University of Lisbon

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Involvement of aquaporins in pancreatic cancer and melanoma: effect of metallodrugs on cell migration

Ana Sofia Generoso Coxixo

Dissertation supervised by Professor Maria da Graça Soveral Rodrigues

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C. Pimpão, **A. S. Coxixo**, M. Aureliano, A. Rompel, G. Soveral, "Screening Polyoxometalates as Aquaporin Inhibitors for Cancer Therapeutics" in *FEBS Congress 2019: From Molecules to Living Systems, 6-11 July, Krakow, Poland.*

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Part of this article was included in section 6.2 in Results.

Abstract

Aquaporins (AQPs) are membrane channels widely distributed in living organisms and generally preserved in mammals, including rodents and humans. Due to their involvement in a wide range of human physiological functions and diseases, these membrane proteins have been found to have great potential for pharmacological targeting and drug discovery.

Clinical and preclinical studies show evidence that AQP expression increases in many different types of cancer. In fact, due to their important role in the transcellular movement of water in response to osmotic gradients, as well as cellular glycerol uptake for lipid synthesis or ATP production, AQPs facilitate tumor growth, local infiltration and metastasis, improving cell migration, angiogenesis, cell matrix adhesion and interaction with oncogenes. *In vivo* and *in vitro* studies have shown attractive opportunities for targeted therapy for AQPs.

Pancreatic adenocarcinoma (PA) is one of the most aggressive diseases that develops without symptoms and is usually already in an advanced stage at the time of diagnosis. As is common in epithelial tumors, carcinogenesis develops through the accumulation of mutations and genetic lesions, leading to the activation of oncogenes and inactivation of tumor suppressor genes. In-depth knowledge of the specific cellular and molecular mechanisms of PA development and progression is needed to identify early detection strategies, preventive measures and effective interventions. The poor prognosis of this disease is essentially due to the lack of molecular information on the development of the disease. Established cell lines remain a useful tool for investigating these molecular events.

Melanoma is a type of cancer that begins in melanocytes, also called malignant melanoma or cutaneous melanoma. Most affected cells still produce melanin, so melanoma tumors are usually brown or black. But some melanomas do not produce melanin and can be pink, beige or even white. Melanomas can develop anywhere on the skin of the human body, but they are more likely to start on the trunk (chest and back) in men and on the legs of women. The neck and face are also very common places.

Previous studies by our research group using immunohistochemical techniques have shown that AQP3 and AQP5 are aberrantly expressed in human tissues of pancreatic adenocarcinoma. Throughout this project, we aim to investigate the involvement of AQP3 and AQP5 in cell membrane permeability, cell proliferation and migration of human pancreatic adenocarcinoma (BxPC3) and human melanoma (MNT-1) cells, to validate their potential as biomarkers and potential targets We evaluated the expression of AQPs in a pancreatic adenocarcinoma cell line and in another human melanoma cell, as well as the membrane's

permeability to water and glycerol. We found that AQP3 is the most abundantly expressed isoform in both cell lines. BxPC3 cells have a higher Pf value than Pgly, as do MNT-1 cells, confirming the predominant role of AQP3 (aquaglyceroporin), which was already expected since both aquaporins present (AQP3 and AQP5) are permeable to water and only AQP3 is an aquaglyceroporin, being permeable to glycerol.

Previous studies have demonstrated the diffusion of hydrogen peroxide across the membrane, and therefore the influence of oxidative stress on BxPC3 has been assessed. It is concluded that, with different incubation times, the effect of H_2O_2 does not present significant differences in cell viability. However, cell migration of the same cells is affected in the presence of hydrogen peroxide, which is reduced.

The effect of selected metallodrugs (potential inhibitors of aquaporins) on the viability and migration of cancer cells was determined in pancreatic cell lines and compared with the same effect in melanoma, to determine their selectivity and evaluate their potential for therapy of AQP-dependent pancreatic cancer. Of all those testes, the tugstate compounds showed the best results.

Finally, the effect of the compounds under study on the permeability of pancreatic cells and human melanoma was evaluated. In BxPC3 cells, when compared to the control, the permeability is drastically reduced, mainly with POT-A, being below 20%.

We have identified new potential aquaporin modulators, which may prove to be therapeutic compounds. The presence of POT-A and POT-C leads to reductions in permeability, migration and cell viability. When compared to Auphen, it reveals better results. However, further studies are needed.

Keywords: aquaporin, cell migration, pancreatic cancer, melanoma, inhibitors.

Resumo

As aquaporinas (AQPs) são canais de membrana amplamente distribuídos em organismos vivos e geralmente preservados em mamíferos, incluindo roedores e humanos. Devido ao seu envolvimento em uma ampla gama de funções fisiológicas e doenças humanas, essas proteínas da membrana foram consideradas com grande potencial como alvos terapêuticos e descoberta de novos fármacos.

A identificação e estudo de inibidores aquaporinas, canais proteicos de membrana altamente conservados permeáveis à água, glicerol, peróxido de hidrogénio e outros pequenos solutos como a ureia, desperta cada vez mais interesse. Os últimos anos foram muitos importantes para a compreensão dos diversos papéis das AQPs na saúde e na doença, particularmente, na capacidade das AQPs de permear não apenas água, mas também outras pequenas moléculas ou solutos, permitindo regular várias funções como volume celular, metabolismo energético, migração, adesão e proliferação. Até ao presente foram identificadas 13 isoformas de aquaporinas (AQP0-12) em mamíferos, distribuídas por todos os tecidos. Com base nas características funcionais e estruturais, as aquaporinas classificam-se em aquaporinas ortodoxas, estritamente envolvidas na permeabilidade à água (AQP0-2, AQP4, AQP5, AQP6 e AQP8) e aquagliceroporinas, facilitando o transporte de pequenos solutos não carregados como glicerol e ureia, adicionalmente à água (AQP3, AQP7, AQP9 e AQP10). As peroxiporinas (AQP1, AQP3, AQP5, AQP8 e AQP9) são uma subclasse de aquaporinas permeáveis ao peróxido de hidrogénio. Por fim, e devido à sua localização subcelular, as AQP11 e AQP12 são classificadas como S-aquaporinas, mostrando menor similaridade de sequência com as outras isoformas. A permeabilidade seletiva da AQP12 ainda não é certa. No entanto, existem indicações de que a AQP11 facilita o transporte de água e glicerol.

Estudos clínicos e pré-clínicos mostram evidências de que a expressão de algumas AQPs aumenta em muitos tipos diferentes de cancro. De facto, devido ao seu papel importante no movimento transmembranar da água em resposta a gradientes osmóticos, bem como na captação de glicerol celular para síntese lipídica ou produção de ATP, as AQPs facilitam o crescimento do tumor, a infiltração local e as metástases, aumentando a migração celular, a angiogénese, a adesão da matriz celular e a interação com oncogenes. Estudos *in vivo* e *in vitro* mostraram oportunidades atraentes para a terapia direcionada para AQPs.

O adenocarcinoma pancreático (PA) é uma doença agressiva que se desenvolve de maneira relativamente assintomática e que geralmente está avançada no momento do diagnóstico. Como é comum em tumores epiteliais, a carcinogénese desenvolve-se através da

acumulação de mutações e lesões genéticas, levando à ativação de oncogenes e inativação de genes supressores de tumores. É necessário um entendimento completo dos mecanismos celulares e moleculares específicos do desenvolvimento e progressão do PA, a fim de identificar estratégias de deteção precoce, medidas preventivas e intervenções eficazes. O mau prognóstico do PA deve-se em parte à falta de informações fisiológicas sobre o desenvolvimento da doença. Linhas celulares estabelecidas continuam a ser uma ferramenta útil para investigar estes processos biológicos moleculares.

O melanoma é um cancro que começa nos melanócitos. A maioria das células de melanoma ainda produz melanina, de modo que este tipo de tumores são, geralmente, castanhos ou pretos. Alguns melanomas não produzem melanina de modo que a lesão pode ser rosa, bege ou mesmo branca. Os melanomas podem desenvolver-se em qualquer parte da pele, no entanto, é mais provável o seu desenvolvimento no tronco (peito e costas) nos homens e nas pernas das mulheres. O pescoço e o rosto são outros locais comuns.

A expressão elevada de AQP3 foi descrita em melanoma, e considerada como potencial alvo terapêutico. Estudos anteriores do nosso grupo, usando técnicas de imuno-histoquímica, mostraram que a AQP3 e a AQP5 são aberrantemente expressas em biópsias humanas de adenocarcinoma pancreático. Assim, neste trabalho investigámos o envolvimento de AQP3 e AQP5 na permeabilidade da membrana celular, proliferação celular e migração de células de adenocarcinoma pancreático humano (BxPC3) e melanoma humano (MNT-1), para validar seu potencial como biomarcadores e potenciais alvos terapêuticos. Para tal avaliámos a expressão de AQPs numa linha celular de adenocarcinoma pancreático e noutra de melanoma humano, assim como a permeabilidade da membrana à água e ao glicerol. Verificámos que a AQP3 é a isoforma mais abundantemente expressa em ambas as linhas celulares, enquanto que a AQP5 também está presente, mas em muito menor quantidade. As células BxPC3 apresentam maior valor de Pf do de que Pgly, tal como as células MNT-1, confirmando o papel preponderante da AQP3 (aquagliceroporina) o que já era espectável uma vez que ambas as aquaporinas presentes (AQP3 e AQP5) são permeáveis à água e apenas a AQP3 é aquagliceroporina, e apresenta permeabilidade ao glicerol.

Estudos anteriores demonstram a difusão de peróxido de hidrogénio através da membrana, e por isso avaliou-se a influência do stress oxidativo nas células de adenocarcinoma pancreático (BxPC3). Conclui-se que, com diferentes tempos de incubação, o efeito do H_2O_2 não apresenta diferenças significativas na viabilidade celular até à concentração de 200mM. No entanto, a migração celular das mesmas células é afetada na presença do peróxido de hidrogénio, sofrendo uma redução de 50% após 12h de exposição.

Posteriormente, foi avaliado o efeito citotóxico de compostos metálicos (ouro, vanádio e polioxometalatos) apontados como potenciais inibidores de aquaporinas e comparados com o efeito de compostos de ouro, reconhecidos como potentes inibidores da AQP3.

Os compostos de ouro demonstraram afetar a viabilidade celular tanto em células pancreáticas como em células de melanoma humano, havendo uma redução de células viáveis para 50% ao fim de 24h de exposição aos mesmos. No ensaio de migração celular com estes compostos, esta também sofreu alterações. Ao fim de 24h, cerca de 40% da lesão das células controlo de adenocarcinoma pancreático continuava aberta enquanto a lesão do controlo fechou na totalidade. Nas células de melanoma humano observaram-se perturbações na velocidade de migração, no entanto, as células não perderam a capacidade de migrar com 5 µM de composto e ao fim de 24h estavam praticamente ao nível do controlo, com 100% da lesão fechada.

Os compostos de vanádio, tal como os de ouro, afetaram a migração e viabilidade celular. Nas células pancreáticas a viabilidade celular, ao fim de 24h, ficou inferior a 50%, por outro lado, nas células de melanoma humano a viabilidade manteve-se acima do nível dos 50%. Estes factos corroboram com os resultados da migração celular, em que as células de melanoma conseguiram capacidade de migração para fechar a lesão tal como o controlo. Nas células pancreáticas, nem o controlo nem as células expostas aos compostos conseguiram fechar a lesão na totalidade.

Os compostos de tungstato, não revelaram muito efeito na viabilidade celular até uma concentração de 20 µM, havendo sempre mais 50% das células viáveis. Na migração celular, tanto o POT-A como o POT-C, afetaram as células pancreáticas e de melanoma. Após 24h de exposição aos compostos, as células pancreáticas migraram cerca de 60% com o POT-A e 80% com o POT-C, comparativamente ao controlo. Já as de melanoma migraram cerca de 70% e 90%, na presença do POT-A e POT-C, respetivamente.

Por último, avaliou-se o efeito dos compostos em estudo na permeabilidade das células pancreáticas e de melanoma humano. Nas células BxPC3, quando comparado com o controlo a permeabilidade é drasticamente reduzida, principalmente com o POT-A, ficando inferior a 20%. As células em junção com o Auphen, inibidor de AQP3 já anteriormente descrito, sofrem uma redução de permeabilidade de 100% para cerca de 20%. Já nas células de melanoma, na presença do Auphen, a permeabilidade é reduzida, mas mantêm-se acima dos 50%.

Em suma, todos os compostos são promissores e merecem um estudo mais detalhado e pormenorizado. Deve-se ainda testar o efeito destes compostos noutros tipos de cancro.

Palavras-chave: aquaporinas, migração celular, cancro do pâncreas, melanoma, inibidores.

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Chegar aqui e olhar para trás é, não sei se gratificante ou assustador. É saber que ao longo destes dois anos o caminho não era para ser bem este, mas que no final está tudo certo, o lugar e as pessoas.

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Chega assim ao fim esta etapa. Que na verdade não é o fim de nada, mas o início de tudo.

Obrigada a todos!

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AQP	Aquaporin
ATP	Adenosine triphosphate
Å	angstrom
BxPC3	Human pancreatic cancer cell line
cDNA	Complementary DNA
DNA	Deoxyribonucleic acid
h	hour
His	Histidine
IC50	The half maximal inhibitory concentration
kDa	Kilodaltons
MNT-1	Human melanoma cell line
mL	milliliter
mM	milimolar
mRNA	Messenger RNA
MTT	(3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide)
µM	Micromolar
ms	miliseconds
nM	nanomolar
nm	nanometer
NDI	Nephrogenic diabetes insipidus
NMO	Optic neuromielitis
NPA	Asn-Pro-Ala
PA	Pancreatic Adenocarcinoma
Pf	Water permeability coefficient (cm/s)
Pgly	Glycerol permeability coefficient (cm/s)
qPCR	Quantitative polymerase chain reaction
Ser	Serine
Thr	Threonine

4. Introduction

Water homeostasis is fundamental to the physiology of all living cells. The exchange of water and solutes between the environment and the intracellular compartments requires their passage across the cell membrane, composed of a hydrophobic lipidic bilayer with specific transmembrane proteins that facilitate the permeation of polar and loaded species [3, 4].

The channels that facilitate the passage of water through the cell membranes were first described in red blood cells in 1957, and years later in the renal epithelium. The first water-channel protein was recognized in red blood cells and called aquaporin-1 (AQP1). It is now accepted that water crosses cell membranes by two parallel pathways, with different mechanisms of permeation: partition/diffusion of water molecules in the lipid bilayer and through aqueous pores formed by aquaporins (AQPs) [4].

The last few years have been very important for the understanding of the various roles of AQPs in health and disease, particularly in the ability of AQPs to permeate not only water, but also other small molecules or solutes, allowing the regulation of various functions such as the regulation of cell volume, energy metabolism, cell migration, adhesion and proliferation [3].

4.1. Aquaporins

Aquaporins (AQPs) belong to a highly conserved group of intrinsic proteins that form a family of more than 1700 integral membrane proteins found in virtually all living things. They are characterized as water channels, with the main function of facilitating the passive transport of water through the plasma membrane in order to respond to osmotic gradients created by the active transport of solutes [5].

To date, 13 isoforms of aquaporins (AQP0-12) have been identified, distributed ubiquitously throughout the body. Based on their functional and structural characteristics, aquaporins are classified (figure 1) as orthodox aquaporins, strictly involved in water permeability (AQP0-2, AQP4, AQP5, AQP6 and AQP8) and aquaglyceroporins, facilitating the transport of small solutes such as glycerol and urea, in addition to water (AQP3, AQP7, AQP9 and AQP10) [6].

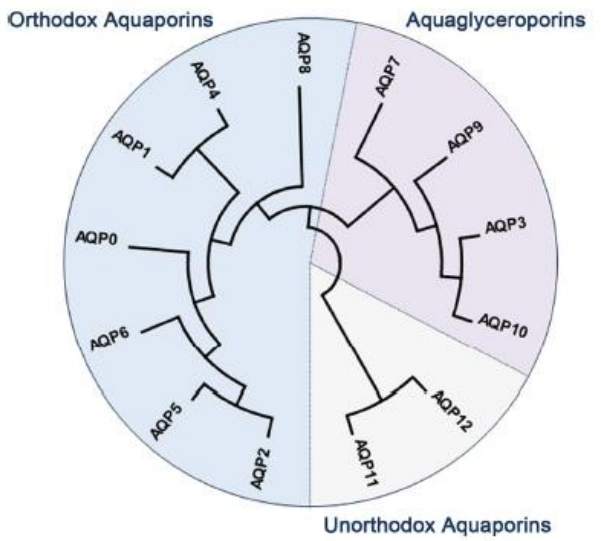


Figure 1 - Dendrogram depicting the phylogenetic relationship of mammalian: orthodox, aquaglyceroporins and unorthodox. Adapted from [2].

Among the aquaporins are peroxiporins, a subclass of hydrogen peroxide permeable aquaporins found in both orthodox (AQP1, AQP5, AQP8) and aquaglyceroporins (AQP3 and AQP9). Finally, due to their sub-cellular location, aquaporins-11 and -12 are classified as S-aquaporins. The nature of the selective permeability of AQP12 is not yet certain, showing less sequence similarity with the other isoforms. However, there are indications that AQP11 facilitates the transport of water and glycerol [7].

Results of experiments with AQP knock-out mice and humans cells with function loss mutations in aquaporin genes suggest that AQP modulators may have broad clinical indications in diseases, including nephrology (for the treatment of hypertension and edema), neurology (for the treatment of epilepsy), oncology (for the treatment of angiogenesis and tumor proliferation), ophthalmology (for the treatment of corneal and crystalline transparency and glaucoma) and the treatment of obesity and dermatological indications (in particular epidermal hydration and proliferation) [8]. In addition, there are two human pathologies directly linked to aquaporins (called aquaporinopathies) that are an opportunity for drug development, including potential therapies for nephrogenic diabetes insipidus (NDI), caused by mutations and loss of function of AQP2 and for optic neuromielitis (NMO), caused by the presence of antibodies against AQP4 [2].

4.1.1. Structure of aquaporins

There is a wealth of information on the molecular structure of AQP, which may be essential for the discovery of small aquaporin modelling molecules. Aquaporins are organized in tetramers, as exemplified in figure 2.

AQP4 can be associated with superior supramolecular assemblies known as orthogonal particle arrays, in which tetramers AQP4 form square arrays that are stabilized by amino-terminals residue interactions in monomer units 3-5 [9]. High-resolution X-ray crystal structures are available for AQP0, AQP1, AQP4, AQP5 and AQP10, which provide a high insight into the atomic mechanisms of water and solute transport and ion and proton exclusion [10]. Based on these data, it was possible to perform simulations of virtual screening and molecular dynamics.

The monomeric units of AQPs consist of six transmembrane alpha-helices (M1, M2, M4-M7 and M8), two half helixes (M3 and M7) and 5 connection loops (loops a-e), presenting a molecular weight of approximately 30 kDa. The N- and C-terminal carboxy domains are located in the cytoplasm[2].

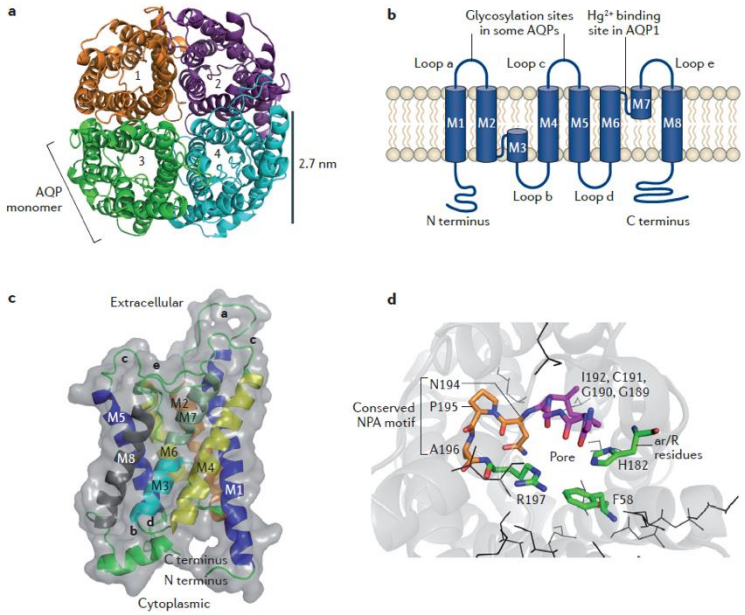


Figure 2 - Structure of aquaporin. A top view of the extracellular face of an aquaporin 1 (AQP1) homotetramer, based on the X-ray structure of bovine AQP1 (Protein Data Bank (PDB) code: 1J4N). b | A schematic of AQP membrane topography. c | Structure of the bovine AQP1 monomeric unit, which shows key helical domains (labelled M1–M8) and connecting linkers (labelled a–e). d | A view into the extracellular vestibule of bovine AQP1. The constriction region (in green) is made up of aromatic and arginine residues (known as the ar/R constriction; residues Phe58, His182 and Arg197); extracellular Asn-Pro-Ala (NPA) residues (Asn194, Pro195 and Ala196) are shown in orange; backbone α-carbonyl hydrogen-bond acceptors (Ile192, Cys191, Gly190 and Gly189) are shown in violet; and hydrophobic side chains comprising nonpolar amphipathic surface are shown in black. Adapted from [2].

[11]The hydrophilic surface consists of group's α-carbonyl from the polypeptide skeleton, and hydrophobic residues from the amphipathic pore prevent permeation of larger molecules. There are also two Asn-Pro-Ala (NPA) motifs preserved in the M3 and M7 half helixes, which contain the inward facing polar side chains of asparagine. Together with the α-carbonyl groups of the spine, the NPA motifs act as donors and hydrogen-binding acceptors that coordinate the transport of water (or glycerol for aquaglyceroporins) through the pore. The extracellular vestibule contains a restriction region composed of aromatic residues and arginine, known as the selectivity filter, which has a narrower region with a diameter of approximately 2.8 Å in human AQP1, 1.5 Å in human AQP4 and approximately 3.4 Å in less selective aquaglyceroporins [2]. The narrow internal pore region of the AQPs presents a potential challenge in identifying small molecules. Molecular dynamics simulation has been useful in elucidating the mechanisms of water and glycerol transport through AQPs, which are the main function of these channels, resulting in many biological activities that have possible clinical applications. There is some evidence that, in addition to transporting water and glycerol, AQPs can also transport gases such as carbon dioxide, ammonia and nitric oxide, as well as polar solutes such as sugars, hydrogen peroxide and even some ions [12].

4.1.2. Biological function in mammals

AQPs are widely expressed in the body, particularly in cell types that are involved in fluid transport such as epithelial cells, and other cell types non-involved in fluid transport, such as adipocytes. Due to the lack of inhibitors, the use of AQP-knockout mice provided most of the information on the physiology of AQP and on the possible indications of aquaporin-oriented modelers [3].

The transport of epithelial fluid is facilitated by aquaporins, responding to osmotic gradients that are created by the transport of active ions and neutral solutes. AQP1 is expressed in the epithelium of the proximal tubule of the kidney and in the thin descending limb of Henle's loop, as well as in the endothelium of the descending vasa straight line. Mice with AQP1 deficiency present difficulties in concentrating the urine, because the reduced permeability of the proximal renal tubule to the water impairs the normal absorption of the water filtered [13]. The reduced permeability of the thin limb of Henle's loop and the straight vessel hinders the counter-current renal multiplication and the exchange systems responsible for urine concentration [7]. Thus, inhibition of AQP1 is expected to produce water diuretics by a different mechanism from conventional diuretics that block salt transport, so it is thought that AQP1

inhibitors may have clinical potential in edema associated with congestive heart failure and cirrhosis. AQP2, AQP3 and AQP4 are expressed in epithelial collecting ductal cells of the kidney. Liquid retention by the kidney requires a high transepithelial permeability to water from the collecting duct, which is achieved by vasopressin-induced traffic of AQP2 from an intracellular vesicular compartment to the plasma cell membrane [14]. AQP3 and AQP4 are active water carriers in the basolateral cell membrane. Mice deficient in these aquaporins consequently have defects in the function of urine concentration and, therefore, inhibition of AQP2, 3 and 4 would produce a positive response similar to that produced by antagonists of vasopressin V2 receptors [15]. When AQP11, whose function is not yet fully known, is deleted, mice develop renal and hepatic cysts, by unknown mechanisms. Aquaporins facilitate the secretion of water in the glands after the creation of an osmotic gradient by the active secretion of solutes. Mice without AQP5 (expressed in salivary and airway submucosal glands) have a defective secretion of saliva and airway mucus [16]. The secretions are hyperosmolar and occur at low levels because the low water permeability of the gland acini, in the absence of AQP5, prevents osmotic balance through the epithelium. In addition, AQP1 facilitates the secretion of aqueous fluid through the ocular ciliary epithelium and of cerebrospinal fluid through the choroid plexus. Therefore, inhibition of AQP1 may reduce intraocular pressure in glaucoma and intracranial pressure in brain trauma or stroke, while inhibition of AQP5 may reduce salivation and mucus production in the airways during anesthesia [2].

4.1.2.1. Tumor angiogenesis and dissemination

Recently, a growing interest has been paid to AQPs and their roles in cancer development. It is well established that tumor growth, development, invasion and metastasis depend on tumor microenvironment and metabolism and it is also known that AQPs play an important role in responding to osmotic gradients, essential for maintaining cell function, including in malignant cells [17, 18]. AQPs can also facilitate tumor growth, local infiltration, and metastasis, improving cell migration and angiogenesis toward chemotactic stimulation.

The mechanism is not yet clear, but it is believed that it may involve rapid changes in cell shape and volume induced by AQP polarization at the front edge of migrating cells, facilitating transmembrane water flows driven by changes in osmolarity (figure 3).

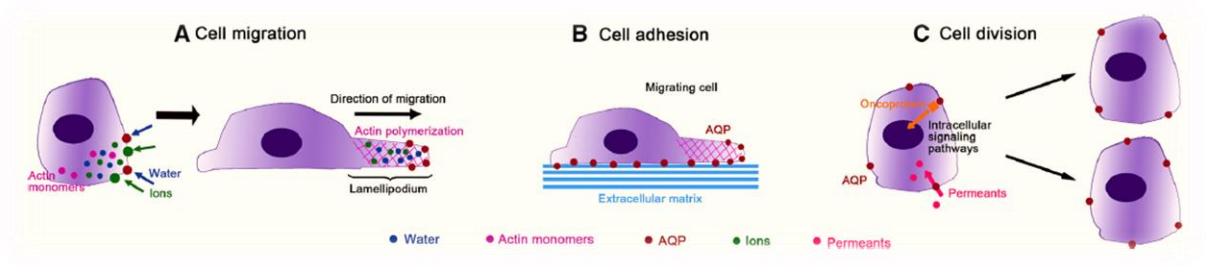


Figure 3 - Role of Aquaporins (AQPs) in the migration, adhesion and division of cancer cells. **(A)** In the membrane of the migrating cell, AQPs facilitate water flows driven by an increase in local osmolarity due to transmembrane ion flows, promoting lamellipodium formation and stabilization by actin polymerization**. (B)** AQPs can increase cell matrix adhesion, which is important for the spread of the tumor. **(C)** AQPs facilitate the capture of permeants (glycerol, hydrogen peroxide) or can interact with oncoproteins, which activate cascades of intracellular signaling promoting the transcription of genes involved in the proliferation of tumor cells. Adapted from [1].

In addition, AQPs may also promote cell matrix adhesion, which is important for tumor cell dissemination and migration, although the underlying mechanism remains uncertain. AQPs have also been associated with tumor proliferation, facilitating glycerol uptake, essential for cell biosynthesis and, consequently, for cell division [18]. Therefore, AQP expression may be advantageous for the high metabolic turnover or tumor-specific metabolic pathways required for malignant cell survival. Moreover, the possible interaction between AQPs and oncogenes / oncoproteins may ultimately activate the transcription of genes involved in cell growth, transformation and survival. AQP1 is highly expressed in microvascular endothelial cells associated with tumors, and numerous studies have shown that AQPs are highly expressed in the non-tumor cells from which the tumor originates [2]. For example, AQP1, 4 and 9 are expressed in normal astrocytes, but are also often found in human astrocitomas, with AQP4 expression correlated with tumor severity. AQP1-knockout mice show reduced growth of implanted and spontaneously generated tumors as a consequence of tumor angiogenesis. In addition, tumors expressing implanted AQP1 have greater local invasion and more metastases than tumors without AQPs [19]. Cell migration is dependent on aquaporins as the polarization of AQPs occurs in migratory cells, facilitating the entry of water into lamellipodia and extending the membrane in the direction of cell movement. Several studies have demonstrated that aquaporins facilitate the proliferation of some types of tumor cells by mechanisms that may involve signaling pathways. AQP3-deficient mice show resistance in the formation of skin tumors, after the administration of chemical stimuli. These observations suggest that aquaporin inhibitors have therapeutic potential in oncology [20].

Figure 4 shows the types of cancer where each of the aquaporins is expressed.

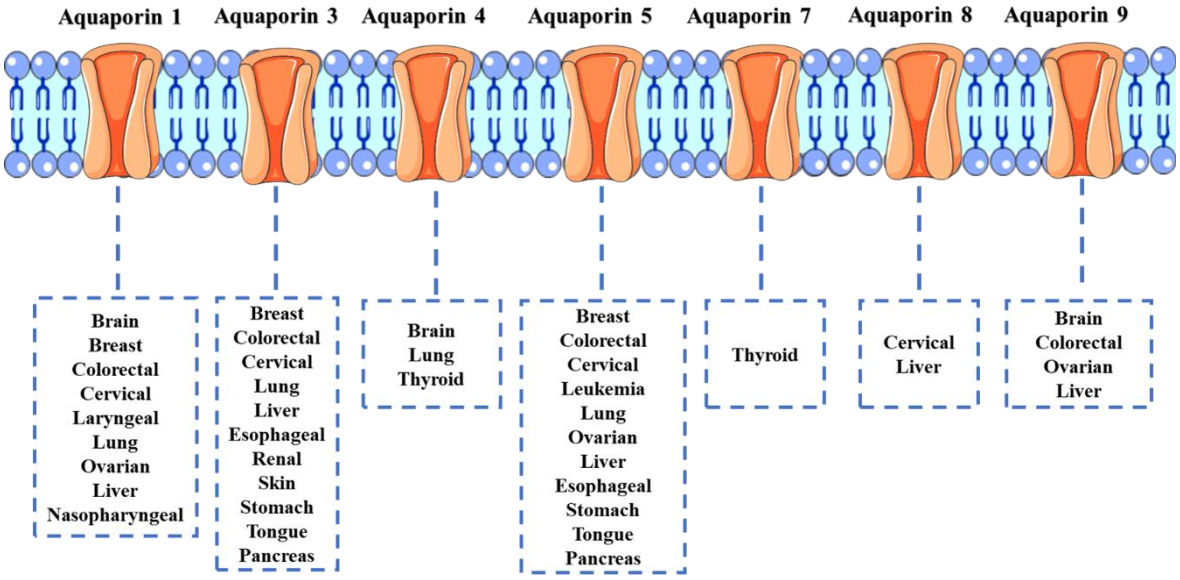


Figure 4 - AQPs display altered expression in various cancer types and are implicated in numerous processes.

Recent studies have demonstrated the aberrant expression of AQP5 and AQP3 in pancreatic adenocarcinoma. Pancreatic cancer is the seventh leading cause of death worldwide, with over 85% of cases being pancreatic adenocarcinomas (PA) [21, 22]. Due to the retroperitoneal localization of the pancreas, the early stages of this cancer usually produce no symptoms. When diagnosed, the disease when diagnosed is already in an advanced state and more than 50% of patients have distant metastases and therefore the five-year survival rate of all stages of pancreatic cancer is approximately 6% [22]. In normal tissue AQP5 is expressed in the apical membrane of ductal cells. On the other hand, in tumor tissue AQP5 is expressed throughout the plasma membrane, eventually diffusing intracellularly. The degree of differentiation is related to immunoreactivity, the more differentiated the more immunoreactive. With regard to AQP3, the expression is practically null in normal pancreatic cells, however, in PA tissues, AQP3 is expressed, although at levels higher than AQP5. In moderately differentiated tissues, aquaporin 3 is expressed in the plasma membrane; on the other hand, in poorly differentiated tissues, aquaporin appears diffused intracellularly [21].

AQP3 has been found expressed in other cancers, including melanoma. Melanoma arises from the malignant transformation of melanocytes, cells responsible for melanin production. Several factors contribute to the onset of the disease, including exposure, but also genetic predisposition and immunosuppressive states [23]. Melanoma is considered the most aggressive and deadly form of skin cancer, with increasing incidence and mortality worldwide [24]. When

detected and treated at an early stage, most cases of melanoma are curable by surgery. However, when evolving to the metastatic state, treatment failures are greater, leading to 80% of skin cancer-related deaths [25]. Melanoma cells are capable of metastasis in nearby tissues and distant major organs, and are the most common metastatic sites located in the lungs, brain, lymph nodes, liver and bone [26]. The AQP3 isoform was found overexpressed in this type of cancer, and recent studies revealed that AQP3-knockout mice develop resistance to the formation of skin tumors highlighting the importance of AQP3 for melanoma therapeutics.

4.2. Peroxiporins

Some aquaporins are capable of transporting hydrogen peroxide (hAQP1, hAQP3, rAQP5 and hAQP8), being called peroxiporins.

Similar to water, H_2O_2 was originally thought to pass freely through the cell membrane by passive diffusion. Experimental studies suggest that rapid diffusion through the lipidic bilayer was probably facilitated by membrane proteins such as aquaporins [27, 28]. Currently ,there is experimental evidence of H_2O_2 permeation by AQP3, AQP5, AQP8 and AQP9, while by AQP1 is still controversial [28, 29]. AQP3, AQP5 and AQP9 can be found in the plasma membrane, while AQP8, in addition to its location in the plasma membrane, is also present in the internal membrane of mitochondria, an important source of reactive oxygen species (ROS) in animals and plants [30]. Hydrogen peroxide belongs to the reactive oxygen species (ROS), which can be oxidizing and react with various cellular targets and can cause their death. However, H_2O_2 also functions as a signal molecule that controls processes essential to living organisms. Because of these opposing functions, the cellular level of H_2O_2 is likely to be subject to strict regulation through processes involved in its production, distribution and removal. Substantial progress has been made in exploring the formation and elimination of H_2O_2, while much remains to be learned about how this signal molecule is transported from its place of origin to the place of action or detoxification. H_2O_2 is suitable for redox signaling due to its relatively slow reactivity with biomolecules.

4.2.1. Redox signaling in cancer

Redox signaling has implications for the physiology of cells and organisms equivalent to other signaling pathways, such as calcium-mediated signaling. H_2O_2 may be involved in both oxidative stress processes and the destruction of biomolecules, depending on concentration. In

concentrations from 1 to 10 nM, it acts as a redox signal, while in concentrations higher than 100 nM it causes the oxidation of biomolecules [31, 32].

It is described that cancer cells are often characterized by the presence of ROS at a higher than normal level, in order to sustain cell growth and proliferation. High intracellular concentrations of ROS have been detected in various types of cancer, with influence increased in survival and cell proliferation. This causes damage to DNA, causing consequently genetic instability [32].

Knowing that specific aquaporins transport H_2O_2, AQPs represent promising new cancer therapeutic targets since their modulation allows the adjustment of H_2O_2 levels in cell compartments, allowing it to function as a signaling molecule [33]. This study focusses on AQP3 and AQP5 which are the peroxiporins expressed in the tumor types under study.

4.2.2. Aquaporin-3 in cancer

AQP3 is widely distributed in human tissues, being one of the most abundant aquaglyceroporins and one of the isoforms with an important role in the kidney [2]. There is great evidence that the transport of H_2O_2 by AQP3 is related to the progression of cancer.

Many reports suggest that there is a relationship between AQP expression and cancer prognosis. Overexpression of AQP3 and AQP5 was found in several types of cancer, such as pancreatic ductal adenocarcinoma, compared to adjacent non-neoplastic tissues [21]. It was observed that the increased expression of both isoforms was associated with the tumor stage, grade, metastasis and prognosis of the patients, which led to the conclusion that the co-expression of these aquaporins may be a marker of diagnosis and prognosis.

In melanoma, it is well-known that AQP3 is overexpressed [34] and researchers have shown that AQP3 knockout mice are resistant to skin tumor formation [35]. A possible mechanism for this impaired skin tumorigenesis is the AQP3-mediated H2O2 transport in AQP3 null mice [36]. Also, Gao and coworkers [34] reported that promoting the overexpression of AQP3 in human melanoma cells would increase chemoresistance to arsenite [34]. Arsenic-based compounds have shown potential as chemotherapeutic agents, with the FDA approval, in 2001, of arsenic trioxide (Trisenox®) for the treatment of acute promyelocytic leukemia [37].

4.2.3. Aquaporin-5 in cancer

In addition to the known expression in the salivary and lacrimal glands, AQP5 is found highly expressed in cancer cells and tumor tissues. This suggests that the expression of this aquaporin is involved in the formation of tumors, cell proliferation and migration and also in

the survival of cells through various mechanisms that are not yet fully understood. The mechanism that explains the contribution of AQP5 to tumorigenesis could be its ability to facilitate the channeling of H_2O_2 through the plasma membrane [1]. The state of phosphorylation was different in normal tissues and cancerous cells, where in cancer AQP5 was preferably found in the phosphorylated form in Ser156 and Thr259, suggesting that the role of AQP5 in tumorigenesis is also dependent on its phosphorylation [1, 38, 39].

It is also known that the AQP5 channel can alternate between different conformations giving rise to different water fluxes. The transition between the open and closed states involves the His67 residue implants and the orientation of the His173 residue located near the ar/R selectivity filter, allowing the transition between the wide and narrow states. However, the trigger for the activation of AQP5 still remains uncertain [40].

Using yeast cells expressing rat AQP5, it was demonstrated that AQP5 is capable of mediating the diffusion of H_2O_2 through plasma membranes, influencing the signaling transduction pathways involved in tumorigenesis [41]. In addition, it was shown that PKA-dependent phosphorylation simultaneously stimulates traffic and the abundance of AQP5 in the plasma membrane and contributes to the opening of its channel at a physiological pH of 7.4.

Considering the participation of AQPs in normal and pathophysiological states, pharmacological modulation of AQPs appears as a promising opportunity for the development of new and innovative therapeutic strategies in a variety of human disorders, namely cancer [42]. However, identification of potent and selective modulators of AQPs has proven to be a difficult task.

4.3. Aquaporin modulators

A few AQP isoforms have been related to cancer. AQP1 overexpression has been identified in brain, breast, lung and kidney, cervical, ovarian and colorectal cancer. AQP3 overexpression was detected in pancreatic, skin, stomach, kidney, liver, colorectal, lung and cervical cancers. Absorption of glycerol through AQP3 is thought to help cancer cell growth. AQP5 overexpression has been found in breast, uterine, colorectal, liver, lung, pancreas, ovarian, and esophageal cancers [20].

There is strong evidence that aquaporins are potential drug targets or diagnostic biomarkers in different diseases, including cancer. In recent years, several molecules have been suggested as inhibitors of various AQPs, especially small molecules. From acetazolamide to micro RNAs, there are several molecules described in the literature.

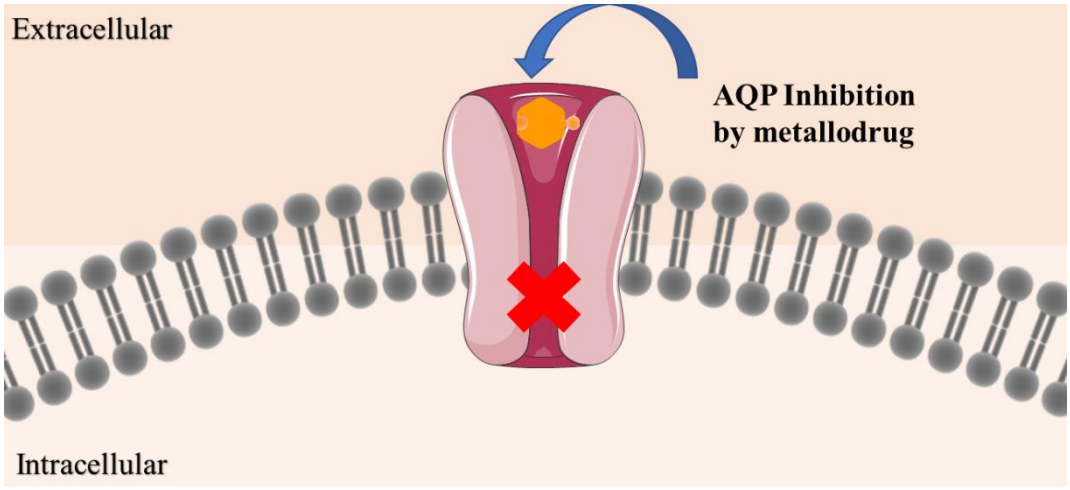


Figure 5 - Schematic representation of AQP inhibition by metal-based complexes in cells

However, the identification of AQP modulators (inhibitors) for therapeutic and diagnostic applications proved extremely challenging. To date, four classes of AQP targeting agents have been defined: (1) metal-based inhibitors; (2) small molecules described as water conductance inhibitors; (3) small molecules aiming at the interaction between AQP4 and the autoantibody for optic neuromyelitis; and (4) agents that act as chemical chaperones, causing AQP2 mutants [25].

Metallodrugs have shown great potential as therapeutic agents in various diseases. The discovery and clinical approval of the cisplatin anticancer drug [cis-diamino dichloride platinum (II)] was a remarkable achievement that led to the search for metal-based complexes as biologically active agents. Currently, three platinum-based drugs, cisplatin, carboplatin and oxaliplatin, are in clinical use in cancer treatment. In addition, ruthenium (Ru) complexes have emerged as promising second-generation metal-based anticancer agents, some of which have entered clinical trials [25].

Over the years, several metal-based compounds for the treatment of human pathologies have been clinically approved, including cancer (platinum and iron), microbial infections (silver), arthritis (gold), ulcers (bismuth), protozoan infections. (antimony), diabetes (vanadium) and malaria (iron) [43]. Unlike organic compounds, metal-based compounds have a wide variety of coordinating numbers and geometries as well as kinetic properties, the new innovative mechanisms of drug action that would otherwise not be available [44].

Copper compounds (mainly Cu^{2+}) are being targeted for studies as new generation metallodrugs, based on the notion that endogenous metals may be less toxic to normal cells

than cancer cells [45, 46]. Copper, a biologically active metal ion, has distinct hydrolytic and redox properties. Cu^{2+} is capable of forming complexes with multiple numbers and coordinated geometries, offering promising therapeutic applications [47]. An interesting approach was the development and study of copper (II) fluorescent mixed ligand complexes. This may be advantageous for detecting compounds at target sites, making it easier to understand their interaction with cells [48].

In addition to copper, gold has also been explored as an antineoplastic agent, as it has been found that rheumatoid arthritis patients receiving gold-based drugs (I) were less likely to develop cancer. In addition, it was later found that such gold (I) compounds inhibit HeLa cell growth [49]. In relation to gold (III) complexes, the design of new binders improved the stability of the complexes in the reducing environment of biological systems. This has led to research into the use of gold (III) compounds as potential antitumor agents [50].

Mercurial compounds were the first to be described as water permeability blockers by AQPs [51]. Other heavy metals, such as silver and zinc, have also been and continue to be studied [52].

Researchers have shown that copper (II) ions inhibit AQP3, reducing cell growth and improving the therapeutic effects of cisplatin [53]. It is also known that copper (II) inhibits AQP3 in a fast and reversible manner and that this effect does not require its internalization by cells [54]. In addition, potent selective inhibitors of AQP3 based on gold (Auphen) [55-58] and copper (Cuphen) [59, 60] have recently been reported. The authors demonstrated that Auphen's antiproliferative activity was positively correlated with AQP3 expression, specifically affecting AQP3-mediated glycerol permeability (figure 5).

Despite the constantly evolving chemical field and attempts to develop new inhibitors, the progress of promising metal-based complexes in clinical trials is often hampered by inherent toxic side effects and "speciation". To overcome these current limitations, new strategies must be adopted, such as using nanotechnology tools for targeted delivery [61, 62].

Thus, the most promising compounds are those of metals, making them the most investigated. In this work, we studied three classes of metal-compounds: gold, vanadium and tungsten [63]. They are touted as very promising, although very little is known about them. The compounds tested were obtained in the frame of external collaborations with our laboratory.

5. Aims

The overall goal of this work is to assess the involvement of AQP3 and AQP5 in pancreatic cancer and in melanoma cancer using as models a human pancreatic adenocarcinoma cell line (BxPC3) and a human melanoma cell line (MNT-1), and to investigate the effect of metallodrugs as AQPs inhibitors on cancer cell migration.

5.1. Specific Aims

- Evaluation of AQP3 and AQP5 expression profile in BxPC3 and MNT-1 cells.
- Evaluation of the effects of oxidative stress on BxPC3 cell viability and migration.
- Evaluation of cell membrane permeability to water and glycerol of BxPC3 and MNT-1 cells.
- Effect of metallodrugs on AQP mediated membrane permeability.
- Evaluation of the effects of AQP3 inhibition with metallodrugs on BxPC3 and MNT-1 cell viability and migration.

6. Methods

6.1. Cell culture

BxPC3 (ATCC® CRL-1687™) were cultured in RPMI 1640 supplemented with 10 % (v/v) heat-inactivated FBS and 1% PenStrep, at 37°C and 5% CO_2 atmosphere.

BxPC3 is derived from a 61-year-old woman with a primary adenocarcinoma of the pancreas. BxPC3 cells produce mucin and the tumor produced in a nude mouse is moderately well-differentiated as the primary adenocarcinoma. Separate sub-confluent cultures (70-80%) 1: 3 to 1: 6, this sowed at 2-40000 cells / cm ^{2} using 0.25% trypsin / EDTA; 5% CO_2; 37 ° C.

MNT-1 (ATCC® CRL-1872™) were cultured in DMEM supplemented with 10% (v/v) heat-inactivated FBS and 1% de PenStrep, denominated complete DMEM and were incubated at 37° C and 5% CO_2 atmosphere.

MNT1 cells are highly pigmented human melanoma cells. Separate sub-confluent cultures (70-80%) 1:3 to 1:8 is recommended using 0.25% trypsin, 0.03% EDTA solution and let the culture sit at room temperature (or 37°C) until cells detach (about 10 minutes). Add fresh medium, aspirate and dispense into new flasks.

6.2. Quantitative PCR

Cells were washed twice with PBS before total RNA purification using the RNeasy mini kit (Qiagen) and treated with RNase-free DNase I (Invitrogen) for 30min to remove any trace of genomic DNA, according to the manufacturer's recommendations. Reverse transcription was performed using an iScriptTM cDNA Synthesis Kit (BioRad). qPCR reactions were carried out using a CFX96 Real-Time System C1000 (BioRad), the TaqMan Universal PCR Master Mix (Applied Biosystems) and the following specific TaqMan pre-designed gene expression primers: AQP3 (Hs01105469_g1), AQP5 (Hs00387048_m1). and ACTB (Hs99999903_m1) (Applied Biosystems, ThermoFisher Scientific, Waltham, MA, USA)

All results were normalized to the level of the reference gene (β-actin) and relative quantification was calculated using a variation of the Livak method. All samples were run in triplicate and the average values were calculated.

6.3. Permeability Assay

Cells were centrifuged at 150 × g to obtain a cellular pellet. The cells were resuspended in isotonic medium (240 mOsm, PBS, pH 7.4) and left for 10 min to reach the equilibrium in

this medium. The cellular preparations were homogeneous and cells were spherical in shape when in suspension, as observed under light microscopy. The diameter of cells was measured for all the preparations with ImageJ software with pictures obtained by light microscopy.

Permeability assays in suspension cells were performed by stopped-flow light scattering [64]. Experiments were performed on a HI-TECH Scientific PQ/SF-53 stopped-flow apparatus, which has a 2 ms dead time and is temperature controlled (24°C), interfaced with an IBM PC/AT compatible 80386 microcomputer. For osmotic water permeability (P_f) measurements, a hyperosmotic shock solution containing a non-permeable solute was used (sucrose 200 mM in 300 mOsm PBS pH 7.4) producing an inwardly directed gradient of solute. To measure glycerol permeability (P_{gly}), a hyperosmotic shock solution containing glycerol (glycerol 200 mM in 300 mOsm PBS pH 7.4) creating an inwardly directed glycerol gradient. Four to eight runs were stored and analyzed in each experimental condition. In each run 0.1 mL cellular suspension was mixed with an equal amount of hyperosmotic solution to reach inwardly directed gradients of solute. After the first fast cell shrinkage due to water outflow, glycerol influx in response to its chemical gradient was followed by water influx with subsequent cell re-swelling. Baselines were acquired using the respective incubation buffers as isotonic shock solutions.

The kinetics of cell shrinking and re-swelling were measured from the time course of 90◦ scattered light intensity at 530 nm until a stable light scatter signal was attained. Water permeability (P_f) was calculated as $P_f = k\ (Vo/A)(1/Vw(osmout)_\infty)$, where Vw is the molar volume of water, Vo/A is the initial cell volume to area ratio, $(osmout)_\infty$ is the final medium osmolarity after the applied osmotic gradient and k is the single exponential time constant (s^{-1}) fitted to the light scattering signal of cell shrinkage; glycerol permeability (P_{gly}) was calculated by $P_{gly} = k(Vo/A)$, where Vo/A is the initial cell volume to area ratio and k is the single exponential time constant (s^{-1}) fitted to the light scattering signal of glycerol influx.

6.4. MTT Assay

Measurement of cell viability and proliferation forms the basis of numerous *in vitro* assays of the response of a cell population to external factors. The reduction of tetrazolium salts is a common and reliable test to assess cell proliferation. The yellow tetrazole MTT (3- (4,5-dimethylthiazolyl-2) -2,5-diphenyltetrazolium bromide) is reduced by metabolically active cells, by the action of dehydrogenase enzymes, generating reducing equivalents such as NADH and NADPH. The resulting intracellular purple formazan can be solubilized with DMSO and quantified by spectrophotometric means.

The MTT Cell Proliferation Assay measures the cell proliferation rate and conversely, when metabolic events lead to apoptosis or necrosis, the reduction in cell viability. The MTT reagent yields low background absorbance values in the absence of cells. For each cell type the linear relationship between cell number and signal produced is established, thus allowing an accurate quantification of changes in the rate of cell proliferation.

Cells are plated (10 000 per well in 96-well plate) under optimum growth conditions (70% confluence), after 24 hours the compound is added and followed by a 24- or 72-hour incubation, depending on the purpose of the work. After the incubation time the MTT reagent is added and resuspended at 37°C, 5% CO_2 for 4h. Finally, DMSO is added and the absorbance is measured at 570 nm.

6.5. Migration Assay

Cell migration is a multi-step process that is essential for the various vital functions in multicellular and unicellular organisms, but is also associated with cancer progression and metastasis.

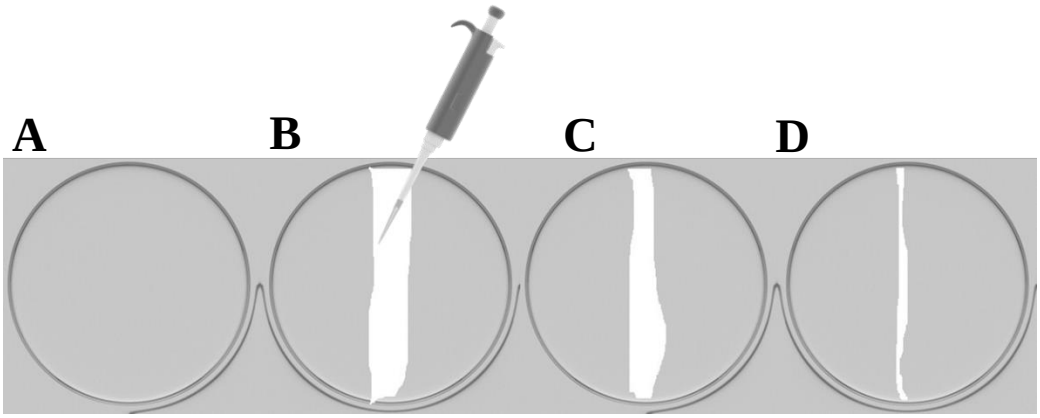


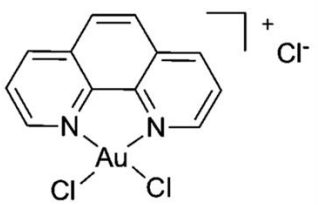
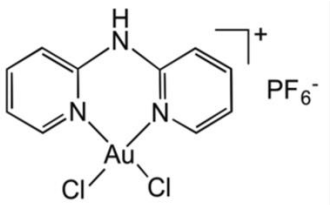
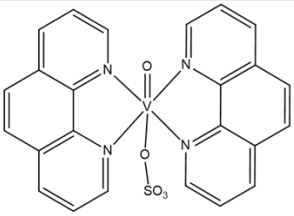
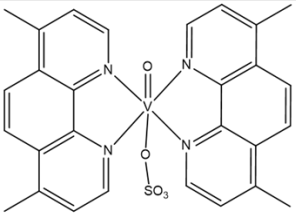
Figure 6 - Scratch test. **(A)** A wound is introduced into a confluent monolayer of cells using a p10 pipette; **(B)** drawing a white tip through the cell layer; **(C)** The naked area is visualized to measure the size of the wound in the pre-migration, that is, in the initial time of the test; **(D)** after the cells have migrated inward to fill the empty space.

One commonly used technique for measuring migration of 2D cells is the scraping wound assay. Scratch assays were first used as models of wound healing for epithelial or mesenchymal cells [22]. In this assay depicted in Figure 6, cells are seeded into a multi-well assay plate and allowed to attach, spread, and form a confluent monolayer. A pin tool or needle is used to scratch and remove cells from a discrete area of the confluent monolayer to form a cell-free zone into which cells at the edges of the wound can migrate [22,23]. Molecules of interest as potential therapeutics are added to the wells and images of cell movement are captured at regular intervals within a 24-hour period for data analysis.

In this study, 800000 cells were plated per well in 12-well plates, grown under ideal conditions, and after 24 hours the wound was made and the compound being tested were added (figure 6). From that moment on, photographs were taken at 3h intervals. At the end of the experiment photos were analyzed using the software Image J.

6.6. Compounds preparation

Table 1 - Compounds and respective anions formula.

Gold Compounds	 **Auphen**	 **AuDipyAm**
Chemical formula	$[Au(phen)Cl2]Cl$	$C_{10}HgAuCl_2F_6N_3P^-$
Vanadium Compounds	 P90	 P91
Chemical formula	$VO(OSO_3)(phen)_2$	$VO(OSO_3)(Me_2phen)_2$
Polyoxometalates	POT-A	POT-C
Chemical formula	$K_6[P_2W_{18}O_{62}].14H_2O$	$Na_{12}[\alpha - P_2W_{15}O_{56}].24H_2O$

6.6.1. Polyoxometalates

The polyoxometalates (POMs) of vanadium and tungstate were sent by a collaborator from Faculty of Sciences and Technology of the University of Algarve, already in solution. Their molecular and anion formulas are summarized in the previous image. The POTs A and C were sent at a concentration of 5 mM that were then diluted to 1 mM in Milli Q water to be used in the stopped-flow experiments and migration and viability assays.

6.6.2. Vanadium compounds

2 compounds from Instituto Superior Técnico were screened by stopped-flow and viability and migration assays: P90 and P91. The structures of these 2 metallodrugs are compiled in the previous image. The metallodrugs arrived in powder to be prepared as a stock solution with DMSO. They were prepared for a final concentration of 10 mM. Subsequently, all the compounds were diluted to 1 mM with phosphate buffer saline (PBS) solution to perform the assays.

6.6.3. Gold compounds

The gold compounds Auphen and AuDipyAm from Cardiff University were screened by stopped-flow. Their structures are summarized in the image. These gold compounds arrived in powder to be prepared at a final concentration of 10 mM. First, according to the instructions from Cardiff University, the compounds were solubilized in water for a final concentration of 10 mM. Then, they were diluted to a concentration of 10 mM to perform the experiments.

6.7. Statistical analysis

Data are presented as mean ± standard error of the mean (SEM). Data were first analyzed with Shapiro-Wilk normality test to understand if the data followed a Gaussian distribution and then analyzed using the student's t-test. For the first test, a value of $P \leq 0.05$ meant that the assumption of a normal distribution had to be rejected and for the second test, a value of $P < 0.05$ meant that the data were statistically significant.

7. Results

7.1. Expression and function of AQPs in cells

The pancreatic adenocarcinoma (BxPC3) and melanoma (MNT-1) cell line was first characterized in terms of AQP3 and AQP5 expression and membrane permeability to water and glycerol.

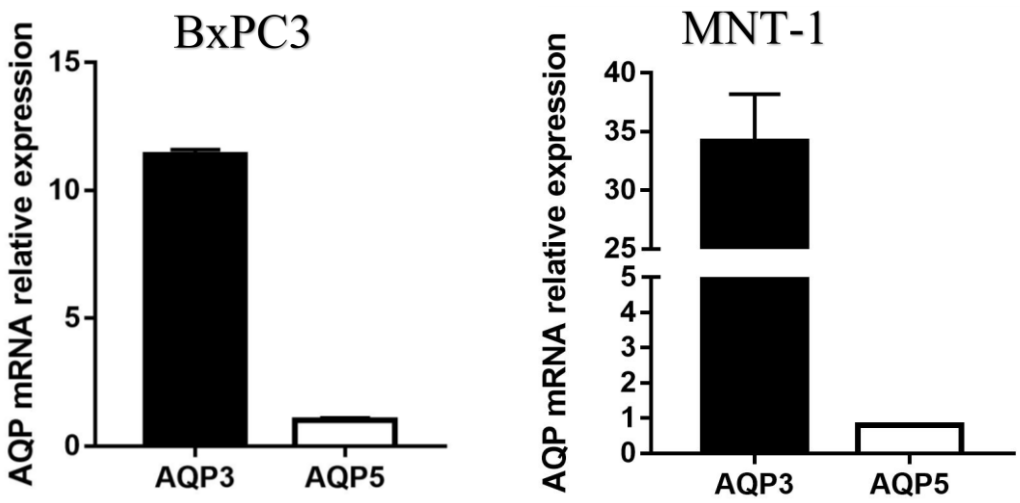


Figure 7 - AQP3 and AQP5 mRNA expressions levels in BxPC3 and MNT-1 cells using β-actin as a reference.

The relative expression of AQP3 mRNA and AQP5 mRNA was evaluated by qPCR, and the results showed that the most expressed aquaporin is AQP3 aquaglyceroporin in pancreatic and melanoma cells. However, in MNT-1 cells the expression of AQP3 is double than in BxPC3 cells. To evaluate membrane permeability to water (P_f) and to glycerol (P_{gly}) we used the stopped flow technique, that allows us to follow the cell volume change after imposing an osmotic gradient with an impermeant solute inducing water outflux (to measure P_f) or with glycerol inducing glycerol and water influx (to measure P_{gly}). Results are shown in figure 8 and figure 9.

Knowing that water crosses the membrane by two distinct pathways, the P_f determined represents the sum of the contribution of the lipid bilayer and the various AQPs expressed in the membrane, and accounts for the water flux through AQP3 and AQP5. Regarding glycerol, since this molecule has a much larger polarity and hydrophilicity, the measured P_{gly} is solely due to the glycerol entrance in the cell via AQP3 and eventually other minor expressed aquaglyceroporins not detected in this work.

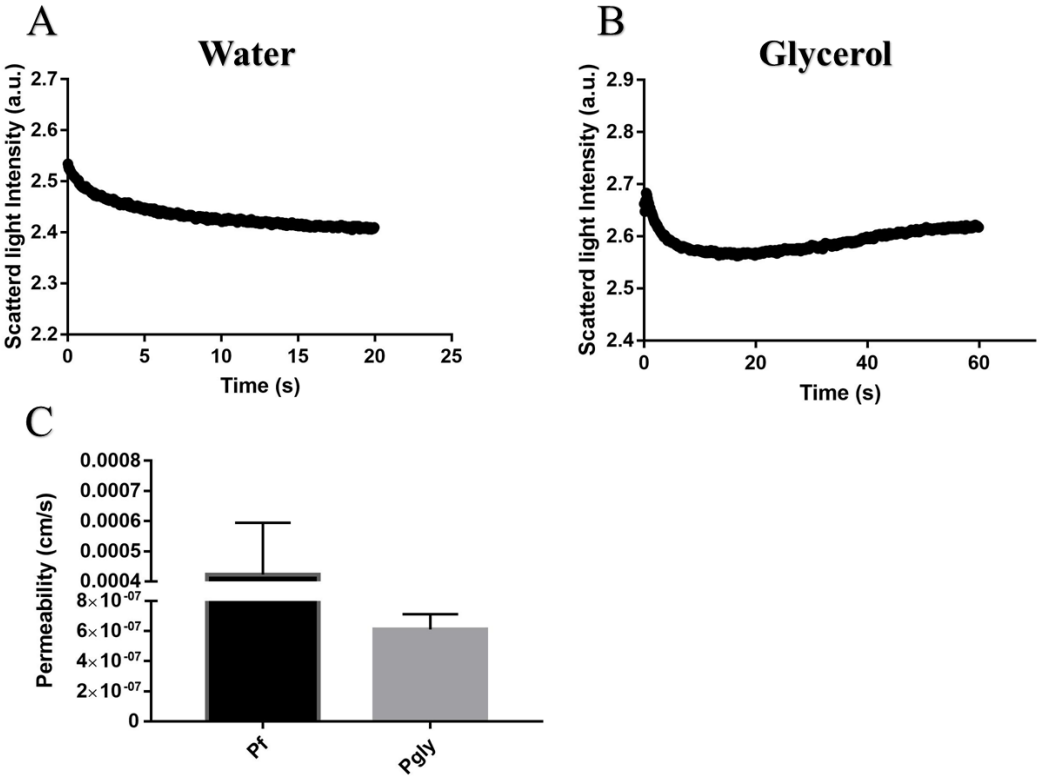


Figure 8 –Study of BxPC3 permeability. (A) Stopped flow representative traces of water; **(B)** Stopped flow representative traces of glycerol; **(C)** Water permeability (Pf) and glycerol permeability (Pgly) of cells.

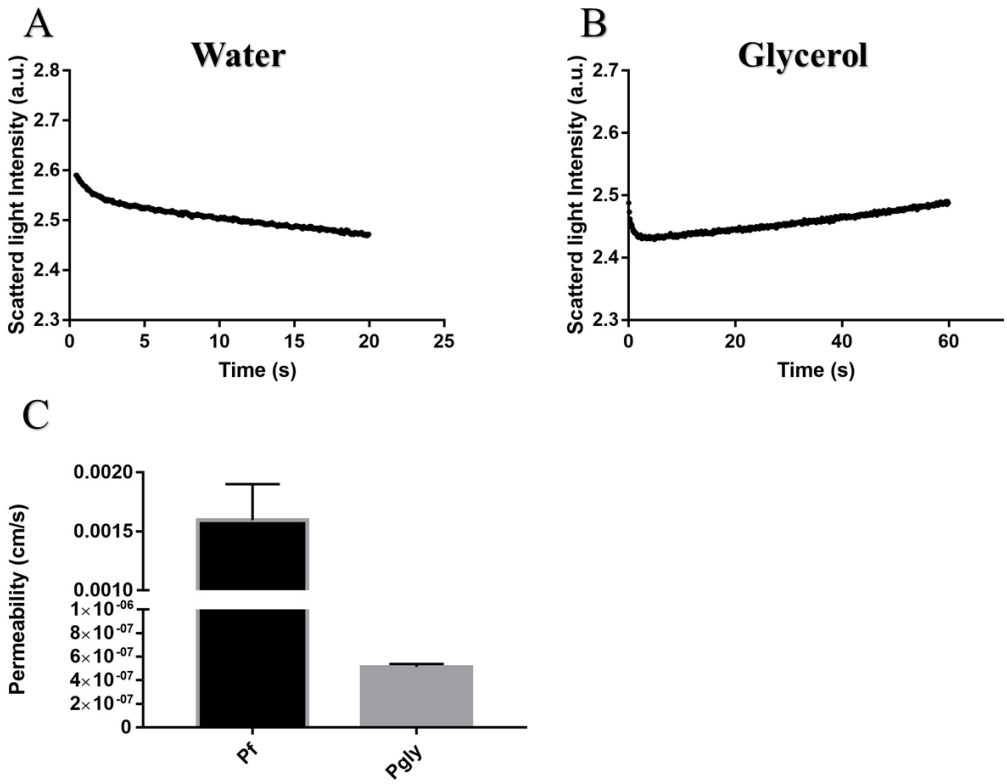


Figure 9 – Study of MNT-1 permeability. (A) Stopped flow representative traces of water; **(B)** Stopped flow representative traces of glycerol; **(C)** Water permeability (Pf) and glycerol permeability (Pgly) of cells.

Comparing the two cell lines of this study, we found that both melanoma and pancreatic cancer cells permeate water and glycerol (Figures 8 and 9). These are the control values for performing the permeability test with metallic compounds.

7.2. Effect of oxidative stress

Previous studies of our group reported that rat AQP5 mediates the diffusion of H_2O_2 in the membrane and is involved in the oxidative cellular response. The transport activity of phosphorylated rAQP5 can be regulated by acidification, a condition favored in all carcinogenic tissues. Thus, we hypothesized that human AQP5 could also channel H_2O_2, and that the high level of expression and activity of this peroxiporin may play an important role in the cellular adaptation to oxidative stress, with influence on the cellular migration of carcinogens. In our most recent study [42], we showed that human AQP5 permeates hydrogen peroxide and modulates resistance to oxidative stress. Thus, here we used the same BxPC3 cell line to evaluate H_2O_2 toxicity and to perform an oxidative migration test on viable cells.

7.2.1. Viability assay

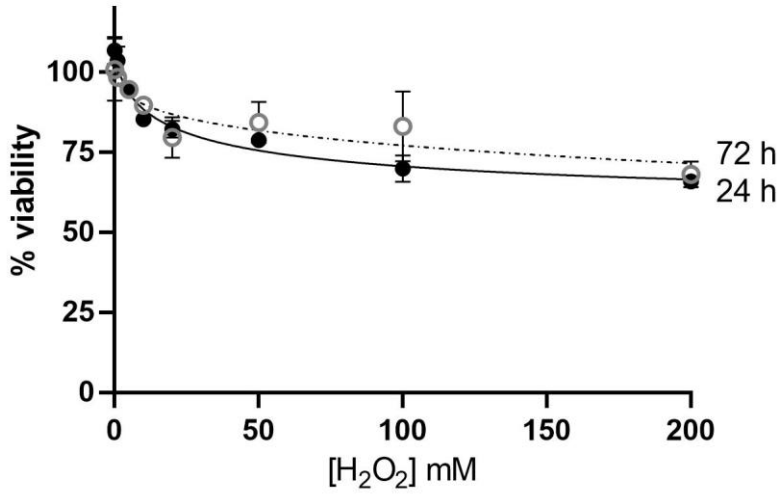


Figure 10 – BxPC3 cell viability determined by MTT assay after cell exposure to range of H_2O_2 concentrations for 24 h and 72 h.

Previous to the oxidative migration assay, we assessed the effect of external H_2O_2 stimulus on the viability of BxPC3 cells. As depicted, the addition of H_2O_2 up to 200 µM induced a maximal 30% loss of cell viability. Thus, in subsequent experiments, we used 100 µM H_2O_2, assuring >70% cell viability for migration assays. Thus, for the cell migration experiment we used 100µM of H_2O_2 to guarantee at least 70% of viable cells.

7.2.2. Migration Assay

To investigate the effect of H_2O_2 on cell migration, we measured the rate of wound closure in cells incubated with 100 µM H_2O_2 and compared with non-treated cells (figure 11).

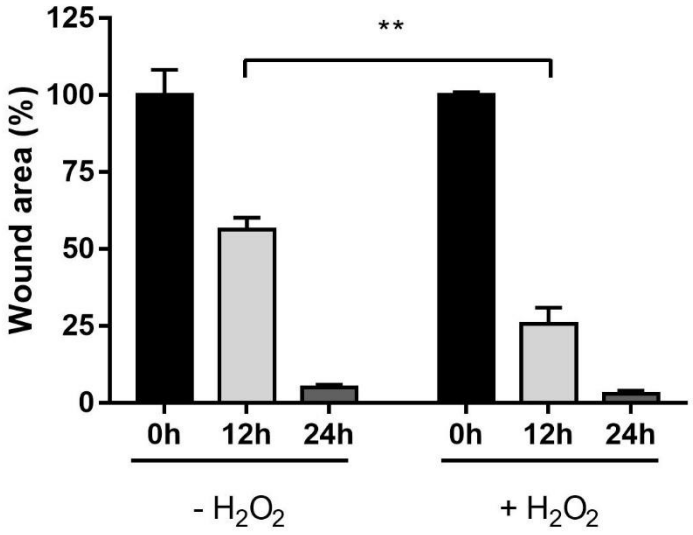


Figure 11 – Cell migration of BxPC3 cells upon oxidative stress. Cell migration of control cells in presence or not of H2O2 (100 µM).

Figure 11 shows that BxPC3 cells treated with H_2O_2 suffer a drastic increase in migration (up to 50% at 12h) compared to control cells (untreated), indicating that diffusion of H_2O_2 through AQP3 and AQP5 may influence tumor formation, cell proliferation, migration and survival across multiple pathways that are not yet fully understood.

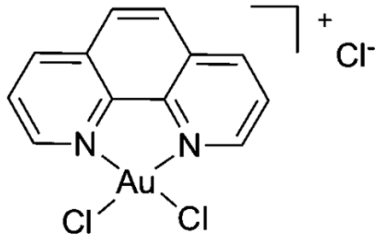
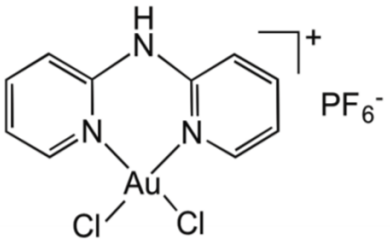
7.3. Effect of metallodrugs

The importance of AQPs in cancer has been widely described, although for some isoforms and/or tumors there is still much to investigate. The identification of aquaporin modulators has become a research goal. Four classes of small molecules targeting aquaporins have been described, with emphasis on gold (III) compounds that inhibit AQP3. In this work, three classes of compounds provided by external collaborators were tested as AQPs inhibitors.

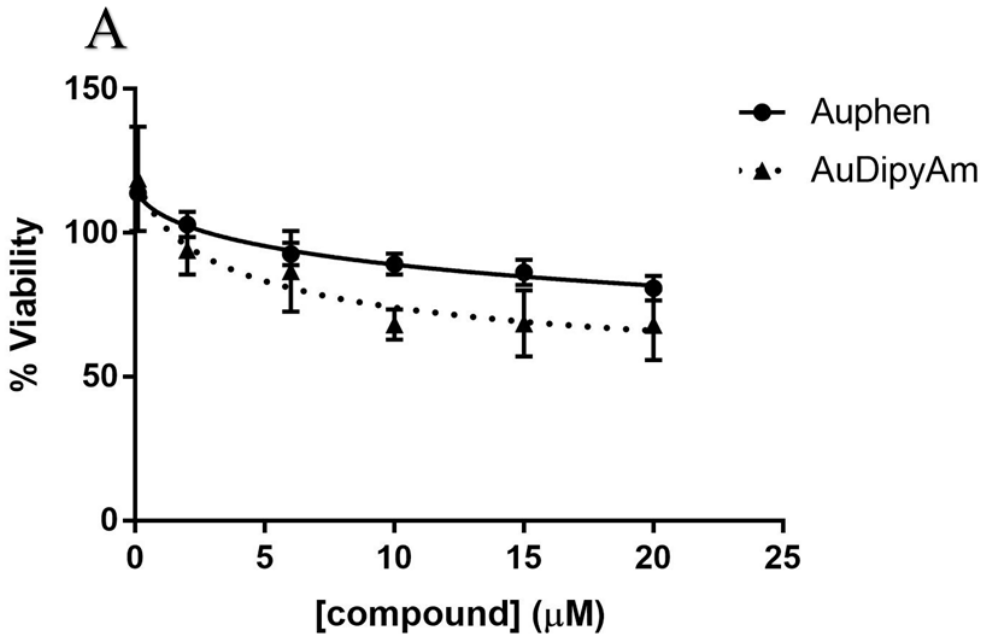
7.3.1. Gold compounds

Gold compounds (table 1) have been reported as AQP3 inhibitors, with Auphen being the most potent described to date. Another gold compound, AuDipyAm, was also tested. Previous to migration assays, a first test of cell viability was carried out to determine the concentration of compound to be used that would still allow to have viable cells for the migration test, because we want to see how drugs influence migration and not prevent the process from happening.

Table 2 - Gold compounds used in this study.

	Auphen	AuDipyAm
		
Chemical formula	$[Au(phen)Cl2]Cl$	$C_{10}HgAuCl_2F_6N_3P^-$

7.3.1.1. Viability assay



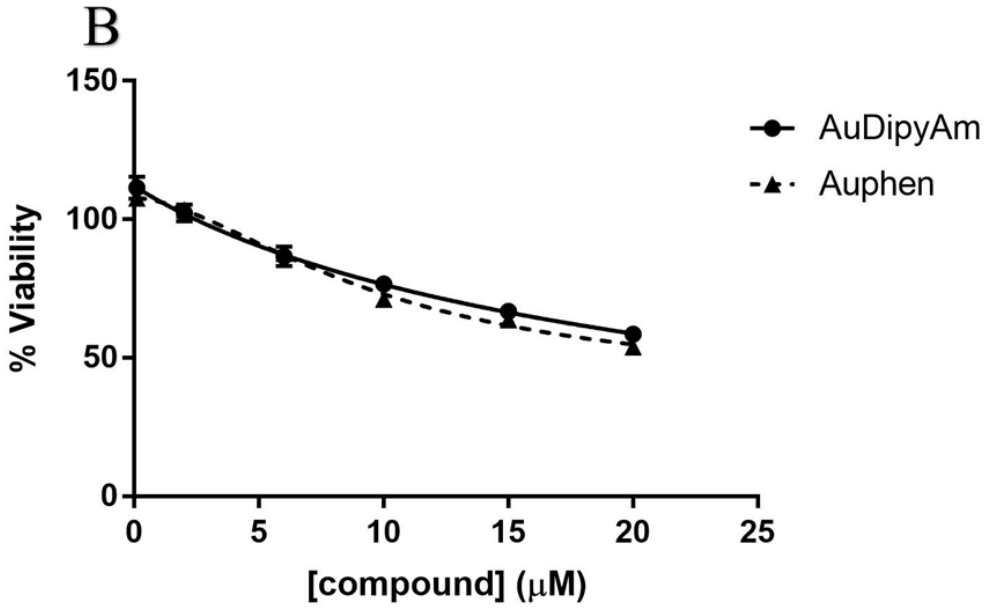


Figure 12 - Cell viability determined by MTT assay after cell exposure to a range of gold compounds concentrations for 24 h. (A) BxPC3, human pancreatic adenocarcinoma cell line; **(B)** MNT-1, human melanoma cells.

In the MTT test, compound's concentrations up to a maximum of 20 µM were used. At the maximum concentration with Auphen, cell viability is 80% in BxPC3 cells, while in MNT-1 it is around 60%. With AuDipyAm, pancreatic cells reach a value of 70%, while melanoma cells only show 60% viability after 24 hours. In both cases it is observed that from 6 µM onwards viability is affected, so it has been determined that the 5 µM will be the ideal concentration to have more than 70% of viable cells and perform a migration test. In the migration test we have to evaluate the effect of the compound on cell migration of viable cells.

7.3.1.2. Migration Assay

To determine the effect of gold compounds on pancreatic adenocarcinoma and human melanoma cell migration, scratch tests were performed.

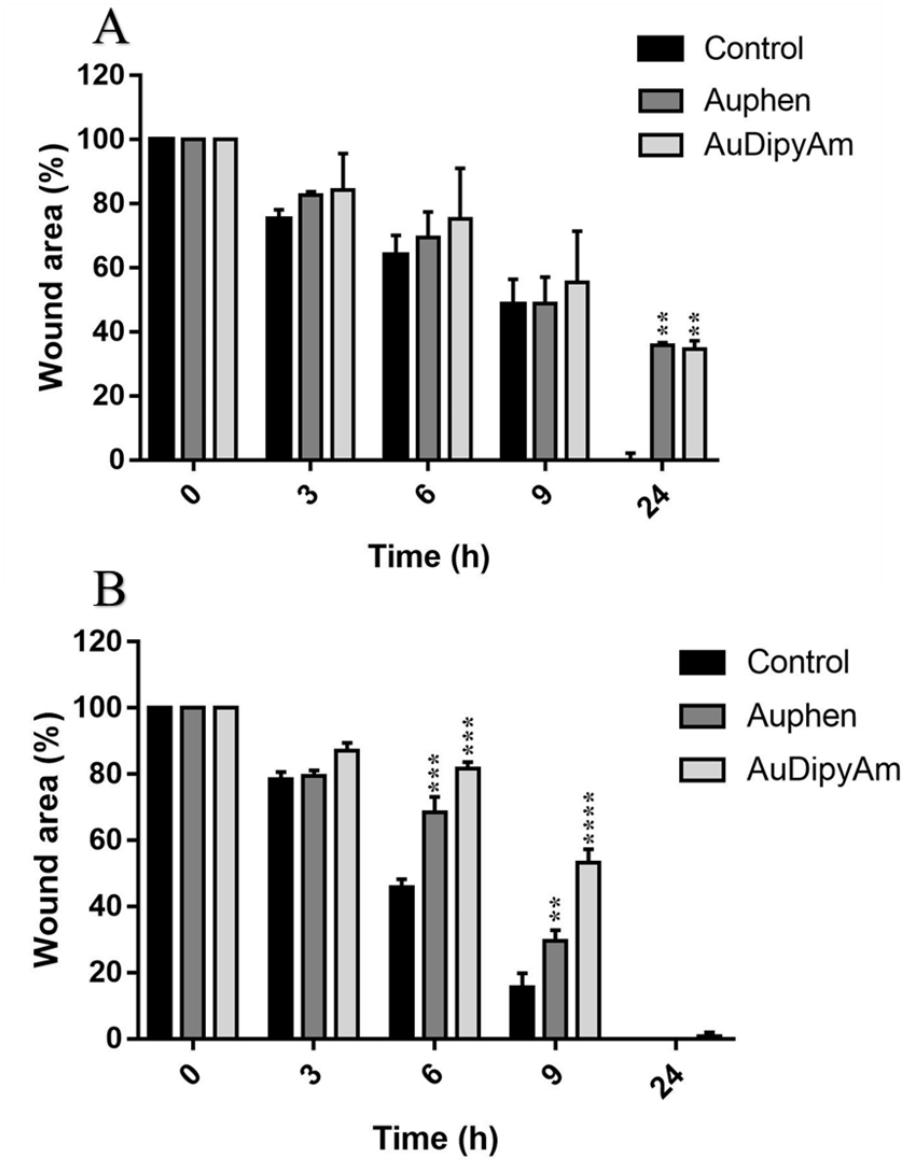


Figure 13 - Cell migration assay with gold compounds. (A) BxPC3, human pancreatic adenocarcinoma cell line; **(B)** MNT-1, human melanoma cells.

Figure 13 shows the effect of Auphen and AuDipyAm on migration of BxPC3 and MNT-1 cell lines. In BxPC3 cells, the effect of the compounds can be seen after 24 hours. While at 24h control cells close the wound completely (0% wound area), the addition of compounds shows that wound area is around 60% at 24 h, with a reduction of 40% in cell migration in human pancreatic adenocarcinoma cells.

In MNT-1 cells, although both compounds affect the rate of migration, we could detect differences in wound area early at 6 h and 9 h where the wound area is higher than control for both gold compounds. Since these cells migrate faster, after 24 h all the wounds are completely closed.

In conclusion, comparing the effects of gold compounds on the cell lines under study, we found that both compounds at 20 μM, cause a measurable decrease in viability of human melanoma cells, being that cellular viability of BxPC3 is more affected for the same compound's concentration. Regarding migration testing, gold compounds at a concentration of 5 μM reduced migration of BxPC3 cells to around 40% of control cells at 24h, while this effect was earlier seen in MNT-1 (6 h and 9 h), indicating an impaired migration rate, but a complete area closure as was observed at 24 h.

Knowing that the two compounds are AQP3 inhibitors, their effect on cell migration indicates that AQP3 is involved in facilitating the process.

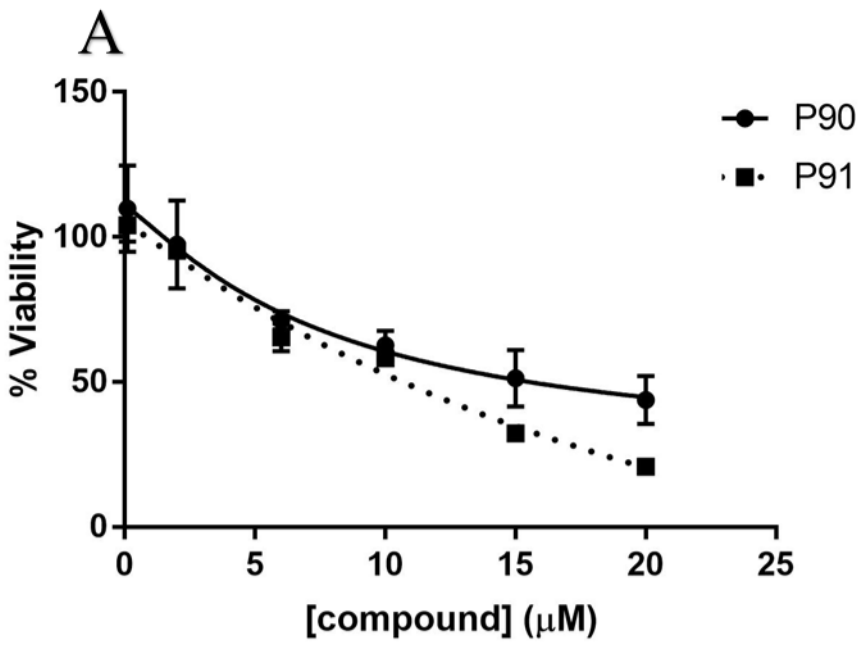
7.4. Vanadium compounds

Table 3 - Vanadium compounds used in this study.

	P90	P91
Chemical formula	$VO(OSO_3)(phen)_2$	$VO(OSO_3)(Me_2phen)_2$

7.4.1. Viability assay

As with gold compounds, a viability test was performed to ensure that the migration test was performed on viable cells. The effect of vanadium compounds (table 2) on what not described. We tested vanadium complexes (P90 and P91) with a structure similar to the gold complexes. The results of the MTT assay for both BxPC3 and MNT-1 cells are shown in figure 14.



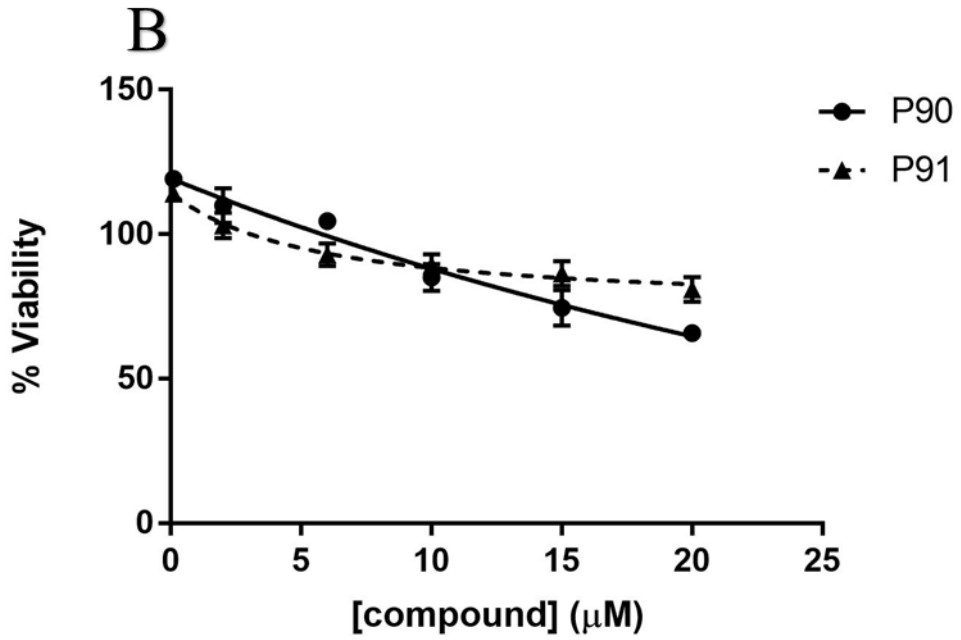


Figure 14 - Cell viability determined by MTT assay after exposure to a range of vanadium compounds for 24 h. (A) BxPC3, human pancreatic adenocarcinoma cells; **(B)** MNT-1, human melanoma cells.

Interestingly, we observed a decrease in viability more pronounced than for gold compounds. Both P90 and P91 at 20 µM cause decrease in viability, and after 24 hours of exposure to the drug, viability was reduced to 50 and 25% respectively in BxPC3 cells (figure 14A). For MNT-1 cells, viability was higher for the same concentration of compound, about 70% for P90 and 60% for P91 (figure 14B). Thus, we chose the concentration of 5 µM of compound to perform the following migration tests, since with this concentration at least 60% of BxPC3 cells and around 90% of MNT-1 are viable.

7.4.2. Migration Assay

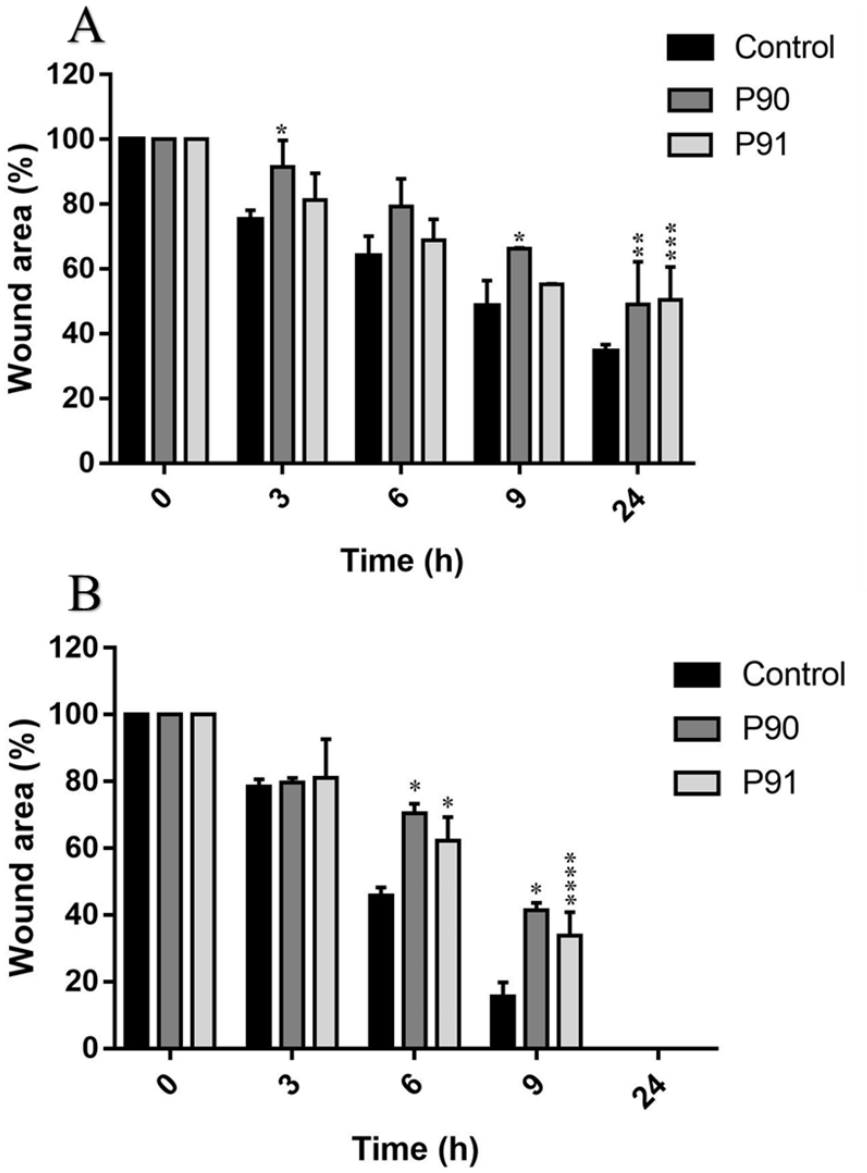


Figure 15 - Cell migration assay with exposure to a range of vanadium compounds. (A) BxPC3, human pancreatic cells; **(B)** MNT-1, human melanoma cells.

After treating cells with P90 and P91, cell migration rate was affected in both BxPC3 MNT-1 cells. After 24 h, while control BxPC3 cells migrated about 60%, treated cells only migrated 40-50% (figure 15A). In MNT-1 cells, the effect is seen earlier and at 9 h there is a significant decrease in the migration rate of treated cells, being the wound area of treated cells approximately 2-fold the control. Since these cells migrate faster than BxPC3, after 24h the wound closure was complete for all the conditions (figure 15B).

7.5. Polyoxometalates compounds

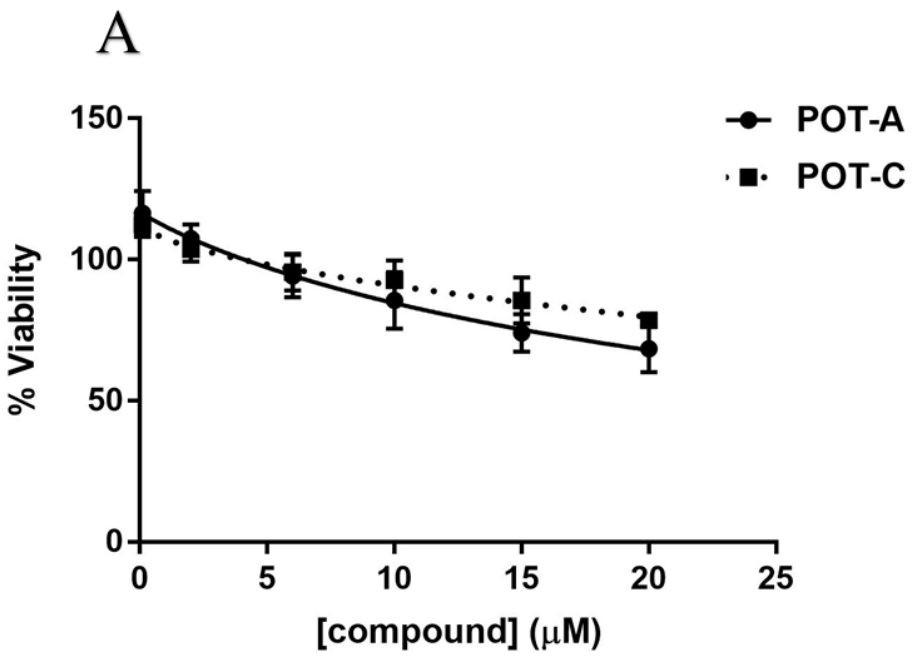
Polyoxometalates are an emerging class of inorganic metal oxides, which over the last decades demonstrated promising biological activities. They possess high potential for the inhibition of various tumor types. The study of these compounds takes place in collaboration with an external group. The polyoxometalate structures are not displayed because we have not had access to them yet.

Table 4 - Polyoxometalates compounds used in this study.

	POT-A	POT-C
Chemical formula	$K_6[P_2W_{18}O_{62}].14H_2O$	$Na_{12}[\alpha - P_2W_{15}O_{56}].24H_2O$

7.5.1. Viability assay

The viability of BxPC3 and MNT-1 cells was tested in the presence of polyoxometalates (POT-A and POT-C). The cell viability after 24 h treatment is shown in figure 16.



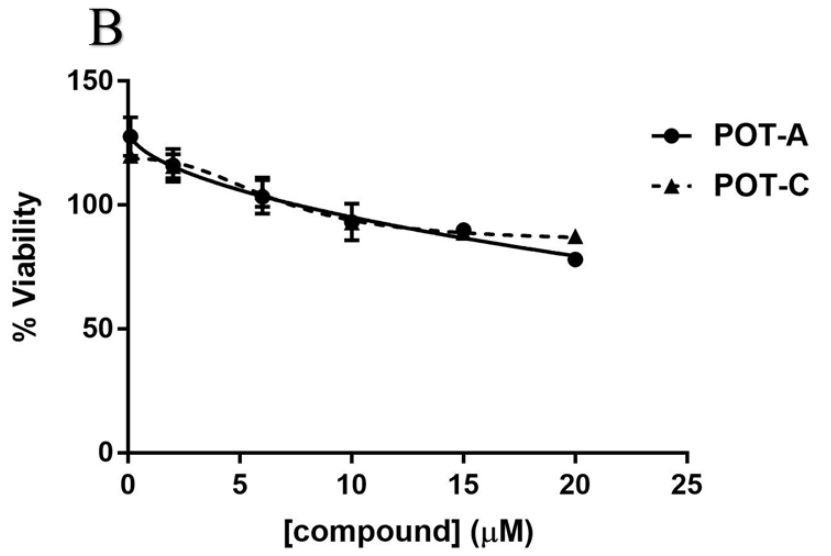
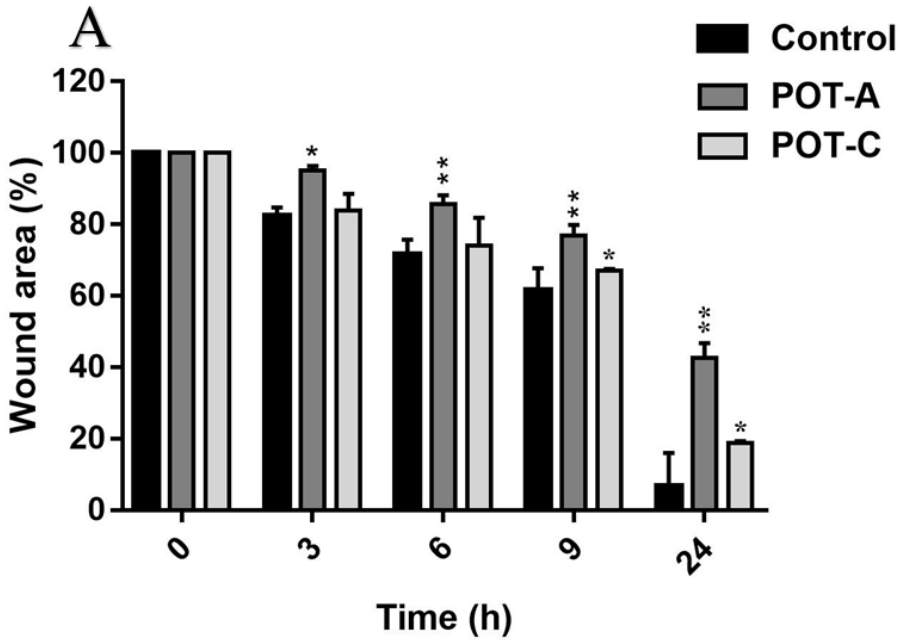


Figure 16 - Cell BxPC3 viability determined by MTT assay after exposure to a range of vanadium compounds for 24 h. (A) BxPC3, human pancreatic adenocarcinoma cells; **(B)** MNT-1, human melanoma cells.

Based on the obtained results, the viability was around 60% for BxPC3 cells treated with both compounds at 20 μM, and was slightly higher for MNT-1 cells, around 80% (figure 16B). Similar to the previous tested compounds, we decided to use a compound concentration of 5 μM for the cell migration assays.

7.5.2. Migration assay



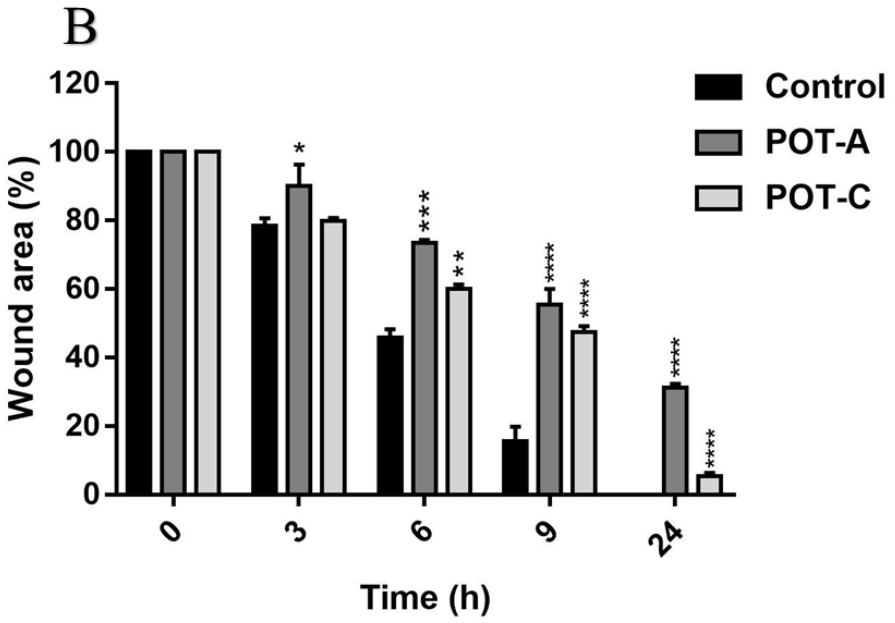


Figure 17 - Cell migration assay with exposure to a range of polyoxometalates compounds. (A) BxPC3, human pancreatic adenocarcinoma cells; **(B)** human melanoma cells.

As depicted in figure 16, a decrease in cell migration rate in both cell lines was observed, with a stronger effect of POT-A. In BxPC3 cells, while the control wound closure is about 90% at 24 h, cells treated with POT-A still show 50% wound area, and cells treated with POT-C present 20% of the open wound. In MNT-1 cells, the scenario is similar, after 24 h the control wound was completely closed, while cells treated with compounds have not been able to do so. Cells incubated with POT-A show around 35% wound area and treatment with POT-C affected around 5% wound closure at 24 h (figure 17A). Again, we can observe that MNT-1 cells migrate at a higher rate than BxPC3 cells, and that both vanadium compounds are more aggressive to these cells than for BxPC3.

7.6. Effect of compounds in permeability

To test the inhibitory effect of the compounds on membrane permeability of glycerol, mediated by AQP3, of BxPC3 and MNT-1 cells, we performed glycerol permeability assays using the stopped-flow technique. Results are presented in figure 18.

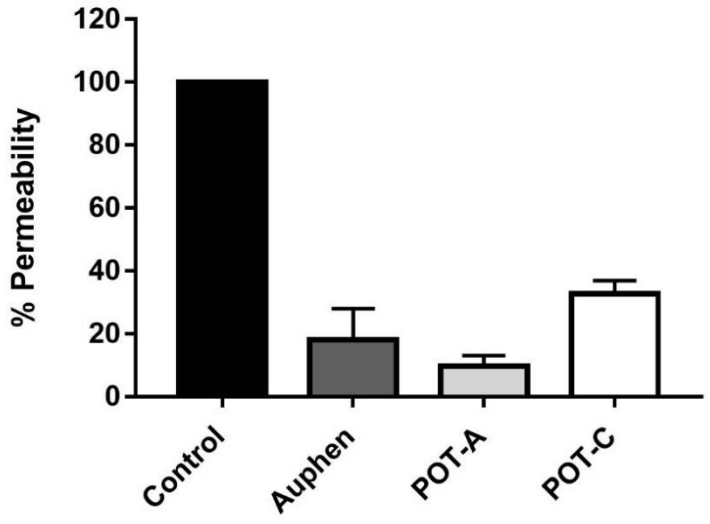


Figure 18 - Inhibition of permeability in BxPC3 cells by metallodrugs (gold, vanadium and tungstate)

Figure 18 shows that glycerol permeability via AQP3 is inhibited by Auphen, POT-A and POT-C compounds. As previously mentioned, the most potent inhibitor of AQP3 described in the literature is Auphen; interestingly, in this study POT-A showed a higher inhibition than Auphen, leading to a decrease in glycerol permeability of over 80% at 5 µM. Auphen inhibits BxPC3 cells by about 80%, while POT-C is the one that shows the lowest inhibition by about 40% at 5 µM.

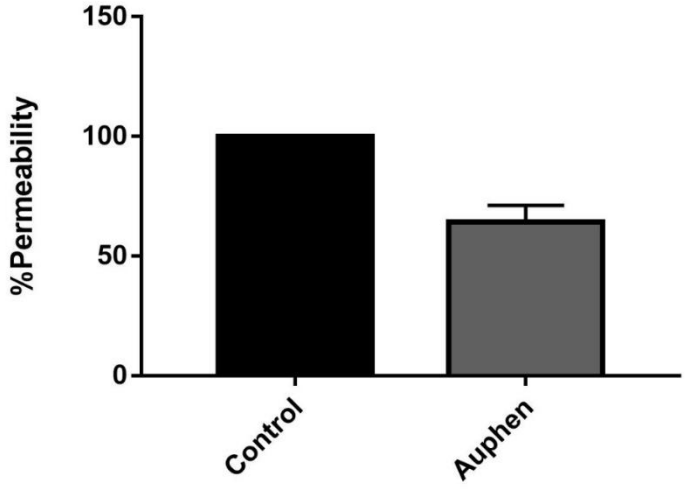


Figure 19 - Inhibition of permeability in MNT-1 cells by metallodrugs

Figure 19 shows that glycerol permeability via AQP3 is inhibited by Auphen. As previously mentioned, the most potent inhibitor of AQP3 described in the literature is Auphen. This compound inhibits MNT-1 cells by about 55%.

8. Discussion

With the main objective of studying the involvement of AQP3 and AQP5 in pancreatic cancer and in melanoma, the present work aimed at evaluating the effect of metallodrugs, inhibitors of AQPs, on the proliferation and migration of cancer cells.

Previous studies from our group have confirmed the expression of AQP3 and AQP5 in pancreatic tumors, and the expression of AQP3 in melanoma has been described in the literature. Based on this, a strategy was devised to study new aquaporin modulators in pancreatic cancer and in melanoma cell lines, and to validate their effect on cell membrane permeability, cell viability and cell migration.

Relative expression studies of aquaporins in cell lines have revealed that the most expressed AQP in both cell lines is AQP3, but the expression of this aquaporin is higher in human melanoma cell line. MNT-1 cells and BxPC3 cells were permeable to both water and glycerol. For this study, we considered the cell volume under study.

AQP3 has already been reported to channel H_2O_2, and previous studies from our laboratory also reported that AQP5 is a peroxiporin. Thus, in this study we evaluated the effect of H_2O_2 on cell viability and migration of pancreatic cancer cells that express AQP3 and AQP5. We found that H_2O_2 treatment has a strong effect on reduction of cell migration in these cells, indication that AQP3 and AQP5 play an important role in tumorigenesis.

As mentioned above, in a parallel study, permeability tests were carried out on RBCs and yeasts to evaluate the inhibitory effect of metallodrugs on AQP activity. From these results, we found that all the compounds tested had a positive effect as aquaporin inhibitors, being the POT-A the more potent, with an IC50 similar to the previously described for Auphen (0.71 µM 0.8 µM respectively). Comparing the results obtained for cell migration in pancreatic cells with those obtained for aquaporin inhibition in RBCs, we can observe that both POT-A and Auphen affect cell migration probably by inhibiting AQP3 activity. Regarding POT-C, it showed to be the less effective as AQP3 inhibitor and also less affected cell migration in BxPC3 cells and MNT-1 cells. POT-C also impaired cell migration speed, suggesting that other participants are involved and should be investigated.

Of the three classes of metallic compounds tested (gold, vanadium and tungstate), all affected BxPC3 and MNT-1 cells, but the most promising were POTs, at a level similar to Auphen.

POT-A and POT-C reduced cell migration in the two cell lines tested. In pancreatic cells, after 24 hours, migration is reduced to 60% in the presence of POT-A and to 80% when cells

are incubated with POT-C compared to control. In melanoma cells, the scenario is equivalent, demonstrating, when compared to control, 68% reduction in migration with POT-A and 88% migration in the presence of POT-C. The biggest difference between gold compounds and POTs is melanoma cell migration, where the presence of gold compounds does not affect migration, after 24 hours all wounds are closed.

In addition, we evaluated the inhibitory effect of POTs in BxPC3 membrane water and glycerol permeability and compared with Auphen. POT-A has been found to reduce glycerol permeability more than 80%, in agreement to the observed effect in BxPC3 cell migration. These data strongly suggest that inhibition of AQP3 is a promising strategy for anti-cancer treatments.

9. Conclusion

This thesis focused mainly on the evaluation of the effect of metallic compounds on cell migration of BxPC3 and MNT-1 cells.

The first part of this work involved the evaluation of AQP3 and AQP5 expression in pancreatic cancer and melanoma cell lines, in which it is concluded that AQP3 is the most expressed aquaporin. Permeability was also evaluated for both cell lines, where MNT-1 cells were found to be more permeable to glycerol. On the other hand, BxPC3 cells are more permeable to glycerol.

In the second part, the effect of hydrogen peroxide on cell viability and migration was evaluated. Up to a concentration of 200 µM, there are no changes in cell viability. After 24h, in the presence of H2O2, migration increases by 50%.

In the third part, the effect of metallic compounds on cell migration was evaluated. In this case, the most promising compound for BxPC3 and MNT-1 cells was POT-A. However, most metallodrugs tested showed a decrease in cell migration; Finally, the effect of some compounds tested on cell permeability was evaluated. In BxPC3 cells, POT-A reduced permeability by more than 80%. In conclusion, as aquaporin inhibitors all the tested compounds are promising candidates for drug development. However, further investigation is needed to evaluate the potency and selectivity of these compounds for specific types of cancer where aquaporins are aberrantly expressed.

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11. Supplementary data

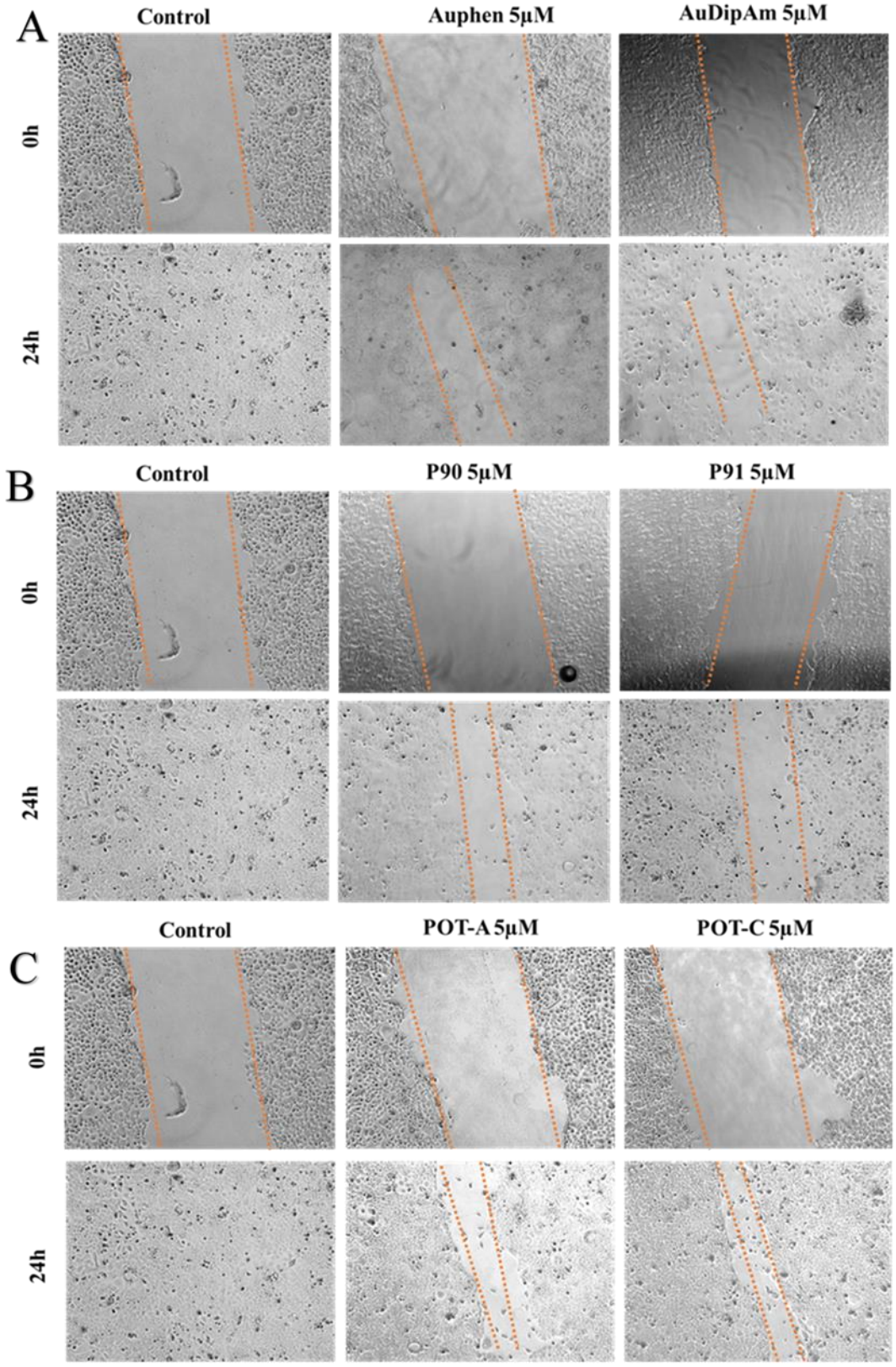

Figure 20 - Supplementary migration figure of BxPC3 cell line. (A) gold compounds; (B) vanadium compounds; (C) polyoxometalates compounds.

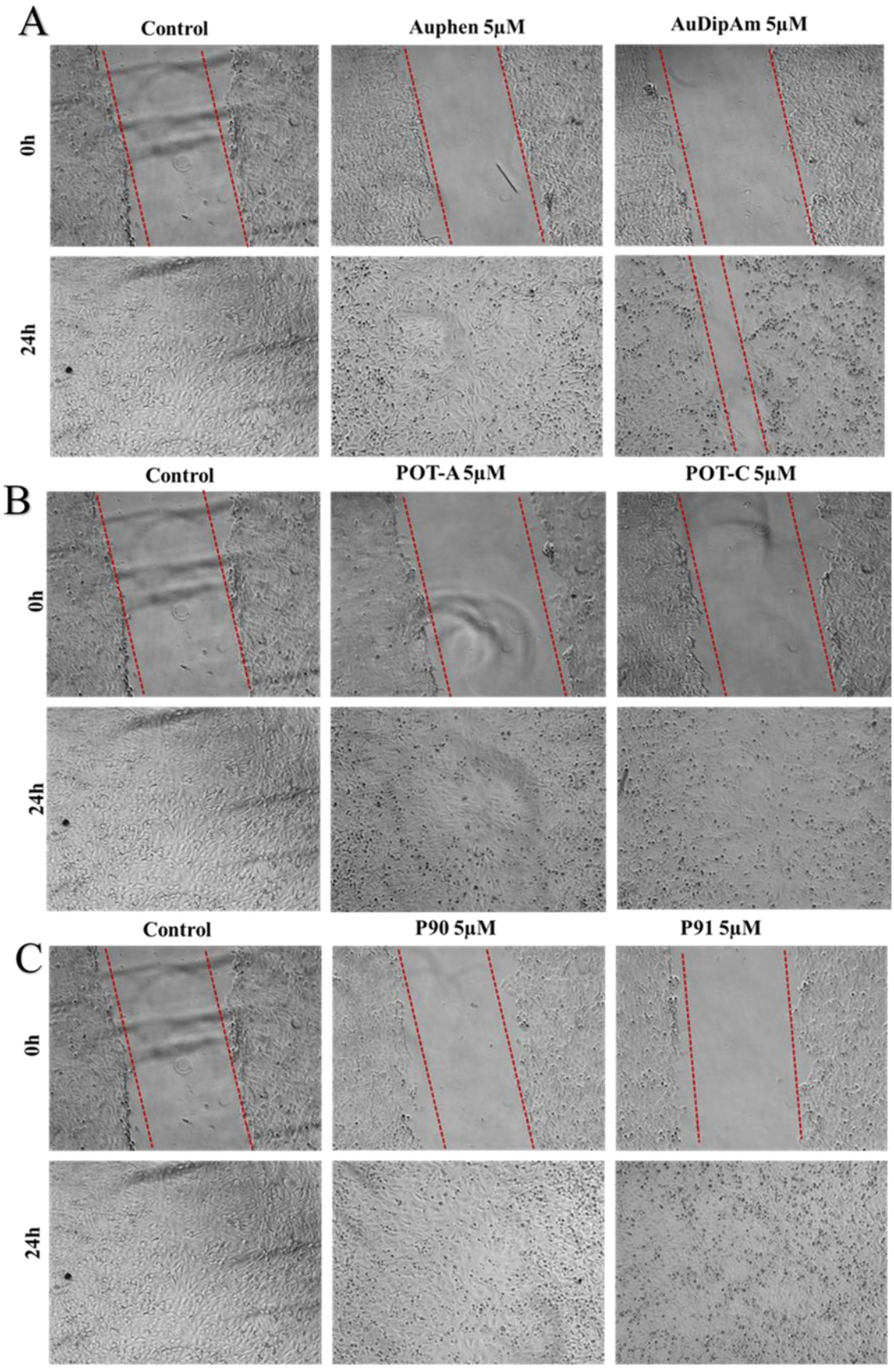

Figure 21 – Supplementary migration figure of MNT-1 cell line. **(A)** gold compounds; **(B)** vanadium compounds; **(C)** polyoxometalates compounds.