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Anti-tumoral effects of an Iron and a Cobalt Scorpionate

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Abstract

The World Health Organization (WHO) reports that cancer is the second leading cause of death globally and there have been several research attempts focused on finding more efficient treatments to deal with this disease, including the area of metal-based drugs for cancer chemotherapy. New transition metal complexes are being continuously designed and tested as anticancer agents, namely complexes obtained with the so called scorpionate ligands, which have provided significant contributions in the fields of catalysis and bioinorganic chemistry, and have also shown interesting antitumoral properties.

This project was focused on the evaluation of the antiproliferative and antimigration effects of two scorpionate metal complexes as a preliminary approach to assess their anti-tumoral properties. An iron scorpionate, $FeCl_2Tpm$ (designated as S6), and a cobalt scorpionate, $CoTpm_2(OH)_2$ (termed S7) with tris(pyrazolyl)methane-derived ligands (Tpm), were tested in various representative cell lines, HCT116 (colorectal carcinoma), B16 (murine melanoma) and HaCaT (a non-tumoral line of spontaneously transformed skin keratinocytes), to evaluate their cytotoxic potential towards these cells and their capacity to inhibit cell migration, two features that would provide some insights regarding their anti-tumoral effects.

The assessment of the cytotoxicity profile of the compounds S6 and S7 was conducted by MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) reduction assays, after confirmation of the purity of the complexes by HPLC (high-performance liquid chromatography). The evaluation of the effects of these scorpionates on cell migration was performed through scratch assays. Samples of cells exposed to S6 and S7 were also prepared and later submitted to metabolomic analyses, that could possibly identify alterations concerning metabolite levels and their respective metabolic pathways.

Results revealed that S6 did not exhibit a significant toxicity towards the HCT116 and HaCaT cell lines under the tested conditions, but displayed the capacity to delay cell migration. S6 also led to an increase of the proliferation and migration of the B16 cell line. By contrast, S7 revealed a higher cytotoxic potential in the cell lines used, as well as the capacity to inhibit cell migration. The results obtained with the tested cell lines suggest that S7 could potentially be used as an anti-tumoral agent, since this scorpionate complex revealed visible effects in two cellular processes that are particularly enhanced in tumoral cells (proliferation and migration). Further studies are needed to confirm these findings, which could involve testing their role in cell invasion in order to allow a better understanding of the anti-tumoral effects of these scorpionate complexes.

Keywords: Scorpionates; antitumoral potential; antiproliferative and antimigration effects

Resumo

As doenças cancerígenas constituem, segundo a Organização Mundial de Saúde (OMS), a segunda causa de mortalidade global. A elevada complexidade destas doenças pode ser evidenciada através dos vários hallmarks que descrevem os diversos aspetos e características biológicas associados ao desenvolvimento destas patologias. Alguns destes hallmarks abordam as capacidades proliferativas mais acentuadas das células cancerosas, aliadas à capacidade destas de evitarem vários mecanismos de controlo e regulação celular, adquirindo assim um potencial replicativo aumentado e resistência a processos de morte celular. Outros hallmarks reforçam a importância da vascularização no contexto tumoral, capaz de favorecer o progresso da doença e adicionalmente fenómenos de metastização, nos quais as células tumorais malignas poderão integrar a circulação e invadir outros locais distantes do tumor primário, onde poderão originar tumores secundários. O perfil metabólico alterado observado nas células cancerosas e a capacidade destas de evitarem determinadas respostas do sistema imunológico constituem outros hallmarks relevantes para o desenvolvimento tumoral. Pode ainda ser destacada a existência de diversas populações celulares (constituídas por células responsáveis pela vascularização, células imunológicas e fibroblastos) que irão proporcionar suporte às células cancerosas e assim estabelecer um meio dinâmico favorável ao desenvolvimento tumoral.

Todos os aspetos descritos anteriormente contribuem para a complexidade e diversidade das doenças neoplásicas, que têm dificultado a aquisição de estratégias preventivas eficazes para estas patologias. Este facto reforça a importância de refinar as estratégias terapêuticas atualmente disponíveis e de procurar desenvolver outros tratamentos mais eficazes. As modalidades mais utilizadas envolvem procedimentos cirúrgicos (que possibilitam a remoção direta de estruturas tumorais), radioterapia (onde se utilizam feixes de radiações ionizantes para obliterar certos tumores) e quimioterapia (que envolve a utilização de variados agentes orgânicos e metálicos para eliminar ou atenuar o desenvolvimento tumoral).

Ao nível da quimioterapia, podem ser realçados certos complexos constituídos por platina (cisplatina, carboplatina e oxaliplatina), cuja elevada eficácia tem favorecido a extensa utilização dos mesmos no tratamento de diversas doenças tumorais, nas quais se incluem tumores testiculares, ováricos, pulmonares e de bexiga. Estes complexos têm manifestado, no entanto, algumas desvantagens, que incluem a elevada toxicidade perante células não tumorais, a qual poderá culminar em diversos efeitos adversos comprometedores da qualidade de vida dos pacientes, destacando a perda de cabelo, nefrotoxicidade e neuropatias periféricas. A possibilidade de se verificarem fenómenos de resistência multifatorial constitui outro aspeto responsável por atenuar o sucesso das terapias de quimioterapia convencionais. Estas considerações têm motivado a investigação e desenvolvimento de outros complexos metálicos

que possam dispor da mesma eficácia revelada pelos agentes de platina, mas que não manifestem os efeitos secundários referidos.

De entre todos os complexos metálicos explorados para este propósito, podem ser destacados os complexos portadores de escorpionatos, que constituem estruturas moleculares geralmente formadas por 3 ligandos que estabelecem ligações entre si e o elemento metálico central segundo uma conformação que se assemelha ao formato de um escorpião, característica que permitiu atribuir a designação sugestiva a estas moléculas. Pode ser destacada a vasta diversidade e variabilidade de complexos formados por diferentes ligandos e centros metálicos que proporcionam diversos efeitos e propriedades relevantes para várias aplicações biomédicas, incluindo aquelas relacionadas a terapias antitumorais.

Certos complexos constituídos por escorpionatos têm sido explorados para a produção de moléculas transportadoras de monóxido de carbono (CO), capazes de libertar esse CO de modo controlado nas imediações das lesões tumorais, onde este poderá ligar-se à hemoglobina e inviabilizar o fornecimento de oxigénio às células cancerosas, exercendo desse modo efeitos citotóxicos. Outros complexos portadores de escorpionatos têm sido estudados como agentes foto-sensibilizadores, utilizados em terapias fotodinâmicas, onde a ativação destes agentes por radiação visível irá promover a interação entre estes e o oxigénio sedeado nos tecidos, da qual resulta a formação de espécies reativas de oxigénio capazes de instigar diversos danos nas células cancerosas e na vasculatura tumoral. Outros complexos têm recebido destaque pela atividade antiproliferativa que poderá estar diretamente associada à capacidade destes de estabelecerem diversos tipos de ligações ao DNA, provocando clivagens e danos estruturais nesta molécula, e noutros casos, que poderá resultar da produção de espécies reativas de oxigénio que por sua vez irão despontar diferentes danos celulares que poderão culminar em processos apoptóticos, manifestando desse modo efeitos antitumorais.

Este projecto de dissertação envolveu o estudo dos efeitos antitumorais de 2 complexos formados por escorpionatos, um deles detentor de ferro ($FeCl_2Tpm$, abreviadamente referido como S6) e outro portador de cobalto ($CoTpm_2(OH)_2$, designado S7). O potencial antitumoral dos complexos S6 e S7 foi avaliado pela análise dos efeitos antiproliferativos e antimigratórios ao nível de 2 linhas celulares tumorais, HCT116 (correspondente a células humanas de carcinoma colo-rectal) e B16 (que corresponde a células de melanoma de murganho). Utilizou-se adicionalmente uma linha celular não tumoral, conhecida por HaCaT (correspondente a queratinócitos humanos espontaneamente imortalizados) de modo a verificar os efeitos que estes complexos teriam ao nível de células normais.

Foram primeiramente realizados ensaios de pureza, recorrendo à técnica de HPLC (high-performance liquid chromatography), em que o cromatograma obtido revelou um sinal

característico do ligando orgânico destes complexos, indicando desse modo que estes se encontravam puros e que não sofreram degradação em solução aquosa.

O perfil citotóxico dos complexos S6 e S7 foi analisado por ensaios colorimétricos de MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium). Os resultados destes ensaios revelaram a ausência de efeitos citotóxicos por parte do S6 sobre as linhas celulares HCT116 e HaCaT, que promoveu, no entanto, um aumento da proliferação das células B16. O complexo S7 revelou, por contraste, um potencial citotóxico mais nítido, que permitiu a obtenção das curvas dose-resposta e os valores de IC_{50} para as várias linhas celulares, podendo destacar a este nível as células HCT116 como as mais afetadas pelos efeitos antiproliferativos deste complexo.

A avaliação dos efeitos dos complexos S6 e S7 ao nível da migração celular foi realizada por ensaios ferida. Nestes ensaios, o complexo S6 revelou capacidades antimigratórias nas linhas celulares humanas HCT116 e HaCaT, tendo manifestado um efeito contraditório nas células B16, nas quais promoveu uma migração mais acelerada. O complexo S7 revelou, por sua vez, capacidades antimigratórias perante todas as linhas celulares testadas, tendo exibido efeitos mais nítidos nas células HaCaT.

Foi adicionalmente realizado um último estudo que envolveu a preparação e recolha de amostras celulares expostas a S6 e S7, as quais foram posteriormente submetidas a análises de metabolómica, de modo a identificar metabolitos e vias metabólicas significativamente alterados, em relação a amostras de controlo, que não tinham sido expostas a estes complexos, tendo o intuito de tentar correlacionar essa informação metabólica aos resultados experimentais obtidos anteriormente. Estas análises permitiram a identificação de vários metabolitos significativamente alterados nas amostras expostas a S6. Não foi possível, no entanto, estabelecer uma ligação direta entre estes resultados preliminares e as observações efetuadas nos ensaios anteriores. Estas análises preliminares não permitiram ainda a identificação de alterações metabólicas significativas nas amostras expostas a S7 em relação às amostras de controlo.

Os resultados obtidos nesta dissertação revelaram a ausência de efeitos citotóxicos por parte do complexo S6, que manifestou, no entanto, efeitos antimigratórios em duas das linhas celulares testadas, o que motiva a realização de outros estudos para melhor esclarecer as eventuais propriedades antitumorais deste complexo. Os efeitos antimigratórios revelados pelo S7, aliados aos efeitos antiproliferativos, essencialmente nítidos nas células HCT116, aparentam favorecer a utilização deste complexo para fins antitumorais, particularmente para o tratamento de tumores colo-rectais. A confirmação destas observações requer a realização de estudos adicionais, que poderiam incluir a análise dos efeitos deste complexo ao nível da invasão celular.

Palavras chave: Escorpionatos, potencial antitumoral, efeitos antiproliferativos e antimigratórios

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General Introduction

The World Health Organization (WHO) reports that cancer is the second leading cause of death globally [1]. The US National Cancer Institute (Rockville, Maryland, USA) recognizes the existence of over 150 different types of human cancers, that can be classified according to the location in the body and the tissue in which they arise [2]. Cancers formed in epithelial tissues, that cover internal or external surfaces of organs, are called carcinomas, and include most of the lung, skin, colon, and breast cancers. Sarcomas are cancers that develop in connective tissues like bone, muscle, and cartilage. Leukemia is a type of cancer that occurs in blood-forming tissues such as the bone-marrow, resulting in the release of large amounts of abnormal blood cells into the bloodstream. Other types of cancer include lymphomas and myelomas, formed in the cells of the immune system, and central nervous system cancers that develop in tissues of the brain and spinal cord [3]. The most common types of cancer in men are lung, prostate, colorectal and liver cancers, while breast, colorectal, lung and cervix cancers are the most common in women [4].

Cancer results from cellular changes that cause the uncontrolled growth and division of abnormal cells. These cells may become cancerous with the subsequent accumulation of mutations in their DNA. Normal cells are usually capable of detecting and repairing DNA damage and can also receive instructions to undergo a programed cell death (apoptosis) when they are worn out or suffer irreversible damage. In contrast, tumoral cells may lack the instructions that induce the programmed cell death, which results in the ongoing and uncontrolled cell division that leads to the formation of more abnormal, mutated cells, that crowd out normal cells and use the oxygen and nutrient reserves meant to nourish those cells. The proliferation of tumoral cells will ultimately prevent the normal physiological activity of the tissues or organ in which those cells emerged [5].

Many factors capable of causing cell abnormalities have been identified and linked to cancer development, which include environmental exposure to sunlight ultraviolet radiation and air pollutants, contact with bacterial and viral pathogens, and risk factors associated to lifestyle (smoking habits, heavy alcohol intake, excess body weight or physical inactivity). Generally, the combination of some these factors, with association to the person's genetics, may contribute to cancer development [3].

Cancer can be perceived as a genetic disease, in the way it results from several changes in genes that control certain aspects of cell physiology, including cell growth and division. Specific gene changes can lead to the formation of cells capable of evading normal growth controls, that become tumoral [6]. Some cancer-causing gene changes can increase the production of proteins that

promote higher cell growth, while others result in the production of misshapen and nonfunctional forms of proteins that would otherwise repair cellular damage.

Genetic changes that promote cancer development can be inherited from the parents or acquired during lifetime, resulting in this case from errors that occur during cell division or from exposure to carcinogenic substances and some forms of radiation capable of damaging the DNA [7]. Some genetic changes can affect nucleotides, that can be replaced by another or may be missing entirely, while other changes can include rearrangements, deletions or duplications of larger stretches of DNA [8]. Other modifications, that do not occur in the actual sequence of DNA, include the addition or removal of chemical marks, designated as epigenetic modifications, that influence gene expression [9]. Cancer cells within the same tumor may also reveal different genetic changes [10].

Table 1 - Examples of known tumor suppressor genes associated to cancer development

Gene	Familial Cancer Syndrome
RB1	Familial Retinoblastoma
p16(INK4a)	Familial melanoma
p14(ARF)	Familial melanoma
CHK 1/2	Li-Fraumeni Syndrome
NF1	Neurofibromatosis Type 1
APC	Familial Adenomatous Polyposis
TSC1	Tuberous sclerosis 1
DCC	Deleted in Colorectal Carcinoma
BRCA1	Familial Breast Cancer
MSH2	Hereditary Nonpolyposis Colon Cancer type 1
MLH1	Hereditary Nonpolyposis Colon Cancer type 2
PTEN	Cowden syndrome
CDH1	Familial diffuse-type gastric cancer
SMAD4	Familial juvenile polyposis syndrome
SMAD2	Juvenile polyposis
P53	Li-Fraumeni Syndrome

Several mutated genes have been linked to cancer development (**Table 1**), namely BRCA1, which is mostly associated with hereditary breast and ovarian cancers, PTEN, that is related to the increased risk of prostate, breast, endometrial and thyroid cancer; and p53 which displays several roles in cell cycle regulation and in the suppression of tumoral growth, being also the most commonly mutated gene found in multiple types of cancer [11].

Hallmarks of cancer

The complexity of cancer biology can be better appreciated through the description of the so-called cancer hallmarks, a set of acquired capabilities that allow the survival, proliferation and dissemination of cancer cells, which include their ability of sustaining proliferative signaling, evading growth suppressors, enabling replicative immortality, resisting cell death, inducing angiogenesis, activating invasion and metastasis, deregulating cellular energetics, and avoiding immune destruction. Genome instability and mutation and tumor-promoting inflammation were also described as two enabling traits that facilitate the acquisition of the cancer hallmarks [5]. All of these different hallmarks and enabling characteristics provide an organizational framework that supports the description of the cellular properties acquired during tumor development [12].

Further developments in tumor biology were also achieved by the recognition of a more complex tumoral content that, instead of solely including proliferating cancer cells, also possesses a wide variety of cell types capable of maintaining dynamic signaling interactions that lead to the establishment of the tumor microenvironment which contributes to the manifestation of certain hallmark capabilities and ultimately to the cancer phenotypes [13]. A concise description of the several cancer hallmarks and the tumor microenvironment will be provided in this section.

Sustaining proliferative signaling and evading growth suppressors

The growth and proliferative signaling responsible for regulating the progression of the cell cycle and for the maintenance of the normal tissue homeostasis is disrupted in the case of cancer [14]. Several alterations within the growth ligands, their respective receptors and the signaling molecules support the increased survival capabilities of cancer cells [15].

Tumor progression can be promoted in an autocrine fashion, where cancer cells directly produce growth factor ligands and express the appropriate receptors to respond to such ligands. Cancer cells may alternatively signal epithelial or stromal cells located in the tumor microenvironment, that will insure the supply of the required growth signals [14]. Deregulation of the receptor signaling may also occur through various processes that involve gene amplification leading to an increased expression of the receptors, mutations resulting in constitutive receptor activation, and inefficiencies in the receptor recycling and degradation machinery [16]. Cancer cells may also achieve growth factor independence, through various alterations in the signaling pathways downstream of receptors [17].

In addition to the deregulation of growth signaling, the disruption of various cell cycle controls and checkpoints constitutes an equally relevant process for tumor development [18]. The tumor suppressor gene retinoblastoma (RB) serves as an example of a crucial regulator, encoding a protein capable of receiving signals from extracellular and intracellular sources, determining whether the cell should proceed through the growth and division cycle, being often inactivated in several tumor types [19]. It should also be mentioned that RB tasks are not exclusive to proliferative control, but also involve processes related to other hallmarks, including maintenance of genomic stability, regulation of apoptosis and suppression of metastasis [20]. The previously mentioned p53 constitutes another critical regulator, capable of detecting and responding to a wide variety of stresses caused by hypoxia, nutrient scarcity and genomic damage. P53 interrupts the cell cycle progression and arrests further proliferation when facing such stresses, being involved in instigating the proper repair mechanisms. When dealing with more extensive and irreversible damage, p53 will instead trigger terminal differentiation states and even cell death [21]. This tumor suppressor displays several other tasks linked to other hallmarks and corresponds to the most commonly mutated gene implicated in cancer development, being mutated in over 50% of the sequenced tumors [22,23].

<u>Resisting cell death and enabling replicative immortality</u>

The third and fourth established hallmarks can be described in the context of various stresses faced by cancer cells during the tumor development process, and overcoming such stresses ensures their survival and proliferation [5].

The apoptotic cell death serves as a response to transformation-associated stress caused by deregulated proliferation, irreversible DNA damage or matrix detachment [24]. This apoptotic process involves an extrinsic pathway related to the interaction between the cell surface receptors and the respective ligands, and an intrinsic pathway capable of sensing internal stress levels and favoring, when necessary, the activity of pro-apoptotic-triggering proteins over the anti-apoptotic counterparts. This process results in the disruption of the integrity of the outer mitochondrial membrane, leading to the release of proapoptotic-signaling proteins that converge in the activation of cleavage proteins known as caspases, capable of disintegrating the cell structure into various fragments later collected by specialized phagocytic cells, efficiently undertaking the execution phase of the apoptotic program [25]. Cancer cells are able to overcome this defensive cellular response through several means, the most common of which resides in inactivating mutations that disrupt the p53 tumor suppressor capacity, elevating cell insensitivity towards various apoptotic signals. Tumors may also reveal increased levels of anti-apoptotic regulators and survival signals, or downregulated pro-apoptotic proteins [24].

Senescence constitutes another defensive mechanism against cellular stress, that serves as a barrier to deregulated and unlimited proliferation, being described as a process that leads to an irreversible departure from the cell cycle into a viable but non-proliferative state [26]. This process is linked to structures responsible for protecting the ends of chromosomes, known as telomeres, which reveal a progressive shortening with the successive cell divisions, that will eventually compromise their protecting capacity and ultimately lead to the exhaustion of the replicative potential. Induction of senescence occurs in order to avoid subsequent cycles of chromosomal fusion and damage, as well as the resulting genomic instability and accumulation of mutations that would ensue the critical shortening of telomeres [27]. Tumor development requires fully transformed cells capable of bypassing senescence-associated stress resulting from short telomeric length, which is mainly achieved by telomerase, the enzyme specialized in countering telomere erosion by restoring the telomeric ends, being upregulated in 85-90 % of human tumors, where it maintains enough telomeric length to extend the replication potential of tumoral cells [27,28]. Other reported stresses capable of triggering the cytoprotective effects of senescence include a more intense and derailed mitogenic signaling, non-telomeric DNA damage (instigated, for example, by anti-tumoral therapies) and activation of tumor-suppressors [26,29].

Vascularization

The formation of new vasculature, capable of ensuring the supply of oxygen and nutrients and of collecting the metabolic wastes and carbon dioxide secreted by cancer cells, stands as an important hallmark of cancer development [5]. Angiogenesis corresponds to the most discussed process of neo-vasculature development, involving the sprouting of new vessels from pre-existing ones through the proliferation and migration of endothelial cells (ECs). This process is controlled by regulatory cell mechanisms formed by anti-angiogenic and pro-angiogenic factors, that will, respectively, disable or activate the angiogenic switch, with specific triggers being required to favor the activity of the pro-angiogenic factors [30]. The several triggers involved in the activation of angiogenic pathways in cancer cells can alternate during tumor development [31], being hypoxia one of the most relevant angiogenic triggers [32]. The hypoxia-inducible transcription factor (HIF) family, linked to oxygen sensing mechanisms found in ECs, is capable of devising adaptive transcriptional responses to hypoxia, resulting in factors involved in enabling the angiogenic switch. HIF levels remain notably higher in various tumors, where hypoxia is a common feature, which favors the chronic activation of the angiogenic process [33].

Several pro-angiogenic molecules have been identified as inducers of this vasculature process, including the ones associated to the vascular endothelial growth factor (VEGF) signaling pathway [34]. The activated VEGF signaling, mediated by HIF activity, increases the proliferation, migration and differentiation of ECs, and regulates the vasculature permeability [35].

The tumor vasculature produced by the angiogenic process often displays several morphological and physiological anomalies, that include dilated, convoluted and excessive vessel branching, distorted vessels characterized by leakiness and an erratic blood flow, which contributes to the establishment of sections of acidosis and hypoxia, resulting in a stressful environment that can further potentiate angiogenesis [36].

Tumor vascularization can also be obtained by non-angiogenic process, that provide alternative survival options to cancer cells and that could be involved in their capacity to evade conventional anti-cancer therapies. These alternative processes include vessel co-option, where cancer cells seize existing vasculature to obtain the necessary oxygen and nutrient supplies [37]; intussusceptive angiogenesis, characterized by a vasculature network that expands within itself by splitting existing vessels into secondary ones, allowing a faster and more energy efficient production of capillaries associated to fewer leakiness [38]; and vasculogenic mimicry that describes the specific capacity of aggressive tumor cells to form new vessel-like networks, promoting the survival of rapidly developing tumors [39].

Activating invasion and metastasis

The capacity of cancer cells to invade and proliferate in other tissues, where they give rise to secondary tumors (metastasis) constitutes the definitive hallmark of malignancy [5]. The invasion-metastasis cascade describes the intertwined process of cell-biologic changes required for the formation of secondary tumors, that starts with local invasion progression through the extracellular matrix (ECM), followed by intravasation into nearby blood and lymphatic vessels, circulation of cancer cells to distant sites, extravasation to the parenchyma of distant organs, formation of micro-metastases and further development to macroscopic tumors in a final step known as colonization [40].

The invasion and metastases processes are mostly regulated by the epithelial-mesenchymal transition (EMT) program, that includes a set of reversible biochemical modifications which lead to cell remodeling, where polygonal epithelial cells acquire a mesenchymal phenotype characterized by a more fibroblastic morphology, associated to elevated migratory and invasive capabilities [41]. Regulation of the EMT dedifferentiation program involves a wide array of transcriptional factors, capable of suppressing epithelial gene activity and enabling mesenchymal gene expression, when triggered by specific signaling pathways, tissue hypoxia and metabolic stress [42,43]. The EMT program should also be described as a set of multiple and dynamic transitional states between the epithelial and mesenchymal phenotypes, replacing the notion that cancer cells undergo a complete EMT with a more modern perspective, in which cancer cells may be submitted to a partial EMT, where they acquire new mesenchymal features while preserving some of the epithelial traits [44,45]. The transcriptional factors associated to EMT will also evoke

the expression of matrix metalloproteinases capable of promoting the degradation of the ECM, that will further contribute to local invasion [46].

Invasive cells can later disseminate to distant sites through intravasation into the lumina of vessels, where they must display several adaptive mechanisms to overcome the selective conditions found in circulation, that include the use of protective platelet shields capable of aiding circulating tumor cells (CTCs) to endure the shear stress while simultaneously providing an immune surveillance escape [47]. CTCs will eventually accumulate in small capillaries where they can cause micro-vessel ruptures or initiate infiltration of the distant tissue through extravasation [40], a process triggered by other circulating non-tumor cells, ligand-receptor interactions and chemokines [48].

After gaining access to the distant tissues, the invasive cells will employ several systemic signals involved in the establishment of the new microenvironment, which will facilitate the formation of micro-metastases and also contribute to the final stage of colonization. Several tumor-derived secreted factors and bone-marrow-derived cells are believed to be involved in this process [49], with increasing evidence also supporting the intervention of exosomes in this regard [50]. These lipidic nanovesicles (of 30-150 nm in diameter) released into the extracellular space enable intercellular communication by transferring their protein and nucleic acid content to recipient cells, where they could modulate pathways related to various biological actions, including invasiveness [51].

Tumoral cells exhibiting a delayed adaptation to the new environment may transition to a state of dormancy that could explain the occurrence of certain cancer relapses, resulting from invasive cells abandoning that dormant state and instigating colonization [52]. This process would require a high self-renewal capability, which supports the idea centered on the existence of a rare population of metastasis-initiating cells, with advantageous stem-cell like features that would allow them to contribute to this final phase of metastasis [53].

Reprogramming energy metabolism

Various adjustments in the cellular energy metabolism are required to fuel the increased proliferation characteristic of tumor development, and to provide cancer cells the capability to adapt to the tumor microenvironment [54,55]. Rewired cancer metabolism was recently classified and organized into six hallmarks that may be fully or partially expressed in different tumor types, being listed as deregulated uptake of glucose and amino acids, opportunistic modes of nutrient acquisition, use of glycolysis and TCA (tricarboxylic acid) cycle intermediates for biosynthesis and NADPH production, increased nitrogen demand, alterations in metabolite-driven gene regulation and metabolic interactions with the microenvironment [56].

Tumoral cell energy metabolism is characterized by displaying several anomalous features, including the reprogramed glucose metabolism, where tumoral cells seem to favor glycolysis over oxidative phosphorylation even when having access to oxygen supplies, exhibiting a state referred to as aerobic glycolysis [54]. An upregulation of the glucose transporter GLUT1 ensures the increased uptake and usage of glucose required for this process reported in multiple human tumor types [57]. It was previously believed that an ineffective mitochondrial oxidative phosphorylation would lead tumoral cells to adopt aerobic glycolysis as the main ATP generator [58], but this notion was discarded after confirming the existence of fully operational mitochondria within tumoral cells [59]. More recent perspectives contemplate instead the use of the two energy-generating pathways to effectively respond to the increased tumoral metabolic demands, with glycolysis providing a faster energy supply and intermediates for anabolic reactions, that favor the biosynthesis of macromolecules and organelles relevant in the tumoral context [54].

Glutamine constitutes another relevant nutrient capable of fueling oxidative ATP production and providing carbon intermediates used in the biosynthesis of macromolecules. Other roles have been attributed to this amino acid regarding its nitrogen-donation capability, which involve supporting the amino acid pool and ensuring nitrogen donation for nucleotide production [60]. Tumoral cells may employ alternative pathways, other than transport, to acquire glutamine under nutrient-depleted conditions, that include scavenging extracellular proteins and collecting nearby apoptotic products. The acquired glutamine may be used to generate glutamate in the mitochondria of proliferating cancer cells, capable of being utilized directly or subsequently converted into α-ketoglutarate (α-KG), involved in the maintenance of the TCA cycle and in fatty acid synthesis [54]. The increased acquisition of lipids observed in proliferating tumoral cells may provide advantages regarding the production and constitution of membranes, which could reveal an increased oxidative-damage-resistant lipidic profile [56,61].

Another relevant aspect of the rewired metabolism lies in the characteristically enhanced accumulation of certain metabolites within the tumoral sites, that also contribute to the molecular processes involved in tumor development, exerting their biological effects outside the conventional metabolic network, essentially through epigenetic regulation [56]. An illustrative example involves the activating mutations in the isocitrate dehydrogenase (IDH) enzymes, that lead to the production of the metabolite 2HG, capable of promoting methylation suppression of several genes that may contribute to tumoral progression [54].

Evading immune destruction

The tumor immune surveillance concept supports the existence of various immunological mechanisms specialized in the continuous monitoring of cells and tissues, and in identifying and eradicating tumoral cells, serving as a barrier to tumor formation and progression. Following this

notion, tumoral development requires transformed cells capable of avoiding immune detection or inhibiting the effectiveness of the immune responses [5].

An effective immune system is capable of continuously eliminating and shaping tumoral diseases through a dynamic process referred to as cancer immunoediting. The first stage of this process corresponds to the elimination phase (cancer immunosurveillance), where a unified effort of the innate and adaptive immune systems contributes to tumor eradication. The following equilibrium phase involves a continuous tumor shaping associated with the selection of less immunogenic tumoral cells that may give rise to more resistant variants, resulting from genetic instability. Some of the tumor cells can transition to a slow-cycling latent state during this phase, with new variants later emerging, displaying different mutations responsible for providing increased resistance towards immune defenses [62]. In the final escape phase of cancer immunoediting, the tumor variants that were capable to evade immune surveillance will later contribute to tumoral development. Disabling the cytotoxic components of the immune system constitutes one strategy employed by tumoral cells to evade immune eradication, which could be achieved by the secretion of cytokines and other factors capable of promoting an immunosuppressive environment, or through the recruitment of immunosuppressive inflammatory cells [63,64]. Tumor-derived exosomes may also be dispatched to suppress the immunological activity and further contribute to immuno-evasion [65].

Tumor Microenvironment

A continuous communication and interaction between tumoral cells and several stromal cell types will lead to the establishment of a dynamic microenvironment required for tumoral development and progression, that will enable and sustain most of the cancer hallmarks. The constituents of the stromal component of the microenvironment can be grouped into three classes that include angiogenic vascular cells, infiltrating immune cells and cancer-associated fibroblasts, with all of them exhibiting broad contributions to the hallmark capabilities [13].

The angiogenic vascular cell class includes ECs and pericytes, that are mainly involved with the vascularization hallmark. Endothelial cell activation is promoted by the angiogenic switch, which will lead to the development of neo-vasculature. Other roles that have been attributed to tumor-associated ECs are related to immune cell recruitment and modulation of cancer cell dissemination [13]. Pericytes constitute a specialized mesenchymal cell type with cytoplasmatic projections that encircle the vessel walls, with literature supporting their involvement in reinforcing the blood vessel barrier in coordination with ECs, contributing to some extent to the prevention of vascular leakage. Pericytes also contribute to the accumulation of cancer stem cells within the tumor microenvironment, and to tumor invasion and metastasis through their differentiation into stromal fibroblasts [66,67].

Various infiltrating immune cells reveal diverse and critical roles in instigating tumor development, that are essentially related to inflammatory processes conducted by a multitude of signaling molecules secreted by the tumor-promoting inflammatory cells [13]. The list of such signaling molecules includes the tumor growth factor EGF, the angiogenic growth factor VEGF, chemokines and cytokines involved in intensifying the inflammatory sate. The expression of these factors reflects the contribution of inflammatory cells in enabling cancer cell proliferation and in supporting tumor vascularization. Infiltrating immune cells may additionally produce proangiogenic and pro-invasive matrix degrading enzymes that will facilitate tissue invasion and support metastatic dissemination [5]. Distinct subclasses of macrophages, neutrophils and myeloid progenitors may be subverted and recruited to support tumoral development, serving as the main sources of the epithelial, stromal and angiogenic growth factors and matrix-remodeling enzymes. The recruitment and activation of such tumor-promoting macrophages and neutrophils can be facilitated by subclasses of B and T lymphocytes. Other immune cell types will display contrasting tumor-antagonizing effects that contradict the activity of the tumor-promoting immune cells, leading to conflicting inflammatory responses that will shape tumor progression [5].

Tumor phenotypes can also be enhanced by reprogramed variants of normal-derived fibroblastic cells found within the tumor microenvironment [13]. These mesenchymal cells referred to as cancer associated fibroblasts (CAFs) display the migratory and contractile properties of myofibroblasts, being characterized by the secretion of collagen, cytokines and chemokines into the tumoral stroma compartment [68]. The secretion of such factors allows the modulation of the microenvironment, through crosslinking of the collagen network capable of increasing ECM stiffness [69]. In tumoral hypoxia scenarios, CAFs generate collagen reticulation that enhances cancer cell contractility, motility and invasiveness [68]. The wide variety of ECM components secreted by CAFs supports the contribution of these cells in cancer cell proliferation, invasion and metastasis.

The origin of the several tumor-associated stromal cells has been linked to various sources, including the surrounding normal tissues that may provide recruited preexisting stromal cells to the developing tumoral environment, and additionally supply local progenitor/stem cells capable of differentiating into the stromal cells. Mesenchymal stem cells arising from the bone-marrow can also serve as an alternative source of stromal cell types, being dispatched to the tumoral progression sites, where they may differentiate into the various stromal components of the tumor microenvironment [5].

The large variety of cellular types found at various stages of differentiation within the stromal compartment lead to a spatial and temporal heterogeneity that raises the difficulty in clarifying

the full extent of microenvironment contributions to the tumoral progression [70]. The additional intra-tumoral heterogeneity defined by the existence of different cancer-cell subpopulations also elevates the challenge of exploring the tumor microenvironment [71], that will consequently exhibit various sections characterized by distinct degrees of differentiation, proliferation, vascularization, dissemination and inflammation. The complexity of the microenvironment can be further extended when taking into consideration the dynamic and reciprocal interactions established between the tumoral cells and the supporting stromal cells, which are also subject to evolve during the course of tumoral development [5].

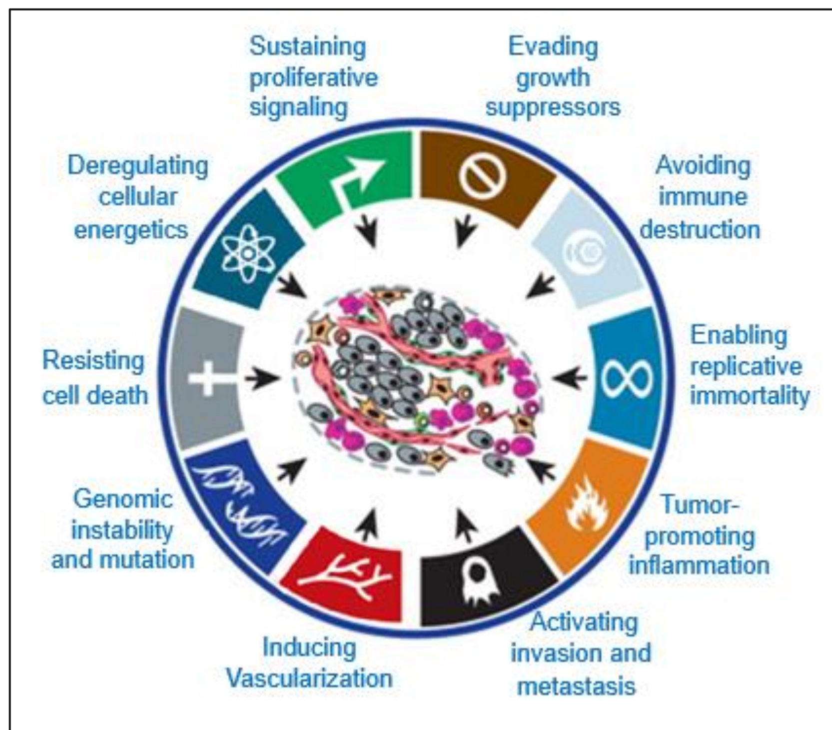


Figure 1 The hallmarks of cancer. The expression of these hallmarks and their intertwined relation with the tumor microenvironment will contribute to tumoral development. (Figure adapted from [5,125]).

The cancer hallmarks (**Figure 1**) have shaped the research focused on cancer biology, encapsulating crucial properties required for tumoral formation and progression. The expression of these interconnected and often overlapping features may differ throughout tumoral development and according to the tumor type. Cancer should be contemplated as a multitude of diseases, with each type capable of exploiting the several hallmarks to various degrees. The description of the cancer hallmarks provided clearer insights regarding the mechanisms of these diseases, that have not been translated into improvements in the prevention capacity, which still remains beyond medical reach. A more solidified comprehension of the hallmarks and of the several aspects surrounding the tumor microenvironment may contribute to improvements in this regard. These considerations further reinforce the relevance of refining the currently available treatments or in investing in the development of more effective anti-cancer therapies.

Current treatments

The type of cancer and the person's overall health are taken into consideration during the selection of the most adequate treatment strategy. The most common approaches include surgery, chemotherapy and radiotherapy [2].

Surgery is generally performed to remove malignant tumors, allowing the determination of the exact tumor size and the extent of invasion to nearby structures or lymph nodes, which may also be removed to reduce or prevent the disease's spread. Chemotherapy involves the use of several therapeutic drugs to eliminate or slow the tumor development, being particularly useful when dealing with metastatic cancer cells. Most of these anticancer agents induce their therapeutic effect by entering cancer cells and binding to DNA [72], distorting its structure and blocking DNA transcription and replication, leading to a network of chemical signals that culminate in apoptotic cell death [73]. Radiotherapy consists on the delivery of ionizing radiation, including X-rays, gamma rays, or other high-energy particles to the affected area, while trying to minimize the damage caused to the surrounding healthy organs. Ionizing radiation is capable of inducing chemical and biomolecular damage that ultimately compromises cell physiology, by either directly interacting with vital biological macromolecules such as DNA and RNA, or by indirect action, through the production of very reactive free radicals capable of causing double-stranded breaks in nuclear and mitochondrial DNA [74]. The delivery of radiation can be done externally, or through the placement of radioactive sources in the vicinity of the cancer cells, a treatment known as brachytherapy, where a high dose of radiation is applied in a localized manner [75].

The various treatment strategies have potential risks and side effects that should be considered when selecting the most appropriate strategy. Chemotherapy side effects include, for example, increased susceptibility to infection, abdominal pain, nausea and loss of hair [76]. Radiation therapy can cause skin irritation, depletion of blood cell counts, tiredness and endocrine dysfunction [75]. The adverse effects related to the high toxicity and relatively low tumor specificity displayed by these modalities [75,76] justify, yet again, the importance of developing new therapy technologies to improve these treatments while exploring alternative therapeutic techniques.

In addition to the conventional approaches, a multitude of other treatment strategies have also been studied and employed, namely angiogenesis inhibitors which correspond to medications used to constrain the formation of new blood vessels required for tumoral growth; cryosurgery that employs the use of low temperatures to eliminate premalignant and tumoral cells, and stem cell transplants, which involve the infusion of stem cells into the body following bone marrow damage caused by high doses of chemotherapeutic agents or radiation [2,3]. Another alternative technique that has been gaining increasing recognition is photodynamic therapy (PDT), that can

be seen has an effective treatment option for localized tumors. PDT involves the use of laser energy of a specific wavelength over tumoral tissues that have been exposed to a photosensitizing agent, capable of triggering a series of photobiological and photochemical processes that could culminate in platelet aggregation, clotting, occlusion of tumor microvasculature and in direct cancer cell damage [77]. The selectivity of this treatment can be improved through the design of targeted photosensitizers capable of identifying tumoral tissues and specifically accumulating in those sites. More than one type of modality is often employed to maximize the effectiveness of the treatment.

Metal-based chemotherapeutic agents

There have been several research efforts focused on improving the efficacy of current treatments and on finding new anti-cancer strategies. The development of metal-based chemotherapeutic agents constitutes an example of such research [72,78,79]. Cisplatin (cis-diamminedichloroplatinum(II)) remains well established in modern chemotherapy, being the most widely known metal-based anticancer drug, with the discovery of its antiproliferative activity significantly contributing to the development of the field of medicinal chemistry. Platinum drugs are frequently used to treat several different cancers, including testicular, ovarian, cervical, bladder, lung, neck and head cancers [80,81], and can be applied with other treatment modalities such as radiotherapy, other antitumoral drugs, or new targeted antitumoral agents. Cisplatin, carboplatin and oxaliplatin (**figure 2**) exhibit various applications as chemotherapeutic agents, but their effectiveness has been stalled by the lesser response to some tumor types, that results from acquired or inherent resistance, and by the incapacity to prevent cancer relapse [82,83].

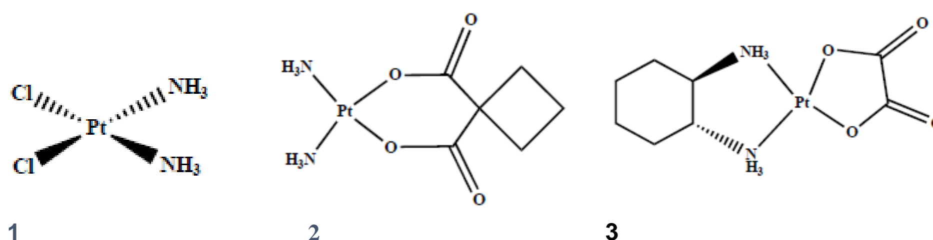


Figure 2 Chemical structure of the platinum chemotherapeutic drugs **1.** Cisplatin, **2.** Carboplatin and **3.** Oxaliplatin (adapted from [122]).

The high toxicity displayed by the currently available platinum anticancer drugs, resulting from their lack of selectivity, constitutes also one of the most critical issues, responsible for several adverse effects, including nephrotoxicity and peripheral neuropathy [84], which has motivated the research for new metal-based drugs for cancer chemotherapy, capable of bypassing the severe toxic effects and induced resistance, while displaying the same or a higher efficiency, revealing in this way a preferable pharmacological profile [78].

Metal complexes of chelating ligands have also been gaining increasing attention due to their physiochemical properties and diversified biologic activities, displaying different mechanisms of action to target various tumoral cells. Some metal complexes reported as potential chemotherapeutic agents are capable of binding to DNA and cleave it under physiological environment, while others act by oxidative mechanisms involving reactive oxygen species, modulated by the ligand responsible for changes in geometry, charge and redox potential around the metal, affecting various organelles [85].

Extensive *in vitro* and *in vivo* investigations have been conducted on several transition metal complexes, to explore their effectiveness as cancer chemotherapeutic agents [86]. Medicinal inorganic chemistry allows the production of new therapeutic agents with unique mechanisms of action, taking into account the wide range of thermodynamic and kinetic characteristics, oxidation states and ligand designs. Understanding the thermodynamics (metal–ligand bond strengths, redox potentials) and kinetics (ligand exchange rates) of reactions of the metal and ligands, that form the therapeutic agent, under biological conditions is a crucial step in medicinal chemistry. In terms of mechanisms of action (MoA), the metal could reveal different functional and structural roles, including the capacity to carry or protect active ligands that are delivered *in vivo*, act as a catalyst responsible for the production of reactive oxygen species or act as a photosensitizer. The choice of the appropriate ligands is also crucial for the determination of the biological features of the metal complexes, considering their capacity to affect their thermodynamic and kinetic stability, solubility and lipophilicity, all of which can be modified by altering the ligand architecture [87]. As such, research in the field of medicinal chemistry has been focused on a multitude of coordination compounds formed by various metals, namely copper, ruthenium, titanium, gold and cobalt [72,86,88]. Exploring alternative chelating systems, including the tridentate ligands referred to as scorpionates, will also contribute to further advances in this field.

Scorpionate metal complexes

The family of scorpionates constitutes an example of metal complexes extensively employed in coordination, organometallic, and bioinorganic chemistry, owing to their molecular design versatility. Scorpionates are accessible coordination compounds usually formed by tridentate ligands that bind the metal with two in-plane donors, with the third donor site reaching over the plane formed by the metal and the other two donor atoms, resulting in an overall scorpion-like conformation (**figure 3**), a distinct feature responsible for the designation of these molecules.

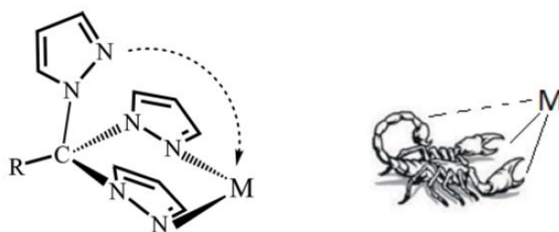


Figure 3 Depiction of the scorpionate ligand tris(pyrazol-1-yl)methane binding to a metal (**M**), exhibiting a conformation similar to that of a scorpion (figure from [89]).

There are multiple classes of ligands that will lead to different types of scorpionates, and those ligands can be modified to fine tune the scorpionate properties and obtain new families of compounds with different activities and effects, which are also dependent on the metal connected to the molecule. Interestingly, the metal and the ligand are sometimes inert when isolated but acquire new activities and effects when connected in a molecular complex. Multiple advances focused on the coordination chemistry of this type of chelators have been made, but research regarding biomedical applications of scorpionate complexes remains scarce, and most of the contributions of this diverse family of ligands for the development of antitumoral agents has been promoted by complexes of lanthanides, copper, ruthenium, silver and cobalt [89,90].

Several scorpionate metal complexes have revealed potential as *in vitro* cytotoxic agents, through intrinsic photonuclease activity via generation and accumulation of reactive oxygen species, with the ligands themselves manifesting antitumor activity [90]. Various studies have been dedicated to the antiproliferative activity of scorpionates, which have also been explored in other clinical treatment approaches, that involve their use as carbon monoxide releasing molecules and photosensitizers, which are equally relevant areas in cancer therapy research [90].

Scorpionate complexes as carbon monoxide-releasing molecules

The development of carbon monoxide (CO) releasing molecules (CORMs) capable of delivering CO to the targeted cells constitutes a strategy to fully explore the therapeutic effects of this molecule. Indeed, CO is recognized as a therapeutic molecule with reported anti-hypertensive, anti-inflammatory and anti-oxidant effects [91], and depending on the concentration, it can be explored as a cytoprotective or cytotoxic agent. The toxicity of CO is manifested in high enough concentrations, where it is capable to bind to hemoglobin, and prevent it from carrying oxygen (O_2) in the body [92], reason why it is crucial to devise CORMs that are able to deliver CO to the target locations without exposing the surrounding tissues to the toxic effects of these molecules. Most of the CORMs used in research and in studies for clinical applications are essentially based on transition metal complexes [91,93]. The release of CO from these complexes can be triggered by several processes, including enzymatic cleavage [94] and light irradiation [95]. One example

of a study focused on CORMs explored the potential of a manganese scorpionate complex (**figure 4**) capable of releasing CO molecules upon irradiation with UV light, acting as a photoinduced CORM [96,97].

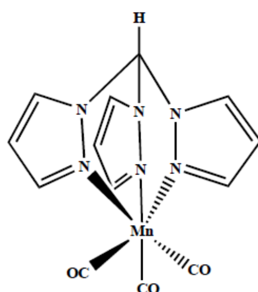


Figure 4 Chemical structure of the manganese scorpionate complex from [96] (adapted from [122]).

Cell viability tests revealed a significant photoinduced cytotoxicity of this manganese scorpionate towards the human colon cancer cells (HT29), comparable to that of 5-fluorouracil, an established clinical agent. The results obtained motivated the study of alternative delivery systems to tumor cells that involve the conjugation of manganese scorpionates to nanoplatforms [98,99], such as silicon dioxide nanoparticles, forming constructs that have the ability to release CO molecules through UV irradiation. Another study focusing on several manganese scorpionates with different ligands (**figure 5**) used UV/Vis spectroscopy-based myoglobin assays to investigate the utility of these complexes as photoinduced CORMs, where some revealed stability in aqueous solution in the dark and the fast release of CO molecules after UV excitation, while others released CO without photoactivation, displaying some instability in solution [100]. These studies highlighted nonetheless the capacity of manganese scorpionates to become efficient photoactivable CORMs, with further evaluations being required to fully explore the potential of these complexes as anticancer agents.

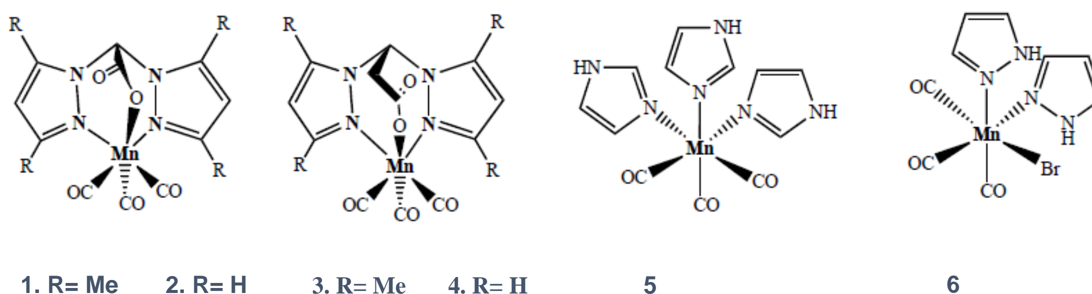
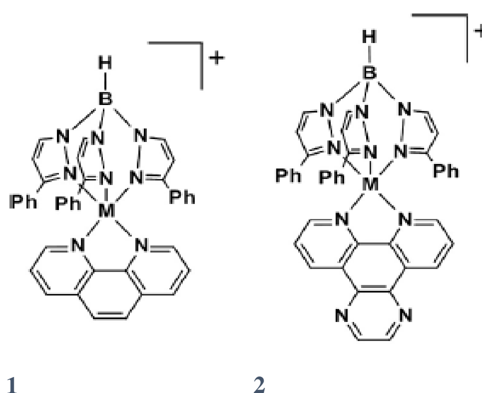


Figure 5 Chemical structures of the. manganese scorpionate complexes from [100]. **1**, **2** and **3** correspond to the complexes that were stable in aqueous solution in the absence of light (adapted from [122]).

Scorpionate complexes as photosensitizers

The use of scorpionate complexes in the field of photodynamic therapy has also been studied, in which they could act as targeted photosensitizers capable of identifying and accumulating in

tumoral tissues, where they could exert their anticancer properties. Several scorpionates containing zinc, nickel, copper and cobalt (**figure 6**) have been analyzed in a study that aimed to ascertain the effects of the electronic structure of such complexes in their photoinduced DNA cleavage capacity [101]. The zinc and nickel scorpionates did not exhibit DNA cleavage effects in visible light, while two of the tested scorpionates, one with a copper center and the other containing cobalt, revealed a positive DNA cleavage activity following irradiation with UV and visible light, motivated by the production of cytotoxic singlet oxygen species. This serves as an illustrative example that reinforces the relevance of the choice of the metal center and the ligand design in the development of effective photosensitizers for clinical purposes.



1 2

Figure 6 Chemical structures of the scorpionate complexes used in [101], with **M= Co, Cu Ni or Zn**, (adapted from [90]). The structures **2, M=Co** and **2**, **M=Cu** correspond to the complexes that revealed a superior DNA cleavage activity with UV-A and visible light irradiation.

Scorpionate complexes as cytotoxic agents

Copper complexes

It was previously mentioned that the cytotoxic activity of a vast array of scorpionate metal complexes has been assessed in various studies, that exploited the properties of multiple metals for the development of viable alternative chemotherapeutic agents. Copper complexes, for example, have been widely investigated for potential anticancer applications [102]. Endogenous metals like copper may exhibit a higher toxicity towards tumoral cells over normal cells, which is a relevant aspect that should be considered to evade some of the concerns raised by platinum drugs. The capacity of copper to produce reactive oxygen species, cleave DNA and RNA and displace other metals justify the toxicity displayed by this metal. The different response between normal cells and tumoral cells to copper could support the synthesis of copper complexes capable of targeting human cancers that have acquired resistance to platinum agents. In 2017, *in vitro* studies were performed in two new mixed-ligand copper complexes (**figure 7**) towards cultured HepG2 liver carcinoma cells [103].These complexes were capable of inhibiting the growth of the liver cancer cells in a dose dependent manner, with one of them revealing effective cytotoxic

activity against the tested cell line, compared to the conventional drug cisplatin. Moreover, in vitro DNA binding studies indicated that the most effective complex was capable of binding to DNA in a specific mode to damage or interfere with the replication of this molecule, which led to the increased cytotoxicity towards hepatocarcinoma cells.

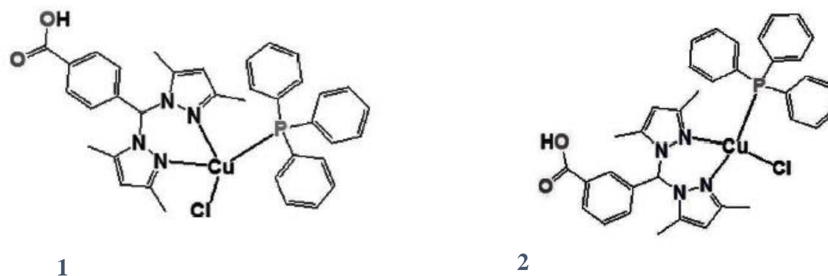


Figure 7 Chemical structures of the mixed-ligand Cu complexes from [103]. **1** corresponds to the complex with superior cytotoxic activity (adapted from [104]).

<u>Ruthenium complexes</u>

Ruthenium complexes have also been studied during the past few years, displaying interesting antiproliferative and antimetastatic properties, with a lower toxicity that could allow them to become suitable alternatives for platinum drugs. Ruthenium exhibits several oxidation states capable of being modified under physiological conditions [104]. Developments in this field resulted in the acquisition of two ruthenium-base complexes, NAMI-A (Imidazolium-trans-tetrachloro(dimethylsulfoxide)imidazole ruthenate(III)) and KP1019 (trans-[tetrachlorobis(1-H-indazole) ruthenate(III)]) (**figure 8**), that reached advanced clinical trials. NAMI-A is capable of interfering with the regulation of the cell cycle and the extracellular matrix, being highly active against metastatic cancer cells [105]. KP1019 reveals instead higher cytotoxicity towards primary tumor cells, causing direct cell apoptosis through the production of reactive oxygen species and the intrinsic mitochondrial pathway [106], exhibiting a particularly higher cytotoxic effect in colorectal cancer which shows some resistance to cisplatin chemotherapy [79].

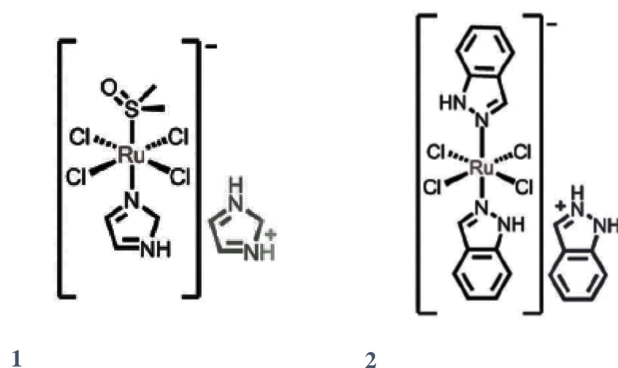


Figure 8 Chemical structures of the ruthenium complexes **1.** NAMI-A and **2.** KP1019 (adapted from [104]).

These two complexes constitute an example of the potential of ruthenium in the development of new chemotherapeutic agents, motivating further research with other complexes, including ruthenium scorpionates. In 2016, one study reported the antitumor capabilities of the complex designated UNICAM-1, [Ru(p-cym)(bis(3,5-dimethylpyrazol-1-yl)methane)Cl]Cl (**figure 9**).

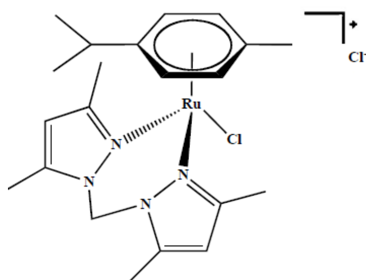


Figure 9 Chemical structure of the organometallic ruthenium (II) complex UNICAM-1 (adapted from [109]).

The cytotoxic effect of UNICAM-1 was evaluated in the human MDA-MB-231 and murine A17 cancer cells and compared with the cytotoxicity of NAMI-A and cisplatin towards the same cells. The selected cell lines served as models of triple negative breast cancer (TNBC), a molecular subtype of breast cancer that does not display common cellular targets for targeted treatment, including estrogen receptors, progesterone receptors and HER2 (human epidermal growth factor 2), restricting the therapeutic options [107]. TNBC is characterized by having the worst negative outcome and prognosis, with an elevated metastatic behavior that justifies the urgency for effective treatment strategies for this disease [108]. In this study, the ruthenium complexes were less effective than cisplatin, but they were nonetheless capable of inhibiting cell proliferation in a dose-dependent manner, with UNICAM-1 being more effective than NAMI-A, leading to apoptosis of TNBC cells. In depth *in vivo* studies of UNICAM-1 against an experimental TNBC model revealed that this complex was capable of suppressing TNBC development through the inhibition of tumor infiltrating regulatory T cells. UNICAM-1 also revealed a low liver toxicity, that was less evident than the one displayed by cisplatin or NAMI-A. The authors attributed the therapeutic efficacy of UNICAM-1 to its unique immunomodulatory action, linked to the capacity

to influence the tumor-host interaction, by triggering immune responses specific to tumoral cells [109]. This example further supported the notion that chemotherapy efficacy can be improved by eliciting anticancer immunosurveillance [110].

Silver complexes

Research concerning the antiproliferative activity of silver-based scorpionate complexes has been conducted to explore the potential of this metal for biological applications. The low toxicity displayed by silver constitutes an advantage over other metals studied in this field. The anticancer properties of silver complexes result from MoA that differ from the ones shown by platinum agents regarding DNA interaction and mitochondrial membrane targeting, being capable of triggering mitochondria initiated apoptosis, in a similar fashion to what has been reported with gold complexes [111–113]. Relevant properties related to the biological activity of silver complexes, including water solubility, stability and rate of release of silver ions, can be modulated by altering the quantity and type of ligands used in these complexes [114]. One particular study reported the synthesis and the antiproliferative activity of new silver scorpionate complexes (**figure 10)** against the A375 human malignant melanoma cancer cell line [115]. These complexes revealed considerable cytotoxic effects that were directly in line with their values of DNA binding constants, indicating that their antiproliferative activity resulted from DNA interactions.

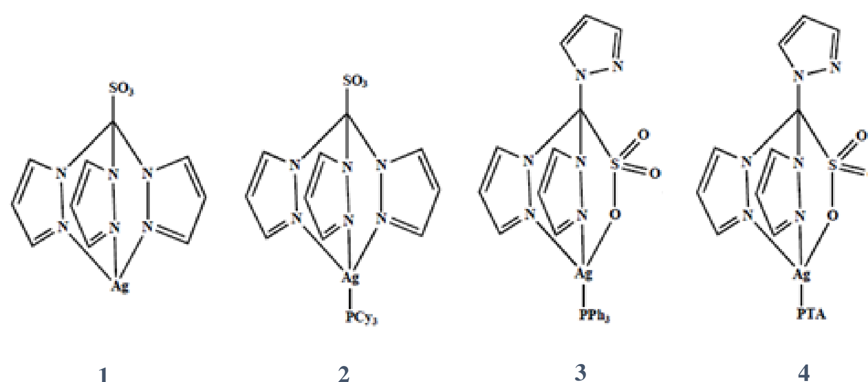


Figure 10 Chemical structures of the silver complexes from [116]. Structures **2** and **3** correspond to the complexes with higher DNA binding constants, and with consequently greater cytotoxicity (adapted from [122]).

Cobalt complexes

The unique redox properties and electronic structures of cobalt complexes justify their potential to be applied as drug delivery devices, enzyme inhibitors and DNA binding and cleavage agents [116]. Some of these cobalt complexes reveal redox-dependent targeting of the tumoral tissues, a feature that contributes to their relevance in anticancer therapy research [89]. A study investigated the antiproliferative activity of three water soluble cobalt scorpionates with tris(pyrazol-1-yl)methane (Tpm) ligands (**figure 11)**, in the human cancer cell lines HepG2 hepatocellular

carcinoma and HCT116 colorectal carcinoma [117]. These complexes revealed moderate cytotoxic effects, leading to a cell viability loss related to an increase in cellular death by apoptosis, but this cytotoxic activity was lower than that induced by cisplatin. In vitro DNA studies also revealed that two of these cobalt scorpionates promoted double-strand plasmid DNA cleavage.

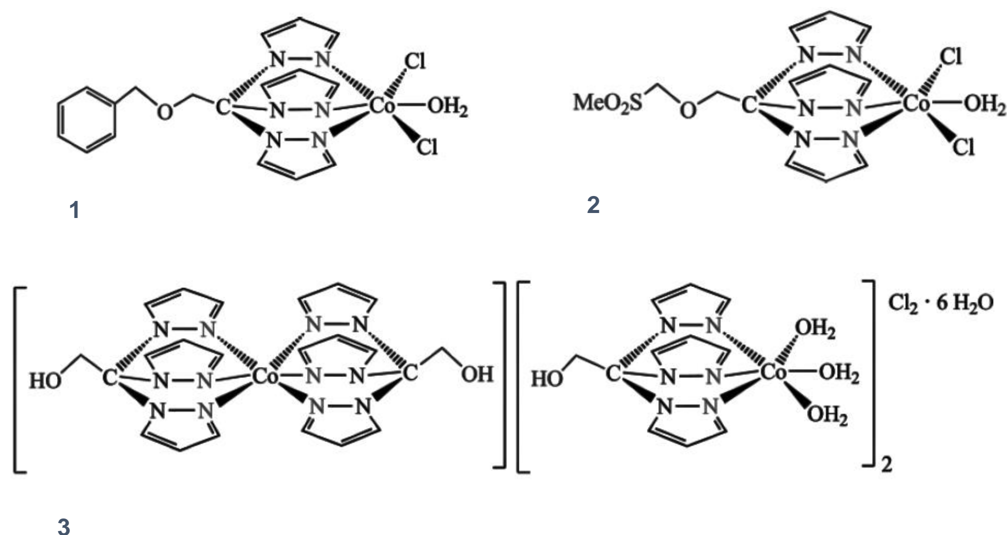


Figure 11 Chemical structures of Cobalt (II) complexes from [117]. Structure **3** corresponds to the complex that displayed higher efficacy concerning cell viability reduction. **1** and **2** indicate the scorpionate complexes capable of causing double-strand plasmid DNA cleavage (adapted from [89]).

Another study tested the *in vitro* cytotoxic activity of a different cobalt scorpionate (**figure 12)**, in the human cancer cell lines MCF-7 breast carcinoma and HCT116, and in one normal human fibroblast cell line [118]. This cobalt complex displayed a higher cytotoxicity in the HCT116 cell line than in the MCF-7 cell line, that was, however, lower than the cytotoxicity exhibited by cisplatin. This scorpionate revealed, nonetheless, a very low toxicity towards the normal human fibroblast cell line, which was considered a promising result, pointing to a certain specificity in terms of cytotoxic activity of this complex towards carcinoma cells.

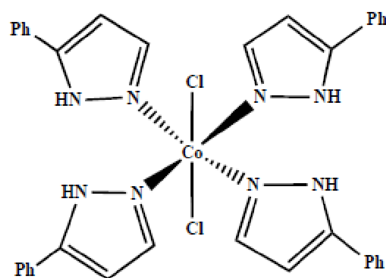


Figure 12 Chemical structure of the Cobalt (II) complex studied in [118] (adapted from [122]).

Aims of the project

Scorpionate metal complexes have revealed, throughout the last few years, a considerable potential to be employed in various biomedical applications. Specifically, the particular properties of transition metal ions with pyrazolyl assisted precursors can be taken into advantage in the production of new potential anticancer agents with several mechanisms of action capable of targeting different cancer cells [102,119].

In particular, two tris(pyrazol-1-yl)methane-type (tpm) scorpionate transition-metal complexes, one containing an iron center ($FeCl_2Tpm$) and the other with a cobalt center ($CoTpm_2(OH)_2$) (**figure 13**), have been synthetized taken into advantage the chemical versatility, high stability and water solubility offered by that ligand, which constitute relevant features that could allow the direct administration of these complexes in several treatment protocols. Similar compounds containing these ligands have shown promising catalytic effects for industrial processes, with others exhibiting antitumoral properties that could be exploited for the development of new chemotherapeutic agents [89], which motivated the antiproliferative and also the antimigration study of these two previously mentioned scorpionate complexes.

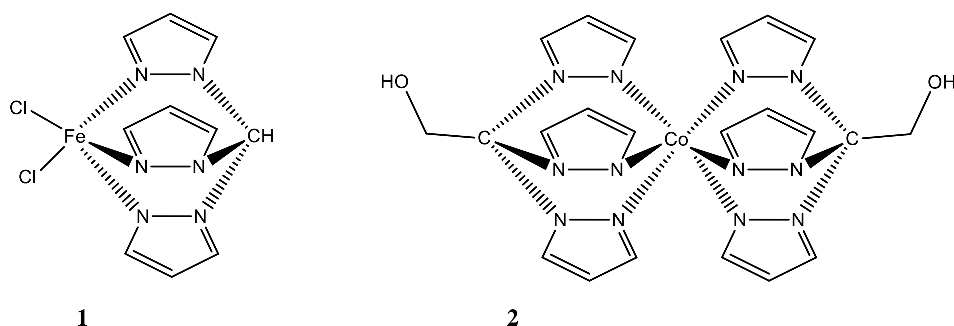


Figure 13 Depiction of the molecular structure of the iron scorpionate **1**. ($FeCl_2Tpm$), and the cobalt scorpionate **2**. ($CoTpm_2(OH)_2$).

This project aimed to analyze *in vitro* anti-tumoral properties of the iron scorpionate ($FeCl_2Tpm$), (S6), and the cobalt scorpionate ($CoTpm_2(OH)_2$), (S7), through the evaluation of their effects on

cell proliferation and cell migration. In particular, this study involved the use of MTS colorimetric assays and scratch assays to assess the effects of S6 and S7 on the viability and migration, respectively, of two tumoral cell lines, HCT116 (human colorectal carcinoma cells) and B16 (cells from murine melanoma) that served as representative models of colon tumor and melanoma. Colorectal carcinoma constitutes one of the four most common cancer types, being also one of the most lethal, having led to approximately 50630 deaths in the USA in 2018 [4]. The death rate has been declining, however, throughout the last years, as a result of the increased screening that may help to avert the occurrence of metastatic colorectal cancer. Invasive melanomas correspond to about 1% of all skin cancer cases, being, nevertheless, responsible for most of the skin cancer related deaths, having resulted in the loss of approximately 9320 lives in the USA in 2018 [4]. The conventional chemotherapeutic approaches often display a lower efficacy towards these two types of cancer, which reinforces the relevance of exploring other metal complexes for the acquisition of newer and more effective treatments.

A non-tumoral cell line designated as HaCaT (human spontaneously transformed keratinocytes), was additionally selected to assess the effects of S6 and S7 over normal cells, to verify if these complexes could reveal more specificity towards cancer derived cell lines. Samples of cells exposed to S6 and S7 were additionally prepared and collected, with the purpose of being submitted to metabolomic analyses, that could identify metabolite profiles altered by these complexes, and subsequently provide further insights regarding their metabolic effects and mechanisms of action.

Materials and Methods

Reagents

Cell culture mediums (McCoy's 5A and DMEM), FBS (fetal bovine serum), trypsin (0.25%), penicillin-streptomycin solution, and DMSO (Dimethyl sulfoxide) were purchased from Sigma-Aldrich. D-Glucose was acquired from AppliChem. CellTiter 96®AQueous Non-Radioactive Cell Proliferation Assay (used for the cellular viability assays) was purchased from Promega.

Preparation of S6 and S7 solutions

The scorpionates S6 ($FeCl_2Tpm$) and S7 ($CoTpm_2(OH)_2$) (**Figure 13**) were prepared in Centro de Química Estrutural, IST (Instituto Superior Técnico, University of Lisbon) as described in the literature [117,120], and were stored in the form of refined powder at room temperature. The purity of each compound was determined by RP-HPLC (reverse-phase high-performance liquid chromatography) analysis (**Figure 16**). HPLC analyses were performed in a modular HPLC system composed of a Varian ProStar 410 autosampler, two 210-LC chromatography pumps and a ProStrar 325 UV detector (Varian, Inc., Palo Alto, CA, USA). Data acquisition and processing were performed using Varian MS Control 6.9.6 software. Samples were prepared in water from concentrated solutions and contained 1 mM of test compound and 0.5 mM of internal standard (4-hydroxytoluene). Samples (5 µL) were injected onto the column via a Rheodyne injector (Rheodyne LLC, IDEX Corp., Lake Forest, IL, USA) with a 100 µL loop in the µL pickup injection mode. Separations were conducted at room temperature, using a ThermoFisher Scientific BDS Hypersil C18 (250 mm x 4.6 mm, 5 µm) reversed phase column and a 1 mL/min flow rate. The mobile phase consisted of 0.1% (v/v) formic acid in water (Eluent A) and acetonitrile (Eluent B). The following elution gradient was used: 0-2 min. isocratic 5% B, 2-22 min. linear gradient to 100% B, 22-28 min. isocratic 100% B, 28-30 min. linear gradient to 5% B, 30-32 min. isocratic 5% B. Chromatograms were recorded at 280 nm.

HCT116, B16 and HaCaT cell cultures

Laboratory activities regarding cell manipulation were performed in a culture room equipped with laminar flow chambers that ensured the sterile conditions required for animal cell cultures. These activities involved the manipulation of different cell lines: HCT116, B16 and HaCaT (**Figure 14**), which were used in several *in vitro* assays that allowed the assessment of the cytotoxicity and cell migration effects of S6 and S7.

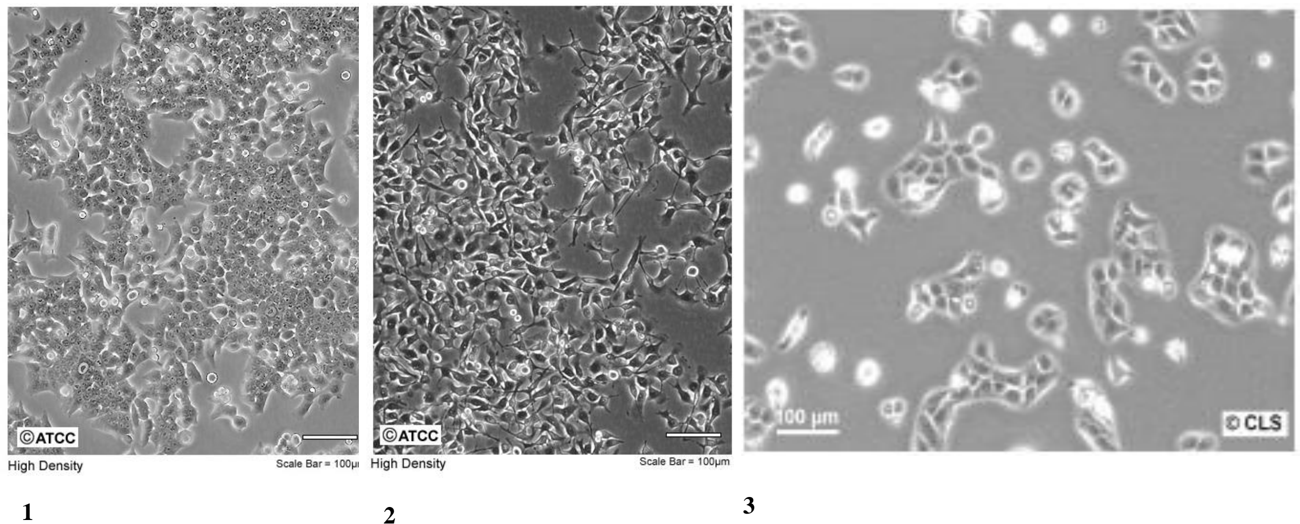


Figure 14 Images from ATTC (American Type Culture Collection) and CLS (Cell-Line-Service) of **1**. HCT116, **2**. B16 and **3**. HaCaT cell cultures.

Cells were seeded in appropriate cell culture t-flasks containing the recommended culture medium (McCoy's 5A for HCT116 cells and DMEM supplemented with 4,5 g/L D-(+)-Glucose (DMEM High Glucose) for B16 and HaCaT cells) with 10% FBS, and incubated at 37 ºC in a humidified atmosphere with 5% CO_2. Cell passages were performed by Trypsin/EDTA 0.25% incubation for 5 min., after which the cells were centrifuged at 200 $\times g$ for 5 min., resuspended in the recommended culture medium. These cell passages were performed every 3 to 4 days, depending on cell confluence. Cell counting was performed using a hemocytometer, and their viability was assessed through trypan blue staining (that stains dead cells with a distinct dark blue coloration, leaving the viable cells transparent).

Cell thawing and freezing

The cell lines used were stored in cryovials in liquid nitrogen (N_2) containers. Cell thawing was conducted by removing the cryovials from those containers and placing them in a water bath at 37 ºC. When almost thawed, the cells were transferred to tubes containing the recommended culture medium. The cells were then centrifuged, after which they were counted and plated in t-flasks (at an approximate density of 2.0×10^4 cells/cm^2). After following the recommended cell thawing procedures, the cells cultivated in the t-flasks with the respective culture medium and 10% FBS were incubated at 37 ºC in a humidified atmosphere with 5% CO_2.

The replenishment of the stocks of the cell lines used was performed by cell freezing, during cell passage, where approximately 2.0×10^6 cells in 1 mL solutions of the recommended culture medium with 10% FBS and 5 % DMSO were placed in cryovials, that were afterwards stored in a cryo-container at -80 ºC, which ensured a 1°C/min. cooling rate, recommended for an effective cell cryopreservation. The cryovials were later moved to the liquid N_2 containers.

Cytotoxicity assays

The HCT116, B16 and HaCaT cells were seeded into 96 well-plates at a density of 3.0×10^4 cells/cm^2 with the respective recommended culture medium with 10% FBS. After 24h, the culture medium was removed from the wells and replaced with 200 µL of complete culture medium supplemented with either S6 or S7, tested at different concentrations ((10-1000 µM) and (3.9-500 µM), respectively). The negative control consisted in cells cultured in complete culture medium with FBS, the solvent control corresponded to culture medium with sterile water and the positive control consisted of culture medium supplemented with DMSO (capable of inducing cellular death). After an incubation period of 48h, the cytotoxicity of S6 and S7 was assessed through MTS assays. MTS is a tetrazolium compound (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) capable of being reduced by metabolically active and viable cells, generating a formazan product (**Figure 15**) soluble in tissue culture medium. The quantity of formazan product formed can be measured through absorbance, and increases directly with the number of viable cells in culture [121].

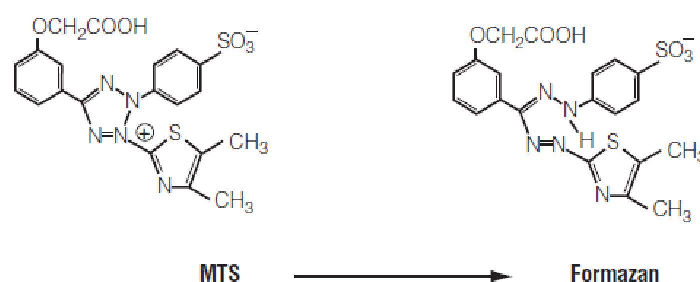


Figure 15 Structures of the MTS tetrazolium salt and its formazan product (figure from [122]).

In the MTS assay, the cells were washed with PBS (phosphate-buffered saline) and subsequently incubated in a solution formed by 100 µL of DMEM with 10% FBS and 20 µL of MTS for 2h (MTS solution). The absorbance of the cells in the 96 well plates was afterwards measured at 690 nm and 490 nm using a spectrophotometer (SPECTROstar Omega Absorbance Microplate Reader).

At least two independent experiments were performed, using 4 replicates for each concentration tested. The results were expressed as percentage based on the solvent control, which in this assay was considered to correspond to 100% of cellular viability / proliferation.

Scratch assays

The evaluation of the effects of S6 and S7 on the migration of HCT116, B16 and HaCaT cells was performed through scratch assays, where these cells were cultivated into 24-well plates at a

density of 6.0×10^4 cells/cm^2 with the recommended culture medium with 10% FBS. When the cells revealed a confluence of approximately 80%, scratches of about 0.5 mm in width were performed on the cell monolayer using a sterile 200 µL pipette tip. After performing the scratch, the cells were washed with PBS and afterwards maintained in a final volume of 500 µL of recommended culture medium without FBS, supplemented with either S6 or S7. In these assays, S6 and S7 were added in concentrations that had not previously manifested cytotoxicity. As negative control, culture medium without FBS was used, while the positive control consisted of culture medium containing 2% FBS (a known motogenic factor). In this assay, the negative control translated the natural cell migration. At least two independent experiments were performed, using 3 replicates for each tested condition.

Digital photographs of the scratches were taken at several time points (0, 2h, 16h, 24h, 40h, 48h and 72h post-scratch) at an amplification of 4× on an Olympus CK30 microscope. The assessment of cellular migration was done through image analysis with the Motic Images Version 3.0 software, where the area of the scratches was measured to calculate scratch closure, given as the percentage of occupied area at each time point, relative to the initial area of the scratch.

Statistical analysis

All data were obtained from at least two independent experiments and were expressed as average ± standard deviation (SD). GraphPad Prism v7.04 software was used to obtain the half maximal inhibitory concentration, IC_{50}, and perform statistical analysis. The two-way ANOVA with multiple comparisons test was used for the *in vitro* cytotoxicity and scratch assays. P-values were represented for statistically significant results (with *$p < 0.1$, **$p < 0.01$, and ***$p < 0.001$).

Preparation of samples for Metabolomic analyses

HCT116, B16 and HaCaT cells were cultivated into t-25 flasks at a density of 2.0×10^4 cells/cm^2 with the recommended culture medium with 10% FBS. After approximately 24 hours, the culture medium was removed from the flasks and replaced with 3 ml of recommended culture medium without FBS, supplemented with 1% Pen-Strep and either S6 or S7, using the same concentrations that were tested in the scratch assays. The negative control consisted of cells maintained in culture medium without FBS. The cells exposed to these conditions during approximately 48h were afterwards removed from the flasks, with trypsin, and transferred to appropriate tubes where they were washed with PBS and centrifuged at 400 ×g for 5 min. After removing the supernatant, the samples of the cells were then stored at -80ºC, where they remained until further use. These samples were later sent to the laboratory of Centro de Química Estrutural, IST, where the metabolomic analyses were performed. For informative purposes, a concise description of the process employed to perform such analyses is provided in the following section.

Metabolomic analyses

The collected cell samples were washed with 500 µL of cold water, and centrifuged at 14 000 ×g for 10 min. at 4 °C. After discarding the supernatant, the cells were resuspended in 500 µL of cold extraction solvent containing water, methanol and acetonitrile. The tubes containing the cells were then transferred to liquid nitrogen for 3 min. after which they were moved to -20 °C to thaw the samples. Following three repetitions of this process of freezing and thawing, the samples were centrifuged at 14 000 ×g for 15 min. at 4 °C, and the supernatant containing the extracted metabolites was afterwards collected into appropriate vials, that were placed in the Elute UPLC (ultra-performance liquid chromatography) (Bruker Daltoniks) interfaced in-line with the mass spectrometer Impact II QqTOF MS (Bruker Daltoniks) equipped with an electrospray source. The metabolites were separated by chromatography based on their chemical properties, being afterwards ionized and discriminated via mass spectrometry according to their molecular mass to charge ratio. The acquired mass spectrometry data were processed with the software XCMS. An untargeted metabolomics approach was employed to provide a comprehensive analysis of all the metabolites that were significantly altered in the samples exposed to S6 and S7 in regards to the control samples, with the additional identification of the altered metabolic pathways associated to those metabolites.

Results

Characterization of S6 and S7

Test compound purity was assessed by RP-HPLC analysis. The chromatograms of each compound prepared in water showed only one signal, eluting at ca. 18 min, compatible with the organic ligand. Retention times are similar for both compounds, despite the difference in formula composition. This arises from the fact that the organic ligands found in S6 and S7 reveal very similar structures, not distinguishable through the use of this chromatographic method. The chromatograms (**Figure 16**) allow, however, to conclude that both compounds were pure and did not suffer degradation when in aqueous solution.

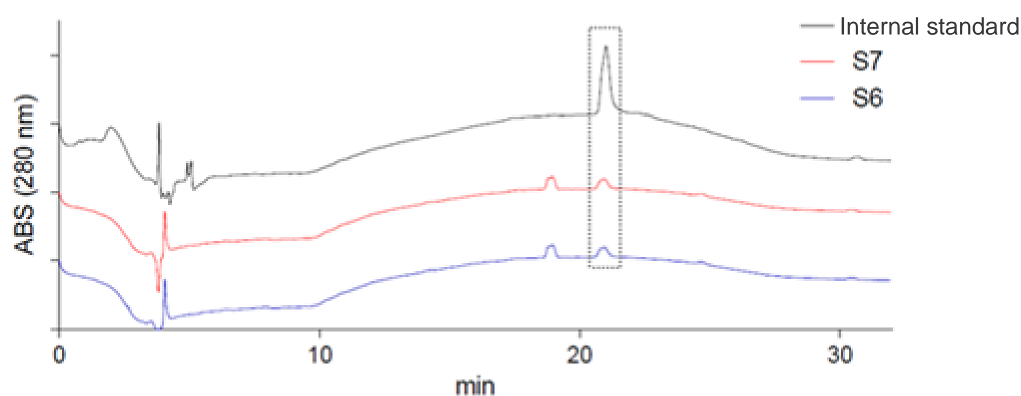


Figure 16 Purity assessment, performed with the HPLC technique, of the solutions of S6 and S7. 4-hydroxytoluene was used as internal standard (IS).

Effects of S6 and S7 on cell proliferation

MTS assays were performed in the HCT116, B16 and HaCaT cells exposed to various concentrations of S6 (10-1000 µM) and S7 (3.9-500 µM) for 48h, to evaluate the cytotoxic potential of these scorpionates (**Figure 17**). The range of concentrations tested was chosen taken into consideration preliminary results obtained with another colorimetric assay (MTT), which revealed to be inappropriate for the cell lines used but that provided nonetheless an indication of the cytotoxic capacity of these complexes.

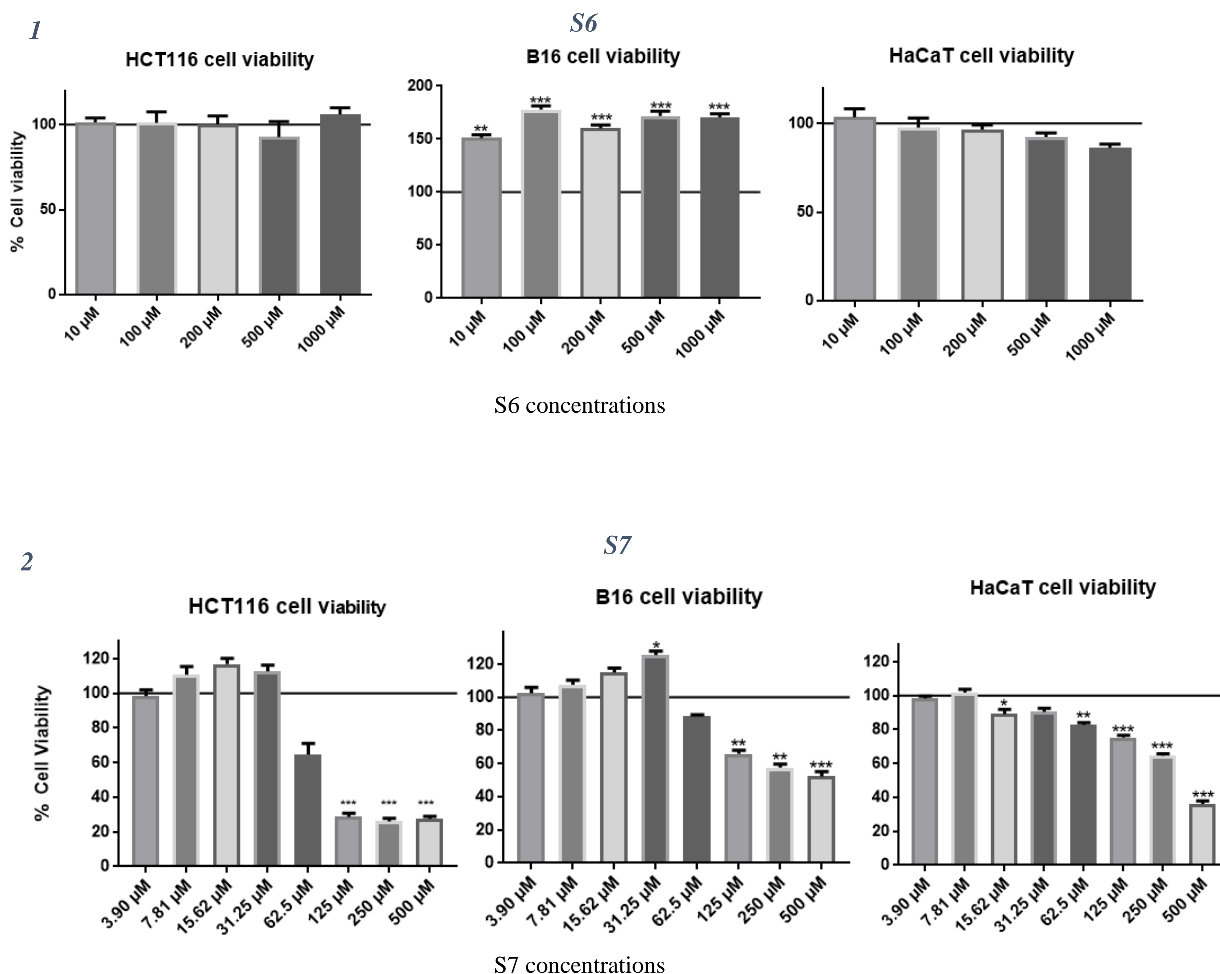


Figure 17 - Graphic representation of the percentage of cell viability after a 48-hour contact with **1**. S6 and **2**. S7, at different concentrations, measured through MTS assay. The symbols * indicate that * $p < 0.1$, ** $p < 0.01$, *** $p < 0.001$.

S6 did not reveal a significant toxicity towards the HCT116 and HaCaT cell lines with the tested conditions, but led to an increased viability in the B16 cell line. By contrast, S7 revealed a higher cytotoxic effect against the tested cell lines, leading to dose-response curves where the half maximal inhibitory concentration (IC_{50}) for each cell line was obtained to provide an indication of the antiproliferative potential of S7. The respective values of IC_{50} for HCT116, B16 and HaCaT were approximately 88 µM, 500 µM and 380 µM.

Effects of S6 and S7 on cell migration

The motogenic effects of S6 and S7 were evaluated through scratch assays, with concentrations that had not previously manifested signs of cytotoxicity. In these assays, S6 was tested with a concentration of 200 µM (**Figure 18**) and S7 was tested at 40 µM (**Figure 19**). These concentrations would allow the assessment of the effect of these complexes on cell migration, while discarting the cytotoxic activity that could otherwise dissimulate their potential antimigration effects.

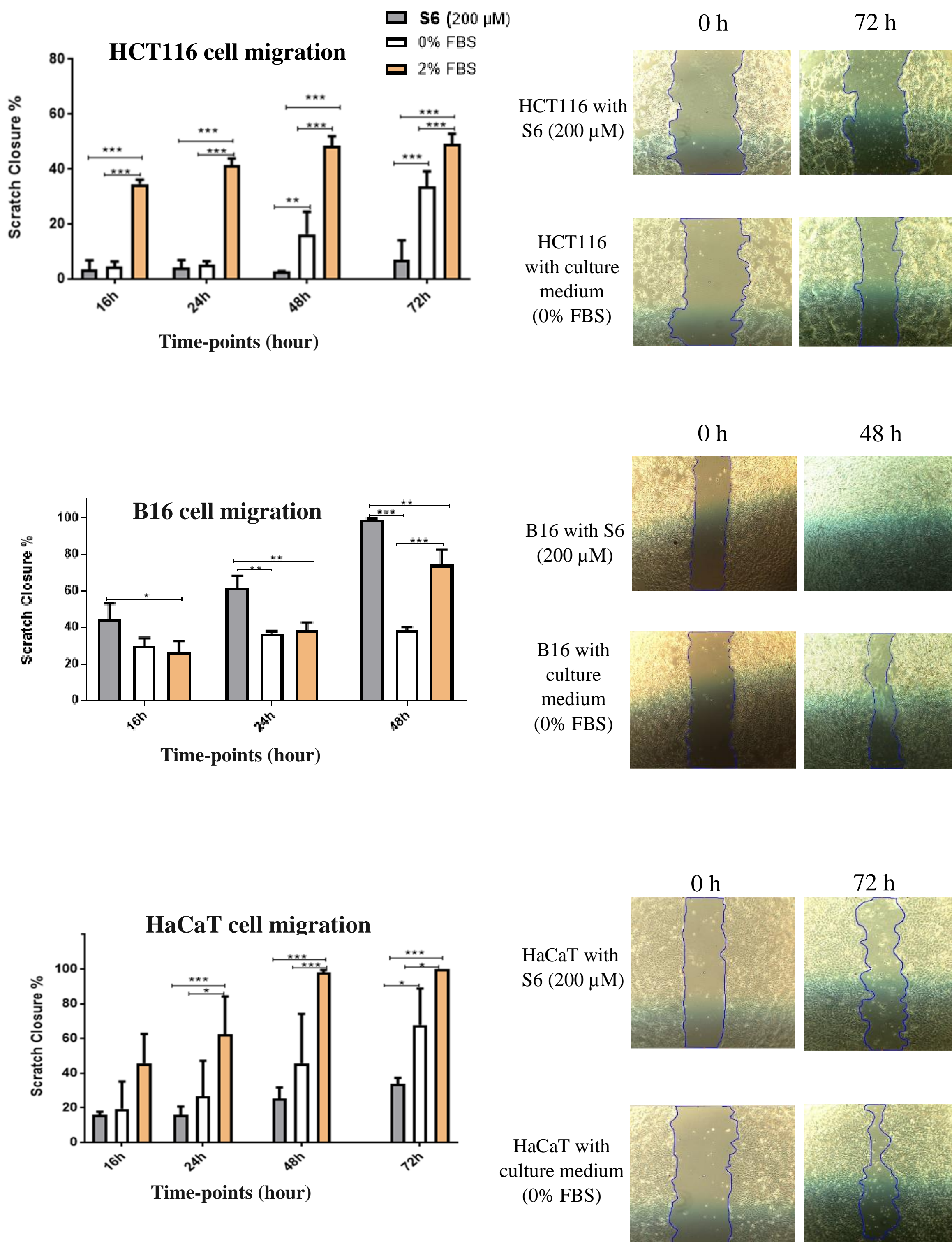


Figure 18 Effects of S6 (200 μM) on cell migration. The scratch closure corresponds to the percentage of occupied area at each time point, relative to the initial area of the scratch. As negative control, culture medium without FBS was used and as positive control, culture media containing 2% FBS was used. (*$p < 0.1$, **$p < 0.01$, and ***$p < 0.001$)

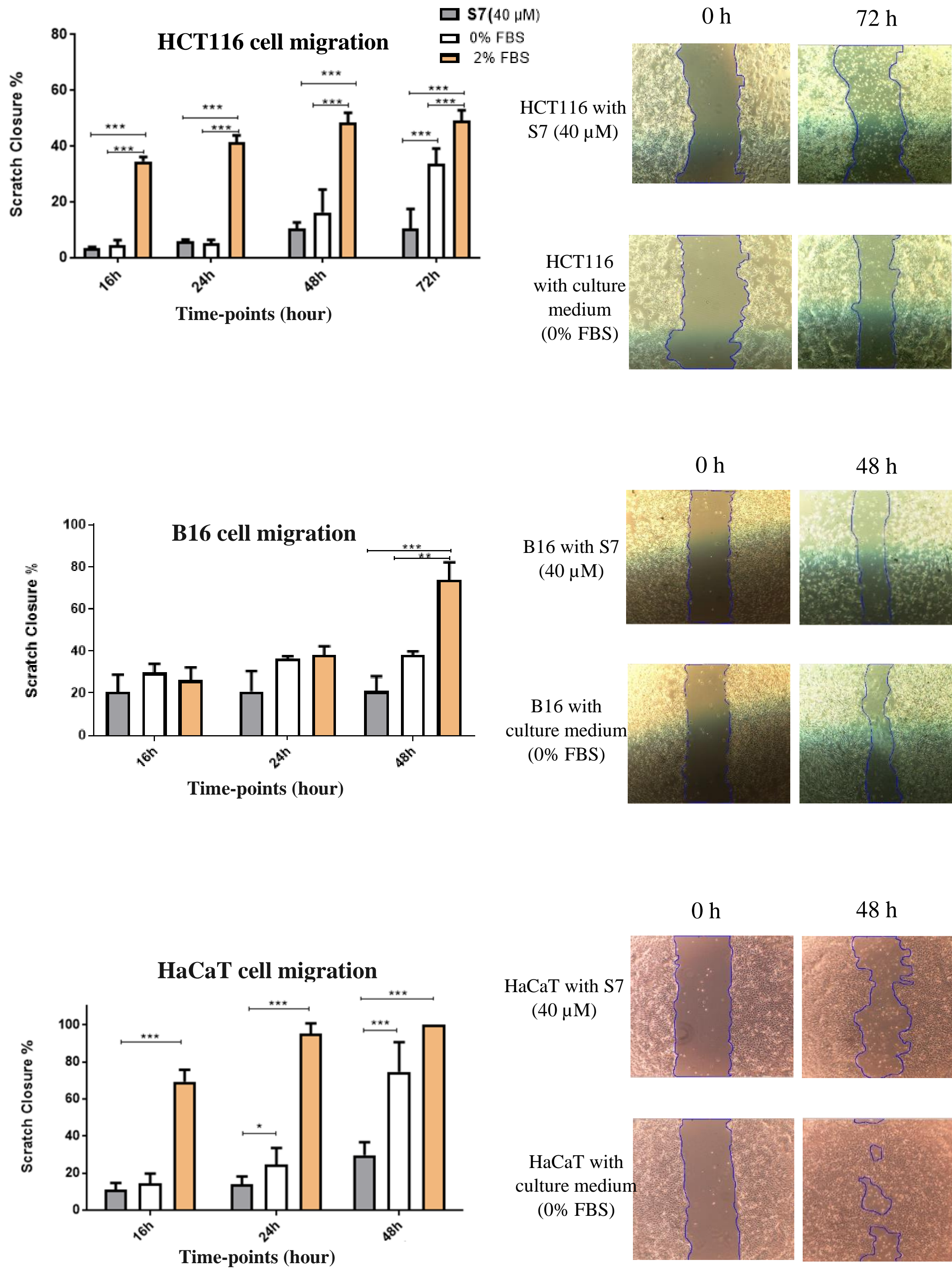


Figure 19 Effects of S7 (40 μM) on cell migration. The scratch closure corresponds to the percentage of occupied area at each time point, relative to the initial area of the scratch. As negative control, culture medium without FBS was used and as positive control, culture media containing 2% FBS was used. (*p < 0.1, **p < 0.01, and ***p < 0.001)

S6 revealed the capacity to delay the migration of the cell lines HCT116 and HaCaT. In the HCT116 cell line, the scratches where S6 was added closed approximately 7% at 72h, while the scratches correspondent to the negative control closed approximately 34%. The scratches performed in the HaCaT cells where S6 was added closed 34%, while the negative control revealed a scratch closure of 68% at 72h. S6 displayed a different effect towards the B16 cell line, promoting a faster scratch closure with these cells, that translates into an increased motogenic effect. At 48h, the scratches treated with S6 revealed an almost full scratch closure, with the scratches correspondent to the negative control displaying approximately 40% of scratch closure.

S7 displayed inhibitory effects in the migration of all the cell lines tested. At 72h, the scratches performed in the HCT116 cells where S7 was added exhibited a closure of 11%, having the scratches of the negative control closed 34%. The scratches performed in the B16 cell line treated with S7 revealed an approximate scratch closure of 20% at 48h, while the scratches corresponding to the negative control exhibited about 40% of scratch closure. At 48h, the scratches made in the HaCaT cells where S7 was supplied closed 30%, with the scratches correspondent to the negative control revealing a closure of 75%.

Metabolomic analyses

The results obtained with the metabolomic analyses are preliminary and have to be confirmed. However, it was possible to observe several metabolite alterations and respective cellular pathways in the samples of cells exposed to S6, in relation to the control samples (**table 2**). These analyses could not identify, however, significant alterations in the metabolic profile of the samples exposed to S7.

Table 2 Altered metabolites and associated cellular pathways identified in the samples exposed to S6.

B16			
Metabolites	Pathway(s) Involved	Dysregulation	Fold Change
adenine	adenine and adenosine salvage I	DOWN	2,8
	purine ribonucleosides degradation to ribose-1-phosphate		
hypoxanthine	adenine and adenosine salvage III	DOWN	1,7
	purine ribonucleosides degradation to ribose-1-phosphate		
D-mannitol	mannitol degradation II	DOWN	2,6
L-thyroxine	thyroid hormone metabolism I (via deiodination)	DOWN	2,6
oleate	oleate biosynthesis II (animals)	DOWN	1,7
3-dehydrosphinganine	ceramide biosynthesis	DOWN	1,7
sphingosine	sphingosine and sphingosine-1-phosphate metabolism	DOWN	1,7
γ-linolenoyl-CoA	γ-linolenate biosynthesis II (animals)	UP	4
AMP	fatty acid activation	UP	2,3
3-oxoadipyl-CoA	3-oxoadipate degradation	UP	3,5
ADP ribose 1",2"-cyclic phosphate	tRNA splicing	UP	1,7
L-γ-glutamylcysteine	glutathione biosynthesis	UP	1,5

HCT116			
Metabolites	Pathway(s) Involved	Dysregulation	Fold Change
α-D-glucose 6-phosphate	GDP-glucose biosynthesis II	DOWN	4
α-D-mannose 1-phosphate	GDP-mannose biosynthesis	DOWN	4
β-D-fructofuranose 1-phosphate	sucrose degradation	DOWN	4
1D-myo-inositol 3-monophosphate	myo-inositol de novo biosynthesis	DOWN	4
1D-myo-inositol 4-monophosphate	D-myo-inositol (1,4,5)-trisphosphate degradation	DOWN	4
myo-inositol	3-phosphoinositide biosynthesis	DOWN	2,9
adenine	S-methyl-5'-thioadenosine degradation	DOWN	2,7
spermine	spermine and spermidine degradation I	DOWN	1,9
α-D-galactose	D-galactose degradation V	DOWN	2,9
β-D-galactose	D-galactose degradation V	DOWN	2,9
L-valine	tRNA charging	DOWN	1,6
2-methylbutanoyl-CoA	isoleucine degradation	DOWN	2
3,3',5-triiodothyronamine	thyronamine and iodothyronamine metabolism	DOWN	3
IMP	adenine and adenosine salvage III	UP	2,2
linoleate	γ-linolenate biosynthesis	UP	1,5
L-lysine	lysine degradation I (saccharopine pathway)	UP	1,5

HacaT			
Metabolites	Pathway(s) Involved	Dysregulation	Fold Change
adenine	S-methyl-5'-thioadenosine degradation	DOWN	2,7
adenosine	adenine and adenosine salvage I	DOWN	1,7
AMP	γ-linolenate biosynthesis	DOWN	4,2
dGMP	purine deoxyribonucleosides salvage	DOWN	4,2
guanine	guanine and guanosine salvage	DOWN	11
S-methyl-5'-thioadenosine	7-(3-amino-3-carboxypropyl)-wyosine biosynthesis	DOWN	21,6
UDP-α-D-galactose	D-galactose degradation V (Leloir pathway)	DOWN	6,3
IMP	adenine and adenosine salvage III	DOWN	5,9
α,α-trehalose	trehalose degradation	DOWN	5,2
L-arginine	citrulline-nitric oxide cycle	DOWN	12,6
β-D-glucose 6-phosphate	GDP-mannose biosynthesis	DOWN	3,7
	pentose phosphate pathway (oxidative branch)		

Discussion

This project was focused on the preliminary assessment of antitumoral properties displayed by two scorpionate complexes that possess the same ligand and a different metal center. This contributed to distinct effects concerning their antiproliferative and antimigration capabilities.

Colorimetric assays have been extensively used to assess the cytotoxic profile of several metal complexes, including those containing scorpionate ligands [122]. In this study, the MTS colorimetric assay was used to analyze the antiproliferative effects of S6 and S7. The similar MTT assay was initially chosen to perform such cytotoxic studies, but the chromogenic product that results from its bio-reduction in viable cells is insoluble, and an additional step to solubilize this product is required before proceeding to the spectrophotometric analysis, which may have contributed to some of the inconsistencies that have been associated to this assay [123]. The more convenient MTS dye was ultimately used to perform all the cytotoxicity assays, where avoiding that additional procedure ensured more consistent results.

The iron scorpionate S6 did not reveal signs of cytotoxicity under the conditions tested in the cell lines HCT116 and HaCaT but led to an increase in the proliferation of the B16 cell line. More

pronounced antiproliferative effects were observed with the cobalt scorpionate S7, which was particularly active in decreasing the viability of the HCT116 cell line (**Figure 17**), a result that is in agreement with previous studies focused on cobalt scorpionate complexes, that tend to display a superior cytotoxic effect towards this colorectal tumor cell line [89,122]. The mechanism associated to the decrease of cell viability caused by exposure to S7 could possibly be similar to that revealed by other cobalt scorpionate complexes described in the literature, whose cytotoxic effects arise from the induction of the apoptotic program. Another cell death mechanism that was recently linked to the antiproliferative effects of a cobalt complex with scorpionate precursors involves the induction of autophagy [124], a process that could also be promoted by S7. Results concerning the cytotoxic effects of S6 and S7 also seem to indicate that the ligand (Tpm) itself does not reveal a significant role in this regard. Therefore, future experiments could include the evaluation of the activation of the apoptotic pathways, namely through the expression levels of p53, bcl2 or bax.

After performing the cytotoxicity assays, the effects of these scorpionate complexes on cell migration were assessed though scratch assays. This *in vitro* experimental technique has provided key contributions to biomedical research, being often employed in studies focused on identifying pharmaceutical agents capable of modulating cell migration. This versatile assay can be applied to a variety of cell types, including tumoral cell lines. The various similarities between chronic wound-healing and cancer, highlighted throughout the scientific literature, have promoted the use of this wound-healing assay in studies dedicated to cancer cell signaling and behavior [125,126]. The quantification method used in the scratch assays performed in this study relied on the recording of wound area changes at different time points. Through this technique, S6 revealed the capacity to delay cell migration in the HCT116 and HaCaT cell lines, manifesting a divergent effect in the B16 cell line, where the migration rate was enhanced (**Figure 18**). The scorpionate S7 was capable of inhibiting the migration of all the cell types used (**Figure 19**), revealing more pronounced effects over the HaCaT cell line.

Several disadvantages have been associated to this procedure, that may decrease the quality and efficacy of the results, with additional efforts being required to minimize such inconveniences. An often-reported issue lies in the accumulation of displaced cells across the scratch edges, where they may potentially reattach and initiate proliferation, biasing the analysis of the gap repair. An effective detection of the migratory process requires the suppression of cell proliferation effects, which was performed by thoroughly washing the scratches with PBS, to avoid the accumulation of displaced cells. Supplying the cells with serum-depleted medium after performing the scratches also contributed to the attenuation of cell proliferation [127]. Another downside of this manual procedure resides in the variation of the size and shape of the obtained scratches, where non-uniform cell-free areas with irregular leading edges may be observed. This can affect the

migration rate measurements, which have also been shown to display sensitivity towards the initial degree of confluence and geometry of the wound [128,129]. A regular monitoring of the cell confluence preceding the scratches, followed by the careful selection of the most centered and straight wounds, may have attenuated to some extent the impact of such issues. Analyzing the gap closure required capturing images of all the scratches at pre-determined time points, a procedure that may be unreliable in the way it involves recapturing the same position and focus on the scratch at several instances, which could not be fully guaranteed. Taking these pictures also requires moving the multi-well plates from the incubator to the camera, which forces the cells to endure cycles of thermal changes, that may also affect cell migration [127]. All these associated limitations may decrease the effectiveness of the scratch assays, but the various strategies employed to diminish such issues supported the acquisition of an important first sign of evidence regarding the migration effects of the tested complexes.

The antimigration effects displayed by S6 towards the HCT116 and HaCaT cell lines might have suggested that this scorpionate complex could still hold some potential regarding its use as an antimetastatic agent. The enhanced proliferation and migration promoted by S6 in the B16 cell line contradict, however, the purpose of an antitumoral agent, which could render this complex obsolete for treatment applications. The murine histological origin of the B16 cell line could also have contributed to the different observations obtained between these cells and the human cell lines HCT116 and HaCaT, and the use of human melanoma cell lines in this analysis may perhaps lead to different results.

The antiproliferative and antimigration effects revealed by S7 in the tested cell lines could favor the use of this complex in future anticancer treatment options. Specifically, HCT116 cells were the most affected by S7 regarding the reduction of viability, that was also capable of interfering with their migration, which could support the particular application of this cobalt complex in the treatment of colorectal tumors, where it could be employed as an agent capable of targeting either primary tumoral cells or metastatic cells.

The *in vitro* cytotoxic and antimigration analyses performed to assess the anti-cancer capabilities of these scorpionate complexes constitute an important step, that serves only as the first in many required to complete the extensive path towards the acquisition of an effective anti-tumoral agent. Additional studies could be performed to obtain a more transparent assessment of the antitumoral activity of these scorpionates, which could include the use of other tumoral and normal (nonmalignant) cell lines, to confirm their specificity towards cancer cells. Other studies could involve cell invasion assays, where the effects of these complexes on the cell capacity to bypass specific barriers may be assessed. Cell cultures provide valuable and informative data regarding the effects of particular molecules over specific cellular mechanisms, but these experimental

models remain far from being capable of replicating the extensive complexity that surrounds tumoral diseases. The assays used in this study allowed the observation of the effects of S6 and S7 over the proliferation and migration of tumoral cells, but their potential interference in other relevant tumoral properties, including vascularization, immune modulation and the microenvironment, remain uncharted. *In vivo* experiments constitute the most effective option to fully assess the anti-tumoral potential of these scorpionate complexes, allowing the exploration of the full extent of their effects over the various cancer hallmarks.

The metabolomic analyses could have provided a better comprehension of the metabolic effects of S6 and S7 in the tested cell lines, through the identification of the altered metabolites and metabolic pathways that would result from the cellular exposure to these metal complexes. These preliminary analyses allowed the identification of various metabolites that were significantly altered in the samples exposed to S6, relatively to the control samples (**table 2**). In the B16 cell line, S6 led to particular deregulations in several pathways involved in the formation of cellular membranes, which may have favored the enhanced proliferation and migration observed in these tumoral cells. S6 interfered with the metabolism of various sugars in the HCT116 cell line, namely inositol and inositol-monophosphate, which are usually involved in several cellular processes, including cell migration. In the HaCaT cell line, S6 promoted several downregulations in the sugar metabolism and in the purine metabolism, and these central and energetic metabolism deregulations might have compromised the migratory capacity of these cells, since much of their metabolism is normally mobilized towards the migratory effects required for their physiological roles in wound healing.

Unfortunately, it was not possible (at the time of writing this report) to establish a more direct connection between the metabolomics data obtained with S6 and the observed effects in the cytotoxic and scratch assays, with a more complete and extensive analysis being required to attain such relation. Additionally, no significant metabolic alterations were found in the samples exposed to S7, which was the most promising complex regarding anti-tumoral properties. The initial washing procedure of the metabolite extraction process might have affected the results, by promoting an early disruption of the cellular membranes, and the subsequent release of a relevant portion of the metabolite content that was discarded and consequently, excluded from this analysis. Avoiding this procedure in future metabolomic analyses may be advised, and could potentially allow the acquisition of more conclusive results. Therefore, future efforts will be performed to reproduce and optimize the assay and obtain more significant data.

Conclusion

The research and development of new and more effective antitumoral agents are crucial steps to obtain more successful anticancer treatments, that could reveal a higher efficacy than the currently available options, while displaying fewer collateral effects. Studies focused on scorpionate metal complexes and their anti-tumoral properties have also provided further contributions and advances to this area.

This project involved the study of an iron and a cobalt scorpionate concerning their *in vitro* cytotoxic and antimigration activities, capable of providing an early indication of their antitumoral potential. These complexes displayed different results in terms of their antiproliferative and antimigration effects, which ultimately, seem to exclude the iron complex S6 from potential treatment options, but could favor the application of the cobalt scorpionate S7 in the treatment of specific tumoral types, including colorectal tumors.

Understanding the anti-tumoral effects of S6 and S7 will require further research capable of clarifying the specific interventions of these scorpionates over the various hallmarks of tumoral development and progression. Employing other assays and *in vivo* models capable of assessing the effects of these complexes on other cellular processes, including cell invasion, constitute relevant strategies for future research. Testing these complexes on other tumoral and normal cell lines could also be an important step to clarify the specificity of these scorpionate complexes. This will provide a better elucidation of their potential as clinical agents for effective anticancer therapy.

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