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MODULATION OF THE MOLECULAR EXPRESSION AND FUNCTION OF BCL-2 FAMILY PROTEINS BY THE ARYL HYDROCARBON RECEPTOR IN BREAST CANCER STEM CELLS OF THE MDA-MB-231

BY

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ABSTRACT

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Title: Modulation of the Molecular Expression and Function of BCL-2 Family Proteins by the Aryl Hydrocarbon Receptor in Breast Cancer Stem Cells of the MDA-MB-231

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Breast cancer (BC) is a frequently occurring neoplasm in women and is a second major cause of cancer related deaths. Despite the remarkable advancements in the understanding of BC and its treatments, the diseases still pose numerous challenges. Many BC patients develop metastasis and advanced tumor which leads to increased morbidity and mortality. There is a substantial evidence that tumor relapse in BC patients is driven by special population of cells called cancer stem cells (CSCs). Breast CSCs confer stemness to BC and survive through the maintenance of several mechanisms among which the involvement of aryl hydrocarbon receptor (AhR) has recently been evaluated. The correlation of this receptor with BCL-2 family proteins for the development of breast CSCs remains unclear. This study is conducted to evaluate the correlation between AhR and BCL-2 proteins *in vitro* and in patient samples.

Breast CSCs were enriched through mammosphere culture and characterized by flow cytometry. BC cells and breast CSCs were exposed to DMBA, αNF and venetoclax (VCX), respectively and the expression of different genes was evaluated through RT-PCR and Western blot analysis. Moreover, determination of cellular content and localization through immunofluorescence and functional assays through muse cell analyser were also conducted. Finally, protein expression of AhR and BCL-2 in 75 human breast tissues was determined.

The constitutive expression of AhR, CYP1B1 and anti-apoptotic BCL-2 proteins

were found to be significantly higher in mammospheres than differentiated cancer cells ($p<0.05$). Moreover, AhR induction and inhibition modulated expression of BCL-2 proteins and inhibition of BCL-2 inhibited AhR and its regulated genes. The expressions of AhR and BCL-2 were also found to be higher in tissues of BC patients compared to the non-cancerous BC tissues.

These findings demonstrated crosstalk between AhR and BCL-2 for development of breast CSCs which offers a strong basis for designing therapeutic strategies to target AhR in order to overcome drug resistance in BC.

Keywords: Aryl hydrocarbon receptor; BCL2 family proteins; Triple negative breast cancer; cancer stem cells; mammospheres; tissue microarray

DEDICATION

This thesis is dedicated to my parents who have been my greatest support

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α-NF: Alpha-naphthoflavone

ABC: ATP-binding cassette

ABCG: ATP-binding cassette super-family G member 2

AhR: Aryl hydrocarbon receptor

ALDH: Aldehyde dehydrogenase

ATCC: American type culture collection

BaP: Benzo[a]pyrene

BAX: BCL-2-associated X

BC: Breast cancer

BCL-2: B-cell lymphoma 2

BCL-xL: B-cell lymphoma-extra large

BID: BH3-interacting domain death agonist

BIM: BCL-2-like protein 11

BRCA1: Breast cancer gene 1

BSA: Bovine serum albumin

CSCs: Cancer stem cells

CTLA4: cytotoxic T-lymphocyte-associated protein 4

CXCR4: Chemokine receptor type 4

CYP1A1: Cytochrome P450 1A1

CYP1B1: Cytochrome P450 1B1

DCV: Dye Cycle Violet

DMBA: 7,12-Dimethylbenz[a]anthracene

DMSO: Dimethyl sulfoxide

DNA: Deoxyribonucleic acid

DNMT1: DNA methyltransferase 1

EMT: Epithelial to Mesenchymal Transition

ER: Estrogen receptor

FBS: Fetal bovine serum

GAPDH: Glyceraldehyde 3 phosphate dehydrogenase

HER2: Human epidermal growth factor receptor type 2

IL-6: Interleukin 6

IL-7: Interleukin 7

IL-12: Interleukin 12

IL-22: Interleukin 22

mRNA: messenger ribonucleic acid

NF-kB: Nuclear factor kappa-light-chain-enhancer of activated B cells

NQO1: NAD(P)H:quinone oxidoreductase 1

MCF-10A: non-tumorigenic breast epithelial cell line

MCL-1: Myeloid cell leukemia 1

MDA-MB-231: Human mammary carcinoma cell line

MYC: Master regulator of cell cycle entry and proliferative metabolism

OCT4: Octamer binding transcription factor 4

PD1: Programmed cell death protein 1

PDL1: Programmed death-ligand 1

PI: Propidium iodide

PR: Progesterone receptor

PTEN: Phosphatase and tensin homolog

PVDF: Polyvinylidene fluoride

RIPA: Radioimmunoprecipitation assay buffer

RNA: Ribonucleic acid

RT-PCR: Reverse transcription polymerase chain reaction

SDS: Sodium dodecyl sulfate

siRNA: Small interfering RNA

SOX: SRY-box transcription factor

SP: Side population

tBID: truncated BH3-interacting domain death agonist

TCDD: 2,3,7,8-tetrachlorodibenzo-p-dioxin

TNBC: Triple negative breast cancer

TPBC: Triple positive breast cancer

TSA: Trichostatin A

VCX: Venetoclax

XRE: Xenobiotic response element

CHAPTER 1: INTRODUCTION

1.1.Cancer

Despite the development of various treatment strategies, cancer remains a major cause of death worldwide [1, 2]. Cancer is generally described as a pathological condition which is characterized by uncontrolled growth and proliferation of cells due to accumulation of genetic mutations [3, 4]. These genetic mutations are generally accumulated upon exposure to chemical compounds and therefore, modify cell's function [4]. The genetic mutations lead to dysfunction of many important genes which results in abnormal cell proliferation [5, 6]. Cancer is mostly curable when it is diagnosed at an early stage where it has not metastasized, however, most of the cancers are diagnosed at a later stage where they have metastasized to other organs and therefore, become aggressive [2]. Cancer is commonly treated using conventional treatments such as radiotherapy, chemotherapy and surgical removal. However, the resistance of cells to these therapies reduce their effectiveness [1]. Even when cancer is treated at an early stage, some residual cells stay quiescent, which later grow and cause tumor relapse [2]. Moreover, cancer is highly heterogenous which is one of the reasons of its treatment failure and disease progression.

In United States (US), in 2012, 23% of deaths were caused by cancer and it is the second major cause of deaths after cardiovascular disorders [7]. According to 2016 statistics, in US, the most common cancers in men were prostate, colorectal, lung and bronchus, and prostate cancer alone accounted for 1 in 5 new diagnosis in men whereas in women, most frequently diagnosed cancers were breast, colorectal, lung and bronchus, where breast cancer (BC) alone accounted for 29% of all new cancer diagnosis in women [7]. In children, most frequently diagnosed cancers were blood cancer, brain tumor and lymphoma [8, 9]. According to World health organization (WHO), in Qatar, 1260 new

cases of cancer were diagnosed in 2018 with 672 total deaths. In Qatar, the incidence of BC is highest in women than any other cancer and it is estimated to increase in the coming years [10].

The development of cancer is a slow stepwise process which takes several years or decades to form. Additionally, there are a number of chemicals that have been studied to cause mutation and therefore lead to carcinogenesis. Chemical carcinogenesis is divided into three phases; initiation, promotion and progression [11, 12].

1.1.1. Initiation

The first step of cancer development is called "initiation". A chemical carcinogen (initiator), or its reactive metabolite, induces a change in chromosomal DNA of target cells which can be repaired or reproduced [13]. If this change in the DNA is not repaired, or the cells containing mutated genes, do not undergo apoptosis, it leads to permanent change in the cells and the target cells are initiated (Fig. 1). Once the target cells are initiated, the change in DNA becomes irreversible [3]. The initiated cells are called preneoplastic cells because they do not have characteristics of uncontrolled growth and proliferation. The experimental animals develop multiple neoplasms upon exposure to chemical initiators and each individual neoplasm arise from a single initiated cell, therefore, they are monoclonal in origin. In order, for an initiated cell to form tumor, promotion is required [3].

1.1.2. Promotion

Promotion is a process in which the initiated (mutated) cells develop clonally to form preneoplastic or benign neoplastic cells [14]. Tumor promotion is an epigenetic process because the promoters bring change, either directly or indirectly, in genetic information without altering DNA sequence [15]. Tumor promoters change the genetic information of cells and inhibit cells from undergoing apoptosis therefore, they induce cell

proliferation. This leads to accumulation of preneoplastic cells in a tissue. Some of the lesions that are formed in promotion, regress, while others acquire further mutations which leads to formation of malignant tumors [3].

Tumor develops when the target tissue is exposed to initiator and then repeatedly exposed to promoter. A tumor still develops when the time between exposure to initiator to exposure of promoter is from weeks to a year but it does not form a neoplasm when the exposure to promoter occurs before the exposure to initiator [3].

1.1.3. Progression

Progression is a stepwise transformation of benign neoplasm to a malignant neoplasm and it causes independent growth and metastasis of malignant tumor [3]. The progression develops a low-grade tumor to a high-grade malignancy which is metastatic and invasive (Fig. 1). It also brings morphological, biochemical and metabolic changes in the cells [14]. Tumor progressors are carcinogens that convert promoted cells to progressed cells i.e., they transform mutated premalignant cells to fully malignant cells. The hallmarks of tumor progression are chromosomal abnormalities such as duplication, deletion and translocation of chromosomal fragments. Moreover, mutation in the tumor suppressor genes and in oncogenes also accumulate during progression [3].

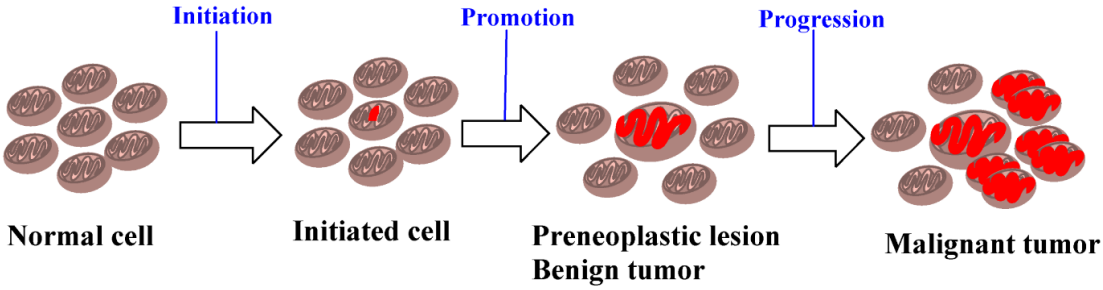


Figure 1. Three phases of carcinogenesis *[16]. Cancer evolve through a process that involves initiation, promotion and progression. This multistep process convert normal cells into malignant tumor.*

1.2.Breast cancer

BC is one of the most serious health problems among women, both in developed and developing countries and it accounts for 30% of all women's cancer [17-19]. BC caused more than 40,000 deaths in US in 2017 [20]. It is a highly heterogenous malignancy due to its pathological characteristics and etiology, some cases of BC show slow growth with better prognosis while others exhibit aggressive growth [21]. Due to being metastatic, BC can travel to different organs such as lung, liver, bones and brain which makes it hard to treat. The possibility of developing BC depends on different risk factors such as family history, age, unhealthy lifestyle and estrogen level [22]. However the risk increases due to environmental factors, genetic mutations and reproductive history [23].

According to American cancer society, in US, one in eight women will develop BC in her lifetime [24]. It is expected that the incidence of female BC globally will reach 3.2 million cases each year by 2050 [25]. These figures represent the seriousness of the disease and urgency for the treatment measures. In Qatar, BC is the most common type of cancer that affects women [18, 26]. The rate of BC diagnosed in Qatari women in far higher than the diagnosis of any other cancer [27]. Arab women in Middle Eastern countries are highly susceptible to deaths caused by cancer because of their late

diagnosis [28, 29]. Even when the cancer is treated at an early stage, some residual cells stay quiescent which later grow and cause tumor relapse [2]. In the Middle Eastern countries, Qatar has one of the highest BC incidence and mortality rate [30, 31]. BC accounts for 39.41% of all female cancers in Qatar. In 2015, 242 new BC cases were recorded with 72.08 per 100,000 people at the risk of developing BC [32].

1.2.1. Breast cancer subtypes

BC is classified into five subtypes based on the expression of three tumor biomarkers estrogen receptors (ER), progesterone receptors (PR) and Her2 oncogene. These subtypes are human epidermal growth factor receptor type 2 (HER2) positive, triple negative breast cancer (TNBC), triple positive breast cancer (TPBC), luminal A and luminal B [33]. ER positive BC is common than the ER negative breast tumors and they are also low grade and smaller than ER negative tumors [34]. ER positive/HER2 negative BC accounts for approximately two-thirds of all invasive BCs [35, 36] and it responds to hormonal therapy. HER2 positive BC comprise 10-20% of breast carcinomas and they have poorer prognosis than HER2 negative BC, however, they respond to HER2 negative targeted therapies such as trastuzumab [37]. 15-20% of BCs that lack ER PR and HER2 expression are classified as TNBC [38].

1.2.1.1.TNBC

TNBC is one of the aggressive forms of BC, and tumors of TNBC are often grade III tumors [39, 40]. This subtype of BC lacks targeted therapies because the cells lack ER, PR and are also negative for HER2. Therefore, TNBC cells cannot be targeted through hormonal therapy or anti-HER2 therapy, thus, it results in poorer outcomes in patients. The survival time of TNBC patients is shorter than the other subtypes of BC and 5-year mortality rate is 40% [39]. TNBC is commonly diagnosed in women younger than 40 years compared to the diagnosis of other BC subtypes [41, 42]. Patients

suffering from TNBC may possess early metastasis than patients suffering from non-TNBC and the median survival time after metastasis is 13.3 months. The rate of recurrence of TNBC after surgery is 25%. The average time of relapse in TNBC patients is 19-40 months whereas in non-TNBC patients is 35-67 months [43]. Although some of TNBC are chemosensitive, a large number of patients who have residual disease following neoadjuvant therapy, have lower overall survival compared to non-TNBC patients [44, 45].

TNBC have similar mRNA expression pattern as the myoepithelial (or basal) cells that are located at the basal side of normal mammary glands, therefore, it is considered as a subtype of basal-like BC [46]. Around 56% of gene expression of TNBC and basal like BC overlap [43]. The earliest molecular studies identified the association of BRCA1 gene mutation with TNBC and also the involvement of non-BRCA mutations that lead to failed DNA repair system [47]. The incidence of TNBC is generally higher in patients with mutated BRCA1, in women with black or Hispanic ethnicity and women of younger age [39, 48].

1.2.1.1.1. TNBC subtypes

In 2011, Lehmann et al divided TNBC into six subtypes upon performing gene expression profiling of tumor samples from 587 TNBC patients. These subtypes are basal-like 1 (BL1), basal-like 2 (BL2), mesenchymal (M), mesenchymal stem-like (MSL), luminal androgen receptor (LAR) and immunomodulatory (IM) [49].

Gene expression profiling of tumors of BL1 subtype of TNBC exhibit abnormal expression of cell-cycle regulating genes and DNA repair system related genes [50]. The BL2 subtype has aberrant regulation of cell signaling pathways [50]. The basal-like subtypes of TNBC are also associated with lower overall survival in patients. Moreover, the basal-like xenografts in animal models of BC exhibit high PI3K activity

compared to luminal-like xenografts [51]. Basal-like subtype of TNBC is particularly common in BRCA1 hereditary tumors and is positive for CK5/6 and/or EGFR [52, 53]. The M subtype of TNBC is also called metaplastic BC and it has sarcoma-like or squamous epithelial cell-like tissue characteristics. This subtype has high activation of signaling pathways related to cell-migration, differentiation and extracellular matrix-receptor interaction pathways. It can be treated through targeting epithelial-mesenchymal transition and through mTOR inhibitors [50, 54]. The MSL subtype expresses more stemness-related genes and exhibit low expression of proliferation-related genes. Moreover, this subtype also expresses mesenchymal stem cell-specific markers and HOX genes [50]. The LAR subtype has a different gene expression profile than the other TNBC subtypes. Although it does not show expression of ER, it exhibits a high expression of Androgen receptor and the expression of androgen receptor in LAR is nine times higher than the other TNBC subtypes [43]. The IM subtype has a high similarity to medullary carcinoma of the breast. It has high expression of immune cell-associated genes and signal transduction pathways such as B cell receptor signaling pathway, NK cell pathway, Th1/Th2 pathway, T cell receptor signaling, dendritic cell (DC) pathway, IL-7 pathway and IL-12 pathway [55]. The immune checkpoint inhibitors such as PD1, PDL1 and CTLA4 are recommended for the treatment of this subtype [50].

These subtypes of TNBC indicate its diversity therefore, the understanding of growth of TNBC and mechanism of metastasis and recurrence is highly important for the development of targeted effective therapies. One mechanism through which BC acquires stemness and causes tumor relapse is formation of CSCs.

1.3.Cancer Stem cells

Tumor is composed of diverse populations of cells. One of these populations is cancer stem cells (CSCs) that play an essential role in cancer initiation and progression [56]. Cancer gains stemness by the formation of CSCs. These cells are generally resistant to conventional therapies due to which they are considered as major reason of tumor recurrence after the halt of radiotherapy and chemotherapy [57]. Moreover, these cells have the unique ability to give rise to heterogenous population of cancer cells and have high DNA repair ability due to dysregulation of a number of genes [49, 58]. Accumulating evidence shows that CSC number is high in highly aggressive and resistant tumors [59, 60].

1.3.1. Biomarkers of CSCs

CSCs are isolated from population of cells through numerous *in vitro* methods based on their distinct features. These cells express certain cell surface markers such as antigens and receptors which can be identified through Fluorescence-Activated Cell Sorting (FACS) [61]. In solid tumors the main CSCs specific surface markers are CD 133, CD44 and CD24. The CSCs of leukemia, that were first studied and understood, exhibit expression of $CD34^{+}CD38^{-}$ surface markers [61, 62]. The expression of surface markers varies depending on the tumor phenotype. There are other characteristics through which CSCs can be identified and isolated such as these cells efflux Hoechst stain and are termed as side population (SP). This exclusion of stain is associated with their increased expression of ATP binding cassette (ABC) transporters [63, 64]. They can also be isolated on the basis of some transcription factors such as Krüppel-like factor 4 (KLF4), Octamer binding transcription factor 4 (Oct4), Nanog and Sox2 [65-67]. In addition, CSCs have ability to grow in spheres, called tumorspheres and they can also be isolated from tumor based on this feature [68].

1.4.Chemoresistance in TNBC

The chemotherapies available for the treatment of TNBC are very limited due to which the development of targeted therapies for TNBC are under intense investigation [69]. The treatment options are limited due to absence of hormonal receptors in this subtype. Moreover, CSCs of BC accumulate after chemotherapy, such as when BC is treated with docetaxel and letrozole, the residual tumor cells exhibit a high number of cells with CD44+/CD24- expression [70]. This indicates that these cells escape chemotherapies and the resulting tumor contains more cells with tumor initiating properties. These findings demonstrate that CSCs play a critical role in pathogenesis of TNBC therefore, targeting these cells is an important approach to reduce chemoresistance and tumor relapse in TNBC.

The chemoresistance of CSC in TNBC is considered a key mechanism of BC relapse [39, 45]. In mice xenografts of human TNBC cell lines, ALDH1 expression was found to be increased upon treatment with sunitinib, which is an antiangiogenic tyrosine kinase inhibitor [71]. The increased expression of ALDH1 in CSCs is also associated with the expression of multi drug resistance protein-1, IL6 and IL8, indicating a number of potential drug targets to target CSCs of TNBC. A number of CSC markers have been identified among which CD44, CD24 and ALDH1 have been extensively studied in TNBC tumors.

1.4.1. CD44+/CD24-

In BC, CD44+/CD24- lineage cells are mainly located peripherally at the edge of tumor [72]. They possess strong tumorigenic ability and have mesenchymal or myoepithelial-like phenotype. Their tumorigenic ability is evidenced from the fact that the transfer of as few as 100 cells of this lineage, in immunodeficient mice was able to form tumor bulk whereas 200-fold more cells that lack these cell surface receptors did not form

tumor [73]. CD44+/CD24- cells are highly enriched in mouse models in which BC cells show lung metastasis which indicate that these cells possess metastatic abilities [74]. Moreover, TNBCs exhibit increased CD44+/CD24- lineage cells compared to HER2 and luminal phenotypes. Immunohistochemistry (IHC) studies by Wang et al., showed that this phenotype of cells is also associated with worse prognosis in patients [52, 75]. In addition, CD44+/CD24- phenotype in TNBC exhibits high expression of androgen receptor compared to CD44+/CD24+, CD44-/CD24- and CD44-/CD24+ phenotypes [52]. Furthermore, CD44+/CD24- phenotype is also associated with lower overall survival in patients compared to patients that have other phenotypes [52].

1.4.2. ALDH1

BCSCs expressing ALDH1 are typically located at the center of tumor bulk and possess luminal or epithelial phenotype [72]. These cells have high proliferation rate and also possess the ability to self-renew. Their tumorigenic ability is evidenced from the fact that the engraftment of 500 cells of this lineage in fat-pad of NOD/scid mice formed tumor, in as few as 40 days whereas ALDH1- cells were unable to generate tumor and the H&E staining of the fat pad sections showed that the tumors formed by ALDH+ cells contained malignant cells. On the other hand, injection of 50,000 ALDH1- cells showed limited tumor formation [76]. The high expression of ALDH1+ cells is also associated with poor prognosis as the tumors that showed highly stained ALDH1+ cells exhibited highly aggressive clinical course [72]. The expression of ALDH1 gene has been shown to be higher in TNBCs compared to luminal A, luminal B and HER2+ BC subtypes [49]. The analysis of ALDH1 protein in tissue microarrays indicated that tumors exhibiting high expression of ALDH1 protein did not express ER and PR which shows that the expression of this CSC marker is higher in TNBC compared to other BC subtypes [76].

1.5.CSCs and their prognostic value

It has been reported that presence of CD44+/CD24- cells is associated with distant metastasis while the CD44+/CD24- cells have no correlation with event free, overall survival and the progression of disease [77]. On the contrary, Lin et al. found that CD44+/CD24 tumor cells in primary and secondary invasive ductal carcinoma samples were associated with worse overall and disease-free survival [78]. Furthermore, Chen et al. found that CD44+/CD24- phenotype in tissues of primary and secondary invasive ductal carcinoma was associated with poor prognosis, recurrent and metastatic tumor. High CD44+/CD24- expression was also associated with poor response to chemotherapy and low disease-free and overall survival [79]. In addition to CD44+/CD24- cells, pluripotency-associated transcription factors such as SOX2 and MYC are overexpressed at the transcriptional level in TNBC and are positively correlated with poor prognosis [80, 81]. Moreover, LRPs, the co-receptors of Wnt, are also associated with poor prognosis in TNBC as Lin et al found a higher mRNA expression of LRP8 in TNBC tissues compared to tissues other subtypes of BC and a positive correlation with poor prognosis [82].

There are various cell signaling pathways which are involved in CSCs development and self-renewal and one of these pathways is mediated through Aryl Hydrocarbon Receptor (AhR). AhR have been implicated in a number of different cancers and cause aggressive tumor.

1.6.Aryl Hydrocarbon Receptor

Aryl hydrocarbon receptor (AhR) is a ligand activated transcription factor which is involved in xenobiotic metabolism [83, 84]. It is found in cytoplasm where it is bound to chaperone proteins such as immunophilin-like protein XAP2 and heat shock protein 90 (HSP90) [85]. It is activated upon binding to some environmental pollutants [85,

86]. Following the binding of AhR to its ligand, it changes its conformation and translocates to nucleus where it dissociated from its chaperone proteins and forms heterodimer with aryl hydrocarbon receptor nuclear translocator (Arnt) (Fig. 2). This complex binds to specific DNA sequence called xenobiotic response elements (XREs), present in the enhancer region of certain genes and therefore, starts the transcription of target genes such as CYP 1A1, CYP1B1, CYP1A2, AhR repressor and anti-oxidant genes such as glutathione s-transferase (GSTA1) and NAD(P)H:Quinone oxidoreductase 1 (NQO1) [86-88].

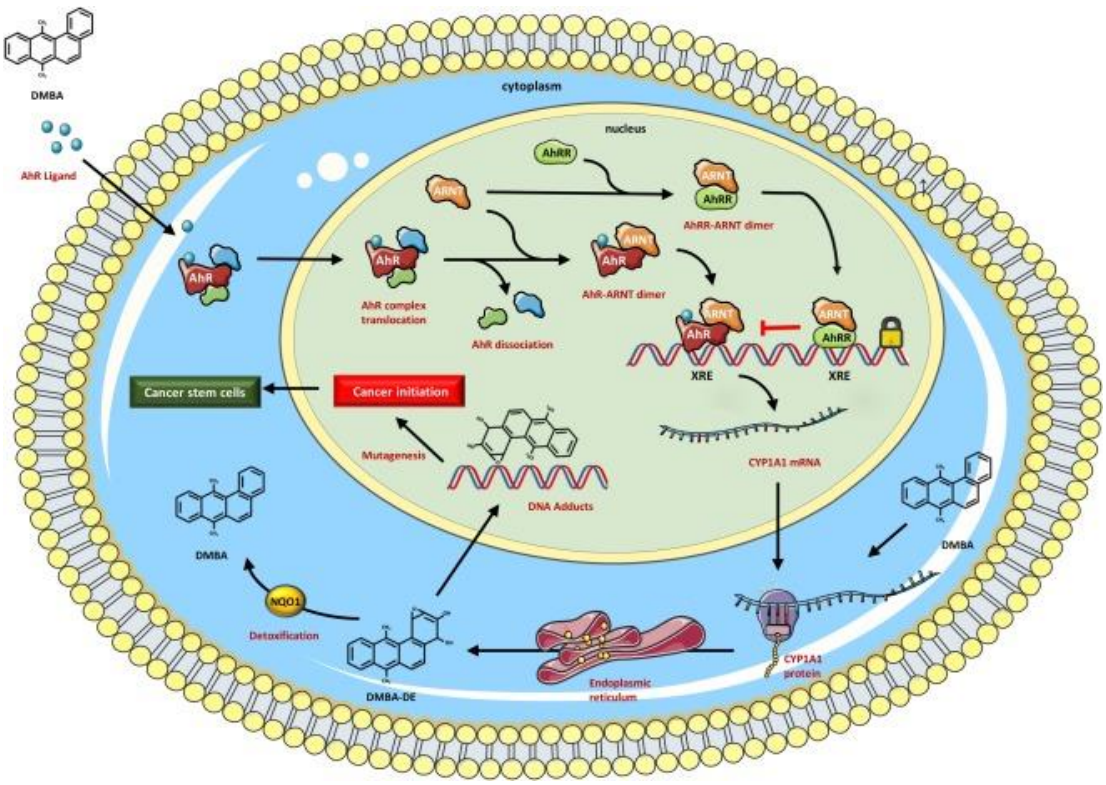


Figure 2. Activation of AhR pathway *[85]. A chemical carcinogen, DMBA, activates AhR and allow its translocation to nucleus from cytoplasm. AhR forms a AhR/ARNT heterodimer complex which binds to xenobiotic responsive element on DNA and transcibes genes which are associated with bioactivation of chemical carcinogens.*

The induction or constitutive expression of AhR has been reported to be associated with

inhibition of apoptosis, cell proliferation, epithelial to mesenchymal transition (EMT), inflammation, cell multiplication [89], differentiation, angiogenesis and migration [90, 91] [91-94]. Recent studies have shown that AhR knockdown inhibits BC cell survival, invasion, proliferation and migration [95, 96] which suggests that this receptor is implicated in promoting BC. In addition, the tumor aggression caused by AhR is mainly through the regulation of CSCs features.

1.6.1. Role of AhR in CSCs

AhR has been found to be highly activated and expressed in CSCs than cancer cells and its activation is associated with increased cancer stem-like characteristics such as chemoresistance, sphere formation, tumorigenic potential and cell proliferation [84, 97]. It regulates the expression of certain genes which eventually enhance CSC features. AhR mediates chemoresistance which is an important marker of cancer metastasis [98]. The knockdown of AhR in breast CSCs, followed by treatment with chemotherapeutic compounds reduced the cell viability and induced apoptosis compared to the cells where the AhR was not knocked down [92]. The knockdown of AhR in TNBC cell line MDA-MB-231, inhibits the expression of many tumor-associated genes such as ABCG3 (multi-drug resistance), MUC1 and IL8 (cell growth), S100A4 and ABI3 (cell migration and invasion), KYNU (tryptophan metabolism), VEGFA and CCL2 (angiogenesis) and BIRC3 and BCL3 (cell survival) [99]. In addition, *in vivo* studies revealed that the inhibition of AhR is associated with reduced tumor size [97].

AhR also induces chemoresistance in CSCs by controlling the expression of ABCG2 [97]. AhR has been reported to be transcriptional activator of ABCG2 [100] as it binds to the XRE binding sequence present on ABCG2 promoter, thus increase its expression and mediate drug resistance [97, 101, 102]. ABCG2 is overexpressed in drug resistant stem-like cells, therefore, harbour more SP [97, 103]. Interestingly, AhR inhibition in

radio-resistant lung adenocarcinoma cell line decreased the expression of numerous stemness marker genes which include ABCG2, ALDH1A1, c-Myc, KLF4, CXCR4 and Lgr6. AhR is a central player in maintenance of stem-like properties as the activation of many stemness-related genes is dependent on AhR activation [84]. Furthermore, Chemokine receptor CXCR4 causes transactivation of AhR in drug resistant BC cells and plays an important role in tumorigenicity [103]. CXCR4 is also associated with the increased ABCG2 expression in chemoresistant cells which is evidenced from the fact that the inhibition of this receptor decreased the expression of ABCG2 in tamoxifen resistant MCF7 xenograft tumors [103]. Additionally, the activation of AhR through TCDD in osteosarcoma cells MG-63, has shown to activate CXCR4 and this change in the expression of CXCR4 was blocked when AhR was blocked using AhR antagonist CH-223191 [104]. These findings demonstrate that AhR promotes CSCs features by interacting and regulating the expression of different receptors.

The activation of AhR through TCDD activates cyclooxygenase-2 (COX-2) in osteosarcoma cell line, MG-63 [104]. COX-2 is overexpressed in cancer and plays a key role in cancer progression, development and metastasis [105]. Moreover, it also confers chemoresistance and radioresistant to cancer cells which is evidenced from the fact that the breast CSCs isolated from primary tumor of transgenic HER2/Neu mice has shown 30-fold increase in COX-2 expression than non-CSCs [106] and the transfection of COX-2 in MCF7 cells increased their spheroid formation ability [107]. These findings demonstrate that AhR can promote CSCs characteristics through the activation of COX-2.

The chemical induction of AhR using 3-methylchloroanthrene is associated with high ALDH+ population in chemoresistant MCF7 cells whereas the ALDH expression is unaffected in chemosensitive cells [103]. Additionally, the inhibition of AhR in

tamoxifen resistant BC cells and subsequent injection of these cells into nude mice delayed tumor formation [103]. It is also a regulator of a number of genes that are involved in drug resistance and tumor progression and its inhibition can reduce the number of cancer progenitor cells [103]. This shows that AhR enhances CSCs features in chemoresistant cells and its knockdown in these cells can reduce tumor aggressiveness. The inactivation of AhR is associated with chemosensitivity, reduced cell proliferation and decreased spheroid size and number in choriocarcinoma cells whereas its activation increases cell proliferation and sphere formation [97]. This indicates the importance of AhR in mediating drug resistance and that its inhibition enhances chemosensitivity in neoplastic cells. Moreover, in a xenograft model of nude BALB/c mice, the injection of AhR shRNA transfected, human choriocarcinoma JEG-3 cells, formed tumors after 30 days, which were lighter in weight and smaller in size compared to negative control group [97].

1.6.2. Effect of AhR/CYP1 in CSC development

The AhR/CYP1 pathway plays a dual role in cancer; as a tumor suppressor or activator, depending on the type of cancer cells [85]. The AhR/CYP1 pathway has been widely studied in numerous cancer types, for its role in carcinogenesis and as a therapeutic target [92, 108-117]. CSCs are a major target of chemical carcinogens and therefore, AhR is considered to play a role in CSC regulation. The expression and functional levels of AhR has found to be higher in CSCs of different cancers compared to their corresponding differentiated non-CSCs which supports the hypothesis that AhR plays a role in self-renewal and progression of CSCs. AhR activation in different neoplastic cells is associated with increased CSC features such as increased ALDH+ cells, high chemoresistance, cell proliferation, tumorigenic potential and number and size of sphere formation [118-121]. In addition, AhR has reported to be constitutively active

in cancerous tissues compared to the normal tissues and the reduced expression of AhR in tumor is associated with reduced tumor size and better overall patient survival [122]. On the other hand, numerous studies have reported anti-neoplastic effect of AhR activation in CSC self-renewal and development. The activation of AhR through β-NF in MCF-7 cells has been found to decrease mammosphere formation ability and also reduces size and rate of secondary spheres [123]. This decrease in mammosphere formation is associated with decreased CSC markers such as expression of Bim1, Nanog, Notch, β-catenin and ALDH+ cells [123]. Furthermore, the activation of AhR/CYP1B1 through 6-formylindolo[3,2-b]carbazole (FICZ) in human acute myeloid leukemia cell lines, MV4-11 and MOLM-14, resulted in reduced CSC population and increased apoptosis. In addition, the chemical/genetic inhibition of AhR/CYP1A1 increased tumorigenicity and CSC population and characteristics [124]. These studies report the anti-tumor effects of AhR which indicate that AhR agonists can be employed to treat cancer.

AhR is involved in maintenance of CSCs characteristics through various cell signaling pathways.

1.6.3. Role of AhR in CSC-related signaling pathways

1.6.3.1.Role of Wnt signalling pathway

Wnt signalling pathway is involved in CSC self-renewal [125]. Activated β-Catenin physically interacts with AhR on its DNA responsive elements thus enhance its activity [126]. Similarly, the activation of AhR through chemical inducers result in nuclear translocation of β-Catenin in breast CSCs and it also increases the expression Cyclin D1 [92]. Due to increased nuclear localization of β-Catenin upon the activation of AhR, wnt/ β-Catenin signalling pathway is considered to cross talk with AhR for regulation CSCs characteristics such as ALDH [92]. The activation of β-Catenin causes

transactivation of AhR but this activation of AhR is not always caused by β-Catenin because there is also an association of other factors [126]. The AhR activation through β-Catenin indicates that activated β-Catenin interacts with certain transcription factors and generally increase their transcriptional activity [127, 128].

In inflammatory and non-inflammatory BC cell lines (SUM149 and MDA-MB-231respectively), the AhR inhibition decreases *Wnt5a* expression at mRNA level. Moreover, in inflammatory BC (IBC), Wnt5a/b and β-Catenin enhances $CD44^{+}/CD24^{-}$ cells and also increases lymph node metastasis. Therefore, activation of AhR causes the stimulation of Wnt5a and progression of CSCs in IBC [129].

1.6.3.2.Role of NF-KB pathway in CSCs

NF-κB signaling pathway regulates radioresistance and cancer progression through IκB kinase α (IKKα). IKKα permits tumor-initiating cell expansion and self-renewal and is also associated with metastasis in prostate and BC [130, 131]. IKKα regulates overexpression of stemness-related genes through its association with AhR. IKKα levels are increased in CSCs and it co-exists with AhR in nucleus. Moreover, the concentration of IKKα increases at the promoter of cancer-initiating genes which indicates that it is involved in regulation of CSCs properties by interacting with tumor-initiating genes. Furthermore, this increase of IKKα at the promoters of stemness markers depends on activation of AhR as the inhibition of AhR decreases IKKα at their promoters. This shows that AhR along with IKKα promotes CSCs features [84].

The activation of AhR through TCDD in MG-63 osteosarcoma cell line is associated with an increase in protein and mRNA level of receptor activator of nuclear factor- κB ligand (RANKL) [104]. RANKL inhibition in mouse models has been reported to decrease breast tumors and pulmonary metastasis [132]. This suggest that AhR participate in cancer development through the RANKL activation which is associated

with cancer invasion and metastasis.

1.6.3.3.PTEN-PI3K/Akt pathway in CSCs

PTEN-PI3K/Akt pathway is involved in chemo-resistance, cell proliferation and survival [133-135]. AhR activation in breast CSCs reduces PTEN expression and its loss along with p53, in neural stem cells, permits self-renewal, cell proliferation and undifferentiation through c-Myc [92, 136]. The activation of AhR in non-small cell lung cancer cells induces resistance to tyrosine kinase inhibitors through Src activation, PI3K/Akt restoration and MEK/Erk signaling pathway [137]. Moreover, the activation of AhR and CYP1A1 results in increased expression of p-Akt in breast CSCs which induces CSCs features and permits mammosphere formation [138]. PTEN/Akt pathway also induces miR-21-induced EMT in BC cells which is a feature of CSCs [139]. The inactivation of Akt is associated with decreased CSCs characteristics which indicate the involvement of this pathway in development of CSCs. Thus, these findings indicate that AhR and PTEN-PI3K/Akt pathway crosstalk for CSCs maintenance [92].

1.6.3.4.Notch signalling pathway

Notch signalling pathway is associated with pro-survival genes and regulate self-renewal and cell proliferation [140-143]. Dysregulation of Notch signalling has been observed in various cancers [144]. Accumulating evidence indicate that Notch signalling is associated with cancer stemness genes and mediate resistance to chemotherapy [143, 145]. Moreover, the activation of Notch signaling pathway reduces the expression of PTEN and overexpress c-myc oncogene, which suggests that this pathway plays an essential role in maintenance of CSCs [133].

The activation of Notch signaling pathway activates AhR by the secretion of endogenous ligands which subsequently stimulates IL-22 secretion from CD4+ T cells [146]. IL-22 is overexpressed in serum and tissue of recurrent NSCLC and permits

cancer cell migration and proliferation which are CSC features [147]. Moreover, it also induces lung cancer cell invasion of A549 cell line, which is an important feature of CSCs [148]. These findings suggest a possible crosstalk between Notch signaling pathway and AhR for maintenance of CSCs characteristics.

Accumulating evidence indicate the involvement of AhR in promoting CSC features. In addition to AhR, there are numerous other genes that have been reported to induce chemoresistance and hence involved in conferring CSCs feature. Among these genes, the modulation of BCL-2 family genes is of paramount importance.

1.7.Anti-apoptotic BCL-2 Proteins

In the breast, as in many other tissues of the body, intrinsic apoptotic pathway is regulated by BCL-2 family of proteins. BCL-2 family of proteins are divided into two main subclasses called anti-apoptotic which include BCL-2, BCL2-A1, MCL-1, BCL-xL and BCL-w and pro-apoptotic which include BIM, BAK, BAD, BAX, BID, BIK, HRK, NOXA and PUMA. The interactions between anti- and pro-apoptotic proteins convey signals to regulate cell death decisions and any dysregulation in these proteins cause the cells to escape apoptosis which is a hallmark of cancer [149].

BCL-2 and other pro-survival proteins Mcl1, Bclxl, Bclw and Bfl1/A1 are known to protect cells from apoptosis until they are neutralised by BH3-only protein. There are two models which explain the mechanisms through which BH3 proteins play a role in loss of mitochondrial membrane integrity [150]. The first model explains that the specific BH3-only proteins have affinities for some members of pro-survival proteins therefore, the cell undergoes apoptosis only when all the pro-survival BCL-2 proteins are neutralised by BH3 proteins that are activated upon an apoptotic signal. The second model subdivides BH-3 only proteins into “direct activators” and “de-repressors” or “sensitizers”. The de-repressors only bind to pro-survival proteins whereas direct

activators such as tBid, Bim and Puma, not only bind to pro-survival proteins but also to Bax and /or Bak [151, 152]. Both models explain that the BH3 protein function leads to the activation of Bax and/or Bak which eventually cause mitochondrial outer membrane permeabilization. This causes release of cytochrome c in cytoplasm that activates caspase cascade and eventually cause cell death [153].

1.7.1. BCL-2 proteins in BC and TNBC

The increased expression of anti-apoptotic/pro-survival BCL-2 protein has been found in numerous cancers. Elevated BCL-2 expression is associated with various mechanisms such as increased gene translation, gene amplification, chromosomal translocation or protein stability. Different assays such as wound-healing, migration and invasion assays performed on medullary BC cell line BCap37 showed that overexpression of BCL-2 is associated with accelerated wound closure, increased cell motility and cell invasion and inhibition of BCl-2 prevents wound closure, cell invasion and migration [154]. Moreover, BCL-2 overexpression also induces lung metastasis of BCap37 cells *in vivo* [154]. The overexpression of BCL-2 in cells expressing BC-Mock BC-Bcl2, BC-shRNA-Bcl2 or BC-shRNA-NC reduces the expression pf epithelial marker E-cadherin and increases the mesenchymal markers N-cadherin and vimentin. Moreover, the inhibition of BCL-2 reduces expression of mesenchymal markers while increases epithelial markers. These findings collectively demonstrate that BCL-2 increases cell invasion and migration by inducing epithelial to mesenchymal transition and inhibition of BCL-2 is can be considered as a potential target to reduce BC metastasis [154].

BCL-2 has been recognized as a major prognostic factor in BC as the levels of BCL-2 are essential in determining the effectiveness of adjuvant chemotherapy in BC patients [155]. In addition, the reduced expression of BCL-2 in BC cells enhances the

effectiveness of chemotherapies [156]. Out of Seventy-seven cases of primary breast carcinomas, BCL-2 has been found to be expressed in 74% of TNBC cases and 70% of non-TNBC cases. In TNBC, the expression of BCL-2 was correlated with invasive ductal subtype [157]. Additionally, the presence of BCL-2 in TNBC patients is associated with poor clinical outcome, poor overall survival, recurrence and mortality [158].

The increased expression of BCL-2 in BC is associated with chemoresistance. The inhibition of BCL-2 through a chemical inhibitor, ABT-199, and the treatment with doxorubicin reduced the cell viability of TNBC cell lines, BT-549 and MDA-MB-231. In addition to reduced cell viability, the combined effect of BCL-2 inhibitor and doxorubicin also induced apoptosis through intrinsic apoptotic pathway in TNBC cells. The genetic knockdown of BCL-2 through siRNA in TNBC cell line BT-549, increased its sensitivity to doxorubicin. Moreover, the colony formation ability of TNBC cells was also reduced when they were treated with BCL-2 inhibitor and doxorubicin. In a subcutaneously developed, xenograft mouse model of MDA-MB-231, the combination of BCL-2 and doxorubicin showed anti-tumor effects by reducing the tumor growth. These findings demonstrate that the enhanced expression of BCL-2 possibly mediate chemoresistance in TNBC and inhibition of BCL-2 enhances the chemosensitivity of TNBC towards doxorubicin [159].

1.7.2. Anti-apoptotic BCL-2 proteins in CSCs

1.7.2.1.BCL-2

The involvement of BCL-2 is maintenance of CSCs is also evidence from the fact that the synergistic effect of BCL-2/BCL-xL inhibitor (navitoclax) and Src inhibitor effectively deplete stem-like cells. In addition to BCL-2, the basal like MDA-MB-468 cells have been found to be more sensitive to MCL-1 inhibitor S63845 whereas they

were less sensitive to combined effect of BCL-2 and Src inhibition. These findings demonstrate that stem-like cells are more dependent on BCL-2 for their survival whereas basal like BC cells are more dependent on Mcl-1 [160].

BCL-2 has also been found to be overexpressed in quiescent leukemia stem cells that also exhibit low level of reactive oxygen species (ROS). Moreover, the gene expression analysis of BCL-2 in the normal marrow specimens showed that normal CD34+ progenitors express high BCL-2 levels compared to highly differentiated CD34 cells. The inhibition of BCL-2 in ROS-low cells increases their oxidative state. Treatment of leukemic stem cells that exhibit low ROS levels, with BCL-2 inhibitor ABT-263, showed increased toxicity compared to ROS-high cells which indicate that BCL-2 plays a significant role in maintaining Leukemic stem cells (LSCs) as the inhibition of BCL-2 induced cytotoxicity. The knockdown of BCL-2 also inhibits the colony formation in primary leukemic blasts. The treatment of LSCs with BCL-2 inhibitor ABT-263 and then their injection into NSG mice, reduced tumor formation. These findings show that targeting BCL-2 could be an effective therapy to eradicate ROS-low LSCs [161]. Furthermore, BCl-2 overexpression in hematopoietic stem cells (HSCs) protects them from apoptosis and leads to increased number of HSCs in bone marrow. It also increases colony formation in vitro and reconstitution capacity in vivo [162, 163].

Sabutoclax, an inhibitor of anti-apoptotic BCL-2 family proteins, reduces the $CD44^{high}/CD24^{low}$ cell population in BC MCF-7 cells and also decreases ALDH+ cells in multidrug resistant derivative of MCF-7 called MCF-7/A02. This indicates that the expression of anti-apoptotic BCL-2 family proteins play a role in maintenance of CSC features in BC. In addition, sabutoclax also decreases sphere formation and colony formation ability in multi drug resistant cell lines, MCF-7/A02 and CALDOX (MDR derivative of human BC Cal51). The expression of BCL-2 is many solid tumors is

associated with IL-6/STAT3 levels and sabutoclax also reduced the IL-6 expression and phosphorylation of STAT3 in MCF-7/A02 and CALDOX cells. The phosphorylation of STAT3 in BC cell lines MCF-7/A02 and CALDOX confers CSC features such as ALDH expression and $CD44^{high}/CD24^{low}$ cell population. This shows that IL-6/STAT3 pathway confers CSC features in BC cell lines and inhibition of anti-apoptotic BCL-2 family of proteins inhibits STAT3 phosphorylation thus reduce CSC formation [164].

1.7.2.2.MCL-1

Mcl-1 is an anti-apoptotic member of BCL-2 family of proteins which sequester pro-apoptotic proteins such as BIM, BID, PUMA and NOXA [165] and is found to cause chemoresistance and tumor relapse [166]. Mcl-1 has also been shown to play a role in the growth of breast CSCs as the attenuation of Mcl-1 in BC cell, SUM159PT and MDA-MB-436, resulted in reduced mammosphere formation, reduced ALDH+ and $CD44^{+}/CD24^{-}$ cells. On the other hand, induction of Mcl-1 in MDA-MB-468 cells through Doxycycline, increased fraction of $CD44^{+}/CD24^{-}$ cells and mammosphere formation. These findings show that Mcl-1 plays a significant role in tumor-initiation and enrichment of breast CSCs of TNBC [167]. In addition, the knockdown of Mcl-1 along with MYC reduced mamopshere formation and ALDH+ cells in MDA-MB-436 and SUM159PT compared to individual knockdown of both genes and the activation of both genes increases $CD44^{+}/CD24^{-}$ cells and also increases the mammosphere formation ability of cells. Mcl-1 and MYC also coorporatively enhance mitochondrial respiration which in turn maintains stemness of cancer cells. Mcl-1 and MYC enriches CSCs in TNBC through the production of ROS which in turn activates hypoxia pathway. The activation of hypoxia pathway is associated with enrichment of CSCs and highly virulent BC biology [167]. In AML patients, expression analysis of pre-leukemic

stem cells showed increased expression of MCL-1 but not BCL-xL and BCL-2 [168].

1.7.2.3.BCL-xL

BCL-xL also promotes tumor sphere formation in melanoma and glioblastoma models of cells. The inhibition of BCL-xL through its chemical inhibitor, WEHI-539, and siRNA and resulted in reduced sphere formation in melanoma cells. BCL-xL enhances the expression of CSC markers such as NANOG, SOX2, HIF-1α, OCT4 and BMI1 which indicates that it plays an important role in maintaining CSC features. Moreover, the cells that express high level of BCL-xL exhibit increased invasive and migratory properties [169].

The expression of Bcl-xL has also found to be higher in spheroids of glioblastoma U-87 MG and patient-derived SC2 cells. However, the knockdown of Bcl-xL reduced the tumor sphere size of U-87 MG without affecting the expression of stemness markers [170]. The increased expression of Bcl-xL in spheroids of glioblastoma sensitize them to BH3 mimetics such as ABT-263 and ABT-737 which target Bcl-xL and Bcl-w/Bcl-xL/BCL-2. Therefore, these BH3-mimetics induce cell death in cells expressing high Bcl-xL[170]. Furthermore, the expression of Bcl-xL protein has been found to be higher in BC cell lines than normal breast epithelial cell line MCF10A which suggests that its expression is associated with neoplastic transformation [171]. Moreover, the expression of Bclx is also higher in breast tumors compared to normal samples and higher expression of Bclx is correlated with lower overall survival. Bclx expression has also found to be higher in tumors invaded with lymph nodes compared to non-invaded sample [171].

Bcl-x is also shown to confer invasive and migratory properties in BC. The invalidation of Bcl-x using CRISPR/Cas 9 system in highly invasive basal BC cell line, Hs578T and TNBC cell line MDA-MB-231, slowed the migration compared to the control cells,

which shows the involvement of Bcl-x in migration of BC cells. The invasive ability of Bcl-x knockout cells was also reduced when they were injected in zebrafish xenograft model [171].

1.8.Pro-apoptotic BCL-2 proteins in CSCs

In CSCs, higher expression of Pro-apoptotic BCL-2 family proteins have been shown to induce apoptosis. In human cervical CSCs, BAX expression is found to be higher upon treatment with morusin which eventually leads to reduced tumor sphere formation, reduced proliferation and migration. Morusin also increased apoptosis in cervical CSCs by increasing BAX levels [172]. In MCF-7 breast CSCs, curcumin enhanced expression of pro-apoptotic BAX, BAD, BIK, BAK and BIM which lead to loss of mitochondrial membrane potential and eventually apoptosis. The activation of BAX through BTSA1 resulted in increased apoptosis in leukemic stem cells ($CD34^{+}CD38^{-}$) isolated from AML patients however the same dose of BTSA1 failed to induce apoptosis in primary bone marrow cell ($CD34^{+}CD38^{-}$) from healthy individuals [168]. This indicates that Bax activation is an effective monotherapy against leukemic stem cells. In human colon cancer stem cells (HCCSCs), increased Bax and reduced BCL-2 protein expression also induced apoptosis upon treatment with Triphala (MET) [173]. Moreover, the reduced Bax expression and inhibition of apoptosis in CSCs is evidenced from the findings of Wei and colleagues that prostate CSCs exhibit lower expression of Bax compared to non-CSCs (Fig. 3) [174].

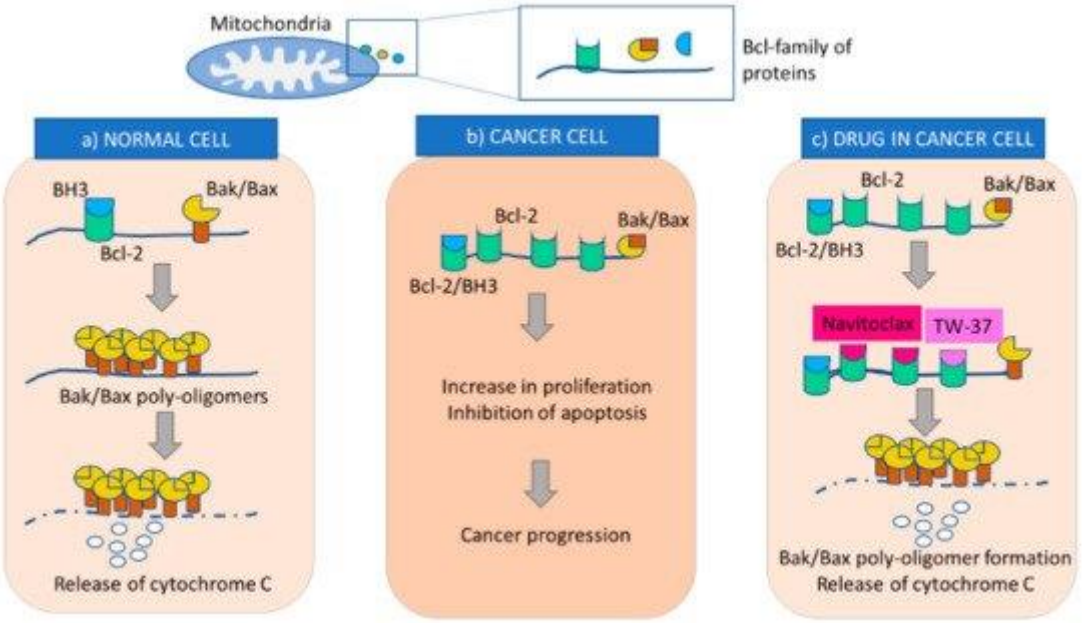


Figure 3. Apoptotic pathway in three different cells *[175]. a) In normal cells apoptosis occurs when Bak/Bax deactivate anti-apoptotic Bcl-2 which allows mitochondrial outer membrane permeabilizaton and release cytochrome C in the cytoplasm which initiates apoptosis. b) In cancer cells the expresison of Bcl-2 is higher whereas Bak/Bax is lower which does not allow apoptosis to occur. c) The anti-neoplastic drugs form compelx with Bcl-2 which allow Bak/Bax to induce apoptois.*

1.9.Role of BCL-2 proteins in CSCs

The hallmark of CSCs is the increased expression of BCL-2 proteins which protect these cells from cytotoxic agents [176]. The importance of BCL-2 and Bcl-xL in breast CSCs is evidenced from the fact that, these proteins interact with dasatinib induced Src inhibition which eventually increases the expression of pro-apoptotic PUMA. PUMA has been shown to interact with BCL-2 and Bcl-xL through its BH3 domains [177]. Therefore, BCl-2 and BCL-xL are considered to mediate resistance in stem-like BC cells by reducing the expression of proapoptotic PUMA [160]. In stem-like BC cells, LM2-4 and BT549, the combined effect of dasatinib and venetoclax (VCX) also increases the levels of free PUMA which eventually leads to activation of intrinsic apoptotic pathway. Therefore, the inhibition of BCL-2 also sensitizes stem-like cells to

induce dasatinib induced apoptosis. Moreover, the synergistic effect of these drugs results in reducing colony formation in self-renewing stem-like cells which indicate that BCL-2 inhibition plays a significant role in reducing stemness properties. These findings show that combined BCL-2/Src inhibition plays an effective role in depleting stem-like cells in BC [160].

Knowing the role of BCL-2 in breast CSCs and role of chemical carcinogens on conferring CSCs features, it is highly likely that AhR/CYP1A signaling pathway crosstalk with BCL-2 proteins for the development of breast CSCs.

1.10. Study rationale and objectives

Although the involvement of AhR has been shown in CSCs, there is a gap in knowledge regarding the expression and function of AhR/CYP1A pathway and its correlation with BCL-2 family proteins in BC and breast CSCs. BCL2 family proteins play a major role in breast CSCs development, which can be characterized by the constitutive, inducible and inhibitory expression levels of AhR in both differentiating and breast CSCs *in vitro*. There is a lack of literature evaluating the correlation between BCL2 proteins and AhR for the growth of CSCs. For this reason, we have hypothesized that anti-apoptotic BCL-2 proteins mediate breast CSCs development and proliferation by AhR activation. This cross talk between AhR and BCL-2 proteins can help in characterizing the mechanism of cell survival in breast CSCs. The results from this study would suggest that the proliferation of CSCs can be controlled by inhibiting AhR, which plays a considerable role in the development of tumor cells.

The aims of the study are summarized as follows

1. To examine the constitutive level of AhR, CYP1B1 and BCL-2 family protein in breast epithelial cells MCF10A, TNBC cells MDA-MB-231 and its counterpart CSCs

2. To determine the inducible and inhibitory expression levels of AhR and its effect on CYP1B1 and BCL-2 proteins in TNBC cells MDA-MB-231 and its counterpart breast CSCs.
3. Evaluate the link between BCL-2 proteins and AhR in MDA-MB-231 and CSCs through inactivation studies.
4. Correlate the expression of AhR and BCL-2 in human BC tissues with cancer stage and subtype

CHAPTER 2: MATERIALS AND METHODS

2.1. Materials

TRIzol reagent, Dulbecco's Modified Eagle's Medium (DMEM), SureCast TEMED, Ultra-Pure Glycine, DNase/ RNase free Distilled Water, DNase/ RNase free Distilled Water were purchased from Invitrogen Co. (Grand Island, NY). MDA-MB-231 (ATCC® HTB-26™) and MCF-10A (ATCC® CRL10317™) were purchased from the American Type Culture Collection (ATCC). StemFlex™ medium, Pierce™ PVDF Transfer Membranes, PageRuler Plus Prestained 10 250kDa Protein Ladder, 20X TBS Tween 20, 10 X Pierce Western Blot Transfer Buffer, 100X Halt Protease Inhibitor Single Use Cocktail, Pierce RIPA Buffer, Nunclon™ Sphera™ Microplates, Dishes, and Flasks, countess cell counting chamber slides, Pierce™ Rapid Gold BCA Protein Assay Kit, Pierce™ Bovine Serum Albumin Standard, LIVE/DEAD™ Viability/Cytotoxicity Kit, Micropipette Tips and PureLink™ RNA Mini Kit were purchased from ThermoFisher Scientific (Waltham, MA USA). Tissue culture flasks were purchased from Corning Incorporated (NY, USA). Aldefluor® kit was purchased from Stem Cell Technologies (Vancouver, CANADA). DMBA and alpha-naphthoflavone (α-NF) were purchased from Toronto Laboratories Research (Toronto, Canada). SYBR® Green master mix, microAmp optical 96-well reaction plate and High Capacity cDNA RT kit were purchased from Applied Biosystems® (Foster city, CA). DNA primers were purchased from Integrated DNA Technologies (Coralville, IA). Dimethyl Sulfoxide (DMSO), Tris base, Cruz Marker,Ñ¢ MW Tag-Alexa Fluor 488, CrystalCruz Plain Micro Slides, 72/pk, CrystalCruz Coverglass, 22 x 22 mm, 1 oz, Primary and Horse Radish Peroxidase (HRP)-conjugated secondary antibodies against target proteins, AhR, CYP1B1, BCL-2, BCL-xL, BID, BIM, MCL-1 and BAX were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The Western blot

detection kits, Clarity Western ECL Substrate, 10X TGS Buffer, 2-Mercaptoethanol, 4x Laemmli Sample Buffer, Ammonium Persulfate, 10% SDS and 30% Acrylamide/Bis Solution, 29:1 were purchased from Bio-Rad Laboratories (Hercules, CA)

2.2. Methods

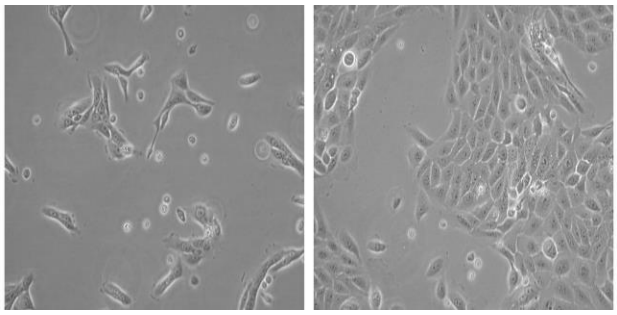
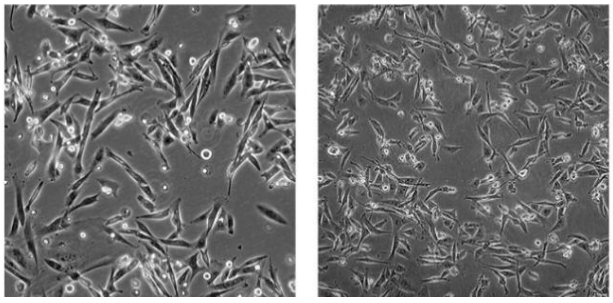
2.2.1. Human Breast Cell Culture Models

Cell culture was first established in early 20th century to study the behaviour of animal cells *in vitro* [178]. It is defined as the removal of animal cells to propagate and cultivate in an artificial environment which is suitable for their growth [179, 180]. A cell line is established when a primary cell culture is grown in a cell culture flask provided with suitable nutrients and growth factors until it reaches confluence and then sub-culturing it into secondary and tertiary culture [181].

Human breast epithelial cell line MCF-10A (CRL-10317 ^{TM}) and breast epithelial adenocarcinoma cell line MDA-MB-231(HTB-26^{TM}) were purchased from American Type Culture Collection (ATCC) (Rockville, MD) (Table. 1). Cells were cultured and maintained in Gibco® DMEM 4.5g/l glucose + glutamax (Thermo Fisher Scientific, USA), provided with 10% fetal bovine serum (FBS) (Thermo Fisher Scientific, USA) and 1X Antibiotic Antimycotic (Thermo Fisher Scientific, USA). The cells were grown in 75 cm^2 cell culture flasks (Corning Incorporated, USA) in humidified incubator at 37°C and 5% carbon dioxide. The cells were routinely sub-cultured in T-75 tissue culture flasks upon confluence. The medium of cells was replaced with fresh medium every second day. The cells were plated in cell culture dishes in their supporting cell culture media for RNA isolation and RT-qPCR, Western blot analysis, and flow cytometry, Immunofluorescence (IF) and Muse cell analysis. All experiments were conducted by treating cells with desired compound in serum-free. The drugs were

freshly prepared in dimethyl sulfoxide (DMSO) just before each experiment where the DMSO concentration did not exceed 0.1% (v/v).

Table 1. Morphology of different human Breast cell lines (www.atcc.org).

Cell line	Morphology	Growing condition
MCF-10A		Normal breast epithelial cells derived from adherent cells in the population. The culturing conditions are 37°C in the presence of 95% air and 5% CO_2. Medium Change: Every 2 to 3 days
MDA-MB-231		TNBC cells derived from metastatic site: pleural effusion. The culturing conditions are 37°C in the presence of 95% air and 5% CO_2. Medium Change: 2 to 3 times per week

2.2.3. Mammosphere (CSCs) formation assay

One of the methods to isolate and enrich breast CSCs is to grow cells in non-adherent, non-differentiating conditions which results in spherical colonies of cells that are termed as "mammosphere" [182, 183]. These colonies of cells are enriched in serum free medium [184]. Mammosphere culture is an effective method to isolate breast CSCs as the transcriptional profiling of cells obtained from mammosphere culture showed an increased overlap with genetic programs of other stem cells [182]. MDA-MB-231 cells were cultured in Nunclon Sphera T75 Cell Culture Flask (Thermo Fisher Scientific, USA) in the presence of StemFlex^{TM} Basal Medium (Thermo Fisher Scientific, USA),

provided with 10X StemFlex supplement and 1X antibiotic-antimycotic, at 37°C in humidified incubator containing 5% CO_2. They were allowed to form mammospheres for 20 days (Fig. 4). 3 millilitres of fresh media were added every 4th day without removing the existing culture media. Breast CSCs were collected after 20 days by gentle centrifugation and dissociation into single cells. They were used to isolate RNA with the help of PureLink™ RNA Mini Kit (Invitrogen, Thermo Fisher Scientific, USA). Equal number of breast CSCs (mammospere) and non-CSCs were collected to perform different assays such as Aldeflour assay, SP assay and cell surface markers.

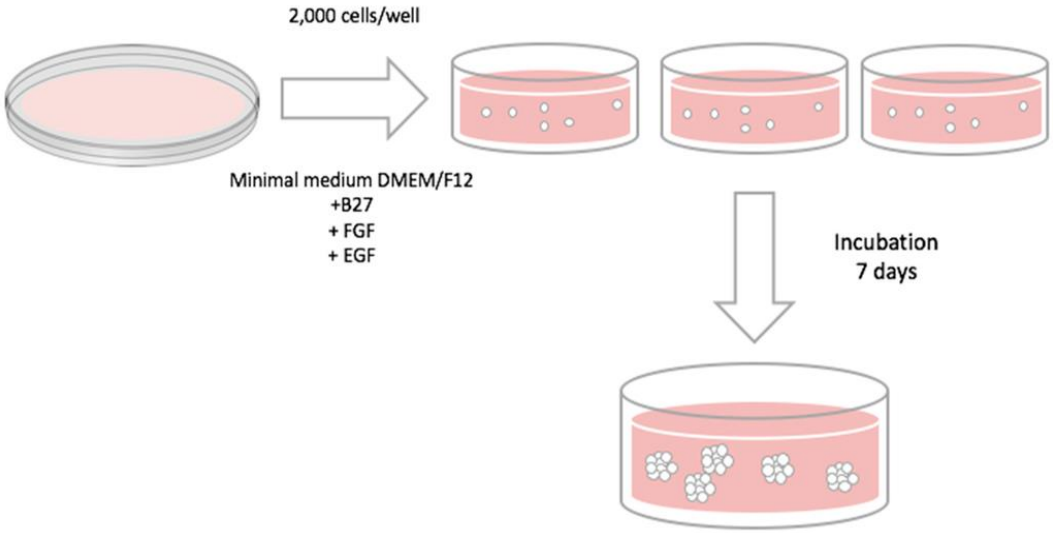


***Figure 4. Mammosphere formation in suspension from adherent cells** [185].*

2.2.3. Aldefluor assay

Aldehyde dehydrogenase (ALDH) is a part of family of enzymes that are located in nucleus, mitochondria or cytoplasm [186]. ALDH is involved in retinoic acid synthesis and aldehyde detoxification. The presence of this enzyme results in drug resistance which is one of the prominent features of CSCs. Recently ALDH has been identified as one of the markers for the identification of CSCs in solid tumors [186, 187]. Aldefluor

assay is a novel non-immunological fluorescent reagent system which identifies, separates and evaluate CSCs based on the enzymatic activity of aldehyde dehydrogenase enzyme [188]. Aldeflour measures the ALDH enzyme activity by fluorescent cleavage of its substrate, BODIPY-Aminoacetaldehyde (BAAA) (Fig. 5). Thus, it has been successfully used to identify and isolate CSC populations from more than 80 tumor tissues [189].

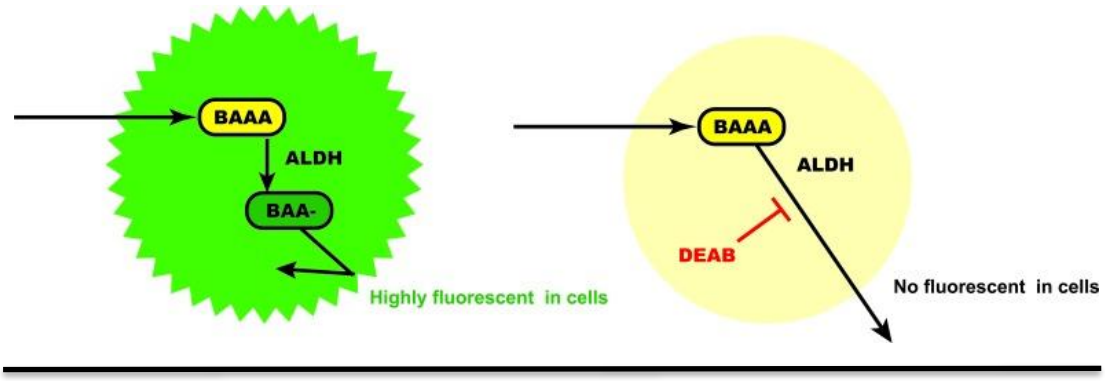


***Figure 5. Principle of Aldefluor assay** [190].*

Aldeflour assay was conducted according to the manufacturing instructions and the previously described method by Al-Dhfyan et al. [92]. MDA-MB-231 cells were collected and centrifuged at 1300 RPM for 5 minutes. The pellet of cells was resuspended in 1 ml of 1X PBS and were counted using Countess™ Cell Counting Chamber Slides (Invitrogen™, Thermo Fisher Scientific, USA) and placed on ice. The non-adherent mammospheres were collected in 50 ml tubes and centrifuged at 1300 RPM for 5 minutes. The pellet was trypsinised to obtain single cells and then they were counted and were kept on ice. Each set of cells was divided into a control tube and a test tube and 1 million cells were taken into each treatment. The cells were then centrifuged at 300g for 5 minutes, and pellet was resuspended in 1ml assay buffer. In the control tube, 5µl of specific ALDH inhibitor, Diethylamino Benzaldehyde (DEAB)

reagent, was added. In the test tube, 5 µl of ALDEFLOUR reagent was added, mixed very well and 500 µl from the test tube was transferred to control tube. The samples were incubated for 45 minutes in a water bath set at 37^{o}C. Thereafter, they were centrifuged at 300g for 5 minutes and pellet was resuspended in 300µl of assay buffer and kept on ice. The cells were then stained with 3 µl of propidium iodide (PI) before reading them on flow cytometer. The percentage of ALDH+ cells was determined through BD FACS flow cytometer. Each treatment was conducted in triplicate.

2.2.4. CD44^{+}/CD24^{-}

In addition to ALDH, BC cells that exhibit high expression of adhesion molecule CD44 and low or no expression of adhesion molecule CD24, have the ability to form mammospheres and show drug resistance. The cells that exhibit high CD44 and low CD24 expression are highly invasive and metastatic [191], and therefore, CD44^{+}/CD24^{-} is associated with CSCs formation [192].

The expression of CD44 and CD24 in MDA-MB-231 and its corresponding mammospheres, was determined as described before [193]. For this purpose, confluent MDA-MB-231 cells were trypsinized using 0.25% trypsin, neutralized with 10% DMEM media, and then collected in 15 ml tube. The cells were centrifuged at 1300 RPM for 5 minutes and the pellet was washed with 1ml 1X PBS and the number of cells was counted using Countess^{TM} Cell Counting Chamber Slides. For the mamospheres (CSCs), the mammospheres were collected in 50 ml tube and centrifuged at 300 g for 10 minutes followed by the addition of 3 ml of trypsin was to dissociate mamospheres. Trypsin was then neutralized using StemFlex media and cells were again centrifuged at 300 g for 10 minutes. The pellet was washed with 1 ml of 1XP BS and cells were counted using Countess^{TM} Cell Counting Chamber Slides. Each set of cells, approximately 1 X10^{6}, was incubated on ice for 45 minutes with *2* µl of CD44 antibody

and 2 µl of CD24 antibody in the dark. Thereafter, the cells were centrifuged at 1300 RPM for 5 minutes and the pellets were washed with 1 ml of 1X PBS and cells were suspended in 200 µl 1X PBS. The expression of CD44/CD24 was then determined using flow cytometer.

2.2.5. Side population

SP is an approach that is employed to sort and identify CSCs in various tissues and species. The presence of multiple drug-resistance proteins, ATP-binding cassette transporter ABCG2, exhibits unique ability to efflux the DNA binding dye such as Dye Cycle Violet (DCV). This leads to formation of SP which forms a 'tail' in the flow cytometry plot (Fig. 6) [194].

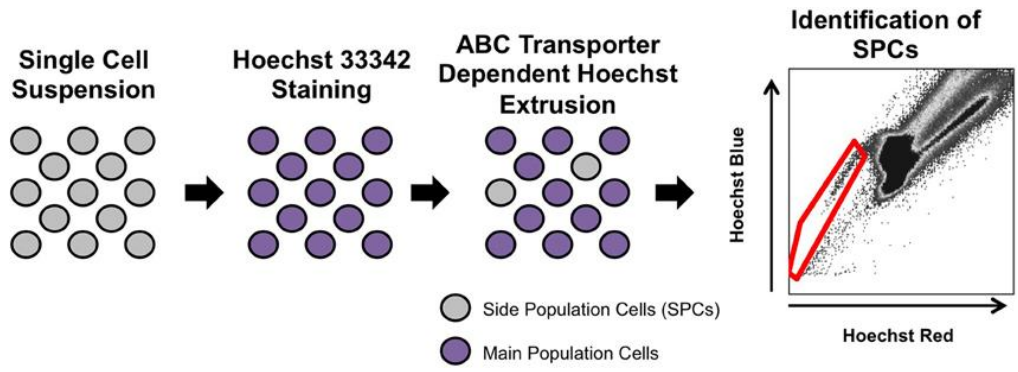


***Figure 6. Representation of SP analyzed by flow cytometry** [195].*

The MDA-MB-231 cells and mammosphere were harvested in the form of pellet and then suspended in their supporting cell culture media. The cells were incubated with a cell permeable dye DCV (10 µM) which binds to the DNA of the cells. The dead cells were eliminated using Propidium iodide (PI) stain. The CSCs were examined using LSRII flow cytometer as they form a distinct tail by excluding DCV. BD flow cytometer, cell sorter was used for the analysis, sorting and separaion of SP and non-

SP. Sidescatter and forward-scatter analyses were used to exclude debris and cell clusters.

2.2.6. RNA isolation and Quantitative Real-Time Polymerase Chain Reaction

2.2.6.1. RNA isolation and Quantification

1.10.1.1.1. 2.2.6.1.1. RNA Extraction from MCF-10A and MDA-MB-231 cells

Total RNA from MCF-10A and MDA-MB-231 was isolated using TRIzol reagent (Invitrogen, Thermo Fisher Scientific, USA) according to manufacturing instructions. MCF-10A and MDA-MB-231 cells were seeded in 6 well plates at a concentration of 200,000 cells/well and then treated with tested compounds for indicated time intervals. At 80% confluency, the culturing media were removed and the cells were collected in 0.5mL of TRIzol to 1.5ml RNase free tube (Thermo Fisher Scientific, USA). Chloroform (200 µl) was added to each 1.5ml for 15 seconds followed by centrifugation at 12000g for 15 minutes, resulted in separation and formation of two layers; upper (aqueous) and lower (liquid) layers. The aqueous layer, which contained RNA, was transferred to new 1.5ml tubes and incubated with 300ul of isopropyl alcohol (*Sigma-Aldrich, USA)* to precipitate RNA followed by centrifugation at 12000g for 10 minutes. The supernatant was discarded and the formed pellet (RNA) was then washed and purified in 500ul of 75% ethanol (prepared in DNase/RNase free water) (*Merck Millipore, USA*), followed by centrifugation at 12000g for 5 minutes. The RNA pellets were allowed to dry before resuspending in approximately 20-50µl of DNase/RNase free water (Thermo Fisher Scientific) followed by heating at 55^{o}C for 10 minutes to ensure complete resuspension. RNA concentration in the samples was quantified by measuring the absorbance at 260 nm using Nanodrop8000 spectrophotometer (Thermo Fisher Scientific, USA). The purity of RNA was determined by measuring 260/280

ratio between 1.7-2.0 using nanodrop spectrophotometer. The RNA was then stored at -20^{o}c for further experiments.

1.10.1.1.2. 2.2.6.1.2. RNA Extraction from mammospheres (CSCs)

RNA from mammospheres (CSCs) was extracted using Purelink RNA mini kit (Invitrogen, Thermo Fisher Scientific, USA) according to the manufacturing instructions. Mammospheres (CSCs) were seeded in 6 well Nunclon sphera plates (Thermo Fisher Scientific, USA), at a concentration of 300,000 cells/well and treated with the desired compound for 72 hours. The mammospheres were collected in 50ml tubes and centrifuged at 300g for 10 minutes. The pellet was incubated in 2 ml trypsin for 3-4 minutes, to dissociate the cells, followed by addition of 3ml of StemFlex Basal Medium and then were centrifuged at 300g for 10 minutes. The pellets were resuspended in 600µl of lysis buffer by gentle pipetting and vortexing followed by homogenization using micropipette. 600µl of 70% ethanol was added and vortexed to disperse white precipitate. 600µl of this was transferred to 2 spin cartridges with collection tube and centrifuged at 12000g for 15 seconds, the liquid in collection tube was discarded. This was followed by two washing steps with 700 µl of wash buffer I and 500ul of wash buffer II respectively and the tubes were then centrifuged at 12000g for 15 seconds and the membrane was then dried by centrifuging at 12000g for 2 minutes. 30 µl of DNase/RNase free water was added to the center of spin cartridge and incubated at room temperature for 1 minute. Spin cartridge was centrifuged at 12000g for 2 minutes and RNA was eluted in the collection tube.

The RNA concentration in each sample was quantified by measuring the absorbance at 260 nm using Nanodrop8000 spectrophotometer (Thermo Fisher Scientific, USA). The purity of RNA was determined by measuring 260/280 ratio between 1.7-2.0. The RNA was then stored at -20^{o}c for further experiments.

2.2.6.2. cDNA synthesis

cDNA synthesis was conducted using the High-Capacity cDNA reverse transcription kit (Applied Biosystems), according to the manufacturer's instructions and as described previously [196]. After total cellular RNA isolation and quantification, approximately 1µg of RNA was used to synthesize cDNA using High Capacity cDNA RT Kit with RNase Inhibitor (Thermo Fisher Scientific, USA). The components of the kit were allowed to thaw on ice and master mix was prepared by mixing the volume of each component of the kit as shown in table 2.

Table 2. Components and their volumes for cDNA conversion.

Components	**Volume per reaction**
10× RT Buffer	2 µl
10× RT Random Primers	2 µl
25× dNTP Mix (100 mM)	0.8 µl
MultiScribe™ Reverse Transcriptase,	1 µl
RNase Inhibitor	1 µl
Nuclease Free Water	3.2 µl

The cDNA conversion was carried by incubating 10 µl of mastermix with 10 µl of RNA in Thermocycler (Applied Biosystems by Thermo Fisher Scientific, USA) at the following cycling conditions (Table 3).

Table 3. Cycling conditions for cDNA conversion

Stage		**Number of Cycles**	**Time**	**Temperature**
Stage 1		1X	10 minutes	25^{o}C
Stage 2		40X	3 minutes	37^{o}C
Stage 3		40X	10 seconds	85^{o}C
Stage 4			∞	4^{o}C

2.2.6.3. Real-Time Polymerase Chain reaction (RT-PCR)

Real-Time Polymerase Chain Reaction (RT-PCR) is a laboratory-based approach that has become a common tool to detect and quantify desired genes and target mRNA expression [197, 198]. The quantitative mRNA expression of target genes was carried out by amplifying the cDNA products using QuantStudio 5 Flex RT-PCR System (Applied Biosystems®). The PowerUp SYBR Green Universal Mastermix was used to perform PCR amplification as described previously [199]. RT-PCR was conducted to examine the expression of target genes in MCF10A, MDA-MB-231 and mammospheres. The 25-µl reaction mixture (Table 4) contained SYBR Green Universal Mastermix, forward and reverse primers of target genes (Table 4), DNase/RNase-free water, and cDNA. The plate was sealed with adhesive film (Thermo Fisher Scientific, USA) and centrifuged at 1500 RPM for 5 minutes. RT-PCR was carried out in QuantStudio 5 (Applied Biosystems by Thermo Fisher Scientific, USA), using the following program: hold stage at 95^0C for 20 seconds followed by 40 cycles of PCR stage which was initially carried at 95^0C for 3 seconds and then at 60^0C for 30 seconds. The fold change in the level of target genes between treated and untreated samples were normalized by the levels of β-actin. The RT-PCR data were analyzed using the relative gene expression (i.e.,ΔΔ CT) method, as explained previously [200]. The sequences of primers used in RT-PCR are given in table 5.

Table 4. Components and their volumes for qPCR

Components	**Volume per reaction**
SYBR green	12.5 µl
Forward primer	0.07 µl
Reverse primer	0.07 µl
DNase/RNase free water	11.15 µl

Table 5. Primers sequences used for Real-Time PCR reactions.

Gene	5'→3' Forward primer	5'→3' Reverse primer
β-actin	GCC CTG AGG CAC TCT TCC A	CCA GGG CAG TGA TCT CCT TCT
AhR	CTG ACG CTG AGC CTA AGA AC	ACC TAC GCC AGT CGC AAG
CYP1A1	CGCAGACCTTGTGATATTCCAG	CGTTTCTTCCATCCTTCC AGG
CYP1B1	ATGGCCTCCCTGTACGACATC	TGTTGCGCTCAATCTCCT CCT
BCL-2	ATG TGT GTG GAG AGC GTC ACC	TGAGCAGAGTCTTCAGA GACAGC
BCL-xL	CTG AAT CGG AGA TGG AGA CC	TGG GAT GTC AGG TCA CTG AA
BAX	TGG CAGCTG ACATGT TTTCTG AC	TCA CCC AAC CAC CCR GGT CTT
SOX9	ATG AAC GCC TTC ATG GTG T	TCT CGC TCT CGTTCA GAAGT
BRCA1	GCAGGAAATGACATTGTAGGAAA A	TGCCAGAATGAGAAAGA ACATCC

2.2.7. Protein Extraction and Western blot analysis

2.2.7.1. Protein Extraction and Quantification

Total proteins from the different breast cell lines were isolated by following the method of Korashy et al. [201]. MCF-10A and MDA-MB-231 cells were seeded in 6 well plates at a concentration of 200,000 cells/well, whereas mammospheres were seeded in Nunclon sphera T75 cell culture flask and allowed to form spheroids for 7 days. MCF-10A and MDA-MB-231 cells were treated with desired compound for 24 hours. After obtaining single cells through trypsinization, mammospheres were seeded in 6-well Nunclon sphere plates at a concentration of 300,000 cells/well and treated with desired compound for 72 hours. After indicated treatment time, MCF-10A, MDA-MB-231, and mammospheres (CSCs) cells were harvested in 50uL of Pierce RIPA lysis buffer (Thermo Fisher Scientific, USA) with 1% of protease inhibitor (Thermo Fisher Scientific, USA). The cells were incubated on ice for 1 hour with intermittent vortexing

every 5 minutes, followed by centrifugation at 12000 RPM for 20 minutes. The supernatant, that contains total proteins, was collected in a new tube and stored at -20 °C for quantification.

Total protein quantification was conducted using Pierce ™ Rapid Gold BCA Protein Assay Kit (Thermo Fisher Scientific, USA), according to manufacturer's protocol. The protein standards of different concentrations were prepared followed by preparation of working reagent by mixing reagent A and B in 50:1 ratio. The required volume of working reagent is calculated using the formula (# standards + # unknowns) × (# replicates) × (volume of WR per sample). 10 µl of protein standards and unknown protein samples were taken in 96 well plate (Corning Incorporated, USA) followed by addition of 200µl of working reagent and incubated for 5 minutes at room temperature. The absorbance was measured at 480nm using Multiskan SkyHigh spectrophotometer. The proteins were stored in -20^{o} C freezer for later use in the Western blot analysis.

2.2.7.2. Western Blot Analysis

Western blot is an important technique that is used in molecular biology, cell biology and biochemistry. It is used to separate and identify protein expression, based on their molecular weight [202] using polyacrylamide gel as separating media [203]. Western blot analysis was conducted according to previously described protocol [92]. Approximately, 30-50 µg of proteins from each group were mixed with loading buffer and then were separated on 10% - 12% (depending on molecular weight of protein) sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE). A prestained PageRuler, Protein Ladder (Thermo Fisher Scientific, USA) was used as an indicator of protein size. The running phase of SDS-PAGE was conducted in two steps where the gel was first run at 60V for 30 minutes followed by 100V for 2 h. Upon complete separation of proteins, they were electrophoretically transferred to PVDF

membrane (Thermo Fisher Scientific, USA) at 120 V for 1 h, in cold transfer buffer (Thermo Fisher Scientific, USA). The membrane was blocked for I hour in blocking solution (5% non-fat dry milk, 1% BSA, 0.5% Tween-20 in Tris buffered saline (TBS)) followed by 3 washes of 10 minutes each in wash buffer (0.1% Tween-20 in TBS). The membranes were then incubated overnight at 4^{o}C with specific primary antibodies against target proteins. Thereafter, the membranes were washed three times with wash buffer (Thermo Fisher Scientific, USA) followed by incubation with specific secondary antibodies for 2 h at room temperature. The membranes were washed several times and the protein bands were visualized and detected by incubating the membranes with Clarity Western ECL Substrate (Bio-Rad, USA) using ChemiDoc imaging system (Bio-Rad, USA). The intensities of protein bands were semi-quantified using ImageJ program (NIH, USA).

2.2.8. Cell viability and Proliferation Assay

In vitro cell viability and cytotoxicity assays are widely used experimental techniques to test chemicals effects based on changes in a number of parameters such as permeability of cell membrane, enzymatic activity, co-enzyme production, ATP production, nucleotide uptake and cell adherence. Recently, these assays are also used in cancer research to examine toxicity of compound and inhibition of tumor growth in drug development [204]. The cell viability and cytotoxicity assay was conducted on MDA-MB-231 cells according to manufacturing instructions and as described before [205]. After treatment with desired compound for 24 h, the cells were centrifuged at 1300 RPM for 5 minutes, and the pellet was then resuspended in 1ml of 1X PBS. The cell suspension (20μl) was incubated for 5 minutes in dark with 380 μl of Muse Count and Viability reagent (Luminex corporation, USA), at room temperature. Both viable and non-viable cells were quantified using Muse cell analyser (Luminex Corporation).

2.2.9. Apoptosis Assay

Multicellular organisms maintain homeostasis by balancing the rate of cell division and cell death. The cell death in these organisms occurs in a precisely coordinated manner which is termed as “apoptosis” [206]. Annexin V is phospholipid-binding protein that has high affinity towards phosphatidylserine (PS) which is component of cell membrane and located on the inner surface of cell membrane. In cells undergoing apoptosis, PS translocate to outside of the cell membrane where annexin V can readily bind them and this conjugate allows analysis of apoptosis (Fig. 7) [207, 208]

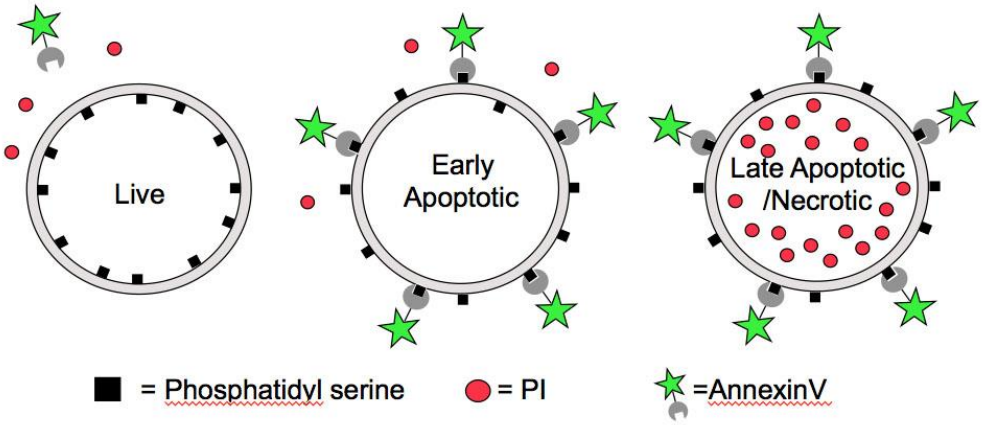


Figure 7. Representation of Annexin V function in apoptosis [209].

The level of apoptosis was determined on Muse Cell Analyser (Luminex corporation, USA) using Annexin V/PI as substrates as described before [205]. Approximately 2 x 10^5 cells /ml counted using Muse™ Count & Viability Kit (Luminex corporation, USA) that were treated with test drugs for indicated time interval and were centrifuged at 1300 RPM for 5 minutes. The pellet was resuspended in 100 µl PBS and 100 µl of Muse Annexin V & Dead cell reagent (Luminex corporation, USA) for 20 minutes in dark at room temperature. The number and percentage of cells undergoing early and late

apoptosis and necrosis were determined.

2.2.10. BCL-2 activation dual detection

BCL-2 is a member of BCL-2 family that regulates apoptosis by controlling proteins that regulate mitochondrial membrane permeability [210, 211]. Phosphorylation of BCL-2 at serine 70 has been found to be critical in apoptosis. The determination of both phosphorylated and total BCL-2 levels in the same cell provides accurate measurement of BCL-2 activation [212].

The level of BCL-2 activation was measured using BCL-2 Activation Dual detection kit (Luminex corporation, USA) according to manufacturing instructions. Treated cells with test compounds for indicated time were centrifuged at 1300 RPM for 5 minutes, and the pellet was resuspended and mixed in 400μl of 1X assay buffer and 400μl of fixation buffer on ice for 5 minutes. The cell suspension was further centrifuged at 300g for 5 minutes, and the pellets were then resuspended in 800μl of ice-cold permeabilization buffer for 5 minutes, followed by further centrifugation at 300g for 5 minutes. The pellets were then resuspended in 1X assay buffer and antibody working cocktail solution (5μl of antiphospho-BCL-2 (Ser70), Alexa Fluor® 555 antibody and 5 μl of anti-BCL-2, PECy5 conjugated antibody) and incubated for 30 minutes in dark at room temperature. Final centrifugation at 300g for 5 minutes was conducted and the pellets were resuspended in 200 μl of 1X assay buffer for analysis using Muse Cell Analyser (Luminex corporation, USA).

2.2.11. Multi-color DNA damage

DNA damage occurs in all cellular organisms and is induced by a number of agents, both natural (reactive oxygen species) and man-made (UV, X-ray, gamma rays). While most of the damaged DNA can be repaired, such repair systems are not 100% efficient and may lead to mutation [213, 214]. DNA damage increases levels of phospho Histone

H2A.X at the site of DNA damage and it is one of the markers to determine the DNA damage inside cells [215].

The level of DNA damage in MDA-MB-231 cells was determined using Muse® Multi-Color DNA Damage kit (Luminex corporation, USA) according to manufacturer's instructions. 3 x 10^5 cells/ml MDA-MB-231 cells were counted using Muse™ Count & Viability Kit and centrifuged at 1300RPM for 5 minutes. Pellets were incubated on ice for 10 minutes after resuspending in 150 μl of 1X assay buffer and 150 μl of fixation buffer followed by centrifugation at 300 g for 5 minutes. The pellets were further centrifuged at 300 g for 5 minutes after incubating for 10 min in 300 μl of ice-cold permeabilization buffer followed by resuspension of pellet in 270 μl of 1X assay buffer and antibody working cocktail solution (5μl of anti-phospho-ATM (Ser1981), PE and 5µL of anti-phospho-Histone H2A.X (Ser139), PECy5 conjugated). Samples were incubated for 30 minutes at room temperature in dark and then 100 μl of 1X assay buffer was added and centrifuged at 300 g for 5 minutes. Finally, pellets were resuspended in 200 μl of 1 X assay buffer and DNA damage was quantified using Muse cell analyser.

2.2.12. Multicaspase

Caspases are proteolytic enzymes that are involved in the execution and propagation of apoptosis in the presence of apoptotic signal. They are synthesized as inactive zymogens and activated by proteolytic cleavage. Most signaling pathways that are activated due to anti-cancer drugs, ultimately results in activation of caspases [216, 217].

Multicaspase profile in MDA-MB-231 cells was determined using Muse® MultiCaspase Kit (Luminex corporation, USA) according to manufacturer's instructions. 2 x 10^5 cells/ml treated cells were centrifuged at 1300RPM for 5 minutes and resuspended in 50 μl of caspase buffer and 5 μl of muse multicaspse reagent

working solution (2 μl of muse multicaspase reagent was dissolved in 318 μl of 1X PBS). The cell suspensions were incubated for 30 minutes at 37^{o}C in the presence of 5% CO_2 followed by addition of 150 μl of muse Caspase 7-AAD working solution (2 μl of muse caspase 7-AAD mixed in 148 μl of 1X caspase buffer) and incubation for 5 minutes. The activation of caspase activation was quantified using Muse cell analyser

2.2.13. Immunofluorescence Analysis

Immunofluorescence (IF) is an IHC technique which is used to identify cellular proteins by using labelled antibodies. This approach can be employed to visualize the localization of cellular components in cells, tissues and in 3D structures. It is commonly used in research as well as in clinical practice to determine protein expression and cellular or intracellular localization [218].

IF assay was conducted as described previously [219]. MDA-MB-231 cells were grown on sterile glass coverslips (Santa Cruz Biotechnology, USA). Approximately, 200,000-250,000 cells/well were seeded in 6-well plate containing coverslips, and the seeded cells were treated with desired drug for 24 hours in a serum free media. The media was removed and cells were washed twice with 1X PBS for 5 minutes at room temperature and then were fixed using 1ml of 4% formaldehyde for 20 minutes at RT. The fixative was removed and cells were again washed twice with 1X PBS for 5 minutes.

For IF assay of mammospheres, treated cells were collected in 50 ml tube and centrifuged at 1300 RPM for 5 minutes, and the pellet was trypsinised to obtain single cells followed by centrifugation at 1300RPM for 5 minutes. Supernatant was removed and pellet were washed with 1X PBS before the addition of ice-cold 70% ethanol. The cells were then poured onto the glass cover slides and allowed to completely dry at -20^{o}C.

Fixative cells were permeabilized using 1ml of 0.2% tritonX-100 (diluted in 1X PBS) for 10 minutes at RT. The cells were then washed twice with 1X PBS for 5 minutes and then incubated with blocking serum (10% FBS diluted in 1X PBS) in a humidified chamber for 1 hour. Thereafter, the cells were washed twice with 1X PBS and then 300μl of desired primary antibody (1:50 dilution, prepared in 1X PBS) was added directly onto the coverslip overnight in a humidified chamber at 4^{o}C. Later, primary antibody was removed and cells were washed thrice with 1X PBS and then 300μl/well of fluorescent secondary antibody (1:1000 dilution, prepared in 1X PBS) was added and incubated for 2 hours at RT in dark. The cells were then washed thrice with 1X PBS at RT and 1 ml of 1μg/ml DAPI (4′-6-Diamidino-2-phenylindole) was added for 2 minutes. The coverslips were removed from 6-well plate and they were mounted on the microscope slides (Santa Cruz Biotechnology, USA) using a drop of mounting media. Each sample was stained in triplicate for each antibody. The slides were allowed to dry in the dark and then imaged using Evos^{TM} M5000 microscope (Thermo Fisher Scientific, USA).

2.2.14. Live/Dead Viability/Cytotoxicity assay

Live/Dead Viability/Cytotoxicity assay is used to visualize living and dead cells based on their plasma membrane integrity and esterase activity. Approximately 250,000 MDA-MB-231 cells were seeded in 6-well plate and treated with desired compound for 24 h. The cells were washed twice with 1X PBS and then incubated at room temperature for 15 minutes with Live/Dead Viability/Cytotoxicity kit (Thermo Fisher Scientific, USA) which is composed of solution (5 µl of Calcein and 5 µl of Ethidium in 10 ml of serum free DMEM media). The cells were then visualized under Evos^{TM} M5000 microscope (Thermo Fisher Scientific, USA).

2.2.15. Immunohistochemistry assay

IHC is a method that is used to determine protein expression in tissue samples. The combination of IHC with tissue microarray (TMA) allows simultaneous determination of protein expression in hundred tissue samples [220]. Immunohistochemical analysis of tissue microarrays was carried out as described previously [221]. The slides were washed twice with xylene and alcohol for 10 minutes each respectively and then blocked for 20 minutes in 5% hydrogen peroxide in methanol. The slides were washed with running tap water and then with distilled water for 5 minutes each followed by 10 minutes treatment with Citrate buffer (pH 6.4). The antigen retrieval was carried out in citrate buffer at 110 °C for 20 minutes followed by cooling the slides to room temperature and washing twice in Tris-buffer saline (TBS) for 3 min each. They were incubated with antibody of interest for 1 hour followed by 2 washes with TBS for 3 min each and then incubated with secondary antibody for 40 minutes. The slides were washed twice for 3 minutes with TBS and treated for 15 minutes in DAB chromogen (20µl of DAB in 1 ml of Substrate buffer) followed by washing with distilled water. They were counterstained with Haematoxylin for 1-2 min and washed in running tap water for 5 minutes followed by dehydration and mounting for inspection.

2.2.16. Statistical Analysis

The results were presented as mean ± SEM (standard error of the mean). Each experiment was repeated either 3 or 6 times. Statistical analysis of the data was performed using Sigma Plot version 11(Systat Software Inc, USA). Differences between control and treatment groups were determined using student's T-test or One-Way Analysis of Variance (ANOVA) followed by Student-Newman-Keuls test. Results were considered significant when $P<0.05$.

CHAPTER 3: RESULTS

3.1. CSCs characteristics and features

3.1.1. MDA-MB-231 mammosphere culture enriches BCSCs

Mammosphere culture is an important approach to enrich CSCs and determine tumor formation of CSCs. Mammosphere assay was developed by Dontu and colleagues to propagate mammary epithelial stem cells which serves as an alternative approach to assess stem cells and CSC activity in mammary gland [182, 222]. When placed in a suspension culture condition provided with serum-free medium, CSCs typically form spherical structures by self-renewing, [223]. MDA-MB-231 cells formed a monolayer adhered to the surface of flask, when they were grown in the presence of serum (Figure 8A) whereas they formed bead like, somewhat, spherical structures when they were grown in suspension conditions in the absence of serum (Figure 8B).

3.1.2. Measurement of CSCs markers, ALDH, $CD44^{high}$ $CD24^{low}$ and SP

To evaluate that our mammosphere culture exhibits characteristics of CSC, we determined CSCs features through four different approaches. These approaches include determination of ALDH+, SP, $CD44^{high}$ $CD24^{low}$ cells, and the gene expression of SOX9 and BRCA1 in CSCs as compared to MDA-MB-231 cells.

ALDH is a CSCs specific marker which was used to confirm presence of CSCs in our mammosphere culture. In order to evaluate the reliability of mammosphere culture approach, the percentage of ALDH+ cells in MDA-MB-231 and its counterpart mammospheres were analysed using flow cytometry. Figure 8C shows that the percentage of ALDH+ cells was 5 times higher in mammospheres (2.6%) than in non-CSCs MDA-MB-231 (0.5%) (Fig. 8D).

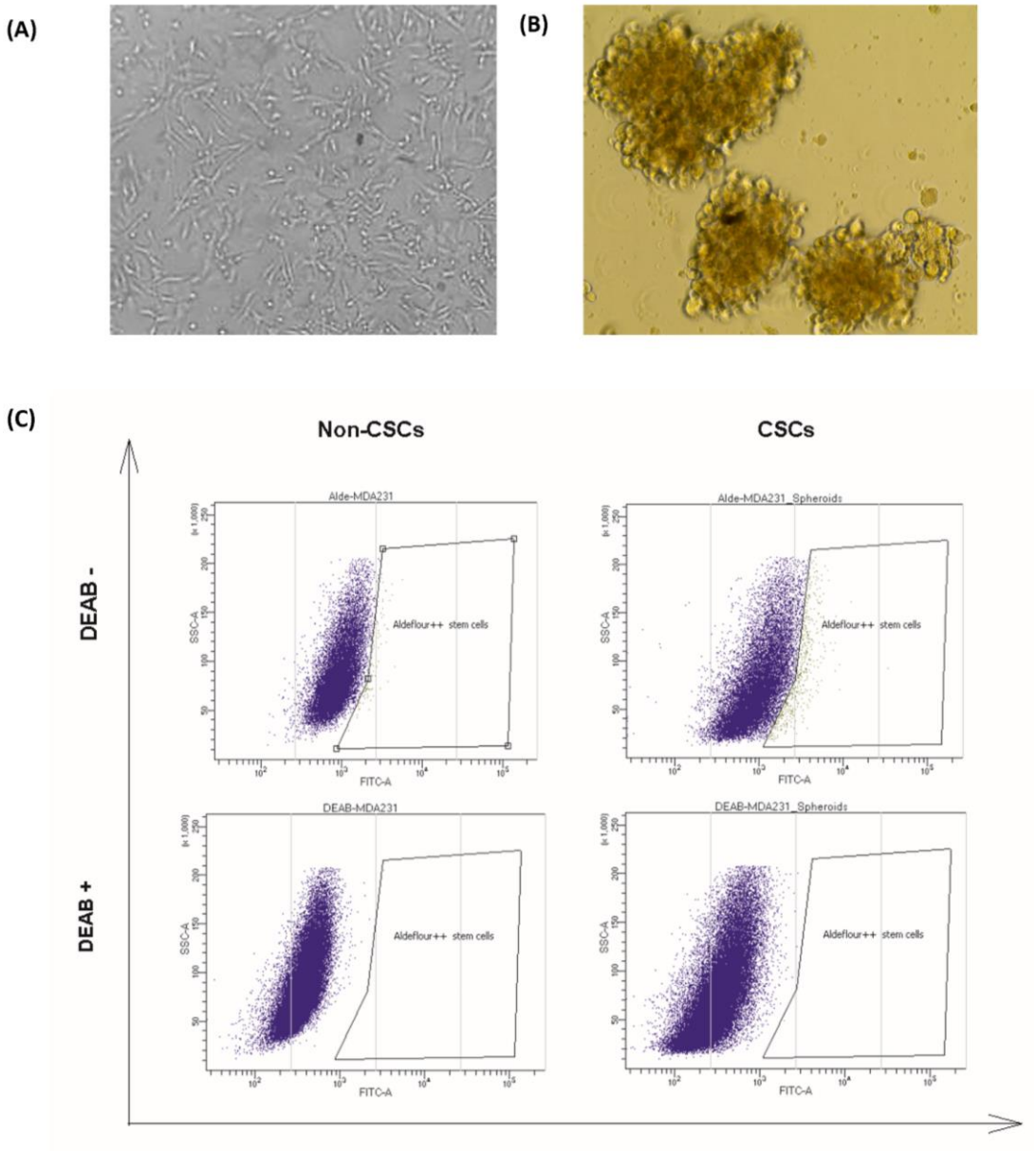


Figure 8. Enrichment of BCSCs through mammosphere formation and characterisation of ALDH+ cells. *The adherent MDA-MB-231 cells were imaged at 70% confluence (A) and mammospheres were imaged at 20th day of culture (B). Both cells were incubated with ALDH substrate in the presence and absence of DEAB, ALDH inhibitor. Thereafter, percentage of ALDH+ cells were determined by flow cytometry (C, D).*

The second approach to confirm the enrichment of the mammospheres with CSCs was to measure the expression of a well-known CSCs cell surface marker, $CD44^{high}$ $CD24^{low}$ using flow cytometry. Figure 9A shows that the percentage of $CD44^{high}$ $CD24^{low}$ in mammospheres (CSCs) were approximately 15 times (16.4%) higher than in non-CSCs (differentiating MDA-MB-231 cells) (0.9%), confirming that mammosphere culture of these cancer cells can enrich CSCs (Fig. 9B).

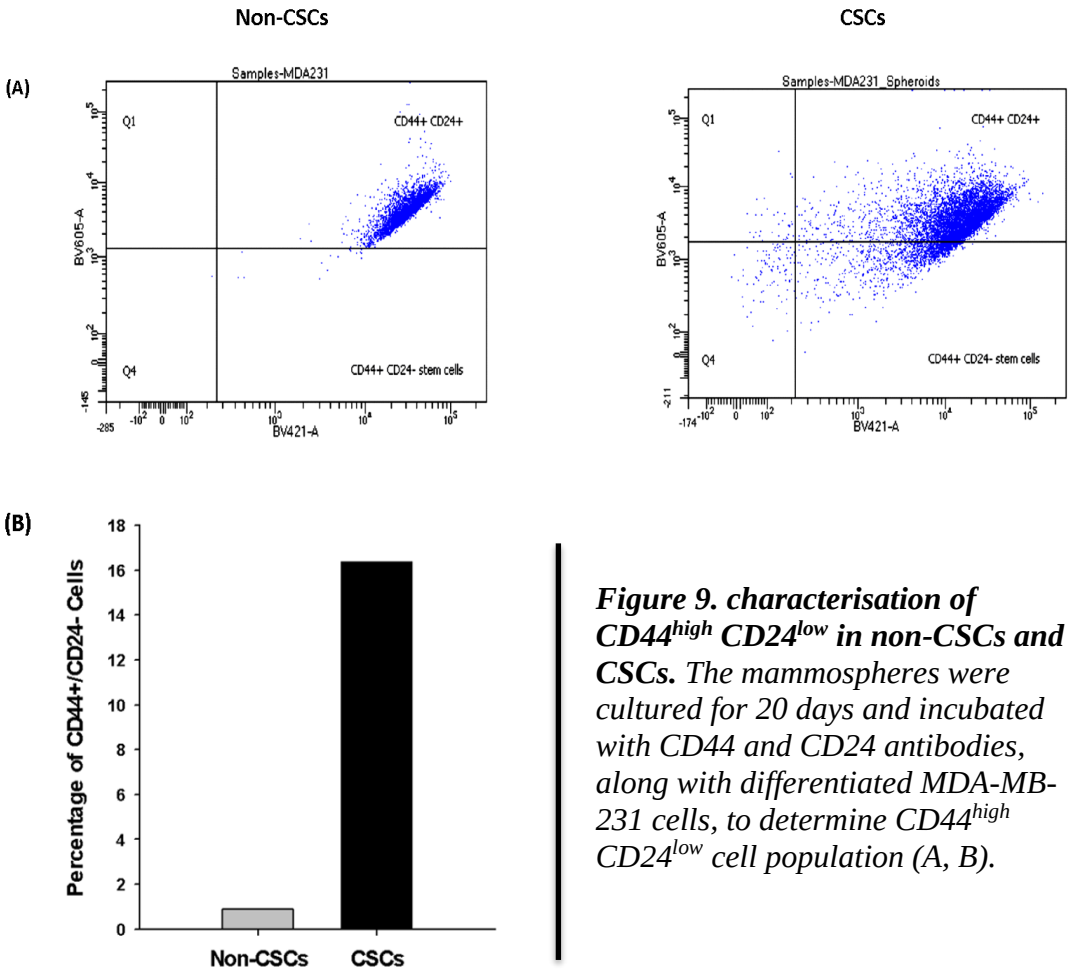


Figure 9. characterisation of $CD44^{high}$ $CD24^{low}$ in non-CSCs and CSCs. *The mammospheres were cultured for 20 days and incubated with CD44 and CD24 antibodies, along with differentiated MDA-MB-231 cells, to determine $CD44^{high}$ $CD24^{low}$ cell population (A, B).*

The CSCs features of the mammospheres were further confirmed by the SP assay, a flow cytometric analysis based on the ability of CSCs to efflux DyeCycle Violet out of the cell, that appears as tail in the flow cytometry plot. The results presented in figure

10A and B were consistent with the previous assays of CSCs determination. In that, the percentage of SP cells in differentiated MDA-MB-231 cells was 0.2% compared to mammosphere which was 0.7%. This further confirms that our mammospheres culture enriched CSCs.

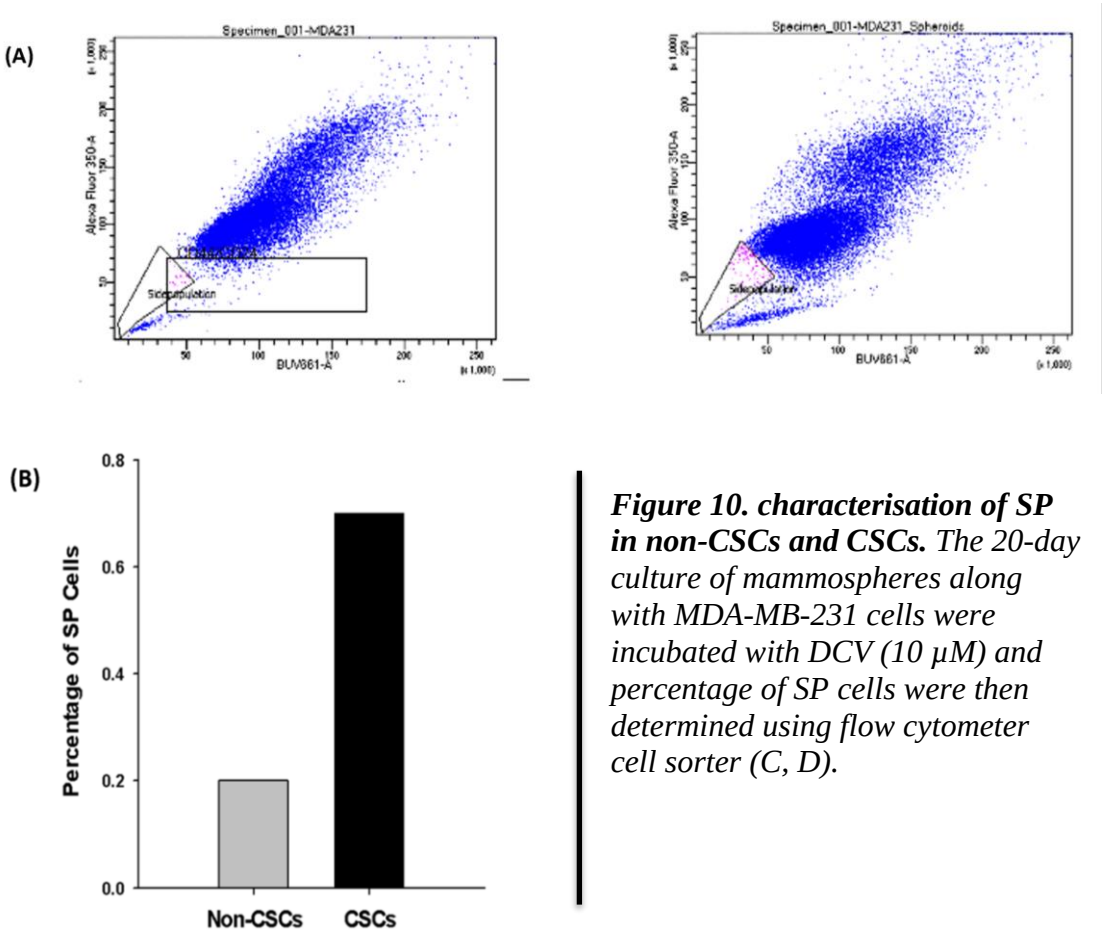


Figure 10. characterisation of SP in non-CSCs and CSCs. *The 20-day culture of mammospheres along with MDA-MB-231 cells were incubated with DCV (10 µM) and percentage of SP cells were then determined using flow cytometer cell sorter (C, D).*

The high levels of ALDH+, SP and $CD44^{high}/CD24^{low}$ cells in mammospheres prompted us to evaluate the constitutive mRNA and protein expression levels of CSC markers, SOX9 and tumor suppressor gene, BRCA1 in MCF-10A, MDA-MB-231 and CSCs by RT-PCR and Western blot analyses, respectively. Figure 11A shows that while SOX9 mRNA levels in MDA-MB-231 cells were 1.5-fold ($p>0.05$) higher than in MCF-10A, the levels in CSCs were significantly higher by approximately 3.7-fold.

At the protein level, similar pattern of expression was observed, in which CSCs exhibited a marked increase of SOX9 protein (1.6-fold) (Fig. 11B). On the other hand, BRCA1, a tumor suppressor gene in BC, was significantly downregulated at the mRNA level, both in MDA-MB-231 (non-CSCs) and CSCs, by approximately 99% and at the protein level, in CSCs, it was decreased by 84% compared to MCF-10A and approximately 89% compared to MDA-MB-231 (Fig. 11C and D).

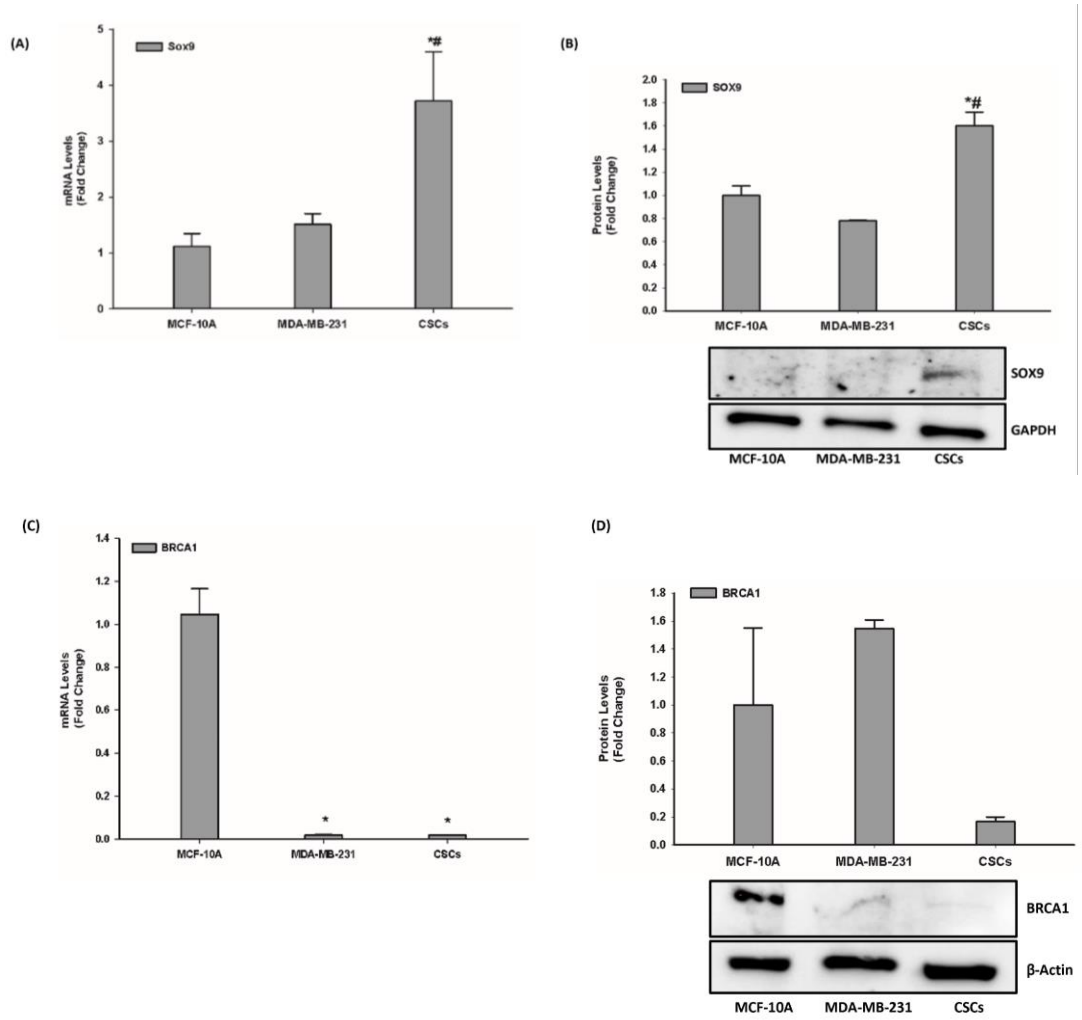


Figure 11. Constitutive expression of SOX9 and BRCA1 in three breast cell lines. *The basal mRNA (A, C) and protein (B, D) expression levels of SOX9 and BRCA1 were determined by RT-PCR and Western blot respectively and normalized to GAPDH or β-ACTIN housekeeping gene. Each experiment was conducted in duplicate and values are presented as mean ± SEM (n = 6). *; p< 0.05 compared to MCF-10A and #; p< 0.05 compared to MDA-MB-231 (non-CSCs) using ANOVA.*

3.2. Constitutive expression of AhR, CYP1B1 and BCL-2 family genes in breast epithelial cell MCF-10A, MDA-MB-231 and CSCs

To examine the constitutive expression of AhR, CYP1B1 and BCL-2 family genes in MCF-10A (normal breast epithelial cells), MDA-MB-231 (non-CSCS) and CSCs, we quantified the mRNA and protein expression levels of these genes using RT-PCR and Western blot. Thereafter, several independent experiments were conducted as follows:

3.2.1. Constitutive expression of AhR and CYP1B1 mRNAs and proteins in MCF-10A, MDA-MB-231, and CSCs

To first explore the basal expression of AhR and its target gene CYP1B1 in CSCs in comparison to normal epithelial MCF-10A and non-CSCs MDA-MB-231, comparative mRNA and protein expression levels of AhR and CYP1B1 in these cells were determined by RT-PCR and Western blot analysis, respectively. Figure 12A shows that the basal expression of AhR and CYP1B1 mRNA levels in MDA-MB-231 cells were 12- and 10-fold, respectively, higher than in normal breast epithelial cells, MCF-10A ($P<0.05$). Importantly, CSCs exhibited a much higher expression level of AhR and CYP1B1 mRNA levels than in MDA-MB-231 cells by approximately 8- and 136-fold, respectively. To further explore whether the changes at the mRNA levels were translated to changes at the protein levels, we examined the protein expression levels of AhR and CYP1B1 using Western blot analysis. Figure 12B shows that the protein levels of AhR and CYP1B1 in MDA-MB-231 were 2.1- and 1.2-fold higher respectively, than MCF-10A. With regards to CSCs, AhR and CYP1B1 protein levels were 2.7-fold and 1.2-fold higher respectively, than MCF-10A.

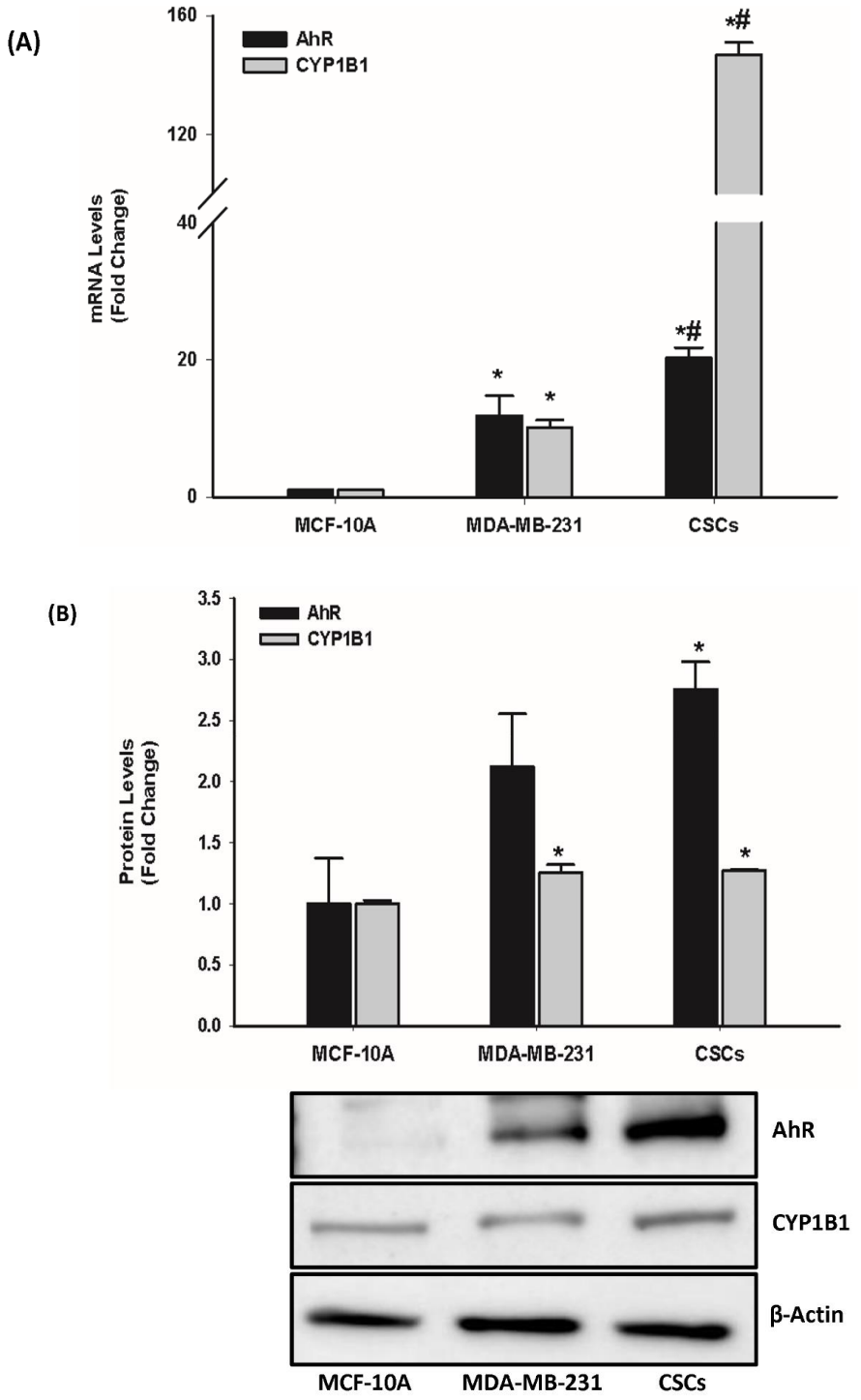


Figure 12. Basal expression of AhR and CYP1B1 in three breast cell lines. *The basal mRNA (A) and protein (B) expression levels of AhR and CYP1B1 were determined by RT-PCR and Western blot respectively and normalized to β-ACTIN housekeeping gene. Each experiment was conducted in duplicate and values are presented as mean ± SEM (n = 6). *; p< 0.05 compared to MCF-10A and #; p< 0.05 compared to MDA-MB-231 (non-CSCs) using ANOVA.*

3.2.2. Constitutive expression of BCL-2 family mRNAs and proteins in MCF-10A, MDA-MB-231, and CSCs

The increase in AhR and CYP1B1 expression in CSCs more than non-CSCs and normal breast epithelial cells prompted us to evaluate whether these increases were associated with significant modulations in the expression of BCL-2 family. To address this point, we examined the mRNA and proteins levels of several anti-apoptotic (BCL-2, BCL-xL, and MCL-1) and pro-apoptotic (BAX, BID, and BIM) BCL-2 family as follows:

3.2.2.1. Constitutive expression of anti-apoptotic BCL-2, BCL-xL, and MCL-1 mRNAs and proteins in MCF-10A, MDA-MB-231, and CSCs

Figure 13A shows that the basal mRNA levels of BCL-2 were higher in both MDA-MB231 and CSCs than in MCF-10A by approximately 3.2- and 0.5-fold, respectively. On the other hand, while BCL-xL mRNA level in MDA-MB-231 cells was not significantly different than MCF-10A, it was higher in CSCs than in both MDA-MB-231 and MCF-10A cells by approximately 3-fold (Fig. 13A).

At the protein expression level, figure 13B shows that the protein levels of BCL-2, BCL-xL and MCL-1 in CSCs were approximately 1.4-, 1.3- and 1.9-fold higher than MCF10A respectively ($P<0.05$) and approximately 17 %, 32% and 47% higher than non-CSCs, MDA-MB-231 ($P<0.05$).

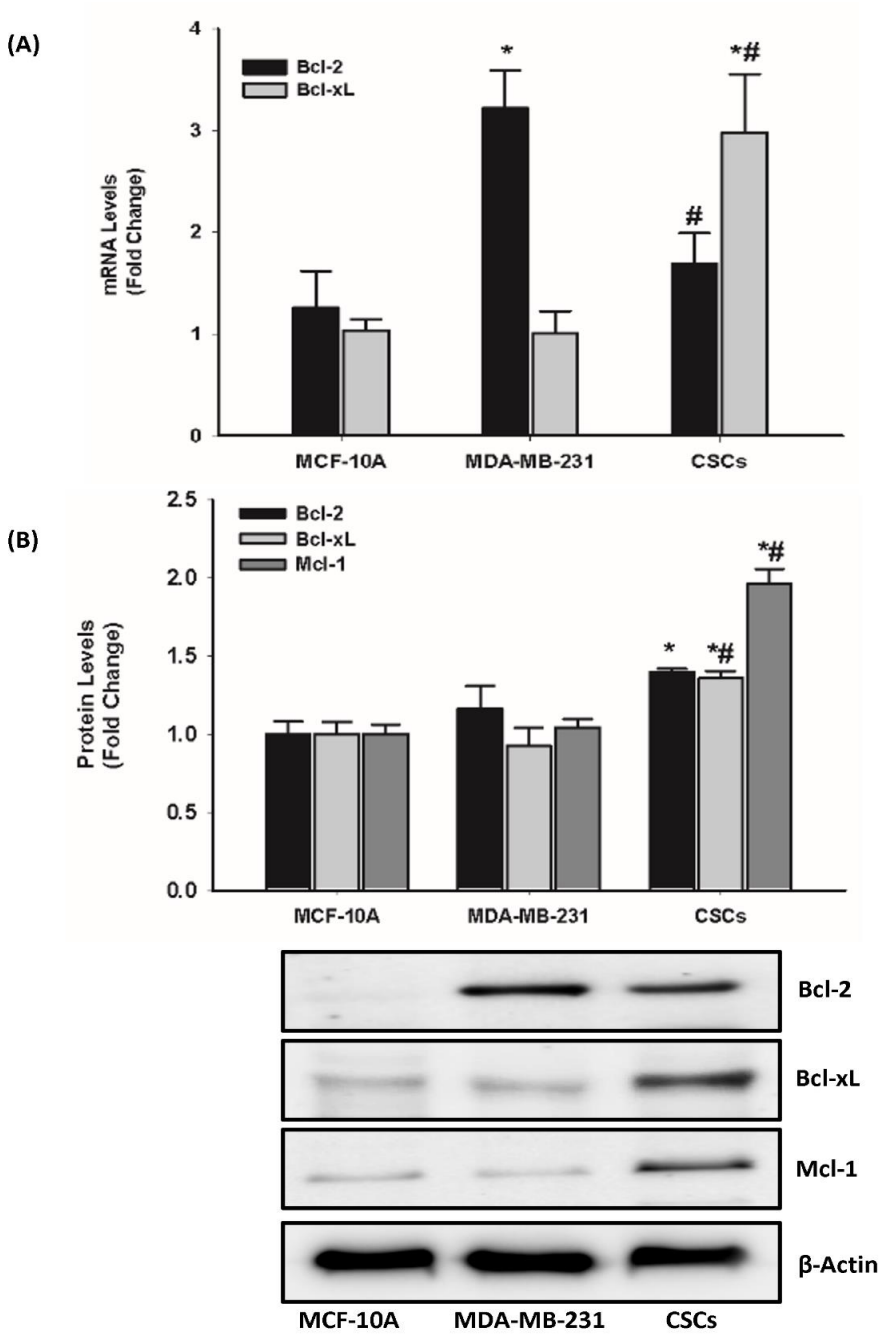


Figure 13. Constitutive expression of BCL-2, BCL-xL and MCL-1 in three breast cell lines. *The basal mRNA (A) and protein (B) expression levels of BCL-2, BCL-xL and MCL-1 were determined by RT-PCR and Western blot respectively and normalized to β-ACTIN housekeeping gene. Each experiment was conducted in duplicate and values are presented as mean ± SEM (n = 6). *; p< 0.05 compared to MCF-10A and #; p< 0.05 compared to MDA-MB-231 (non-CSCs) using ANOVA.*

3.2.2.2. Constitutive expression of pro-apoptotic BAX, BID, and BIM mRNAs and proteins in MCF-10A, MDA-MB-231, and CSCs.

The mRNA and protein levels of pro-apoptotic members of BCL-2 family were also

determined using RT-PCR and Western blot analyses, respectively. Figure 14A shows that the mRNA levels of BAX in CSCs and MDA-MB-231 cells were significantly downregulated by approximately 50%. Similarly, the protein expression levels of BAX and BIM were significantly downregulated in CSCs as compared to MCF-10A and similar pattern compared to MDA-MB-231 cells. With the exception, BID protein levels in CSCs were 1.3-fold higher than MCF-10A and MDA-MB-231 cells (Fig. 14B).

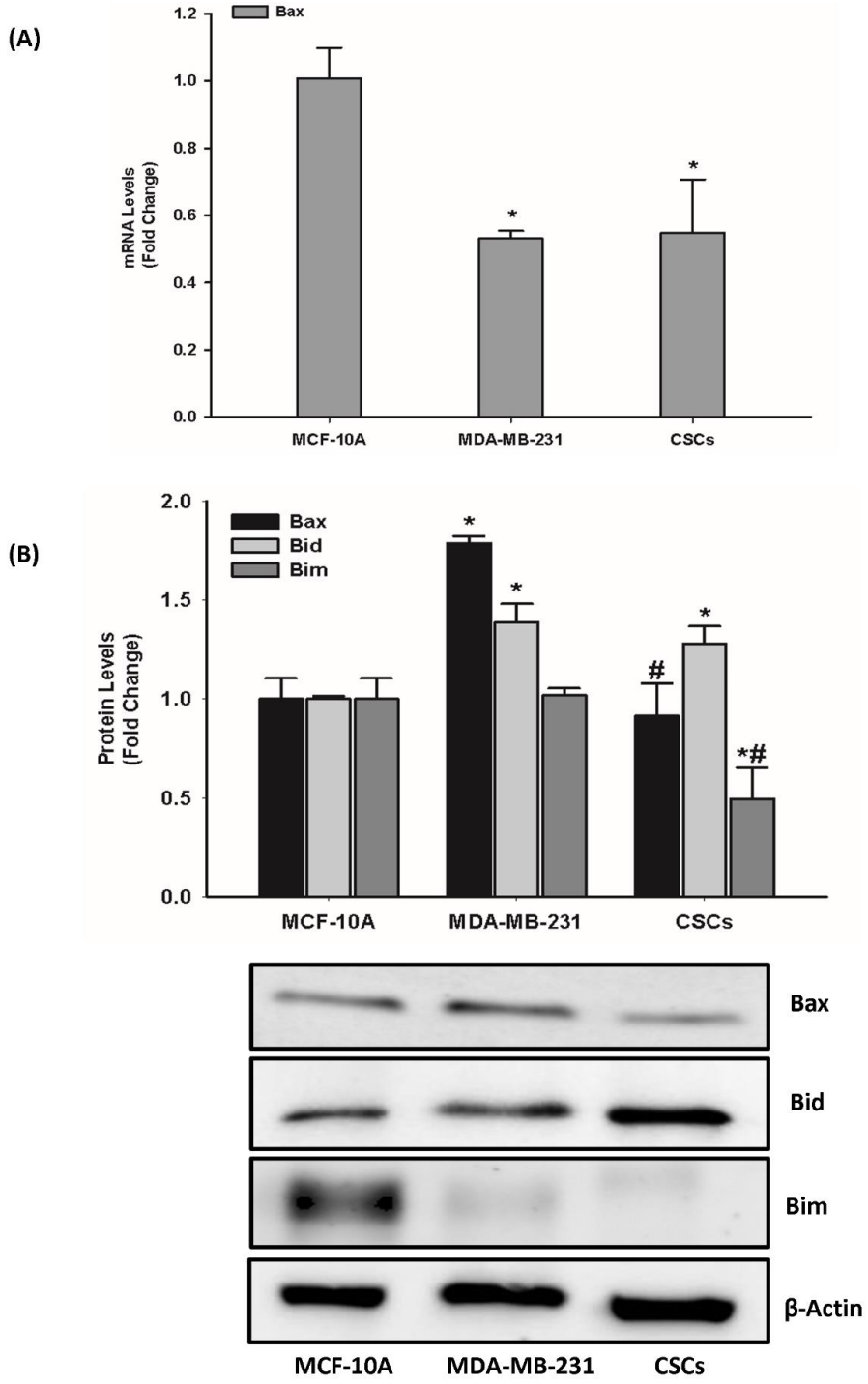


Figure 14. Constitutive expression of BAX, BID and BIM in three breast cell lines. *The basal mRNA (A) and protein (B) expression levels of BAX, BID and BIM were determined by RT-PCR and Western blot respectively and normalized to β-ACTIN housekeeping gene. Duplicate experiments were performed and the values are presented as mean ± SEM (n = 6). *; p< 0.05 compared to MCF-10A and #; p< 0.05 compared to MDA-MB-231 (non-CSCs) using ANOVA.*

3.3. Effects of AhR activation on mRNA levels of BCL-2 family proteins in MCF10A, MDA-MB-231 and CSCs

To examine the effect of AhR activation on BCL-2 family, a well-known AhR agonist, DMBA (5 µM) was used. MCF-10A and MDA-MB-231 cells were treated *for* 24 h, whereas mammospheres (CSCs) were treated for 72 h. Thereafter the mRNA levels of AhR, CYP1A1, CYP1B1, BCL-2, BCL-xL and BAX were determined. Figure 15A shows that the treatment of MCF-10A with DMBA resulted in no significant change in CYP1B1 mRNA levels which was associated with insignificant change in BCL-2 mRNA expression. However, the treatment in MDA-MB-231 cells with DMBA, resulted in activation of CYP1A1 and CYP1B1 by 3- and 2.7-fold, respectively. Importantly, activation of AhR regulated genes by DMBA was associated with a proportional increase in the mRNA levels BCL-2, BCL-xL and BAX by approximately, 3.6-, 3.6- and 2.9-fold, respectively (Fig. 15B). This association of AhR activation with BCL-2 was further confirmed in CSCs. Figure 15C shows that DMBA treatment in CSCs caused approximately 1.3-fold increase in CYP1B1 mRNA levels which resulted in proportional and significant increase in BCL-2 mRNA expression (1.4-fold).

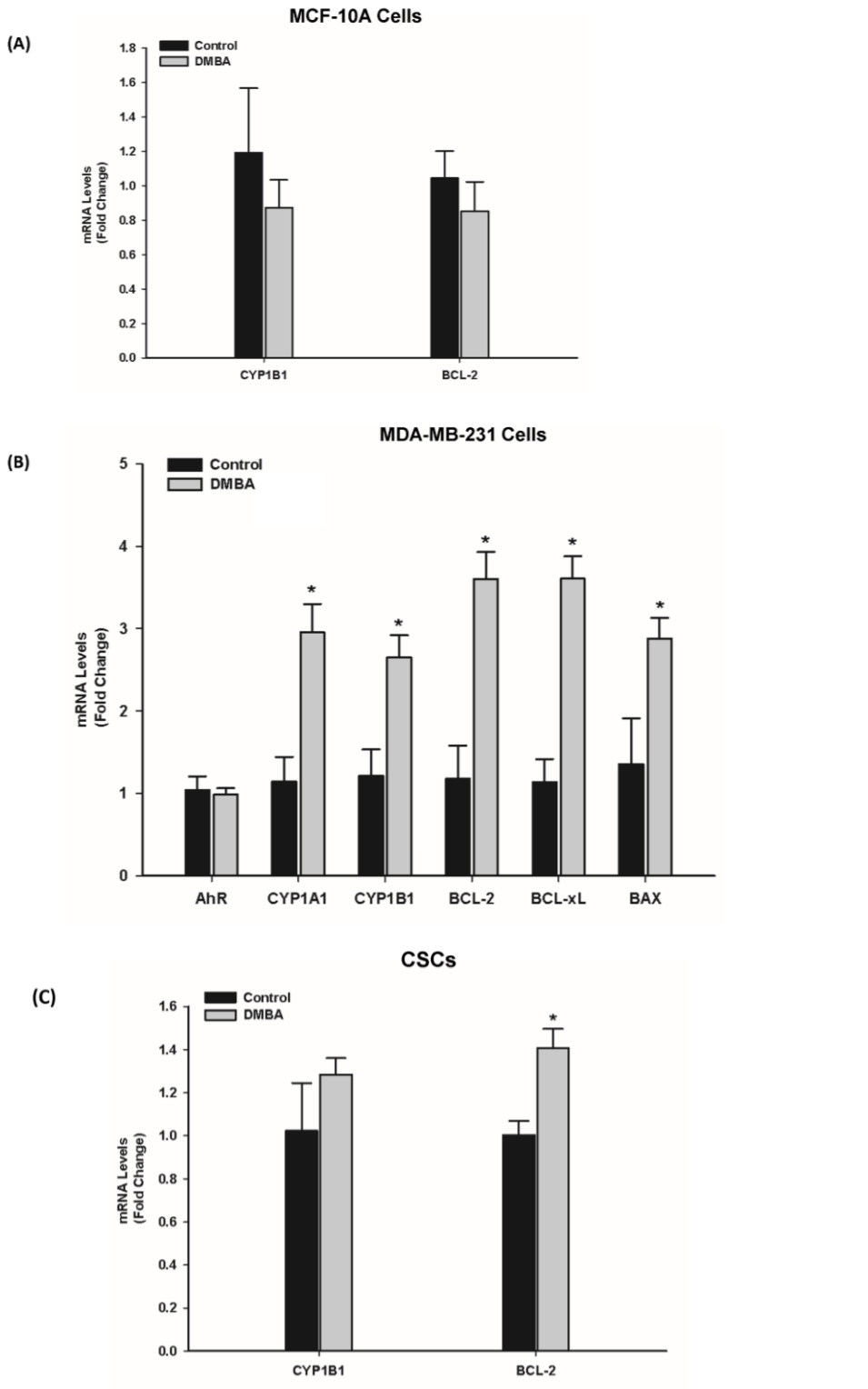


Figure 15. Effects of AhR activation on mRNA levels of BCL-2 family proteins in MCF-10A, MDA-MB-231 and CSCs. *MCF-10A and MDA-MB-231 cells were treated for 24 h and mammospheres were treated for 72 h with 5 µM DMBA. The mRNA expression levels of AhR, CYP1A1, CYP1B1, BCL-2, BCL-xL, and BAX were determined by RT-PCR and normalized to GAPDH or β-ACTIN housekeeping gene. Each experiment was conducted in duplicate and the values are represented as mean of percentage ± SEM (n = 6). *p<0.05 compared to control using t-test.*

3.4. Effects of AhR inhibition on the expression and function of BCL-2 family proteins in MDA-MB-231 cells

To explore the crosstalk between AhR and BCL-2 family in cancer, we tested whether inhibition of AhR by α-NF (10 μM) would modulate the expression and function of BCL-2 family proteins. For this purpose, several independent experiments were performed as follows:

3.4.1. Effects of AhR inhibition on the mRNA and protein levels of BCL-2 family

To examine the effect of AhR inhibition on BCL-2 family of proteins, MDA-MB-231 cells were treated with α-NF 10 μM, thereafter the mRNA and protein levels of AhR, BCl-2, BCL-xL, BIM, BID, MCL-1, and BAX were determined. Figure 16A shows that treatment of MDA-MB-231 cells with α-NF resulted in approximately 15% decrease in BCL-2 mRNA levels whereas mRNA levels of BAX remained unchanged. Figure 16B shows that the inhibition of AhR by α-NF resulted in approximately 31% decrease in protein levels of AhR. This inhibition of AhR resulted in a proportional reduction in protein levels of BCL-2, BCL-xL, MCL-1, BID and BIM by approximately 25%, 9%, 27%, 22% and 25% respectively whereas, the protein levels of pro-apoptotic BAX remained unchanged (Fig. 16B).

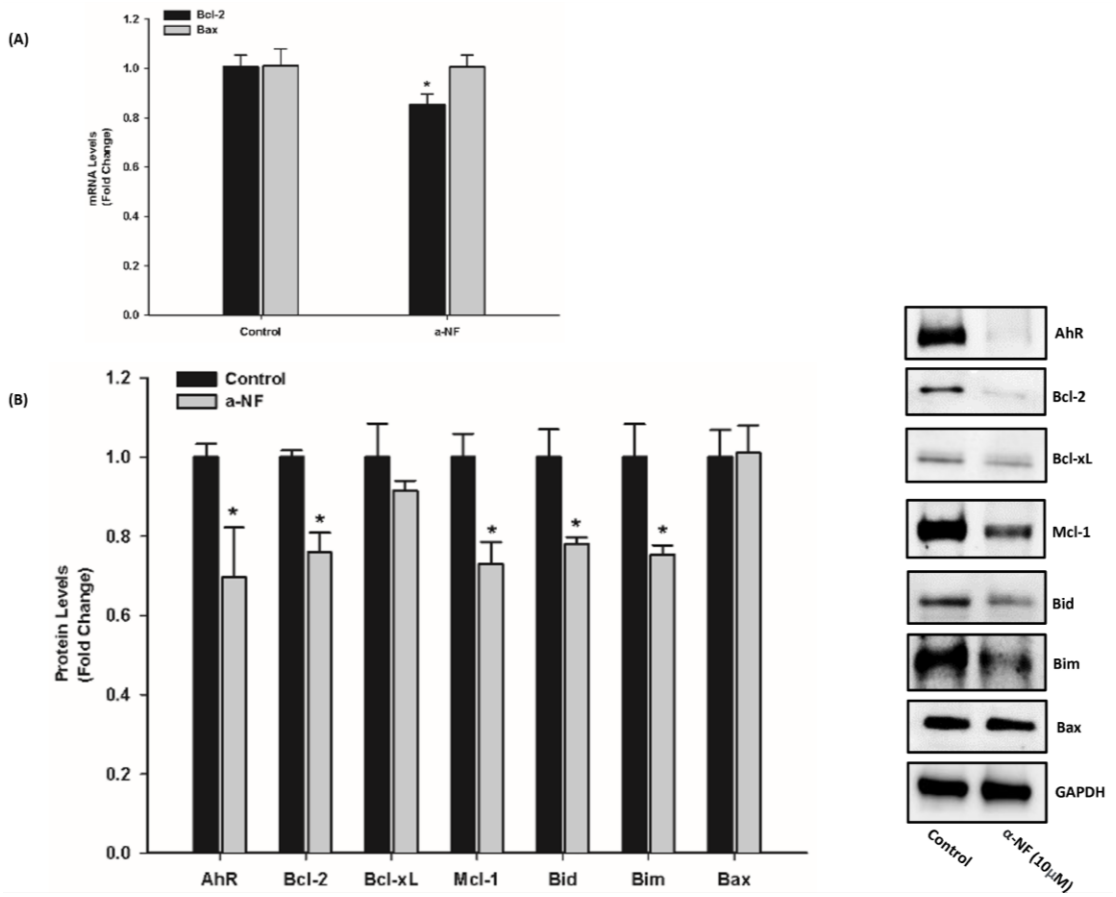


Figure 16. Effects of AhR inhibition on BCL-2 family proteins. *MDA-MB-231 cells were treated for 24 h with 10 μM α-NF. The mRNA (A) and protein (B) expression levels of AhR, BCL-2, BCL-xL, MCL-1, BID, BIM and BAX were determined by RT-PCR and Western blot respectively and normalized to GAPDH or β-ACTIN housekeeping gene. Each experiment was conducted in duplicate and the values represent mean of percentage ± SEM (n =6). *p<0.05 compared to control using t-test.*

3.4.2. Effects of AhR inhibition on cellular contents and localization of AhR and BCL-2 protein

To further confirm that inhibition of AhR by α-NF decreases BCL-2 in MDA-MB-231, we measured the cellular content and localization of both genes using IF analysis. As shown in Fig. 17A, α-NF markedly decreased the cellular content and localization of AhR as compared to control. This reduction in AhR was associated with a reduction in the cellular content and localization of BCL-2 protein (Fig. 17B).

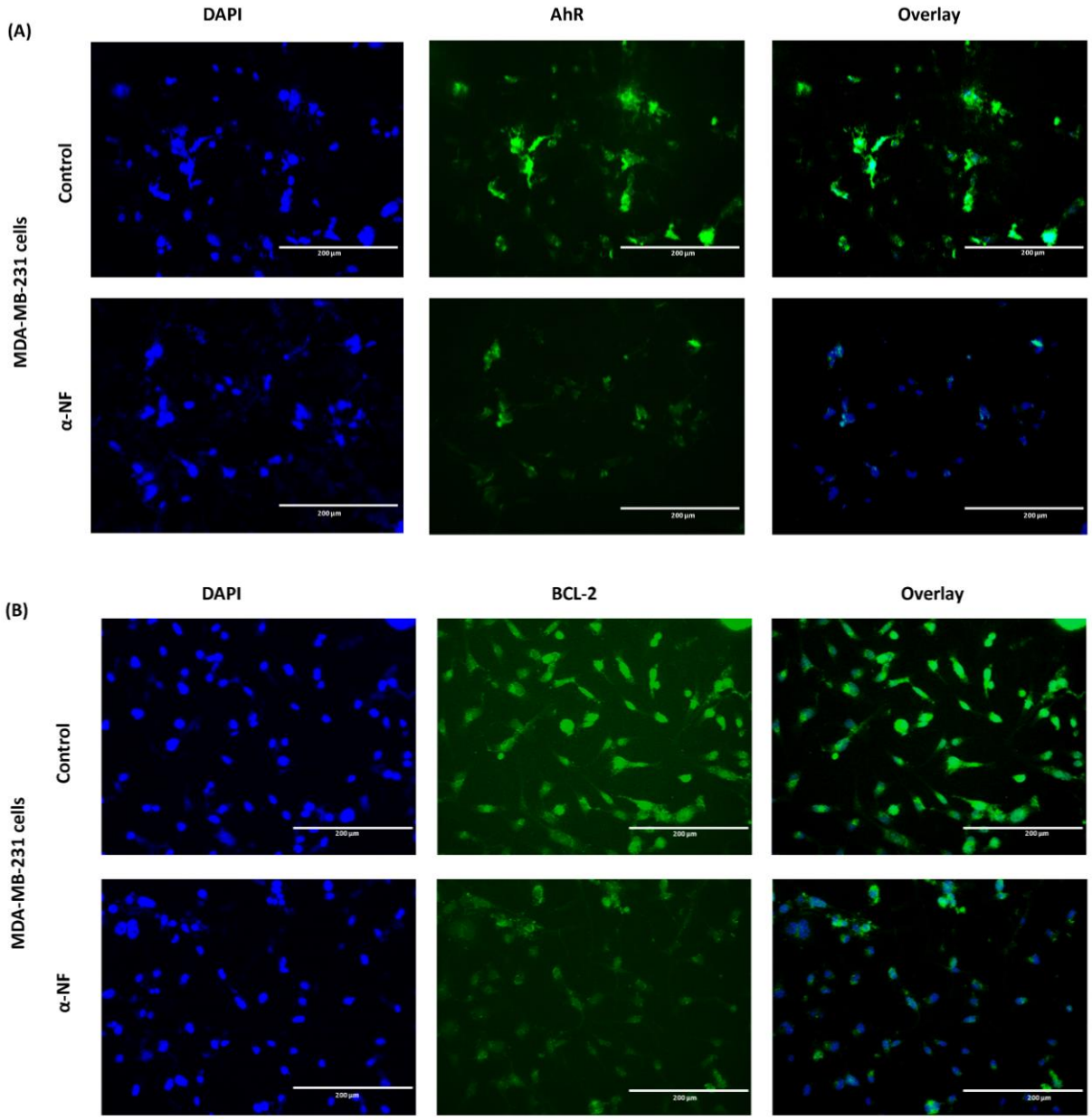


Figure 17. Cellular localization and expression of AhR and BCL-2 in α-NF treated vs. control group. *MDA-MB-231 cells were treated with 10 μM α-NF and stained with primary antibodies against AhR (A) and BCL-2 (B) (green) followed by fluorescent secondary antibodies and DAPI (blue). Thereafter, the AhR and BCL-2 proteins expression was determined by IF analysis. Each treated sample was stained in triplicate for each antibody.*

3.4.3. Effects of AhR inhibition on BCL-2 activation and inactivation, apoptosis and cell death

To further examine whether the inhibitory effect of AhR inhibitor, α-NF on BCL-2 family proteins is associated with reduced drug chemoresistance and cell proliferation, a set of different functional assays using Muse Cell Analyzer were conducted to examine the levels of BCL-2 activity, apoptosis, and cell viability/proliferation as follows:

3.4.3.1. Effects of AhR inhibition on BCL-2 activation

Figure 18 shows that the treatment of MDA-MB-231 cells with α-NF resulted in approximately 8% reduction in activated (phosphorylated) BCL-2 cells compared to control whereas the percentage of BCL-2 inactivated (dephosphorylated) cells increased approximately 7% in the treatment group compared to the control group.

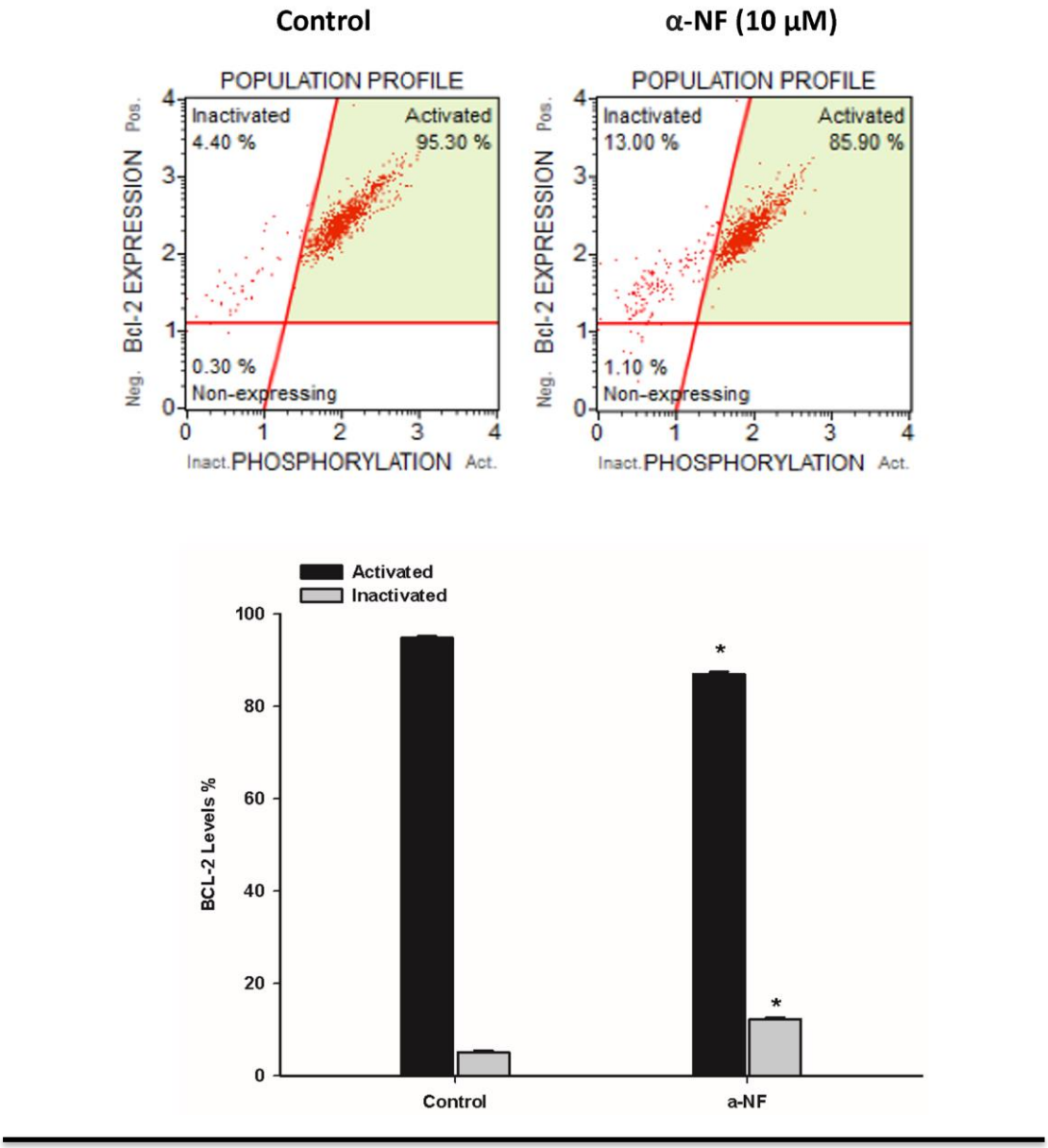


Figure 18. Effect of AhR inhibition on BCL-2. *MDA-MB-231 cells were treated for 24 h with 10 µM α-NF. Thereafter, the percentage of cells expressing activated (p-BCL-2) and inactivated BCL-2 were determined using Muse® BCL-2 activation dual detection kit. The values are presented as mean ± SEM (n = 3). *p<0.05 compared to control using t-test.*

3.4.3.2. Effects of AhR inhibition on apoptosis

To further investigate that the AhR inhibition and inactivation of BCL-2 pathways are associated with reduced chemoresistance of MDA-MB-231 cells, we measured the percentage of cell undergoing apoptosis/necrosis. Figure 19 shows that the treatment of MDA-MB-231 cells with α-NF resulted in a significant decrease in the percentage of

healthy cells by 55% and a marked increase in the percentage of early and late apoptotic cells by approximately 3-fold as compared to control.

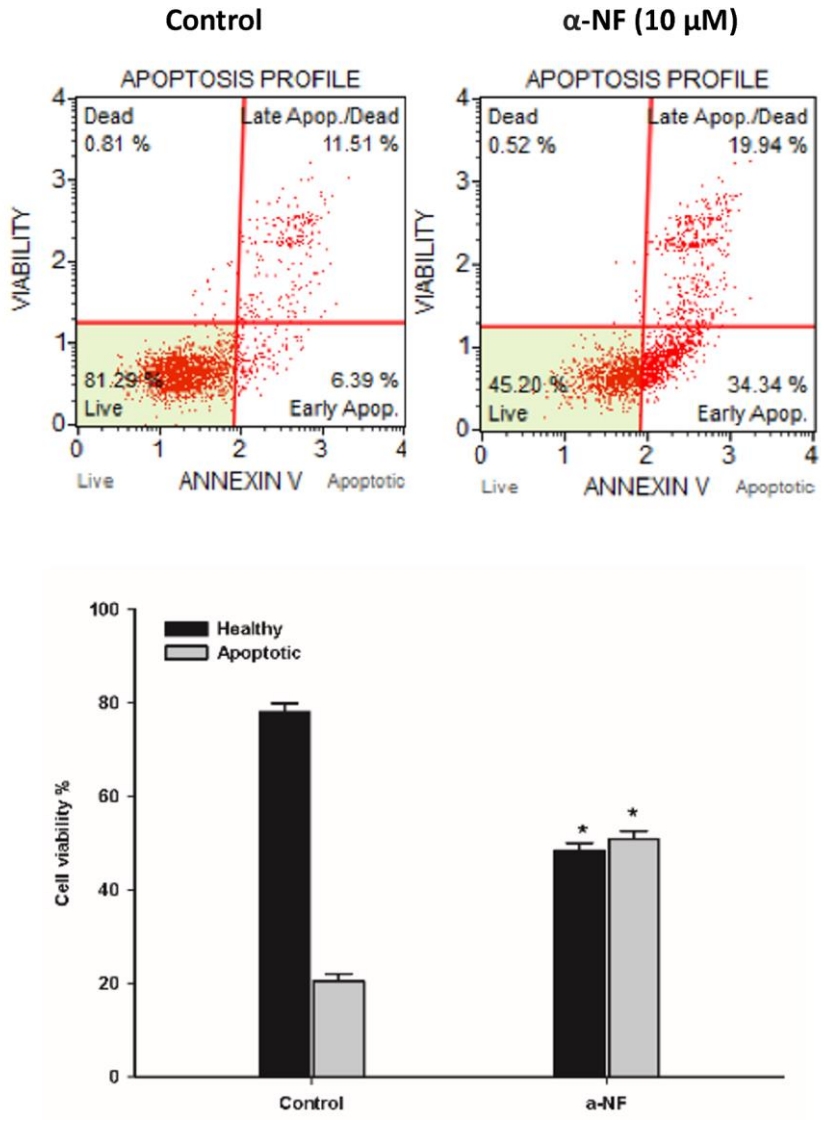


Figure 19. Effect of AhR inhibition on apoptosis. *MDA-MB-231 cells were treated for 24 h with 10 µM α-NF. Thereafter, the percentage of cells undergoing apoptosis was determined using Muse® Annexin V & Dead Cell Kit. The values are presented as mean ± SEM (n = 3). *p<0.05 compared to control using t-test.*

3.4.3.3. Effects of AhR inhibition on cell viability

We further investigated whether AhR inhibition in MDA-MB-231 cells also reduces cell viability and eventually leads to cell death. To test this hypothesis, we determined

the cell viability using Muse® Count & Viability Kit and LIVE/DEAD™ Viability/Cytotoxicity Kit. Figure 20A shows that α-NF treatment caused approximately 2.5-fold increase in the cell death of MDA-MB-231 cancer cells. Moreover, live and dead cells were also visualized through fluorescent microscope. Figure 20B shows that AhR inhibition in MDA-MB-231 cells also increased dead cells which is evidenced by increased red-fluorescence and reduced live cells (green-fluorescence).

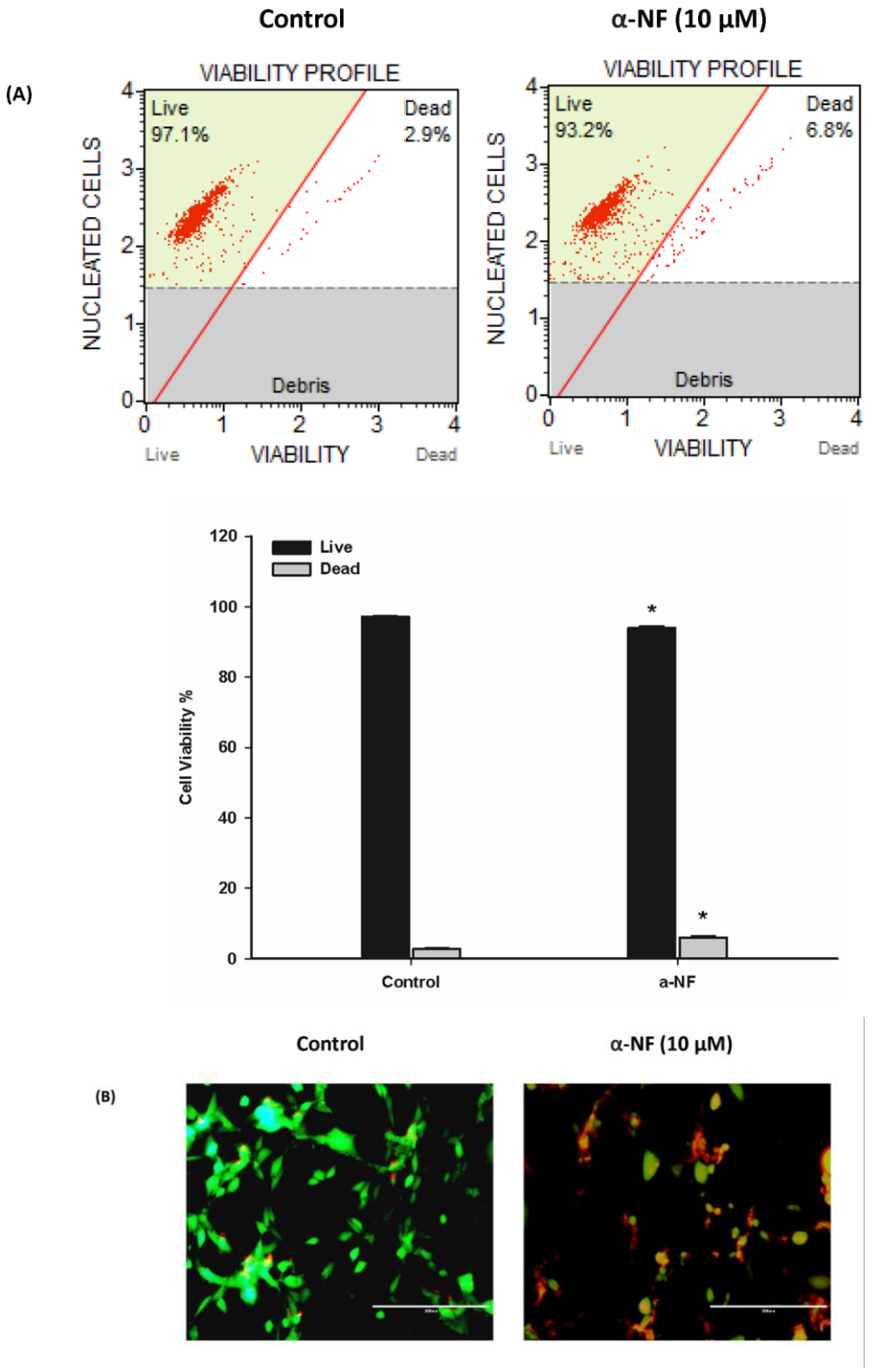


Figure 20. Effect of AhR inhibition on cell viability. *MDA-MB-231 cells were treated for 24 h with 10 µM α-NF. Thereafter, the percentage of Live and dead cells were determined using Muse® Count & Viability Kit (A) and the loss of plasma membrane integrity and cell death was also determined through LIVE/DEAD™ Viability/Cytotoxicity Kit (B). The values represent mean ± SEM (n = 3). *p<0.05 compared to control using t-test.*

3.5. Effects of AhR inhibition by α-NF on BCL-2 family of proteins in CSCs

The inhibition of AhR and its resulting effect on BCL-2 family prompted us to determine the effect of AhR inhibition on BCL-2 family proteins in CSCs. Therefore, a set of different experiments were performed.

3.5.1. Effects of AhR inhibition on mRNA and protein levels of BCL-2 family

To examine the effect of AhR inhibition on BCL-2 family proteins, mammospheres (CSCs) were treated with α-NF 10 µM for 72 h, thereafter the mRNA and protein levels of AhR, CYP1A1, CYP1B1, anti-apoptotic and pro-apoptotic members of BCL-2 family were determined by RT-PCR and Western blot analyses, respectively. As shown in figure 21A, treatment of CSCs with α-NF resulted in a significant decrease in AhR, CYP1A1 and CYP1AB1 mRNA levels by approximately 24%, 52%, and 55%, respectively. Importantly, this inhibition in AhR/CYP1 pathway was associated with a proportional reduction in the mRNA expression of BCL-2, BCL-xL, and BAX by 16%, 10% and 17%, respectively. Furthermore, the effects of AhR inhibitor α-NF were also confirmed at the protein level. Figure 21B shows that the α-NF treatment of CSCs resulted in decrease in AhR, BCL-2, BCL-xL and MCL-1 protein levels by 35%, 70%, 40% and 18% respectively while the protein levels of BIM and BAX were increased by 1.2-fold and 1.3-fold respectively.

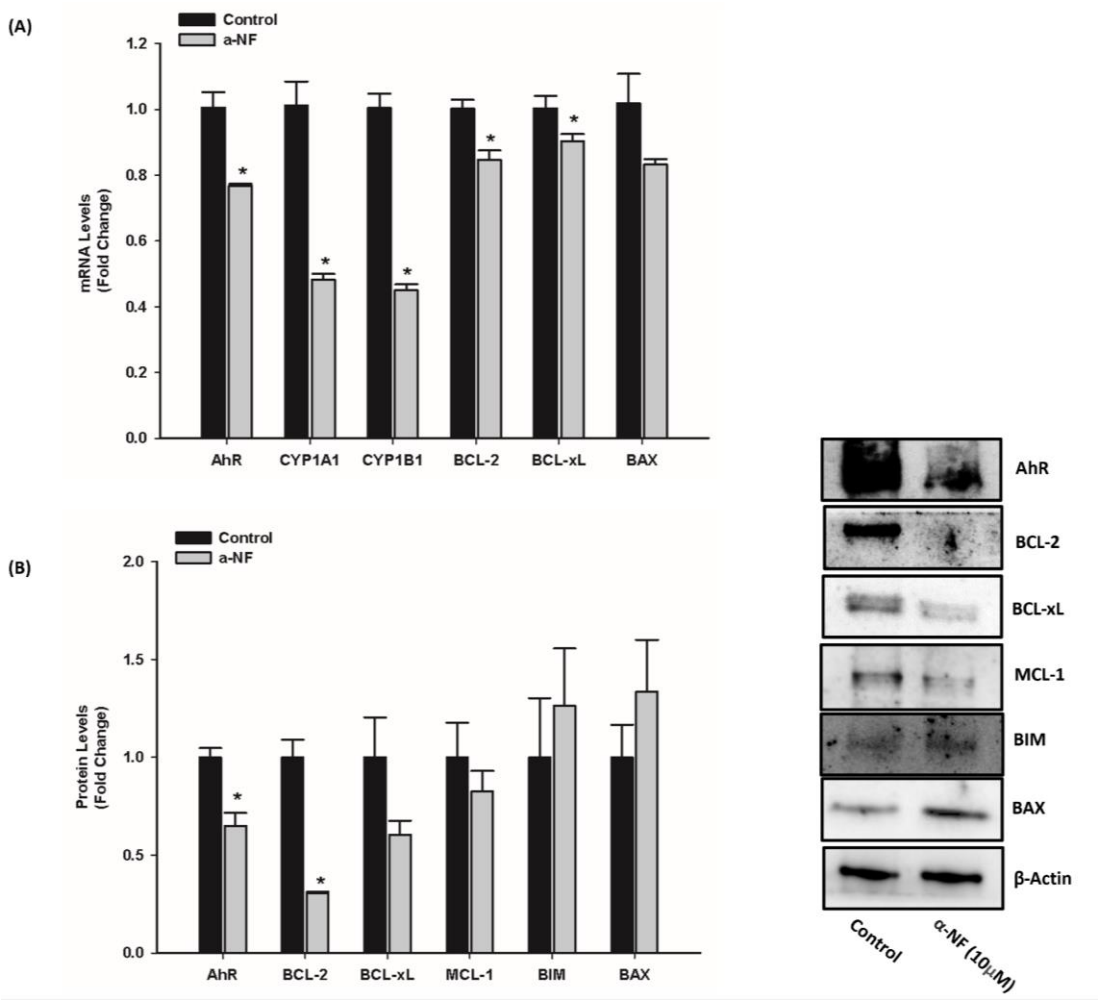


Figure 21. Effects of AhR inhibition on BCL-2 family proteins in CSCs. *CSCs were treated for 72 h with 10 μM α-NF. The mRNA (A) and protein (B) expression levels of AhR, CYP1A1, CYP1B1, BCL-2, BCL-xL, BIM and BAX were determined by RT-PCR and Western blot respectively and normalized to GAPDH or β-ACTIN housekeeping gene. Each experiment was performed in duplicate and the values represent mean ± SEM (n = 6). *p<0.05 compared to control using t-test.*

3.5.2. Effects of AhR inhibition on cellular contents and localization of AhR and BCL-2 protein

To further confirm the inhibitory effects of AhR by α-NF on BCL-2 in CSCs, we measured the content and cellular localization of both genes using IF analysis. As shown in figure 22A, α-NF markedly decreased the cellular content and localization of AhR as compared to the control. This effect was associated with a proportional

reduction in the cellular content and localization of BCL-2 protein (Fig. 22B).

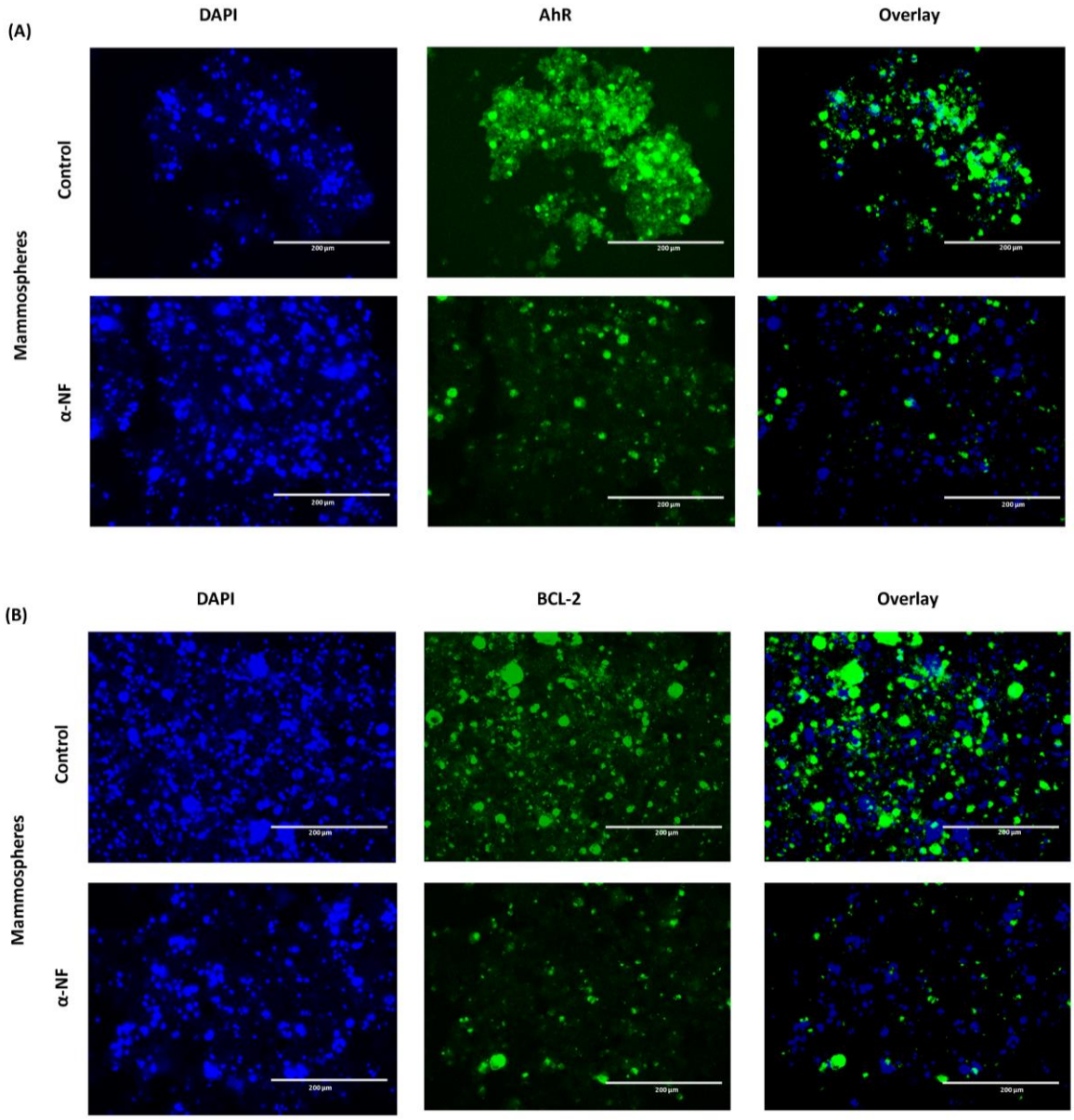


Figure 22. Cellular localization and expression of AhR and BCL-2 in α-NF treated vs. control group. *Mammospheres (CSCs) were treated with 10 µM α-NF for 72 h and stained with primary antibodies against AhR (A) and BCL-2 (B) (green) followed by fluorescent secondary antibodies and DAPI (blue). Thereafter, the AhR and BCL-2 proteins expression was determined by IF analysis. Each sample was stained in triplicate for each antibody.*

3.6. Effects of BCL-2 inhibition on caspase activation, DNA damage, apoptosis and cell death in MDA-MB-231 cells

The main purpose of this study was to further explore the crosstalk and interaction of AhR and BCL-2. For this purpose, we tested the effects of BCL-2 inhibition on AhR/CYP1A pathway and its impact on cancer proliferation and apoptosis. Therefore, we evaluated this through a set of different experiments using muse cell analysis.

3.6.1. Effects of BCL-2 inhibition by VCX on BCL-2 activation

BCL-2 levels were examined using Muse® Bcl-2 activation dual detection kit and RT-PCR. Figure 23A&B shows that untreated MDA-MB-231 cells exhibited 89% activation (phosphorylation) of BCL-2 and only 10% cells were expressing inactive (total) BCL-2. Treatment of MDA-MB-231 cells with VCX resulted in a significant decrease (35%) in BCL-2 activation (p-BCL-2) and a significant increase (4-fold) in BCL-2 inactivation (dephosphorylated). Similarly, the mRNA levels of BCL-2 also show a significant decrease (47%) upon treatment with VCX (Fig. 23C).

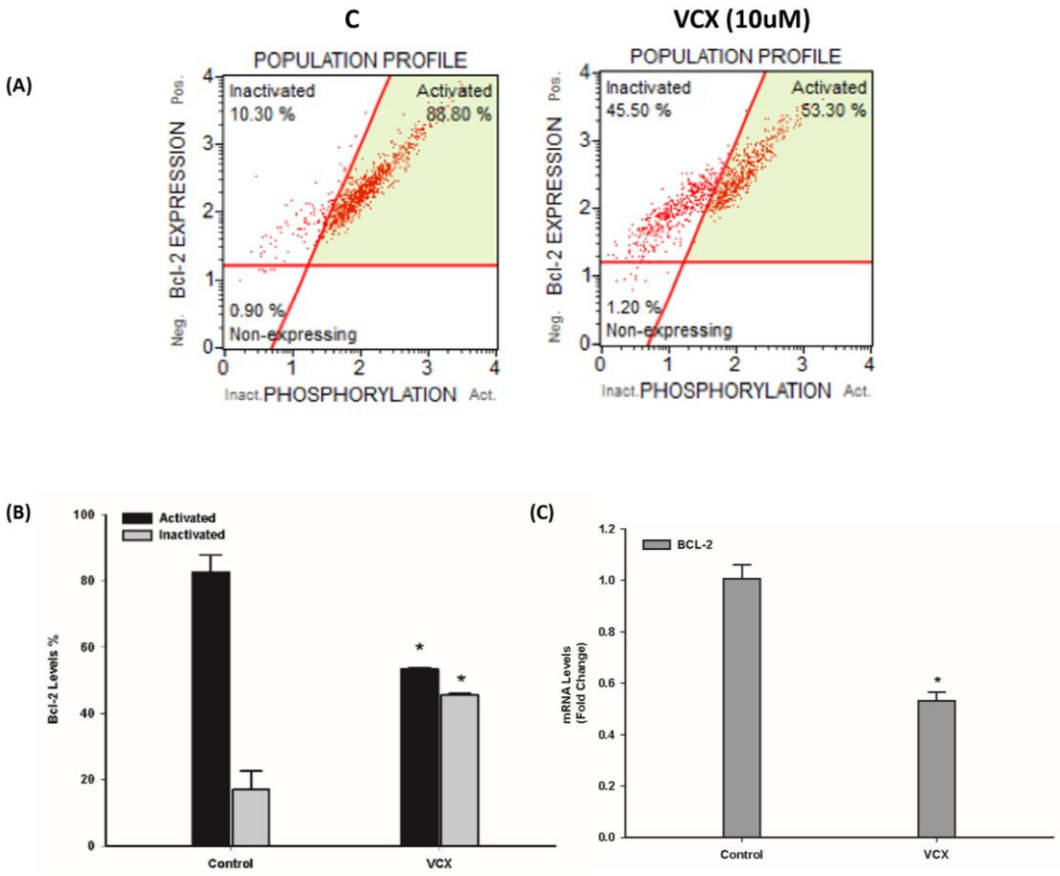


Figure 23. Effect of VCX on BCL-2 inhibition. *MDA-MB-231 cells were treated for 24 h with 10 µM VCX. Thereafter, the percentage of cells having activated and inactivated BCL-2 were determined using Muse® BCL-2 activation dual detection Kit (A, B) and percent decrease of BCL-2 mRNA was determined using RT-PCR. The values represent mean ± SEM (n = 3). *p<0.05 compared to control using t-test.*

3.6.2. Effects of BCL-2 inhibition on apoptosis and cell death

To further explore the effect of VCX on apoptosis and cell death, we conducted independent experiments using Muse® Annexin V & dead cell kit, RT-PCR, Muse® count & viability kit and LIVE/DEAD™ Viability/Cytotoxicity Kit. Figure 24A&B shows that VCX treatment decreases the healthy cell population by 40% whereas it increases the apoptotic cells by approximately 3-fold. This was associated with approximately 3-fold increase in the mRNA levels of BAX (Fig. 24C), increase in the dead cells by 3.4-fold (red-fluorescence) and decrease in living cell population by approximately 9% (green-fluorescence) (Fig. 24D & E).

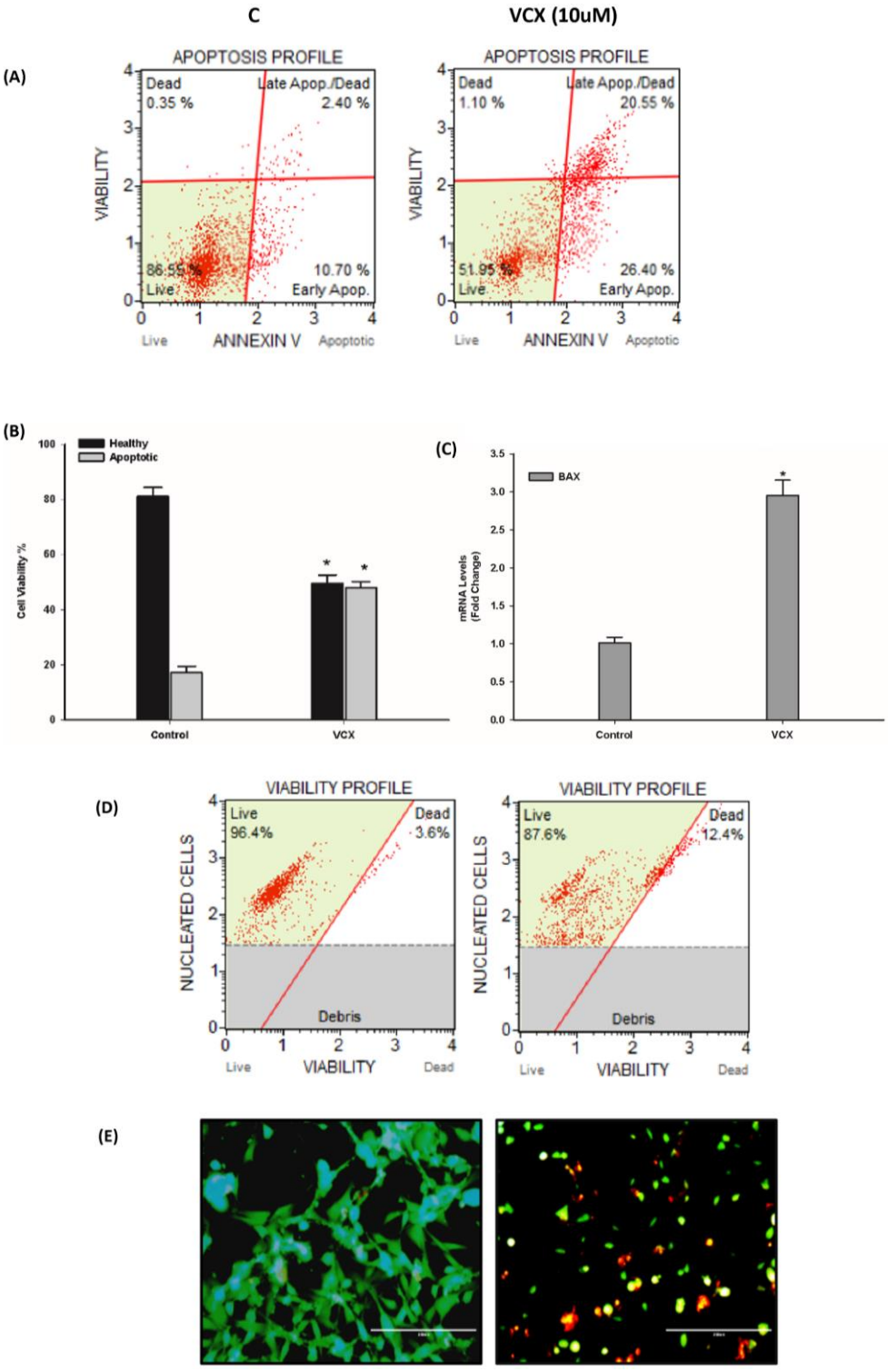


Figure 24. Effect of BCL-2 inhibition on apoptosis and cell death. *MDA-MB-231 cells were treated for 24 h with 10 µM VCX. Thereafter, the percentage of cells undergoing apoptosis was determined using Muse® Annexin V & dead cell kit (A, B) and expression of BAX was determined through RT-PCR (C). Live and death cells were determined using Muse® count & viability kit and LIVE/DEAD™ Viability/Cytotoxicity Kit (D, E).. The values represent mean ± SEM (n = 3). *p<0.05 compared to control using t-test.*

3.6.3. Effects of BCL-2 inhibition on caspase activation

To investigate that if VCX, in addition to its inhibitory effect on BCL-2, could cause caspase activation in MDA-MB-231 cells, Muse® MultiCaspase Kit was used. Figure 25 shows that VCX treatment caused a 50% decrease in the percentage of healthy cell population, whereas increased the caspase activity of MDA-MB-231 cells by 3.5-fold.

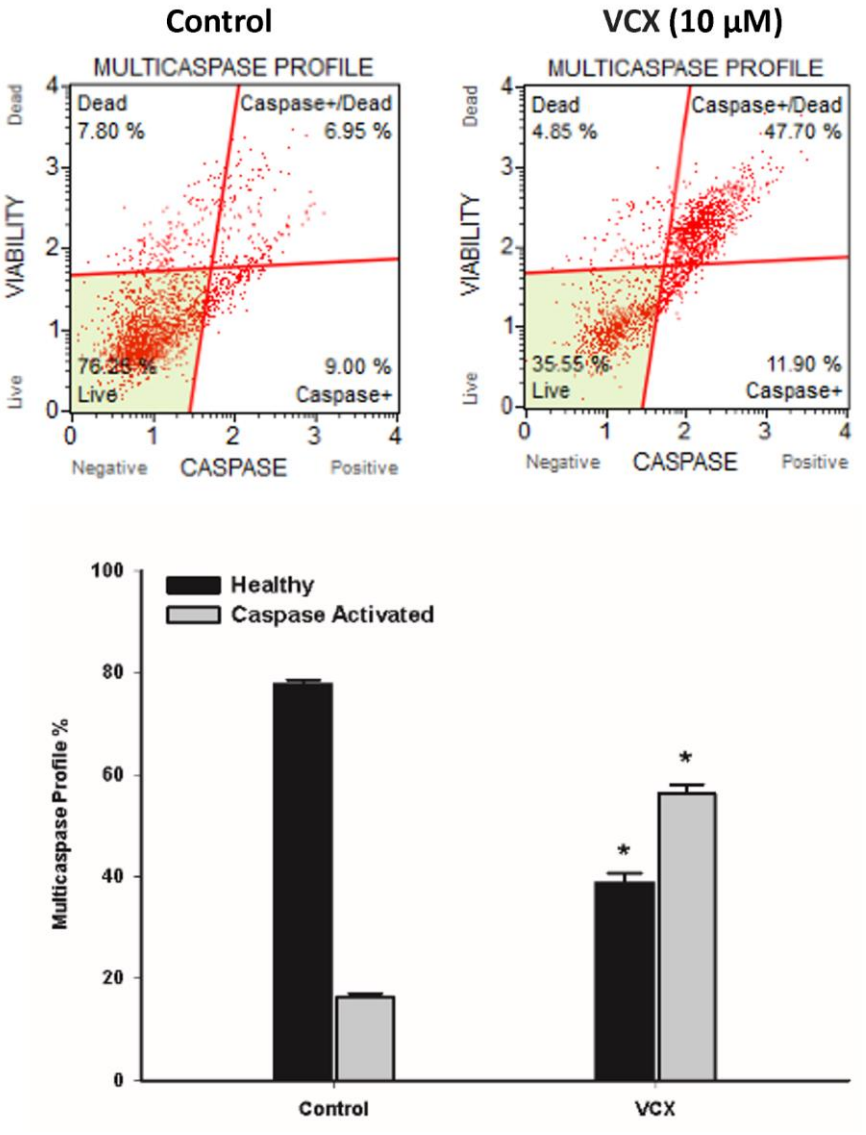


Figure 25. Effect of BCL-2 inhibition on multicaspase. *MDA-MB-231 cells were treated for 24 h with 10 µM VCX. Thereafter, the percentage of caspase positive cells was determined using Muse® MultiCaspase Kit. The values represent mean ± SEM (n = 3). *p<0.05 compared to control using t-test.*

3.6.4. Effects of BCL-2 inhibition on DNA damage

We further investigated if VCX causes any damage to the DNA in MDA-MB-231 cells. To evaluate the DNA damage in the cells, Muse® Multi-color DNA damage kit was used. Figure 26 shows that VCX treatment caused a 5% decrease in the percentage of healthy cell population compared to the control. Whereas, caused a significant (1.5-fold) increase in the DNA damage compared to untreated cells.

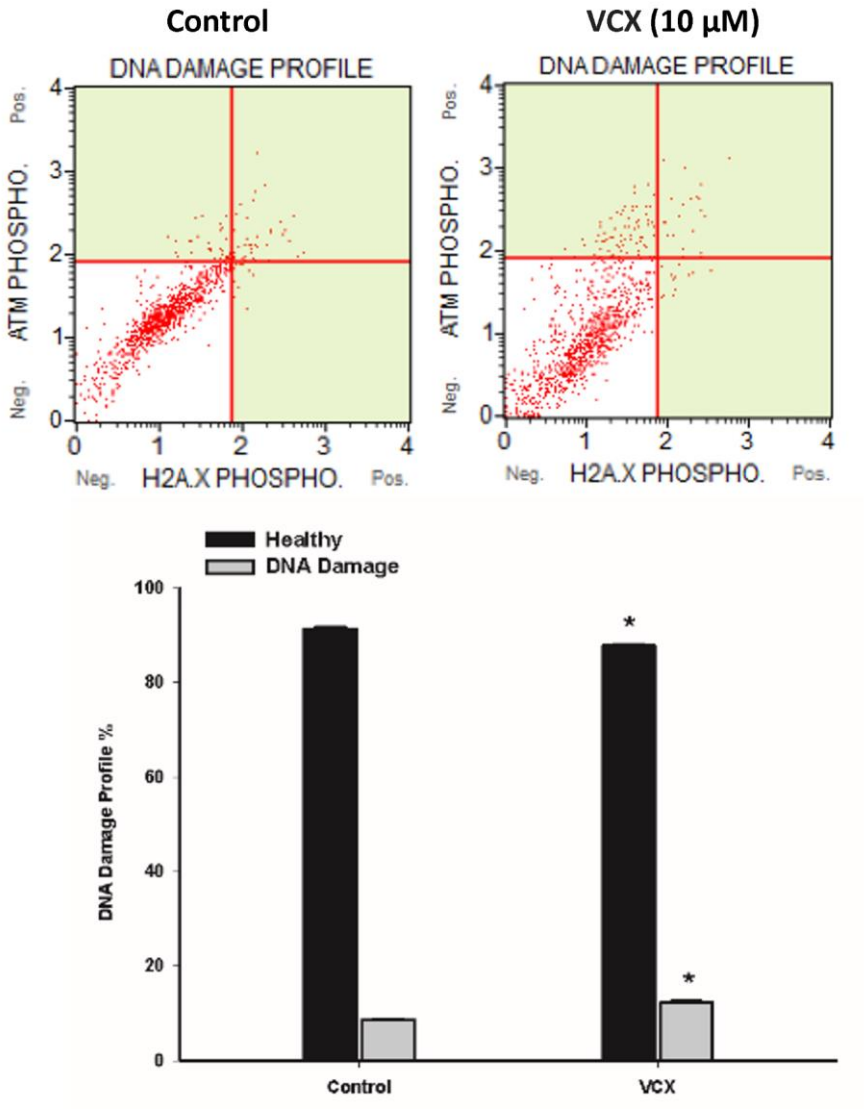


Figure 26. Effect of BCL-2 inhibition on DNA damage. *MDA-MB-231 cells were treated for 24 h with 10 µM VCX. Thereafter, the percentage of cells having damaged DNA were determined using Muse® Multi-color DNA damage Kit. The values represent mean ± SEM (n = 3). *p<0.05 compared to control using t-test.*

3.7.Effects of BCL-2 inhibition on mRNA levels of AhR/CYP1A in CSCs

To further explore the crosstalk between AhR and BCL-2 pathways in CSCs, we considered that what will be the effect of BCL-2 inhibition on CYP1A1 and CYP1B1. To address this question, mammospheres (CSCs) were treated with VCX 10 μM for 72 h, thereafter the mRNA levels of AhR, CYP1A1, CYP1B1, BCL-2, BCL-xL and BAX were determined by RT-PCR. Figure 27 shows that the treatment with VCX resulted in a significant reduction in mRNA levels of BCL-2 by 16%, while did not significantly affect BCL-xL or BAX levels. Importantly, inhibition of BCL-2 by VCX caused a significant and proportional decrease in the mRNA levels of AhR, CYP1A1, and CYP1B1 by approximately 20%, 29%, 20% respectively.

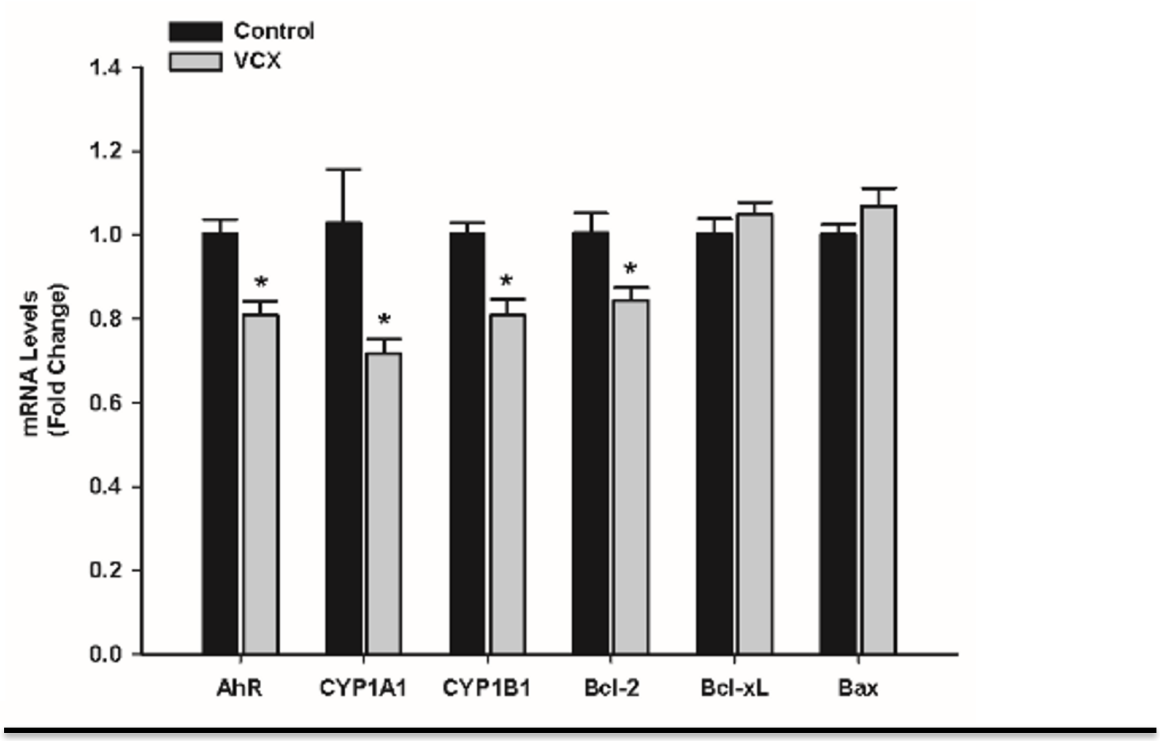


Figure 27. Effects of BCL-2 inhibition on mRNA levels of AhR/CYP1A in CSCs. *CSCs were treated for 72 h with 10 μM VCX. The mRNA expression levels of AhR, CYP1A1, CYP1B1, BCL-2, BCL-xL, and BAX were determined by RT-PCR and normalized to β-ACTIN housekeeping gene. Duplicate reactions were performed for each experiment and the values represent mean of percentage ± SEM (n = 6). *p<0.05 compared to control using t-test.*

3.7.AhR and BCL-2 protein expression in human BC tissues

To further determine the role and correlation of AhR and BCL-2, tissues from 72 different BC patients and 3 normal breast tissues were characterized for relative protein expression of AhR and BCL-2 (Fig. 28). The expression of AhR and BCL-2 was assessed through IHC, in tissue microarrays containing BC tissues from 75 different patients. Table 6 describes patient characteristics.

Table 6. Patients and tumor characteristics

Characteristics	Groups	Number of patients
Types	Control patient Cancer patients	3 (4%) 72 (96%)
Age	≤50 >50	44 (58.6%) 31 (41.3%)
Estrogen Receptor Status	ER-positive ER-negative Not tested	32 (49.2%) 33 (50.8%) 4
Progesterone Receptor Status	PR-positive PR-negative Not tested	38 (58.5%) 27 (41.5%) 4
HER2 Expression Status	HER2-positive HER2-negative Not tested	21 (31.8%) 45 (68.1%) 3
TNBC	TNBC-Positive TNBC-Negative Not tested	12 (18.4%) 53 (81.5%) 4
T-Stage	T0 T1 T2 T3 T4 Not tested	7 (10.44%) 3 (4.47%) 36 (53.7%) 13 (19.4%) 8 (11.9%) 2
N-Stage	N0 N1 N2 Not tested	43 (64.1%) 11 (16.4%) 13 (19.4%) 2
Metastasis	No Yes	69 0
Grade of Tumor	Grade I Grade II Grade III Not tested	4 (6.8%) 39 (67.2%) 15 (25.8%) 11
Ki67 Status	≤40% >40% not tested	37 (63.7%) 21 (36.2%) 11

3.7.1. Association of AhR and BCL-2 with breast cancer subtypes, tumor grade and Ki67 status

Using IHC, a stronger immunostaining of AhR and BCL-2 proteins was detected in BC patients than the normal patients. The results revealed that AhR and BCL-2 protein

expressions were positively correlated. Table 7 describes the association between AhR and BCL-2 and currently established prognostic markers. They were scored based on their staining; 0 for no staining, 1; <10% stain, 2; 11-25 %, 3; 26-50%, 4; 51-75%, 5 > 75%. The scoring was used to categories them in to high positive and low positive. Our results showed that the expression of AhR and BCL-2 in cancer patients is almost the double as compared to healthy subject. Table 7 shows that percentage of BC patients exhibited high-positive expression of AhR and BCL-2. For example, 87% of ER-positive cancer patients expressed high level of both AhR and BCL-2 compared to only 25% of the healthy control tissues. Similar pattern of expression was also observed with all molecular subtypes. For TNBC patients, we also report here that the levels of AhR and BCL-2 in high-positive level is higher than in the control. Importantly, the high expression level of AhR and BCL-2 in low-positive samples in TNBC might indicate a high expression of their basal levels.

Table 8 shows that the expression of AhR and BCL-2 proteins is higher in all the BC tumor grades compared to the expression in the control breast tissues. The highest expression of AhR as well as BCL-2 was observed in cancer grade I.

The direct correlation of AhR and BCL-2 expression with the Ki67 status was not observed, however all tumor tissues showed a high expression of AhR and BCL-2 (Table 9).

Table 7. Association between AhR and BCL-2 and BC subtypes

	Groups	**Classification**	**Percentage of patients**
AhR Status	Control	High positive Low positive	2 (25%) 4 (75%)
	ER-positive	High positive Low positive	28 (87.5%) 4 (12.5 %)
	PR-positive	High positive Low positive	33 (86.85%) 6 (15.78%)
	HER2-positive	High positive Low positive	16 (76.2 %) 5 (23.8%)
	TNBC	High positive Low positive	7 (58.33%) 5 (41.66%)
BCL-2 Status	Control	High positive Low positive	40 (40%) 60 (60%)
	ER-positive	High positive Low positive	28 (87.5%) 4 (12.5 %)
	PR-positive	High positive Low positive	32 (84.21%) 6 (15.78%)
	HER2-positive	High positive Low positive	17 (80.95%) 4 (19.05%)
	TNBC	High positive Low positive	8 (66.67%) 4 (33.33%)

Table 8. Association of AhR and BCL-2 with tumor grade

Tumor Grade	**AhR**	**BCL-2**
Normal breast tissue	1	1
Breast cancer grade I	2.3	1.4
Breast cancer grade II	2.2	1.3
Breast cancer grade III	1.6	1

Table 9. Association of AhR and BCL-2 with Ki67 expression

Ki67	**AhR**	**BCL-2**
Normal breast tissue	1	1
Low expression	2.16	2.46
Medium expression	2.3	2.42
High expression	2.21	2.56
Highest expression	1.89	2

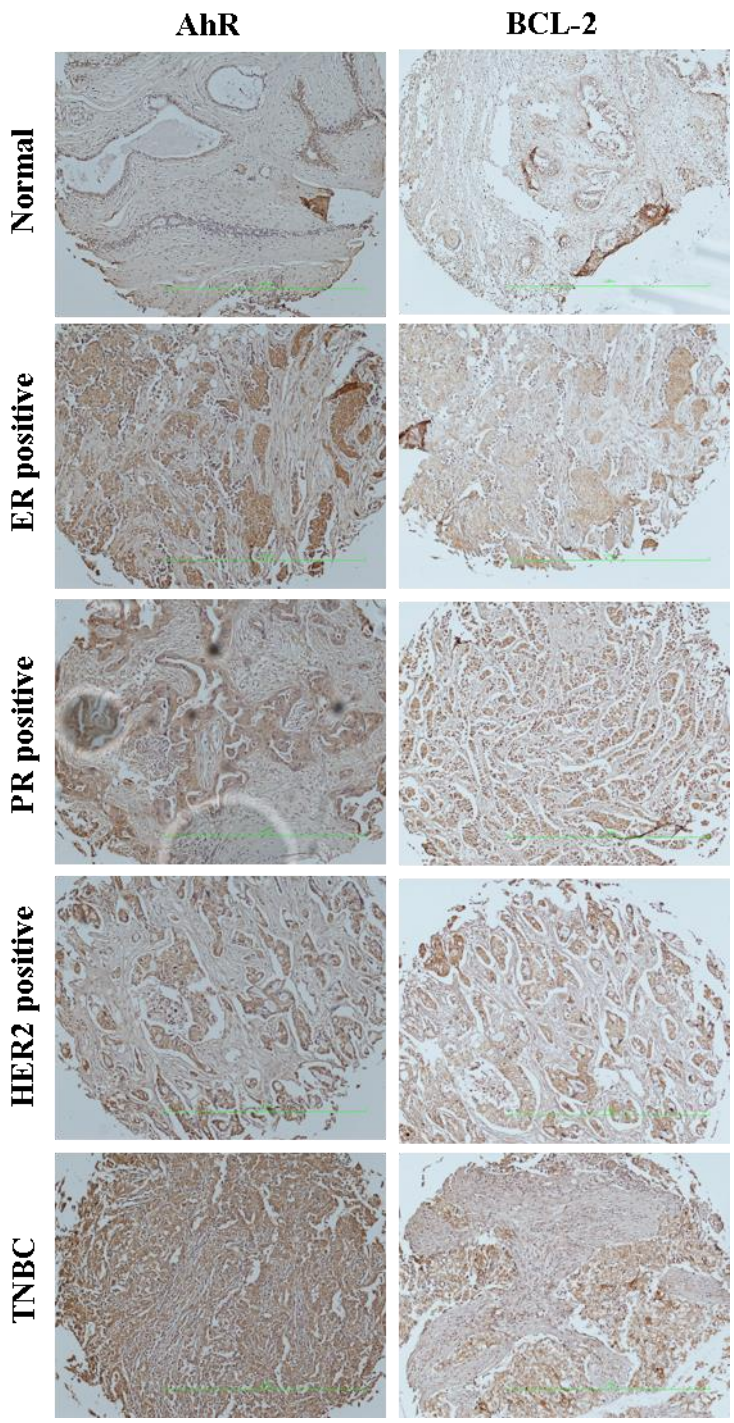


Figure 28. Immunostaining of human breast tissues. *Tissue microarrays containing tissues from 75 different patients were stained with AhR and BCL-2 (brown) antibodies followed by secondary antibody. Thereafter, the relative protein expresison of AhR and BCL-2 was determined and scored. Each tissue sample was stained in triplicate for each antibody.*

CHAPTER 4: DISCUSSION

The present study provides the first evidence that AhR/CYP1A pathway is correlated with the BCL-2 family proteins for the development of CSCs of TNBC. This correlation is supported by the following observations. First the constitutive expression of AhR, CYP1B1 and anti-apoptotic BCL-2 family proteins is markedly higher in CSCs compared to differentiating cancer cells (non-CSCs) and normal breast epithelial cells (MCF-10A). Second, activation of AhR/CYP1A by DMBA increased mRNA expression of BCL-2 in non-CSCs and CSCs, whereas inactivation of AhR signaling pathway by α-NF significantly reduced the expression and cellular localization of anti-apoptotic BCL-2 proteins in non-CSCs and CSCs. Third, the inactivation of AhR and the consequent inhibition of BCL-2 in non-CSCs resulted in induction of apoptosis and eventually cell death. Fourth, the inhibition of BCL-2 by VCX in CSCs was associated with significant reduction in the expression of AhR and its regulated genes.

Studies have shown that exposure to environmental pollutants results in activation of AhR which has been studied to play a role in numerous cancers [85]. Moreover, high AhR activation has been observed in choriocarcinoma spheroid which causes tumorigenesis through the conferring CSCs characteristics [84, 97]. In a previous work from our laboratory, we have reported that activation of AhR/CYP1A1 pathway in human breast MCF-7 cells increased the spheroid formation and CSCs features which was associated with increased the chemoresistance, whereas genetic and chemical inhibition of the AhR/CYP1A1 pathway significantly increased apoptosis [92]. The involvement of AhR in apoptosis is evidenced by the fact that the knockdown of AhR in non-immunized mice resulted in markedly reduced expression of BCL-2 and upregulated expression of Bax [224]. These studies suggest a possible interaction between AhR and BCL-2 in cancer cells, however there is not enough information

about this interaction in CSCs and the mechanisms involved. In the light of information described above, we hypothesize that AhR/CYP1A signaling pathway is correlated with BCL-2 family proteins for the development of CSCs of TNBC. To test our hypothesis, we a) examined the functional relevance of constitutive AhR, CYP1B1 and BCL-2 family expression in MCF-10A, MDA-MB-231 and CSCs, b) investigated the crosstalk between AhR and BCL-2 in MDA-MB-231 and CSCs and their impact on CSCs using chemical inducer of AhR, DMBA and inhibitors α-NF and VCX, c) Correlate the expression of AhR and BCL-2 proteins in human BC tissues with BC subtype and cancer grade/stage.

4.1. Characterization of CSCs of TNBC

Initially we cultured mamopsheres of MDA-MB-231 cells in suspension conditions for 20 days and to confirm the presence of CSCs in mammopshere, we determined their features through flow cytometry. The presence of ALDH+ cells, $CD44^{high}/CD24^{low}$ cells and SP were found to be higher in mamopsheres compared to differentiated cancer cells which indicated that our mammopshere culturing assay was a reliable method to enriched CSCs. This is consistent with the earlier findings of Zhang and colleagues who found a higher ratio of $CD44^{high}/CD24^{low}$ in mammosheres of MDA-MB-231 cells [225] and higher levels of ALDH+ and $CD44^{high}/CD24^{low}$ cells in BC are associated with metastasis, chemoresistance and high recurrence rate. Moreover, the xenotransplants from MDA-MB-231 cells also exhibit high tumorigenicity and metastasis [226]. In addition to ALDH+ and $CD44^{high}/CD24^{low}$, another characteristic of CSCs has also been studied that is based on their high expression of ATP-binding cassette transporters which efflux drugs out of the cells and thus confer multidrug resistance [227]. We observed increased SP in our mammopshre culture which is indicative of high efflux activity in these cells.

4.2. Basal Expression of AhR and BCL-2 Genes in CSCs

After characterizing CSCs features, we investigated the basal expression of AhR, CYP1B1 and BCL-2 family genes in three different cell types. At the constitutive level, three cell lines, MCF-10A, MDA-MB-231 and its corresponding mammosphere (CSCs) were used to explore the differential constitutive expression of AhR, CYP1B1 and anti-apoptotic and pro-apoptotic BCL-2 family genes. The results from set of these experiments demonstrated for the first time that basal expression of AhR and CYP1B1 were markedly higher in CSCs compared to MDA-MB-231 and MCF-10A, and this overexpression was associated with a proportional increase in the expression of the anti-apoptotic BCL-2 family proteins (BCL-2, BCL-xL and MCL-1). Whereas, the basal expression of pro-apoptotic BCL-2 proteins, BAX and BIM was reduced in CSCs, with the exception of BID which showed an increased expression. In an agreement with previous work, Colak et al., reported that colon cancer spheroids highly expressed anti-apoptotic BCL-2 proteins and they were also resistant to oxaliplatin which suggests that the apoptotic balance in chemoresistance cancer cells is shifted more towards anti-apoptotic proteins [228]. These results may indicate that modulation of BCL-2 proteins expression in CSCs could be a novel target therapy for chemoresistance property of these cells.

The involvement of AhR/CYP1 pathway in breast carcinogenesis, cancer initiation, and CSCs proliferation has been studied by Al-Dhfyan et al., who reported that activation of AhR/CYP1A1 pathways is associated with CSC development, proliferation, chemoresistance and self-renewal [92]. The increased expression of CYP1B1 in breast CSCs in the current study is aligned with the previous studies showed that CYP1B1 expression is associated with high grade tumor (grade III and IV) and increased chemoresistance of ovarian [229] and renal [230] cancers, suggesting that increased

CYP1B1 expression in our CSCs could mediate chemoresistance. One of the important mechanisms of chemoresistance is overexpression of anti-apoptotic protein family BCL-2 as illustrated in current results. BCL-2 has also been associated with the modulation of BC cell behavior and contributes to malignant phenotype of BC [231]. Another evidence that supports this hypothesis, is that high expression of anti-apoptotic BCL-2 proteins has been reported in CSCs of haematologic and solid tumors and this increased expression of these proteins reduces apoptosis and confer chemoresistance [232, 233]. However, the cross-talk between AhR and BCL-2 in modulating cancer CSCs development and chemoresistance is still unrevealed.

The expression of pro-apoptotic protein BID has been found to be higher in our mammospheres. BID has an active truncated form called truncated BID (tBID) which is generated from its precursor BID and is involved in intrinsic apoptotic pathway. Upon apoptotic signal, BID is cleaved and gives rise to tBID that translocates to mitochondria and results in release of cytochrome c which eventually leads to apoptosis through activation of caspase cascade [234, 235]. Therefore, the pro-apoptotic activity of BID is highly dependent on its cleavage [236]. The high expression of BID in our mammopsheres indicates that it has not been converted to its active form tBID which shows that the level of apoptosis in CSCs is lower than the MDA-MB-231 and MCF-10A. The CSCs exhibited lower constitutive expression of BIM. BIM is a pro-apoptotic protein and tumor suppressor in epithelial solid tumors. It promotes tumor suppression in nude mice upon treatment with paclitaxel [237]. Gargini and colleagues reported that loss of BIM in mamopshere is associated with increased mesenchymal phenotype and reduced epithelial phenotype indicating that the reduced expression of BIM confers metastatic features in mammospheres [238]. Moreover, they also reported that mammosphere formation in breast epithelial cell line MCF-10A is increased when the

expression of BIM is reduced. Together these findings demonstrate that loss of BIM expression plays an essential role in conferring aggressive characteristics to CSCs.

BAX is another pro-apoptotic protein that showed reduced expression in CSCs. This decreased expression of BAX can be associated to the findings of Sharifi et al., who found that the expression of BAX is reduced in MCF-7 paclitaxel resistant cell line which is indicative of decreased apoptosis in chemoresistant cells [239]. Moreover, BAX levels have also been found to be reduced in cisplatin resistant ovarian cancer cells [240]. These findings show that loss of BAX leads to drug resistance in cancer and overexpression of BAX sensitizes drug resistant cancer cells to chemotherapy [240]. In B-cell chronic lymphocytic leukemia, high BCL-2 and low BAX expression was associated with high resistance. Moreover, the cells that undergo apoptosis upon treatment with chlorambucil in B-cell lymphocytic leukemia had only marginal decrease in BCL-2 but BAX expression were found to be markedly lower which indicates that decreased expression of BAX is highly essential in inducing apoptosis in cancer [241].

4.3. AhR Pathway Controls Cancer Cell and CSC Survival Through Modulation of BCL-2 Family Genes

The exact effect and role of AhR on BCL-2 expression and the impact on CSCs development were investigated in MDA-MB-231 and CSCs by two approaches; a) AhR activation by using DMBA and b) inhibition using α-NF and its effect on BCL-2 family. The carcinogenicity and tumorigenicity of DMBA is attributed to its ability to induce CYP1A1/CYP1B1. In the present study, activation of AhR pathway by DMBA resulted in increased expression of proto-oncogenes of BCL-2 family. In that DMBA, not only increased AhR regulated genes CYP1A1 and CYP1B1 but also increased the expression of anti-apoptotic BCL-2 and BCL-xL. These results indicate that

AhR/CYP1A activation increases the cancer cell survival by increasing the expression of anti-apoptotic proteins. Currier and colleagues reported that environmental carcinogens such as DMBA leads to mammary carcinogenesis by disrupting apoptosis. DMBA is associated with the increased expression of cell cycle regulator genes c-myc and cyclinD1 and also activates Wnt signaling pathway in mammary tumors [242]. Studies have shown that Polychlorinated biphenyls (PCBs), that share chemical characteristics with dioxins, initiate carcinogenesis through activation of CYP enzymes (CYP2B1/2B2) in rat hepatocytes therefore reduce cell apoptosis [243]. Moreover, induction of CYP1A1 through PCBs is also involved in lowering apoptosis in human BC MCF-7 cells [244]. Bekki and co-workers reported that activation of AhR by TCDD reduced UV induced apoptosis in BC cell lines MCF-7 and MCF10AT1 whereas knockdown of AhR in BC cells using siRNA or treatment with an AhR antagonist, 3′methoxy-4′nitroflavone (MNF), resulted in UV-induced apoptosis. Similar results were reported by Vogel and colleagues that in human lymphoma cell lines, activation of AhR by TCDD reduced UV induced apoptosis and AhR antagonist, 3′-methoxy-4′-nitroflavone, prevented TCDD induced anti-apoptotic effect [245]. These findings demonstrate that AhR interacts with cancer cell's apoptotic pathway and its activation confers anti-apoptotic effects in BC cells [246].

In other words, AhR induces chemoresistance in BC through suppression of apoptosis therefore, inhibition of AhR through its antagonists can serve as an effective approach to sensitize BC cells to chemotherapeutics [246].

The AhR antagonist, α-NF. is a prototype flavone which directly binds AhR and decreases its transcriptional activation and affinity towards DNA by modulating its conformation and therefore, is considered as an antagonist of AhR [247-249]. α-NF has also been reported to induce apoptosis through activation of caspase 3 and 12 in mouse

hippocampal neuronal cells [248]. Our results indicate that inhibition of AhR activates intrinsic apoptotic pathway by downregulating the expression of anti-apoptotic proteins while keeping pro-apoptotic BAX unchanged. The inactivation of BCL-2 family proteins in MDA-MB-231 cells upon treatment with α-NF was confirmed at different molecular levels. In that, blocking of AhR/CYP1B1 significantly reduced the mRNA and protein expression levels of (BCL-2, BCL-xL and MCL-1) family proteins, decreased cellular localization and expression of BCL-2, and reduced its enzyme activity. In addition, inhibition of AhR pathway by α-NF did not significantly modulate the expression of pro-apoptotic BAX however, the expression of pro-apoptotic BID and BIM were reduced. These results indicate that AhR inhibition promotes cancer apoptosis cell death by decreasing the expression of anti-apoptotic genes. Our observations are in agreement with previous studies, in that Dubrovska et al., reported that preincubation of tamoxifen resistant MCF-7 cells with AhR antagonist and then subcutaneous injection of these cells in nude mice resulted in reduced tumor growth [103]. In addition, Xiao-Fei and colleagues reported that AhR knockdown in Gastric cancer cell lines MKN45 and SGC7901 resulted in increased apoptosis and reduced cell proliferation [250].

Perhaps the most interesting results are the effects of AhR inhibition on BCL-2 family in CSCs. To the best of our knowledge, we are the first to report that inhibition of AhR in CSCs using α -NF significantly reduced the expression of anti-apoptotic BCL-2 proteins without significantly affecting BAX expression. Failure to modulate BAX expression by AhR inhibition in CSCs could be explained by the findings of Zhang and co-workers, who reported that BAX plays an essential role in inducing apoptosis in HCT116 compared to other pro-apoptotic BCL-2 proteins. They reported that knockdown of BCL-xL and MCL-1 in wild type and triple knockout

(*Bid*$^{-/-}$*Bim*$^{-/-}$*Puma*$^{-/-}$) HCT116 cells induced increased apoptosis but this knockdown of BCL-xL and MCL-1 did not induce apoptosis in double knockout (*Bax*$^{-/-}$*Bak*$^{-/-}$*)* and Penta knockout (*Bid*$^{-/-}$*Bim*$^{-/-}$*Puma*$^{-/-}$ */Bax*$^{-/-}$*/Bak*$^{-/-)}$ cells. This shows that triple knockout cells exhibit BAX/BAK mediated apoptosis. These results indicate that the inactivation of anti-apoptotic BCL-xL and MCL-1 leads to activation of BAX/BAK which induces apoptosis in the absence of BID, BIM and PUMA. This shows that the activation of BAX/BAK plays an essential role in inducing apoptosis even in the absence of other po-apoptotic proteins. This indicates also that suppression of anti-apoptotic BCL-2 family proteins is sufficient to induce apoptosis through BAX activation in the absence of BID, BIM, BAK and PUMA [30]. The α-NF induced downregulation of BCL-2 in CSCs can also be explained by the findings of Yiangou, Maloof and Dubrovska et al., that AhR antagonist increases basic fibroblast growth factor-2 (FGF-2) in drug resistant MCF-7 cells which eventually decreases BCL-2 levels, induces apoptosis and reduces cancer progenitor population [103, 251, 252]. Collectively, these findings demonstrate carcinogenic role of AhR in BC and that chemoresistance in CSCs can be targeted through AhR and BCL-2 inhibition.

In CSCs, α-NF treatment also resulted in reduced expression of CYP1B1. Zhu and co-workers reported that inhibition of CYP1B1 through α-NF in drug resistant ovarian cancer cells A2780TS increased their sensitivity towards paclitaxel which indicates that CYP1B1 is a critical indicator of chemoresistance in cancer cells [229]. Moreover, the combination of α-NF and paclitaxel in drug resistant ovarian cancer cells A2780TS induced apoptosis [229] which is in alignment with our findings that inhibition of CYP1B1 caused inhibition of anti-apoptotic BCL-2 proteins in CSCs. These findings were also confirmed in xenograft mouse model of ovarian cancer in which A2780TS cells were injected. The treatment of these mice showed inhibition if ovarian tumor in

the presence of both α-NF and paclitaxel whereas the treatment with only paclitaxel did not significantly inhibit tumor formation. These findings confirm that CYP1B1 confer chemoresistance in ovarian cancer and inhibition of CYP1B1 sensitizes ovarian cancer cells and *in vivo* tumor to paclitaxel [229].

4.4. BCL-2 modulates the expression of AhR/CYP1A pathway

High expression of BCL-2 has been implicated in development of chemoresistant cells and these cells possess characteristics of CSCs. The inhibition of anti-apoptotic BCL-2 family has been suggested as a possible antineoplastic approach as different BCL-2 inhibitors have been tested for the treatment of various cancers. Hu and co-workers reported that Subotoclax, a pan-active BCL-2 family antagonist, reduced cell growth and induced apoptosis in chemoresistant BC cell lines. Moreover, inhibition of anti-apoptotic BCL-2 family proteins by subotoclax also resulted in reduced CSCs characteristics such as reduction of ALDH+ cells, $CD44^{high}/CD24^{low}$ subpopulation and also decreased sphere formation ability of the chemoresistance BC cells [164]. Furthermore, Song and co-workers reported that in gastro-oesophageal cancer, downregulation of BCL-2 through AT101 resulted in reduction of CSC markers, YAP1/SOX9. They indicate that BCL-2 inhibitor AT101 exhibits anti-CSCs properties as it reduces formation of tumor spheroids. Moreover, AT101 also improved clinical response in patients and improved overall survival [253]. This raises the question of whether BCL-2 pathway would modulate the expression of AhR. This question was addressed by investigating the effect of BCL-2 inhibition using VCX, a clinically used BCL-2 inhibitor, on AhR/CYP1B1 in both cancer cells and CSCs. In the present study, inhibition of BCL-2 by VCX in CSCs resulted in decreased expression of AhR and its regulated genes (CYP1A1 and CYP1B1) while keeping the BAX unaffected which indicates that BCL-2 inhibition is also correlated with AhR/CYP1A pathway. In

addition, in non-CSCs (MDA-MB-231), BCL-2 inhibition caused DNA damage and activation of caspases, which eventually led to apoptosis and cell death.

4.5. Correlation of AhR and BCL-2 activity in human BC tissues

In the present study, expression of AhR and BCL-2 was determined in 75 human BC tissues through IHC and whether these expressions were correlated. Different human BC tissues were compared to normal breast tissues and expression of AhR and BCL-2 was found to be higher in BC patients compared to the control group. Similar results were demonstrated by Mohamed and colleagues who found that expression of AhR is directly correlated with tumor aggressiveness in inflammatory BC. Additionally, they also found that AhR expression was positively correlated with Ki67 expression [254]. Moreover, Li and coworkers also reported high immunoreactivity in BC tissues compared to normal tissues and they found that high AhR expression is associated to invasive ductal BC [255].

CHAPTER 5: CONCLUSION

The current study provides first evidence of the crosstalk between AhR and BCL2 proteins for development of BC cells and breast CSCs. Our findings demonstrate that there is an association of AhR with BCL2 proteins for the development of CSCs as the inhibition of AhR using chemical antgonists affect the expression of BCL2 proteins. AhR and BCL-2 control each other and confer CSCs characteristics in BC. Hence, it was concluded that AhR is implicated in the development of breast CSCs through regulation of BCL2 proteins, indicating that it an important regulator of breast CSCs. These findings indicate that AhR could be a novel target for development of anti-neoplastic drugs. Therefore, the results from this study form a strong basis in the field of recognizing molecular targets for the treatment of BCSCs.

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