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THE ROLE OF HYPOXIA DRIVEN ADENOSINERGIC PATHWAY IN THE MALIGNANT FEATURES OF BLADDER CANCER CELLS

Dissertação no âmbito do Mestrado em Bioquímica orientada pela Doutora Célia Maria Freitas Gomes e pela Professora Doutora Paula Cristina Veríssimo Pires e apresentada ao Departamento de Ciências da Vida da Faculdade de Ciências e Tecnologia da Universidade de Coimbra.

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Maria Margarida Ribeirinho Pereira

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Resumo

Introdução: A hipóxia é uma característica comum dos tumores sólidos e desempenha um papel crítico nas neoplasias malignas, incluindo o cancro da bexiga (CB). O factor induzível por hipóxia-1α (HIF-1α) desempenha um papel importante na regulação da resposta das células tumorais sujeitas ao stress hipóxico que resulta em alterações metabólicas e na ativação de mecanismos de sobrevivência. As células hipóxicas sobreexpressam as ecto-nucleotidases CD39 e CD73 que estão envolvidas na geração de adenosina extracelular. Este nucleosídeo atua como um importante regulador de processos inflamatórios em condições fisiológicas mas no cancro, inibe o sistema imunitário permitindo que as células cancerígenas escapem ao controlo do sistema imunitário. Além disso, existem evidências que associam a ativação da via adenosinérgica em resposta à hipóxia com a agressividade tumoral. No entanto, os mecanismos que estão subjacentes a este processo não estão completamente clarificados.

Objetivo: Explorar o papel patofisiológico da via adenosinérgica mediada por hipóxia nas características malignas do CB e avaliar a contribuição do HIF-1α e da adenosina nesses processos biológicos.

Métodos: Duas linhas celulares humanas de CB, UM-UC3 e HT-1376, foram expostas a hipóxia usando um sistema anaeróbio GasPak^{TM} EZ na ausência e na presença do inibidor do HIF-1α (Digoxina). A expressão do HIF-1α, CD39, CD73, dos receptores de adenosina A2A e A2B e do PD-L1 foi avaliada por Western blot. Os níveis extracelulares de adenosina no sobrenadante foram medidos usando um kit comercial de medição de adenosina. A proliferação celular e a quimiossensibilidade à cisplatina foram avaliadas pelo ensaio de WST-1. A migração celular foi determinada pelo ensaio de *scratch*. Os marcadores de superfície e os fatores de transcrição da transição epitelial-mesenquimal foram analisados por qPCR. Estas experiências foram realizadas em condições de normóxia na presença de adenosina.

Resultados: Ambas as linhas celulares em condições de hipóxia estabilizaram o HIF-1α e ativaram a via adenosinérgica como demonstrado pela sobreexpressão das ecto-nucleotidases CD39 e CD73, produção de adenosina extracelular e sobreexpressão do receptor A2B. A hipóxia diminuiu a susceptibilidade das células de CB à cisplatina e aumentou a expressão do PD-L1, antecipando o desenvolvimento de mecanismos de evasão do sistema imunológico. A hipóxia induziu a transição epitelial-mesenquimal em ambas as linhas celulares e aumentou a capacidade de migração das células HT-1376, mas não das UM-UC3. O tratamento com adenosina exacerbou as características malignas das células de CB, semelhante às induzidas pelas condições de hipóxia. A Digoxina atenuou a expressão de HIF-1α nas células de CB em

condições de hipóxia sem contudo prevenir a produção de adenosina extracelular e os seus efeitos pro-tumorais.

Conclusão: A hipóxia ativou a via adenosinérgica nas células de CB e promoveu a agressividade tumoral ao interferir com a proliferação celular, quimiorresistência e capacidade de invasão. A adenosina desempenha um papel importante no efeito pro-tumoral da hipóxia. Assim sendo, estratégias terapêuticas incorporando inibidores da via hipóxia-CD39-CD73-A2BR podem ser benéficos no controlo da progressão tumoral e na resposta à terapia.

Palavras-chave: carcinoma da bexiga; hipóxia; adenosina; ecto-nucleotidases; receptores da adenosina.

Abstract

Introduction: Hypoxia is a common feature of solid tumors and a critical hallmark of malignant disease, including bladder cancer (BC). The hypoxia-inducible factor-1α (HIF-1α) is a crucial regulator of cancer cells response to hypoxia stress and results in metabolic changes and activation of survival mechanisms. Hypoxic cells upregulate the ecto-nucleotidases CD39 and CD73 that are involved in the generation of extracellular adenosine. This nucleoside in physiological conditions acts as an important regulator of inflammatory processes but in cancer, dampen the immune system allowing cancer cells to escape the immune control. Moreover, accumulating evidences suggest a link between the adenosinergic response to hypoxia and tumor aggressiveness. However, the mechanisms behind this process are not completely clarified.

Objectives: To explore the pathophysiological role of the hypoxia-driven adenosinergic pathway on the malignant features of BC and to unravel the contribution of HIF-1α and of adenosine in those biological processes.

Methods: Two human BC cell lines, UM-UC3 and HT-1376, were exposed to hypoxia using the GasPak ez anaerobe system in the absence and in the presence of a HIF-1α inhibitor (Digoxin). The expression of HIF-1α, CD39, CD73, adenosine receptors A2A and A2B and of PD-L1 were measured by Western blot. Extracellular levels of adenosine in supernatants were measured using an adenosine assay kit. Cell proliferation and chemosensitivity to cisplatin were evaluated using the WST-1 assay. Cell migration was assessed via a wound-healing assay. Epithelial-to-mesenchymal transition surface markers and transcription factors were detected by qPCR. A parallel set of experiments were conducted under normoxic conditions in the presence of adenosine.

Results: Both cell lines under hypoxic conditions stabilized HIF-1α and activated the adenosinergic pathway as shown by the upregulation of CD39, CD73 and extracellular generation of adenosine, accompanied by an upregulation of the A2B receptor. Hypoxia impaired the susceptibility of BC cells to cisplatin and upregulates the expression of PD-L1, anticipating the development of an immune escape mechanism. Hypoxic stress induced epithelial-to-mesenchymal transition in both cell lines and increased the migratory rate of HT-1376 cells, but not of UM-UC3 cells. Treatment with adenosine exacerbated the malignant features of BC cells in a fashion similar to those induced by hypoxic conditions. Digoxin attenuated the protein expression of HIF-1α in BC cells under hypoxic conditions without preventing the extracellular production of adenosine and its pro-tumoral effects.

Conclusion: Hypoxia activated the adenosinergic pathway in BC cells and promoted tumor aggressiveness by interfering with cell proliferation, chemoresistance and invasiveness. Adenosine has a high contribution to the tumor-promoting effects of hypoxia. Therefore, therapeutic strategies incorporating inhibitors of the hypoxia-CD39-CD73-A2BR pathway might be beneficial in controlling tumor growth and response to therapy.

Key words: bladder cancer; hypoxia; adenosine; ecto-nucleotidase; adenosine receptors.

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List of acronyms

ADA	Adenosine Deaminase
ADO	Adenosine
APS	Ammonium Persulfate
ARNT	Aryl Hydrocarbon Nuclear Translocator
ATP	Adenosine Triphosphate
BC	Bladder Cancer
BCA	Bicinchoninic Acid
bHLH-PAS	Basic Helix-loop-helix-Per-ARNT-Sim
BSA	Bovine Serum Albumin
CD39	Ectonucleotidase Triphosphate Diphosphohydrolase
CD73	Ecto-5'-nucleotidase
CIS	Cisplatin
CNT	Cation-linked concentrative Nucleoside Transporters
CT	Computed Tomography
CXCR4	C-X-C Motif Chemokine Receptor 4
DC	Dendritic cell
DTT	Dithiotreitol
ECL	Enhanced Chemiluminescence
ECM	Extracellular Matrix
EDTA	Ethylenediaminetetraacetic Acid
EMT	Epithelial-to-mesenchymal Transition
ENT	Equilibrative Nucleoside Transporter
FBS	Fetal Bovine Serum

FIH	Factor Inhibiting HIF
HDI	Human Development Index
HIF-1α	Hypoxia Inducible Factor-1 alpha
HRE	Hypoxic Response Elements
IC_{50}	Half Maximal Inhibitory Concentration
IP_3	1,4,5-triphosphate
LOX	Lysyl Oxidase
MET	Mesenchymal-to-epithelial Transition
MIBC	Muscle-invasive Bladder Cancer
MMP	Metalloprotease
MRI	Magnetic Resonance Imaging
NAD^+	Nicotinamide Adenine Dinucleotide
NAT2	N-acetyltransferase 2
NFDM	Non-fat Dry Milk
NMIBC	Non-muscle-invasive Bladder Cancer
ODDD	Oxygen-dependent Degradation Domain
PBS	Phosphate Buffered Solution
PD-1	Programmed Cell Death-1
PD-L1	Programmed Death-Ligand 1
PHD	HIF Prolyl Hydroxylase Domain
PKC	Calcium Dependent Kinase
PLC	Phospholipase C
PVDF	Polyvinylidene Difluoride Membrane
pVHL	Von-Hippel Lindau protein

qPCR	Real Time-Polymerase Chain Reaction
RIPA	Radio Immuno Precipitation Assay Buffer
ROS	Reactive Oxygen Species
SAH	S-adenosylhomocysteine
SCC	Squamous Cell Carcinoma
SDF-1	Stromal Cell-derived Factor-1
SDS	Sodium Dodecyl Sulphate
SDS-PAGE	Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis
Snail	Zinc Finger Protein SNAI1
TBS	Tris Buffered Solution
TEMED	Tetramethylethylenediamine
TCC	Transitional Cell Carcinoma
TNM	Tumor, Node, Metastasis
TURBT	Transurethral Resection
Twist	Twist-related Protein 1
VEGF	Vascular Endothelial Growth Factor
WST-1	4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate
ZEB1	Zinc Finger E-box-binding Homeobox 1
ZO1	Zonula Occludens 1

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Chapter 1

Introduction

1.1 Bladder cancer

1.1.1 Epidemiology and risk factors

Bladder cancer (BC) is the 10th most incident cancer worldwide, with approximately 550 000 people diagnosed in 2018, which corresponds to 3% of all new cancer diagnosis. In Portugal is the 4th most common cancer among men and the 11th most common cancer among women, and killed about 1000 patients in 2018 (Figure 1.1). [1,2,76]

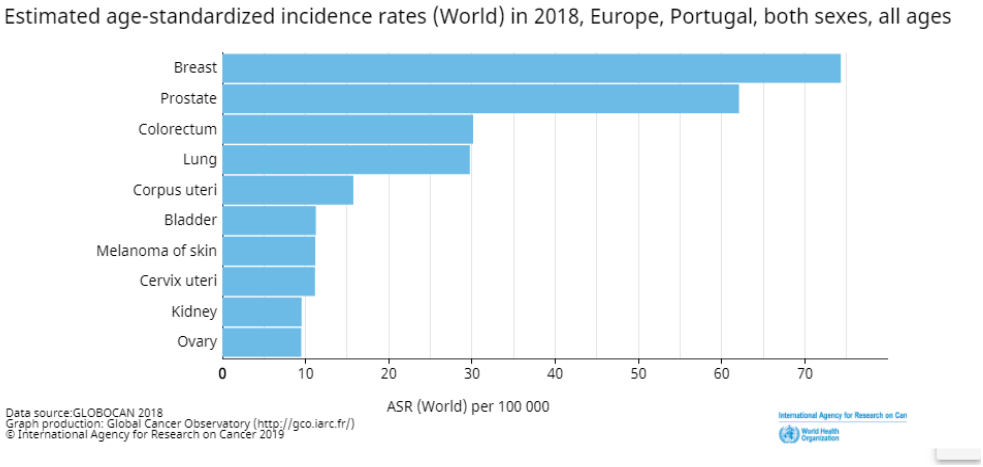

Figure 1.1 – WHO statistics on the most incident cancers in Portugal in the year 2018 (Adapted from Global Cancer Observatory)

The lower urinary tract is divided in the urinary bladder and urethra. Bladder is a hollow smooth muscle organ whose main purpose is to store urine received from the kidneys filtration until micturition. It consists in several layers, including a muscular wall and a mucous membrane. Urothelial cells, specialized cells that lining the urinary tract, hold the volume of urine and are in a constant danger of suffer mutations that could lead to cancer, since bladder is a reservoir organ exposed to multiple nefarious agents that are filtered into the urine. [77,78]

BC has a worldwide incidence rate three times higher in men when compared to women. This gender discrepancy is associated with the higher exposition to potential carcinogens involved in the development of the malignancy, namely the differential rates of tobacco smoking. [3,4,9]

The majority of cases are diagnosed above 50 years old and the average age at diagnosis is 73 years old. Several studies revealed that the development of this disease occurs decades after the exposure to carcinogenic agents, which indicates that mutagens need to overcome multiple cellular tumor-suppressor mechanisms before it culminates in carcinogenesis. However, BC appears also in children and young adults, but usually in less aggressive forms. [3,5,79]

This pathology is more incident in developed countries and has a higher incidence in white race individuals. BC is highly correlated with human development index (HDI), due to higher industrial chemical exposure and easy access to tobacco by individuals from countries with above average on HDI. [4,80]

Incidence of BC is related with genetic factors, lifestyle, diet, environmental pollution and chronic infections. [4] The main risk factor for the development of this pathology is tobacco, accounting for nearly 50% of new cases each year. Tobacco smoke contains a set of compounds that can modify cell cycle through activation of multiple pathways that can culminate in uncontrolled cell proliferation. These compounds promote inflammation and genetic mutations, leading to carcinogenesis. Some of these compounds are β-naphthylamine and polycyclic aromatic hydrocarbons, which are excreted into the urine and potentially carcinogenic to the bladder. [6,81]

The second main cause of BC is occupational exposure to urothelial carcinogens, including aromatic amines and polycyclic aromatic and chlorinated hydrocarbons. These compounds can be found in industrial productions of paints, dyes, metal, rubber and petroleum products. Occupational exposure is responsible for 18% of BC new cases; although, it takes decades after exposure until the disease develop. [4,82]

Genetic abnormalities that affects N-acetyltransferase 2 enzyme (NAT2) are also an important risk factor, since this enzyme acts as an acetylator and converts carcinogenic compounds in acetylated compounds, less carcinogenic. There are two variants of this enzyme: a rapid variant and a slow variant, wherein the gene that codifies slow NAT2 is autosomal dominant. Individuals expressing slow NAT2 have difficulties in degrade carcinogenic compounds excreted by the urinary system. Mutations in MYC, a cell-signaling molecule and oncogene, and GSTM1, an enzyme involved in detoxification of environmental carcinogens, are also genetic alterations that increase susceptibility to develop BC. Individuals with Cowden's syndrome and Lynch syndrome have higher predisposition to BC; these syndromes are associated with tumor-suppressor genes and repair mechanisms and these patients are usually good candidates for checkpoint inhibitor immunotherapy. [7,83-86]

Although not statistically relevant, high alcohol, red meat and processed meat consume could increase the risk of BC. Obesity is also a risk factor, since leads to insulin production that modifies cell proliferation and apoptosis, and still promotes chronic inflammation. [87-89]

Schistosomiasis is the main cause of BC in developing countries, especially in African countries. This parasitic disease is caused by *Shistosoma haematobium* which colonize host blood vessels and releases thousands of eggs that are excreted into the urine, leading to a chronic infection. Furthermore, the infection may promote inflammation, synthesis of N-nitrosamines and free oxygen radicals (ROS). [8,90]

On the other hand, the frequent ingestion of liquids shows a protector effect because of the effect on voiding. The ingested fluids dilute the urine and increase the frequency of voiding, which decrease the potential effects of carcinogens on bladder. Intake of selenium, vitamins A, D and E, and folate are also associated with decrease in BC incidence. [10,91]

1.1.2 Symptoms and diagnosis

The most frequent symptoms of BC are painless macroscopic hematuria (presence of erythrocytes into the urine), accounting with 70% of patients presenting this symptom. Other symptoms correlated with non-specific lower tract infections include urgent and frequent urge to urinate and irritation to voiding, although they can be ambiguous and frequently associated with other diseases, hindering the diagnosis. Cystitis (chronic inflammation), dysuria (difficulty in urination) and nocturia are also commonly observed. In patients with advanced disease, upper tract obstruction and pain may occur. [11,12]

In patients with suspected BC, serum blood urea nitrogen and creatinine levels are evaluated by laboratory analysis. In case of suspected metastatic cancer, a complete blood count and metabolic panel is accomplished. Cytology is a non-invasive method for detection of BC based on the microscopic examination of urine samples for tracking malignant cells. It has a great sensibility to high-grade urothelial tumors and carcinoma in situ, however fails in detect low grade tumors and recent lesions. Despite that, is used in the follow-up of BC patients, combined with cystoscopy. [13,14,93]

Transabdominal ultrasound is commonly used to investigate hematuria and bladder associated lesions. However, its use is patient dependent, so a negative result does not exclude bladder tumor. [93]

Computed tomography (CT) allows detecting masses in the urothelium; however its role in detecting small lesions and evaluating the depth of tumor invasion is poor. Magnetic resonance imaging (MRI) is a promising tool, since allows to detect tissue differentiation, facilitating the distinction of noninvasive from invasive disease; however, it remains in study the advantages of using this technique in BC diagnosis. [14,93]

When patients have gross hematuria, a cystoscopy should be accomplished; this is the gold standard test in case of BC suspect, with high sensibility and specificity. It consists in the direct visualization of the bladder using a cystoscope. In case of cystoscopy with abnormal results, transurethral resection should be performed; this allows an improved diagnosis and the removal of small tumors. [13,15,93]

In order to improve diagnosis tools and reduce patient discomfort during examinations, new cancer biomarkers, less invasive, have been investigated in the last years (Table 1.1). Although not every test have been validated for the responsible entities, they represent promising targets for BC detection. [94]

1.1.3 Staging

BC is a heterogeneous disease characterized by different stages and subsequent specific molecular alterations and therapeutic responses to chemotherapy [16, 20]. The most common form of BC is the transitional cell carcinoma (TCC), accounting for 90% of cases in the Western world; however, non-urothelial carcinomas may also be detect, including squamous cell carcinoma (SCC), adenocarcinoma, sarcoma and neuroendocrine tumors. SCC is more common in countries where schistosomiasis is highly present. [77]

TCC can be divided into two grades, according to the depth of tumor lesions and invasion on bladder wall: non-muscle-invasive bladder cancer (NMIBC), accounting for 70% of the patients, and muscle-invasive bladder cancer (MIBC), that affect 30% of diagnosed patients. [77]

When the tumor produces an epithelial layer along the bladder wall, causing a papillary lesion, is classified as stage Ta. However, when the tumor infiltrate the lamina propria is called stage T1; this type of tumor is more likely to progress to muscle invasion, classified as stage T2. If tumor invades perivesical soft tissue is classified as stage T3, and if invades adjacent organs

is stage T4; this stage can be divided into T4a, if tumor invades prostatic stroma, uterus or vagina, and T4b, if invades pelvic or abdominal wall (Figure 1.2). [17-19]

Table 1.1 – Non-invasive bladder cancer biomarkers (Adapted from Batista, Rui, et al. *Diagnostics*, 2020)

Name	**Marker**	**Type of biomarker assessed**	**Sensitivity (%)**	**Specificity (%)**
Cytology	Tumor cells into urine	Cell phenotype	38	98
NMP-22	Nuclear matrix protein involved in mitosis process	Peptides	40	99
BTA Stat and BTA-TRAK	Detection of nCFHrp in urine	Proteins	70	75
UroVysion	FISH probes to chromosomes 3,7, 17 and 9p21	DNA (aneuploidies)	72	83
ImmunoCyt/uCyt+	Fluorescent-labeled antibodies to 3 proteins	Antigens/Metabolites	73	66
Uromonitor	Detects hotspot mutations in *TERTp* and *FGFR3* genes	Tumor cell DNA	74	93
UroSEEK	Targets typical mutations of BC	Tumor cell DNA	95	93
EpiCheck	Analyses 15 DNA methylation	DNA methylation	68	88

markers

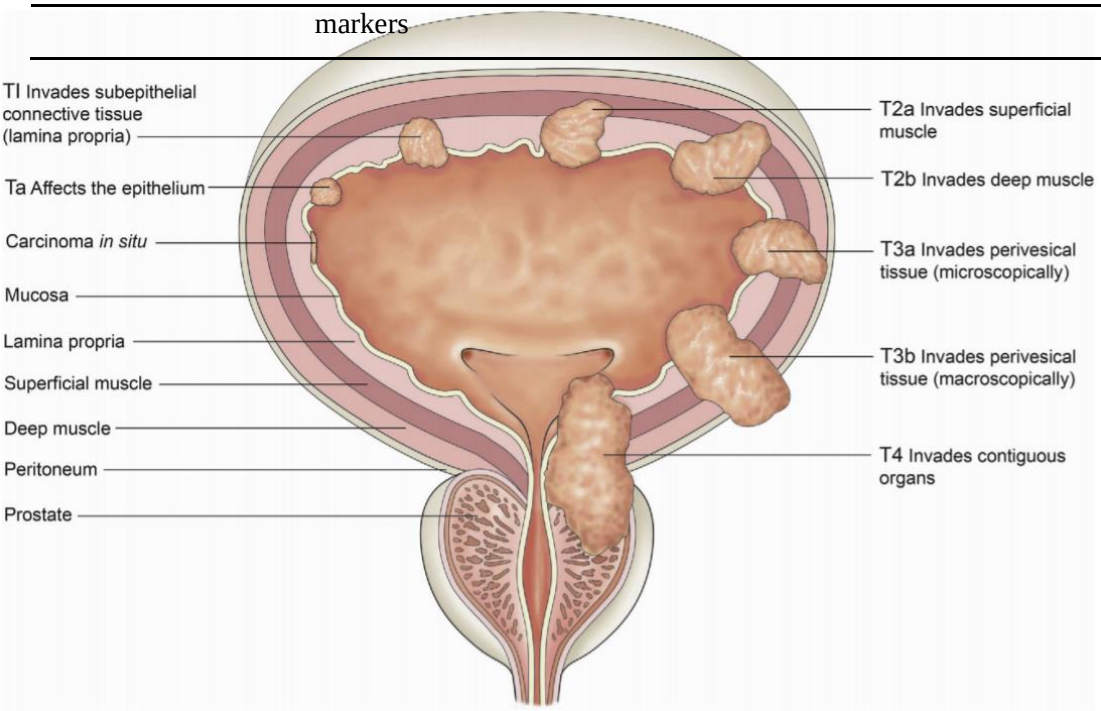

Figure 1.2 – Bladder staging according to TNM system (Adapted from Mushtaq, Jameel et. al, *Surgery*, 2019)

TCC can also be classified according to their ability to invade lymph nodes or develop distant metastasis (Table 1.2). Lymphatic invasion occurs via the iliac, presacral, obturator and para-aortic lymph nodes. About 10-15% of patients with BC present metastasis at diagnosis, with the most common form of metastatic disease in BC being bone metastasis, accounting approximately 40% of metastatic patients; however, cancer cells may also spread to the liver, lungs and adrenal glands. [18,77,]

Although the majority of BC patients are diagnosed with NMIBC, nearly 80% of these patients have at least one recurrence and 30% progress to MIBC. BC is the 13th most deadly cancer worldwide, corresponding to 2.1% of all cancer deaths. Mortality rates are higher in countries where schistosomiasis infections increase incidence rates. Despite the substantial increase in incidence, there has been a decrease in mortality. [80]

Patients with carcinoma in situ have a five-year survival rate of 96%. The survival rate drops with the progress of the disease. For localized disease is 70%, 35% for regional disease and only 5% for metastatic disease. These values reflect the need to improve diagnostic tools, in order to detect early lesions, since poor prognosis is correlated with disease progression. [77]

Table 1.2 – Staging system TNM from American Joint Committee on Cancer (AJCC) (Adapted from Magers *et al, Histopathology*, 2019)

Primary tumor (T)		
TX		Primary tumor cannot be assessed
T0		No evidence of primary tumor
Ta		Non-invasive papillary carcinoma
Tis		Carcinoma in situ
T1		Tumor invades subepithelial connective tissue (lamina propria)
T2		Tumor invades muscularis propria
	T2a	Tumor invades superficial muscularis propria
	T2b	Tumor invades deep muscularis propria
T3		Tumor invades perivesical tissue
	T3a	Microscopically
	T3b	Macroscopically
T4		Tumor invades prostatic stroma, seminal vesicles, uterus, vagina, pelvic wall, abdominal wall
	T4a	Tumor invades prostatic stroma, uterus, vagina
	T4b	Tumor invades pelvic wall, abdominal wall
Regional lymph nodes (N)		
NX		Lymph nodes cannot be assessed
N0		No lymph node metastasis
N1		Single regional node metastasis in the true pelvis
N2		Multiple regional lymph node metastasis in the true pelvis
N3		Lymph node metastasis to the common iliac lymph nodes
Distant metastasis (M)		
M0		No distant metastasis
M1		Distant metastasis

1.1.4 Therapeutic approaches

Treatment guide for BC is applied in agreement with tumor stage, namely if its muscle invasive or not [21]. Treatment of NMIBC consists in transurethral resection (TURBT) followed by immunotherapy with Bacillus Calmette-Guerin (BCG) vaccine or intravesical chemotherapy using mitomycin-C (MMC). BCG strongly binds to tumor cells due to presence of fibronectin receptors in bacilli surface and stimulates an antigen-mediated immune response against cancer cells. In other hand, MMC is an antibiotic chemotherapeutic agent able to inhibit DNA synthesis and promote cell death. Both MMC and BCG reduce the risk of recurrence and progression after TURBT, and are recommended as routine treatment. [15, 22, 23]

According to the risk of progression to MIBC, NMIBC can be divided into three groups, based on prognostic and specific follow-up. Despite the risk, all TURBT performed to NMIBC should be follow-up by a cystoscopy examination 3 months after treatment. Low-risk tumors should perform a cystoscopy at 12 months and if the patient is disease free at this stage, annually for 5 years. Intermediate-risk tumors should have cytology at 3 months and then cystoscopy is recommended at 3-6 monthly intervals for 5 years and then annually. In case of high-risk tumors, cytology should be performed at 3 months. If the patient receive a negative result, should have cystoscopy and cytology 3 monthly for 3 years, 6 monthly for 5 years and then 12 monthly thereafter. [95]

Patients with MIBC are submitted to radical cystectomy (bladder removal) followed by cisplatin-based neoadjuvant chemotherapy, to reduce the risk of recurrence and to improve 5-year survival rate. Most patients develop complications after urinary diversion, including obstruction and infections, potentially fatal. Recurrence rate of MIBC is 48.6%, 20 years after radical cystectomy. Tumor recurrence occurs most of the times locally in the pelvis, upper urinary tract and urethra. Some patients may develop lung, liver or bone metastasis. [96]

Cisplatin (CIS) is a metal-based chemotherapeutic drug that diffuses across the plasma membrane and form DNA adducts and inter-strand crosslinks, leading to cell damage and death apoptosis. Moreover, induce mitochondrial damage via generation of ROS and induce changes in membrane organization and fluidity that activate apoptotic pathway. [97-100]

When bladder is preserved, patients are submitted to a combination of chemotherapy with radiotherapy. Patients with metastatic disease are treated with palliative chemotherapy, to increase survival up to 12-14 months. Non-urothelial bladder cancer is treated with cystectomy or radiation. [22,24,25]

Despite cisplatin-based chemotherapy is the first-line treatment for MIBC, not all patients are eligible for this therapy, due to the high secondary effects. Patients with impaired renal function, bilateral obstruction of the upper urinary tract, intractable hematuria or severe lower urinary tract symptoms are not suitable for neo-adjuvant chemotherapy and have poor prognosis. Despite that, not all BC are chemosensitive to cisplatin, which leads to a need to develop new effective anti-tumor therapies to treat this type of pathology. [24, 26,77]

Immunotherapy has been applied to BC patients for several years, with the intravesical infusion of BCG. Recently a new immunotherapeutic approach targeting the PD-1/PD-L1 axis was approved to treat BC patients not responding or not eligible to first-line cisplatin-based chemotherapy. [29,30]

Programmed cell death protein 1 (PD-1) is a transmembrane protein expressed on T cells, B cells, NK cells, activated monocytes and DCs and limits T cells function in peripheral tissues. PD-1 is involved in cytokine production and cell proliferation, and inhibits transcriptional factors associated with effector T cells. This receptor has two main ligands, PD-L1 and PD-L2, both transmembrane proteins that after PD-1 ligation on T cells delivers an inhibitory signal that suppress T cells activation. PD-L1 is often upregulated in tumor cells and inhibits T cells activation after binding to PD-1 receptor. [28, 31]

High levels of PD-L1 have been found to correlate with the overall poor outcome in BC patients being associated with progression to high-grade tumors, recurrence and metastasis. Nevertheless, BC is the type of tumor that benefits from immunotherapy with immune checkpoint inhibitors, showing clinical benefits. Several monoclonal antibodies are currently approved for clinical use in the treatment of BC as listed in Table 1.3 and many other are in clinical trials. [104,105]

Despite the exciting results of the anti-PD-1/PD-L1 therapies only 30% of BC patients expressing PD-L1 demonstrated clinical benefits. This suggests that there are other mechanisms behind the expression of PD-L1 that can compromise the efficacy of checkpoint inhibitors. [28, 32]

Table 1.3 – Approved monoclonal antibodies for bladder cancer immunotherapy. (Adapted from J. Bellmunt et al., Cancer Treatment Reviews, 2017)

Treatment	Mechanism	Population
Atezolizumab	Monoclonal antibody that inhibits PD-L1	Inoperable, platinum-treated, locally advanced/metastatic carcinoma
Nivolumab	Monoclonal antibody against PD-1	Progression to metastatic disease after 1 year of platinum chemotherapy
Pembrolizumab	Monoclonal antibody targeting PD-1	Previously treated metastatic cancer
Durvalumab	Monoclonal antibody inhibiting PD-L1	Inoperable/metastatic cancer

1.2 Hypoxia

Tumor microenvironment is a dynamic and complex milieu that surrounds and interacts with tumor cells and influences the malignant behavior and progression of cancer. It is composed of tumor cells and non-malignant stromal cells including cancer associated fibroblasts, endothelial cells, adipocytes and infiltrating immune cells and also extracellular matrix components, that overall creates a specific environment favorable to tumor growth and progression. Cell populations in the tumor microenvironment are in permanent dynamic interactions adapting its behavior to the environmental changes, such as the metabolic status that is influenced by the O_2 content, pH deregulation or nutrients availability. [33,34,106]

Solid tumors after reaching a certain size (> 50 μM) are characterized by the presence of hypoxic regions with low oxygen content, arising from an imbalance between increased oxygen consumption and inadequate oxygen supply, as a consequence of the rapid cancer cell proliferation and limited access to oxygen (Figure 1.3). Hypoxia is caused by poor blood flow and limitations in oxygen diffusion resulting in an inefficient vascularization and scarcity of nutrients. Hypoxic cells have genetic instability leading to resistance to apoptosis and impairment on DNA repair mechanisms. [106]

Cells with permanent oxygen failure provoked by prolonged vascular inadequacy are exposed to chronic hypoxia and repeated inflammation, while cells in acute hypoxia are characterized by temporary low intracellular oxygen levels followed by reoxygenation. This dynamic fluctuation in oxygen promotes distinct biological effects and biochemical reactions, such as changes in epigenome, signaling pathways and homeostatic mechanisms that will ensure cell survival under hypoxic stress conditions. [106,107]

The hypoxic response is mainly ascribed to hypoxia-inducible factors (HIFs) which are transcription factors that promote the expression of certain genes necessary for cells to adapt to hypoxic environments. In fact, oxygen-sensitive genes targeted by HIF are involved in adaptation to lack of oxygen. However, repeated activation of these genes can result in several pathologies. [35,36]

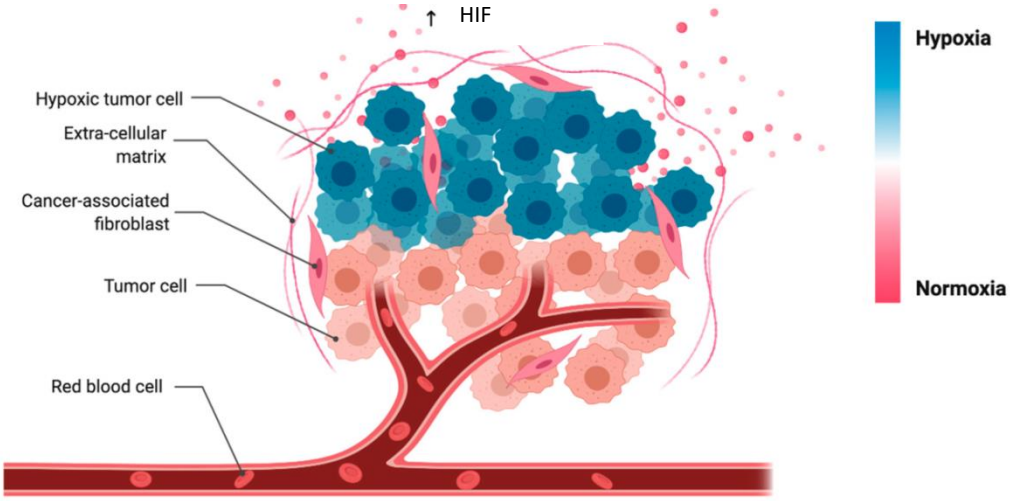

Figure 1.3 – Hypoxic tumor microenvironment. (Adapted from Vito, Alyssa et. al, *Cells*,2020).

1.2.1 Hypoxia-Inducible Factor 1-Alpha

HIF-1 is a major regulator of oxygen homeostasis within cells. It is a heterodimeric transcription factor synthetized in the cytosol, constituted by an oxygen-dependent α-subunit and a constitutively oxygen-independent β-subunit. [37]

There are three isoforms of the oxygen-labile α subunits, HIF-1α, HIF-2α or HIF-3α, and an oxygen-insensitive β subunit, HIF-1β, also known as the aryl hydrocarbon nuclear translocator (ARNT). Both HIF-1α and HIF-1β belong to the basic helix-loop-helix-Per-ARNT-Sim (bHLH-PAS) protein family, since bHLH and PAS motifs are necessary for heterodimer formation and binding to hypoxia response elements (HRE). HIF also possesses two transactivation domains, N-TAD and C-TAD, this last one with particular interest in binding to CBP/p300 coactivactor to activate transcription. Despite that, HIF-1α also contains an oxygen-dependent degradation domain (ODDD), implicated in stabilization of HIF in normoxia. HIF-2α and HIF-3α share structural similarities with HIF-1α, but their distribution along the body is not continuous, being predominantly expressed in certain tissues over others. [108-111]

The regulation of HIF-α subunit stability is dependent on an adequate supply of oxygen, while HIF-1β is constitutively expressed regardless of oxygen levels. HIF-1α is constantly synthesized, but rapidly degraded under normoxic conditions. In hypoxia, the absence of cellular mechanisms capable of HIF-1α degradation allows the stabilization of the protein. HIF-1α degradation succeeds after several post-translational modifications, such as hydroxylation, ubiquitination, acetylation and phosphorylation. [111-113]

In normoxia, von-Hippel Lindau protein (pVHL) ubiquitinates HIF-1α leading to its proteasomal degradation (Figure 1.4). Interaction between α subunits and VHL requires hydroxylation of proline residues in the ODDD and is catalyzed by HIF prolyl hydroxylase domain family enzymes (PHDs). There are three PHD enzymes capable to hydroxylate HIF-1 and 2α, but PHD2 is the primary and key enzyme in this reaction and in the control of HIF-1α degradation. Furthermore, Factor inhibiting HIF (FIH) modulates transactivation domains and hydroxylates HIF-1α in asparagine residues, leading to a decrease in transcriptional activity, by inhibition of CBP/p300. [109,114-117]

Under hypoxic conditions, HIF-1α is expressed, since PHDs and FIH are oxygen dependent. Hypoxia prevents hydroxylation and proteasomal degradation and allows stabilization and accumulation of HIF-1α in cell cytoplasm. Furthermore, the absence of oxygen prevents hydroxylation of asparagine residues and activation of CBP/p300. HIFα is then translocated to the nucleus and heterodimerizes with HIF-1β, forming active complexes. [38-41]

After activation, HIF-1α binds to a 50-base pair HRE located in the promoter region, targeting a large number of genes implicated in several events that favor cell survival and adaptation to lack of oxygen. [42,118]

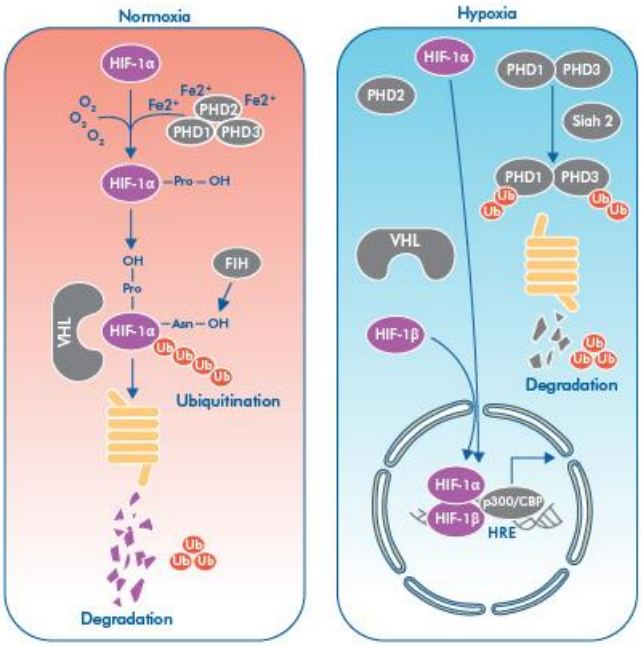

Figure 1.4 – Molecular events involved in HIF-1α degradation in normoxia and stabilization in hypoxia. In normoxia, von-Hippel Lindau protein (pVHL) ubiquitinates HIF-1α leading to its proteasomal degradation, after hydroxylation of proline residues in the ODDD by PHD. FIH hydroxylates HIF-1α in asparagine residues, inhibition CBP/p300 and decreasing transcriptional activity. In hypoxia, PHD and FIH are inhibited and HIF-1α is expressed and heterodimerize with HIF-1β. [119]

The activation of HIF-1α in response to oxygen deficiency promotes tumor aggressiveness by acting on multiple molecular pathways such as angiogenesis, metabolic alterations, invasion, metastasis and immune escape. [42]

The lack of oxygen caused by the restricted distance in which simple diffusion occurs lead to the formation of new blood vessels from pre-existing vessels, a process called angiogenesis. In solid tumors, cells are initially oxygenated by simple diffusion; however when tumor grow beyond the limit of oxygen diffusion, formation of new vessels became necessary. HIF-1α regulates genes involved in angiogenic process, such as Vascular Endothelial Growth Factor (VEGF), the key transcription factor involved in this process. VEGF stimulates angiogenesis either in physiological or pathological situations, leading to tumor vascularization that allows then cell proliferation. Nonetheless, the new vessels formed are often poorly organized and dysfunctional: endothelial cells of tumor-associated vessels have gaps between them which lead to variability in flow velocity and local tissue edema and also decrease the efficiency of drug delivery, compromising chemotherapy. [43,120-122]

Cancer cells undergo metabolic reprogramming wherein the rate of glucose uptake and lactate production increases, even in the presence of oxygen, named as the Warburg Effect. Tumor cell populations are heterogeneous regarding the oxygen availability, with cells surrounding the blood vessels being well oxygenated and utilize oxidative phosphorylation as the main metabolic pathway to produce ATP. However, poorly oxygenated cells without vascularization switch their metabolism from oxidative phosphorylation to glycolysis. Although glycolysis is not such a profitable process it leads to a faster production of ATP. To compensate the hypoxic cellular energy demands, HIF-1α mediated the overexpression of glucose transporters and glycolytic enzymes. Furthermore, these cells are able to inhibit the conversion of pyruvate to acetyl-CoA, and consequently reducing TCA cycle. For example, hypoxia controls the expression of several biomarkers of BC, including GLUT1, the primary glucose uptake transporter, which is upregulated in malignant tissue compared to normal bladder tissue. Overexpression of GLT1 is associated with poor outcome and disease progression, by increasing glucose uptake, contributing to the glycolytic metabolism. [44,92,123,157]

Autophagy can be induced by hypoxia to maintain the cellular metabolic homeostasis. Autophagy is a mechanism by which old and damaged cell organelles are eliminated to promote cell survival. The main triggers of autophagy are nutrient starvation, metabolic stress and hypoxia. Survival of tumor cells under hypoxia has been attributed to autophagy and is correlated with poor prognosis in multiple cancers predictive of resistance to therapy. HIF-1α target genes, such as *BNIP3* and *BNIP3L* responsible for promoting autophagy under hypoxia, increasing cell survival and tumor progression. HIF-1α also promotes mitophagy, autophagy of

mitochondria, leading to the downregulation of oxidative phosphorylation as described before. Mitophagy prevents the accumulation of ROS in cells, maintaining redox homeostasis. [124-126]

Interestingly, recent studies indicate that a decrease in O_2 availability is not accomplished by a decrease in ROS. In fact, cellular metabolism is modulated to maintain redox homeostasis. HIF-1α intervenes in respiratory chain function by switching subunits in cytochrome c oxidase, to maintain the efficiency of electron transfer without an increase in ROS levels in hypoxia. [127-129]

Hypoxic cells are supposedly less proliferative than oxygenated cells. To continue growing under hypoxia, cancer cells need to activate adaptive mechanisms to overcome anti-proliferative signals. Expression of HIF-1α alone is sufficient to induce cell cycle arrest; in fact, HIF-1α induces activation of inhibitor of cyclin-dependent kinases, responsible to regulate cell cycle. [133-135] Alterations on *Myc* expression, an oncogene, are involved in cell proliferation by hypoxic cells. Under normal conditions, HIF-1α suppresses cell proliferation and levels of Myc are low; however hypoxic cancer cells upregulate the expression of Myc, that will overwhelm the effects of HIF-1α, allowing cells to continue proliferating. High levels of both Myc and HIF-1α in cancer cells promote transcription of target genes that encode enzymes that facilitate proliferation. [136,137,140]

The majority of chemotherapeutic drugs applied to cancer therapy causes cell damage by inducing apoptosis or another type of cell death. Apoptotic pathway is regulated by the balance between Bcl-2 family of proteins. Bcl-2 family is divided into pro and anti-apoptotic proteins. During apoptosis, there is a downregulation of anti-apoptotic proteins and an overexpression of pro-apoptotic proteins. Hypoxia regulates Bcl-2 family either at transcriptional level or protein interactions; impairment in Bcl-2 family expression causes resistance to apoptosis. [140,158]

Epithelial-to-mesenchymal transition (EMT) is a reversible biologic mechanism in which cells switch their epithelial phenotype to a mesenchymal-like phenotype. This process requires biochemical changes such as inhibition of epithelial markers and upregulation of mesenchymal markers, loss of cell-cell adhesion and apical-basal polarity, remodeling of extracellular matrix (ECM), reorganization of cytoskeleton and alterations on cell morphology. All these changes lead to an increase in migration capacity and resistance to senescence and apoptosis [141]. There are three types of EMT: the first is associated with embryogenesis and tissue development in fetus. The main objective is to generate different cell types with the same mesenchymal phenotype to occur the opposite process, mesenchymal-to-epithelial transition

(MET). EMT type 2 is important to tissue regeneration and forms fibroblasts that allow recuperation after trauma or inflammatory process. The type 3 of EMT is associated with neoplastic cells in which already occurred genetic and epigenetic alteration, facilitating migration and invasion. EMT helps in tumoral progression, favoring metastization [142-144]. Activation of EMT leads to multiple cellular and molecular events. Cell surface protein complexes constitute cell-cell junctions for epithelial integrity. They are dismantled with subsequent relocalization and degradation of these cellular junctions. This degradation is caused by a decrease in claudin and occluding expression (components of tight junctions) and dispersion of Zonula Occludens 1 (ZO1). Despite that, dissociation of adherent junctions causes degradation of E-cadherin, an epithelial marker, and implies loss of apical-basal polarity. Actin cytoskeleton reorganization induces alterations that allow elongation and motility. Degradation of ECM facilitates cell invasion. Regulation of EMT is divided into regulation of transcription factors and EMT markers. Several protein complexes are implicated in this process with distinct functions, for example Twist1, Snail, ZEB1 and ZEB2 are known transcription factors that regulate EMT and when expressed are correlated with metastatic phenotypes. On the other hand E-cadherin is an epithelial marker while N-cadherin and Vimentin are surface markers that when are overexpressed suggests a mesenchymal-like phenotype. [145,146]

Under hypoxic conditions, the tumor microenvironment activates EMT-triggering pathways, such as transforming growth factor beta (TGF)β and Notch signaling pathways, contributing to tumor progression and metastasis. Activation of TGFβ pathway induces the expression of multiple EMT-associated transcription factors, including Snail and ZEB1. Notch-signaling pathway regulates stem cell renewal and is influenced by hypoxia. Notch activation increases Snail expression and can also interact with TGFβ to induce EMT and to increase motility and invasiveness of tumor cells. HIF-1α intervenes in Twist1, Snail, ZEB1 and ZEB2 regulation and facilitates the switch from an epithelial to a mesenchymal-like phenotype. Subsequently, expression of N-cadherin and Vimentin are amended. Attenuation of E-cadherin expression is induced by HIF-1α via activation of lysyl oxidase (LOX)-Snail pathway. Hypoxia regulates the process of disruption of ECM, by increasing the levels of metalloprotease (MMP), and establishes premetastatic niches, which is correlated with poor prognosis. Despite that, the fact that hypoxia leads to chronic inflammation enhances the expression of inflammatory cytokines, such as IL-1, IL-6 and IL-8, which are frequently associated with tumor progression, metastasis and poor prognosis by participating in hypoxia-induced EMT. Co-expression of both hypoxia and EMT markers is highly correlated as an indicator of worse clinical prognosis in multiple kinds of human cancers. [44, 46]

Finally, HIF-1α also plays an important role in immunosuppression. Hypoxia is capable to induce the expression of PD-L1, an immune checkpoint, since PD-L1 is a target of HIF-1α. This means that under hypoxic conditions, cancer cells have a greater ability to escape surveillance by the immune system. High expression of PD-L1 is commonly associated with poor outcome. [47,48,150,151]

As a common solid tumor, BC presents hypoxic regions and accumulation of HIF-1α. The expression of HIF-1α is considered an adverse prognosis factor in MIBC patients being associated with high rate of recurrences and poorer overall survival rate. In NMIBC, the expression of HIF-α is associated with high risk of progression to a more aggressive phenotype and increased resistance to chemotherapy. BC at advanced stages express a wide variety of hypoxia-related genes involved in immune tolerance, enhanced invasion and cell-matrix interaction. Curiously, overexpression of HIF-1α is considered a potential prognostic marker of disease progression for NMIBC, through its effects in stimulating angiogenesis and metabolic adaptations. In addition to contribute to chemoresistance in BC, hypoxia also promotes resistance to radiotherapy. Drug resistance is associated with expression of several micro-RNAs that participate in multiple mechanisms capable to promote tumor aggressiveness. [71,130,154-157]

1.3 Adenosinergic pathway

Being hypoxia a common feature of solid tumors, oxygen deprivation is associated to metabolic changes that favor tumor progression. One of them is the activation of the adenosinergic pathway that culminates with the production of extracellular adenosine (ADO). Hypoxia induces an increase in extracellular ATP, the primary metabolite of this pathway, that ends with the production of adenosine in the tumor microenvironment. HIF-1α is a strong inducer of adenosine-generating enzymes CD39 and CD73 which catabolize ATP to adenosine [75].

Describing in more detail, ATP works as a cellular messenger, through the ionotropic (P2X) or metabotropic (P2Y) purinergic receptors. [171, 172] Cells release ATP after exposure to physical or chemical stress, including mechanical distortion, membrane damage, hypoxia or treatment with cytotoxic agents. The ATP release from cells occurs in a controlled manner through several mechanisms, such as exocytosis of vesicles containing ATP, secretion through

pannexin chanel or hemi-channel connexins, transporters of the ATP-binding cassette and P2X7 receptors. Autophagy is also necessary for optimal ATP release. [173,175]

CD39, also known as ecto-nucleoside triphosphate diphosphohydrolase, is a cell surface enzyme with a catalytic site on the extracellular side. CD39 binds to extracellular ATP, previously released, and converts it to AMP. CD39 is regulated under hypoxic conditions by Sp1, a ubiquitously expressed transcription factor implicated in hypoxic gene transcription by binding to a site in CD39 promoter. However, HIF-1α is also capable to regulate the expression of CD39 in hypoxia. [131,132,138,139] Ecto-5'-nucleotidase, commonly known as CD73, is the critical enzyme of this metabolic process and a glycosyl phosphatidyl inositol (GPI)-anchored protein capable to convert AMP to adenosine. CD73 is a HIF-1α target gene and has a HRE binding site in the promoter. The hypoxia-promoted adenosine accumulation is the result of extracellular catabolism of released ATP by hypoxia-sensitive ecto-nucleotidases leading to a rise in the extracellular levels of adenosine (Figure 1.5). [53,167,168]

Adenosine (ADO) is a purine nucleoside with a key role in regulating inflammatory responses in different tissues and preventing the auto-immunity and tissue damage. ADO participates in several aspects of cellular physiology, such as neuronal activity, vascular function, platelet aggregation and blood cell regulation. Activation of the adenosinergic pathway within hypoxic tumors dampens the immune system allowing cells to escape the immune control [49-51, 165,166]. Under physiological conditions, biological levels of ADO are very low (< 1 μM); however, during some pathological conditions, such as hypoxia, ADO levels increases in tissues to levels capable to hamper immune cell activation and infiltration (may increase up to 5-100 μM). Moreover, several recent studies suggest a link between the hypoxia-driven adenosinergic pathway and tumor aggressiveness. [49,50,57]

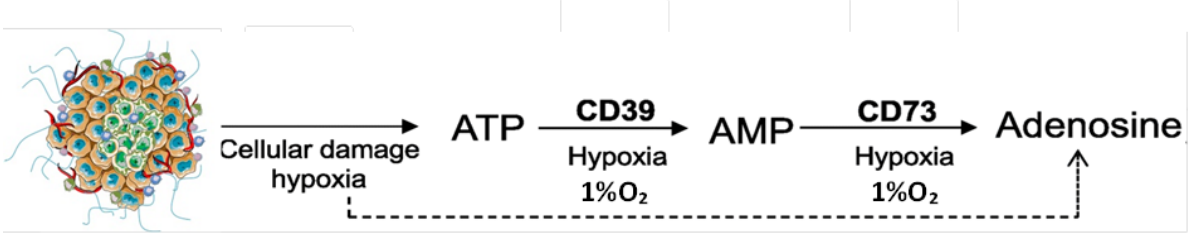

Figure 1.5 – Hypoxia-driven adenosinergic pathway. Hypoxia induces an increase in extracellular ATP. The adenosine-generating enzymes CD39 and CD73 are upregulated in hypoxia and catabolize ATP to adenosine (Adapted from Leone *et al*., *Journal of ImmunoTherapy of cancer*, 2018).

Adenosine binds to P1 purinergic receptors in an autocrine or paracrine manner. There are four receptors for ADO, all G-protein coupled: A1R, A2AR, A2BR and A3R. They are characterized by either high affinity (A1R, A2AR and A3R) or low affinity (A2BR). The high levels of adenosine under pathological conditions are capable to activate the low-affinity A2BR. [52,75]

Adenosine can be produced either in the intracellular medium or in the extracellular medium through another pathway. In the last, known as non-canonical pathway, NAD^+ is converted by NAD^+-glycohydrolase - CD38 - to ADPR, and then converted by CD203a to AMP, which in turn is transformed into ADO. [50, 52]

Intracellularly, AMP is converted to S-adenosylhomocysteine (SAH) by endo-5'-nucleotidase and SAH to adenosine by SAH hydrolase. ADO is then released to extracellular medium via cation-linked concentrative nucleoside transporters (CNTs) and equilibrative nucleoside transporters (ENTs). [169]

ADO has a short half-life (less than 1 second). After uptake, ADO is deaminated to inosine by ADA or phosphorylated to AMP by adenosine kinase (AK). Although ADO can be converted to inosine by ADA, cancer cells have the ability to downregulate this enzyme, which maintains the high levels of ADO in tumor microenvironment. [50,52,170]

1.3.1 Adenosine receptors

Adenosinergic receptors belong to the P1 purinergic receptors family and subdivided into four subtypes (A1, A2A, A2B and A3), according to the cAMP signaling pathway downstream effects. The A2R and A3R trigger signals through the coupled G_i and G_o proteins, which reduce the intracellular levels of cAMP triggering an immune response, while the A2AR and A2BR are coupled to G_s-protein and stimulate cAMP levels conferring immunosuppressive functions. [52,75,176]

A1R is a high affinity receptor mainly present in the central nervous system and mediates the effect of ADO in brain cortex, cerebellum, hippocampus, autonomic nerve terminals, spinal cord and glial cells. The mechanism behind A1R function is through activation of phospholipase C (PLC)-β, increasing inositol 1,4,5-triphosphate (IP_3) and intracellular Ca^{2+} levels. This stimulates calcium-dependent kinases (PKC) and calcium-binding proteins [68,170,177]. In glioblastoma, ADO binding to A1R reduced cell proliferation, increased

chemosensitivity and apoptosis. However, in breast cancer, A1R stimulates proliferation and angiogenesis, which indicates that A1R action is cancer-type dependent. [178,179]

A2AR is expressed on postsynaptic neurons, leukocytes, platelets, vasculature and tumor cells. It plays an important role in blood-brain barrier permeability, immune cell migration, anti-inflammatory response and vasodilatory effects [170,180]. In normal conditions, the release of ADO and its binding to A2AR protects the tissues from an excessive immune response. However, when overexpressed in cancer cells, A2AR acts as mechanism to escape immune system by upregulating PD-L1. Furthermore, when adenosine binds A2AR in tumor cells may promote cell proliferation [68, 69]. A2AR also plays an important role in stimulating Treg responses and inhibiting NK activity, further promoting immune escape and metastasis formation. [170]

A2BR is a low affinity receptor, which means that requires a higher concentration of ADO for activation. Since this receptor is not stimulated by physiological concentration of ADO in tissue during normal conditions, it is associated to pathological conditions, when there is a strong release of ADO [68-70]. A2BR is expressed in several tissues, such as bowel, bladder and lung, and also in different cell types, including fibroblasts, endothelial and immune cells, smooth muscle, platelets and tumor cells. [170]

Recently, it was reported that A2BR has a functional binding site for HIF-α, which means that hypoxia may regulate the expression of this pathological receptor. Signaling through A2BR involves PKA phosphorylation, is activated by cAMP and can stimulate Ca^{2+} mobilization and regulation of ion channels [170,181,182]. A2BR inhibits immune responses, activates M2 macrophages, known to help tumor progression and promotes an anomalous phenotype with pro-angiogenic effect in DCs. A2BR plays an important role in tumor growth, migration and metastasis, particularly because when expressed, A2BR induces angiogenesis by releasing cytokines and growth factors, such as VEGF. ADO binding to A2BR suppresses cell-cell adhesion, facilitating cell migration [68-70,183,184]. A2BR is also involved in EMT and is associated with the appearance of cancer stem cells. Taking together these considerations, A2BR is the receptor most associated with pathological conditions and may be responsible for an aggressive phenotype, increasing spontaneous metastasis. [68-70]

A3R have a selective tissue distribution. Low levels are located in the thalamus, hypothalamus, hippocampus, cortex, retinal ganglion cells, motor nerves and intercerebral arteries. A3R plays a cardioprotective role in coronary and carotid artery and inflammatory cells [68,170]. ADO binding to A3R reduces cAMP levels and induces PLC and calcium, leading to an increase in glycogen synthase kinase-3β. A3R regulates various signaling pathways,

including MAPK, PI3K/Akt and NF-κB [170,185]. It's highly expressed in tumor cells, when compared with normal tissues. Contrary effects have been observed in different tumors; however A3R is associated with the degree of severity of the disease. In fact, A3R is overexpressed in primary and metastatic tumors. Activation of A3R showed an increase in tumor migration and invasion, and also demonstrates an ability to regulate cell cycle and pro and anti-apoptotic effects. [68, 69]

1.3.2 Effects of adenosinergic pathway in promoting tumor progression

ATP has an immunostimulatory effect in tumor microenvironment, since it is capable to induce an immune response against tumor cells. Increasing levels of ATP released by damage cells promotes immunogenic cell death and chemotaxis of monocytes, macrophages and neutrophils to tumor site. Nonetheless, the conversion of ATP to AMP and posteriorly to ADO ends up having an immunosuppressive role and a decrease in the immunogenic effects of chemotherapy-induced cell death. [167,186,187]

Expression of CD39 by T cells is associated with dysfunction and inhibition of $CD4^+$ T cells and suppression of NK cells activity in metastatic tumors. Preventing the depletion of ATP of the tumor microenvironment might be helpful in preserving inflammatory immune responses and the inhibition of CD39 decreases the levels of the immunosuppressive ADO in the tumor microenvironment. [167,188,189]

CD73 is expressed in lymphocyte, endothelial and epithelial cells, and is overexpressed in certain types of tumor cells, including bladder, breast cancer and melanoma [54, 55]. CD73 expression is associated with a poor prognosis, increased risk of relapse and increased probability of metastasis formation. CD73 regulates cell-cell and cell-matrix adhesion, which may have influence in cell migration. Several experiments have demonstrated that the metastatic potential of CD73 is independent of the immune system and is related to adhesion molecules. Silencing of CD73 showed a decrease in VEGF and angiogenic potential of endothelial cells. [56,63,190-192]

There is evidence that CD73 can affect the proliferation of T cells, downregulates NK and $CD8^+$ T cells and contributes to M2 macrophages polarization. Some clinical trials demonstrate that CD73 expression interferes with the anticancer immune response in breast cancer patients treated with anti-HER2 targeted therapeutics [192,193]. In fact, the inhibition of

CD73 and A2BR strongly contributes to an improvement in disease prognosis, by recruiting immune cells to act against tumor cells. Overexpression of CD73 is also correlated with proliferation of cancer cells by regulating cell-cycle. [56, 63]

As described above, the adenosinergic pathway is involved in promoting tumor progression by regulating several important biological processes associated with malignant behavior, including cell proliferation, cell-cycle progression, cell motility and adhesion properties [56,57,159,190,193]. Apart from being one of the most relevant immunosuppressive regulatory molecules in the tumor microenvironment, ADO also plays a role in tumor angiogenesis, through activation of the A2B receptor that mediates the secretion of VEGF, of bFGF and of angiopoietin by endothelial cells. Tumor-derived CD73 also increases VEGF production by tumor cells, contributing for an optimal angiogenic response [54,62]. The role of ADO in tumor progression is dependent on the expression and activity of CD73 in tumor cells, and has been linked with the cancer progression, chemoresistance, migration, and angiogenesis in several tumors such as ovarian, gliomas and breast cancer. [64,65,67]

In the context of BC, the role of adenosinergic pathway is not yet fully understood and deserves investigation. However, some findings associate the activation of this pathway to a more aggressive phenotype. In BC according to tumor stage, the association between prognosis/expression of CD73 differs. In NMIBC, expression of CD73 is considered a favorable prognosis; however, during the course of the disease progression, CD73 expression increases, and is therefore associated with a poor outcome in patients with MIBC. [200-202]

In addition to the role of the this enzyme in the prognosis of the disease, signaling of ADO itself through the A2BR promotes tumor progression in BC, not only by modulating immune responses via the A2A receptor, which is associated with a failure in PD-1/PD-L1 immunotherapy, but also for exerting a pro-tumoral effects in the cancer cells. In fact, high expression of A2BR is correlated with poor outcome and blockade of A2BR inhibits proliferation, migration and invasion of BC cells. Therefore, the adenosine pathway appears to exert a crucial role in promoting BC progression. [50,147-149]

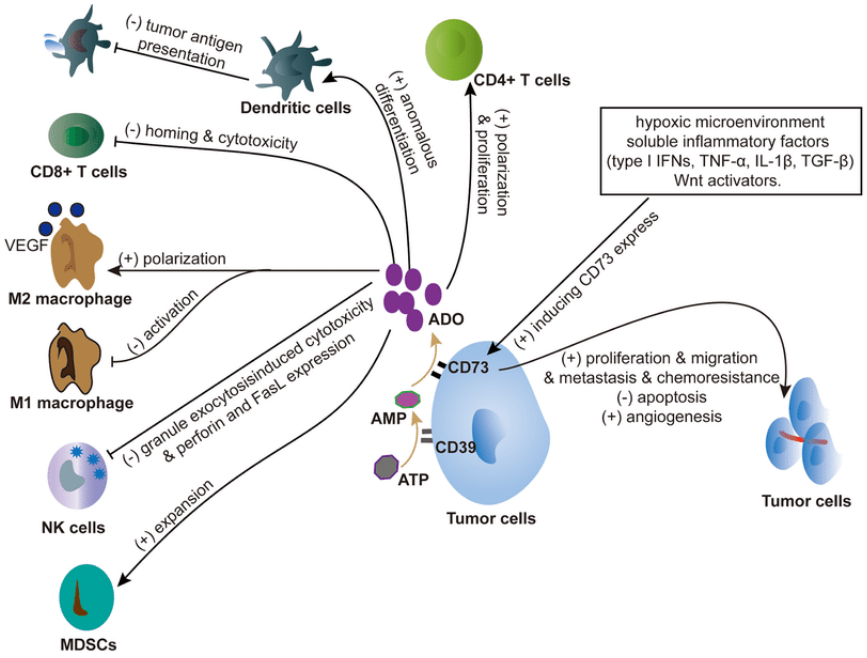

Figure 1.6 – Effects of adenosine on tumor microenvironment (Adapted from Wang et al, Nature 2017)

1.4 Objectives

Hypoxia is a common feature of most solid tumors, being considered an adverse prognostic factor in cancer patients, with negative implications in the disease outcome, and bladder cancer is not an exception. Tumor cells, while adapting to hypoxia, presents metabolic changes through the activation of the adenosinergic pathway and subsequent accumulation of extracellular adenosine in the tumor microenvironment.

The adenosinergic pathway is strongly linked with the inhibition of an immune response, but it is also involved in promoting tumor progression through direct effects on tumor cells. However, despite the large number of studies that link immunosuppression with adenosine, little is known about the specific contribution of this pathway to the malignant features of bladder cancer cells.

The main goal of this project is to explore the role of the hypoxia-driven adenosinergic pathway on the malignant features of BC cells and to investigate the contribution of HIF-1α and of adenosine in those biological processes.

Hereupon, to address these questions, we propose to:

- Evaluate the role of hypoxia in the regulation of adenosinergic pathway in BC cell lines, by measuring the expression of HIF-1α and adenosine-generating enzymes CD39 and CD73 and measuring of extracellular adenosine levels;
- Investigate the role of hypoxia in promoting a more aggressive phenotype in BC regarding cell proliferation, chemoresistance and cell motility;
- Understand the contribution of adenosine in the hypoxia-induced effects in BC cells;
- Evaluate the role of a HIF-1α in the hypoxia-inducible malignant features in BC.

Chapter 2

Methods

2.1 Cell culture

All the assays were performed using two human bladder cancer cell lines, UM-UC3 and HT-1376 (American Type Culture Collection, Manassas, VA, USA). Both cell lines were derived from high grade III transitional cell carcinoma.

Cells were cultured in RPMI-1640 medium (R4130, Sigma-Aldrich), supplemented with 10% (v/v) heat inactivated fetal bovine serum (FBS; 10500, Gibco) and 1% (v/v) antibiotic/antimycotic solution (A5955, Sigma-Aldrich) containing 10 000 units of penicillin, 10 mg streptomycin and 25 µg amphotericin B per mL. Cells were maintained in a humidified atmosphere at 37º C with 5% CO_2. When cells reached an 80-90% confluence they were detached with TrypLE Express (12604-021, Gibco), centrifuged at 1500 rpm during 5 min and subcultivated at an appropriate ratio. All procedure involving cell manipulation was performed in sterile condition inside a laminar flow chamber.

2.1.1 Cell counting

Cell counting and cell viability was determined before each experiment through Trypan Blue exclusion method. In this method, a cell suspension is mixed with trypan blue dye and then visualized at the microscope in order to determine the number of viable cells. Viable cells have intact membranes that prevent cell staining and appear clear at the microscope, while dead cells have a blue cytoplasm.

Equal volumes of trypan blue solution (T8154, Sigma-Aldrich) and cell suspension were mixed (dilution 1:2) and transferred into a Neubauer chamber, containing four quadrants. Cells were observed immediately and counted in the four quadrants using an inverted microscope. It was calculated the average of live cells in each quadrant and then it was applied the following equation to determine the total number of cells *per* milliliter:

$$Number\ of\ cells/mL = Average\ of\ cells\ in\ the\ four\ quadrants \times dilution\ factor \times 10^4$$

Dead cells were also counted in order to determine cell viability. Cell viability was calculated as the percentage of viable cells relative to the total number of cells. Only cell suspensions with viability higher than 90% were used in all the experiment.

2.1.2 Generation of hypoxic conditions

Hypoxic conditions were induced using a BD GasPak EZ Anaerobe Pouch System with Indicator (260683, BD Biosciences). This system consists of a plastic pouch and sachet containing inorganic carbonate, activated carbon, ascorbic acid and water, to scavenge oxygen and generation of an anaerobic environment. To achieve hypoxic conditions, cells were seeded in a dish and were maintained in a 5% CO_2 incubator overnight to adhere, and then placed inside the pouch with the sachet. The pouch was sealed by pressing the zipper and placed in the 5% CO_2 incubator at 37º C during 24 h (Figure 2.1). The sachet is activated by exposure to air and appear white and turns blue when anaerobic conditions are lost. Using this system, the oxygen levels inside the pouch decrease rapidly within 1 h to a percentage of 1% with a CO_2 levels around 10%, mimicking hypoxic conditions.

To evaluate whether the effects of hypoxia on tumor cells were mediated via upregulation of the transcription factor HIF-1α cells were maintained in the Anaerobe Pouch System in the presence of Digoxin. Digoxin is a clinically approved cardiac glycoside used to treat heart failure that inhibits the accumulation of HIF-1α under lower percentages of oxygen [153]. Digoxin was added to the cells at a concentration of 200 nM immediately before being placed inside the plastic pouch.

Figure 2.1 – BD GasPak EZ Anaerobe Pouch System with the Indicator, containing two cell dishes.

2.2 Western blotting

To evaluate whether cells under hypoxic conditions activates adenosinergic pathway, we measure the levels of HIF-1α (NB100-479, Novusbio), of adenosine-generating enzymes CD39 (ab223842, Abcam) and CD73 (D7F9A, Cell Signaling), and of the A2A and A2B adenosine receptors (GTX132217, GeneTex) by western blot. We also analyzed the levels of the immune checkpoint PD-L1 (E1L3N, Cell Signaling).

2.2.1 Preparation of cellular extracts

UM-UC3 and HT-1376 were plated in 100 mm polystyrene cell culture dishes (353003, Corning) at a density of $1.5x10^6$ cells/dish and allowed to attached overnight. Next day cells were maintained under normoxic or hypoxic conditions as previously described in section 2.1.2 using the GasPak EZ Anaerobe Pouch System during 24h.

After the incubation, culture medium was discarded and cells were washed with cold Phosphate buffered saline (PBS; 8 mM NaH_2PO_4 (6346.1000, Merck), 3 mM KH_2PO_4 (1.04873.1000, Merck), 140 mM NaCl (1.06404.5000, Merck), 5 mM KCl (1.04936.0250, Merck), pH = 7.3-7.4), scraped using 3 mL of cold PBS and collected to microcentrifuge tubes. All the procedure was performed with dishes on ice. Then, cells were centrifuged at 1500 rpm, during 10 min at 4º C. The supernatant was discarded and the cell pellets were incubated with 200 µL of lysis buffer RIPA (Radio Immuno Precipitation Assay buffer), containing 150 mM NaCl, 1% Triton X-100 (X-100-500ML, Sigma-Aldrich), 0.5% sodium deoxycholate (D6750, Siga-Aldrich), 0.1% SDS (161-0301, BioRad), 50 mM Tris (T1378, Sigma-Aldrich) (pH=8) and 2 mM EDTA (EDS-100G, Sigma-Aldrich) and a mixture of proteases and phosphatases (04906845001, Roche) inhibitors and 1 mM of dithiotreitol (DTT; D0632, Sigma-Aldrich) during 30 min at 4º C. After incubation samples were sonicated dipped on ice in an ultrasound device (Vibra cell Sonics and Materials Inc. Danbury, CT, USA) at 40 MHz with 3-5 pulses for 5 seconds.

Protein concentration was quantified using the bicinchoninic acid (BCA) method. This method is based in the formation of complexes protein/Cu^{2+} that leads to the reduction of Cu^{2+} to Cu^{+}. The reduction is proportional to the amount of protein present in the sample. Both protein standards with bovine serum albumin (BSA; A9418, Sigma-Aldrich) and samples are incubated with a mixing BCA (B9643, Sigma-Aldrich) reagent and Copper (II) sulfate solution

(C2284, Sigma-Aldrich) for 30 min. Absorbance was read at a wavelength of 570 nm. A standard curve is performed and protein concentration of samples was calculated.

Protein samples were denatured using 4x denaturation solution (0.25 M Tris pH 6.8, 8% (w/v) SDS, 200 mM DTT, 20% (v/v) glycerol and bromophenol blue (B-8026, Sigma-Aldrich)) in a dilution of 1:4. Samples are heated at 100º C during 10 min and were stored at -20º C until usage.

2.2.2 Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Electrotransference

SDS-polyacrylamide gels with 8 or 12% of 30% acrylamide/bisacrylamide solution (E344, VWR), 20% SDS, 10% ammonium persulfate (APS; 0486, AMRESCO) and tetramethylethylenediamine (TEMED; T9281, Sigma-Aldrich) were prepared. Samples with 30-60 μg and a protein marker (Precision Plus Protein All Blue Standards, 161-0373, BioRad) were loaded and the proteins were separated by electrophoresis during 90 min at 110 V in buffer solution [100 mM Tris-HCl, 100 mM bicine (B3876, Sigma-Aldrich) and 0.1% (w/v) SDS] using Bio-rad Mini-Protean Electrophoresis System.

After electrophoresis, proteins were transferred onto a polyvinylidene difluoride membrane (PVDF; 10600023, GE Healthcare) previously activated in methanol (20837.360, VWR). Electrotransference was performed also in an electrotransfer buffer solution [12.5 mM Tris-HCl (pH 8.0-8.5) containing 96 mM glycine (G8898, Sigma-Aldrich) and 20% (v/v) methanol] applying a current of 150 V during 2 h at 4º C in a Bio-Rad Mini-Protean Electrophoresis System.

2.2.3 Immunoblotting and quantification

After electrotransference, to avoid non-specific interactions between the membrane and the antibody, membranes were blocked in 5% (w/v) non-fat dry milk (NFDM) or 5% BSA in Tris-buffered solution T (TBS-T; 20 mM Tris-HCl, 137 mM NaCl, 0.1% (v/v) Tween 20 (437082Q, VWR)) for 1 h with soft agitation at room temperature.

After blocking membranes were incubated with primary antibody overnight at 4º C with appropriated dilutions in 1% (w/v) (A2AR and A2BR) or 5% (w/v) (HIF-1α) non-fat dry milk in TBS-T, or in 5% BSA (CD39, CD73 and PD-L1) in TBS-T.

On the following day, membranes were washed 3 times for 10 min in TBS-T with soft agitation and then incubated for 1 h at room temperature with the appropriate secondary antibody (Immun-Star Goat Anti-Mouse (GAM)-HRP Conjugate, 170-5047, BioRad; Immun-Star Goat Anti-Rabbit (GAR)-HRP Conjugate, 170-5046, BioRad). Membranes were washed again 5 times for 5 min, incubated with ECL reagent (Clarity Western ECL Substrate, 170-5060, BioRad) and revealed using LAS.

After analyzing the levels of proteins of interest, membranes were washed twice for 15 min with TBS-T or stripped using 0.2 M NaOH (1.06498.1000, Merck) and incubated with β-actin primary antibody (8H10D10, Cell Signaling) for 1 h at room temperature, following the protocol previously described. β-actin is a loading protein from the cytoskeletal and is highly conserved in cells, reason why is commonly used as a control.

Protein quantification was performed using Image J software. Band intensity of target proteins was normalized to their corresponding β-actin controls.

Table 2.1 summarizes the conditions used in Western Blotting for each protein measured.

Table 2.1 – Parameters used in Western Blot

Protein	Molecular weight (KDa)	% of acrylamide used in electrophoresis gel	Blocking solution (in TBS-T)	Dilution of primary antibody	Secondary antibody	Dilution of secondary antibody
HIF-1α	93	8	5% NFDM	1:500	Anti-Rabbit	1:10000
CD39	78	8	5% BSA	1:500	Anti-Rabbit	1:10000
CD73	70	8	5% BSA	1:1000	Anti-Rabbit	1:10000
A2AR	42	12	5% NFDM	1:1000	Anti-Mouse	1:10000
A2BR	36	12	5% NFDM	1:1000	Anti-Rabbit	1:10000
PD-L1	40	12	5% BSA	1:1000	Anti-Rabbit	1:10000
B-actin	43	8-12	5% NFDM	1:1000	Anti-Mouse	1:10000

2.3 Adenosine levels measurement

Extracellular levels of adenosine were measured using a fluorometric Adenosine Assay Kit (MET-5090, Cell Biolabs). Adenosine levels are detected based on the conversion of adenosine into xanthine and then in hydrogen peroxide, through the sequential activity of several enzymes. The resulting hydrogen peroxide was detected using a highly specific fluorometric probe. Sample adenosine levels were calculated by comparison with a standard curve between 0 and 100 μM of adenosine.

To perform this assay, BC cells were plated in 6-well-plate (Orange Scientific) in a density of 100 000 cells/mL and allowed to attach overnight. On the following day, medium was removed and replaced by 1.5 mL of fresh medium and cells were incubated in normoxic and hypoxic conditions during 24 h. In order to evaluate if digoxin could inhibit the production of adenosine, cells were maintained under hypoxia during 24 h in the presence of digoxin 200 nM.

After the incubation, conditioned medium was collected and centrifuged at 10 000 rpm during 5 min at 20º C, to remove insoluble particles. Conditioned medium was used directly, without dilutions, and the protocol was performed according to manufacturer instructions.

2.4 WST-1 assay

The WST-1 assay was performed to evaluate the effects of adenosine or hypoxia in BC cell proliferation and also the cytotoxicity of cisplatin in BC cells under normoxic and hypoxic conditions. 4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate (WST-1) is a water formazan dye used to evaluate cells viability by measuring the production of superoxide dismutase. CIS is the most commonly chemotherapeutic drug used to treat BC.

Cells were seeded in 96-well plates (Orange Scientific) at a density of $3.0x10^3$ cells/well and allowed to attach overnight. Next day cells were incubated with ADO (A9251, Sigma-Aldrich) at 10 μM and plated in a CO_2 incubator during 48 h. This assay was also performed in the presence of increasing concentrations of CIS (Teva).

For cytotoxicity assays to CIS, adherent cells were incubated with CIS in a range of concentrations from 0-50 μM during 48 h under normoxic and hypoxic conditions. Proliferation

under normoxic and hypoxic conditions was also evaluated in the same period of time. Stock solutions of CIS were diluted in PBS and stored at -20º C until usage.

To evaluate the effect of digoxin in preventing chemoresistance to CIS, cells were incubated and maintained under hypoxia with digoxin 200 nM during 24 h. Next day, the medium was replaced and the cells were incubated with CIS 10 μM in the presence of digoxin during 48 h.

After the incubation period 10 μL of the WST-1 reagent (05015944001, Roche) were added to each well and the plate was maintained in a humidified atmosphere at 37º C for 3 h. Thereafter, the plate was agitated in an orbital shaker for 1 min to dissolve the crystals and the absorbance was read at a wavelength of 450 nm and 620 nm (reference filter) in an ELISA microplate reader. Values of absorbance were normalized to untreated control. Dose-response curves were performed and half-maximal inhibitory concentration (IC_{50}) was calculated from a non-linear regression analysis using GraphPad Prism Software.

2.5 Scratch-wound healing assay

To evaluate whether hypoxia increases the motility of BC cells, we performed a scratch-wound healing assay, culturing cells under normoxic and hypoxic conditions.

Both UM-UC3 and HT-1376 BC cell lines were seeded in 24-well plates (Orange Scientific) at a density of 350 000 cells/mL, and placed in a CO_2 incubator until reaching 100% confluency. A scratch along the cell monolayer was performed using a 200 μL pipette tip. The medium was removed to eliminate cell debris and replaced by fresh medium. After scratching, cells were cultured under hypoxic or normoxic conditions during 24 h. Images were captured using an inverted microscope at a magnification of 5x, immediately after scratching and after 24 h of wound healing. To evaluate whether the wound-healing under hypoxic conditions is affected by HIF-1α deficiency, cells were incubated with digoxin 200 nM during the 24 h of hypoxia. In another set of experiments, cells under normoxic conditions were exposed to exogenous adenosine at 10 μM during the course of the experiment.

The wound area was drawn and calculated using Image J software. Areas of each experiment were normalized to their respective initial wound healing area. Each time point and condition was captured in triplicate.

2.6 Real Time-Polymerase Chain Reaction (qPCR)

2.6.1 RNA extraction and cDNA synthesis

UM-UC3 and HT-1376 cells were plated in culture dishes at a density of $1.5x10^6$ cells/dish and allowed to attach overnight and then exposed to normoxic or hypoxic conditions using GasPak EZ Anaerobe Pouch System during 24 h, as previously described.

After the 24 h, cells were washed with PBS, detached using trypsin-ethylenediaminetetraacetic acid (tripsin-EDTA; T4049, Sigma-Aldrich) and centrifuged at 1500 rpm during 5 min at 20º C. The supernatant was discarded, and the pellet was transferred to 1.5 mL RNase-free microcentrifuge tube and washed twice with 1 mL of PBS.

RNA extraction was performed using the TRIzol reagent. After washing, the cellular pellet was dissolved in 1 mL of TRIzol (15596026, Invitrogen) and incubated during 5 min with regular vortex. After this period, 200 μL of chloroform (1.02444.2500, Merck) were added to each tube and shacked vigorously during 15 seconds. The mixture was centrifuged at 13 000 rpm during 10 min at 4º C for separation of the aqueous and organic phases.

Aqueous phase, which contains RNA, was transferred to a new RNAse-free microcentrifuge tube, mixed gently with 500 μL of isopropanol and centrifuged at 13 000 rpm, during 10 min at 4º C for the precipitation of RNA.

Supernatant was removed, and the pellet was washed with 500 μL of ethanol 75% and centrifuged at 13 000 rpm, during 5 min at 4ª C. Supernatant was discarded again and the pellet was left to air dry. Pellet was then dissolved in 50 μL of nuclease free water (MB1101, NZYtech) and samples were quantified using nanodrop. RNA quality was evaluated based on $A_{260/280}$ and $A_{260/230}$ ratios. Samples were stored at -20º C until usage.

cDNA synthesis was performed with 2 μg of RNA and using the NZY First-Strand cDNA synthesis kit (MB12501, NZYtech) according to manufacturer instructions in a GeneAmp.PCR system 9700 thermal cycler (Applied Biosystems). The final concentration of cDNA was 10 ng/μL.

2.6.2 Real Time-PCR

Real Time-PCR was performed in duplicate in CFX Connect Real-time PCR detection system (Biorad) using the KAPA SYBR FAST Universal qPCR Kit (KK4602, KAPA Biosystems) in a final volume of 12.5 μL containing 2.5 μL of 25x diluted cDNA, according to the following protocol. Forward and reversal primer sequences of the target and housekeeping genes used are listed in Table 2.2 and were synthetized by Eurofins Genomics.

qPCR conditions were as follows: 95º C for 3 min for denaturation, followed by 40 cycles of 30 s at 95º C for denaturation, 20 s at 55º C for primer annealing, 20 s at 72º C for extension cycle at 72º C during 10 min. Transcript levels were first normalized to the housekeeping gene 18S and then to the control group using the ΔΔCt method and the Bio-RAD CFX Maestro software.

Table 2.2 – Primers sequences.

Gene	Primer forward	Primer reverse
E-cadherin	5'-GCCTCCTGAAAAGAGAGTGGAAG-3'	5'-TGGCAGTGTCTCTCCAAATCCG-3'
N-cadherin	5'-CCTCCAGAGTTTACTGCCATGAC-3'	5'-GTAGGATCTCCGCCACTGATTC-3'
Vimentin	5'-AGGCAAAGCAGGAGTCCACTGA-3'	5'-ATCTGGCGTTCCAGGGACTCAT-3'
Twist	5'-GCCAGGTACATCGACTTCCTCT-3'	5'-TCCATCCTCCAGACCGAGAAGG-3'
ZEB1	5'-GGCATACACCTACTCAACTACGG-3'	5'-TGGGCGGTGTAGAATCAGAGTC-3'
Snail	5'-AATCGGAAGCCTAACTACAGCG-3'	5'-GTCCCAGATGAGCATTGGCA-3'
18S	5'-GAAGATATGCTCATGTGGTGTTG-3'	5'-CTTGTACTGCGCTGGATTCTG-3'

2.7 Statistical analysis

Graphical artwork and statistical analysis were computed using GraphPad Prism Software version 5.0. Significant values were considered with $p<0.05$.

Chapter 3

Results

3.1 Hypoxia activates the adenosinergic pathway in bladder cancer cells

As already mentioned, solid tumors have restricted access to oxygen caused by poor vascularization and improper diffusion, which leads to a hypoxic state, characterized by a drop in oxygen levels when compared to normal tissue. Under hypoxic conditions, tumor cells express the hypoxia-inducible factor-1α (HIF-1α) that intervenes in several molecular and cellular mechanisms favorable to tumor progression. One of the mechanisms regulated by hypoxia and HIF-1α is the adenosinergic pathway that consists in a sequence of reactions by which ATP is converted into adenosine.

To further evaluated whether BC cells under hypoxia activates the adenosinergic pathway, both BC cells were exposed to either normoxic or hypoxic (in BC GasPak EZ Anaerobe Pouch System) conditions for 24 h. Western blot analysis revealed a significant ($P < 0.05$) upregulation for HIF-1α in the two cell lines in hypoxia as compared to normoxic conditions, confirming the generation of a reduced-oxygen atmosphere (<1% O_2) in the GasPak EZ Anaerobe Pouch System. The activation of the adenosinergic pathway was checked by measuring the levels of the adenosine-generating ecto-nucleotidases CD39 and CD73 by western blot and of the extracellular levels of adenosine in the culture medium. Both ecto-nucleotidases are upregulated in the two cell lines under hypoxic conditions, although a more pronounced effect has been observed for CD73, which is considered the rate-limiting enzyme in the generation of extracellular adenosine that converts AMP into ADO (Figure 3.1).

The extracellular levels of ADO were measured by ELISA in the conditioned medium collected after 24 h under hypoxic or normoxic conditions. Data were normalized *per* milligram of total protein (Figure 3.2.a). Although not statistically significant, it was observed a trend towards an increase in the ADO concentration in conditioned medium of both cell lines when cultured in hypoxic conditions. The ADO levels released by UM-UC3 cell line under normoxic were of 0.69 ± 0.07 μM and increased to 1.67 ± 0.15 μM in hypoxia. For the HT-1376 cell line, extracellular ADO levels in normoxia were 1.22 ± 0.32 μM and in hypoxia were of 2.78 ± 1.06 μM. We also measured the pH of conditioned medium in normoxia and hypoxia after 24 h of incubation. We observed a trend toward a decrease in pH in samples cultured under hypoxic conditions in both cell lines (Figure 3.2.b).

Taken together these results confirmed the activation of adenosinergic pathway in the two BC cell lines when subjected to a hypoxic stress.

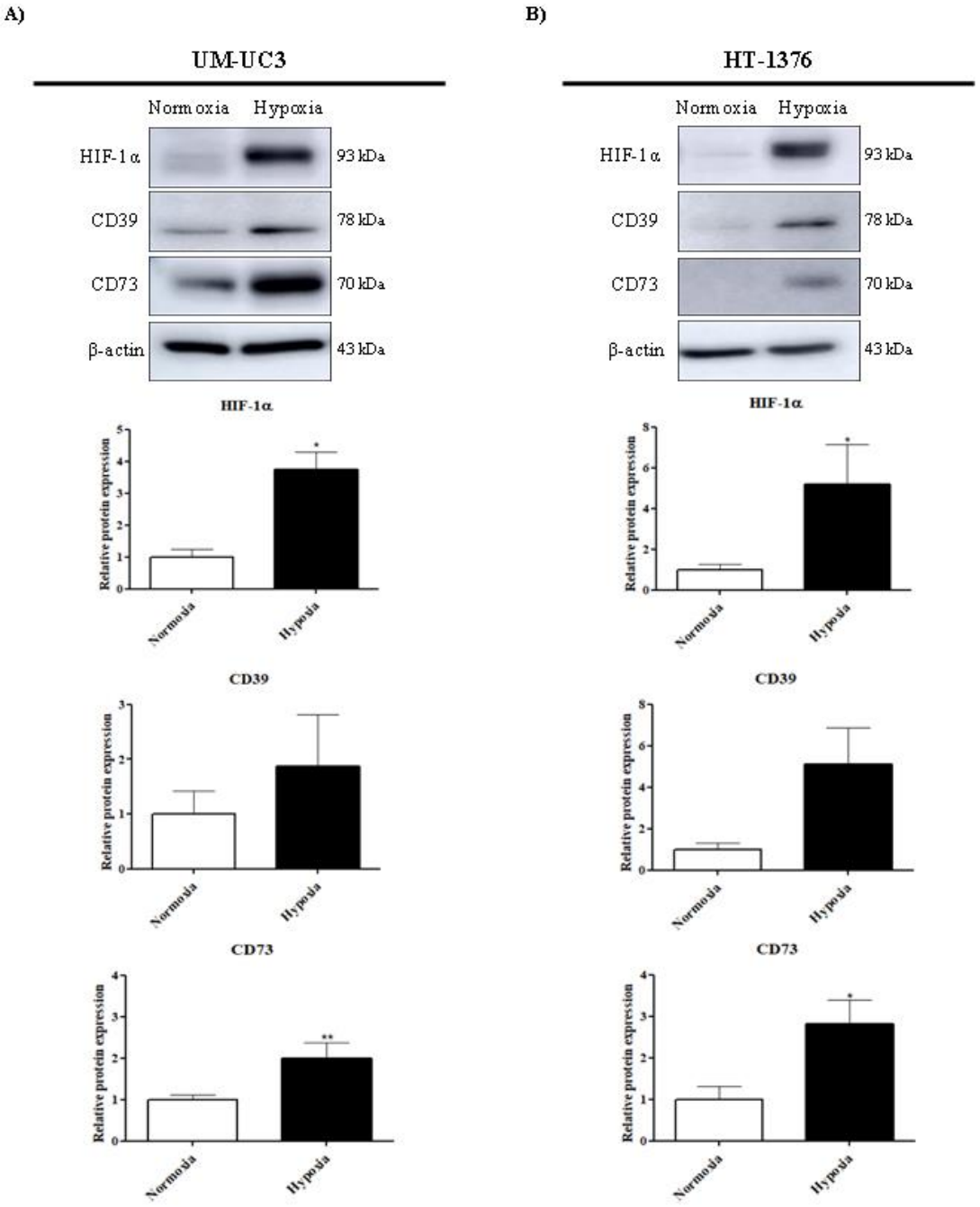

Figure 3.1 – Effects of hypoxia on HIF-1α, CD39 and CD73 expression levels in BC cells. Quantitative analysis of HIF-1α, CD39 and CD73 after a 24 h incubation under normoxic and hypoxic conditions, using the Gaspak EZ Anaerob Gas Generating Pounch System in **A)** UM-UC3 and **B)** HT-1376. Representative western blot images of analyzed protein, respective β-actin control and graphic representation of relative protein expression of HIF-1α, CD39 and CD73. All values were normalized to their respective constitutive protein β-actin and then to untreated control. All results are shown as mean ± SEM, n=3-6. **p*<0.05 as compared to normoxic conditions (Student's *T-test*).

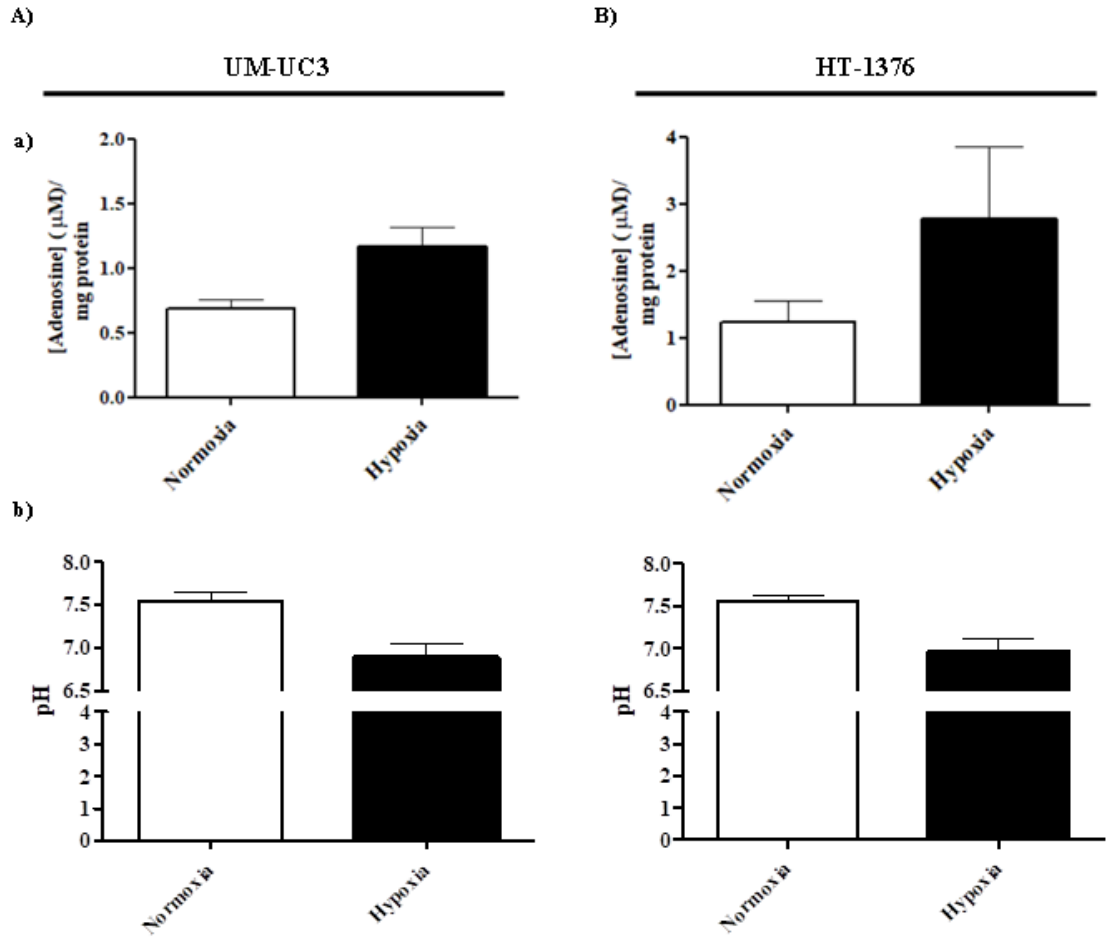

Figure 3.2 – Effects of hypoxia in A) UM-UC3 and B) HT-1376. a) on extracellular adenosine levels. Adenosine concentration (µM) *per* milligram of protein after a 24h incubation under normoxic and hypoxic conditions, using the Gaspak EZ Anaerob Gas Generating Pounch System. Adenosine levels in the supernatant were measured using an adenosine kit assay. All results are shown as mean ± SEM, n=3. **b) on pH.** Cells were incubated under normoxic and hypoxic conditions during 24 h using the Gaspak EZ Anaerob Gas Generating Pounch System and the pH of conditioned medium was measured using a pH meter. All results are shown as mean ± SEM, n=3.

3.2 Hypoxia regulates A2B adenosine receptor expression in bladder cancer cells

Being ADO a signaling molecule that mediate its physiological effects by interacting with P1 purinergic receptors, next we analyze the levels of two subtypes of ADO receptors A2AR and A2BR. The A2AR is a high affinity receptor for ADO that promotes tumor proliferation, while A2BR possess low affinity, but is upregulated under pathological conditions, such as cancer and is involved in tumor growth, cell migration and metastization.

This analysis was performed by western blot in extracts of cells cultured in normoxic and hypoxic conditions.

As depicted in Figure 3.3, the A2A receptor is constitutively expressed in both BC cells, and was not significantly affected by hypoxia, although a trend to a downregulation was noted in the HT-1376 cell line. The A2B receptor is less expressed in BC cell than the A2AR at the same normoxic conditions, but is upregulated in a hypoxic environment, at least 2-fold in UM-UC3 and a 6-fold in HT-1376.

These findings suggest that hypoxic induction of ADO upregulates the expression of the A2B receptor and might act in an autocrine fashion on BC cells.

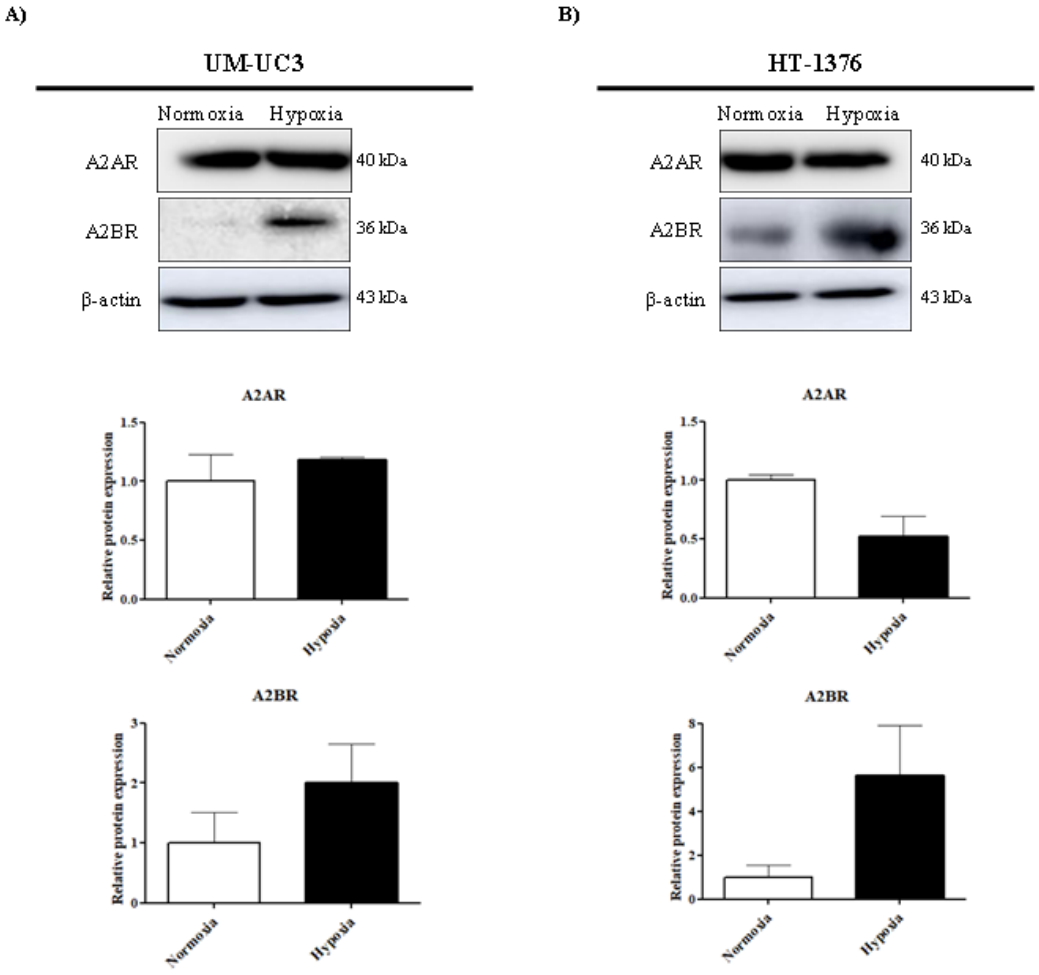

Figure 3.3 – Effects of hypoxia on A2AR and A2BR expression levels in BC cells. Quantitative analysis of A2AR and A2BR after a 24h incubation under normoxic and hypoxic conditions, using the Gaspak EZ Anaerob Gas Generating Pouch System in **A)** UM-UC3 and **B)** HT-1376. Representative western blot images of analyzed protein, respective β-actin control and graphic representation of relative protein expression of A2AR and A2BR. All values were normalized to their respective constitutive protein β-actin and then to untreated control. All results are shown as mean ± SEM, n=3.

3.3 Hypoxia exposure induced resistance to cisplatin

To evaluate whether hypoxia could change the chemosensitivity of cells to CIS, both cell lines were subjected to hypoxic conditions for 24 h and then incubated with increasing concentrations of CIS (0 μM to 50 μM) under hypoxia during 48 h. Cell viability was evaluated using a WST-1 assay and the half-maximal inhibitory concentration (IC_{50}) was calculated from a non-linear regression (sigmoidal) analysis.

Both BC cell lines in hypoxia display a high chemoresistance profile to CIS in comparison to their normoxic counterparts, as depicted in Figure 3.4.a. For the same CIS concentration, the percentage of viable cells under hypoxic conditions was higher compared to those in normoxia, mainly for the highest CIS concentration.

The IC_{50} values (Table 3.1) obtained from the concentration-response curves (Figure 3.4.b) was found to be increased by approximately 2-fold in cells exposed to hypoxia in comparison to normoxic cells, highlighting the crucial role of hypoxia in the development of resistance to CIS in these cells.

3.4 Hypoxia induces differential effects in the proliferation rate of bladder cancer cells

One of the speculated mechanisms by which hypoxia induces chemoresistance is by decreasing the proliferation rate of tumor cells, as CIS like other conventional chemotherapeutics target proliferating cells. To verify this hypothesis, we measured the cell proliferation after a 48 h incubation under hypoxic conditions, which correspond to the same period of the chemosensitivity assay. Cell proliferation was measured using WST-1 assay. Results are shown as the percentage of absorbance values measured and the reference value is the control group, set as 100% (Figure 3.5).

We observed a significant increase ($P < 0.05$) in the proliferation rate by 60% in the UM-UC3 cells when exposed to hypoxia. In the case of HT-1376 cells, the effect was the opposite, having observed a decrease in the proliferation rate by 20% in hypoxic conditions compared to normoxia. These results suggest that hypoxia interferes with the proliferation mechanisms, although in a cell-type dependent manner. These distinct effects suggest that other mechanisms are underlying the chemoresistance profile induced by hypoxia.

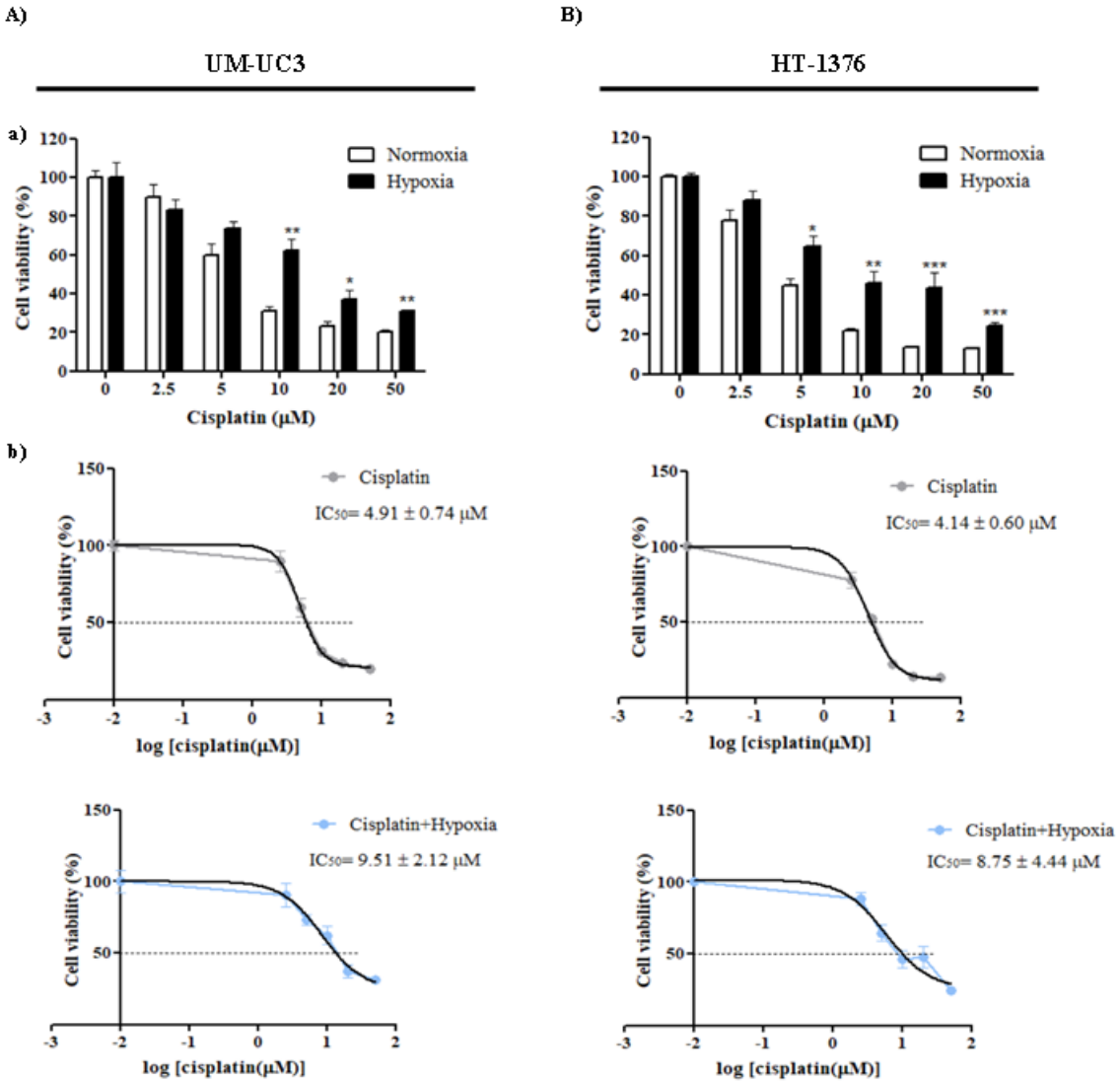

Figure 3.4 – Effect of hypoxia in A) UM-UC3 and B) HT-1376. a) on cell viability. Percentage of viable cells after induced hypoxia with the Gaspak EZ Anaerob Gas Generating Pounch System for 24 h and subsequent treatment with hypoxia and increasing concentrations of CIS (0-50 μM) during 48 h. Cell viability was measured with WST-1 assay. All values were normalized to untreated control. All results are shown as mean ± SEM, n=3. *p<0.05; **p<0.01; ***p<0.001 as compared with cells treated with the same CIS concentration in normoxic conditions (Two-Way ANOVA with a Bonferroni post-test). **b) Graphic representation of dose-response curves and IC_{50}.**

Table 3.1 – Comparison between IC_{50} for control and hypoxic cells from BC. All results are shown as mean ± SEM, n=3. *p<0.05 as compared with cells treated with CIS in normoxic conditions (Student's *T-test*)

	CIS (IC_{50}, µM)	
	Normoxia	**Hypoxia**
UM-UC3	4.91 ± 0.74	9.51 ± 2.12*
HT-1376	4.14 ± 0.60	8.75 ± 4.44*

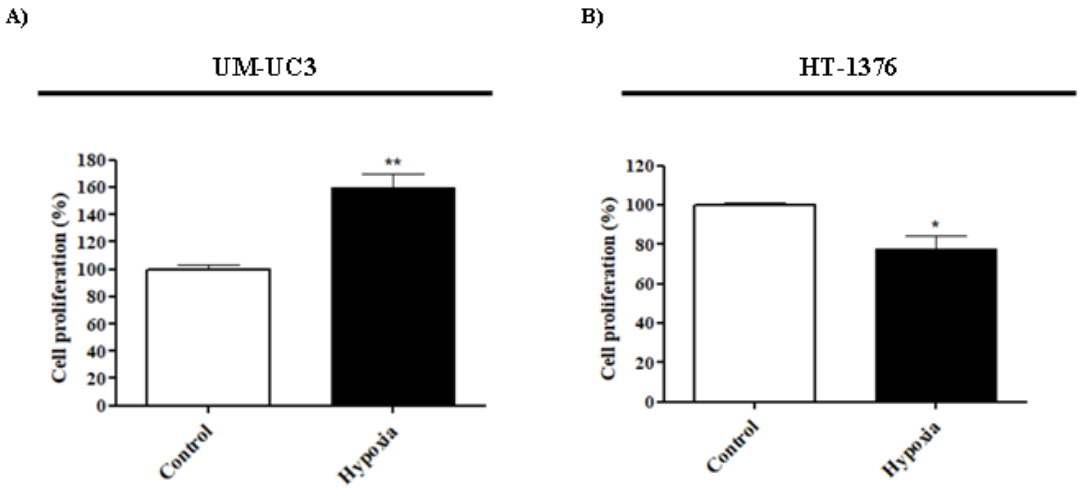

Figure 3.5 – Effect of hypoxia on cell proliferation in A) UM-UC3 and B) HT-1376. Cells were plated and incubated in normoxia and hypoxia using the Gaspak EZ Anaerob Gas Generating Pounch System during 48 h and cell proliferation was measured with WST-1 assay. All values were normalized to untreated control. All results are shown as mean ± SEM, n=3. *p<0.05; **p<0.01 compared with normoxic conditions (Student's *T-test*).

3.5 Hypoxia induces alterations on cell motility

As already mentioned, hypoxia interferes with several cell events, contributing to poor prognosis in different ways. One of them is to promote the cell dissemination from the primary tumor and facilitate the formation of metastasis at distant sites

We analyze the effects of hypoxia in the BC cells migration ability by performing a wound-healing assay. BC cells were incubated in normoxia until they reached 100% confluence. Upon reaching confluence scratch was performed using a P200 pipette tip. The scrapped cells were removed and the plate was placed inside the GasPak EZ Anaerobe Pouch System for 24 h. The pictures were taken immediately after scratching (t = 0 h) and 24 h later to determine the

extent of the wound closure. This assay was also performed in cells maintained in normoxic conditions as a control.

The migration ability of the UM-UC3 cell line was reduced under hypoxic conditions, as depicted in Figure 3.6. The percentage of wound closure after 24 h was about 30%, significantly lower ($P < 0.05$) as compared to control normoxic conditions, which was approximately 60%. An opposite effect was observed in the HT-1376 cell line. In this case, the wound gap close after 24 h, while in normoxic conditions the percentage of wound closure for the same time-point was about 50%. Apparently these differences are not related with cell proliferation, since hypoxia-induced opposing effects on cell proliferation in the cell lines.

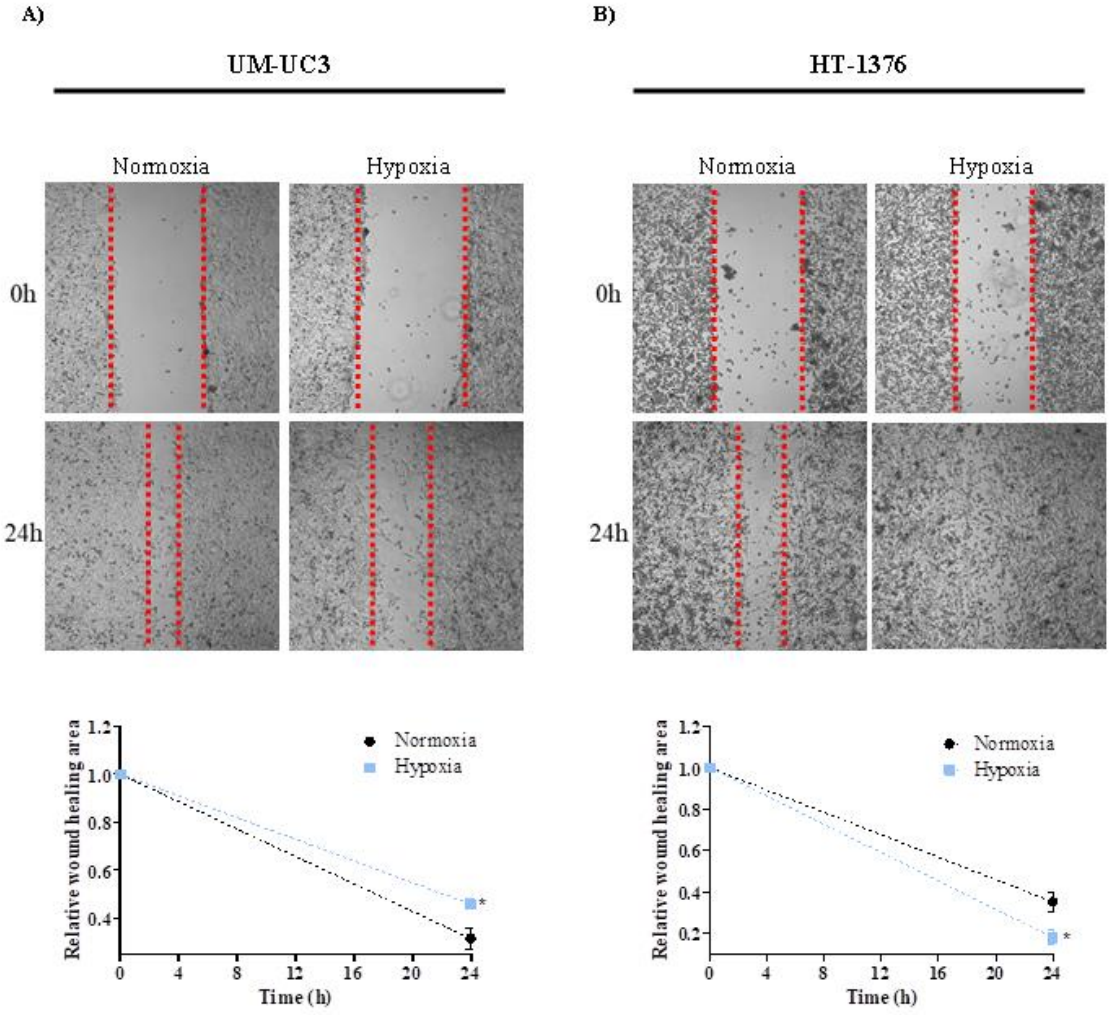

Figure 3.6 – Effects of hypoxia on cell migration in A) UM-UC3 and B) HT-1376. Cells were plated and incubated in hypoxia using the Gaspak EZ Anaerob Gas Generating Pounch System during 24 h. Motility of BC cells was accessed at 0 h and 24 h after the scratch was made. Microscopic images of wound gap at the described time points. Graphic representation of relative wound healing area. All results were normalized to 0 h respective wound healing area. All results are shown as mean ± SEM, n=3. *p<0.05 as compared with normoxic conditions (Student's *T-test*).

3.6 Hypoxia induces epithelial-to-mesenchymal transition

A cellular mechanism involved in cell migration and invasion is the EMT, during which cells lose their epithelial phenotype losing their morphology and switch to a mesenchymal-like state. This process involves the loss of epithelial markers like E-cadherin and the gain or upregulation of N-cadherin or Vimentin, and is regulated by a network of EMT transcription factors (Snail, Twist and ZEB1), in response to environmental factors such as hypoxia.

Based on this assumption, we analyzed whether hypoxia could induce EMT in BC cells by evaluating the expression levels of EMT-transcription factors (Snail, Twist and ZEB1) and some cell surface markers (E-cadherin, N-cadherin and Vimentin) (Figure 3.7). This analysis was performed by qPCR in mRNA extracted from cells subjected to hypoxia and normoxia during 48 h.

Overall, it was observed an upregulation of the three transcription factors (Snail, Twist and ZEB1) in both BC cell lines exposed to hypoxia in comparison to normoxic cells, although with different intensities.

The expression levels of the cell adhesion molecule E-cadherin required for the formation of stable adherens junctions and maintenance of an epithelial phenotype, decreased in the two BC cell lines exposed to hypoxic conditions as compared to untreated normoxic cells. The two mesenchymal markers N-cadherin and Vimentin, showed a trend towards an upregulation, mainly for Vimentin, whose levels increased about 4-fold in hypoxic cells.

Altogether, these results suggest that hypoxia contribute to the induction of EMT in BC cells.

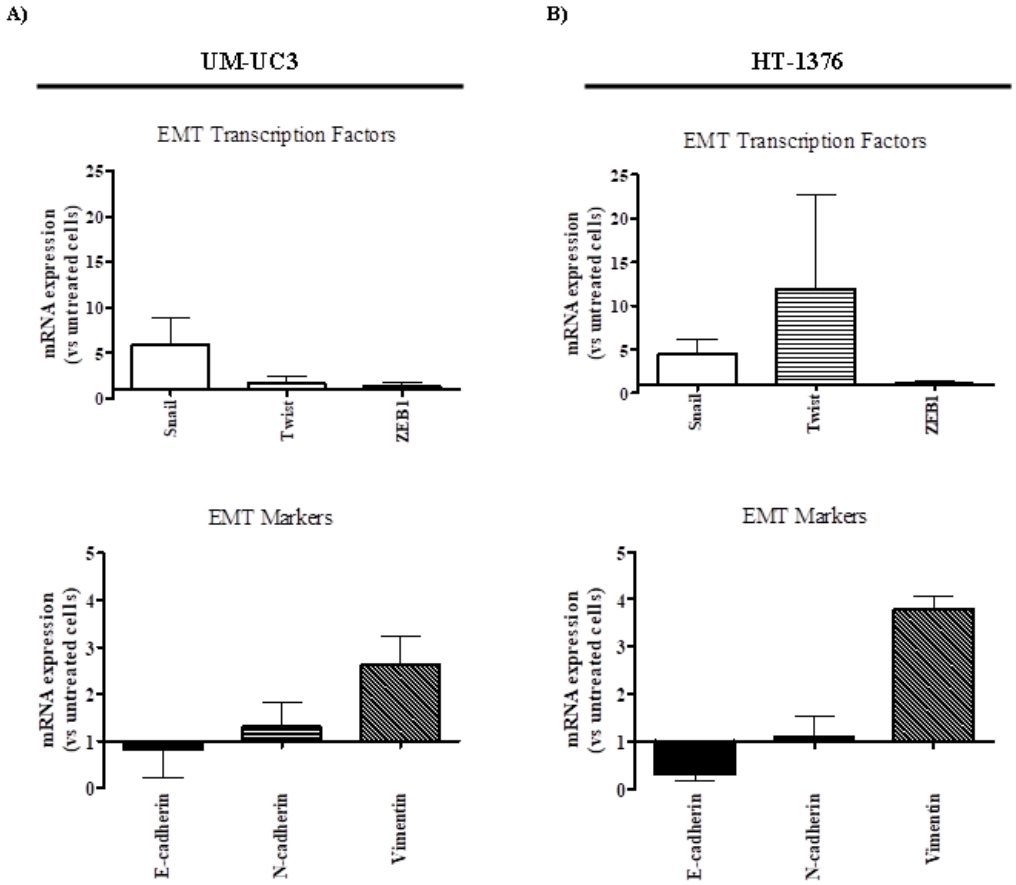

Figure 3.7 - Effects of hypoxia on EMT surface markers and transcription factors in A) UM-UC3 and B) HT-1376. Cells were plated and incubated in hypoxia using the Gaspak EZ Anaerob Gas Generating Pounch System during 48h. Gene expression of E-cadherin (epithelial marker), N-cadherin and Vimentin (mesenchymal markers) and Snail, Twist and ZEB1 (transcription factors) were accessed through qPCR. All results were normalized to untreated control. All results are shown as mean ± SEM, n=3.

3.7 Hypoxia increases PD-L1 expression in bladder cancer cells

Since hypoxia is also considered a key player in antitumor immune response, we also analyzed how tumor cells regulate the expression of the Programmed death-ligand 1 (PD-L1) when subjected to a hypoxic microenvironment. PD-L1 is a membrane-bound protein that is expressed by tumor cells to inhibit the activity of T cells expressing PD-1. So far, it is the most common clinically detected biomarker for predicting patient response to immunotherapy targeting the PD-L1/PD-1 axis.

In this context, we analyzed the influence of hypoxia in the expression levels of PD-L1 in the two BC cell lines. Cells were incubated in normoxia and in hypoxia during 24 h as

previously described. Levels of PD-L1 protein were accessed through western blot (Figure 3.8). Both cell lines upregulated the levels of PD-L1 when subjected to hypoxia, further highlighting the importance of hypoxia in driving cancer immune escape from cytotoxic T lymphocytes.

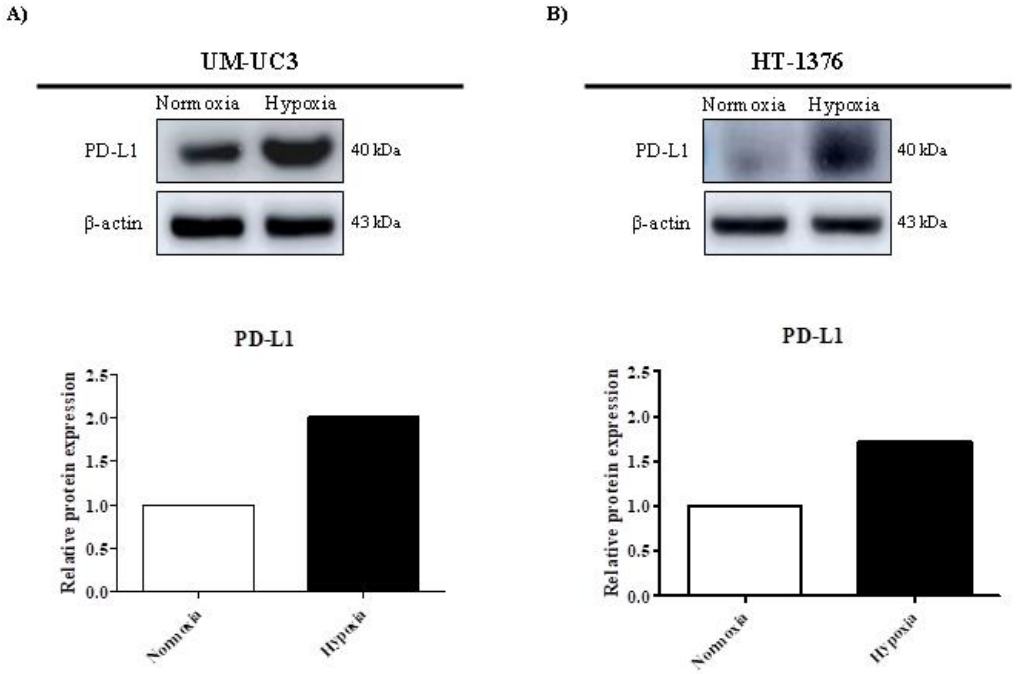

Figure 3.8 – Effects of hypoxia on immune checkpoint PD-L1 expression. Quantitative analysis of PD-L1 after a 24h incubation under normoxic and hypoxic conditions, using the Gaspak EZ Anaerob Gas Generating Pounch System in **A)** UM-UC3 and **B)** HT-1376. Representative western blot images of analyzed protein, respective β-actin control and graphic representation of relative protein expression of PD-L1. All values were normalized to their respective constitutive protein β-actin and then to untreated control. n=1.

3.8 Exogenous adenosine exacerbate the malignant phenotype of bladder cancer cells

Adenosine is the end-product of the adenosinergic pathway activation in response to hypoxic conditions. This purine nucleoside is commonly found in the tumor microenvironment to suppress anti-tumor immune responses and stimulate cancer progression. After verifying that BC cells under hypoxic conditions activates the adenosinergic pathway and released ADO to the extracellular medium, we decided to evaluate the effects of ADO *per se* in the previously studied malignant features of BC cells, namely proliferation, migration and chemosensitivity.

To this end, BC cells were incubated under normoxic conditions with ADO 10 μM that is generally found in the tumor microenvironment as reported in the literature. [57]

The proliferation rate of both BC cell lines increased significantly when incubated with ADO at 10 μM in comparison with corresponding untreated controls. It is noticed that both cell lines express constitutively the A2A receptor which is a high-affinity receptor of ADO (Figure 3.9.a).

The effects of exogenous ADO in cells migration were evaluated through a wound-healing assay as illustrated in Figure 3.9.b. Incubation with ADO at 10 μM stimulate cell migration of both BC cell lines, as shown by the increased percentage of gap closure after wounding as compared with untreated controls at 24 h.

In agreement with the previous data obtained with BC cells subjected to hypoxic conditions, co-incubation with ADO also decreased the chemosensitivity of BC cells to CIS as compared with CIS-treated cells in normoxic conditions. As can be seen from Figure 3.10 the cytotoxicity of CIS in this cell lines decreased significantly when combined with ADO for all tested combinations. The chemoresistance-inducing effect of ADO was less pronounced in the UM-UC3.

The mean IC_{50} values obtained from concentration-response curves are shown in Table 3.2, and confirmed the increased chemoresistance of both BC cell lines to CIS when incubated in the presence of ADO.

Altogether these results suggest that adenosine exacerbates the malignant features of BC cells in the range of concentrations typically found in the tumor microenvironment. Table 3.3 summarizes this set of results.

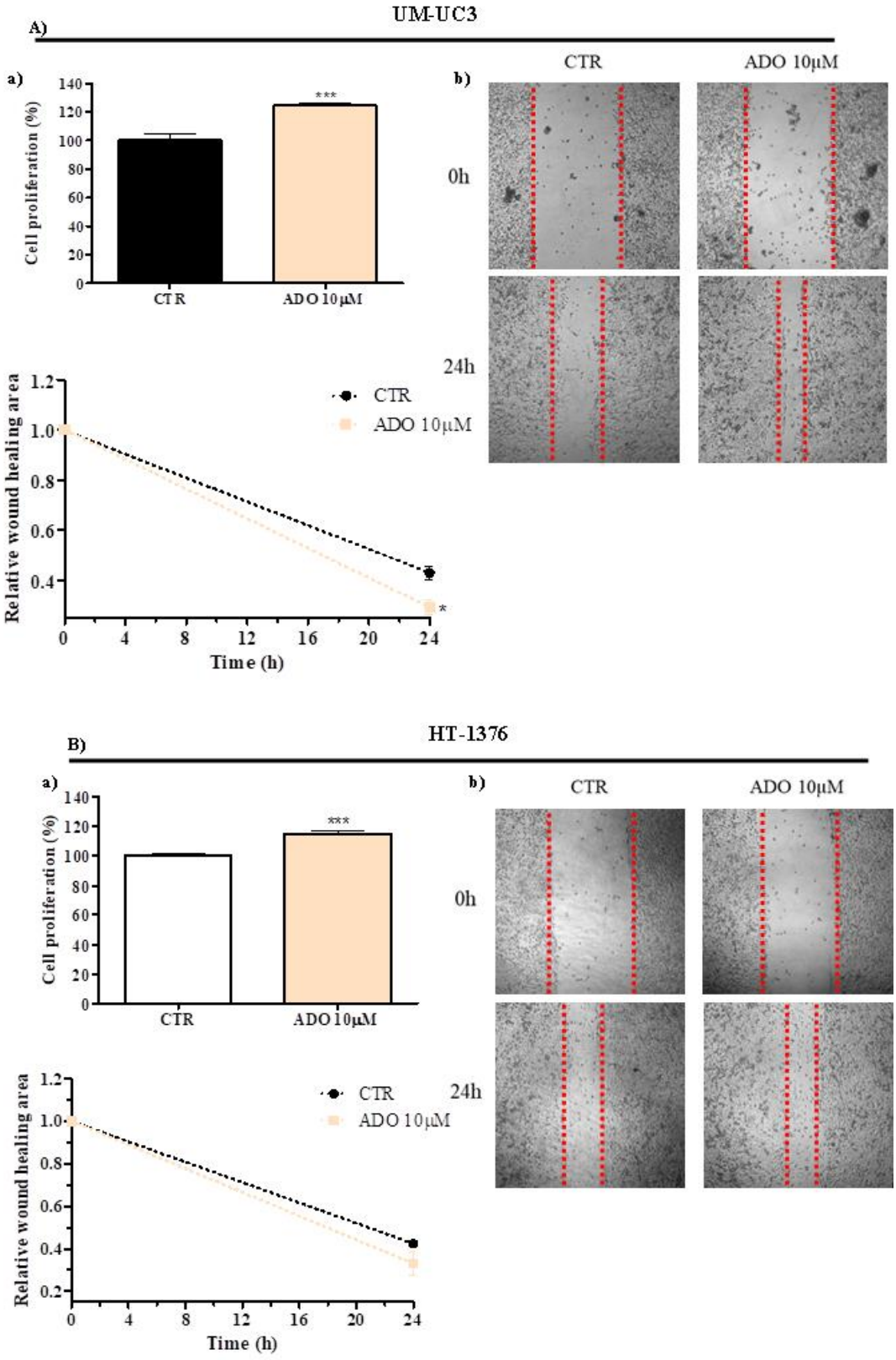

Figure 3.9 – Effects of exogenous adenosine in A) UM-UC3 and B) HT-1376. a) on cell proliferation. Cells were plated and incubated with adenosine 10µM during 48h and cell proliferation was measured with WST-1 assay. All values were normalized to untreated control. All results are shown as mean ± SEM, n=3. *p<0.05; **p<0.01;

[continuation] ***p<0.001 as compared with untreated cells (Student's *T-Test*). **b) on cell migration.** Cells were treated with adenosine 10µM for 48h. Migration of BC cells was accessed at 0h and 24h after the scratch was made. Microscopic images of wound gap at the described time points. Graphic representation of relative wound healing area. All results were normalized to 0h respective wound healing area. All results are shown as mean ± SEM, n=3. *p<0.05 as compared with untreated cells (Student's *T-Test*).

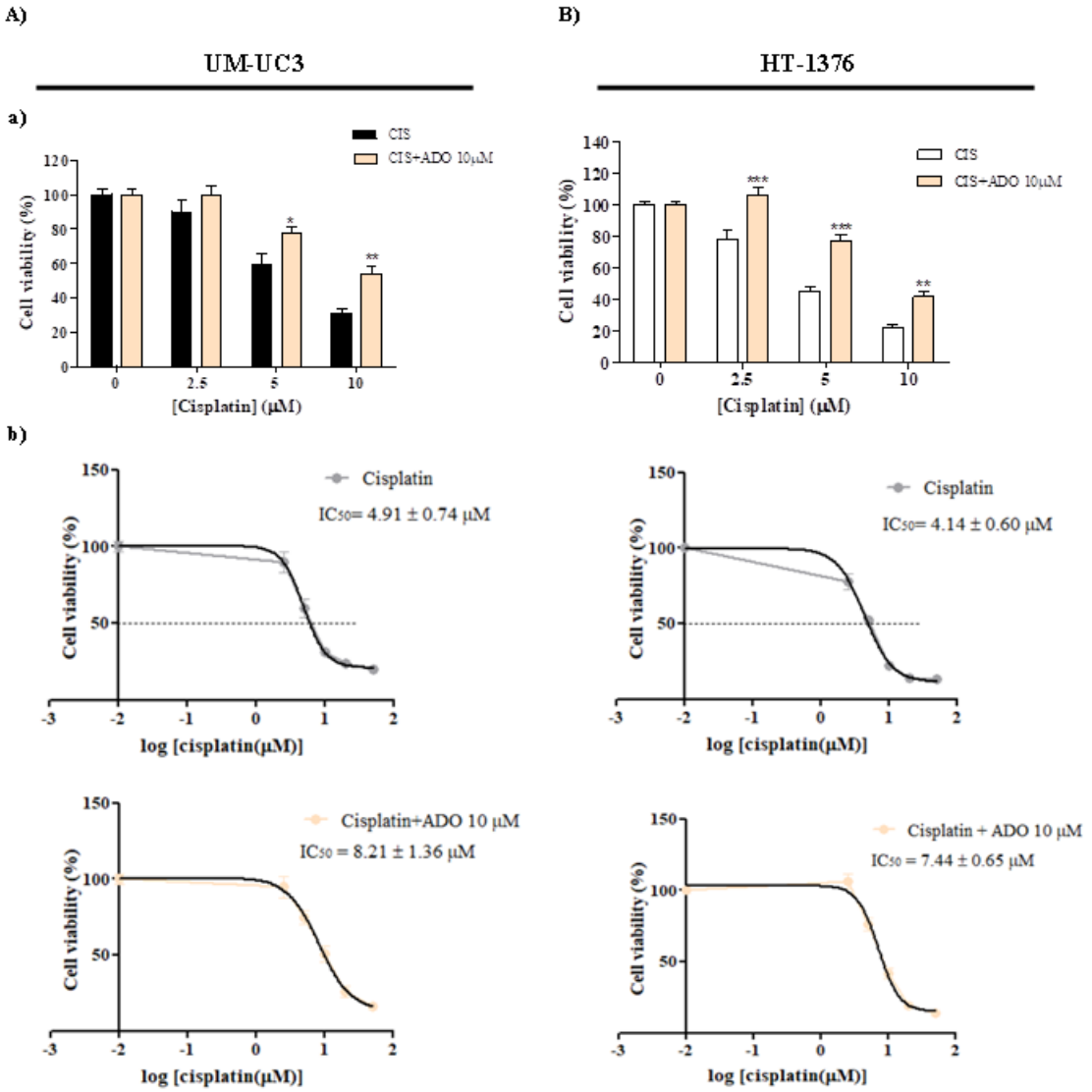

Figure 3.10 – Effects of exogenous adenosine in A) UM-UC3 and B) HT-1376 a) on cell viability. Percentage of viable cells after treatment with adenosine 10µM and subsequent treatment with increasing concentration of cisplatin (2.5-10µM) during 48h. Cell viability was measured using WST-1 assay. All values were normalized to untreated control. All results are shown as mean ± SEM, n=4. *p<0.05; **p<0.01; ***p<0.001 as compared with untreated cells (Two-Way ANOVA with a Bonferroni post-test) **b) Graphic representation of dose-response curves and IC_{50}**

Table 3.2 – Comparison between IC_{50} for control and BC cells incubated with exogenous adenosine. All results are shown as mean ± SEM, n=3. *p<0.05 as compared with cells treated with CIS without ADO (Student's *T-test*)

	CIS (IC_{50}, μM)	
	Cisplatin	**Cisplatin + ADO 10 μM**
UM-UC3	4.91 ± 0.74	8.21 ± 1.36
HT-1376	4.14 ± 0.60	7.44 ± 0.65 *

Table 3.3 – Effects of exogenous adenosine on cell proliferation, on chemosensitivity and on cell migration in BC cells compared to untreated control.

	Cell proliferation rate	**IC_{50} Cisplatin (μM)**	**% of wound closure**
UM-UC3	24%	8.21 ± 1.36	70%
HT-1376	15%	7.44 ± 0.65 *	67%

3.9 Digoxin inhibits hypoxia-induced HIF-1α accumulation, but not the hypoxia-inducible changes in bladder cancer cells

To further evaluate whether the hypoxia-induced effects previously described were mediated by HIF-1α, cells were subjected to hypoxic conditions in the presence of Digoxin (Digo), which is a cardiac glycoside that inhibit the accumulation of HIF-1α. HIF-1α is a transcriptional activator of various genes that has been shown to play an important role in the growth and malignant progression of tumors related to adaptive responses to hypoxia.

Our data confirmed that Digo at a concentration of 200 nM during 24 h prevented the upregulation of HIF-1α in BC cells subjected to hypoxic conditions (Figure 3.11.a and 3.12.a). Moreover, at this concentration, Digo has no significant effects in the viability of BC cells (Figure 3.11.d and 3.12.d). Next we examined, whether the pharmacological inhibition of HIF-1α with Digo prevents the activation of the adenosinergic pathway and attenuate the malignant features induced by hypoxia, namely chemoresistance and migratory ability.

Treatment of UM-UC3 and HT-1376 cells with Digo blocked largely hypoxia-induced CD73 expression, suggesting that hypoxia is an inducer of CD73 through the activity of HIF-1α (Figure 3.11.a and 3.12.a).

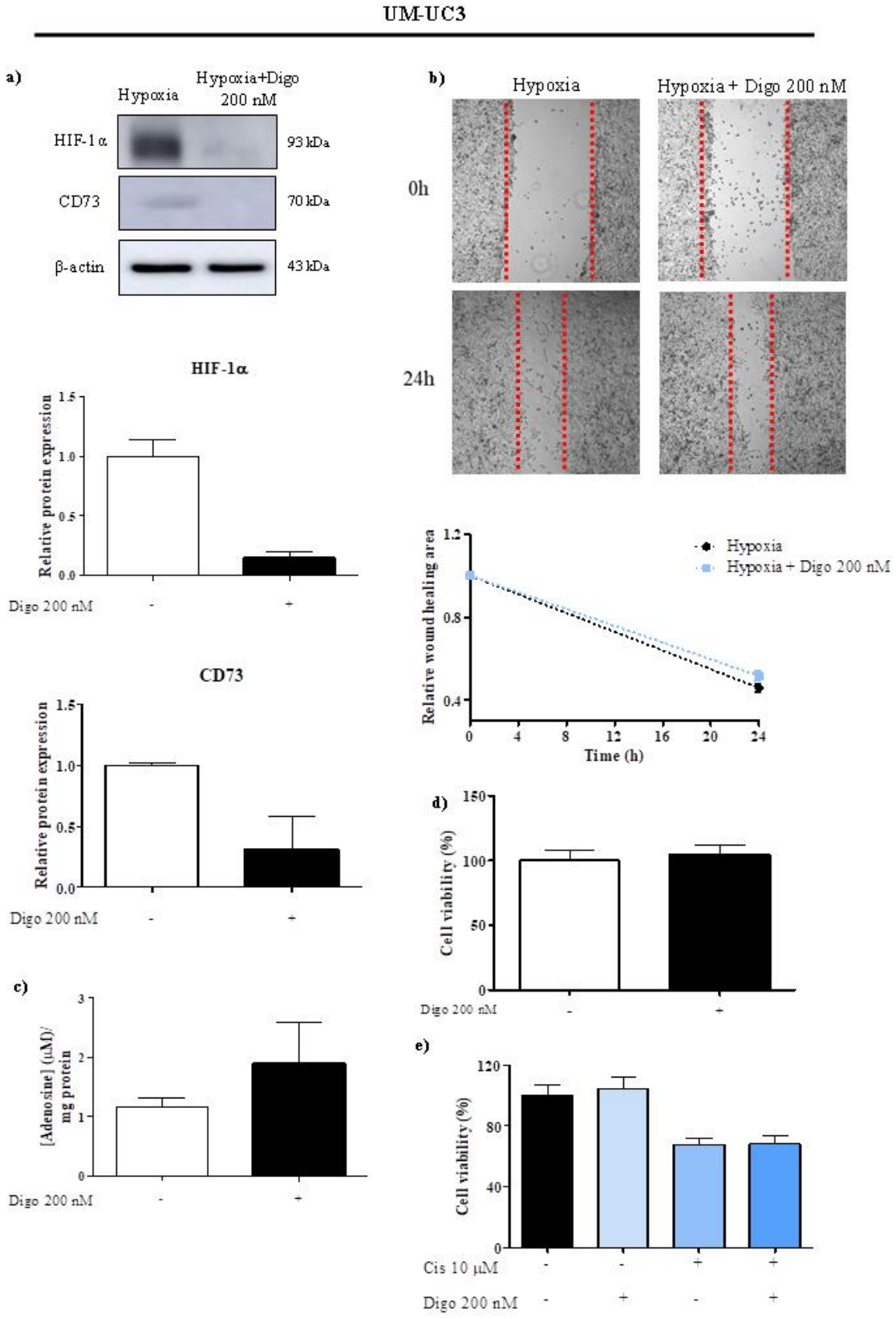

Figure 3.11 – Effects of digoxin in UM-UC3 cells. a) on HIF-1α and CD73 expression. Quantitative analysis of HIF-1α and CD73 after a 24h incubation with Digoxin 200 nM under hypoxic conditions, using the Gaspak EZ Anaerob Gas Generating Pounch System. Representative western blot images of analyzed protein, respective β-actin

[continuation] control and graphic representation of relative protein expression of HIF-1α and CD73. All values were normalized to their respective constitutive protein β-actin and then to untreated control. All results are shown as mean ± SEM, n=2-3. **b) on cell migration.** Cells were plated and incubated with Digoxin 200 nM in hypoxia using the Gaspak EZ Anaerob Gas Generating Pounch System during 24 h. Motility of BC cells was accessed at 0 h and 24 h after the scratch was made. Microscopic images of wound gap at the described time points. Graphic representation of relative wound healing area. All results were normalized to 0 h respective wound healing area. All results are shown as mean ± SEM, n=3. **c) on extracellular adenosine levels.** Adenosine concentration (μM) *per* milligram of protein after a 24 h incubation under hypoxic conditions with Digoxin 200 nM, using the Gaspak EZ Anaerob Gas Generating Pounch System. Adenosine levels in the supernatant were measured using an adenosine kit assay. All results are shown as mean ± SEM, n=3. **d) on cell viability.** Cells were incubated in hypoxia using the Gaspak EZ Anaerob Gas Generating Pounch System and with Digo 200 nM during 48 h. Cell viability was measured with WST-1 assay. All values were normalized to untreated control. All results are shown as mean ± SEM, n=3. **e) on chemosensivity to cisplatin.** Percentage of viable cells after induced hypoxia with the Gaspak EZ Anaerob Gas Generating Pounch System and incubation with Digoxin 150 nM for 24 h and subsequent treatment with Digoxin and CIS 10 μM during 48 h. Cell viability was measured with WST-1 assay. All values were normalized to untreated control. All results are shown as mean ± SEM, n=3.

However, this effect was not followed by a decrease in the generation of extracellular adenosine, since no significant differences were observed in relation to untreated hypoxic cells (Figure 3.11.c and 3.12.c).

The migratory ability of UM-UC3 cells that decreased in hypoxia, was not significantly affected by the pharmacological inhibition of HIF-1α, suggesting this transcription factor is not required for the cell migration under hypoxic conditions (Figure 3.11.b).

We also evaluated whether inhibition of HIF-1α could reverse chemoresistance of hypoxic cells to CIS. In this assay we also used CIS at a concentration of 10 μM which correspond approximately to the IC_{50} of cells in hypoxic conditions. Also here, the inhibition of HIF-α with Digo did not reverse the chemoresistance of UM-UC3 cells to CIS (Figure 3.11.e).

Results obtained with the HT-1376 cell line were quite similar (Figure 3.12). The pharmacological inhibition of HIF-1α with Digo 200 nM prevented the HIF-1α protein expression in response to hypoxia and almost abrogated the expression of CD73, without compromising the generation of ADO in the extracellular medium. Rather, a trend towards an increase in the secreted levels of ADO was observed in relation to untreated hypoxic cells.

Likewise, treatment with Digo had no significant effects on the migratory ability of HT-1376 cells nor in the chemosensitivity to CIS in hypoxic conditions, suggesting none of these malignant features of BC cells depends on HIF-1α in hypoxic conditions.

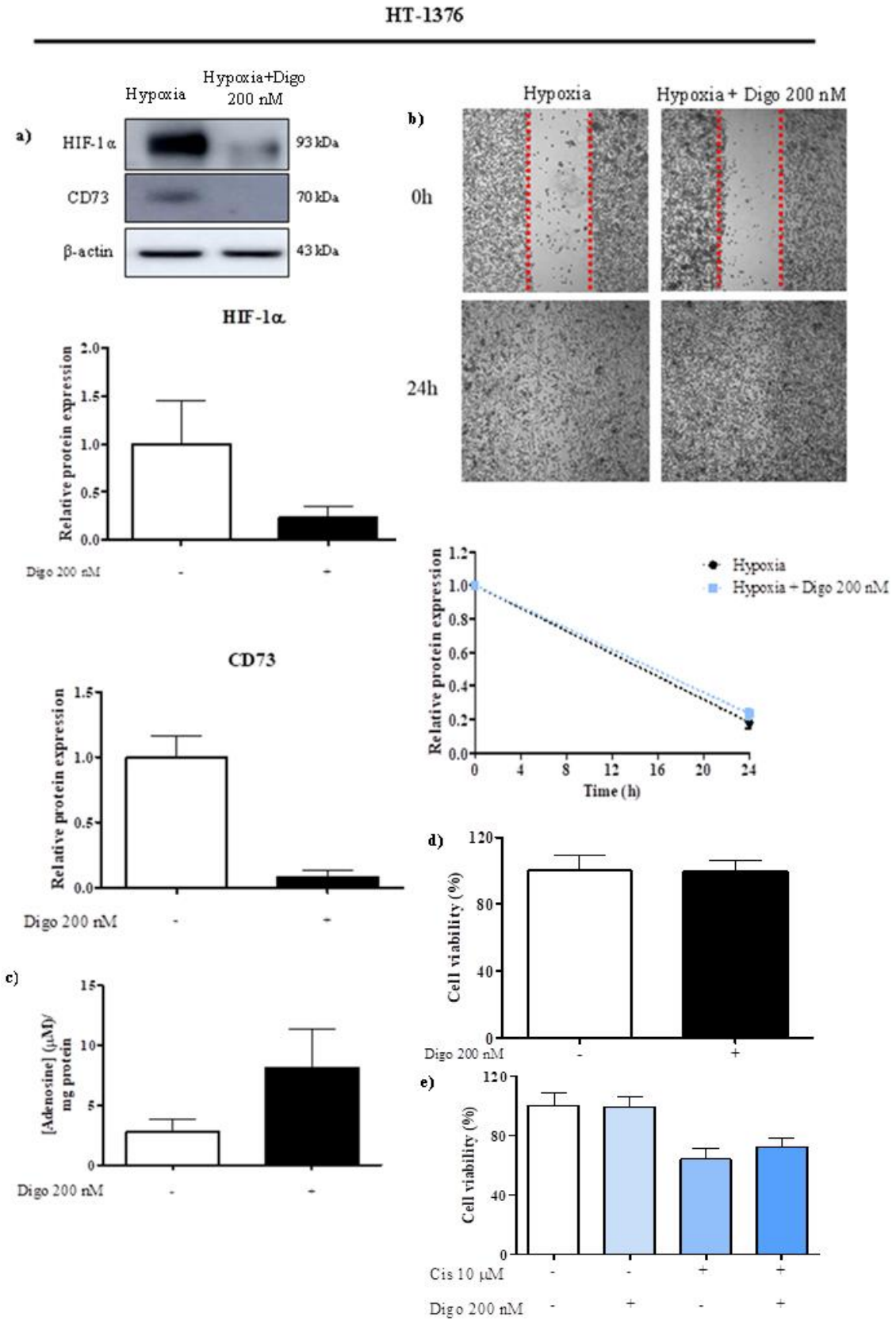

Figure 3.12 – Effects of digoxin in HT-1376 cells. a) on HIF-1α and CD73 expression. Quantitative analysis of HIF-1α and CD73 after a 24h incubation with Digoxin 200 nM under hypoxic conditions, using the Gaspak EZ Anaerob Gas Generating Pounch System. Representative western blot images of analyzed protein, respective β-actin

[continuation] control and graphic representation of relative protein expression of HIF-1α and CD73. All values were normalized to their respective constitutive protein β-actin and then to untreated control. All results are shown as mean ± SEM, n=2-3. **b) on cell migration.** Cells were plated and incubated with Digoxin 200 nM in hypoxia using the Gaspak EZ Anaerob Gas Generating Pounch System during 24 h. Motility of BC cells was accessed at 0 h and 24 h after the scratch was made. Microscopic images of wound gap at the described time points. Graphic representation of relative wound healing area. All results were normalized to 0 h respective wound healing area. All results are shown as mean ± SEM, n=3. **c) on extracellular adenosine levels.** Adenosine concentration (μM) *per* milligram of protein after a 24 h incubation under hypoxic conditions with Digoxin 200 nM, using the Gaspak EZ Anaerob Gas Generating Pounch System. Adenosine levels in the supernatant were measured using an adenosine kit assay. All results are shown as mean ± SEM, n=3. **d) on cell viability.** Cells were incubated in hypoxia using the Gaspak EZ Anaerob Gas Generating Pounch System and with Digo 200 nM during 48 h. Cell viability was measured with WST-1 assay. All values were normalized to untreated control. All results are shown as mean ± SEM, n=3. **e) on chemosensivity to cisplatin.** Percentage of viable cells after induced hypoxia with the Gaspak EZ Anaerob Gas Generating Pounch System and incubation with Digoxin 150 nM for 24 h and subsequent treatment with Digoxin and CIS 10 μM during 48 h. Cell viability was measured with WST-1 assay. All values were normalized to untreated control. All results are shown as mean ± SEM, n=3.

Chapter 4

Discussion

4. Discussion

Hypoxia is a widely recognized critical hallmark of solid tumors and a major contributor for tumor aggressiveness. Hypoxia is caused by inadequate oxygen delivery due to poor blood flow, low oxygen content and diffusion-restricted causes [106]. The hypoxia-inducible factor-1α (HIF-1α) is a master regulator of cancer cells response to hypoxic stress that results in adaptive metabolic changes and activation of pro-survival mechanisms, such as cell proliferation, acquisition of a more invasive phenotype via epithelial-to-mesenchymal transition, induction of angiogenesis and drug resistance [35,36]. The majority of muscle-invasive bladder cancers are highly hypoxic just like the advanced stage of other solid tumors that evolved adaptive responses to survive [55].

The adenosinergic pathway is a major immunosuppressive mechanism that allow tumor cells to escape to immune system and is considered a major contributor of resistance to checkpoint inhibitors recently approved for the treatment of MIBC [28,32,50,52]. This signaling pathway is active within hypoxic tumors as a consequence of the extracellular accumulation of adenosine. The master transcriptional regulator of hypoxia (HIF-1α) is a stronger inducer of the ecto-nucleotidases CD39 and CD73 that are critical mediators of the generation of the extracellular ADO. This purinergic pathway can be observed on tumor, stromal and immune cells within the tumor microenvironment, with a negative impact on anti-tumor immune response [49-51]. The knowledge that adenosine can act directly on tumor cells, prompted us to investigate the role of the adenosinergic pathway in the malignant behavior of BC cells when subjected to a hypoxic environment [55,57,66]. We use two commercially established BC cell lines, the UM-UC3 and HT-1376 both representative of grade III transitional cell carcinoma.

To induce hypoxic culture conditions, we used a model from GasPak, the BC GasPak EZ Anaerobe Pouch System. This system was originally developed to incubate anaerobe bacteriology specimens [163], but it can be adapted to culture cells in hypoxia. As described for the manufacturer, the Anaerobe Generating Pouch System produces a reduced-oxygen atmosphere of 1% O_2 and 10% CO_2 after 1h of incubation. Hypoxia with 1% O_2 is described as a severe hypoxia. The choice of the hypoxic model fell in this system since it mimics a physiologic hypoxia, without the use of chemical compounds such as $CoCl_2$.

The exposure of BC to severe hypoxia during 24 h, induced a pronounced upregulation of HIF-1α at protein level in the two BC cell lines, further confirming the activation of this transcription factor in response to the low availability of oxygen and its post-translational stabilization. Next, we evaluated whether BC cells activate the adenosinergic pathway under hypoxic conditions, by measuring the levels of ecto-nucleotidases CD39 and CD73 that are the

main enzymes responsible for generating adenosine. The former catalyzes the sequential hydrolysis of ATP to AMP while the later converts AMP to adenosine, being considered the rate-limiting enzyme in this pathway. Our results confirmed the upregulation of both enzymes in BC cell lines subjected to hypoxic conditions, as compared with normoxia, further suggesting a metabolic adaption of BC cells via activation of adenosinergic pathway during hypoxia. Studies on transcriptional effects of hypoxia revealed that these ecto-nucleotidases are transcriptionally induced by hypoxia through subsequent induction of transcripts and protein levels [167,168]. Under normal physiological conditions, ATP is mostly located in the intracellular compartment, but rises in the extracellular medium when cells are subjected to hypoxic stress, being rapidly converted into adenosine via the concerted enzymatic activity of CD39 and CD73 [50,52].

We measured the levels of adenosine in the extracellular medium of hypoxic cells by ELISA, and it was observed an increase compared with normoxic conditions, although not significant. According to the literature, the ADO concentrations in homeostatic situations range from 10 to 200 nM, whereas in stress conditions such as hypoxia may be as high as 10 to 100 μM [57]. The levels we measured in the supernatants were in the range of 1-3 μM. This measurement was performed only at 24 h of incubation in hypoxic conditions, and obviously more time-points would be necessary to better understand the kinetics of adenosine production.

We observed a trend toward a decrease in pH in cell incubated under hypoxic conditions, when compared to untreated cells. Hypoxic cells switch their metabolism from oxidative phosphorylation to glycolysis, which leads to an increase in the lactate production and a subsequent decrease in pH [44].

The extracellular adenosine elicits its regulatory functions via binding its cognate receptors. We measured the levels of two ADO receptors (A2AR and A2BR) in BC cells under normoxia and hypoxia by western blotting. ADO receptors belong to the P1 purinergic G-protein coupled receptors family, and are divided in A1R, A2AR, A2BR and A3R. A2AR is a high affinity receptor that promotes tumor proliferation, while A2BR is a low affinity receptor associated to pathological conditions where ADO levels are increased [52,176]. Both cell lines under normoxia constitutively express the A2A receptor and its expression levels were not significantly affected in hypoxic conditions. The A2BR expression was induced by hypoxia in both cell lines although a more pronounced effect has been observed in the HT-1376 cell line. Critical roles of A2BR in stimulating tumor progression have been reported recently in several tumors. In prostate cancer cell lines, adenosine signaling via A2BR increased cell proliferation

and tumor growth and in breast cancer promoted filopodia formation, invasion and metastasis [70].

These data suggests that BC cells when subjected to a hypoxic environment activates the adenosinergic pathway as demonstrated by the upregulation of the ecto-nucleotidases CD39 and CD73, generation of extracellular adenosine and upregulation of the A2B receptor.

Overexpression of proteins involved in adenosinergic pathway has been found in various tumors and is often correlated with a poor outcome in cancer patients. In fact, Monteiro *et al.* verified that melanoma patients' expressing CD73 had a decrease in overall survival [51]. Similarly, Yu *et al.* described that this ecto-nucleotidase is involved in renal cell carcinoma progression [194]. Other studies indicate CD73 has a surface marker of malignant tumors, such as leukemia [195], glioma [196], ovarian [197], breast [198] and prostate cancer [199]. Curiously in BC, expression of CD73 in NMIBC is associated with a favorable prognostic [200,201]. However, Stella *et al.* described that expression of CD73 increases with the progression of the disease and is associated with a poor outcome [202]. Yan *et al.* described not only the role of CD73 in the aggressiveness of glioblastoma, but also the pathogenicity of A2BR signaling [203]. Overexpression of A2BR was associated with poor outcome in triple negative breast cancer, multiple myeloma, acute myeloid leukaemia, liposarcoma and gastric cancer. Knockdown of A2BR in triple negative breast cancer cells reduced lung metastases [75].

Our data confirmed the exacerbation of the malignant features of BC cells when exposed to a hypoxic environment concerning cell proliferation, chemoresistance and invasiveness.

Cisplatin is one main chemotherapeutic drug used in the treatment of patients with MIBC. It is a platinum-based compound that exerts a broad spectrum of anti-tumor activities via the formation of both inter- and intra- strand DNA adducts. Despite the high cytotoxicity, most patients developed resistance to this drug and is still a major obstacle to overcome [99,100].

We evaluated if the adaptive response to hypoxia and subsequent activation of the adenosinergic pathway may contribute to cisplatin resistance in BC cells. For that, BC cells were incubated with increasing concentrations of CIS under hypoxic and normoxic conditions for 48 h. Overall it was observed a decrease in the chemosensitivity of BC cells in hypoxia, when compared to normoxic cells as confirmed by the high IC_{50} values of cisplatin for hypoxic cells. These results are in accordance with those previous reported by Su *et al.* in BC cell lines. They found that under hypoxia BC cell proliferation and drug resistance were greatly promoted compared with cells under normal oxygen conditions and attributed those effects to circELP3

which is a circular RNA that is upregulated in the cancer stem cell population during the adaptive response to hypoxia [45].

In fact, chemotherapy resistance is strongly linked to cancer stem cells (CSCs) and HIF-1α has been implicated in the maintenance of the stemness phenotype [152]. We evaluated the effects of hypoxia in the expression levels of some pluripotency transcription factors SOX2, Oct4 and Nanog, but there were no differences between cells under hypoxic and normoxic conditions (data not shown), suggesting the increase resistance we observed is not attributed to the acquisition of a stem-like phenotype. Adenosine signaling through the A2B adenosine receptor (A2BR) has been implicated and a key contributor to the development of chemoresistance in several other tumors such as glioblastoma, lung cancer and melanoma [203,206,207]. Our data are in line with these findings; both cell lines upregulated the levels of the A2BR and increased the release of adenosine in the extracellular medium when subjected to hypoxic conditions, which can account for the less sensitivity of hypoxic cells to cisplatin. Additional studies blocking the A2BR would be helpful to confirm this hypothesis. The mechanism linking the adenosine/A2BR pathway to chemoresistance is not completely clarified. However, some studies suggest that A2BR promotes P-gp/MRP1 expression, which increases chemoresistance [203].

Hypoxic cells are usually less proliferative in comparison to normoxic cells by activation of anti-proliferative mechanisms, through cell cycle arrest, inhibition of Myc expression (oncogene involved in cell proliferation), and inhibition of cyclin-dependent kinases that control cell cycle. However, hypoxic cancer cells can overcome these anti-proliferative signals and continue to progress [133-137].

Curiously in this study, the BC cell lines we have used were differentially affected by the hypoxic conditions. Hypoxic UM-UC3 cells significantly increased cell proliferation, while the HT-1376 cell line experienced a decrease in the proliferation rate as compared with normoxic conditions. The effects of adenosine on cell proliferation depend on the engagement of different receptor subtypes, and on the cell line itself. Therefore, differences in the density of receptors and the extracellular concentration of adenosine can contribute to these discrepancies. We cannot exclude that UM-UC3 might have mechanisms able to overcome the suppressive effect of hypoxia in cell proliferation. Further experiments are necessary to understand the mechanism behind BC cells proliferation under hypoxia.

Hypoxia signaling is thought to unleash the invasive and metastatic potential of tumor cells. Data obtained from the wound-healing assay showed an increase in the migratory ability of the HT-1376 under hypoxic conditions as indicated by the complete closure of the wound gap

by 24 h, while in normoxia the percentage of the wound closure was of 50% for the same time-point. An opposite effect was seen in the UM-UC3 cells, that migrated more slowly when maintained in hypoxia. One of the mechanisms underlying the enhanced migratory ability of tumor cells is the EMT, which is a process whereby epithelial cells are transcriptionally reprogrammed, resulting in decreased adhesion and enhanced migration or invasion. We examined the expression of EMT-linked transcription factors (Twist, ZEB1 and Snail) and of epithelial and mesenchymal surface markers (E-cadherin, N-cadherin and Vimentin). Although not significant, the results showed a trend towards an increase in the transcriptional levels of Twist, Snail and ZEB1 in both cell lines subjected to hypoxia, when compared to control cells. It was also observed a loss of epithelial marker E-cadherin, considered the guardian of the epithelial phenotype and an increase in N-cadherin and Vimentin levels (canonical markers of EMT), suggesting at least a partial transition to a mesenchymal-like phenotype.

These results might explain the increased motility of the HT-1376 cells in hypoxia, but not of the UM-UC3 cell line, suggesting other mechanisms is behind motility of this cell line under hypoxic conditions. Other mechanisms EMT-independent might underlie the invasive behavior of tumor cells [27]. For example, the cytokine IL-6 and the activation of the WNT5A signaling pathway drive cells migration and invasion via EMT-independent route [73,74,101]. The interaction of the stromal cell-derived factor-1 (SDF-1) with its C-X-C motif chemokine receptor 4 (CXCR4) has been shown to regulate cell migration. The A2AR receptor activation suppresses the SDF-1/CXCR4 interaction leading to decreased cells motility and invasion in some tumor [204,205]. This receptor is highly expressed in the UM-UC3 cells in both hypoxic and normoxic conditions. It is possible that the extracellular adenosine released during hypoxia activates the A2AR and block the SDF-1/CXCR4 axis attenuating the migratory ability of the UM-UC3 cells. Although the A2AR is also expressed in the HT-1376 cell line, there was a downregulation in hypoxic conditions that can attenuate the feedback in the SDF-1/CXCR4 signaling. Although, further studies are needed to clarify these mechanisms, our data clearly suggest that tumor cells respond differently to decreased oxygenation levels, highlighting the heterogeneous nature of tumor cells.

Being adenosine the end-product of the hypoxia-driven adenosinergic pathway, we evaluated the contribution of adenosine *per se* on the hypoxia-induced malignant features in BC cell lines. To this end, cells were maintained in normoxia and treated with ADO at a concentration of 10 μM, which is the one generally found in the tumor microenvironment [57]. Incubation with adenosine at this concentration exerted similar effects in the two cell lines. Both displayed higher proliferation rates, increased migratory ability, and increased chemoresistance to cisplatin, as compared with untreated control cells. In this case, ADO had a similar effect

with respect to proliferation and migration in the two cell lines. These data suggest potential contributions by other signaling pathways that are activated in response to hypoxia and might affect cells differentially with regard to proliferation and migration. Another explanation is the concentration of ADO used in these experiments, which was of 10 μM, about 3-6 fold higher than the one released in the extracellular medium by cells under hypoxia.

The adaptive cell response to hypoxia is mediated by HIF-1α through transcriptional activation of downstream pathways which regulate essential processes for survival and proliferation of tumor cells with reduced O_2 availability. To further evaluate whether the hypoxia-induced effects were mediated by HIF-1α, we used Digoxin, a cardiac glycoside that inhibit the accumulation of HIF-1α in cells [153]. Digoxin was recently clinical tested in cancer therapy to improve prognosis by eliminating HIF-1α effects [160,164]. Our data confirmed that Digoxin at a concentration of 200 nM during 24 h prevented the upregulation of HIF-1α in BC cells exposed to hypoxic conditions and decreased the expression of CD73, which is the rate-limiting enzyme for adenosine generation. Surprisingly, the production of adenosine into the extracellular medium maintained unaltered, observing even a tendency for an increase with the treatment with adenosine. These findings suggest the reduced CD73 expression was not enough to prevent the adenosine generation, under these experimental conditions. The influence of digoxin in the adenosinergic pathway is still unclear, so we cannot exclude that other compensatory mechanisms are activated.

For instance, in the heart, high concentrations of Digoxin were found to increase the levels of ADO [161,162]. Several reports indicated that Digoxin, through the inhibition of HIF-1α under hypoxic conditions, leads to a decrease in ATP by interfering with autophagy processes. The exact mechanism behind the increase of adenosine is not completely clarified. A possible explanation is that Digoxin decreased the ATP concentration and the loss of ATP leads to a decrease in extracellular ADO production. However, due to concentration gradients, the adenosine produced intracellularly is released [161,174]. Therefore, the nucleoside is not produced in the extracellular medium, but inside the cell. Even using an inhibitor of HIF-1α accumulation, higher concentrations of ADO are found in the tumor microenvironment.

We also analyzed the effects of Digoxin 200 nM in the migratory ability and susceptibility of BC cells to cisplatin 10 μM in hypoxic conditions, and curiously in none of the assays were observed significant alterations in comparison with those in untreated hypoxic cells. A possible explanation for these results is the fact the extracellular levels of adenosine released by BC cells were not altered in the treatment with digoxin. Besides that, we cannot exclude that

hypoxic cells activate other mechanisms independently of HIF-1α, which contribute equally to chemoresistance and increased migratory ability.

Accumulating evidence suggests that a hypoxic microenvironment protects tumors by inhibiting anti-tumor immune effector cells and facilitating immune escape. Herein, we verified that hypoxia upregulates the levels of PD-L1 in BC cells. This immune checkpoint is a transmembrane protein that binds to its receptor PD-1 in T cells, inhibiting its activity [28,31]. Several other studies have demonstrated that hypoxia, via HIF-1α, upregulates PD-L1 in various cancers (melanoma, renal cell carcinoma, breast and prostate cancer) by direct binding the hypoxia responsive elements (HRE) in the promoter of the PD-L1 gene to block immune effector cells [208,209]. A number of studies demonstrate that the adenosine signaling, via activation of A2AR, plays an important role in immunosuppression. A positive correlation between the expression of PD-L1 and A2AR has been found in tumor samples of colon cancer and correlated with a poor prognosis [102,103], highlighting the role of extracellular adenosine in promoting immune tolerance. Although preliminary, our results are in line with these findings, suggesting that hypoxic tumors upregulate PD-L1 in BC cells to escape from cytotoxic lymphocytes, an aspect that deserves future investigation.

Chapter 5

Main conclusions

5. Main conclusions

Based on the results obtained in this thesis, we conclude:

- BC cells under hypoxic conditions stabilize HIF-1α and adapt to low oxygen levels by activating the adenosinergic signaling pathway, as demonstrated by the increased expression of adenosine-generating enzymes CD39 and CD73 and extracellular ADO levels.
- The protein levels of the adenosine A2BR receptor, but not of the A2AR, increased in both BC cell lines when subjected to hypoxic conditions, suggesting a regulatory role of the A2BR signaling in promoting cellular adaptations to hypoxia.
- Hypoxia impairs the susceptibility of BC cells to CIS and upregulates the expression of PD-L1, anticipating the development of an immune escape mechanism.
- Hypoxia induces a partial epithelial-to-mesenchymal transition in both BC cells, which resulted in increased migration of the HT-1376 cell line but not of the UM-UC3, suggesting a tumor cell-dependent effect.
- Treatment with adenosine exacerbated the malignant features of BC cells in a fashion similar to those induced by hypoxic conditions.
- Digoxin attenuated the protein expression of HIF-1α in BC cells maintained under hypoxic conditions without interfering with the extracellular production of adenosine.

In sum, our results demonstrated the relevance of the adenosinergic pathway as an adaptive mechanism to hypoxia in advanced tumors, and emphasized the critical role of adenosine in promoting cancer progression and resistance to therapy. Therapeutic strategies incorporating inhibitors of the hypoxia-CD39-CD73-A2BR pathway holds great promise to improve patient's outcome and deserves further investigation.

Chapter 6

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