in a sterile filtered solution of sodium chloride (NaCl) 0.9% (w/v) in deionized water and stored at -20 °C, protected from light.

The $IC_{50}$ values for PMC78, PMC79, PMC85 and cisplatin were previously determined for the two cell lines by our group, through Sulphorhodamine B (SRB) assay, and are showed in **Table 1**.

**Table 1.** $IC_{50}$ values of PMC78, PMC79, PMC85 and cisplatin for RKO and SW480 cell lines.

| | **SW480** | **RKO** |
|---|---|---|
| **Compound** | $IC_{50}$ (μM) | $IC_{50}$ (μM) |
| **Cisplatin** | 7 | 12.5 |
| **PMC78** | 6 | 4 |
| **PMC79** | 40 | 3 |
| **PMC85** | 4 | 3.5 |

## 3.4. Cell cycle analysis

In order to determine if the compounds induce cell cycle arrest, we performed cell cycle analysis by flow cytometry using propidium iodide (PI) to stain the DNA content.

Both cell lines, SW480 and RKO, were seeded at a concentration of $2\times10^5$ cells/ml and $8\times10^4$ cells/ml, respectively, in 6-well plates. For each cell line, two wells per condition were

used, except for the negative control, where only one well was used. To evaluate the autofluorescence of the compounds, the cell lines were also seeded in 60 mm petri dishes, at a final concentration of $2.5\times10^5$ cells/ml for RKO and $6.3\times10^5$ cells/ml for SW480, one per condition. After 24 hours, cells were exposed to the $IC_{50}$ values of cisplatin, PMC78, PMC79, and PMC85. The negative control cells were treated with DMSO 0.1% (vehicle). Cells were incubated 48 hours with the treatment, after which the medium was collected to 15 ml tube, cells were washed with phosphate buffered saline (PBS) 1× and harvested with trypsin- ethylendiamine tetraacetic acid (EDTA) 0.05% (v/v). All solutions used for washing cells were collected for the respective falcon to collect dead and living cells of the same condition. The suspension was then centrifuged at 500 g for 3 minutes, the pellet was resuspended and washed with PBS, and kept on ice for 15 minutes. After incubation on ice, 1.5 ml of ice-cold 96% ethanol was added and mixed until air bubbles formed in the suspension, to a final concentration of 70% of ethanol, and the cell suspension was kept on ice for further 15 minutes. Thereafter, 4 ml of PBS were added, and the cells were centrifuged at 500 g for 3 minutes, three times. Between centrifuges, the cells were washed with PBS 1×. After the last wash, 50 µl of RNase A at 200 µg/ml in sodium citrate (1% w/v) were added, vortexed and incubated at 37 °C for 15 minutes. Prior to analysis on the COULTER® EPICS® XL™ Flow Cytometer (Beckman Coulter©), it was added 30 µl of propidium iodide staining solution at 0.5 mg/ml in sodium citrate (1% w/v), vortexed and incubated at room temperature (RT) for 30 minutes. The autofluorescence conditions were only incubated with RNase A.

The analysis of the results was made using FlowJo 7.6 software. Values represent mean ± standard deviation (SD) of at least three independent experiments. Statistical analysis was performed using two-way ANOVA followed by Tukey's multiple comparisons test with GraphPad Prism version 6.01 for Windows, GraphPad Software, La Jolla California USA, www.graphpad.com.

## 3.5. Apoptosis assay

To determine if the compounds induce apoptosis we performed the Terminal transferase dUTP nick end labeling (TUNEL) assay using *In Situ* Cell Death Detection Kit, Fluorescein (Roche©).

The cell lines SW480 and RKO were seeded, in 6-well plates, at a concentration of $1\times10^5$ cells/ml and $4\times10^4$ cells/ml, respectively. For each cell line, two wells per condition were used

except for the negative control where only one well was used. Cisplatin was used as positive control since it is known to induce apoptosis.

Cells were exposed 24 hours after seeding, to the $IC_{50}$ values of cisplatin, PMC78, PMC79, and PMC85. The negative control cells were treated with DMSO 0.1% (vehicle). After 48 hours, the medium was collected to 15 ml tube, cells were washed with PBS 1× and harvested with trypsin-EDTA 0.05% (v/v). After detachment, the wells were washed again with PBS 1×. All solutions used for washing cells were collected to the respective falcon, in order to gather both dead and live cells from the same condition.

The suspension was then centrifuged at 2000 rpm for 10 minutes, the pellet was resuspended and washed with PBS 1×, and centrifuged again under the same conditions. To the resuspended pellet was added paraformaldehyde 4%, for 15 minutes to fix the cells, which were then washed with PBS and centrifuged for 10 minutes, at 2000 rpm. The pellet was resuspended in 500 µl of supernatant, transferred to 1.5 ml microtubes and storage at 4°C where it could be stored for several weeks.

Cytospins were obtained in the Cytospin™ 4 Cytocentrifuge (Thermo Fisher Scientific©), using 250 µl of cell suspension after centrifugation for 5 minutes, at 500 rpm. After centrifugation, the cytospin was demarcated using a hydrophobic pen. The slides were washed 3 times in PBS 1×, for 5 minutes each wash, and then placed on ice to incubate with 50 µl of permeation solution (0.1% Triton X-100 in 0.1% sodium citrate), for 2 minutes. After that, the slides were washed again.

The TUNEL reaction mixture was prepared in a ratio of 10:9:1 (dilution buffer, label solution and enzyme solution, respectively). 20 µl of this mixture were added to each slide which were then incubated, for 1 hour, at 37 °C, in a box wrapped in aluminum foil with wet paper inside to prevent dehydration of the slides.

Following incubation, the slides were washed 3 times in PBS 1×, for 5 minutes each wash, in an immunohistochemical box wrapped in aluminum foil. 2 µl of VECTASHIELD Antifade Mounting Medium with 4',6-diamidino-2-phenylindole (DAPI) (Vector Laboratories®) were used to mount with a coverslip on the top. The slides were storage at -20 °C to preserve the fluorescence until visualization. Representative images were obtained in a Confocal Microscope Olympus FluoView™FV1000 (Olympus©) at a magnification of 600×.

The results were obtained from at least three independent experiments and expressed as mean ± SD. For each condition were counted at least 500 cells using ImageJ 1.50i software (Schneider, Rasband, & Eliceiri, 2012).

One-way ANOVA followed by Dunnett's multiple comparisons test was performed using GraphPad Prism version 6.01 for Windows, GraphPad Software, La Jolla California USA, www.graphpad.com.

## 3.6. Protein extraction

RKO and SW480 cell lines were seeded, in 60 mm petri dishes, two per condition, at a final concentration of $2.5\times10^5$ cells/ml and $6.3\times10^5$ cells/ml, respectively. 24 hours later, cells were treated with the $IC_{50}$ of each ruthenium compound and cisplatin, and in the negative control cells were treated with DMSO 0.1% (vehicle).

After 48 hours of incubation, the medium was collected to 15 ml tube and cells were washed with PBS 1×, at 37 °C. To collect both live and dead cells from each condition, 300 µl of trypsin-EDTA 0.05% (v/v) was added to each petri dish. After detachment, the petri dishes were washed with PBS 1×, at 4 °C. The cell suspension was centrifuged at 2000 rpm for 10 minutes, at 4 °C, and the supernatant discarded.

The pellet was then resuspended, transferred to 1.5 ml microtubes, washed with 500 µl PBS, at 4 °C, and centrifuged at 1200 rpm for 5 minutes, at 4 °C. The supernatant was discarded, and the pellet resuspended in RIPA buffer (tris(hydroxymethyl)aminomethane (Tris) hydrochloric acid (HCl) 50 mM pH 7.5, NaCl 150 mM, EDTA 2 mM, NP-40 1% (v/v)) supplemented with inhibitors (sodium fluoride (NaF) 20 mM, sodium orthovanadate ($Na_3VO_4$) 20 mM, phenylmethylsulfonyl fluoride (PMSF) 1 mM, cOmplete™, Mini, EDTA-free, protease cocktail inhibitor (Roche©) (4%)) and incubated on ice for 20 minutes. Samples were then centrifuged at 14000 rpm, for 10 minutes, at 4 °C, and the supernatant containing the protein extract were transferred to new 1.5 ml microtubes and stored at -20 °C.

Protein quantification of the samples was performed using *DC*™ Protein Assay kit (Bio-Rad©). In order to know the protein quantification of each sample, a bovine serum albumin (BSA) standard curve with 0.25 mg/ml, 0.50 mg/ml, 1.0 mg/ml, 2.0 mg/ml, 3.0 mg/ml and 5.0 mg/ml concentrations was performed. The protein content of each sample was calculated by the equation of the standard curve obtained. The validity of the results was assumed for an $R^2 > 0.98$ of the standard curve.

## 3.7. Western blotting analysis

Expression of several proteins was assessed by Western blot analysis. Protein samples were prepared using 25 µg of protein extract, deionized water and loading buffer 6× (0.5 M Tris HCl pH 6.8, 30% (v/v) glycerol, 10% (w/v) sodium dodecylsulfate (SDS), $\beta$-mercaptoethanol 6.8% (v/v), bromophenol blue 12% (w/v)) in a final volume of sample to be loaded onto the gel of 25 µl. To denature the proteins, the samples were heated at 95 °C, for 5 minutes. Before being loaded onto the gel a short spin was performed to concentrate the samples at the bottom of the microtubes.

Protein samples were separated by 10% acrylamide SDS/polyacrylamide gel electrophoresis (PAGE) (resolving gel 10%: 40% acrylamide, 1.5 M Tris HCl pH 8.8, 10% SDS, 10% ammonium persulfate (APS) and 1% tetramethylethylenediamine (TEMED); separating gel 5%: 40% acrylamide, 0.5 M Tris HCl pH 6.8, 10% SDS, 10% APS and 1% TEMED). In each gel, 3 µL of molecular marker (BioRad® Precision Plus™ Protein Standards Dual Color) was loaded. Gels were emerged in running buffer 1× prepared from a stock solution of 10× (0.25 M Tris base, 1.92 M glycine, 1% (w/v) SDS), and run for 90 minutes at 100 volts. After proteins separation in the gel, they were transferred to a polyvinylidene fluoride (PVDF) membrane (previously activated in methanol for 10 seconds) emerged in transfer buffer 1×, prepared from a stock solution of 10× (0.25 M Tris base, 1.92 M glycine) and 100% methanol, at 100 volts for 1 hour with a Bio-Ice cooling unit.

When the transfer was complete, membranes were washed in PBS with 0.05% Tween 20 (PBS-T) and blocked in 5.0% BSA for 1 hour at RT. The membranes were further incubated for 1 hour at RT or overnight at 4 °C with primary antibody (**Table 2**). After incubation, the membranes were washed twice with PBS-T, for 5 minutes and 15 minutes. Then the membranes were incubated with the secondary antibody conjugated with IgG horseradish peroxidase, for 1 hour at RT (**Table 2**). Before the immunoreactive visualization the membranes were washed twice for 5 minutes and 15 minutes with PBS-T. Protein detection was performed using a commercial kit (Milipore® Immobilon™ Western Chemiluminescense Detection HRP Substrate) using the BioRad® ChemiDOC™ XRS imaging system.

The antibodies used, their characteristics and dilutions used are summarized in **Table 2**.

To visualize proteins of the same molecular weight it was performed a stripping protocol. After protein detection, the membranes were washed twice for 10 minutes with stripping solution

pH 2.2 (1.5% (w/v) glycine, 0.1% (w/v) SDS, 1% (v/v) Tween 20), then the membranes were washed twice, for 10 minutes with PBS 1×, and twice, for 5 minutes, with PBS-T. At the end, to confirm if the stripping worked well, the procedure to visualize proteins was performed again.

To determine the volume intensity of which band was used Image Lab software version 3.0. The results were obtained from at least three independent experiments and expressed as mean ± SD.

One-way ANOVA followed by Dunnett's multiple comparisons test was performed using GraphPad Prism version 6.01 for Windows, GraphPad Software, La Jolla California USA, www.graphpad.com.

**Table 2.** List of antibodies used, target size and incubation conditions.

| Antibody | Host | Protein Molecular Weight (kDa) | Dilution | Temperature (°C) | Incubation time | Manufacturer (Reference) |
|---|---|---|---|---|---|---|
| Anti-$\beta$-Actin | Mouse | 42 | 1:5000 | RT | 1 hour | Sigma-Aldrich® (A5441) |
| Anti-GAPDH | Rabbit | 36 | 1:10000 | RT | 1 hour | Gene Tex® (GTX100118) |
| Anti-GLUT1 | Rabbit | 55 | 1:500 | 4 | Overnight | abcam® (ab15309) |
| p44/42 MAPK (ERK1/2) | Rabbit | 42/44 | 1:1000 | 4 | Overnight | Cell signaling® (#4695) |
| Phospho-p44/42 MAPK (ERK1/2) (Thr202/Tyr204) | Rabbit | 42/44 | 1:2000 | 4 | Overnight | Cell signaling® (#4370) |
| AKT (pan) | Rabbit | 60 | 1:1000 | 4 | Overnight | Cell signaling® (#4691) |
| Phospho-AKT (Ser473) | Rabbit | 60 | 1:2000 | 4 | Overnight | Cell signaling® (#4060) |
| Anti-Rabbit IgG | Goat | - | 1:5000 | RT | 1 hour | Sigma-Aldrich® (A9169) |
| Anti-Mouse IgG | Rabbit | - | 1:5000 | RT | 1 hour | Vector Laboratories® (PI-2000) |

## 3.8. F-actin staining with Phalloidin

To study the effect of the different compounds on the cytoskeleton of cells, namely on actin, a F-actin staining assay was made. In this assay, RKO and SW480 cell lines were seeded at a concentration of $8\times10^4$ cells/ml and $1\times10^5$ cells/ml, respectively, in 12-well plates with one coverslip per well. Two wells per condition were used, for each cell line. After 24 hours, cells were exposed to the $IC_{50}$ values of cisplatin, PMC78, PMC79, and PMC85, and the cells of negative control were treated with DMSO 0.1%.

After 48 hours of incubation, cells were washed twice with PBS 1×, for 5 minutes, and fixed with paraformaldehyde 4% (w/v) for 10 minutes. Then cells were washed three times with PBS 1×, for 5 minutes. After fixation, cells were incubated, for 10 minutes, with ammonium chloride ($NH_4Cl$) 50 mM (to block the aldehyde groups left by paraformaldehyde), and washed twice with PBS 1×, for 5 minutes. Cells were permeabilized with Triton X-100 0.2%, for 5 minutes, washed twice with PBS 1×, for 5 minutes, and blocked with PBS 1× - BSA 3% for 20 minutes. Then cells were incubated with Alexa Fluor™ 568 Phalloidin (ThermoFisher Scientific®), which is a high-affinity F-actin probe conjugated to the red-fluorescent Alexa Fluor™ 568 dye, diluted in PBS 1× (1:40), for 1 hour in the dark and washed twice with PBS 1×. To finalize coverslips were mounted using, 5 µL of VECTASHIELD Antifade Mounting Medium with DAPI (Vector Laboratories®) in microscope slides. The slides were reserved at -20 °C, protected from light until needed.

The results were obtained from at least three independent experiments. Representative images were obtained in a Confocal Microscope Olympus FluoView™FV1000 (Olympus©) at a magnification of 600×.

## 3.9. Sulphorhodamine B assay

### 3.9.1. Determination of STF-31 $IC_{50}$

In order to determine the $IC_{50}$ of 4-[[[[4-(1,1-dimethylethyl)phenyl]sulfonyl]amino]methyl]-N-3-pyridinyl-benzamide (STF-31) (Sigma-Aldrich®), a well-known inhibitor of GLUT1 it was performed a Sulphorhodamine B (SRB) assay. STF-31 was dissolved in DMSO and stored at -20 °C, protected from light.

Both RKO and SW480 cell lines were seeded, in 24-well test plates, three wells per condition, at a concentration of $1\times10^5$ cells/ml and $4\times10^4$ cells/ml for SW480 and RKO, respectively. After 24 hours of seeding, cells were incubated with different concentrations of STF-31, for 48 hours. For each cell line, two negative controls were used: one control in which cells were incubated only with growth medium and the second control, to discard any influence of the DMSO in the results, in which the cells were exposed to the concentration of DMSO corresponding to the highest concentration at which the compound was dissolved (maximum of 0.1% of DMSO per well (v/v)).

After 48 hours of treatment, cells were washed once with PBS 1× and fixed in ice-cold methanol containing 1% acetic acid (v/v) for at least 90 minutes at -20 °C. Fixing solution was then removed, and the plate was left air-dry at RT, then the fixed cells were incubated with 0.5% (w/v) SRB dissolved in 1% acetic acid (v/v) for 90 minutes at 37 °C protected from light. Then SRB was removed and the plate was washed with 1% acetic acid (v/v), to remove excess of SRB that was not bound to proteins, and air-drying at RT. SRB was solubilized with 10 mM Tris pH 10, in agitation and protected from light. The samples of each well were transferred into a 96-well microplate and absorbance was read at 540 nm in SpectraMax® Plus 384 Microplate Reader (Molecular Devices©).

The results were obtained from at least three independent experiments and expressed as mean ± SD. One-way ANOVA followed by Dunnett's multiple comparisons test was performed using GraphPad Prism version 6.01 for Windows, GraphPad Software, La Jolla California USA, www.graphpad.com. The $IC_{50}$ was determined applying a dose vs response (variable slope) non-linear regression using GraphPad Prism version 6.01 for Windows, GraphPad Software, La Jolla California USA, www.graphpad.com.

### 3.9.2. Study the effects on cell proliferation of the combination of PMC79 and STF-31

SW480 cell line was seeded, in 24-well test plates, three wells per condition, at a concentration of $1\times10^5$ cells/ml. 24 hours after seeding, cells were incubated with different concentrations of PMC79 and STF-31, for 48 hours, using the same negative controls and the SRB protocol described previously.

The results were obtained from at least three independent experiments and expressed as mean ± SD. One-way ANOVA followed by Tukey's multiple comparisons test was performed using

GraphPad Prism version 6.01 for Windows, GraphPad Software, La Jolla California USA, www.graphpad.com.

## 3.10. Statistical analysis

The results were obtained from at least three independent experiments and expressed as mean ± SD. All different statistical analyses were performed using GraphPad Prism version 6.01 for Windows, GraphPad Software, La Jolla California USA, www.graphpad.com.

# 4. Results

## 4.1. RuPMC compounds induce apoptosis without affecting cell cycle in colorectal cancer cells

To evaluate the effect on cellular proliferation of RuPMC compounds in colorectal cancer cell lines, RKO$^{BRAFV600E/\ PIK3CAH1047R}$ and SW480$^{KRASG12V/\ TP53R273H/P309S}$, we performed cell cycle analysis by flow cytometry. For this purpose, we used the $IC_{50}$ values of PMC78 (4 μM for RKO and 6 μM for SW480), PMC79 (3 μM for RKO and 40 μM for SW480), and PMC85 (3.5 μM for RKO and 4 μM for SW480), previously determined by our group using the SRB assay (Moreira, 2016). Cisplatin, a classical chemotherapy drug used in the clinics, was also used as a model of comparison. The $IC_{50}$ values of cisplatin (12.5 μM for RKO and 7 μM for SW480), were also previously determined using the SRB assay (Moreira, 2016).

In RKO cell line, the results showed that the $IC_{50}$ values of Ru compounds PMC78, PMC79 and PMC85 have no effect on the cell cycle phases when compared to the negative control (**Figure 13**). However, cisplatin induced an increase in the percentage of cells in G2/M phase of the cell cycle (44%) comparing with the negative control (24%) what is in accordance with a cell cycle arrest at that phase. Cisplatin showed an increase in the number of cells in hypodiploid sub-G1 cell-cycle phase, which is associated with cell death. These changes consequently led to a decrease in the percentage of cells in G0/G1 and S cell cycle phases.

In the case of SW480 cell line, PMC78 compound decreased the percentage of cells in the G0/G1 cell cycle phase (35%) compared to the negative control (**Figure 14**). Relatively to PMC79 there was a decrease in the percentage of cells in G0/G1 (29%) and G2/M (25%) cell cycle phases and a significant increase in the hypodiploid sub-G1 cell-cycle phase (35%) when compared to the negative control (G0/G1 - 44%; G2/M – 43%; sub-G1 – 1%). As to compound PMC85, this had no effect on the cell cycle distribution, which is common to both cell lines. Contrary to what happens in RKO cell line with cisplatin, in SW480 cell line, there was a decrease in the percentage of cells in G2/M cell cycle phase.

To assess the effect on cell death of RuPMC compounds in colorectal cancer cell lines, we performed TUNEL assay, after 48 h of incubation with the $IC_{50}$ values of each compound. In both cell lines, in all conditions treated with Ru compounds or cisplatin, we could observe the presence of apoptotic bodies, phenotypic alterations typical of apoptosis (O'Brien & Brown,

2006; Velma, Dasari, & Tchounwou, 2016) (**Figure 15A**). This phenotypic alteration is one of the hallmarks of apoptosis and the evidence of being apoptosis the cell death induced by these compounds.

In RKO cell line, there was an increase in the levels of TUNEL positive cells after the incubation with the $IC_{50}$ values of PMC78 (3.7%), PMC79 (4.2%) and PMC85 (5.8%) compared with negative control (0.4%) (**Figure 15**). Cisplatin presented the highest percentage of apoptosis in this cell line (9.3%).

In SW480 cell line, PMC78 (10.7%), PMC79 (17.0%) and PMC85 (12.5%) demonstrated to induce high apoptosis levels comparing with the negative control (0.5%) (**Figure 16**). PMC79 showed the highest percentage of apoptotic cell death in this cell line. Cisplatin (11.6%) also induced a high percentage of apoptotic cell death compared with the negative control.

Ru compounds showed to be more efficient in SW480 cell line than in the RKO because they induced higher percentages of apoptosis determined by TUNEL assay.

## RKO

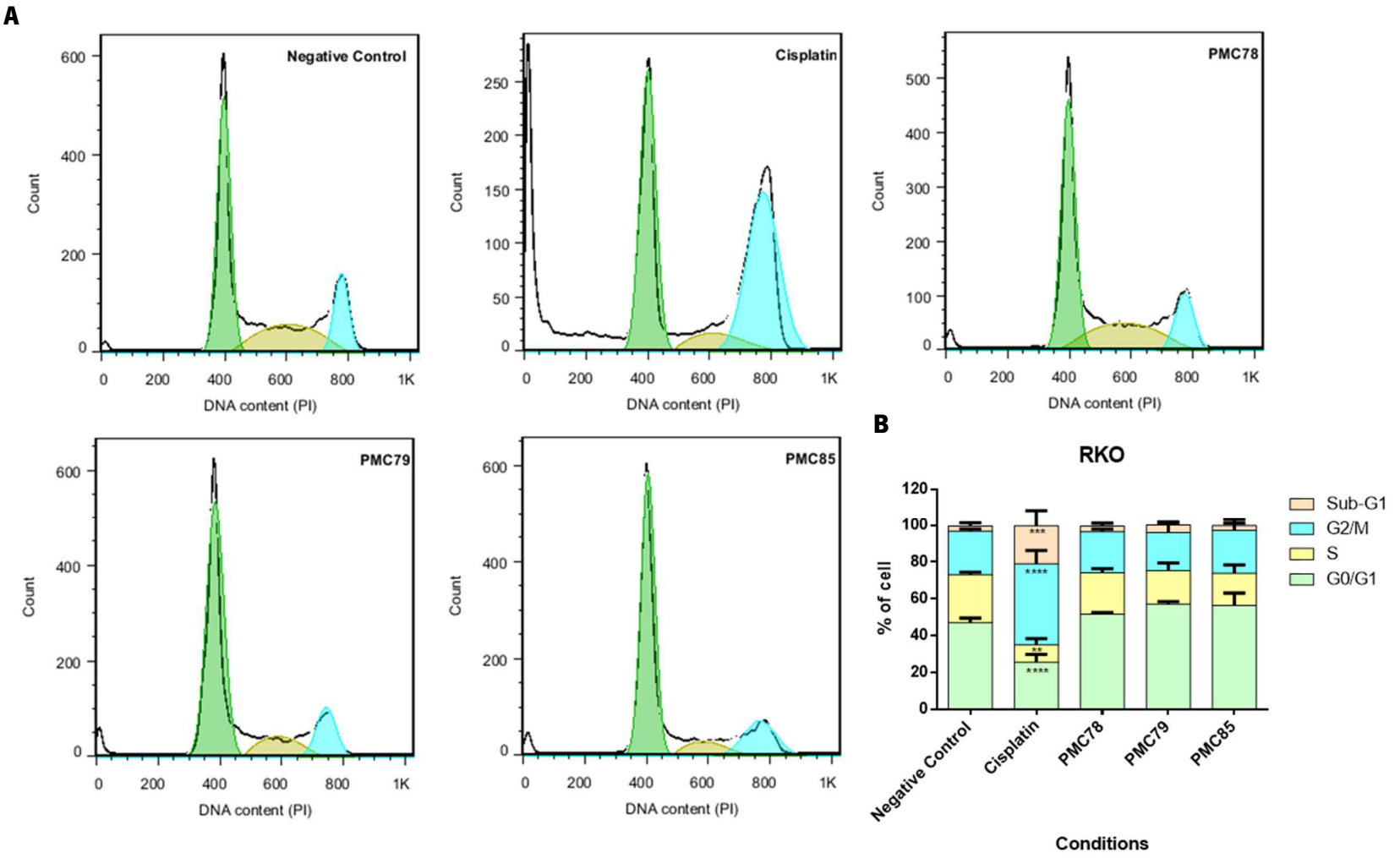



**Figure 13. RuPMC compounds do not affect cell cycle in RKO colorectal cancer cell line.** Cell cycle assay in RKO cells were performed by flow cytometry, after incubation with $IC_{50}$ concentrations for 48 h. **(A)** Representative graphs were obtained using FlowJo 7.6 software. **(B)** Analysis of cell cycle in RKO cells. Values represent mean ± SD of at least three independent experiments. ** $P \leq 0.01$; *** $P \leq 0.001$; **** $P \leq 0.0001$ compared with negative control.

## SW480

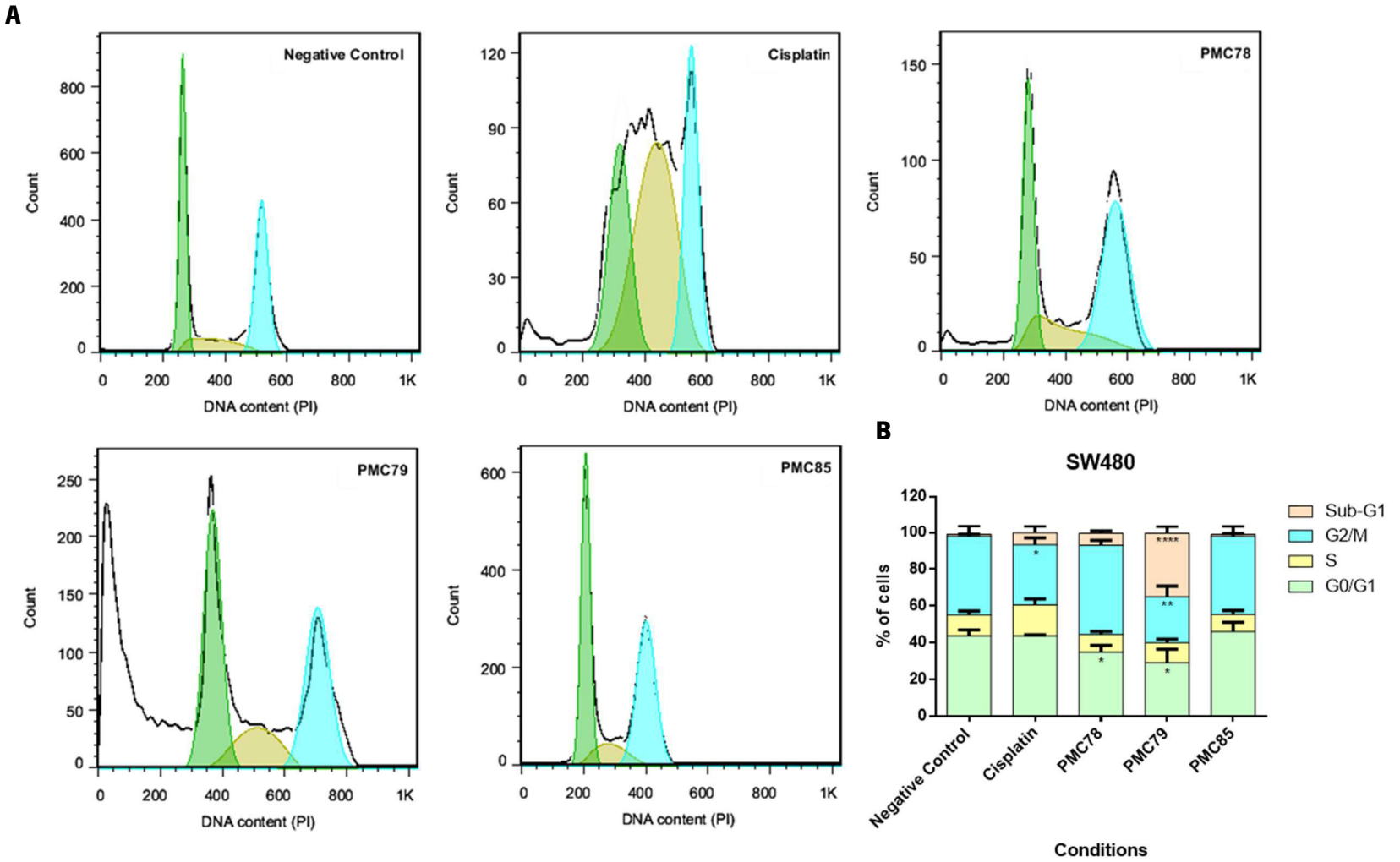



**Figure 14. RuPMC compounds do not affect cell cycle in SW480 colorectal cancer cell line.** Cell cycle assay in SW480 cells were performed by flow cytometry, after incubation with $IC_{50}$ concentrations for 48 h. **(A)** Representative graphs were obtained using FlowJo 7.6 software. **(B)** Analysis of cell cycle in SW480 cells. Values represent mean ± SD of at least three independent experiments. * $P \leq 0.05$; ** $P \leq 0.01$; **** $P \leq 0.0001$ compared with negative control.

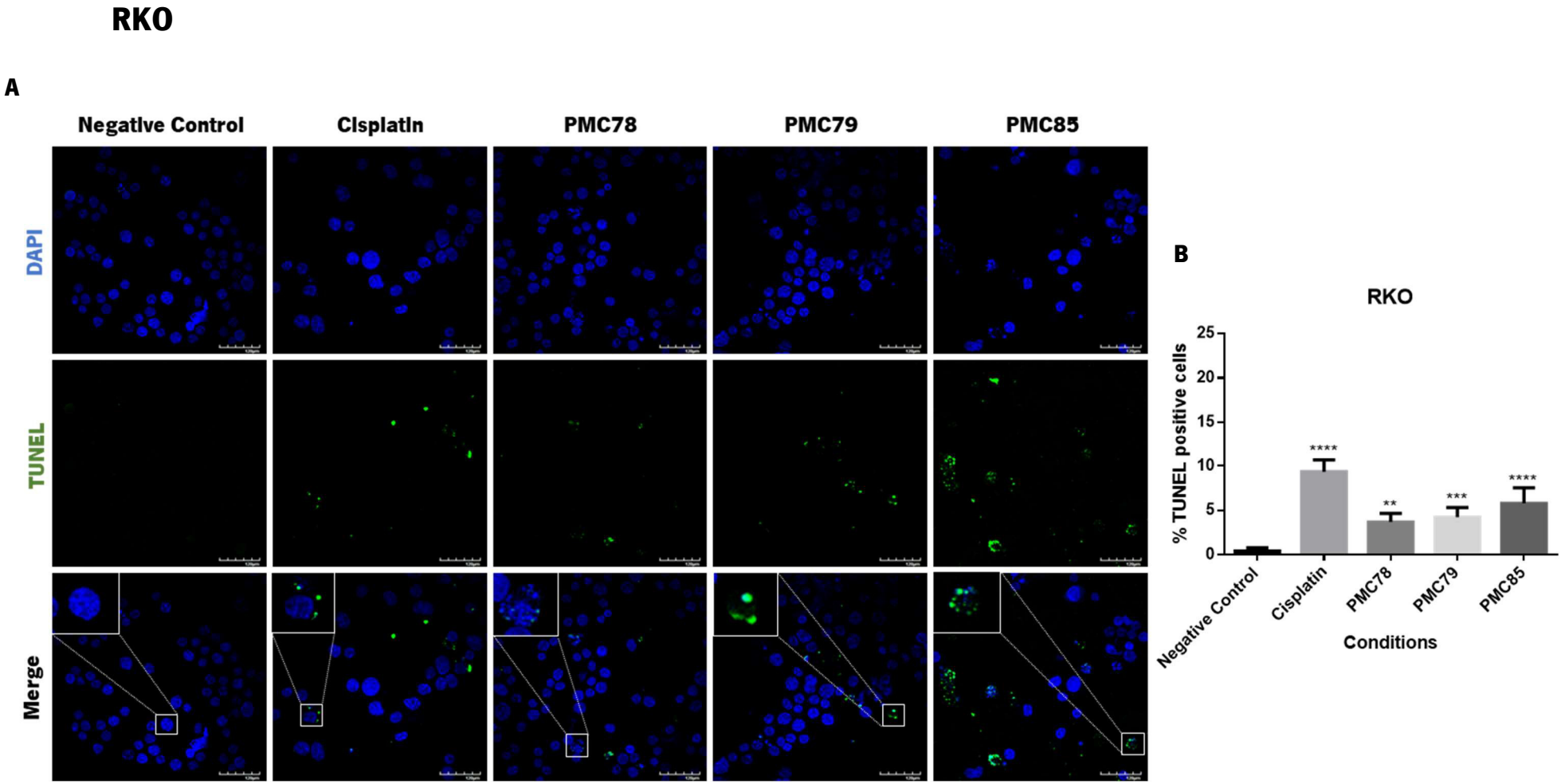



**Figure 15. RuPMC compounds induce apoptosis in colorectal cancer cell lines.** RKO cells were analyzed by TUNEL assay, after incubation with $IC_{50}$ concentrations for 48 h. **(A)** Representative images (×600) of DAPI (4',6diamidino-2-phenylindole), FITC (fluorescein isothiocyanate) and merged were obtained by confocal microscopy. **(B)** Analysis of TUNEL assay in RKO cells. Values represent mean ± SD of at least three independent experiments. ** $P \leq 0.01$; *** $P \leq 0.001$; **** $P \leq 0.0001$ compared with negative control.

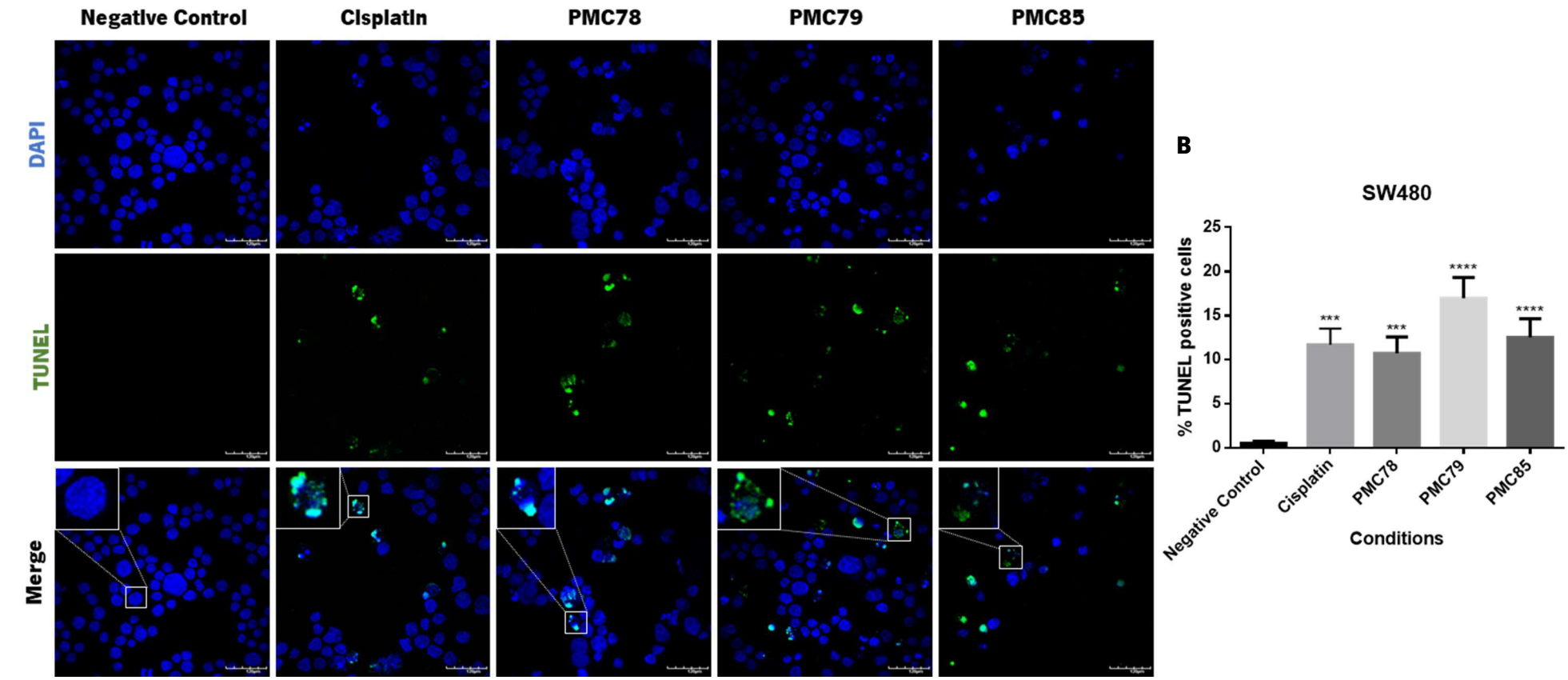



**Figure 16. RuPMC compounds induce apoptosis in colorectal cancer cell lines.** SW480 cells were analyzed by TUNEL assay, after incubation with $IC_{50}$ concentrations for 48 h. **(A)** Representative images (×600) of DAPI (4',6diamidino-2-phenylindole), FITC (fluorescein isothiocyanate) and merged were obtained by confocal microscopy. **(B)** Analysis of TUNEL assay in SW480 cells. Values represent mean ± SD of at least three independent experiments. *** $P \leq 0.001$; **** $P \leq 0.0001$ compared with negative control.

## 4.2. RuPMC compounds induce changes in AKT and ERK1/2 expression levels in colorectal cancer cells

SW480 and RKO cell lines used in this work harbor mutations in *KRAS*$^{G12V}$, and *BRAF*$^{V600E}$ genes, the most common mutations in CRC (Laurent-Puig et al., 2009). KRAS and BRAF downstream signaling pathways, PI3K and MAPK, are involved in survival and proliferative processes that influence the progression of the disease (Fruman & Rommel, 2014). PI3K-AKT and MAPK-ERK signaling pathways are the two most important pathways implicated in CRC (Ersahin, Tuncbag, & Cetin-Atalay, 2015; S. Hong et al., 2016; Porta, Paglino, & Mosca, 2014; Temraz et al., 2015).

Here we wanted to understand whether the Ru compounds might affect KRAS and/ or BRAF signaling pathways by analyzing the expression of the downstream regulators AKT and ERK1/2, proteins involved in PI3K and MAPK pathways respectively. We performed a Western blot assay using protein extracts of RKO and SW480 CRC cell lines treated with RuPMC's $IC_{50}$ values.

Our results showed that in RKO cell line harboring *BRAF*$^{V600E}$ and *PIK3CA*$^{H1047R}$ mutations there was a significant increase in the expression of phosphorylated-AKT (p-AKT) with PMC85 and also an increase in the expression of total AKT (tAKT) with PMC78 and PMC79 (**Figure 17A**). These changes were not significant on the p-AKT/tAKT quantification but only when the quantification of the two proteins was performed normalized for the levels of GAPDH. In the case of ERK proteins, the Ru compounds did not induce significative changes in the expression of these proteins (**Figure 17B**).

Interestingly, in the SW480 cell line harboring *KRAS*$^{G12V}$ and *TP53*$^{R273H/P309S}$ mutations there was a significant decrease in the levels of both p-AKT, tAKT and phosphorylated-ERK (p-ERK), total ERK (tERK) proteins when treated with PMC79 (**Figure 18**). PMC78 and PMC85 do not affect the levels of p-AKT, tAKT and p-ERK, but showed a statistical decrease in the expression of tERK protein when normalizing it expression for GAPDH (**Figure 18B**).

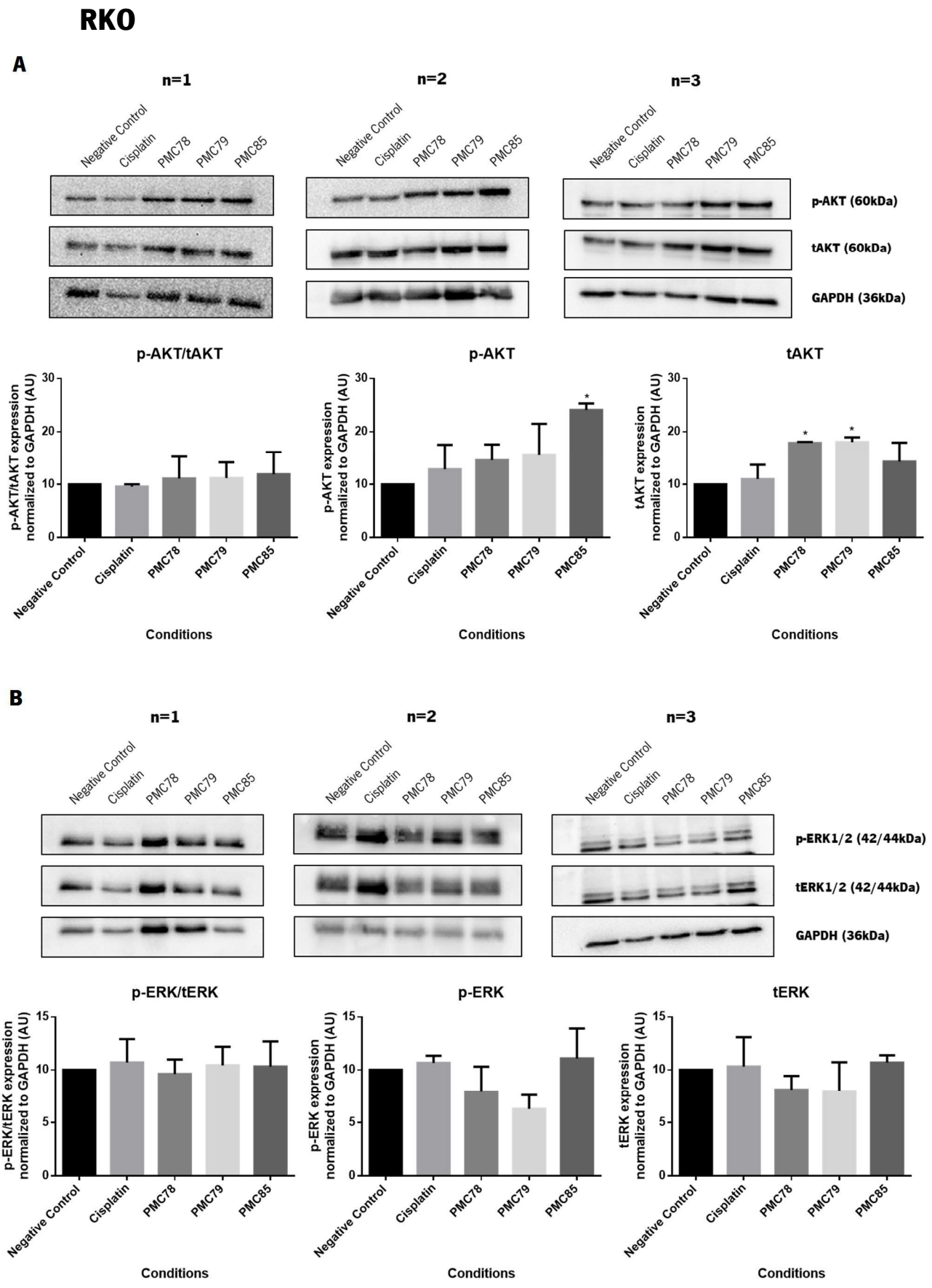



**Figure 17. RuPMC compounds modify the levels of AKT and ERK1/2 expression in RKO cancer cell line.** Western blot analysis of RKO cell line after 48 h of exposure with the $IC_{50}$ concentrations. (**A**) Blots of AKT proteins and analysis of quantification. Values represent mean ± SD of at least three independent experiments. * P≤ 0.05 compared with negative control. (**B**) Blots of ERK1/2 proteins and analysis of quantification. Values represent mean ± SD of at least three independent experiments.

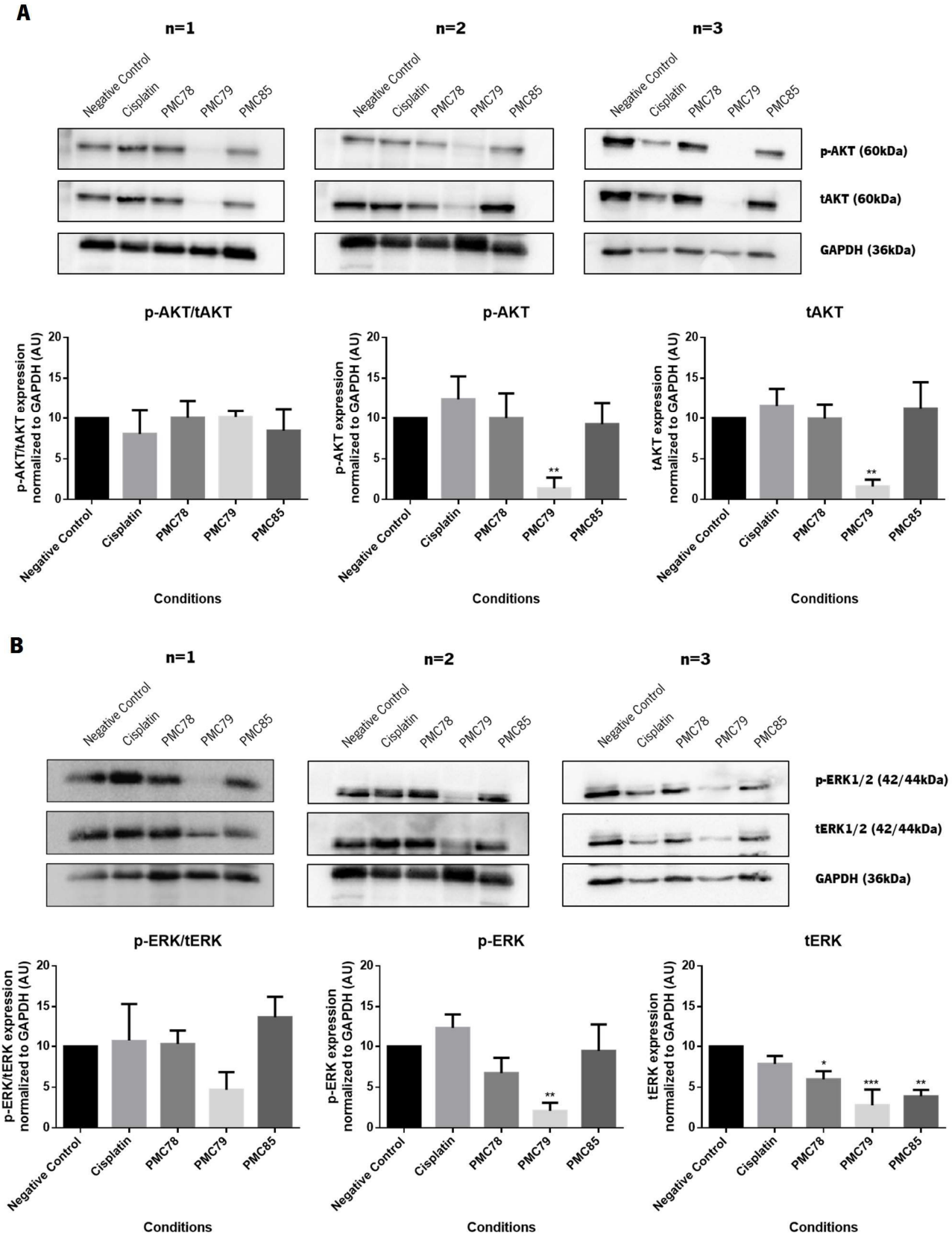



**Figure 18. RuPMC compounds modify the levels of AKT and ERK1/2 expression in SW480 cancer cell line.** Western blot analysis of SW480 cell line after 48 h of exposure with the $IC_{50}$ concentrations. (**A**) Blots of AKT proteins and analysis of quantification. Values represent mean ± SD of at least three independent experiments. ** P≤ 0.01 compared with negative control. (**B**) Blots of ERK1/2 proteins and analysis of quantification. Values represent mean ± SD of at least three independent experiments. * P≤ 0.05; ** P≤ 0.01; *** P≤ 0.001 compared with negative control.

## 4.3. RuPMC compounds affect the actin cytoskeleton of colorectal cancer cells

Previous results of our group showed that the different ruthenium-derived compounds seem to have a direct effect on the cytoskeleton (Moreira, 2016; unpublished data). With the purpose to evaluate if the Ru compounds also induced changes in the cytoskeleton structure of RKO and SW480 colorectal cancer cell lines, we analyzed F-actin organization in the cell using phalloidin. Cells were treated with $IC_{50}$ concentrations of RuPMC compounds, for 48 h, and then were stained with Phalloidin-AlexaFluor® 568 and DAPI (for staining the nucleic acids at the nucleus).

In RKO cells, the $IC_{50}$ values of cisplatin seemed to influence cell number and cell size without changes in F-actin organization (**Figure 19**). Treatments with the PMC78 and PMC85 compounds did not seem to affect cell-cell adhesion and cell junction establishment. Nevertheless, PMC79 appeared to affect cell cytoskeleton organization with cell dispersion and evident filopodia-like protrusions (**Figure 19**).

In SW480 cells, cisplatin $IC_{50}$ had the same effect as in RKO cells, influencing the number of cells (**Figure 21**). However, contrary to what happened in RKO cells, treatments with cisplatin, PMC78, and PMC79 appeared to affect cell-cell adhesion and intercellular contacts establishment, accompanied by alterations in cell phenotype and roundness. PMC79 also seem to induce filopodia-like protrusions although less evident when comparing with RKO (**Figure 21**). While treatment with PMC85 did not seem to affect cell-cell contact and intercellular contacts establishment, it affected cell actin cytoskeleton organization, with gaining of evident actin belts at the cell periphery and with formation of lamellipodia.

Since Ru compounds induced alterations in the cell cytoskeleton (F-actin) we decided to assess the expression levels of $\beta$-actin, by Western blot analysis, using the same conditions. In contrast to what happens in the F-actin staining assay, in RKO cells, Western blots did not show any significant variation in $\beta$-actin protein levels, even in cisplatin and PMC79 conditions (**Figure 20**).

On the other hand, SW480 cells showed a decreased in the levels of expression of $\beta$-actin only in PMC79 treatment (**Figure 22**). The remaining conditions showed no effect on $\beta$-actin levels compared to the negative control.

# RKO

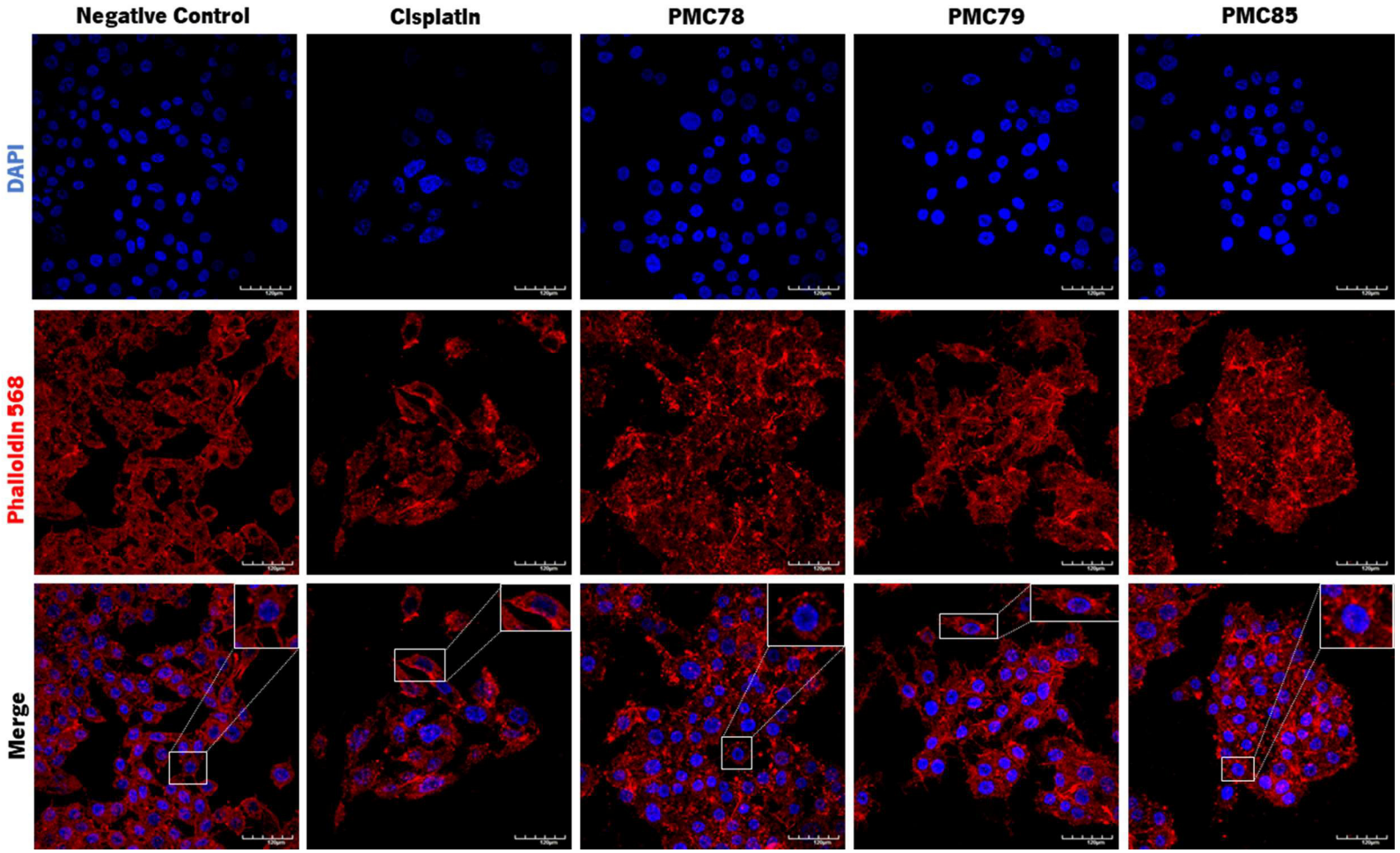



**Figure 19. RuPMC compounds affect the cytoskeleton of RKO cancer cells.** The analysis of F-actin staining was performed using the $IC_{50}$ values of each compound, for 48 h. Representative images (×600) of DAPI (4',6diamidino-2-phenylindole), Phalloidin-AlexaFluor® 568 and merged were obtained by confocal microscopy. The results were obtained from at least three independent experiments.

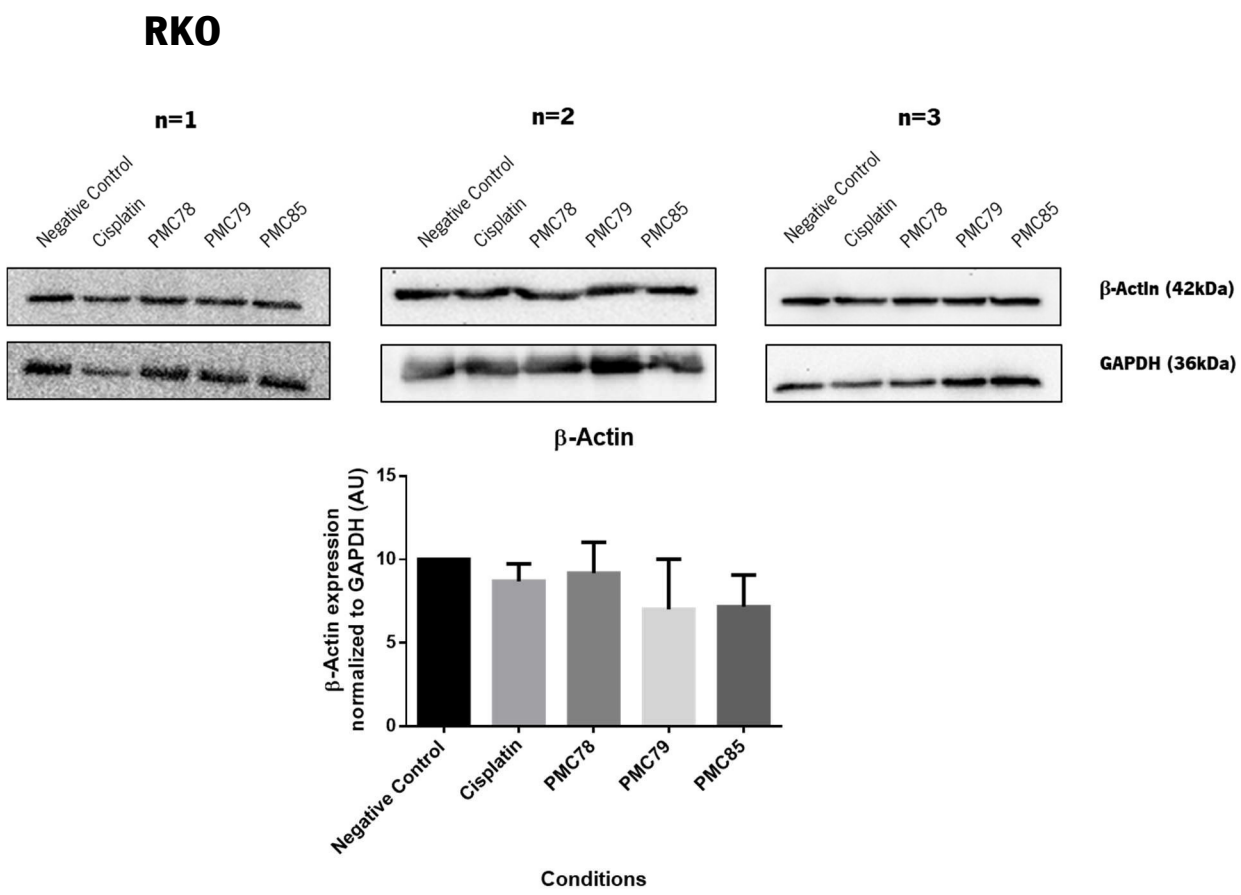



**Figure 20. RuPMC compounds do not affect the levels of $\beta$-actin in RKO cells.** Western blot analysis of RKO cell line after 48 h of exposure with the $IC_{50}$ concentrations. Blots of $\beta$-actin expression and analysis of quantification. Values represent mean ± SD of at least three independent experiments.

## SW480

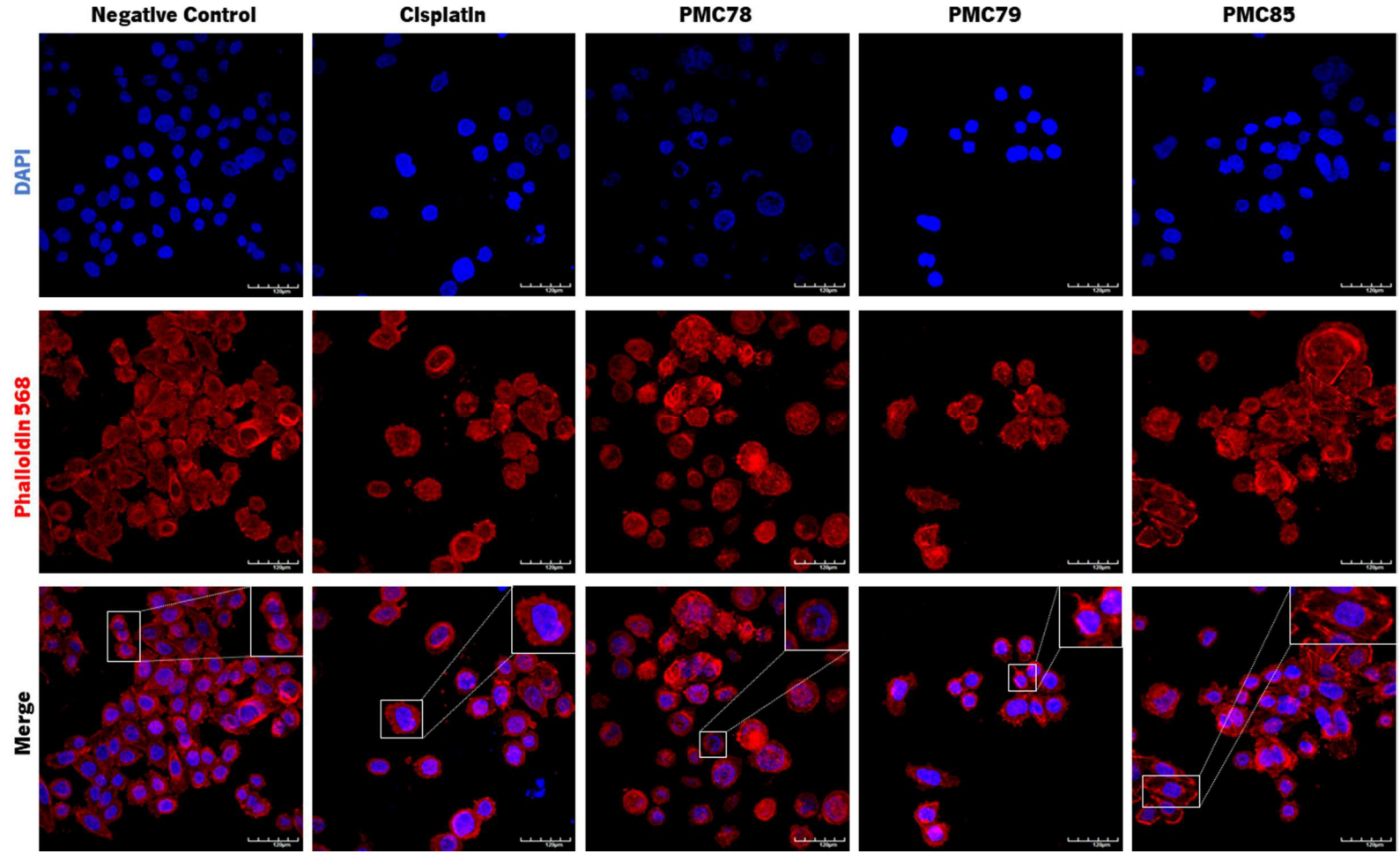



**Figure 21. RuPMC compounds affect the cytoskeleton of SW480 cancer cells.** The analysis of F-actin staining was performed using the $IC_{50}$ values of each compound, for 48 h. Representative images (×600) of DAPI (4',6diamidino-2-phenylindole), Phalloidin-AlexaFluor® 568 and merged were obtained by confocal microscopy. The results were obtained from at least three independent experiments.

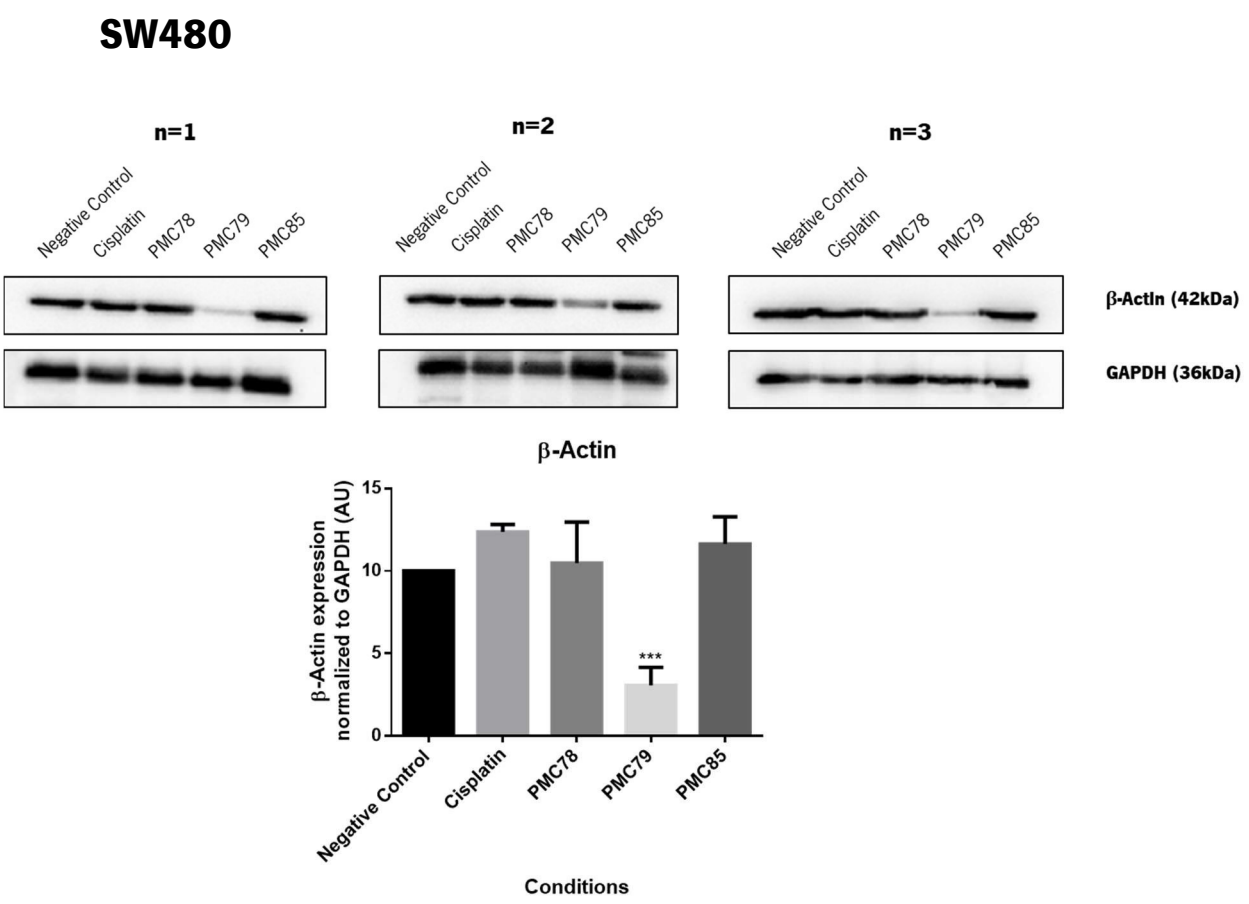



**Figure 22. PMC79 affects the levels of $\beta$-actin in SW480 cells.** Western blot analysis of SW480 cell line after 48 h of exposure with the $IC_{50}$ concentrations. Blots of $\beta$-actin expression and analysis of quantification. Values represent mean ± SD of at least three independent experiments. *** $P \leq 0.001$ compared with negative control.

## 4.4. PMC79 induces overexpression of GLUT1 in SW480 cells

Previous results of molecular docking have shown that a model of PMC85 structure is capable to be identified by GLUT1 receptor (Antunes, 2016). This interaction is dependent on the size of the polymer chain of PMC85. It is also known that this protein is overexpressed in CRC, being one of the main transporters of glucose in this type of cancer. In order to study this correlation, we assessed if the compounds interfere with the expression levels of GLUT1 by Western blotting analysis.

The results showed that, in RKO cell line, treatment with the $IC_{50}$ values of cisplatin and with the different ruthenium compounds, during 48 h, did not lead to significant changes in the expression of this receptor (**Figure 23**).

In the case of SW480 cell line, the PMC79 compound induced an increase in GLUT1 expression (**Figure 24**).

In order to understand if the effect of PMC79 compound might be correlated with GLUT1 protein we decided to test the effect of STF-31, a well-known GLUT1 inhibitor (Chan et al., 2011; Granchi, Fortunato, & Minutolo, 2016). In order to determine the $IC_{50}$ values of STF-31, concentrations in the micromolar range were tested for both cell lines, using SRB assay. The $IC_{50}$ concentrations determined after 48 h of exposure to STF-31 were 17.0 μM and 29.0 μM, respectively for RKO and SW480 cells (**Figure 25**).

We assessed the co-incubation of PMC79 and STF-31 in SW480 cells, which showed that the combination of PMC79 and STF-31 was more efficient than the two compounds alone (**Figure 26**). These results suggest that inhibition of GLUT1 receptor might potentiate the effect of PMC79 thus the two compounds appeared to have a synergistic effect.

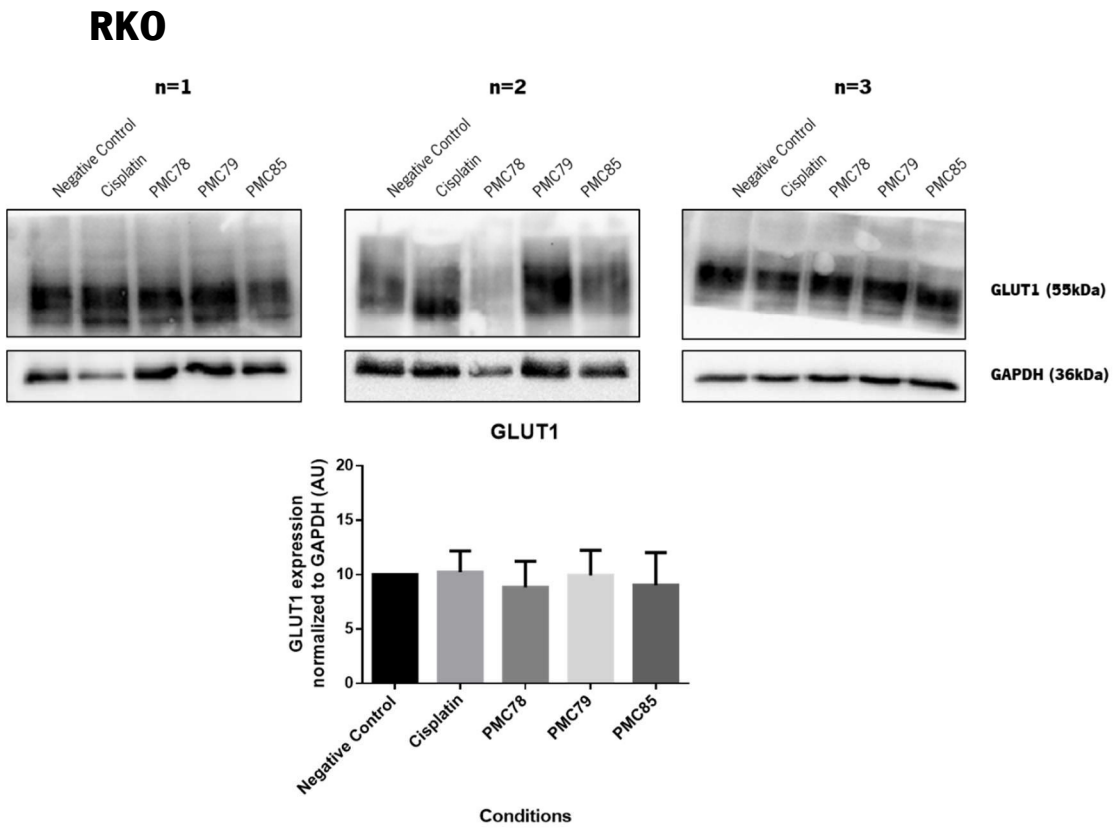



**Figure 23. Ru compounds did not affect the levels of GLUT1 in RKO cells.** Western blot analysis of RKO cell line after 48 h of exposure with the $IC_{50}$ concentrations. Blots of GLUT1 expression and analysis of quantification. Values represent mean ± SD of at least three independent experiments.

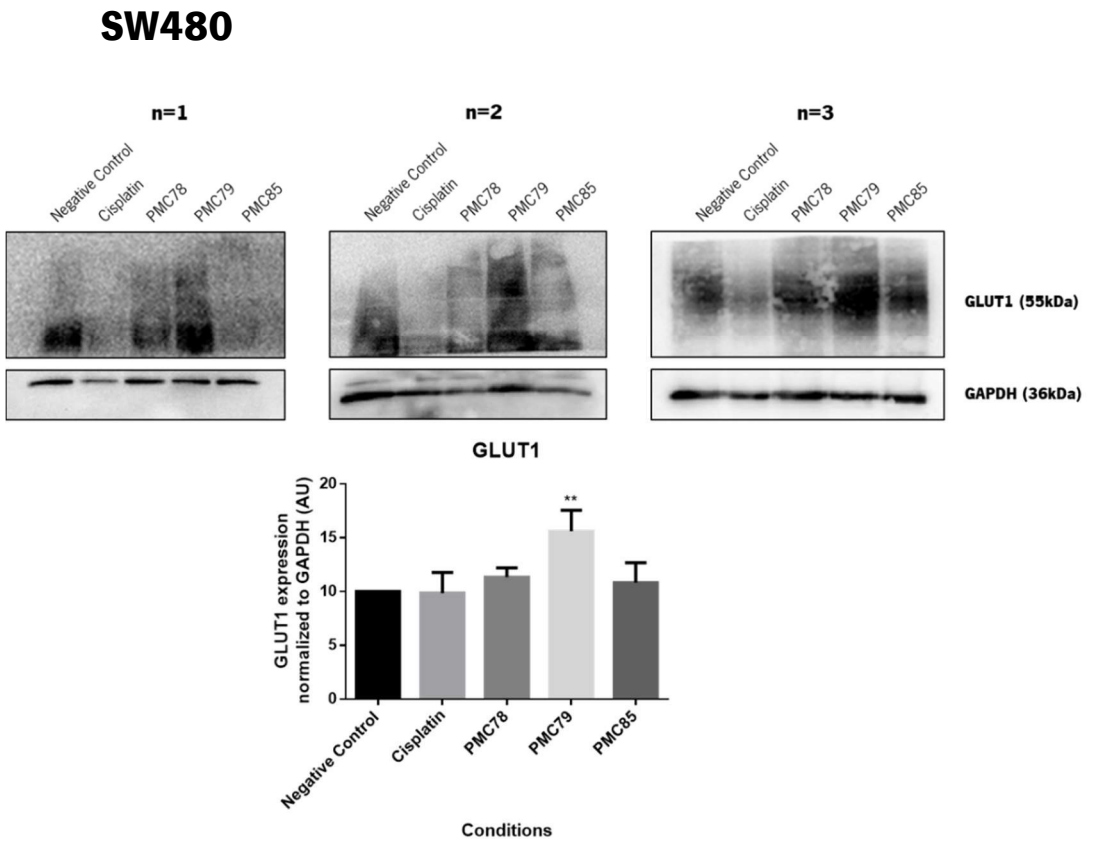
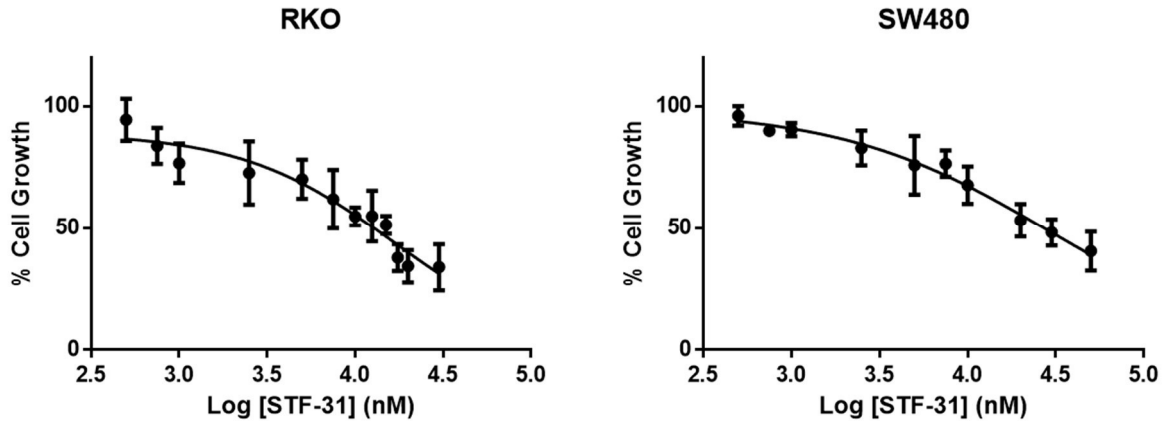



**Figure 24. PMC79 induces overexpression of GLUT1 in SW480 cell line.** Western blot analysis of SW480 cell line after 48 h of exposure with the $IC_{50}$ concentrations. Blots of GLUT1 expression and analysis of quantification. Values represent mean ± SD of at least three independent experiments. ** $P \leq 0.01$ compared with negative control.

**A**




**B**

| | $IC_{50}$ (µM) |
|---|---|
| **RKO** | 17,0 ± 1,0 |
| **SW480** | 29,0 ± 3,0 |

**Figure 25. Effect of STF-31 on cell proliferation of RKO and SW480 colorectal cancer cell lines, determined by SRB assay.** RKO and SW480 cell lines were incubated, for 48 h, with increasing concentrations of STF-31. **(A)** The percentage of cell growth relatively to the negative control was determined after the period of incubation and is expressed as a mean ± SD for each treatment, from at least three independent experiments. **(B)** $IC_{50}$ values determined using GraphPad Prism 6 software, applying a dose vs response non-linear regression (n=3).

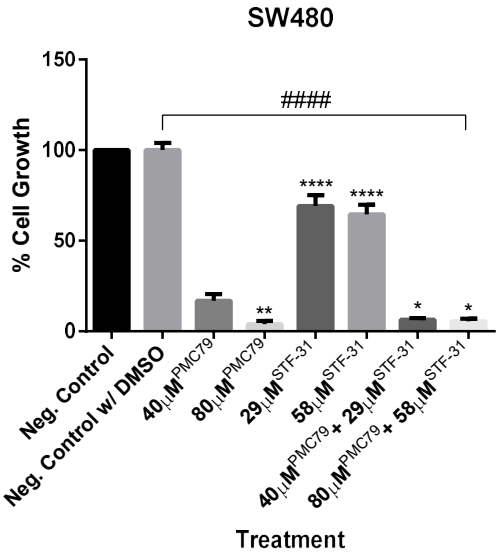



**Figure 26. PMC79 and STF-31 have a synergistic effect in SW480 colorectal cancer cell line.** SW480 cell line were incubated, for 48 h, with PMC79 and STF-31. The percentage of cell growth relatively to the negative control was determined after the period of incubation and is expressed as a mean ± SD for each treatment, from at least three independent experiments. * $P \leq 0.05$; ** $P \leq 0.01$; **** $P \leq 0.0001$ compared with 40 µM$^{PMC79}$. #### $P \leq 0.0001$ compared with negative control w/ DMSO.

# 5. Discussion

CRC is one of the main causes of cancer-related morbidity and mortality being of extreme importance to understand its biology with the purpose of trying to find more targeted therapies (Fitzmaurice et al., 2015). Nowadays, there are few chemotherapeutic agents available for the treatment of CRC. Despite the chemotherapeutic drugs available, the percentage of patients with metastatic CRC who responds to available targeted drugs when used as monotherapy is as low as 20% (S. Hong et al., 2016; Temraz et al., 2015). Moreover, colorectal patients harboring mutations in *KRAS* or *BRAF* and/or *PIK3CA* do not respond to EGFR antibodies, which creates a relevant clinical problem that needs to be overcome (S. Hong et al., 2016; Temraz et al., 2015). 5-FU is the standard chemotherapy treatment for CRC, however, the success rates are as low as 10%–15% because of their severe side effects and resistance (Pardini et al., 2011; Provenzale et al., 2015; Eric Van Cutsem et al., 2016). Cisplatin, a classical chemotherapy drug used in the clinics, also present severe side effects and intrinsically resistance in most cancers, and because of that, and contrarily to what happens with 5-FU, is not used in the treatment of colorectal cancer (Raymond et al., 2002).

Recently, three new multifunctional polymer-ruthenium conjugates were synthesized and tested as anticancer agents in an attempt to produce new specific drugs for cancer (Garcia et al., 2016; Valente et al., 2013). These compounds bring together several characteristics that make them very promising, taking advantage of ruthenium's anticancer properties (PMC79) as well as its functionalization in order to improve the targeting approach (PMC78 and PMC85). An earlier study with the first polymer ruthenium-cyclopentadienyl complex (RuPMC) had already shown the potential of these compounds (Valente et al., 2013). More recently, we performed another study with a new ruthenium methylcyclopentadienyl complex (Ru2) highlighting again the anticancer properties of Ru compounds. In that work, the complex exhibits lower $IC_{50}$ values than cisplatin for CRC RKO and SW480 cell lines, and greater cytotoxicity in cancer cells compared to noncancerous cell line, NCM460 (Teixeira et al., 2018). The effect of the Ru compounds used in our study (PMC78, PMC79 and PMC85) was already determined by the group in MCF7 and MDA-MB-231 breast cancer cell lines and in RKO and SW480 colorectal cancer cell lines, in which the $IC_{50}$ values of the compounds were determined (Garcia, Valente, Morais, & Tomaz, 2016; Moreira, 2016; unpublished data). Cisplatin was used as a comparison, to understand if the ruthenium compounds are more appropriated than this platinum-based

drug. All Ru compounds showed $IC_{50}$ doses lower than $IC_{50}$ values determined for cisplatin, in each cell line, except the compound PMC79 in SW480 cells, which $IC_{50}$ was higher than cisplatin. It has been shown that the $IC_{50}$ values for 5-FU, determined for SW480 and RKO cell lines, respectively, were 360 μM and 5 μM (Thant et al., 2008; Zhao et al., 2017). It is important to note that the $IC_{50}$ of 5-FU (360 μM) is 9-fold higher than the $IC_{50}$ of PMC79 in SW480 cells (40 μM), determined by the group (Moreira, 2016), which in turn was greater than the $IC_{50}$ value of cisplatin (7 μM) and also the highest concentration determined for all cell lines used in our work. It should also be noted that all values were in the micromolar range, which increases the potential interest in exploring the use of the Ru compounds in cancer therapy.

Here, we wanted to uncover the mechanism of action of these ruthenium-cyclopentadienyl derived compounds and their effects in proliferation and cell death of colorectal cancer cells. We started by assessing the effects of the three Ru compounds (PMC78, PMC79 and PMC85), comparing with cisplatin, in the cell cycle. The results showed that RuPMCs did not induce cell cycle arrest, in any of the cell lines tested. Only cisplatin induced a cell cycle arrest in G2/M phase, in RKO cells. This result is consistent with previous work, that showed the treatment of HL-60 human leukemic cells with low doses of cisplatin resulted in a significant impact on cell viability through modulation of gene expression, cell cycle arrest at G2/M phase and apoptosis (Velma et al., 2016). Moreover, another work showed that cisplatin cytotoxic effect is primarily due to its well-described formation of adducts with DNA which leads to replication arrest, cell cycle checkpoint activation and sustained G2 arrest and, if the damage is too severe, cell death (O'Brien & Brown, 2006). Concerning our results, there was also an increase in the percentage of cells in sub-G1 under conditions treated with cisplatin in RKO, and PMC79 in SW480 cells. In the remaining conditions, it is possible to observe a slight increase not reaching statistical significance in the percentage of cells in sub-G1 compared to the negative control, in both cell lines what indicates that the cells may be undergoing apoptosis/necrosis. These results are in accordance with our previous report showing that a new ruthenium methylcyclopentadienyl compound (Ru2) also induce a slight tendency to increase the number of cells in the sub-G1 phase relative to the negative control, and the $2\times IC_{50}$ value induced a cell cycle arrest in G0/G1 phase, in RKO cells (Teixeira et al., 2018). Moreover, a proteomic study in breast cancer cells, MDA-MB-231, showed that PMC78 is able to induce a cell cycle arrest at the beginning of mitosis (maybe in G2/M cell cycle phase) because there were several proteins involved in the subsequent stages that were under-expressed (our unpublished data).

The anticancer effect of a compound is largely based on the ability of the drug to induce apoptotic cell death in cancer cells. This ability is correlated with good results in most preclinical and clinical studies (R. Kim, 2005). Therefore, due to the importance of the type of cell death induced by an anticancer drug, we evaluated whether our compounds induce apoptosis by TUNEL assay. The results showed that PMC78, PMC79 and PMC85, in RKO cells, induced increasing percentages of cell death nevertheless, cisplatin was the most efficient drug, presenting the higher percentage of cell death. In SW480 cell line, Ru agents showed to be more efficient, namely PMC79 which showed the highest percentage of cell death. It is important to note that the results obtained are consistent with the results of the cell cycle analysis relative to the increase of cells in sub-G1 phase in RKO cells treated with cisplatin and in SW480 cells treated with PMC79 compound. These conditions also presented the highest percentage of cell death in TUNEL assay. Our results support the evidence that the accumulation of cells in sub-G1 phase might be due to the cells undergoing apoptosis. Previous studies, in MCF7 breast cancer cell line, had already shown by annexin V/PI assay that the main type of cell death induced by the compounds is apoptosis (our unpublished data). In that work, PMC79 showed similar results to cisplatin and, as happened with SW480 cells in our work, was the most efficient Ru compound. In a recent study of the group, a new Ru complex (Ru2) also showed to induce apoptosis, in RKO and SW480 colon cancer cells (Teixeira et al., 2018). Overall, our results obtained in colorectal cancer cell lines using ruthenium-metal based complexes suggest that these compounds appear to inhibit cell growth namely due to cell death induction.

CRC are characterized by the presence of several alterations, 40% of CRC cases hold a *KRAS* mutation, 15% hold a *BRAF* mutation, and 20% hold a *PIK3CA* mutation (Laurent-Puig et al., 2009). Moreover, *KRAS* and *BRAF* mutations occur in different CRC subtypes and were never found simultaneously in same cancer (De Roock et al., 2010; Sartore-Bianchi et al., 2009). Mutations in *PIK3CA* can occur together with *KRAS* or *BRAF* mutations (De Roock et al., 2010; Sartore-Bianchi et al., 2009). The MAPK and PI3K pathways are the two-major oncogenic signaling pathways activated in colorectal cancer. MAPK and PI3K pathways control fundamental cellular processes being compensatory pathways that mediate cell survival through co-regulated proteins (Ersahin et al., 2015; S. Hong et al., 2016; Porta et al., 2014; Temraz et al., 2015). The carcinogenic role of these signaling pathways is almost always associated with *KRAS* or *BRAF* mutations which signal to these pathways and lead to their constitutive activation. The CRC derived cell lines used in this work have different genetic backgrounds and thus different

pathways might be differently activated. Moreover, they encompass the two major molecular subtypes of colorectal cancer and present the mutations most commonly found in CRC (Ahmed et al., 2013). Cancer-derived cell lines have emerged as important models for the study of this disease, providing important information about the molecular and cellular biology of the tumor. They are a fundamental tool to test novel anticancer agents, and for the discovery of biomarkers of drug sensitivity, resistance, and toxicity. The identification of molecular markers associated to the response of classical chemotherapy and targeted agents has shown clinical utility (Barretina et al., 2012). PI3K and MAPK regulate positively and negatively each other depending on the type of signals they receive and the location of the effector proteins in the cell (Mendoza, Er, & Blenis, 2011). Thus, activating mutations in effector proteins of one of the pathways lead to the activation of the two signaling pathways. For example, mutations in KRAS lead to the activation of MAPK and PI3K through direct interaction with its catalytic subunit (Rodriguez-Viciana et al., 1994; Steelman et al., 2011). Furthermore, both pathways are responsible for several processes of cell proliferation and cell death. Nothing was known about the interaction of polymer-metal complexes of ruthenium with *KRAS* or *BRAF* and/or the activated signaling pathways, however, the major goal of any therapy is to inactivate relevant signaling pathways which might simultaneously result in inhibition of cellular proliferation and induction of apoptosis. Thus, with the purpose to uncover some targets of RuPMCs we evaluated the expression of proteins involved in these pathways. Our results showed that in RKO cells there were no changes in ERK1/2 expression, but there was a slight increase in the levels of p-AKT and total AKT in the conditions treated with all Ru compounds. Elevated levels of activated components of these pathways are often associated with poor prognosis, altered sensitivity to targeted therapy and resistance mechanism (Z. Liu, Zhu, Getzenberg, & Veltri, 2015; Martelli et al., 2010). Interestingly, in the case of SW480 cells, PMC79 showed decreased levels of p-AKT, total AKT as well as p-ERK1/2 and total ERK1/2. There was also a decrease in the expression levels of total ERK1/2 with PMC78 and PMC85 compounds. The results point for a possible specific inhibition effect of PMC79 in mutated *KRAS*, because the effect of PMC79 both in cell death and MAPK and PI3K signaling pathways is more evident in SW480 cell line harboring *KRAS* mutated. Moreover, this result was consistent with previous results showing the effect of PMC79 on the induction of cell death. Considering that the inactivation of the MAPK and PI3K signaling pathways simultaneously results in inhibition of cellular proliferation and induction of apoptosis and, given that the effector proteins studied are closely correlated with apoptosis effector molecules, this may be one of the

mechanisms of action that triggers the cell death process by PMC79 compound. Indeed, it is known that both ERK and AKT proteins (downstream effector proteins of MAPK and PI3K pathways, respectively) can phosphorylate transcription factors that influence the transcription of important apoptotic effector molecules as B-cell lymphoma 2 (Bcl-2) and myeloid cell leukemia 1 (Mcl-1) (anti-apoptotic proteins), Bcl-2-associated death promoter (Bad) and Bcl-2-like protein 11 (Bim) (pro-apoptotic proteins), cAMP response element-binding protein (CREB), forkhead box O (FOXO), cysteine-aspartic acid protease 9 (caspase-9) (McCubrey et al., 2011). At the end, the different interactions between these molecules trigger the mechanism of cell death.

CRC with *KRAS* mutations and EGFR activation are a clinical problem that needs to be solved. Our results on PMC79 are quite promising as we showed that this compound is able to target CRC cells harboring $KRAS^{G12V}$ mutation by inhibiting the MAPK and PI3K pathways, the two major pathways in CRC carcinogenesis (Ciardiello & Tortora, 2008). The observation that PMC79 does not decrease the levels of ERK and AKT proteins in RKO cells harboring *BRAF* activated mutation, suggests that it does not directly interact with ERK and AKT proteins and that the PMC79 mechanism leads to the suppression of the expression of these proteins by the interaction with other cellular targets. Although more studies will be needed to find out which effector molecules are the targets of this compound, our results suggest that PMC79 compound maybe inhibiting KRAS, an effector protein upstream MAPK and PI3K which then leads to inhibition of these pathways. Furthermore, it is also important to stress the fact that these compounds might act directly on the MAPK-ERK or in the PI3K-AKT pathway and the reason why it has no effect on RKO cells might be due to the fact that RKO cells have a double *BRAF* and *PIK3CA* mutations and thus the pathway is highly activated, and the dose needed to inhibit the pathways might be higher. It would be interesting to evaluate the effects of these novel compounds in a cell line that possessed mutations in *KRAS* and *PIK3CA* (HCT116 harbor mutations in $KRAS^{G13D}$ and $PIK3CA^{H1047R}$). Moreover, the fact that the PMC78 and PMC85 compounds, in SW480 cells, also decrease total ERK expression may indicate a major sensitivity of tumors with a mutation in *KRAS* to these type of compounds. A recent study showed that one ruthenium complex with phenylterpyridine derivatives also suppressed the expression of AKT, p-AKT, ERK and p-ERK in A375 malignant melanoma cells harboring a $BRAF^{V600E}$ mutation (Deng et al., 2017). In this work, it was showed that the compound firstly accumulates on the cell membrane and targets death receptors to activate extrinsic apoptosis signaling pathway. Then, the compound enters the cell cytoplasm through transferrin receptor (TfR)-mediated endocytosis,

decreasing the level of cellular ROS, which results in the activation of Jun N-terminal kinase (JNK) and p38, inhibition of ERK and AKT, and release of proapoptotic proteins, which can activate caspase-9 and then intensify apoptosis. The rest of the compound interacts with DNA inside the nucleus, inducing DNA damage, activating p53 signaling pathway and enhancing apoptosis (Deng et al., 2017). However, PMC79 could also induce the suppression of ERK and AKT proteins in SW480 cells through the increase of ROS levels in the cell, as demonstrated by Huang with selenium nanoparticles, which induced apoptosis through activation of intrinsic and extrinsic pathways with consequent ROS overproduction, which leads to DNA damage and the decrease of ERK and AKT expression levels (Huang et al., 2013). This hypothesis corroborates with the fact that our compound accumulates preferentially in the membranes and not in the nucleus (unpublished data) and in that way, DNA damages are not a direct consequence of the effect of PMC79 in DNA but a result of the effect of PMC79 in other effector molecules. More studies will be necessary to uncover the targets of our compounds and the cell death pathways activated. In the case of cisplatin, we know that preferentially accumulates in the nucleus and its cytotoxicity is related to its interaction with DNA (Shaloam & Tchounwou, 2014). Furthermore, the mechanism of cell death comprises several molecular mechanisms of action that are activated, such as ROS production and lipid peroxidation, induction of p53 signaling and cell cycle arrest, down-regulation of proto-oncogenes and anti-apoptotic proteins, and activation of both intrinsic and extrinsic pathways of apoptosis (Shaloam & Tchounwou, 2014). Nevertheless, none of these mechanisms of action induce a suppression of AKT and ERK expression when cells are treated with cisplatin, which is consistent with our results.

In a recent work, our group showed that in MCF7 cells, PMC78 and PMC85 accumulate in the cytoskeleton (Garcia et al., 2016), while PMC79 accumulate in the membranes (unpublished data). The cytoskeleton is responsible for maintaining the cellular shape and aiding in communication between cells (Alberts et al., 2008). In addition, the cytoskeleton is also involved in processes of cell motility, endocytosis, cell division and intracellular transport (Fletcher & Mullins, 2010; Herrmann, Bär, Kreplak, Strelkov, & Aebi, 2007). Taking into account previous results of our group, that suggested that RuPMCs interacted with the cytoskeleton, we decided to analyze F-actin using phalloidin, which is a high-affinity F-actin probe (Garcia et al., 2016; Moreira, 2016; unpublished data). The results showed that in RKO cells the treatments with Ru compounds did not affect the communications between cells however, PMC79 seemed to affect cell cytoskeleton organization with cell dispersion and evident filopodia-like protrusions.

These motility like-structures may be related to cell progression and migration, or cellular retraction. The results of Tiago Moreira, in the wound healing assay, showed that Ru compounds have no influence on cell migration (Moreira, 2016). This may indicate that the compound PMC79 induces cell retraction rather than stimulating cell progression which is a good indication of the anticancer activity of these compounds. Anyway, other assays, such as time-lapse microscopy, would help to better understand these results. In contrast to RKO cells, in SW480 cell line, treatment with the $IC_{50}$ value of cisplatin, PMC78, and PMC79 seemed to affect cell-cell adhesion and intercellular contacts establishment, accompanied by alterations on cell phenotype and roundness. It is not the first time that our compounds affect the intercellular contacts establishment. In a recent work, carried out in MDA-MB-231 breast cancer cell line, was observed that our compounds avoid the formation of tunneling nanotubes, communication structures involved in the carcinogenic process of this cells (Moreira, 2016). Despite these structures are not present in SW480 cells, after incubation with the compounds both SW480 and MDA-MB-231 cell lines no longer display structures involved in intercellular communication and the cells become more isolated. These results highlighted again the anticancer properties of these new complexes of ruthenium. PMC85 did not seem to affect cell-cell contact and intercellular contacts establishment in SW480 cells, although it affected cell actin cytoskeleton organization with the presence of focal adhesions and lamellipodia. These cytoskeleton structures mainly formed by F-actin are involved in the maintenance of cell structure and cell motility, respectively (Alberts et al., 2008). Studies showed that new adhesions formed at the leading edge of migrating cells (in lamellipodia) suggest cell migration and spreading (Partridge & Marcantonio, 2006). However, in stationary cells, they serve as anchoring devices that maintain cellular morphology (Morgan, Humphries, & Bass, 2007). These results may indicate that compound PMC85 does not affect cell migration, which is consistent with previous results obtained in our group (Moreira, 2016). Nevertheless, as mentioned above, other assays, as time-lapse microscopy, would help to understand the results. In addition to what has already been stated above, cisplatin and PMC79 also affected the cell number, in both cell lines what may be related to the percentage of cell death induced by these drugs. In previous work, PMC79 also showed to have an effect on the cell number in MCF7 cell line, and also have the highest percentage of cell death among the Ru compounds in the same cells (unpublished data). In summary, RuPMCs showed to interact with cell cytoskeleton of colorectal cancer cell lines in different ways. These results are in accordance with previous results of our group that showed that the Ru compounds also affected the

cytoskeleton of MDA-MB-231 and MCF7 breast cancer cell lines (Garcia et al., 2016; Moreira, 2016; Valente et al., 2013; unpublished data).

$\beta$-actin is a housekeeping gene which is constitutively expressed in all human cells, being an actin isoform responsible for cellular motility. Our results on $\beta$-actin expression showed no differences in $\beta$-actin expression levels in RKO cell line and in SW480 cells upon cisplatin, PMC78, and PMC85 treatment. Only PMC79, in SW480 cell line, presented a significant decrease in $\beta$-actin expression. This result may be related to the changes induced in the cytoskeleton by this compound. However, we also observed that PMC79 induced the higher percentage of cell death, in SW480 cell line. The relationship between apoptosis and cytoskeleton is well known since the actin cytoskeleton has been implicated in regulating apoptosis at different stages (Hacker, 2000). Actin is the major component of the cytoskeleton and has a role in the initiation and mediation of apoptosis via the intrinsic and extrinsic pathways (Desouza, Gunning, & Stehn, 2012). Behind that, at the final stages of apoptosis, the actin cytoskeleton is degraded resulting in the formation of two actin fragments (31 kDa (Fractin) and 14 kDa (tActin)) (Mashima, Naito, & Tsuruo, 1999), which could explain the almost absence of band in compound PMC79, in SW480 cell line. In this way, the changes that are observed in the cytoskeleton and $\beta$-actin expression could be a direct effect of this compound in cytoskeleton and/or a consequence of cell death mechanism (Desouza et al., 2012; Ndozangue-Touriguine, Hamelin, & Breard, 2008). Taking into account the previous results of PMC79, SW480 cell line appears to be the most sensitive cell line to this compound. Interestingly, it has been shown that actin cytoskeleton is important in KRAS clustering which is dependent on actin (Plowman, Muncke, Parton, & Hancock, 2005). We might hypothesize that affecting actin might be a way of indirectly inactivate KRAS signaling pathway and thus explain the effect of PMC79 in ERK and AKT levels. Considering the results of cell cycle and cell death, we could also suggest that we are dealing with an actin-mediated apoptosis pathway which arises from a tight interaction between the cytoskeleton and the RAS signaling pathway (Desouza et al., 2012; Gourlay & Ayscough, 2006).

The glucose metabolism is known to be altered in cancer cells, exhibiting overexpression of several glucose receptors and glycolytic enzymes involved in this process (Hsieh, Yang, & Fu, 2014; Song et al., 2016). Glucose transporters, mainly GLUT1, are frequently overexpressed in several types of cancer, including colorectal cancer (Amorim et al., 2015; Yang et al., 2016). GLUT1 is also the predominant glucose transporter in many types of cancer cells (Amann et al., 2009). As already mentioned, PMC85 present in its structure a sugar-like molecule with the

purpose to target cancer cells. Previous studies showed that a model of PMC85 structure is able to be identified by GLUT1 receptor in a manner dependent on the polymer chain size (Antunes, 2016). Therefore, we assessed the effect of Ru compounds in GLUT1 expression and observed that in SW480 cell line, PMC79 showed an up-regulation of the GLUT1 receptor. GLUT1 overexpression is associated with a poor prognosis and an indicator of chemoresistance (Evans et al., 2008; Harshani, Yeluri, & Guttikonda, 2014; Maki et al., 2013). Several authors also reported that there is a link between GLUT1 expression and resistance to chemotherapy, more precisely with resistance to apoptosis (Amann et al., 2009; W. Liu et al., 2014). Although this evidence seems contradictory concerning to PMC79, since this compound induced the higher percentage of cell death, in SW480 cell line. Nowadays, it is known that several oncogenes and tumor suppressor genes play a key role in glycolytic metabolism by regulating the expression of glucose receptors and glycolytic enzymes. Some of these genes are involved in MAPK and PI3K pathways, two crucial signaling pathways involved in colorectal carcinogenesis. *AKT*, *RAS, RAF*, and *EGFR* are some of the most mutated genes found in CRC, that play a key role in cancer metabolism (Chen, Li, Guan, Yang, & Cheng, 2016). A study showed that cells harboring mutations in *KRAS* and *BRAF*, expression higher levels of the GLUT1 receptor (Yun et al., 2009). Another study also showed that AKT has an essential role in the localization of GLUT1 at the plasma membrane (Airley & Mobasheri, 2007). In addition, loss of p53, overexpression of Myc and hypoxia-inducible factor (HIF) up-regulation under hypoxic conditions are also involved in glucose uptake and overexpression of GLUT1 (Airley & Mobasheri, 2007; Chen et al., 2016). In this way, the GLUT1 up-regulation associated to PMC79 could be related with some of the genes above mentioned like a feedback loop in response to p-ERK and p-AKT inhibition, but more studies will be needed to uncover the mechanism that triggers the GLUT1 overexpression. Liu et al. demonstrated that overexpression of GLUT1 was associated with 5-FU resistance, in colon cancer cells (W. Liu et al., 2014). They also showed that inhibiting GLUT1 receptor sensitizes colorectal cells to 5-FU treatment. Several studies have proposed the inhibition of GLUT1 receptor as an option to overcome drug resistance (Chen et al., 2016; W. Liu et al., 2014; Matsumoto, Jimi, Migita, Takamatsu, & Hara, 2016). In this way, we used STF-31, a well-known synthetic GLUT1 inhibitor, which had already shown to inhibit glucose uptake in colorectal cancer cells (Tyagi et al., 2016; Wexler, 2003). STF-31 is predicted to interact within the central pore of the solute carrier, inhibiting its activity (Chan et al., 2011; Granchi et al., 2016). The STF-31 mechanism of action decreases the glycolytic metabolism only targeting the glucose transport

(Chan et al., 2011). This inhibitor is also known to induce apoptosis in multiple myeloma cells and sensitize these cells to classical chemotherapeutic drugs (Matsumoto et al., 2016). Thus, we decided to evaluate whether GLUT1 might influence the effect of PMC79, in SW480 cells. Previous reports from the literature showed that STF-31 synergistically enhanced the cytotoxic effect of melphalan, doxorubicin, and bortezomib when used in combination, in multiple myeloma cells (Matsumoto et al., 2016). Nomura et al. also showed that STF-31 treatment of pancreatic $CD133^{+}$ cancer cells determined an increased sensitivity to cytotoxic agents like gemcitabine, paclitaxel and 5-FU, leading to extensive cell death (Nomura et al., 2016). Thus, our results are in accordance to these data showing that the inhibitor enhances the action of our drug PMC79. STF-31 interacted synergistically with PMC79, and the combination of the two drugs potentiated the action of the Ru compound more than the action of the two drugs alone. This could reverse the effect of the overexpression of GLUT1 in SW480 cells treated with PMC79 relative to possible resistance mechanisms. Another GLUT1 inhibitor also showed similar results, Liu et al. demonstrated that WZB117, a GLUT1 inhibitor, interacted synergistically with paclitaxel or carboplatin in lung and breast cancer models (Y. Liu et al., 2012). Identical results have been reported in colon cancer and leukemic cells, using WZB117 and daunorubicin (Cao et al., 2007). One hypothesis could be that the overexpression of GLUT1 may be a result of PMC79 action on another cellular target which, in turn, leads to the up-regulation of this glucose transporter as a feedback loop to compensate. Another hypothesis will be the acquisition of resistance by SW480 cell line to this compound, as was demonstrated in the work of Liu et al., in which colorectal cancer cells overexpressing GLUT1 receptor were sensitized for treatment with 5-FU using a GLUT1 inhibitor (W. Liu et al., 2014). In this way, the use of GLUT1 inhibitors could be a good strategy to overcome possible problems of resistance to PMC79. However, a more detailed study of glycolytic metabolism will be necessary in order to better understand the mechanisms that lead to the overexpression of this transporter and, whether its expression is related to some mechanism of acquired resistance. Regarding RKO cell line, none of the compounds lead to GLUT1 overexpression, however, an increase in AKT expression levels was observed in the study of signaling pathways. A recent work showed that hexokinase 2 (HK2), a glycolytic enzyme, induce drug resistance in cancer cells, by AKT/mTOR pathway (Min et al., 2013). In another work, increased pyruvate kinase isozymes 2 (PKM2) levels, other enzyme involved in cancer metabolism, were reported to be linked with 5-FU resistance in patients with colorectal cancer (Shin et al., 2009). Because of AKT is closely related to drug resistance mechanisms, further

studies of glycolytic metabolism (mainly the assessment of glycolytic enzymes) will help to uncover if there is any mechanism of resistance behind the up-regulation of AKT in RKO cell line. Previous results of our group showed that the rational functionalization of our compounds, potentiated the action of PMC85 in MDA-MB-231 breast cancer cell line, through downregulation of GLUT1 expression (Moreira, 2016). In fact, and as already noted, this compound proved to be the most efficient in this cell line. However, in colon cancer cells, Ru compounds did not lead to a decrease in the expression levels of this glucose receptor, suggesting that the structure of the compounds, particularly in the case of PMC85, are not the only ones to influence GLUT1 expression, and that other mechanisms are behind the downregulation or up-regulation of this receptor (as in PMC79).

# 6. Conclusion

In this work, the first steps were taken towards the discovery of the effect, mechanisms of action and molecular targets of three new ruthenium cyclopentadienyl compounds, using colorectal cancer cell lines.

Our results showed that RuPMC compounds induce apoptosis but do not interfere with cell cycle, being PMC85 (in RKO cells) and PMC79 (in SW480 cells) the most efficient Ru compounds.

Here, we showed that the different genetic background which activates different signaling pathways in the cell, influence the effect of the compounds. Moreover, the structure of the compounds also affects the mechanisms of action and the cellular targets in the cells. We evidenced that cells harboring *KRAS* mutations seem to be more sensitive being the effects on the suppression of ERK and AKT expression levels the mechanism by which the compounds might trigger cell death.

Our data also demonstrated that RuPMCs seem to interact with actin filaments from the colorectal cancer cells, suggesting that the cytoskeleton might be a target of our compounds. Interestingly, as actin seem to be important for KRAS cluster signaling, these compounds by affecting actin might indirectly interfere with KRAS signaling inside the cell.

Moreover, we also observed that PMC79 induced an up-regulation of GLUT1 receptor in SW480 cells, which is often associated with resistance mechanisms, and showed that GLUT1 inhibitor, STF-31, potentiated the effect of PMC79.

Overall, our results showed that Ru compounds seem to affect SW480 and RKO cell lines in a different manner, being SW480 the most sensitive cell line, what seems to be associated to the different signaling pathways activated in the cells. Compounds PMC78 and PMC85 also demonstrated promising anticancer activity, however, more studies will be needed to better understand their mechanisms of action and main targets.

# 7. Future perspectives

Despite the promising results obtained there is still much to discover about the mechanisms of action of our compounds and their cellular targets. Beginning with cell death induced by RuPMC compounds, in order to complement the apoptosis assay, other assays should be performed to confirm the type of cell death induced by Ru compounds, like annexin V/PI; cysteine-aspartic acid protease (caspase) expression namely cleavage of pro-caspases 3, 8 and 9; caspase activity assays; cytochrome c release and poly (ADP-ribose) polymerase (PARP) degradation by Western blot analysis. Uncover the cell death signaling pathway triggered will help us to understand some cellular targets. In this way, we could study the expression of some proteins of the Bcl-2 family. Study of the induction of the autophagy pathway may also be performed due to the importance of this type of programmed cell death/survival mechanism in cancer. Therefore, microtubule-associated proteins 1A/1B light chain 3A (LC3II/I) conversion, autophagy protein 5 (ATG5) and Beclin1 expression by Western blot analysis could be some ways to assess autophagy.

With the purpose to evaluate other possible targets of the different compounds, we could study their effects in some organelles as mitochondria (by ROS production and mitochondria membrane potential analysis), lysosomes (lysosome membrane permeabilization and cathepsins release) and DNA (assess DNA damage by comet assay). We could also increase the concentration of each compound in order to assess changes in the expression of proteins involved in MAPK and PI3K signaling pathways, in cancer cell lines which present double mutations in genes involved in these signaling pathways, as RKO cell line. Regarding the cytoskeleton, which was proved to be a target of our compounds, a time-lapse microscopy assay would be helpful to clarify some effects of the Ru compounds on the cell motility. A proteomic assay will also help to identify the main targets of our compounds maybe through a two-dimensional gel electrophoresis (2-DE), which allow comparing protein expression between samples (Klose, 1975; O'Farrell, 1975).

With regard to glycolytic metabolism, uncover the molecular targets behind the up-regulation of GLUT1 in SW480 and AKT in RKO should be performed through the evaluation of some molecules involved in the glycolytic metabolism as HIF, c-Myc by Western blot analysis or even the assessment of gene expression profiling under conditions of combined treatments. This will help to discover some possible resistance mechanisms. The combination of STF-31 with

RuPMC compounds may be extended to the other compounds and to RKO cell line, in order to understand whether this inhibitor also potentiates the action of the other compounds. GLUT1 inhibition by siRNA could also be important to perform with the purpose to assess whether other ways of inhibition of GLUT1 also potentiates the effect of the compounds. Furthermore, metallothioneins and glutathione are already known to be overexpressed in cells resistant to metal compounds (Basu & Krishnamurthy, 2010). In this way, will be important assess metallothioneins and glutathione expression by Western blot analysis, which will show whether colon cells are resistant to RuPMC or if the resistance is developed later.

The assessment of the effects of our compounds in noncancer colon cells (NCM460 cell line) should also be performed in order to understand whether RuPMCs affect normal cells to the same extent they affect cancer cells.

The next step could also be taken toward pre-clinical *in vivo* studies, with the purpose to evaluate the effects of the different compounds in mice with tumor xenografts and understand the effect on cancer size reduction and if any side effects are observed in the mice.

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