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Impact of chemotherapeutic drugs in NK-mediated killing by over expression of death receptors on cancer cells

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IMPACT OF CHEMOTHERAPEUTIC DRUGS IN NK-MEDIATED KILLING BY OVEREXPRESSION OF DEATH RECEPTORS ON CANCER CELLS

Dissertação de Mestrado em Investigação Biomédica, na especialidade de Oncobiologia, apresentada à Faculdade de Medicina da Universidade de Coimbra para obtenção do grau de Mestre.

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“For every fact there is an infinity of hypothesis”

Robert M. Pirsig

À Beatriz e à Ermelinda,

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LIST OF ABBREVIATIONS

^{51}Cr	Chromium-51
ABC	ATP-Binding Cassette
ACT	Adoptive cell transfer
ADCC	Antibody-dependent cellular cytotoxicity
ALDH	Aldehyde desidrogenase
BC	Bladder cancer
BCG	Bacilus Calmette-Guérin
bFGF	Basic fibroblast growth factor
BID	BH3-interacting domain
BRCP	Breast Cancer Resistance Protein
BTZ	Bortezomib
CAM	Cell adhesion molecules
CIS	Carcinoma in situ
CMA	Concanamycin A
CSCs	Cancer-stem cells
DISC	Death-inducing signaling complex
DMSO	Dimethyl sulfoxide
DOX	Doxorubicin
E:T	Effector:Target
EGF	Epithelial growth factor
EMT	Epithelial to mesenchymal transition

ESA	Epithelial specific antigen
FADD	Fas-associated death domain
FasL	Fas ligand
FBS	Fetal bovine seum
FSC	Forward scatter
GSTM1	Glutathione-S-transferase Mu 1
HLA	Human leucocyte antigen
HSCT	Hematopoietic stem cell transplant
IFNγ	Interferon-gamma
ILCs	Innate lymphoid cells
ITAM	Immunoreceptor tyrosine-based activation motifs
ITIM	Immunoreceptor tyrosine-based inhibition motifs
MHC-I	Major histocompatibility complex-I
MMC	Mitomycin
MRPs	Multidrug Resistance-associated proteins
NAT 2	N-acetyltransferase 2
PBL	Peripheal blood lympchocytes
PBL	Peripheral blood lymphocyte
PBMCs	Peripheral blood mononuclear cells
P-gp	P-glycoprotein
SCID	Severe combined immunodeficiency disease
sFas	Soluble Fas
SSD	Side scatter

SULT	Sulfotransferases
TCC	Transitional cell carcinomas
TIL	Tumor infiltrating lymphocytes
TNF	Tumor-necrosis factor
TNM	Tumor-Node-Metastasis
TUR	Transurethral resection

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ABSTRACT

Background: Bladder Cancer (BC) is the fifth most incident cancer worldwide. It is characterized by a high recurrence rate, probably due to the presence of a small population of cells within the tumor, named cancer stem cells (CSCs), responsible for therapeutic resistance and relapse after initial therapy. Therefore, new therapeutic approaches are needed to eliminate all tumor cells, preventing the tumor recurrence. In this context, natural killer(NK) cells-based therapy has emerged as an alternative strategy, alone or in combination with other therapeutic modalities, to treat BC. Herein, we evaluate the effect of sub-apoptotic concentrations of doxorubicin (DOX) and bortezomib (BTZ) in sensitizing tumor BC cell lines to NK-mediated killing, through death receptors (Fas and DR5) upregulation.

Methods: Two BC cell lines (UM-UC3 and HT-1376) and their corresponding CSCs, isolated through the sphere-forming assay, were incubated with sub-apoptotic concentrations of DOX (0.05µM and 0.1µM) and BTZ (5nM and 10nM) for 24h or 48h to measure the gene expression of death receptors, using the qRT-PCR technique. Subsequently, a 20h ^{51}chromium release assaywas performed to evaluate the NK-mediated killing, via death receptors signaling, in pre-treated tumor cells. Additionally, qRT-PCR was performed to assess the death receptors expression pattern among different stages of BC, using clinical samples.

Results: Pre-treatment with sub-apoptotic concentrations of DOX and BTZ induces a significant upregulation of both death receptors in BC parental cells. Compared to untreated cells, the pre-treatment of parental cells with 0.05µM DOX and 0.1nM BTZ promotes a significant increase in NK cells anti-tumor response, through a perforin-independent mechanism. The CSCs population was less responsive to DOX- or BTZ-induced death receptors upregulation, and consequently less susceptible to NK cells mediated killing. Furthermore, death receptors gene expression increases progressively with the BC tumor stage.

Conclusions: Our data demonstrates that a pre-treatment with low concentrations of DOX and BTZ sensitizes parental BC tumor cells to NK-mediated killing by inducing an

upregulation of death receptors. The CSCs populations express low basal levels of death receptors and is less sensitive to NK cell-mediated killing by death-receptors.

Altogether these results suggest that a combination of DOX or BTZ with NK-based immunotherapy may increase the antitumor efficacy against differentiated BC cells but are less effective against CSCs. The expression of death receptors in BC correlates with tumor stage and aggressiveness, suggesting that it might be used as a prognostic marker in BC.

Keywords: Bladder cancer; Doxorubicin; Bortezomib; NK cells; death receptors; Fas; DR5; immunotherapy;

RESUMO

Introdução:O cancro da bexiga (BC, do inglês *bladder cancer*)é a quinta neoplasia mais comum em todo o mundo. É caracterizado por uma alta taxa de recidiva, provavelmente devido à presença de uma pequena população de células, designada de Células Estaminais Cancerígenas (CSCs, do inglês Cancer Stem Cells), responsável pela resistência à terapêutica e a recidiva após a terapia inicial. Assim sendo, são necessárias novas abordagens terapêuticas de modo a eliminar completamente o tumor, prevenindo o seu reaparecimento. Neste contexto, a terapia com base em células natural killer (NK) tem sido estudada como uma estratégia alternativa, sozinha ou em combinação com outras terapias, para o tratamento do BC. Neste trabalho avaliamos o efeito de concentrações sub-apoptóticas de doxorubicina (DOX) e bortezomib (BTZ) na sensibilização das células tumorais do BC à morte mediada pelas células NK, através do aumento de expressão dos receptores de morte.

Métodos: Duas linhas celulares de BC (UM-UC3 e HT-1376) assim como as suas respectivas CSCs, isoladas através do método de formação de esferas, foram incubadas com concentrações sub-apoptóticas de DOX (0.05µM e 0.1µM) E BTZ (5nM e 10nM) durante 24h e 48h de modo a medir a expressão génica dos receptores de morte, usando a técnica de PCR em tempo real. De seguida foi realizado um ensaio de libertação de ^{51}crómio de forma a avaliar a morte mediada pelas células NK, através da via dos receptores de morte, em células pré-incubadas com DOX e BTZ. Adicionalmente, um ensaio de qRT-PCR, utilizando amostras clínicas, foi elaborado de modo a avaliar o padrão de expressão dos receptors de morte nos vários estadios do BC.

Resultados:O pré-tratamento com concentrações sub-apoptóticas de DOX e BTZ induz um aumento significativoda expressão dos receptores de morte nas células parentais do BC. Comparando com células não tratadas, o pré-tratamento de células parentais com 0.05µM DOX e 0.1nM BTZ promove um aumento significativo da resposta anti-tumoral das células NK, através de um mecanismo independente da perforina. A população de CSCs foi menos sensível à regulação dos receptors de de morte induzida pela DOX e BTZ e, consequentemente menos sensível à morte celular mediada pelas células NK.

Conclusão:O nosso estudo demonstra que o pré-tratamento com baixas concentrações de DOX e BTZ sensibiliza as células tumorais de BC para a morte mediada pelas células NK devido ao aumento da expressão dos receptores de morte. As populações de CSCs expressam baixos níveis basais de receptores de morte e são menos sensíveis à morte mediada pelas células NK, através destes receptores.No seu conjunto, estes resultados sugerem que a combinação de DOX e BTZ com a immunoterapia baseada em células NK pode aumentar a eficácia da resposta anti-tumoral contra células parentais mas é menos sensível contra CSCs. A expressão de receptores de morte no BC correlaciona-se com o estadio e agressividade tumoral, sugerindo que os receptores de morte poderão ser usados como marcadores de prognóstico.

Palavras-chave: Cancro da bexiga; Doxorubicina; Bortezomib; células NK; Receptores de morte; Fas; DR5; Imunoterapia;

CHAPTHER 1

INTRODUCTION

1.1 Bladder cancer

1.1.1 Epidemiology

In Western World, bladder cancer (BC) represents the fourth and ninth most common cancer in men and women, respectively[1]. Overall BC is the fifth most incident cancer in both sexes, only surpassed by lung and bronchus, colorectal, prostate or breast cancers[2].

Although the incidence rates of the 4 major cancer types have been declining over the last 20 years, the incidence rate of BC has remained relatively stable[2].

BC appearance is strongly related to age, with the highest rates of incidence being in the elderly population. Approximately 66% of BC cases are diagnosed among individuals older than 65 years[3].

There are substantial differences in the incidence and severity of BC between genders and ethnicities. Men have a higher risk of BC than women in a 3:1ratio. This is mainly due to tobacco smoking and occupational exposure to aromatic amines, the major risk factors in BC. Furthermore, white population presents a higher risk of BC compared to the black population[4].

The prognosis of individuals diagnosed with BC is better in younger ages. Overall, the relative 5-years survival after BC diagnosis was described as 72% in men and 67% in women. Patients diagnosed at 15-44 years of age present 90% and in patients older than 75 years of age, the 5-year survival rate drops to 61%[5].

1.1.2 Etiology

The relationship between BC and potential risk factors that influence bladder carcinogenesis has long been studied. The development and progression of BC appear to

be multifactorial processes and the contributing factors can be differentiated into external exposure and inherited genetic alteration[6].

External exposure

The cigarette smoking is recognized as the major risk factor for BC and is estimated to account for approximately 66% of new cases of BC in men and 30% cases in women in industrialized population. The tobacco smoking has the same effect in men and women, as well as in different ethnic populations[7]. The BC risk is 2-4 times higher in smokers than in nonsmokers[8,4].

Tobacco contains aromatic amines, which are excreted renally and exert a carcinogenic effect in the entire urinary system. Cessation of smoking can reduce BC risk around 30% after 1-4 years of cessation and the decrease in risk continues with time until 60% after 25 years of cessation. People who stopped smoking also demonstrate improvements on outcome and overall survival of BC patients. Environmental exposure to smoke during childhood or adulthood has been associated with BC, as well. Interestingly, women who had never smoked are the more susceptible[1,6].

Following smokers, occupational exposure to carcinogenic agents is viewed as the second most important risk factor for BC. BC-associated carcinogens described in literature include aromatic amines, polycyclic aromatic hydrocarbons and chlorinated hydrocarbons. Industrial areas, such as processing paint, dye, metal and petroleum products, had been associated with BC cases. Other jobs that might increase the risk of BC include hairdressers and barbers, painters and coke-production workers[1,4,6,8].

Although diet might also influence bladder carcinogenesis, no consistent association between the intake of a specific nutrient or micronutrient and BC has emerged so far[1]. Previous studies associated the intake of fruits and vegetables as a protective effect against BC[9,10]. Other lifestyle factors have been investigated, like the consumption of coffee, tea or alcohol, but no meaningfully association has been reported[11,12]. In relation to fluid consumption, the high intake of tap water has been

positively associated with BC, probably due to an exposure to arsenic and by products of disinfection[13].

Urinary tract diseases can play an important role in the development of BC, particularly in developed countries. In these areas of the world, there is a strong association between urinary *schistosomiasis*, caused by trematode *Schistosoma haematobium*, and BC. Other infections in urinary tract can lead to a chronic irritation of the bladder epithelium, and thereby increase the risk of BC[4–6].

Genetic susceptibility

Familial bladder cancer is considered a rare phenomenon comparing with other types of tumor. However, some genetic factors had been established for BC carcinogenesis. Polymorphisms in genes coding for enzymes involved in the metabolism of urothelial carcinogens highly contribute to susceptibility to BC[8].

The enzyme N-acetyltransferase 2 (NAT2) is involved in detoxification of bladder carcinogens, such as aromatic amines and has long been known to be polymorphic. Some individuals present an enzyme variant associated with slow metabolization of carcinogens. Several point mutations in NAT2 gene associated with the slow acetylator phenotype have been identified. Individuals with this polymorphism have 40% higher risk of developing BC than individuals with the normal enzymatic variant. The increased risk of developing BC seemed to be stronger in cigarette smokers[3,4,6,8].

The Glutathione-S-transferase Mu 1 (GSTM1) belongs to a large family of enzymes involved in the detoxification of aromatic amines, a group of bladder carcinogens. About 50% of people have an inherited deletion in both copies of GSTM1 gene, responsible for loss of the enzymatic activity. This polymorphism has been associated with higher risk for BC in several studies, although it has no interaction with smoking profile[3,4,6,8].

Sulfotransferases (SULT) and cytochrome P450 enzymes are also involved in the metabolism of aromatic amines but the relation between gene mutations and BC development is not fully established yet[6].

1.1.3 Urothelial carcinogenesis

Bladder cancer is a morphologic highly heterogeneous disease. Approximately 90% of bladder malignant tumors are transitional cell carcinomas (TCC), which arise from transitional cells in the bladder urothelium. Other forms of BC include primary squamous cell carcinoma, adenocarcinoma, sarcomatoid carcinoma and small cell carcinoma[14].

Although non-urothelium variants of BC are rare, they are at higher stages and grades at presentation and are associated with poor prognosis outcomes, being low sensitive to current therapies[14].

Clinically, bladder TCC are divided into superficial and invasive urothelial carcinomas. Superficial carcinomas, also called non-muscle-invasive bladder cancers, are confined to mucosa or submucosa layers of the bladder. In contrast, invasive carcinomas are defined as tumor that reached muscular layers of the bladder wall, through *lamina propria* invasion[15,16].

Superficial bladder carcinomas include papillary tumors and carcinoma *in* situ (CIS) limited to mucosal layer as well as tumors that invade sub-epithelial connective tissue, defined in Tumor-Node-Metastasis (TNM) classification as Ta, Tis and T1, respectively. On the other hand, the same classification divides muscle-invasive carcinomas by grade in T2, T3 and T4, according with the depth of invasion[16–18].

The superficial and invasive tumors are represented in Figure 1.

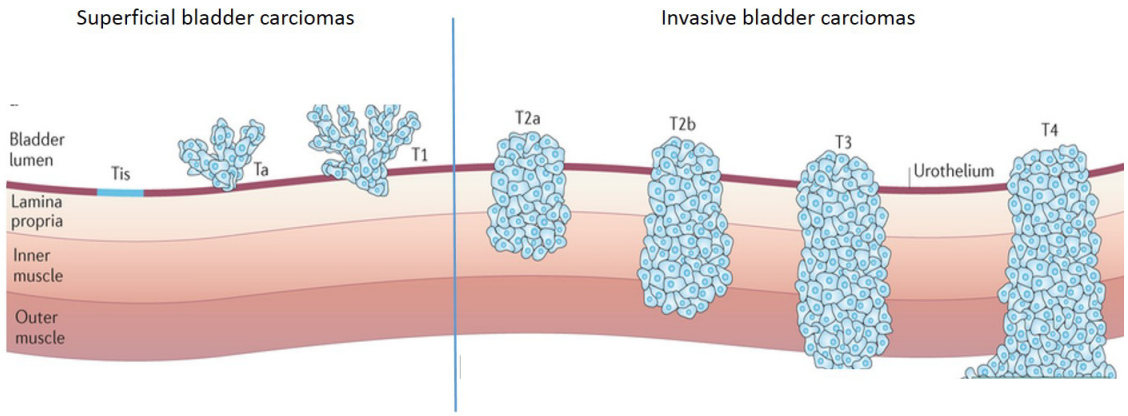

FIGURE 1.1 GRADES OF BLADDER CANCER.
Bladder carcinoma is a heterogeneous disease that is divided into 7 sub-types, accordingly with TNM classification. Low grade tumors correspond to superficial or non-muscle invasive carcinomas, while invasive carcinomas represent tumors in higher grades. (Adapted from Hurst et. al., 2015)

The majority of the BC cases corresponds to papillary lesions as a result of a hyperplasia in normal epithelium. Some papillary tumors present a dysplastic phenotype, which corresponds to an early stage of progressive alterations between normal epithelium and urothelial CIS. The transition of normal urothelium cells to dysplastic cells is usually subtle. The majority of cellular alterationsare comprised between basal and intermediate cell layers. Dysplastic cells frequently show genetic alterations, such as in p53 gene. Only approximately 15% of papillary tumors evolve into a higher stage, an invasive TCC. The reason why some TCC cases become invasive is currently unknown[19].

In contrast to low-grade papillary tumors, CIS presents genetic instability caused by alterations in tumor suppressor genes expression, including TP53, RB and PTEN. These mutations lead to a genetic instability and an anti-apoptotic phenotype, causing alterations in physiological functions and accumulation of genetic mutations. The majority of invasive BCs occur in patients without prior history of papillary tumors[19,20]. CIS evolves to invasive TCC in 60-80% of the cases if left untreated, being the most clearly defined precursor of bladder cancer[20]. The mechanism of tumor progression described is illustrated in figure 1.2.

The risk of tumor recurrence or progression depends on several histopathological factors, such as grade, tumor size, depth of invasion, multiplicity, presence or absence of lymph or vascular invasion and presence or absence of CIS[16,18].

Because superficial tumors, by definition, tend to be limited to the basement membrane without access to the bloodstream, these tumors have a tendency to be smaller and remain localized. In contrast, muscle-invasive tumors represent larger and more aggressive malignancies with the capacity to invade adjacent tissues or other organs of the body. The last phenomenon occurs due to the presence of lymph and blood vessels in muscular layers and is called metastization[15–18].

Since superficial carcinomas correspond to a large spectrum of malignancies, their prognosis is also very variable. Low-grade papillary tumors have a low progression rate despites their high risk of recurrence, while high-grade TCC lesions, especially T1 cancers, have a high potential to recur and progress, becoming invasive[17].

Muscle-invasive tumors have a worse prognosis than superficial tumors, being intrinsically related with poor outcomes. Within invasive tumors, the probability of develop a metastasis disease increases progressively with the stage, while 5-years survival rate decreases with the tumor aggressiveness[16,18].

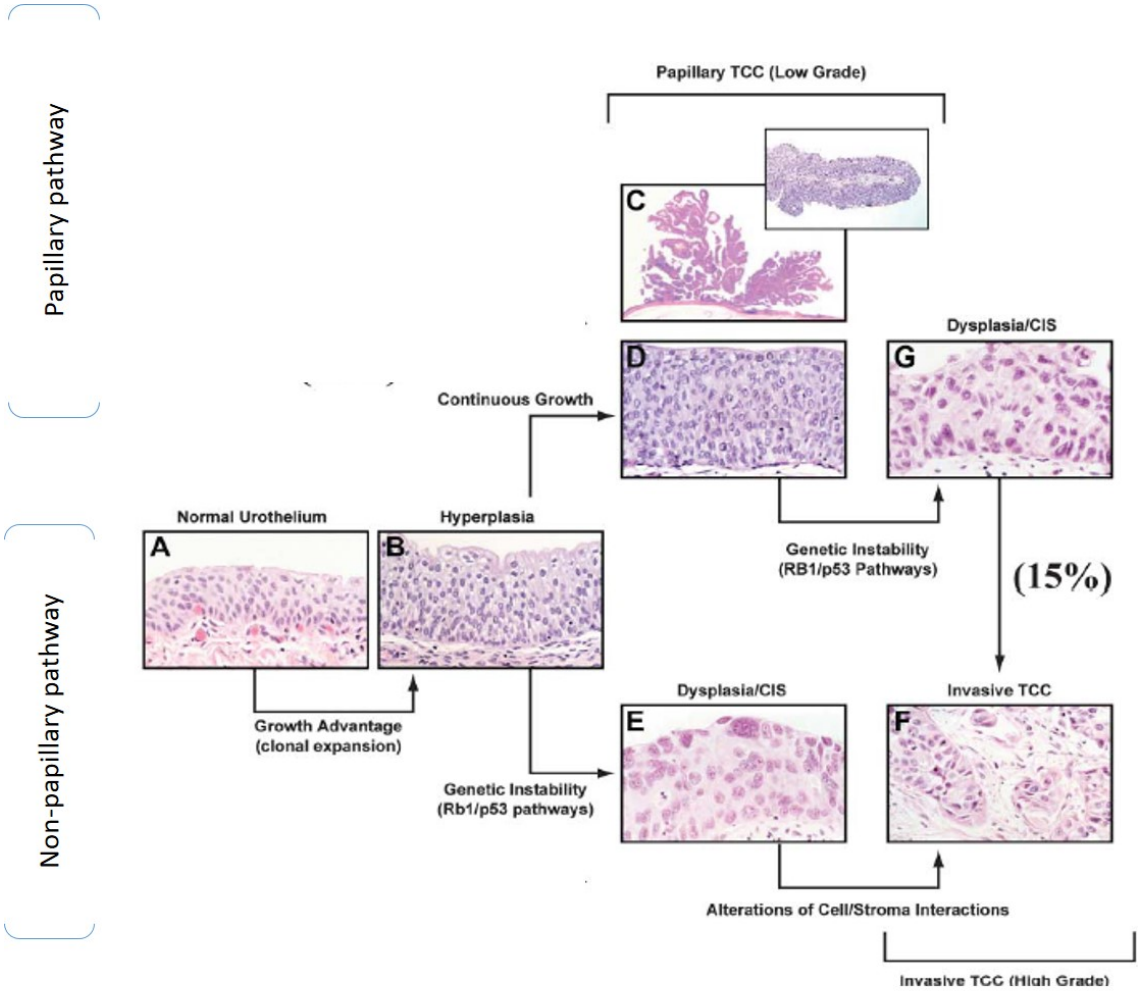

FIGURE 1.2 BLADDER CARCINOGENESIS.
Bladder tumors follow papillary pathway, leading to superficial tumors in most of the cases. Only 15% of tumors that follow the papillary pathway will progress to invasive tumors. Non-papillary pathway occurs in a small percentage of cases and associated with more aggressive tumors. (Adapted from Czerniak et al., 2014)

1.1.4 Diagnosis and Treatment

Currently, the diagnosis of BC is based on imaging techniques, such as magnetic resonance imaging (MRI), and an endoscopy test followed by histological examination of the material obtained by biopsy. Unfortunately, these diagnosis tools do not detect all BC cases[4]. Over the past decades, the scientific community has been able to identify biomarkers to enhance the detection of BC. These group of biomarkers have higher

sensitivity and include cytokeratines, hyaluronic acid or antiapoptotic proteins, like survivin[21].

The choice of an appropriate treatment for BC is essential for overall survival given the high tendency to progress and metastasize in the absence of a correct management. The first procedure after the diagnosis of BC is the transurethral resection (TUR) of all the visible lesions in the bladder wall[14].

For low-grade non-invasive tumors, the surgical resection is considered sufficient, however most of them will recur within a 5-years period, frequently without tissue invasion. These cases rarely result in death of the patients. Intravesical chemotherapy or immunotherapy are performed to reduce the risk of progression or recurrence[4,14,22].

Although a variety of intravesical therapies are used to treat BC, one of the standard procedures is Bacilus Calmette-Guérin (BCG) therapy. Briefly, administrated live BCG interacts with urothelial cells and is internalized by cancer cells through macropinocytosis. Then, BCG internalization lead to an overexpression of MHC-II and ICAM-I molecules, which facilitate the antigen presentation to immune cells, and a secretion of cytokines, such as IL-6 and IL-8, that recruit immune cells to the site. Subsequently, infiltrating immune cells exert their cytotoxic effect against tumor cells by several mechanisms[23].

Interestingly, natural killer (NK) cells, among other immune cells, are capable to reach bladder tissue after BCG therapy. The cytotoxicity of BCG-specific NK cells, which occurs essentially by perforin mechanism (explained in detail in NK section), is augmented by IL-12 and interferon (IFN)-γ and inhibited by IL-10. BCG therapy is used in high-risk superficial tumors upon TUR and it is associated with decreased risk of recurrence and progression to an invasive stage[23].

In an attempt to decrease the risk of recurrence and progression, the intravesical therapy with chemotherapeutic drugs is widely used in clinical practice. Mitomycin (MMC), doxorubicin (DOX) and epirubicin are the most common drugs used, which are administrated immediately after resection[14,24].

In most aggressive cases, the standard care involves the radical cystectomy. Due to the high risk of recurrence, chemotherapy has been recommended as an adjuvant therapy to occult micro metastases. Several combinations of chemotherapeutic agents have been tested in clinical trials, presenting similar effects on patient's survival. The group of chemotherapeutics includes cisplatin, doxorubicin, methotrexate and vinblastine[14].

Cisplatin-based chemotherapy has remained as the first-line treatment for BC for decades due to its lower cytotoxic effect comparing with other drugs. As a result of it, little progress has been made in developing novel agents for BC treatment and medium survival for patients with high-grade BC has not change significantly, remained at about 12 months[14].

Currently available therapies for BC consistently fail in eradicating permanently the tumor and the relapse often occurs. Chemotherapeutic drugs used in cancer usually induce tumor shrink but it grows back after some time[18]. This phenomenon may be explained by the presence of a small population of cells with drug-resistant phenotype and ability to induce tumor growth, named cancer stem cells (CSCs)[25,26]. Therefore, it has been suggested that CSCs-targeted therapies could provide a long-term disease-free survival when associated with non-CSCs therapies[27].

1.2 Cancer stem cells

1.2.1 Definition and Discovery

Normal stem cells are defined as undifferentiated cells capable to generate mature cells through a differentiation process. Essentially all tissues in the human body present a pool of adult stem cells that possess the ability to self-renew and generate an organ-specific cell following a hierarchical model[28,29].

It has been proposed a cancer hierarchy model for carcinogenesis, which postulates that a tumor is organized like a normal tissue with a subset of stem cells responsible for the generation of identical daughter stem cells as well as cells that undergo differentiation to become specialized tissue cells. In this model, CSCs are lying in the apex of the tumor hierarchy and they are responsible for sustaining tumor growth[26].

The CSCs frequency is highly variable among tumors with a relative low frequency in solid tumors. The CSCs proportion also varies widely in tumors of the same type. Recent evidences indicate that advanced tumors might have a higher CSCs fraction[27,30].

CSCs were first identified in 1994 in acute myeloid leukemia (AML). This initial report demonstrated that only a small population of cells, characterized as $CD34^{+}CD38^{-}$ cells, was able to reproduce a heterogeneous tumor when transplanted to a severe combined immunodeficiency disease (SCID) immunodeficient mice. The formed tumor presented cells in various stages of differentiation and contained multiple mature blood cell types. Therefore, the authors demonstrated the existence of a subset of cells with the ability of self-renewal and extensive proliferation[31].

Since this discovery, cells with stemness features were found in several solid tumors, such as breast, brain or colon cancer[32–34].It has been used several terms to describe CSCs, namely cancer initiating or cancer propagating cells.

1.2.2 Cancer Stem Cell hypothesis

The majority of the tumors presents a functionally and phenotypically heterogeneity among cancer cells. Two models have been proposed two explain the cell heterogeneity within the tumor: the stochastic/clonal evolution model and the CSCs model, which are illustrated in Figure 1.3[35,36].

The stochastic model is a nonhierarchical model, which assumes that any cell within the tumor has the same tumorigenic potential. This model postulates that the

tumor heterogeneity is caused by genetic and epigenetic changes leading to a selection process. Thus, the appearance of advantageous genetic/epigenetic changes tends to cause more aggressive tumors over the time, whereas disadvantageous genetic/epigenetic alterations contribute to the loss of tumorigenic potential. The stochastic model predicts that the heterogeneity could lead to differences in therapy response[37–39].

The CSC model postulates that only a small group of cancer cells can proliferate extensively given rise to non-tumorigenic tumor cells, which compose the bulk of the cells in the tumor. Unlike CSCs, non-tumorigenic tumor cells have little capacity to contribute to the disease progression[40]. In this model, the differentiation of CSCs can provide a mechanism of generation phenotypic and functional heterogeneity. Cancers that follow the CSC model are also subject to clonal evolution as well as environmental pressures[35,36,38].

Several data support the CSCs model in several types of cancer, such as leukemia, breast or brain[31,32,34]. In each case, the ability to propagate the disease upon transplantation into an immunocompromised NOD/SCID mice appeared to be restricted to a small population of tumor cells. These studies also prove that tumorigenic and non-tumorigenic cancer cells have a distinct immunophenotype[41].

Although recent studies in many types of cancer strong suggest that these tumors follow the CSC model, it seems that this model cannot be applied to all types of cancer[35,38].

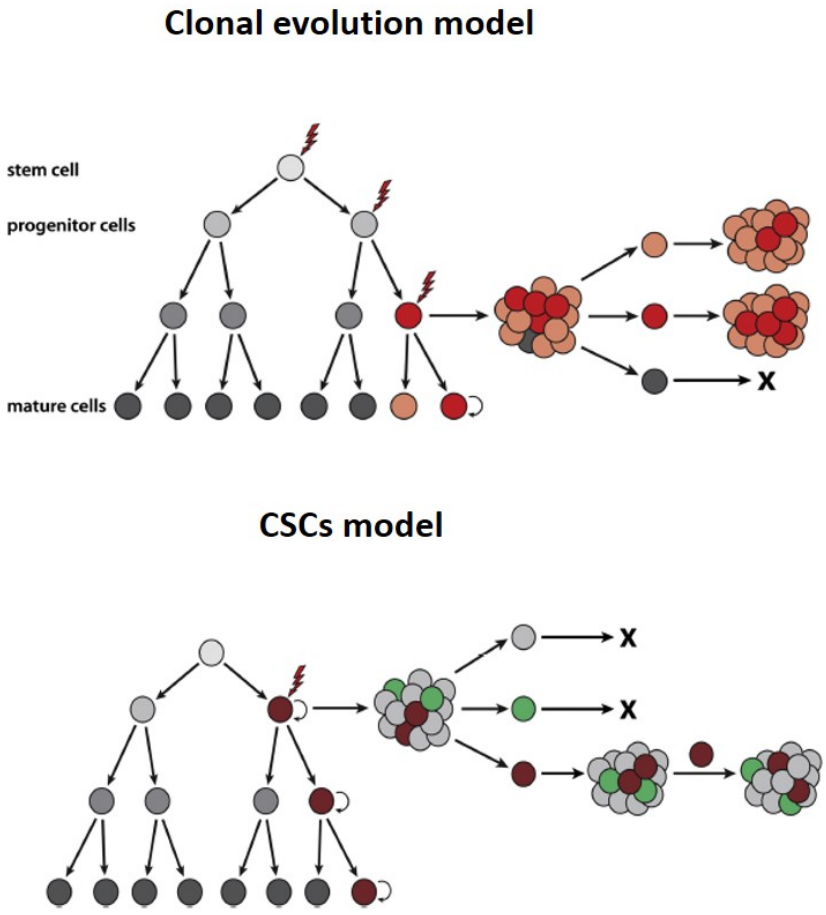

FIGURE 1.3 CLONAL EVOLUTION MODEL AND THE CANCER STEM CELLS MODEL.
Clonal evolution model states that tumor heterogeneity is caused by the occurrence of series of genetic mutations that confer a selective growth advantage, while CSCs model propose the existence of a pool of CSCs in the ability of generate heterogeneity through a differentiation process. (Adapted from Lindeman et al., 2012)

1.2.3 Cancer Stem Cells biology

Despites the name, a CSC is not necessarily derived from a normal tissue stem cell that undergo trough mutations giving rise to a malignant clone. CSC can arise from differentiated cells as a result of mutations that activate self-renewal mechanisms, thus acquiring the properties of a stem cell[42].

The location of CSCs within the tumor is not fully known. Like adult stem cells, CSCs may exist in a physiological limited and specialized microenvironment, named CSC niche. The CSCs niche has the functions of maintain the stem cell pool, varying in nature and location with the tumor type. The existence of a CSCs niche has been associated

with CSCs quiescent state since the microenvironment would produce molecules with proliferation and growth inhibitory functions. Several data reinforce the idea that the niche may be important for maintaining the asymmetric division and consequently the self-renew property of CSCs but there is no agreement if CSCs require a specific niche or they are capable of survive in normal stem cell niche[43,44].

Over the last years, several groups have been trying to identify CSCs markers among diverse types of tumor in order to distinguish CSCs from cancer cells with more limited proliferative potential. Due to the highly inter- and intratumoral heterogeneity, the identification of CSCs markers has been difficult. Fortunately, the rapid development of the CSCs field combined with genome screening techniques has allowed the identification of new CSC markers in a wide range of tumors[45,46].

Each type of cancer has distinct combination of surface markers that demark the CSCs pool. CD44, CD24, CD133, EpCAM and ALDH1 have been commonly used as CSC markers in many types of cancer however they are not considered universal markers. Nonetheless, these markers are not exclusive for CSCs in any tumor[26,45,46].

The existence of a CSCs subset has been extensively studied in breast, prostate, glioblastoma and lung cancer among other malignancies. The range of markers used to identify CSCs in several solid tumors are described in table 1.

TABLE 1.1Phenotype of cancer stem cells among several malignances.

Type of tumor	CSCs phenotype	Reference
Breast	CD44+CD24-/lowCD133+	32,47
Prostate	CD44+CD133+	48
Lung	CD133+	49
Glioblastoma	CD133+CD15+	50
Melanoma	CD20+CD271+	51
Pancreas	CD44+CD24+ESA+	52
Liver	CD133+	53

ESA: epithelial specific antigen

Other markers have been used to isolate CSCs from the remainder cell population, particularly pluripotency-related genes such as Oct4, Sox2, Nanog or Nestin that cooperate to maintain the self-renewal and pluripotency properties of stem cells[54].

CSCs can be isolated by other assays than flow cytometryaccording to CSC-specific surface markers. These functional assays include the sphere-forming assay, the assessment of aldehyde dehydrogenase (ALDH) activity and evaluation of cell tumorigenicity in SCID immunodeficient mice[55].

The sphere-forming assay is one of the most used techniques to isolate CSCs from progeny, based on the capacity of CSCs to grow in non-adherent conditions, forming colonies from a single cell. To perform this assay, a low number of tumor cells (to assure that each colony derived from a single cell) are cultured in serum-free medium, usually using soft agar or matrigel as support. During culture periods, some molecular factors are added to promote the maintenance of the CSCs-like phenotype, such as EGF, bFGF or B27[55–57].

Although the mechanism underlying clonal spheres formation is poorly understood, this technique pretends to generate populations of tumor cells with the capacity to self-renewal and grow in suspension under serum-free conditions. This procedure might be repeated to produce a more purified population of CSCs[55–57].

The measurement of ALDH activity is another technique that allows the isolation of CSCs. ALDH is an enzyme responsible of detoxification of aldehydes, leading to formation of retinoic acid, which confers resistance to some chemotherapeutic agents like cyclophosphamide. High levels of ALDH activity has been associated with low survival rates and chemo resistance and identified tumor cells with stem cells features in some tumors, like breast or colon. Although ALDH family consist in a group of enzymes, the well-studied member is ALDH1A1[55,58,59].

To isolate CSCs using ALDH activity, tumor cells are sorted in a flow cytometer accordingly with their ALDH activity into $ALDH^{bright}$ and $ALDH^{low}$ cells. The $ALDH^{bright}$ cells usually exhibit CSCs-like features[55,58,59].

Ultimately, the main principle of CSCs is their capacity to form a heterogeneous tumor when a small group of cells is transplanted to a SCID immunocompromised mice, resembling the original tumor. Theoretically, a single cell is able to generate a whole tumor, however none of the previous studies achieved that purification state[60,61].

Previous study reported that non-CSC populations also form heterogeneous tumors SCID immunocompromised mice, but in a much lower frequency and requiring a higher cell number. Two models are currently used toevaluate the tumorigenic potential of CSCs, namelythe subcutaneous and the orthotopic models. In subcutaneous xenograft, tumor cells are injected subcutaneously and the tumor grows in a different organ of origin, while in orthotopic model cells are directly delivered to the normal organ niche[60,61].

1.2.4 Drug resistance and therapeutic implications

Even though current cancer therapies, such as chemotherapy and radiotherapy, are responsible for killing the majority of cells in the tumor, frequently the tumor relapses. This might be explained by the presence of a CSC pool within the tumor mass, which are less sensitive to these therapies. Thus, CSCs remain viable after the treatment and have the ability to re-establish the tumor[35,62].

Anticancer therapies mostly fail to eradicate the CSCs pool instead favor their expansion and/or selection of resistant clones. Thus, the successful elimination of a cancer requires an anticancer therapy focused in differentiated cancer cells and potential CSCs[35,62].

It has been suggested that CSCs are resistant to diverse therapy approaches in the same way that normal stem cells are protected against injuries. The protection mechanisms include quiescence status, expression of ATP-Binding Cassette (ABC) transporters, high expression of antiapoptotic proteins and enhanced DNA-damage repair mechanisms, previously described[25,63].

One particularly characteristic of CSCs is their ability to enter in a quiescent stage, dividing infrequently. Quiescence might be a crucial mechanism to drug resistance since chemotherapeutic drugs act mainly in highly proliferating cells. Further, the dormancy state might explain the tumor recurrence or metastasis after long lag periods[64].

Another mechanism of CSCs drug resistance is their augmented expression of ABC transporters members, such as P-glycoprotein (P-gp), Breast Cancer Resistance Protein (BCRP) and Multidrug Resistance-associated proteins (MRPs). These receptors are responsible for the drug efflux in an ATP-dependent mechanism. Thus, chemotherapeutic drugs that are substrates of ABC transporters do not remain in the interior of cells long enough to induce apoptosis[63].

The escape from induction of apoptosis has been considered one of the hallmarks of CSCs, representing a key mechanism against oncogenic events. CSCs present compensatory mechanisms to escape from apoptosis fate otherwise they were not capable of survive and self-renew. The apoptosis escape could be achieved specially by increasing levels of anti-apoptotic proteins or decreasing the quantity of pro-apoptotic members[65].

Further, CSCs have a higher activation and expression of genes and proteins with DNA-repair functions than non-CSCs. Thus, CSCs use more genetic repair mechanisms, allowing the maintenance of their functional abilities[66].

It has been proposed that cancer therapies currently employed might cause a positive selection of resistant cancer cells and dissemination of CSCs from their niche. When subjected to a new set of microenvironmental selection pressures, CSCs acquire a different phenotype, leading to CSCs heterogeneity. CSCs heterogeneity was recognized in many types of cancer, including BC, using karyotyping approach and suggested as a cause to differences in patients therapeutic responses[40].

Despite the true clinical relevance of the CSCs population is yet to be revealed, it seems that therapies directed against CSCs might be essential to long-term disease-free survival[28]. The tumor progression after current therapies are illustrated in figure 1.3.

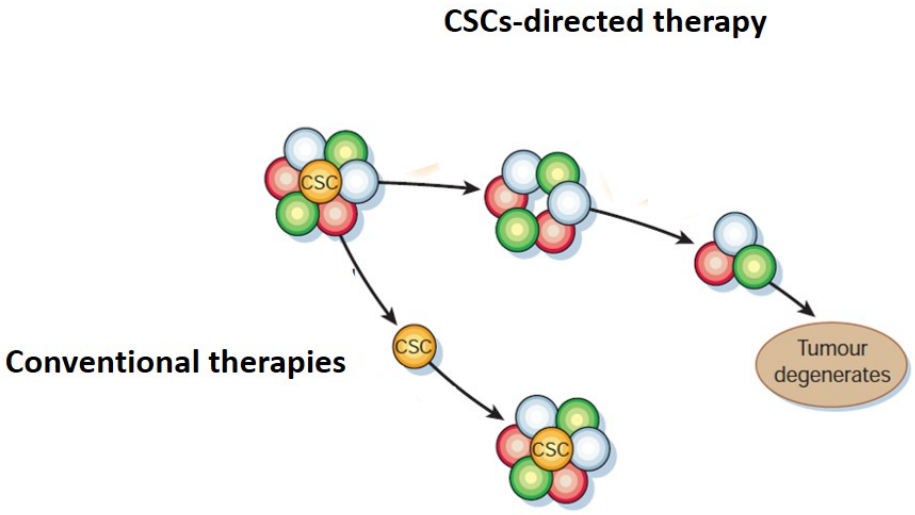

FIGURE 1.4 TUMOR PROGRESSION AFTER CHEMOTHERAPY.
Currently therapies do not affect the CSC pool, which re-establish the tumor. In contrast, therapies directed to CSCs promotes their elimination prevent tumor grow and reappearance. (Adapted fromWeissmanet al., 2001)

1.2.5 Bladder Cancer Stem Cells

The human urothelium is constantly renewing itself during normal homeostasis. This ability is achieved by the presence of basal stem cells with a high capacity for cell division. Urothelium stem cells are protected from differentiation, loss of self-renewal capacity and apoptosis by a specific anatomical and functional microenvironment, called niche[29,67].

Although their origin remains unknown, it has been described a tumorigenic basal cell population in the interface between tumor and stoma, that resemble benign urothelial stem cells. This population might represent bladder CSCs[68,69].

Like in other types of cancer, bladder CSCs can give rise to a heterogenic tumor population by differentiating in down-stream tumor cells[67].

Several studies demonstrate that a cell population identified as bladder CSCs is more resistant to cisplatin-based treatments, which are the most commonly used drugs

to advanced BC. This phenomenon might be due to CSCs characteristics described above[70].

In last years, the epithelial to mesenchymal transition (EMT) has been associated with stemness and CSCs origin in epithelial tumors, playing a role in cancer progression and metastasis[3]. Several recent studies propose that the generation of bladder CSCs occurs during tumorigenesis following EMT, through a cell selection mechanism[71,72].

Although the bladder CSC molecular profile is not fully characterized yet, some biomarkers have been identified in cancer stem-like cells. CD44 is one of the most prominent molecules implicated in cell adhesion and used for bladder CSCs isolation. The tumorigenic potential of $CD44^+$ tumor cell is 10-200 times greater than that of $CD44^-$ cells[73]. Several studies demonstrate that intracellular cytokeratin 5 (CK5) is usually co-localized with CD44 in tumor cells. In contrast, in immunofluorescence assays $CD44^-$ cells are often co-localized with CK20 molecule, which is the differentiated molecule of CK5[74,75].

The ALDH1A1, previously described, has been used to isolate CSCs in BC due to its higher expression in bladder cancer stem-like cells. A recent study demonstrates that bladder $ALDH1A1^+$ cells has 100-fold greater potential of tumor formation than bladder $ALDH1A1^-$ cells. Interestingly, $ALDH1A1^+$ cells appear to be enriched in $CD44^+$ tumor cell with more primitive CSC features[76]

1.3 Natural Killer cells

1.3.1 Definition, Origin and Types

Natural Killer (NK) cells are a subpopulation of innate lymphoid cells (ILCs) with cytotoxic potential. NK cells represent approximately 15% of all lymphocyte in bloodstream and have a crucial role in the innate immune system, which is responsible for the rapid response and protection against host injuries[77].

NK cells are developed in bone marrow from common lymphoid progenitor cells. After development, these cells distribute throughout tissues, namely bone marrow, spleen, lymph nodes and peripheral blood[77].

The human NK cells usually are characterized phenotypically as $CD56^{+}CD3^{-}$. These cells can be divided in two subsets as $CD56^{dim}$ and $CD56^{bright}$ based on the relative expression of the CD56 marker. $CD56^{dim}$ NK cells comprise the majority of circulating cells, whereas $CD56^{bright}$ set represent only 5-15% of total NK cells, being mainly localized in lymph nodes[77,78].

$CD56^{dim}$ NK cells are highly cytotoxic cells upon interacting with target cells but have a lower cytokine production potential, when compared with $CD56^{brigh}$ NK cells. In contrast, the $CD56^{brigh}$ subpopulation exhibits predominantly immune regulation properties by producing high amounts of cytokines, such as interferon-γ (IFNγ) in response to stimulation by several interleukins like IL-12 or IL-15[78].

1.3.2 Recognition and killing pathways

Natural killer cells have the capability to recognize stressed cells, such as tumor cells or virus-infected cells from normal cells in the absence of pre-immunization or stimulation. NK cells have a large group of receptors, which enable them to recognize altered cells as targets. The group of NK receptors consists of activating, inhibitory, adhesion molecules and cytokine receptors, as listed in table 1.2. The combination of all signals determines whether an NK cell becomes activated or not[79].

Inhibitory receptors prevent the activation of NK cells and, consequently, their action against cells. The inhibition of the lytic process is due to the expression of one or more immunoreceptor tyrosine-based inhibition motifs (ITIM), which upon activation recruits SHP1, SHP2 and SHIP phosphatases to prevent activation cellular signaling cascades[80].

In contrast, activating receptors are associated with immunoreceptor tyrosine-based activation motifs (ITAM), which recruit Syk upon phosphorylation. Consequently,

Sky leads to activation of cellular signaling cascades responsible for degranulation and transcription of chemokines and cytokines. Moreover, NK cells exhibit also a group of co-stimulatory receptors. Although these molecules have no capacity to trigger NK cells activation by itself, they provide further stimulation. The group of co-stimulatory receptors include DMAN-I and NKR-P1[80].

The cell adhesion molecules (CAM) are receptors on NK cells surface responsible for the interaction with accessory and target cells, being essential players to an effective NK cells response[81].

NK cells also exhibit a range of cytokine receptors on their membrane that interact with cytokines released by neighbor immune cells, especially dendritic cells. Cytokines influence the cytotoxic activity and NK cells[82].

TABLE 1.2List of the most import natural killer cells receptors organized by function

Inhibitory receptors	Activating receptors	Adhesion receptors	Cytokines receptors
Ly49A **Ly49C** **KIR2DL1** **KIR2DL2** **NKG2A** **LIR** **KLRG1**	NKG2C NKG2D NKp30 NKp44 NKp46 DMAN-I NKR-P1	LFA-1 CD54 CD58 NKH-1	IL-2/15r IL-12R IL-18R IL-21R IFNAR

The NK cell recognition of a stressed cell is a complex mechanism, mainly explained trough two different models: *missing-self* and stress-*induced self* recognition[79,83].

The major histocompatibility complex-I (MHC-I) is a group of glycoproteins expressed by nearly every cell of the body with the function of display cell antigens. Every NK cell expresses inhibitory receptors that recognize MHC-I and protects normal cells from NK-mediated killing. In cancer, the expression of MHC-I is often reduced or even lost, allowing NK activation and action upon these cells (*missing-self model)*[79,83].

Besides the inhibitory receptors that identify self, NK cells also have activating receptors on their membrane. These activating receptors are able to detect molecules that in a steady-state condition would be barely detectable. The upregulation of activating receptors ligands is usually associated with various forms of stress, including cancer *(stress-induced self model)*[79,83].

Therefore, the recognition of a tumor cell by a NK cell is a tightly regulated process, involving the integration of both positive and negative signals. Whether NK cells receive more positive signals, delivered by activating receptors, than negative signals, from inhibitory receptors, they become activated, resulting in a target-cell killing. NK cells can also be activated by various kinds of cytokines[79].

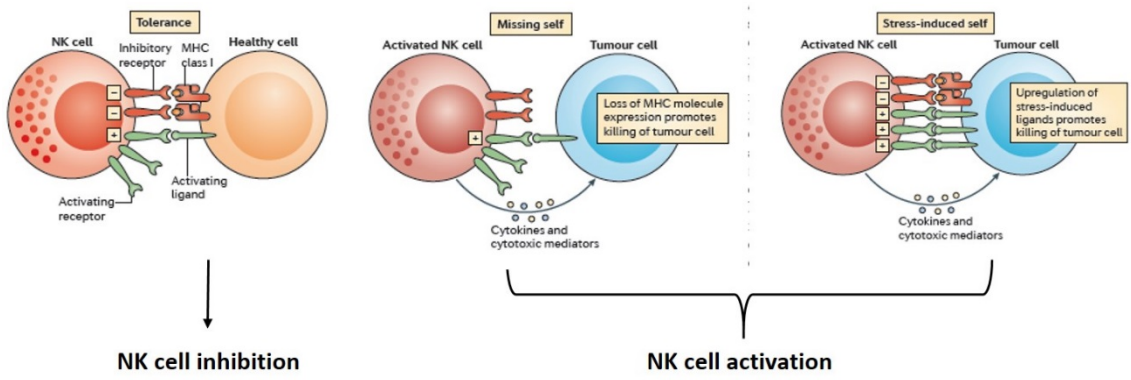

FIGURE 1.5Recognition mechanisms of NK cells.
Tumor cells alterations deregulates the expression of activating and inhibitory receptors, allowing the recognition by NK cells and consequently their activation. The recognition occurs when tumor cells loose expression of inhibitory ligands, like MHC-I, or due to the upregulation of stress-induced ligands. (Adapted from Brossay et al., 2012)

After recognition, NK cells kill directly target cells mainly through by several mechanisms, namely the perforin/granzyme system, IFNγ-mediated pathway and the death receptors-mediated apoptosis[83].

One way of killing target cells is by releasing cytoplasmic granules, containing perforin and granzymes, by exocytosis. Granzymes enter in target cells through pores in plasma membrane formed by perforin and then induce cell death in a caspase-dependent or independent manner. Granzyme-A activates caspase-independent cell death via single stranded DNA damage, while Granzyme-B cleaves caspase-3 resulting in

DNA fragmentation, degradation of cytoplasmatic and nuclear proteins. Both molecules lead to innumerous biochemical alterations, such as DNA fragmentation, proteins cleavage and expression of ligands for phagocytic cells receptors. This killing pathway is considered the main mechanism used by NK cells to eliminate target cells[84,85].

NK cells can kill target cells by promoting the secretion of various effector molecules, such as IFNγ. The production of IFNγ by mature NK cells is involved in the suppression of pathogens as well as in antitumor functions, like inhibiting angiogenesis and stimulating adaptive immunity. A recent study demonstrate that IFNγ contributes to control the spread of the tumor[85].

Another mechanism of NK cell-mediated killing is through death receptors (DRs), a large group of cell membrane proteins of tumor-necrosis factor (TNF) family[86,87]. Since DR-mediated apoptosis is the main NK killing pathway studied in this work, the DRs are described more extensively below.

Lastly, NK cells are also able of destroy tumor cells by antibody-dependent cellular cytotoxicity (ADCC). In this mechanism, CD16 on NK cells membrane binds to an antigen-associated antibody on target cells surface and, causing NK cells activation and resulting in lysis of antibody coated cells. Although this mechanism was observed in *in vitro* models, an effect in anti-tumor response in human remains to be show[88].

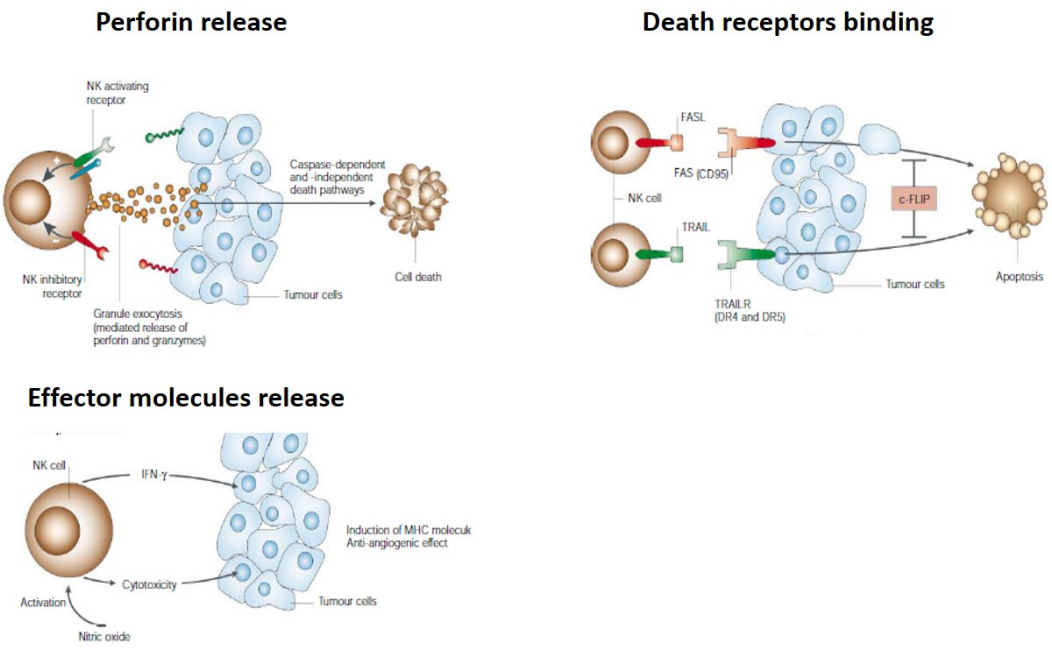

FIGURE 1.6 NATURAL KILLER CELLS EFFECTOR FUNCTIONS.
Upon activation, NK cells exert their cytotoxic action through several mechanisms, such as release of granules containing perforin, engagement with death receptors on target cells and also the release of effector molecules like IFN-γ. (Adapted from Yagita et al., 2002)

During tumor progression, cancer cells promote some mechanisms to escape from NK cell recognition and attack. These defense mechanisms include a downregulation of molecules that promote the NK-mediated killing, like co-stimulatory ligands or ligands for activating receptors, or an upregulation of inhibitory signals, such as MHC I. Moreover, tumor cells can protect themselves from an immune response by secreting immunosuppressive molecules, including IL-10 and TGF-β, and resisting Fas- or perforin-mediated apoptosis[89].

1.3.3 Death receptors

Death receptors belong to the tumor necrosis factor (TNF) superfamily, which includes more than 20 proteins with a wide range of biological functions, including cell death regulation and immune regulation[87].

The best recognized death receptors include TNF-R1, Fas (also called CD95 or APO-1) and DR5 (or TRAIL-R2). These receptors are expressed in a broad range of cells,

including cancer cells, and become activated upon binding to their ligands, expressed constitutively in immune cells, like NK cells. The ligands on NK cells which binds to TNF-R1, Fas and DR5 on tumor cells are TNFα, Fas ligand and TRAIL, respectively[87,90].

The activation of death receptors triggers the apoptotic cellular process. Apoptosis is considered a controlled cellular mechanism capable of eliminate altered cells and characterized by morphological and biochemical alterations, such as DNA fragmentation, membraneblebbing and cell shrinkage[87].

The apoptosis machinery can be activated by two main pathways: the intrinsic pathway, mainly engaged by chemotherapeutic agents, and the extrinsic pathway, associated with death receptors. Unlike intrinsic pathway, the extrinsic pathway is independent of mitochondria and leads to apoptosis in a p53-independent manner. Although different molecules play a role in both pathways, there is a crosstalk at multiple levels[86].

The ligand-receptor binding induce the formation of a macromolecular complex, named DISC, which recruits procaspase-8, an initiator caspase. In death-inducing signaling complex (DISC), procaspase-8 is converted into caspase-8, its active form, which is released into the cytosol where directly cleaves effector caspases-3 and -7, and consequently leads to apoptosis. In the other hand, activated caspase-8 cleaves BID, a BH3 domain-containing proapoptotic Bcl2 family member, which in turns interacts with BAX and BAK proteins to release mitochondrial cytochrome c. Once cytochrome c is released from the mitochondrial compartment, it binds to APAF-1, forming the apoptosome. This complex induce activation of effector caspases -3 and -7 mediated by caspase-9 action[86].

Interestingly, death receptors expression may vary between cell types and is often downregulated or absent in resistant tumor cells. This phenomenon has been associated with the tumor immune escape[90].

Several studies have demonstrated that a death-receptor-based therapy exert a robust anticancer therapy. In this direction, some death receptors agonists (dulanermin, mapatumuab or lexatumumab) have been followed in clinical trials for multiple cancer

types, such as multiple myeloma, breast, leukemia or BC in single treatments or in combination with chemotherapeutic drugs[91].

Some studies with DR5 are already in phase II and results are promising in terms of safety and efficacy. In the other hand, administration of soluble Fas ligand was reported as highly cytotoxic due to its systemic consequences[87,91].

Unfortunately, TNF-α has been used in several clinical trials in a cancer treatment context but the results have been disappointed. Besides its severe toxic effects, several data indicate that TNF-α has a low efficacy rate. Besides this disappointment, the idea of target death receptors to trigger apoptosis in tumor cells remains as attractive antitumor approach, especially because occurs independent of p53 tumor suppressor gene, which is deleted or inactivated in many tumors. Thus, death receptor approach is focused on DR5 and Fas receptors[86,91].

So far, there is no report of ongoing clinical trial that explores the death receptors potential in NK-mediated killing, however *in vitro* and animal model experiments in leukemia, prostate and multiple myeloma demonstrated that an upregulation of death receptors on tumor cells increases the cytolitic potential of NK cells. Thus, the increase of death receptors in tumor cells comes up as an attractive approach to sensitize tumor cells to NK-based immunotherapy[86,91].

Recently, several data indicate that doxorubicin, a chemotherapeutic drug, and bortezomib (BTZ), also called PS-341, a proteasome inhibitor, induce Fas and DR5 expression in tumor cells, which could lead to sensitization of tumor cells to NK cell-mediated killing[92–97].

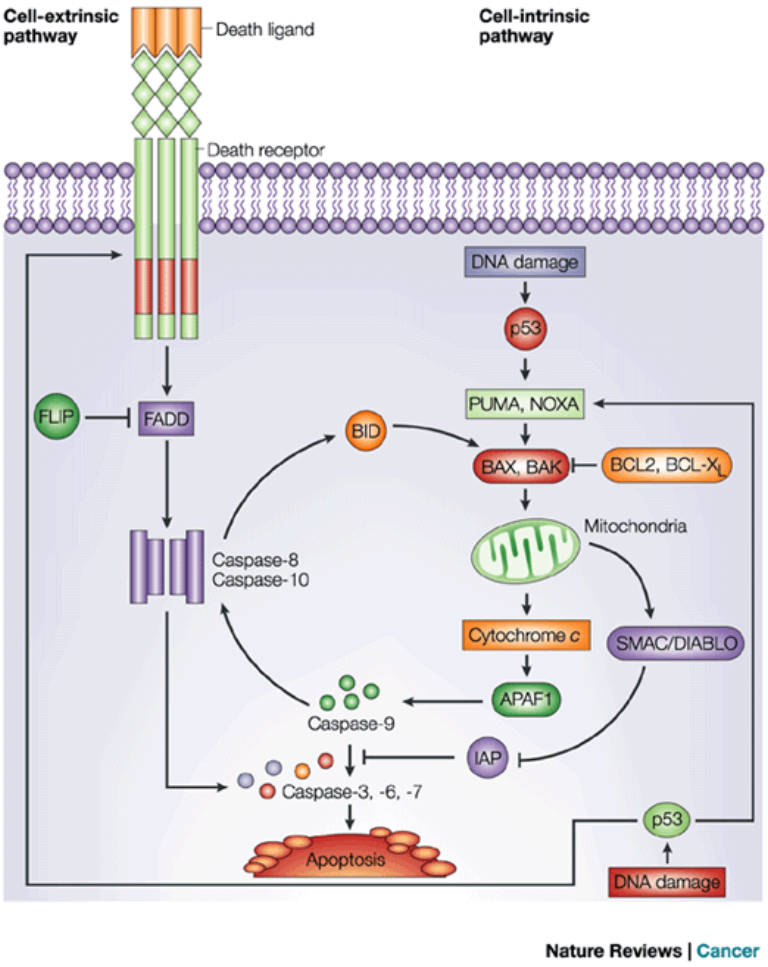

FIGURE 1.7DEATH RECEPTOR SIGNALING PATHWAY
The pro-apoptotic receptors of NK cells bind to death receptors on tumor cells. ligand binding induce the activation of extrinsic apoptosis signaling cascade by recruitment of Fas-associated death domain (FADD) and initiator procaspase-8 and procaspase-10, forming DISC. This complex triggers activation of caspase-3 and -7, the effector caspases. Caspase-8 can also cleave BH3-interacting domain (BID), which promotes mitochondrial release of cytochrome c and, consequently formation of apoptosome and activation of caspase-3 and -7. (Adapted from Krammer et al., 2002)

1.3.4 Immunotherapy

Although immune surveillance of tumor has been a controversial subject for decades, there is evidence that immunosuppressed patients have a tendency to develop some kinds of malignancies[89]. Furthermore, some NK abnormalities have been observed, such as lower proliferation rates, reduced cell cytotoxicity, overexpression of inhibitory receptors and defective cytokine production[83].

Low levels of NK cells in peripheral blood has been associated with higher risk of cancer while enhanced levels of NK cells in peripheral blood have a significantly longer metastasis-free survival time[78,83,98].

The presence of NK cells within the tumor tissue appears to be a good prognosis factor, at least for several types of cancer. Further, some studies have demonstrated that NK cells are highly effective at eliminating tumors, especially small tumor grafts and metastasizing cells[99–101].

Due to their great potential to recognize and eliminate tumor cells, NK cells appear to be one of the most likely effectors for an effective antitumor immune therapy. Thus, they have been widely studied in several immunotherapeutic approaches for cancer and recently trialed in a clinical context[78,98,102].

The NK cell-based therapies have been most successful in hematopoietic cancer but more recently the immunotherapies using NK cells have been more studied in solid tumors and metastatic cancers[78,83].

The use of NK cells in an immunotherapy approach has many advantages in a clinical context. NK cells are antigen non-specific and do not require a specific donor HLA allotype, avoiding rejection risk. These immune cells recognize a broad group of ligands that induce a cytolitic response. Thirdly, NK cells can be easily isolated and expanded *ex vivo*, allowing their use in both adoptive and autologous cell therapies[78].

Importantly, some recent studies suggest that NK cells appear to kill CSCs more effectively than chemo and radiotherapy, the common therapeutic approaches for cancer. Since NK cells are capable of target and eliminate both CSC and non-CSC population, NK immunotherapy is theoretically an effective antitumor therapy[103,104].

There are several approaches to NK-based immunotherapy. One of the strategies already studied in a clinical trials is the administration of cytokines, which promotes the proliferation and activation rates of endogenous NK cells through a direct or indirect mechanism. Activated NK cells present a higher cytotoxic potential than non-stimulated cells. The group of cytokines used in cancer treatment includes IL-2, IL-12 and IL-15. These cytokines bind to specific receptors on NK cells (previously described) and

promote an increase in NK cytotoxic potential due to an augment of IFNγ production and also NK cell survival[78].

The obtained results from these clinical trials vary accordingly with the kind of tumor and cytokines administration conditions. The use of cytokines in cancer therapy has two main limitations, namely the toxicity of their systemic administration and cytokine-activated NK cells apoptosis[83].

Immunomodulatory drugs, like thalidomide and their analogues used in some cancers, such as myeloma, can also activate NK cells by increase IL-2 secretion in T-cells, which explain in part the mechanism of these anticancer drugs[105].

Other therapeutic strategies for cancer include the use of exogenous NK cells via hematopoietic stem cell transplant (HSCT) or adoptive cell transfer (ACT)[78].

HSCT is most common used in hematological malignancies, being divided according to the donor cell source in autologous (the donor and the recipient are the same person) and allogeneic (the donor and the recipient are different persons). After the HSCT, NK cells are the first lymphocytes to repopulate, being capable of mediate graft-vs-tumor, which occurs when graft recognizes the tumor population. NK cells don't contribute to graft-vs-host disease in contrast to other lymphoid populations, like T cells[78].

The administration of autologous NK cells with high doses of IL-2 were tested in models of glioma, renal carcinoma or melanoma, but results were disappointing since subsequently studies showed similar effect when IL-2 were administrated alone. Although both autologous and allogeneic NK cells can be used for ACT, the lack of significant results with autologous NK cells led to shift the focus to allogenic cells[98].

The administration of allogenic NK cells must be associated with intravenous injection of activating cytokines, otherwise NK cells undergo apoptosis before of interact with the tumor. The group of cytokines that regulates survival, proliferation and function of NK cells include IL-2, IL-12, IL-15 and IL-18, resulting in a far greater NK cells activation and cytotoxicity that can be achieved *in vivo*. It has not been reported adverse side

effects of NK cell allogenic transfer. The main obstacle of ACT is the low number of NK cells in peripheral blood mononuclear cells (PBMCs) and effector cells preparation[78,98].

As already mentioned, the increase in the death receptors levels in tumor cells appear to be a possible alternative to currently NK cells immunotherapies. This approach has been poorly explored to improve NK cells cytotoxic activity in the presence or absence of activating cytokines[106].

It is likely that combinatory therapies, in which NK cells are one of components, will become the main therapeutic approach for many types of cancer in the near future. The combination of therapies with NK cell therapy can promote synergetic antitumor effects[102].

CHAPTHER 2

OBJECTIVES

The main goal of this work is to investigate the effects of sub-apoptotic concentrations of DOX and BTZ in the sensitization of bladder cancer cells (parental cells and corresponding CSCs) to NK cells-mediated cytotoxicity through upregulation of death receptors. To achieve this goal we propose to:

- Isolate subpopulations of CSCs from two human bladder carcinoma cell lines (UM-UC3 and HT-1376), using the sphere-forming assay;
- Perform the characterization of parental bladder cancer cells and CSCs regarding expression of death receptors Fas and DR5;
- Identify the concentrations of DOX and BTZ that promote the upregulation of the two major death receptors Fas and DR5 in bladder cancer cells;
- Assess the effects of DOX and BTZ in bladder cancer cells susceptibility to NK-mediated killingthrough death receptor-mediatedapoptosis*;*
- Perform the characterization of death receptors expression in specimens of human bladder cancer tumors;

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CHAPTHER 3

MATERIAL AND METHODS

3.1 Bladder carcinoma cell lines

The human UM-UC3 and HT-1376 bladder carcinoma cell lines were purchased from the American Type Culture Collection (ATCC, Rockville, MD, USA). The cell lines grow in monolayer and were routinely cultured in RPMI 1640 medium (R4130, Sigma-Aldrich, USA) supplemented with 10% fetal bovine serum (FBS, Gibco, Invitrogen Life technology), 1% antibiotic/antimicotic containing 100U/mL penicillin, 100µg/mL and 0,2 µg/mL amphotericin B (Sigma-Aldrich, USA) and 1% L-glutamine (Sigma-Aldrich, USA). Cells were maintained at 37^{o}C in a 5% CO_2-95% air atmosphere and passaged in sterile conditions when reaching 80% of confluence. Cells were discarded after 40 passages.

3.1.1 Cell viability

The viability of cells was assessed before all experiments through the trypan blue method. Trypan blue is a stain that confers a distinct blue color to non-viable cells when observed at microscope, while viable cells remain unstained. This phenomenon occurs due to the fact that non-viable cells do not exhibit an intact and functional membrane to prevent the dye uptake.

To perform this technique, equal volumes of cell suspension and 0,4% trypan blue solution (Sigma Chemicals) were mixed and transferred by capillary action into a Neubauer hematocytometer (Optic Labor). Then, cells were observed and counted in an inverted microscope (Nikon, Eclipse TS 100). Both viable (unstained) cells and non-viable (stained) cells were counted in two or more of four corner quadrants.

Cell viability was calculated using the following expression:

$$number\ of\ viable\ cells = cell\ average\ \times\ 10^4 \times dilution\ factor$$

Only cellular suspensions with viabilities higher than 90% were used in all experiments.

3.2 Reagents

The proteasome inhibitor, BTZ, was provided by Cell Signaling Technologies. Stock BTZ solution (6,5mM) was prepared in sterile dimethyl sulfoxide (DMSO) and stored at -80^{o}C. The stock solution was diluted to 1mM solution in DMSO and stored at -80^{o}C as well. Aliquots from 1mM solution were stored at -20^{o}C for up to months. The 1μM solution was prepared from 1mM solution and diluted in Dulbecco's PBS solution, immediately before the use. BTZ solutions were protected from the light at all times.

Doxorubicin was obtained from DOXO-CELL. Stock solution (3,45Mm) was diluted in Dulbecco's PBS solution and stored at -20^{o}C. Any diluted solution was prepared immediately before use.

Concanamycin A (CMA) was obtained from Santa Cruz Biotechnology. Stock solutions (57,7μM) were prepared in sterile DMSO and maintained tightly sealed at -20^{o}C. The 2 μM solutions were prepared from stock solution, aliquoted, stored at -20^{o}C and thawed only once.

3.3 Sphere-forming assay

The isolation of CSCs from UM-UC3 and HT-1376 cell lines was performed using the sphere-forming assay. For this assay, a cell suspension containing 2x10^{5}cell/mL were mixed with Matrigel (BD Biosciences). Then, 100μL of the cell/matrigel mixture were added to each well in a 12-well plate (Orange Scientific). Cells were incubated for 30m at 37^{o}C before the addition of 1mL of serum-free DMEM/F12 medium supplemented with 2μl/mL bFGF (Peprotech), 2μl/mL EGF (Sigma-Aldrich) and 20μl/mL B27 (GIBCO, Life technologies). The medium was changed every three days.

The spheres were collected after 7-9 days of culture by addition of 1mg/ml of dispase solution (GIBCO, Invitrogen Corporation) and incubation for 45m-1h at 37^{o}C.

After that, the cell aggregates were transferred to a tube and centrifuged at 1500RPM for 5m and then incubated with 500µL of accutase solution (Sigma-Aldrich, USA) for cells dissociation. After 5m, RPMI medium containing 10%FBS was used to stop the accutase reaction and the cell suspension was centrifuged at 1500RPM for 5m. Once centrifuged and the supernatant was removed, CSC were prepared to use.

3.4 Cytotoxic assays

The cytotoxicity of DOX and BTZ was accessed using the [3-(4, 5-Dimethylthiazol-2-yl)-2, 5-Diphenyltetrazolium Bromide] (MTT) colorimetric assay. When the cell confluence reached 80%, cells were detached using trypsin/EDTA (Sigma-Aldrich), counted and seeded in 96-multiwell plates.

The cell density plated *per well* was determined accordingly with the period of incubation with the drugs and the cell growth rate in order to avoid cells reaching full confluence. Briefly, for the 48h incubation assay, cells were plated at a density of 10.000 cells/well or 12.000 cells/well for UM-UC3 and HT-1376, respectively. In 24h incubation assay, cells were seeded at 7500cells/well or 9500 cells/well for UM-UC3 and HT-1376, respectively.

After 24h incubation, to allow cells attachment to the wells, drug solutions or fresh RPMI 1640 medium (control) were added to each well at different concentrations, in duplicate. The DOX concentrations added to tumor cells vary between 0.01µM and 0.1 µM (0.01µM, 0.025µM, 0.05µM, 0.075µM and 0.1µM) while the range of BTZ concentrations include 5nM, 10nM and 20nM. Plates were incubated for an additional 24h or 48h in the CO_2 incubator.

After the incubation periods, the culture medium was removed and 50µl of MTT solution (Sigma-Aldrich) at 0.05mg/mL was added to each well. Then cells were incubated for 4h at 37^{o}C protected from the light. Subsequently, the formazan crystals were dissolved in 100µl of 0,04M Isopropanol and the absorbance was measured at

570nm and 620nm in Synergy™ HT multi-detection microplate reader (BioTeck) using Gen 1.09 software.

The signal intensity generated is dependent of the quantity of formazan, the product of MTT reaction, which is directly proportional to the number of viable cells since only viable cells can reduce MTT to formazan. The cell viability was calculated as percentage of the average absorbance of treated cells relative to the absorbance of untreated control cells using the following formula:

$$\%\ cellular\ viability = \frac{Abs\ (sample)}{Abs\ (control)} \times 100$$

3.5 Gene expression analysis

3.5.1 RNA extraction

The RNA extraction is the first step to investigate the gene expression of cell lines. The extraction of total RNA was performed using TRIzol reagent (Invitrogen). Briefly, 1 ml TRIzol was added to samples in order to promote biological material solubilization and protein denaturation. Samples were incubated for approximately 5 min with constant agitation before the addition of 200µL chloroform. Then, samples were centrifuged at 13 000RPM for 10 min at 4°C.

The centrifugation process promotes the formation of three layers: the lower layer constituted by chloroform, an interphase layer containing proteins and lastly an aqueous layer with RNA molecules. Thus, aqueous (upper) layer is transferred into another tube.

Then, 200µL isopropanol was added to wash and precipitate RNA present in the solution. Subsequently, the sample was centrifuged at 13 000RPM for 10 min at 4°C and

the formed pellet was washed with 500µL of ethanol. After removing the main supernatant, the pellet was left at room temperature to dry and 100µL solution of H_2O mQ was added to dissolve the obtained RNA pellet.

The concentration and purity of isolated RNA were assessed using a NanoDrop ND-1000 spectrophotometer (Thermo Scientific, USA). The ratio of absorbance at 260nm and 280nm is used to assess the purity of the RNA preparation. Only samples with A260/A280 ratio higher than 1.6 were used in qRT-PCR technique.

3.5.2 Complementary DNA synthesis

The RNA was reverse transcribed using NZY First-Stand cDNA Synthesis kit (Nzytech), accordingly with the manufacturer's indications. Briefly, 1µg of RNA was mixed with 10µL NZYRT 2X master mix, 2µL NZYRT enzyme mix and H_2O mQ until make up 20µL.

The master mix solution contains dioxylnucleotides (dNTPs) that are used two the new strand and MgCl2, which acts as a co-factor of reverse transcriptase action. In the other hand, the enzyme mix include reverse transcriptase, enzyme that binds dNTPs to single strand of RNA in a complementary-manner, as well as ribonuclease inhibitor to protect the degradation of RNA.

Samples were incubated at 25°C for 10 min, followed by 50°C for 30 min for cDNA synthesis, and then at 85°C for 5 min for reverse transcriptase inactivation, using system 9700 thermal cycler (Applied Biosystems).

Finally, RNA strand is degraded by adding 1 µL RNase H (*E. Coli*) and incubated at 37°C for 20 min, leaving a single stranded cDNA ready for qRT-PCR. Obtained samples were diluted 25x and stored at -80^{o}C until use.

3.5.3 qRT-PCR

Quantitative real-time PCR was performed using a Perfecta SYBR Green Fast Mix (Grisp®) in a CFX96 real-time PCR detection system (Bio-Rad, Irvine, CA, USA). GAPDH and 18S were used as housekeeping genes for normalization.

The sequences of sense and antisense primers for human FasR, DR5, GAPDH and 18S are described in table 3.1 and were purchased from NZYTech. Water was used as a negative control. BLAST® searches were performed to confirm the total gene specificity of the primer sequences.

To execute the experiment, we mix 2.5µL of cDNA with 10µL of a master mix, containing 6.25µL SYBR Green Fast Mix, 3.25 µL H_2O mQ and 0.5µL primer mix. Each sample was tested in duplicate. After all samples have been plated, plate was centrifuged at 4000RPM for 2min. Then, plate was ready to proceed to the protocol run.

The protocol applied for FasR started with an initial incubation at 95°C for 5 min followed by 39 cycles at 95^{o}C for 10s, 55,7^{o}C for 10s and 72^{o}C for 10s. In the case of DR5 the protocol was 1 cycle at 95°C for 5 min, 9 cycles at 95^{o}C for 10s, 55^{o}C for 10s and 72^{o}C for 10s. Target gene expression levels were normalized to housekeeping genes using the $\Delta\Delta$Ct method and Bio-Rad CFX manager software.

TABLE 3.1Primers sequences used in QRT-PCR

DR5 (forward)	5'-GCACTCACTGGAATGACCTC-3'
DR5 (reverse)	5'-GCCTTCTTCGCACTGACAC- 3'
FasR (forward)	5’-AGCTTGGTCTAGAGTGAAAA -3’
FasR (reverse)	5’-GAGGCAGAATCATGAGATAT -3’
GAPDH (forward)	5’-ACAGTCAGCCGCATCTTC-3’
GAPDH (reverse)	5’-GCCCAATACGACCAAATCC-3’
18S (forward)	5’-GAAGATATGCTCATGTGGTGTTG-3’
18S (reverse	5’-CTTGTACTGGCGTGGATTCTG-3’

3.6 Evaluation of NK-mediated killing

3.6.1 Isolation of NK cells

Freshly NK cells were isolated from peripheral blood mononuclear cells (PBMCs) from healthy donors by negative selection using the NK cell isolation kit (Miltenyi Biotec) according to the manufacturer's protocol. Isolated NK cells were assessed for purity by flow cytometry using human anti-CD3 and CD56 monoclonal antibodies.

NK cells were maintained in RPMI 1640 medium supplemented with 10% FBS and antibiotics as described above. For expansion and activation of NK cells, the culture medium was supplemented with 250 IU/ml recombinant human IL-2 (Peprotech) and 100 IU/ml recombinant human IL-15 (Peprotech) at 37^{o}C in a 5% CO_2-95% air atmosphere. IL-2 and IL-15 were added to NK cells on days 1 and 2 and then NK cells were used in killing assays.

3.6.2 Concanamycin A inhibition assay

Concanamycin A (CMA) is a potent and selective inhibitor of the perforin/granzyme cell death pathway in NK cells, which is the main pathway used by cytotoxic NK cells. To determine the functional contribution of the perforin pathway in fresh and IL-2 activated NK cells, NK cells were incubated with 10μM CMA for 20h before co-culture with target cells. CMA was kept in the medium during the experiment. The cytotoxicity of NK cells against tumor cells was performed after 4h co-culture using the chromium-51 (^{51}Cr) release assay as described below in section 3.6.4.

3.6.3 Cytolytic activity of NK cells against tumor cells

The cytolytic activity of NK cells against adherent and spheres was analyzed using the ^{51}Cr release assay. This method has been considered the "gold standard" assay to accurately evaluate the NK cells cytotoxic activity against tumor cells. Chromium release assay is based on fact that NK cells exert cytotoxic mechanisms, which ultimately promote the disruption of cell membrane of ^{51}Cr pre-labeled target cells, causing the release of the radioisotope to the surrounding medium. Thus, the ^{51}Cr activity released to supernatants allows the quantification of cell death, compared to control conditions.

For testing the effects of DOX and BTZ on cells' susceptibility to NK cells, tumor target cells were pretreated with 10nM of BTZ or 0.05µM of DOX for 24h before incubation with NK cells.

3.6.4 Chromium-51 release assay

A tumor cell suspension containing $1x10^6$ cells/mL in 500µL RPMI medium was incubated with 50µCi of 51Cr (PerkinElmer) for 1h at 37^{o}C. After the incubation period cells were washed twice to remove the unincorporated 51Cr and plated in a round-bottom 96-MW (Orange Scientific) plate at a density of 5.000 cells/well and co-cultured with fresh or IL-2 activated NK cells in a effector:target (E:T) ratio of 10:1. Cells were maintained in co-culture for 4h or 20h at 37^{o}C. Afterwards plates were centrifuged and the supernatants were transferred to tubes and assayed for radioactivity in a radioisotope well counter (CRC-55tW Capintec®) within the 51Cr sensitivity energy window.

The amount of radioactivity released in supernatant is taken as an indicator of the amount of lysis occurred. The percent specific lysis was calculated using the following formula:

$$Specific\ lysis\ (\%) = \frac{test\ sample\ release - spontaneous\ release}{maximum\ release - spontaneous\ release} \times 100$$

Spontaneous release represents ^{51}Cr released from tumor cells in medium in the absence of NK cells and maximum release corresponds to ^{51}Cr release from cells lysed in medium containing 4% SDS.

3.7 Statistic

Statistical analysis was performed using GraphPad Prism 6.0 software (San Diego, CA). Data was presented as the mean ± standard error of the mean (SEM) of the indicated number of independent experiments (n).

The Mann-Whitney non-parametric test was performed for comparison of cells under different treatment conditions. The same test was used to compare between cell types maintained in the same conditions.

The one-way ANOVAtest with Dunnet's correctionwas used for multiple comparisons between multiple samples under the same conditions. Results were considered statistical significant when p-value was lower than 0.05.

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CHAPTHER 4

RESULTS

4.1 Bladder cancer cell lines contain sphere-forming stem -like cells

The isolation of CSCs from BC cell lines was performed using the sphere-forming assay that is a functional approach useful to retrospectively isolate and enrich potential CSCs subpopulations when specific surface markers are not identified. This assay relies on the ability of undifferentiated cells to proliferate and grow as spherical colonies in serum-free medium containing growth factors and supplements whereas more differentiated cells stop proliferating and die under these culture conditions.

Both UM-UC3 and HT-1376 cell lines, when cultured in serum-free medium containing EGF, bFGF and B27, formed visible spherical colonies that continued to grow until day 10 reaching 50 µM diameter, which is indicative for the presence of a stem-like cell population in both cell lines. Figure 4.1showed spherical colonies generated from single-cell suspensions of UM-UC3 and HT-1375 after 9 days. These cells, termed as CSCs, were further collected, gently dissociated and used in subsequent studies.

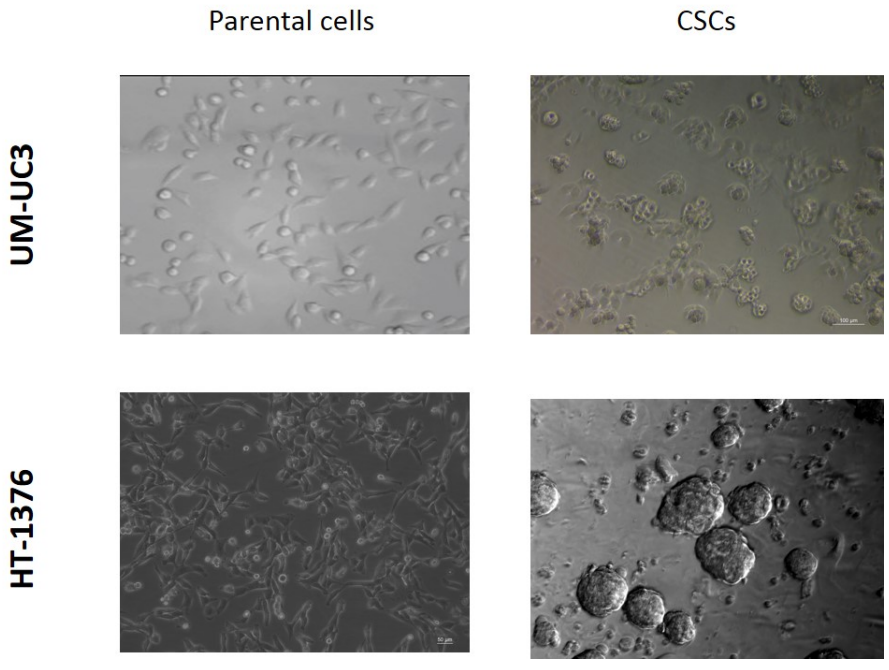

FIGURE 4.1 BLADDER CANCER CELL LINES CONTAIN A POPULATION OF STEM-LIKE CELLS.
UM-UC3 and HT-1376 cell lines (left panel) grew as monolayers in cell culture flasks with serum containing medium. Spherical colonies generated from single-cell suspensions of UM-UC3 and HT-1376 cells (right panel) cultured in a mixture of Matrigel and DMEM/F12 medium supplemented with EGF, bFGF and B27 after 9 days.

4.2 Effect of doxorubicin in the viability of BC cell lines

Since DOX is a chemotherapeutic agent highly cytotoxic against tumor cells, we performed a dose- and time -response study in both BC cells lines and corresponding CSCs. This assay was performed to determine the drug range concentrations to be used in further experiments, in which we will investigate the effect of DOX in the upregulation of death receptors in tumor cells, without affecting significantly the cell viability.

Both parental cells and corresponding CSCs were incubated with 0.025µM, 0.5µM, 0.75µM and 0.1µM of DOX during 24h and 48h. Then, cell viability was evaluated using the MTT colorimetric assay.

The dose-response curves of BC cell lines and their paired CSCs to DOX at 24h and 48h are shown in Figure 4.2. The incubation with DOX for 24h had practically no effects on cell viability nor in parental HT-1376 and UM-UC3 cells neither in corresponding CSCs.

After 48h, it was observed a decrease in the percentage of viable cells mainly in the HT1376 cell line. The percentage of viable cells reaches the 70% when cells were incubated with 0.1µM DOX. For the UM-UC3 cell line,increasing the incubation period to 48h did not affect significantly the cell viability over the range of DOX concentrations used. No significant differences were observed between parental and CSCs.

These results showed that both BC cell populations are relatively tolerant to the DOX concentrations tested. Based on this, we decided to test the effects of 0.05µM and 0.1µM DOX during 24h or 48h on the regulation of death receptors at mRNA level in tumor cells.

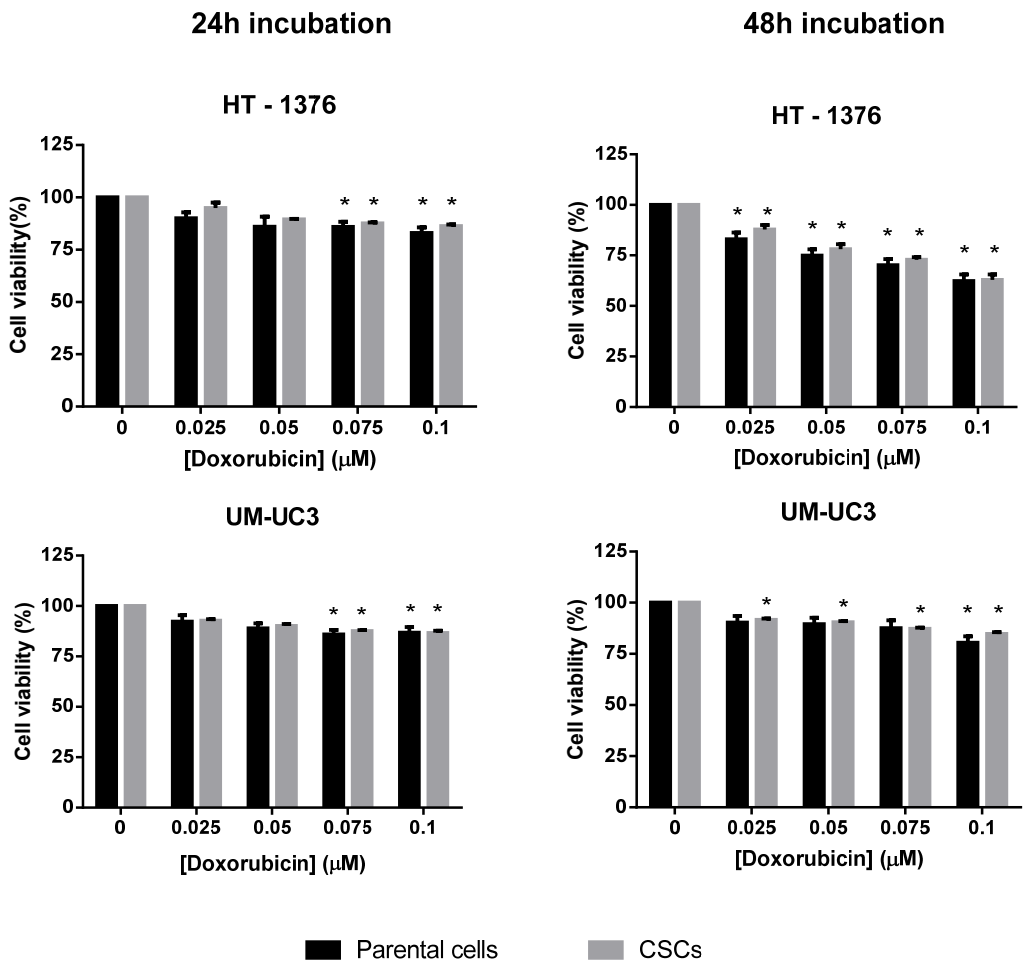

Figure 4.2 Effects of doxorubicin in the viability of BC cell lines.
Both UM-UC3 and HT-1376 cells and corresponding CSCs were incubated with varying concentrations of DOX for 24h or 48h.The percentage of viable cells was determined using the MTT colorimetric assay. Results were presented as mean ± SEM from three independent assays performed in duplicate. For statistical analysis, Mann-Whitney non-parametric test was applied to compare treated cells with a control condition. *p-value<0.05; **p-value<0.01; ***p-value<0.001

4.3 Effect of bortezomib in the viability of BC cell lines

Bortezomib (BTZ) was recently tested as the first proteasome inhibitor with anti-tumor activity in hematological and solid tumors. We sought to determine the susceptibility of parental UM-UC3 and HT-1376 cell lines and corresponding CSCs to different concentrations BTZ ranging from 5nM to 20nM for up to 24h or 48h of incubation. The cell viability was evaluated using the MTT colorimetric assay.

The effects of increasing concentrations of BTZ in cells' viability at different incubation periods are shown in Figure 4.3. The treatment of HT-1376 cell lines with BTZ showed a significant decrease in cell viability for concentrations above 10 nM at 24h.

At 48h of exposure the effects become more pronounced with a significant decrease being observed at the lowest tested concentration (5nM). The UM-UC3 cell line was relatively less sensitive to BTZ as compared with the HT-1376 cell line. Only for concentrations of 20nM at 24h it was observed a pronounced decrease in cell viability that was more pronounced at 48h post-incubation. Similar to DOX viability assay, there was no significant difference between parental cells and CSCs.

Taking these results into account, we decided to evaluate the effects the lower concentrations of BTZ (5nM and 10nM) during 24h or 48h on the mRNA expression levels of death receptors in parental and CSCs.

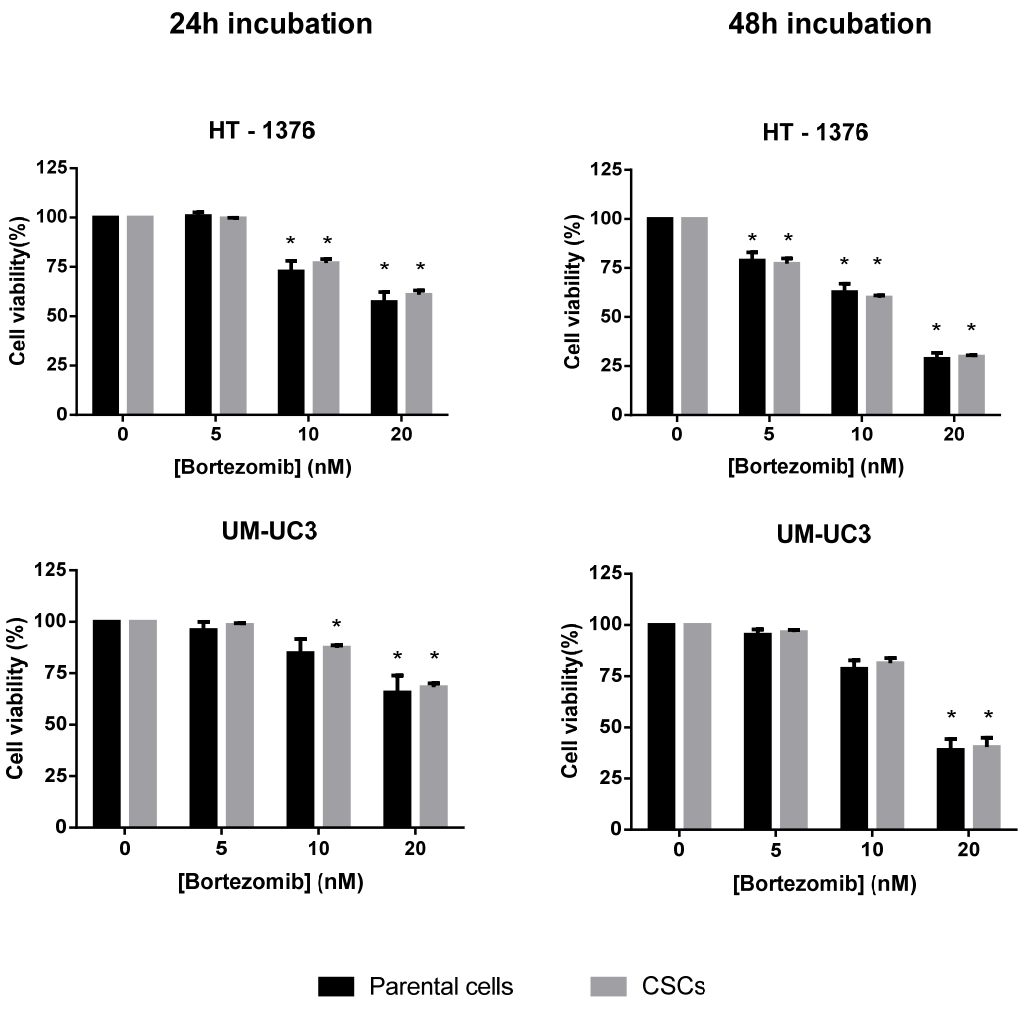

FIGURE 4.3 EFFECTS OF BORTEZOMIB IN THE VIABILITY OF BC CELL LINES.
Both UM-UC3 and HT-1376 cells and corresponding CSCs were incubated with varying concentrations of BTZ for 24h or 48h. The percentage of viable cells was determined using the MTT colorimetric assay. Results were presented as mean ± SEM from three independent assays performed in duplicate. For statistical analysis, Mann-Witney non-parametric test was applied to compare treated cells with a control condition. *p-value<0.05; **p-value<0.01; ***p-value<0.001

4.4 Constitutive expression levels of death receptors in adherent and corresponding CSCs

Although some genes have been associated with parental or stem cells in several cancer types, the difference in death receptors expression between CSCs and non-CSCs is poorly understood.

To further evaluate whether CSCs might differ from parental cells in the expression levels of death receptors, we performed a quantitative qRT-PCR analysis for measuring the transcript levels of Fas and DR5 in spheres and adherent cells. The expression levels of target genes in each cell population were normalized to two housekeeping genes (GAPDH and 18S) and are expressed in arbitrary units.

Fas and DR5 genes are constitutively more expressed in parental cells than in CSCs, with the exception of DR5 in the HT-1376 cell line, whose levels were similar in adherent and spheres. However, only the differences of Fas expression in UM-UC3 achieve the statistical significance, as depicted in Figure 4.4.

Overall, the death receptor Fas was constitutively more expressed (10- to 20-fold) in the two tested BC cell lines than DR5. This higher expression of Fas mRNA relatively to DR5 was observed in parental cells as well as in sphere-forming cells.

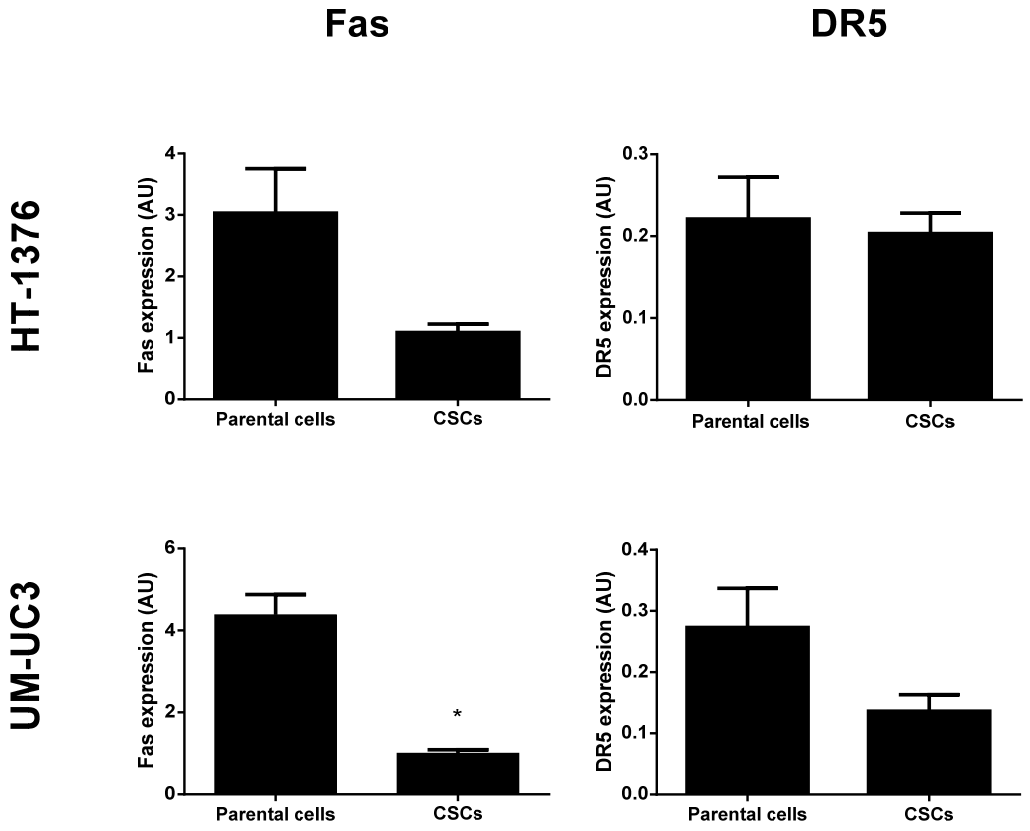

FIGURE 4.4qRT-PCR ANALYSES OF FAS AND DR5 IN PARENTAL AND SPHERES FROM UM-UC3 AND HT-1376 CELL LINES.

The mRNA expression levels of Fas and DR5 were normalized to two housekeeping genes (18S and GAPDH). The results are expressed in arbitrary units (AU) ± SEM from two independent assays performed in duplicate. For statistical analysis, t-student test was applied to compare CSCs with parental cells. *p-value<0.05;

4.5 Regulation of Fas and DR5 expression by DOX and BTZ

Recent studies have demonstrated that some chemotherapeutic drugs have the ability to increase the surface expression of apoptosis-related receptors in some types of tumors. It has been proven that this effect could be due to a decrease of degradation rate and an upregulation of gene expression.

We though to examined the effects of DOX and BTZ on mRNA expression of Fas and DR5 in two BC cell lines and in their corresponding CSCs, using a QRT-PCR assay. The results are shown in Figure 4.5.

Both UM-UC3 and HT-1376 parental cells were incubated with two different concentrations of DOX (0.05µM and 0.1µM) and BTZ (5nM and 10nM) during 24h or 48h, previously selected in sections 4.2 and 4.3, respectively. The selection of these concentrations was based on their small effects on cell viability.

The mRNA expression levels of the two genes of interest, Fas and DR5, in pre-treated cells were normalized to two housekeeping genes (GAPDH and 18S) and expressed as fold change relative to untreated control cells that were set as 1.

We start by testing two different drug concentrations in parental cells. Both drugs induced a significant increase in the expression of Fas and DR5 mRNA levels relatively to control untreated cells. However this augment did not occur in a dose- or time-dependent manner. For instance, in parental cells the most pronounced increase in Fas expression was mostly achieved at 24h incubation with 0.05µM DOX, while the highest Fas upregulationwas observed using 5nM or 10nM BTZ in UM-UC3 and HT-1376 cell lines, respectively. Increasing the incubation for 48h did not resulted in pronounced increments in Fas expression.

The death receptor DR5 that was expressed at very low levels in control conditions increased significantly after treatment with both drugs after 24h incubation and persisted or increased even more after 48h incubation. In both BC cell lines the highest upregulation of DR5 was observed when cells were treated with 0.05μM DOX for 48h. The treatment with 10nM BTZ for 24h or 48h promotes the highest expression of DR5 gene in UM-UC3 cells and HT-1376, respectively. Based on these observations we selected two drug concentrations (0.05μM DOX and 10nM BTZ) for testing in CSCs.

The effects of DOX or BTZ on death receptors expression in sphere-forming cells were not so pronounced. Any of the drugs exerted significant increments on Fas expression, with the exception of a significant upregulation in UM-UC3 cells treated with 0.05μM DOX during 48h. The expression of DR5 gene in CSCs was significantly increased upon DOX and BTZ exposure being more marked after 48h.

Taken together, these results suggest that sub-apoptotic doses of DOX and BTZ promote Fas and DR5 transcription in BC tumor cells. Moreover, bladder CSCs are less responsive to DOX and BTZ sensitizing effect, when compared to their corresponding parental cells.

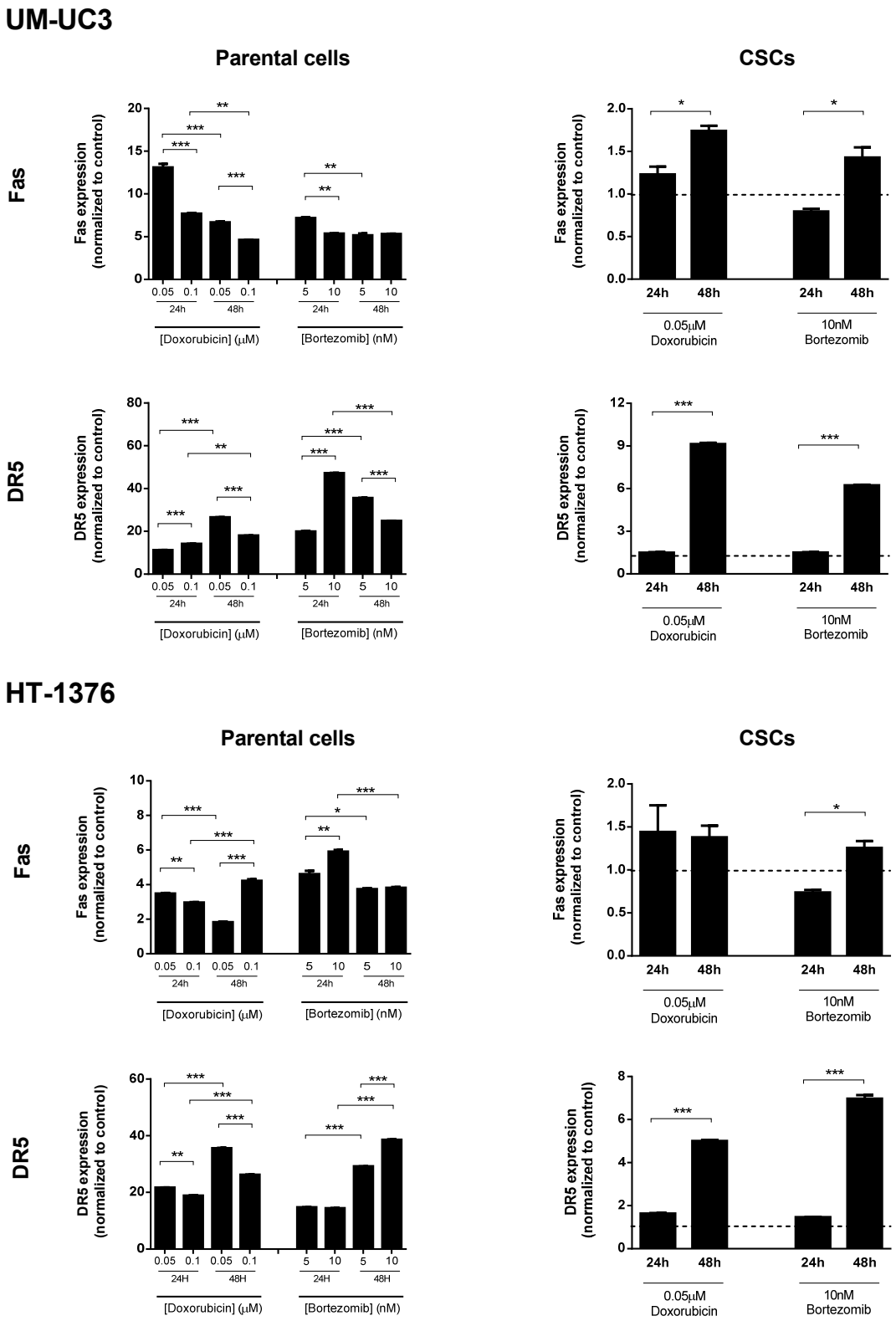

FIGURE 4.5 EFFECTS OF DOX AND BTZ IN DEATH RECEPTORS FAS AND DR5 GENE EXPRESSION.
mRNA expression levels of Fas and DR5 in parental and CSCs of UM-UC3 and HT-1376 cell lines, upon incubation with DOX (0.05μM and 0.1μM) and BTZ (5nM and 10nM) during 24h and 48h. The results are expressed as fold difference of the control (untreated cells) ± SEM. For statistical analysis, one-way

Kruskal-Wallis non-parametric ANOVA test with Dunnet's correction was applied to compare expression levels of Fas and DR5 between treatment conditions. *p-value<0.05; **p-value<0.01; ***p-value<0.001

4.6 Isolation of a highly purified NK cell population

Purified NK cells were isolated from PBMCs of healthy donors using a commercial isolation kit, according to the manufacturer instructions. The purity of the resulting cell populations was checked by flow cytometry using a gated strategy. The gating strategy isshown in figure 4.6. Briefly, the lymphocyte population was identified based on forward (FSC) and side-scatter (SSC) parameters, which are indicators of relative particle size and complexity. Then single cells were selected examining the relative signal height *vs.* area of cells to exclude doublets. Subsequently, $CD3^{+}$, $CD14^{+}$ and $CD19^{+}$ expressing cells representing T-cells, B-cells or monocytes, were excluded. Pure NK cells subset was selected based on a $CD56^{+(dim)}/CD16^{+}$or $CD56^{+(bright)}/CD16^{+/-}$ profile. The isolated NK cell population purity was generally above 90%.

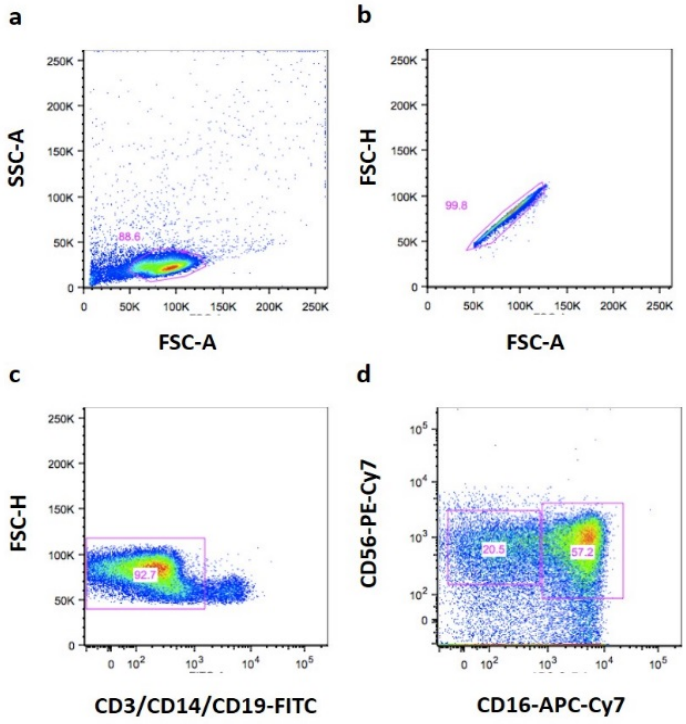

FIGURE 4.6 GATING STRATEGY TO IDENTIFY NK CELLS SUBSET.
Representative flow cytometry plots from the healthy donors blood to demonstrate NK cells gating strategy. NK cell subsets were isolated by sequentially gating on (a) lymphocytes, (b) single cells, (c) CD3/CD14/CD19- negativepopulation and finally (d) NK cells expressing CD56 and/or CD16. NK cell subsets were identified as $CD56^{+(bright)}/CD16^{-}$ (upper left gate): and $CD56^{+(dim)}/CD16^{+}$. (upper right gate)

4.7 Inhibition of perforin killing pathway by concanamycin A

It has been proven that NK cells exhibit two major killing pathways, namely the release of granules containing perforin and granzymes and activation of death receptors signaling on tumor cells by interaction with NK ligands.

Since we intended to study exclusively the role of death receptors in NK-mediated killing it was necessary to block the perforin-based cytotoxic activity of NK cells. There are some described compounds with the capacity to reduce the perforin content and inhibit the *de novo* synthesis in immune cells. Concanamycin A (CMA) has been extensively used as a selective inhibitor of perforin-mediated cytotoxicity in immune cells. Importantly, previous studies have demonstrated that CMA does not affect the expression and cellular localization of Fas ligand or TRAIL[107].

Previous studies demonstrate that the perforin pathway is the major NK cells killing mechanism occurring in earlier phases while death receptors killing pathway take place predominantly at later time points. To confirm whether CMA inhibits the perforin-mediated killing, we measured the lytic capacity of untreated or CMA treated NK cells in a short-term cytotoxic assay using the UM-UC3 cell line.

Briefly, tumor cells were labeled with ^{51}Cr isotope for 1 h and then co-cultured with NK cells in a E:T ratio of 10:1. To block perforin killing pathway, NK cells were incubated with CMA for 2h before the co-culture with tumor cells. After 4h cells-free supernatants were collected and measured in a gamma counter for ^{51}Cr activity. This study was performed with fresh NK cells and with 24h IL-2-activated NK cells.

As expected, fresh NK cells exhibited a weak cytolytic activity against tumor cells, being not significantly affected by pre-treatment with CMA. When we used NK cells previously stimulated with IL-2 for 24h, these immune cells became more able to induce lysis in tumor cells, than freshly isolated cells. Moreover, the inhibition of perforin activity with CMA almost abolished the lysis of tumor cells by NK cells as indicated by the ^{51}Cr-release assays in Figure 4.7

These results indicate that inhibition of perforin/granzyme-mediated pathway with CMA strongly inhibits cytolytic activity of IL-2-activated NK cells against tumor cells.

In contrast, pre-treatment with CMA had no significant effect on the lytic activity of fresh NK cells, suggesting fresh NK cells have low lytic ability mediated by perforin/granzyme pathway, probably due to low expression of activating receptors on their surface. Moreover, CMA appears to be an important tool to distinguish the contribution of each cytolytic pathway to NK cells killing.

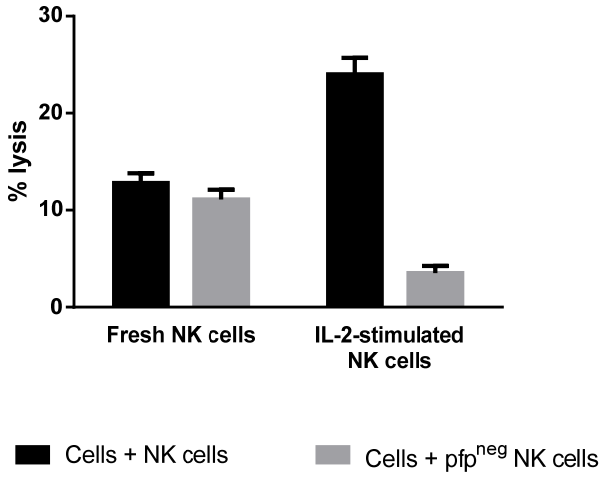

FIGURE 4.7 INHIBITION OF PERFORIN/GRANZYME PATHWAY BY CMA IN NK CELLS
Tumor cells were labeled ^{51}Cr for 1h and then incubated with NK cells in a 4h cytotoxic assay in a E:T ratio of 10:1. Fresh and IL-2 stimulated NK cells were pre-treated with CMA for 2h before the co-culture to deplete perforin. Apoptotic cell death was calculated by collecting the cells supernatants and measuring their ^{51}Cr activity in a gamma counter. The experiment was performed in duplicate.

4.8 Pre-treatment with DOX or BTZ sensitize BC cells to fresh NK-mediated cytotoxicity

To verify whether upregulation of cell death receptors sensitizes BC cells to NK-mediated killing NK cells, we next performed a ^{51}Cr-release assay against BC cell lines pre-treated with sup-apoptotic doses of DOX and/or BTZ.

Since NK cells are capable of using death receptors ligands to induce cytotoxicity in the absence of perforin, we measured the effects of DOX (0.05µM) and/or BTZ (10nM) in death receptors-mediated killing using fresh NK cells and a long-term cytotoxicity assay. To address this, both parental and CSCs were treated with 0.05µM DOX and/or

10nM BTZ during 24h before the 20h co-culture with freshly isolated NK cells. To test exclusively the influence of death receptors signaling in NK antitumor response, NK cells were incubated with 10nM CMA immediately before the co-culture with tumor cells.

The results presented in Figure 4.8, demonstrated that fresh NK cells exert a weak anti-tumor activity, causing less than 10% of lysis either in both BC parental cell lines as well as in corresponding CSCs, under controls conditions. Pre-treatment with DOX, BTZ or both drugs in combination induced a significant increase in the killing activity of NK cells against parental cells that reached nearly 50% of tumor lysis. Only 10% of cell death was elicited by drug side-effects. No significant effects were observed in the % cell lysis between cells treated with DOX or BTZ.

In contrast, pre-treatment of CSCs with DOX, BTZ or both during 24h did not increase significantly their susceptibility to the anti-tumor activity of NK cells. Overall, the small increase observed in lysis rate of CSCs in relation to untreated control cells, is mainly caused by the drug-side effects and not by the NK-mediated killing, as depicted in Figure 4.8.

Remarkably, the addition of CMA to NK cells did not significantly affect their capacity to kill tumor cells in a long-term assay using fresh NK cells, suggesting that death receptors-mediated pathway compensate the lack of perforin-dependent pathway to induce cytotoxicity.

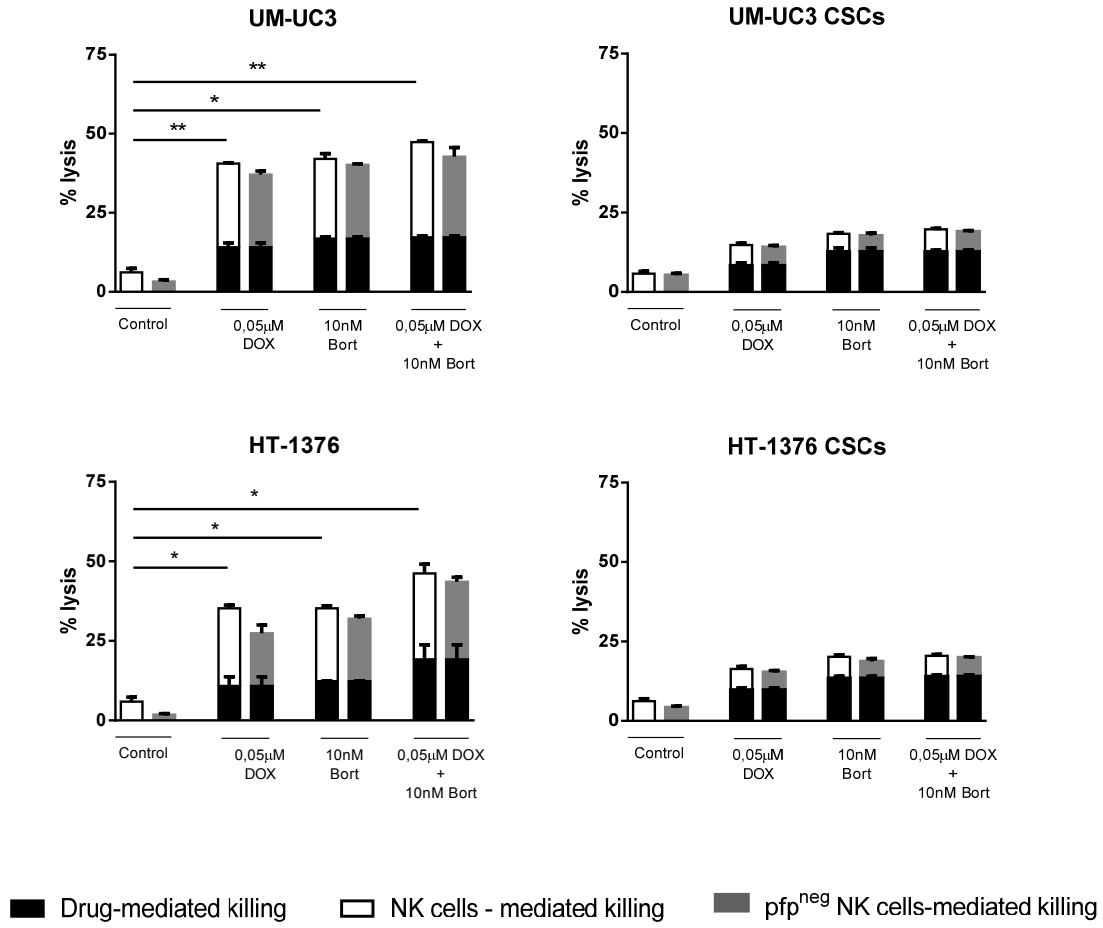

FIGURE 4.8 PRE-TREATMENT WITH DOX AND BTZ SENSITIZES PARENTAL BC CELL LINES BUT NOT CSCS TO NK-MEDIATED KILLING.
Fresh NK cell killing of UM-UC3 and HT-1376 parental cells and their corresponding CSCs in a 20hr ^{51}Cr release assay, using a E:T ratio of 10:1. Results were presented as mean ± SEM from two independent assays performed in duplicate. For statistical analysis, t-student test was applied to compare treated cells with a control condition. *p-value<0.05; **p-value<0.01;

4.9 Pre-treatment with DOX or BTZ sensitize BC cells to IL-2-stimulated NK-mediated cytotoxicity

It has been suggested that IL-2 stimulation promotes an increase in NK cells lytic activity, in part due to the upregulation of FasL and TRAIL, ligands to Fas and DR5 receptors on tumor cells, making them more able to kill tumor cells than freshly isolated NK cells[108,109].

Taken this into account, we considered to test whether the increase levels of death receptors ligands on NK cells promote a greater tumor killing through death receptors signaling.

To address this, NK cells were incubated with 250 IU/mL of IL-2 for 24h before the co-culture with tumor cells, which is carried out for 20h. Once again, 10nM of CMA was added to NK cells before the co-culture with tumor cells in order to inhibit the formation of perforin/granzyme granules and, subsequently their release to the medium. This study was only performed with the parental UM-UC3 cell line

IL-2-activated NK cells revealed higher cytotoxicity activity than fresh NK cells (observed in figure 4.8) even when the perforin pathway was blocked with CMA. As we observed with fresh NK cells, pre-treatment with DOX and BTZ also improved the anti-tumor response of IL-2-stimulated NK cells through death receptors signaling pathway. When tumor cells were treated with drugs during 48h, it was observed a slightly increase in the percentage of lysed cells resulting from the direct effects of drugs on tumor cells as well of the cytolytic activity of NK cells.

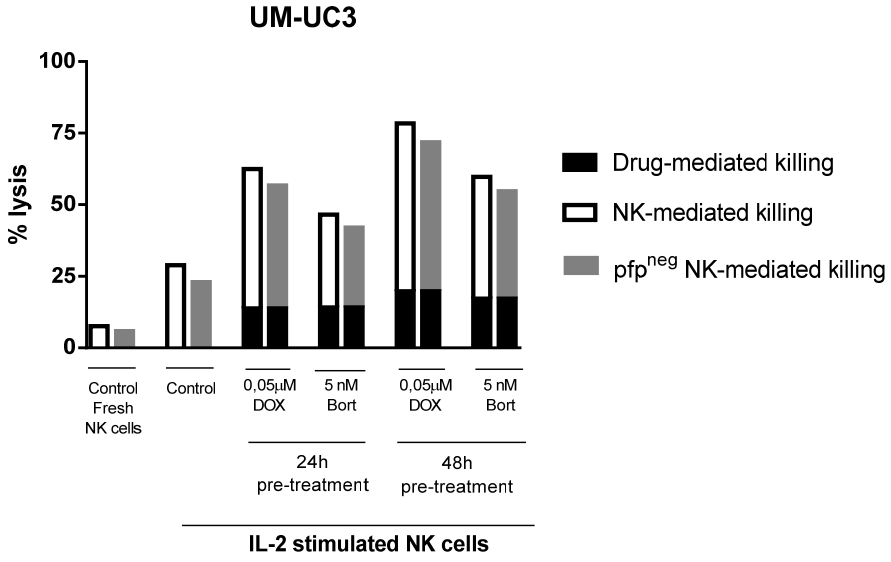

FIGURE 4.9 PRE-TREATMENT WITH DOX OR BTZ SENSITIZES UM-UC3 CELLS TO IL-2-ACTIVATED NK-MEDIATED KILLING

IL-2 –activated NK cells were co-cultured withUM-UC3 cells pre-treated with 0.05µM or 5nM for 24h or 248h with a E:T ratio of 10:1. The % of specific lysis were determined using the ^{51}Cr-release assay after 20h of co-culture. To block of perforin-mediated cytotoxicity, NK cells were pre-treated with 10nM of CMA. This experiment was performed once, in duplicate.

4.10 Expression levels of Fas and DR5 in bladder cancer specimens

Although death receptors have been proven to play an important role in tumor killing by immune cells, their variation of expression in BC is poorly known. Hence, we though to evaluate the association between mRNA levels of apoptosis-related receptors and the tumor stage in BC.

With this intend, we used mRNA isolated from 14 clinical samples of BC patients collected during TUR. These tumors were classified by a clinical pathologist as non-invasive low grade (n=3), non-invasive high grade (n=7) and muscle invasive (n=4). Then, mRNA was reverse transcribed into complementary DNA in order to perform the qRT-PCR technique. The expression levels of target genes were normalized to two housekeeping genes and expressed as arbitrary units (Figure 4.10).

Our results showed that both Fas and DR5 genes transcripts are constitutively expressed in BC samples, and exhibit a similar expression pattern among the set of samples analyzed. Despite the few number of cases studied, there is evidence of an increase of death receptors with the tumor stage. The expression levels of death receptors are lower in low grade non-muscle invasive tumors and increased progressively with the tumor stage, being higher in the muscle-invasive tumors, which are associated with a poor prognosis.

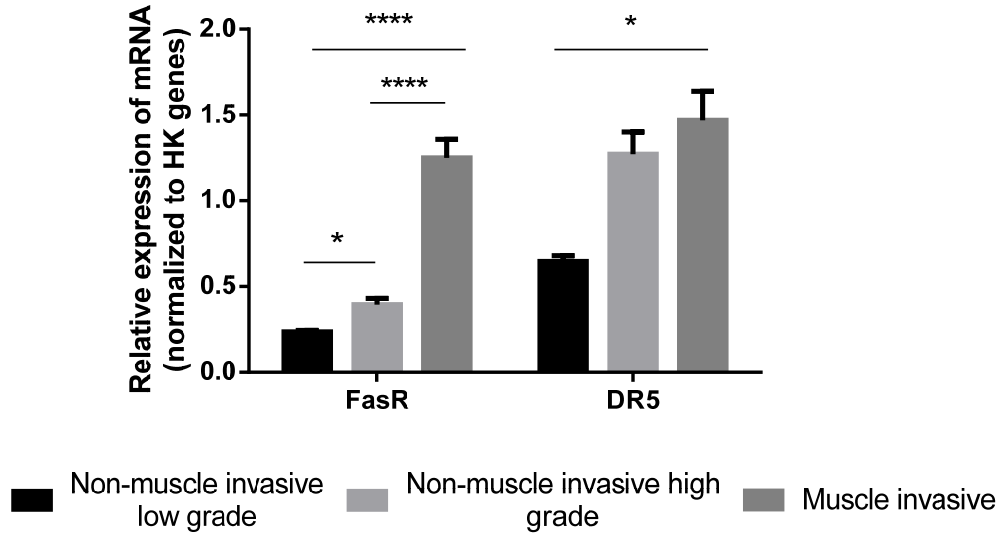

FIGURE 4.10FAS AND DR5 MRNA EXPRESSION IN BLADDER CANCER CLINICAL SAMPLES ACCORDING WITH THE TUMOR STAGE.

Expression of Fas and DR5 was normalized to housekeeping genes, namely 18S and GAPDH. Results are expressed as mean ± SEM. For statistical analysis, one-way ANOVA test was applied to compare Fas and DR5 expression among BC stages. (*P<0.01, ****P<0.0001

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CHAPTHER 5

DISCUSSION

In this work, we aimed to enhance the efficacy of a NK-based therapy against BC cell lines through upregulation of death receptors Fas and DR5 induced by treatment with sub-apoptotic concentrations of DOX and BTZ.

Bladder cancer is the one of the most common cancers in the world, being present in one of two forms, namely superficial and muscle-invasive tumors. The former, often with a good prognosis have a high propensity to relapse and to progress into muscle-invasive forms with an overall poor prognosis[14]. Indeed, increased tumor invasiveness is associated with poor patients' outcomes[29,67]. A highly tumorigenic population of cells, named CSCs, has emerged as key drivers of carcinogenesis and tumor progression in BC as well as in other types of malignancies[110].

Chemotherapy remains a first-line treatment for BC advanced stages, however recent evidences demonstrate that standard chemotherapy regiments promote a CSCs enrichment, by survival of chemoresistant clones and active production of PGE_2, which induces the proliferation of bladder stem-like cells[111,112].

Therefore, new therapeutic approaches are needed to eliminate all tumor cell populations. In this context, cancer management has dramatically changed over the past decades, from conventional cytotoxic agents that nonspecifically kill tumor cells,to targeted therapies designed to interfere with specific molecules or pathways that are required to tumor cells growth[113].

With the advanced knowledge of immunology, immunotherapy has become a promising and effective modality to fight cancer. Indeed, the ability of several immune cells, like NK cells, to recognize and kill tumors is well documented. Several advantages to use NK cells as immunotherapeutic agents rather than other immune cells have been described, including their antigen-independent response capacity and their simple isolation and expansion method[78].

Unfortunately, tumor cells may develop strategies to evade the NK-mediated killing by increasing expression of MHC-I or anti-apoptotic proteins, avoiding NK recognition and protecting apoptosis activation,or establishing an immune-privileged

environment impairing NK functions.To counterbalance this phenomenon, several strategies have been developed to enhance NK cells anti-tumor efficacy, improving antigen presentation, inhibiting tumor-mediated immunosuppression and sensitizing tumor cells to NK-mediated killing[106,113,114].

We focused our attention on the ability of some chemotherapeutic agents to induce an upregulation of death receptors genes in BC tumor cells, which can lead to an increase in NK anti-tumor response efficiency. Gene expression of death receptors has been assessed in several tumor cell types upon incubation with several drugs, such as Ibuprofen, melatonin, etoposide, DOX and BTZ among others[85-89]. Owing to their ability to upregulate death receptors expression in other malignancies, we chose to test the effects of DOX and BTZ in BC cell lines.

The cell lines used in this study (UM-UC3 and HT-1376) were both established from tumor samples of patients diagnosed with muscle-invasive TCC. Despite displaying different cell morphology and growth rate, they respond similarly to the chemotherapeutic agents cisplatin and methotrexate (unpublished data). We have successfully isolated a population of stem-like cells from both UM-UC3 and HT-1376 cell lines using the sphere-forming assay. Indeed, previous experiments performed in our lab (submitted for publication) showed these cells express pluripotent stem cell markers (SOX2, Nanog and Oct4), basal urothelium surface markers (CD44 and KRT14) and exhibited a chemoresistant phenotype to chemotherapeutic agents used in the treatment of invasive BC, which fulfills the criteria for functional CSCs.

We started to evaluate the constitutive expression levels of Fas and DR5 in both BC cell lines and in their corresponding CSCs using the QRT-PCR technique. Our results showed that both death receptors are constitutively expressed in BC cell lines. However the expression levels of DR5 are considerably inferior to that of Fas. The expression levels of Fas in both parental cells were confirmed at protein levels by flow cytometry (data not shown). Additionally, Królh *et* al. confirmed the DR5 surface expression on other BC cell lines (SW780, 647V, T24) using the same method of detection [90].

Remarkably, we demonstrate for the first time that both death receptors aredown-regulated in CSCs population when compared to their parental cells;

however,statistical significance only was achieved for Fas gene in UM-UC3 cells. These results suggest a novel mechanism of CSCs resistance to prevent the attack of immune system by NK cells and consequently cell death. Other mechanisms of CSCs to escape from immune cells have been described, namely down-regulation of MHC-I, a receptor responsible for antigen presentation, preventing the T-cell mediated recognition[115].

Before examining the effects of DOX and BTZ on regulation of death receptors genes, we first analyzed the dose- and time-dependent effects of both agents in the viability of BC cells using a MTT colorimetric assay. This assay measures the mitochondrial activity, which is intrinsically related to the number of viable cells. With this experiment, we intended to determine the concentrations of DOX and BTZ that cause a small reduction in cell viability to be used in subsequent studies.

To address this, cells were incubated with DOX and BTZ for 24h or 48h in a concentration range of 0.01-0.1µM and 5-20nM, respectively. Results demonstrated that BC cells are relatively tolerant to DOX concentrations tested, and more susceptible to BTZ especially the HT-1376 cells. Since BTZ is a potent proteasome inhibitor, its sensitivity on BC lines might at least in part be due to the accumulation of misfolded proteins that promote the misfolded protein response, leading to apoptosis activation [92]. Moreover, previously literature also suggests that the proteasomal inhibition induces ER stress, causing calcium release. Consequently, the mitochondrial uptake of calcium leads to cytochrome c release, followed by Bid cleavage, resulting in activation of apoptosis system [93].

Based on theresultsobserved, we decided to test the effect on 0.05µM and 0.1µM of DOX and 5nM and 10nM of BTZ in the regulation of Fas and DR5 genes expression, since under these concentrations cell viability was not reduced below 70%. The transcripts levels of each death receptors after DOX and BTZ treatments in parental cells and their corresponding CSCs, were measured using the QRT-PCR technique.

We observed a considerable and significant increase of each receptor expression in both cell lines upon treatment with DOX and BTZ that varied with concentrationand period incubation. Overall, the maximal expression of Fas in parental cellswas observedafter 24h incubation with 0.05µM DOX or 10nM BTZ incubation while maximal

increaseof DR5 was observed with higher drug concentration or longer incubation times. The mRNA levels of DR5, whose basal expression levels were very low, increased at much higher extent than Fasin response to drugs treatment in both BC cell lines.

The mechanisms by which DOX and BTZ upregulate death receptors appear to be complex. Doxorubicin and BTZ trigger the activity of c-Jun N-terminal kinase (JNK), which in turn enhances the activation of the tumor suppressor p53[116,117]. Subsequently, p53 migrates to the nucleus and binds to elements within the promoters and intronic sequences of Fas and DR5 genes, inducing gene expression[118,119].

In addition to promoting death receptors transcription, DOX and BTZ influence the levels of death receptors by stimulating the association of mRNA stabilizing factor HuR protein to 3'UTR of death receptors transcripts, increasing their half-life and stimulating trafficking from the Golgi to the membrane in a p53-dependent manner[118,120].

In contrast, DOX and BTZ treatments slightly induced an upregulation of Fas as well as DR5 in CSCs populations, with greater increases being observed in 48h treatments. This is likely due to the fact that CSCs populations frequently exhibit mutations in p53 gene, which plays a crucial role in the transcription of death receptors, as explained above. Strictly, several reports have demonstrated a correlation between tumors with p53 mutations and their undifferentiated phenotype, suggesting that p53 mutations are major players in the formation of the CSCs population[119].Moreover, p53 mutations have been widely associated with increase tumor aggressiveness and progression in several types of malignancies[119].

Nevertheless, Virseda-Rodriguez et al. observed numerous point mutations in p53 gene in BC patients, responsible for genetic instability and alterations in p53 function, implying p53 mutations in tumor progression and invasiveness. Higher doses or longer exposure times to chemotherapeutic agents might induce a greater expression of death receptors, however this could compromise the CSCs viability[121].

While BTZ has been described as a cancer-specific agent not affecting normal cells, DOX is very cytotoxic and commonly affects normal and tumor cells[122,123]. In fact, the studies of Fernandez *et al.*have demonstrated that BTZ selectively induces the

expression of apoptosis-related proteins, such as NOXA, in tumor cells but not in normal cells[124]. However, the selective upregulation of death receptors by BTZ is yet to be demonstrated. Nevertheless, their possible unspecific effects in death receptor regulation should not affect the viability of normal cells, since NK cell-mediated cytotoxicity depends on a previous tumor cell recognition by these immune cells, which is independent of death receptors expression.

Considering the previous results, we decided to test the effects of 0.05µM DOX and 10nM BTZ during 24h in the sensitization of tumor cells to NK-mediated killing, using the long-term ^{51}Cr release assay. To perform this, ^{51}Cr-loaded tumor cells were co-cultured with NK cells for 20h in a E:T of 10: 1. This ratio was selected within a range of effector/target proportions previously tested between 1:1 and 1:10. The 10:1 ratio was the one with high lytic efficiency and was selected to our studies. This technique allows the measurement of the ^{51}chromium activity in the supernatant, which is an indicator of the amount of lysis occurred in target cells.

The cytolytic activity of NK cells against BC cells pre-treated with DOX or BTZ wastested using a long-term assay in alternative to the classical short-term assay of 4h that measures perforin/granzyme-mediated cell death pathway, which is the predominant pathway used by NK cells. The co-cultures were maintained for 20h since death receptor-mediated cell killing occurredat later time points. Moreover a long-term assay may better represent the *in vivo* potential and anti-tumor capacities of NK cells, allowing the observation of several lytic pathways.

We focused our attention on the cytotoxic activity of fresh NK cells due to the fact that IL-2 stimulation induces the gene expression of perforin and granzymes genes, increasing the perforin and grazymes content[125]. Thus, it is expected that in assays where target cells are surrounded by perforin-enriched NK cells the effects of death receptors-mediated killing would not be observed. Additionally, we measured the surface expression levels of Fas ligand on NK cells, which engages with Fas receptor on tumor cells, upon 48h IL-2 stimulation and we did not observed a significant increase in NK stimulated cells (data not shown).

To avoid the contribution of the perforin/granzymes pathway on NK cells-mediated cell death, NK cells were pre-treated with 10nM CMA. This agent prevents perforin-based cytotoxicity by increasing pH inside of perforin-containing vesicles, leading to the enzymatic degradation without affecting death receptors ligands[107]. The inhibitory effect of CMA was confirmed in a 4h ^{51}Cr release assay, in which it was observed a decrease in the lytic activity of stimulated NK cells treated with CMA, suggesting the cytotoxicity of IL-2 stimulated NK cells dependson perforin content. On the other side, the cytolytic activity of fresh NK cells was very low and appears to be independent of perforin, since we did not observed a significant decrease in the cytolytic activity of NK cells after treatment with CMA.

Results from long-term cytotoxic assay using fresh NK cells revealed that pre-treatment of tumor cells with DOX and BTZ augments significantly the NK cells anti-tumor response, resulting in a significant increase of tumor lysis as a consequence of the upregulation of death receptors. We cannot exclude the contribution of other mechanisms beside death receptors upregulationto the increase cell death of target cells. In fact, previous studies have demonstrated that DOX and BTZ influence the expression of other apoptosis-related proteins, increasing the expression of pro-apoptotic molecules, such as caspase-8 and Bid, and/or reducing expression of anti-apoptosis elements, like Bcl-XL and c-FLIP.

Furthermore, previous reports suggest that engagement death receptors to their respective ligands on NK cells can also induce an even higher expression of death receptors on tumor cells and, consequently a more efficient NK cells-mediated killing. The drugs by themselves also contribute to an increase in cell death despite the low concentrations we used. The fact the cells remained in culture for an additional 20h (following 24h drugs incubation) during the long-term assay might explain the percent of lysis that was observed.

Since death receptors signaling is one of the two major killing pathways,acting hand in hand with perforin/granzymes mechanism, it is likely that effect in tumor lysis when perforin is depleted is mainly due to Fas and DR5 activation. However, recent studies suggest the possibility that both chemotherapeutic agents studied promote an augment in cytokines production (IFN-γ) by NK cells, causing cell death by other killing

pathway[126,127]. Interestingly, the Fas-mediated killing is apparently dependent of IFN-γ[128].

These results are in line with previous studies that demonstrated the increase in tumor killing with DOX and BTZ in several types of tumors. Indeed, Mizutani *et al*. has demonstrated that DOX can sensitize BC cells to lysis, an effect mediated by peripheral blood lymphocytes (PBL) and tumor infiltrating lymphocytes (TIL)[129].

It was also shown that the combination of several chemotherapeutic agents causes similar effects to DOX and BTZ treatment alone. This fact is likely to be due to the redundant mechanisms by either DOX or BTZ cause death receptors upregulation.

In CSCs, we observed an increase of tumor lysis, an effect that was mainly caused by exposure to chemotherapeutic agents and not by NK cells. In fact the percent of tumor cell lysis resulting from NK cells killing activity did not change significantly in comparison to the control condition. These results can be explained by the slightlyupregulation of both death receptors induced by the concentrations of DOX and BTZ we used. In this experiment CSCs were pre-treated with 0.05μM DOX and/or 10nM BTZ during 24h, which were the same conditions we used in parental cells. These experimental conditions were less effective in upregulating death receptors in CSCs than that seen for parental cells. Future studies using increased drug exposure testing will be required to clarify this hypothesis.

We conducted another experiment in which we used IL-2 activated NK cells to further evaluate whether upregulation of death receptors in tumor cells improve the antitumor activity of activated NK cells. This assay was performed only with one cell line (UM-UC3). We observed that DOX and BTZ treatments (alone or in combination) improve the anti-tumor response of NK cells, as occurred when we used resting NK cells. Nevertheless, since IL-2 activated NK cells are intrinsically more cytotoxic than resting NK cells, the relative changesobserved in lysis activity versus control conditions were higher when we used freshly isolated NK cells.

Once we proved that death receptors play an important role in anti-tumor response of NK cells, we tested the expression levels of both death receptors, using samples from dissected material of 14 BC patients in a qRT-PCR experiment. The clinical

samples were classifiedby a pathologist in non-invasive low grade, non-muscle-invasive high grade and muscle-invasive. Surprisingly, the results revealed a progressive increase in the expression of both death receptors transcripts with the tumor stage, being higher in muscle-invasive tumors with poor prognosis. Studies performed in other types of malignancies, such as breast, renal carcinoma or non-small lung cancer, also demonstrated the same tendency regarding DR5 expression[130–132]. On the contrary, a study performed in BC samples correlated DR5 protein levels with a better prognosis[133]. This discrepancy might be explained by the fact that we measured DR5 at mRNA level, while this study measured DR5 at protein levels and their distribution within BC cells. Moreover, recent reports demonstrated the presence of DR5 receptors in cytosolic and nuclear compartments, besides the plasma membrane, where they trigger apoptosis signaling[134]. Although the functions of intracellular DR5 remain unclear, a correlation of apoptosis resistance with intracellular presence of DR5 in breast cancer cells was also proposed in a study from Zang *et al*.[135].

Moreover, the role of Fas in tumor cells apoptosis has been a subject of intense debate, with some studies suggesting a pro-survival function, in contrast to others that propose a pro-apoptosis action[136,137]. Ultimately, the role of Fas in cancer regression or progression is related with cellular localization and types of tumor cells. A mechanism of alternative splicing during transcription of Fas gene generates a soluble form of Fas (sFas), which antagonizes the function of Fas on the plasma membrane. In fact, Yajima et al. related the levels of sFas in advanced stages of gynecological malignancies[138].Therefore, it would be important to evaluate the cellular distribution of both death receptors and associate them with BC stage

Impact of chemotherapeutic drugs in NK-mediated killing by overexpression of death receptors on cancer cells

CHAPTHER 6

CONCLUSION AND FUTURE WORK

In this study we proposed to explore an alternative therapeutic approach to BC treatment, using cytotoxic agents to improve the NK cells immune response against cancer cells (parental and CSCs). Therefore, the main goal of this work was to evaluate the effect of low concentrations of DOX and BTZin sensitizing human BC cells to NK cells-mediated killing by inducing death receptors (Fas and DR5) expression.

Our results provide evidence that NK cells antitumor response can be enhanced by a previous exposure of tumor cells to sub-apoptotic concentrations of DOX and BTZ, as a result of an upregulation of death receptors mainly in differentiated parental tumor cells. The CSCs population exhibited low basal levels of death receptors and were less sensible to an upregulation induced by DOX and BTZ, becoming less sensitive to NK-mediating killing. Additionally, the expression of death receptors in BC was associated with tumor stage and aggressiveness, suggesting that they might be used as a prognostic marker in BC.

Altogether, these results suggest that DOX and BTZ pretreatment might be used to enhance the efficacy of NK cells-based therapies, improving clinical outcomes.

In future experiments we intend to evaluate the effect of higher concentrations and longer exposure times of DOX and BTZ in sensitizing the CSCs population to death receptors-mediated killing. Furthermore, we are interested in knowing the contribution of each receptor individually to NK-mediated tumor cell lysis, by blocking individually TRAIL and FasL on NK cells surface.

Additionally, we aim to explore the effects of DOX and BTZ in the regulation of apoptosis-related proteins, either pro- and anti-apoptotic molecules, as well as in other NK cells killing pathways, like perforin and IFNγ mechanisms. Results from both experiments would provide new insights into DOX and BTZ-induced effects in sensitizing tumor cells to NK cells-mediated killing.

Lastly, giving the interesting results we observed in BC cell lines, the next step will be to perform this therapeutic approach in *in* vivo studies in an animal modelBC.

CHAPTHER 7

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