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DISSERTATION

Ansprechen auf und Resistenz gegen BET-Inhibitoren bei triple-negativem Brustkrebs

Response and Resistance to BET inhibitors in Triple Negative Breast Cancer

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List of abbreviations

AML	acute myeloid leukemia
AMPK	AMP-activated protein kinase
ATR	ataxia-telangiectasia and Rad3-related
AURKA	aurora kinases A
AURKB	aurora kinases B
BCL-2	B-cell CLL/lymphoma 2
BD	bromodomain
BET	bromodomain and extra-terminal domain
BRCA	breast cancer
BRD2	bromodomain-containing protein 2
BRD3	bromodomain-containing protein 3
BRD4	bromodomain-containing protein 4
BRDT	bromodomain-containing protein T
BSA	bovine serum albumin
CA9	carbonic anhydrase 9
CCLE	cancer cell line encyclopedia
CDK9	cyclin-dependent kinase 9
CDKs	cyclin-dependent kinases
CI	combination index
CTD	c-terminal domain
CXCR2	chemokine receptor 2
DLBCL	diffuse large B-cell lymphoma
DMSO	dimethyl sulfoxide
DPBS	dulbecco's phosphate-buffered saline
DRI	drug resistance index
EDTA	ethylenediaminetetraacetic acid

EGFR	epidermal growth factor receptor
EGTA	ethylene glycol-bis (β-aminoethyl ether)-N,N,N,N-tetraacetic acid
ER	estrogen receptor
ET	extra-terminal domain
FACS	fluorescence-activated cell sorting
FBS	fetal bovine serum
FDA	Food and Drug Administration
FPKM	fragments per kilobase million
FSC	forward scatter
FSC-A	forward scatter area
FSC-H	forward scatter height
HDAC	histone deacetylase
HEPES	4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid
HER2	human epidermal growth factor receptor 2
HNSCC	head neck squamous cell carcinoma
HPA	human protein atlas
IC50	the half maximal inhibitory concentration
LA	luminal A
LB	luminal B
M	mol/L
MW	molecular weight
MEK	mitogen-activated protein kinase enzymes
MOM	mitochondrial outer membrane
MOMP	mitochondrial outer membrane permeabilization
MTT	3- (4.5-dimethylthiazol-2-yl) -2.5-diphenyl tetrazolium bromide
NHEJ	non-homologous end joining
NSCLC	non-small cell lung cancer
PAGE	polyacrylamide gel electrophoresis

PBS	phosphate buffered saline
PBST	phosphate buffered saline with tween® 20
PI	propidium iodide
PP2A	protein phosphatase 2A
PR	progesterone receptor
pSer2	phosphorylation of serine 2
P-TEFb	positive transcription elongation factor-b
PVDF	polyvinylidene difluoride
RT	room temperature
S2	serine 2
SASP	senescence-associated secretory phenotype
SDS	sodium dodecyl sulfate
SSC	side scatter
TCGA	the cancer genome atlas
TNA	triple negative A
TNB	triple negative B
TNBC	triple negative breast cancer
TPM	transcripts per million
USP17	ubiquitin-specific protease 17
UV	ultraviolet
VEGF-A	vascular endothelial growth factor A

Abstract

Introduction: Triple-negative breast cancer (TNBC) is the most aggressive subtype of breast cancer, and urgently needs new potent therapies. Bromodomain and extra-terminal proteins (BET) are primary regulators of RNA polymerase II, which also regulate gene transcription. For cancer patients, numerous BET inhibitors are now undergoing clinical trials. Unfortunately, the responses to BET inhibitors varied between preclinical and clinical investigations, necessitating more research into their functions in cancer.

Materials and methods: Gene expression data were acquired from The Cancer Genome Atlas the (TCGA), Cancer Cell Line Encyclopedia (CCLE), and the Human Protein Atlas (HPA) to analyze the gene and protein expression of BET family members in TNBC patients and cell lines. One cell line (MDA-MB-231R) resistant to the BET inhibitor JQ1 was established from the parental cell line MDA-MB-231. The cytotoxicity of the medications following treatments was assessed using the MTT test. The cell cycle, cell apoptosis, and BH3 profiling analyses were carried out using FACS analysis. Western blotting was used to detect protein expression before and after treatments.

Results: Particularly in TNBC tissues and cell lines with the low rate of mutations, BRD4 was overexpressed in breast cancer. The BET inhibitor JQ1 could induce cell cycle G1 arrest, senescence, and minor cell apoptosis in TNBC. When combined with CDK9 inhibitors, JQ1 showed a strong synergistic effect in TNBC cell lines by inducing apoptosis. It was discovered through the use of the BH3 profiling assay and the MTT assay that BCL-xL was necessary for the survival of MDA-MB-231 and MDA-MB-231R cells, whereas that of MDA-MB-436 cells was dependent on BCL-xL and MCL-1. According to Western blot analysis the combination of JQ1 and CDK9 inhibitor dramatically reduced the expression of BCL-xL and MCL-1 in TNBC cell lines. Therefore it is likely that apoptosis induced by combination of both substances is a result of reduction of BCL-xL and/or MCL-1.

Summary/conclusions: Promising Targets in TNBC include BET proteins, particularly BRD4. The BET inhibitor JQ1 is a potential therapeutic agent in treatment of TNBC treatment. Moreover, the combination of BET and CDK9 inhibitors has a synergistic inhibitory effect in TNBC treatment

not only in JQ1-sensitive cell but also in primary and secondary resistant cell lines by inducing cell apoptosis possibly through suppressing MCL-1 and BCL-xL.

Zusammenfassung

Einleitung: Das triple-negative Mammakarzinom (triple negative breast cancer, TNBC) gilt als der aggressivste Subtyp von Mammakarzinomen und es werden für diese Entität dringend neue wirksamere Therapieansätze gesucht. Bromodomain und Extra-Terminal (BET)-Proteine sind Schlüsselregulatoren der RNA-Polymerase II und kontrollieren die Gentranskription. Derzeit werden BET-Inhibitoren in klinischen Studien für Krebspatienten untersucht. BET-Inhibitoren zeigten bis dato unterschiedlichen Ergebnissen in präklinischen Studien untersucht, weshalb ihre Rolle bei Tumortherapien, insbesondere beim TNBC, weiter erforscht und definiert sollte.

Material und Methoden: Genexpressionsdaten wurden vom Cancer Genome Atlas (TCGA), der Cancer Cell Line Encyclopedia (CCLE) und dem Human Protein Atlas (HPA) heruntergeladen, um die Expression der BET-Familie bei TNBC-Patienten und Zelllinien zu analysieren. Eine Zelllinie (MDA-MB-231R), die gegen den BET-Inhibitor JQ1 resistent war, wurde aus der ursprünglichen Zelllinie MDA-MB-231 entwickelt. An TNBC-Zelllinien wurde die Zytotoxizität der Substanzen mittels MTT-Assay untersucht. Zellzyklus, Zellapoptose und BH3-Profiling wurden mittels FACS analysiert. Die Proteinexpression nach der Behandlung wurde mittels Western Blot analysiert.

Ergebnisse: BRD4 wird bei Brustkrebs überexprimiert, besonders in TNBC-Gewebe und Zelllinien mit zugleich niedriger Mutationsrate. Der BET-Inhibitor JQ1 induzierte Zellzyklus-G1-Arrest, Seneszenz und geringe Zellapoptose in TNBC. In Kombination mit CDK9-Inhibitoren zeigte der Bromodomain-Inhibitor JQ1 jedoch einen starken synergistischen Effekt auf TNBC-Zelllinien bei der Apoptose-Induktion. Mit Hilfe des BH3-Profiling-Assays und MTT-Assays konnte gezeigt werden, dass das Überleben von MDA-MB-231- und MDA-MB-231R-Zellen von BCL-xL abhängt, während die Überlebensrate der MDA-MB-436-Zellen von BCL-xL und MCL-1 abhängig ist. Die Western-Blot-Analyse zeigte, dass die Kombination von JQ1 und CDK9-Inhibitoren eine signifikante Herabregulierung der BCL-xL- und MCL-1-Expression in TNBC-Zelllinien bewirkte. Daher wat es wahrscheinlich, dass die durch die Kombination der beiden Substanzen induzierte Apoptose auf eine Verringerung von BCL-xL und/oder MCL-1 zurückzuführen ist.

Zusammenfassung/Schlussfolgerungen: BET-Proteine, insbesondere BRD4, sind vielversprechende therapeutische Zielstrukturen beim TNBC. Der BET-Inhibitor JQ1 erwies sich als effektiv in die Behandlung von TNBC-Zelllinien. Darüber hinaus hat die Kombination von BET- und CDK9-Inhibitoren eine synergistische hemmende Wirkung bei TNBC, nicht nur bei JQ1-empfindlichen Zellen, sondern auch bei primär und sekundär resistenten Zelllinien, möglicherweise durch Apoptoseinduktion über die Unterdrückung von MCL-1 und BCL-xL.

1. Introduction

1.1 Triple negative breast cancer (TNBC)

The greatest cause of death and a major obstacle to extending life is cancer. According to global cancer statistics, female breast cancer has surpassed lung cancer as the most commonly diagnosed cancer, with an estimated 2.3 million new cases (11.7%) in 2020 (Sung et al., 2021). Despite a high incidence of breast cancer, the mortality has decreased significantly in most subtypes due to new therapeutic treatments, such as targeted therapy (Harbeck et al., 2019). However, one subtype of breast cancer, the Triple Negative Breast Cancer (TNBC), still has a poor prognosis and more effective therapeutic options are needed. According to da Silva et al. (2020), the most aggressive subtype of breast cancer is TNBC, which is a breast cancer that lacks Estrogen Receptor (ER), Progesterone Receptor (PR), and Human Epidermal growth factor Receptor 2 (HER2) expression. It frequently affects young women under 40 years old and is extremely invasive (Yin et al., 2020). Systemic Chemotherapy is still the primary medication option for patients with TNBC in the early and late stages of the disease, despite the fact that several target treatments are on the horizon (Bianchini et al., 2016). The lack of targeted therapies and the poor prognosis of TNBC patients has prompted the search for molecular targets to treat patients with these tumors.

1.2 Bromodomain and extraterminal domain (BET) protein

Cancer has a complicated etiology that is influenced by both hereditary and environmental factors. In cancer cells, alterations in genomic information such as genomic instability and mutations are frequently found. Cancer is also frequently caused by reasons other than changes to the genetic code, such as deregulation of gene expression. Epigenetics, according to scientists, is the study of changes in gene expression adjustments to the underlying sequence. Epigenetic changes can affect gene expression in several ways such as DNA methylation, histone modification and Non-coding RNA. One of the dynamic histone modifications that controls the transcription of genes is histone acetylation (Kelly and Issa, 2017).

The process of acetylation and deacetylation of lysine residues within the N-terminal tail extending from the histone core of the nucleosome are referred to as histone acetylation and deacetylation.

The nucleosome is the fundamental unit of chromatin. There are two major structures, heterochromatin and euchromatin. Acetylation reduces the contact between the N terminus of the histone and the negatively charged DNA phosphate group by removing the positive charges from histones. As a result, condensed chromatin changes into a looser structure linked to higher levels of gene transcription (Woodcock and Ghosh, 2010). These changes in gene expression are modulated by three categories of epigenetic regulatory proteins, which are commonly known as "writers", "erasers", and "readers" (Figure 1) (Verdin and Ott, 2015).

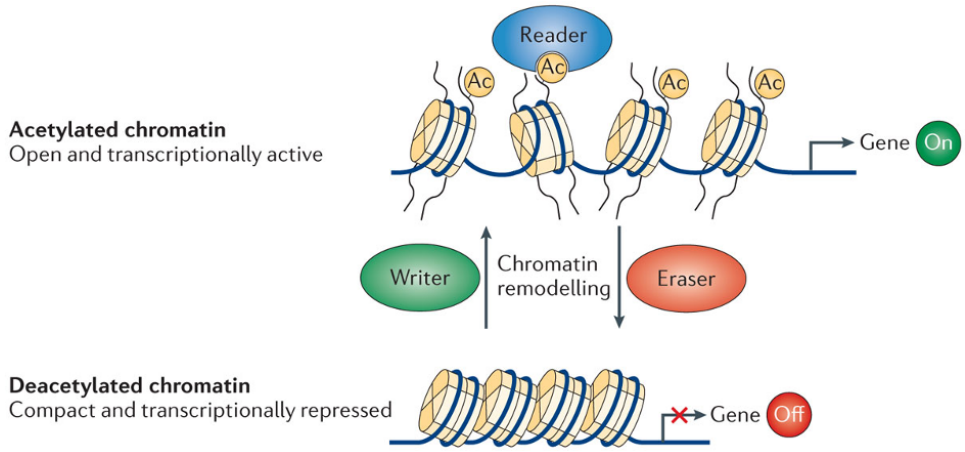


Figure 1. Programming of the epigenome during development occurs via the activity of a variety of epigenetic modifiers, including "readers, writers and erasers" of histone.

Bromodomain and extraterminal domain (BET) proteins are a class of proteins that function as chromatin "readers" and recruit chromatin-modifying enzymes to target gene promotors (Park and Han, 2019). BET proteins family is composed of four proteins, namely bromodomain-containing protein 2 (BRD2), BRD3, BRD4, and BRDT (Stathis and Bertoni, 2018). The positive transcription elongation factor-b (P-TEFb), which phosphorylates serine 2 (S2) on the carboxy-terminal domain of the RNA polymerase II, and is necessary for transcription elongation, is recruited and activated by the BET proteins, which are composed of two bromodomains (BD1 and BD2), an extra terminal domain (ET), and a c-terminal domain (CTD). BET proteins recognize acetylated (Barrero, 2017). The only ubiquitously expressed member of the BET family that directly binds to P-TEFb is BRD4, which has received the most research attention (Perez-Salvia and Esteller, 2017). BRD4 protein regulates the transition of cells from M to G1 phase throughout

the cell cycle and is necessary for appropriate chromatin stability. This is done in part through the recruitment of P-TEFb (White et al., 2019).

1.3 **BET inhibitor in TNBC**

Breast tumors have been found to have amplification or overexpression of BET proteins, highlighting their clinical significance. The crucial role of BET proteins in transcription has led to the development of small molecule inhibitors that target their functions, known as BET inhibitors (Filippakopoulos et al., 2010).

BET inhibitors, have demonstrated encouraging outcomes in breast cancer cells and xenograft models by interfering with the binding of BET proteins to acetylated lysine residues in chromatin and consequently inhibiting the transcription of multiple oncogenes (Klauber-DeMore et al., 2018, Delmore et al., 2011, Mertz et al., 2011, Shu et al., 2016, Maggisano et al., 2019). Since then, numerous selective and pan-BET inhibitors have been developed, including JQ1, I-BET762, OTX-015, etc (Zhao et al., 2013, Vazquez et al., 2017, Filippakopoulos et al., 2010).

It has frequently been confirmed that BET inhibitors reduce the expression of genes important for cell proliferation, such as c-myc, thereby inducing G1 cell cycle arrest. C-myc is a potent MYC oncogene and is overexpressed in 30-50% of high-grade cancers (Wang et al., 2018, Delmore et al., 2011, Li et al., 2019, Klauber-DeMore et al., 2018). In contrast to other malignancies, TNBC does not require c-myc suppression to respond to BET inhibitors. BET inhibitors repress the growth of multiple *in vitro* and *in vivo* types of TNBC by inducing two responses: apoptosis and senescence by the suppression of Aurora kinases A and B (AURKA/AURKB) (Sahni et al., 2016). Moreover, BET inhibitors suppress carbonic anhydrase 9 (CA9) hypoxia-responsive genes, thereby preventing hypoxia-induced upregulation of genes involved in angiogenesis (da Motta et al., 2017).

1.4 **BET inhibitor resistance**

Currently, there are many clinical trials of BET inhibitors are currently the subject of numerous clinical trials in a variety of malignancies, while TNBC research is less common (Table 1). The preliminary results of these research have not been encouraging. The majority of these clinical studies have revealed BET inhibitor resistance.

BET inhibitor resistance does not seem to be due to changes in gene expression patterns of BET proteins, such as copy number alterations induced by particular BET inhibitors or somatic mutations in gatekeeper genes. For instance, resistance to BET inhibitors in preclinical proteomics studies in TNBC is thought to be caused by downregulation of protein phosphate 2A (PP2A), which results in hyperphosphorylation of BRD4, and enhanced interaction and activity with MED1 (mediator of RNA polymerase II), which then cause an upregulation of transcriptional machinery and cell proliferation (Shu et al., 2016). Deubiquitinating enzyme DUB3 binds to BRD4 and enhances its deubiquitylation and stability. DUB3 inhibiting substances, such as CDK4/6 inhibitors, reduce this action (Jin et al., 2018, Ge et al., 2020). Similarly, in other solid cancers, the mechanism of resistance to BET inhibitors in lung adenocarcinoma was mediated by casein kinase phosphorylation of BRD4 (Calder et al., 2021). Furthermore, BET inhibitor resistance is demonstrated to evolve from leukemia stem cells, but not mediated through enhanced drug efflux or metabolism (Fong et al., 2015). JQ1 produced little apoptosis in acute myeloid leukemia (AML) leukemia progenitor cells that were resistant to the drug, but it promoted autophagy by upregulating BECN1 and increasing the production of autophagosomes. Drug-induced autophagy inhibition or BECN1 knockdown enhanced JQ1-induced apoptosis in resistant cells (Jang et al., 2017). A similar result was obtained in ovarian cancer cell (Luan et al., 2019).

1.5 **Combination of BET inhibitors for TNBC**

Focusing on the combination of BET inhibitors with other agents, such as targeted agents and/or other epigenetic drugs, is necessary because to the low activity of monotherapy. BET inhibitors have been demonstrated to significantly increase the effectiveness of many prior anticancer chemotherapeutics and immunotherapeutic agents in preclinical investigations using different cancer models (Fiskus et al., 2014, Bauer et al., 2018, Heinemann et al., 2015, Shahbazi et al., 2016, Lee et al., 2015).

First, BET inhibitors alone may simply have effects that restrict cell development without induction of cell death, but, when combined with other targeted agents, they may have extremely produced massive cytotoxic effects. For instance, JQ1 caused cell cycle arrest in osteosarcoma cell lines and xenografts without change of c-myc. Growth was inhibited and c-myc was significantly

downregulated when rapamycin was administered. On the other hand, BET inhibitors might improve the effectiveness of chemotherapy agents. It impaired the toleration of TNBC cells to DNA damage following cisplatin exposure, leading to massive cell death (Mio et al., 2019). Moreover, JQ1 combined with docetaxel or vinorelbine exhibited a synergistic cytotoxic impact (Perez-Pena et al., 2016).

Secondly, in the case of acquired resistance to targeted therapy, combination therapies seem to have particular promise. BET inhibitors may re-sensitize resistant cells to other inhibitors, including mTOR inhibitors (Bihani et al., 2015), PI3K inhibitors (Stratikopoulos et al., 2015), MEK inhibitors (Zawistowski et al., 2017), tamoxifen (Feng et al., 2014), lapatinib (Matkar et al., 2015), etc. BET inhibitors have also demonstrated synergistic benefits for mutation-induced resistance, such as BRA Fmut (Paoluzzi et al., 2016) and PI3KCA (Stratikopoulos et al., 2015).

Thirdly, BET inhibitors might enhance the activity of cell cycle inhibitors. For instance, the BET inhibitor BI 894999 demonstrated synergistic tumor growth inhibition and c-myc suppression when combined with CDK9 inhibitor in AML xenografts (Gerlach et al., 2018). Similar results were observed when BET inhibition was combined with CDK2 inhibitor (Bolin et al., 2018), CDK7 inhibitor (Guo et al., 2020, Zhang et al., 2020), and CDK4/6 inhibitor (Ge et al., 2020).

Finally, combination therapies may be used to treat patients who have developed resistance to BET inhibitors through combined with AKT inhibitors (Zhang et al., 2017) in SPOP-mutant prostate cancer, NF-κB inhibitors in uveal melanoma cells (Ambrosini et al., 2019), AMP-activated protein kinase (AMPK) inhibitors in AML (Jang et al., 2017) and PLK1 inhibitors in TNBC (Nieto-Jimenez et al., 2020).

1.6 **Aims of this study**

The clinical consequences of Triple negative breast cancer (TNBC) are a highly heterogeneous disease with the poorest outcome of all breast cancer subtypes and still lacks efficient targeted treatment, leading to dismal clinical outcomes. BET proteins are a group of epigenetic targets and BET inhibitors have demonstrated potential therapeutic benefits in various hematological and solid cancers. Unfortunately, due to drug resistance and/or toxicities of the drugs, preliminary data from various clinical trials using BET inhibitors fail to show encouraging results. The clinical

applications of BET inhibitors may be improved by reducing drug cytotoxicity by lowering the doses and enhancing drug activity by combination therapy with other drugs. In this study, it is first analyzed the expression of the BET protein family in breast cancer cells and tissues, as well as their mutations, using public database, to confirm its reliability as a drug target. The second goal is to determine the sensitivities of TNBC cells to BET inhibitors and to establish resistant cell lines to analyze the possible resistant mechanisms. Thirdly, screening for possible combination partners of BET inhibitors to enhance their efficacies is performed. Finally, the combination effects are analyzed in detail to identify the possible underlying mechanisms.

Table 1. Clinical trials relate to BET inhibitor according to ClinicalTrials.gov

BET inhibitor	NCT Number	Status	Results	Disease	Phases	No.	Combination
PLX51107	NCT04910152	Not recruiting	No Results	Acute Graft Versus Host Disease	Phase1	34	N/A
				Steroid Refractory Graft Versus Host Disease	Phase 2		N/A
FT-1101	NCT02543879	Completed	No Results	Haematological malignancies	Phase 1	94	Azacitidine
RO6870810	NCT03068351	Completed	No Results	Multiple Myeloma	Phase 1	24	daratumumab
ZEN-3694	NCT04840589	Not recruiting	No Results	Metastatic Malignant Solid Neoplasm	Phase 1	51	Ipilimumab
				Recurrent Malignant Solid Neoplasm			Nivolumab
				Recurrent Platinum-Resistant Ovarian Carcinoma			

ZEN-3694	NCT04471974	Recruiting	No Results	Refractory Ovarian Carcinoma Castration-Resistant Prostate Carcinoma Metastatic Prostate Adenocarcinoma Metastatic Prostate Small Cell Carcinoma Stage IV Prostate Cancer AJCC v8 Stage IVA Prostate Cancer AJCC v8 Stage IVB Prostate Cancer AJCC v8	Phase 2	54	Enzalutamide Pembrolizumab
CPI-0610	NCT02157636	Completed	No Results	Multiple Myeloma	Phase 1	30	N/A
CPI-0610	NCT01949883	Completed	No Results	Lymphoma	Phase 1	64	N/A
BMS-986158	NCT03936466	Recruiting	No Results	Solid Tumor, Childhood	Phase 1	34	N/A

	5			Lymphoma Brain Tumor, Pediatric			
CPI-0610	NCT02158858	Recruiting	No Results	Myelofibrosis	Phase 1	271	Ruxolitinib
				Leukemia, Myelocytic, Acute Myelodysplastic/Myeloproliferative Neoplasm Myelodysplastic Syndrome (MDS)	Phase 2		
GSK525762	NCT03266159	Withdrawn	No Results	Solid Tumors	Phase 2	0	Trametinib
GSK525762	NCT01943851	Completed	No Results	Neoplasms	Phase 2	111	N/A
GSK525762	NCT01587703	Completed	PR: 2, n = 17	Carcinoma, Midline	Phase 1	196	N/A
ZEN003694	NCT02711956	Completed	No Results	Metastatic Castration-Resistant Prostate Cancer	Phase 1 Phase 2	75	Enzalutamide

ZEN003694	NCT02705469	Completed	No Results	Metastatic Castration-Resistant Prostate Cancer	Phase 1	44	N/A
INCB054329	NCT02431260	Terminated	PR: 1,n=54	Solid Tumors and Hematologic Malignancy	Phase 1 Phase 2	69	N/A
Birabresib	NCT02259114	Completed	PR (NMC): 3,n=46	NUT Midline Carcinoma Triple Negative Breast Cancer Non-small Cell Lung Cancer with Rearranged ALK Gene/Fusion Protein or KRAS Mutation Castrate-resistant Prostate Cancer Castration-resistant Prostate Cancer Pancreatic Ductal Adenocarcinoma	Phase 1	47	N/A
CPI-0610	NCT02986919	Withdrawn	No Results	Peripheral Nerve Tumors	Phase 2	0	N/A

Birabresib	9 NCT02698189	Not recruiting	NR, n=9	AML Including AML de Novo and AML Secondary to MDS DLBCL	Phase 1	9	N/A

*CR complete response, PR partial response, NR no response.

2 Materials and Methods

2.1 Materials

2.1.1 Media

Table 2. Cell culture media and supplements

Product	Cat #	Company
RPMI1640 medium Supplement*	21875-034	Gibco
Fetal Bovine Serum (FBS)	10270-106	Gibco
Penicillin/streptomycin.	11548876	Gibco
Amphotericin B	11120009	Corning

*The media were mixed with 10% FBS, 1% (w/v) penicillin/streptomycin, and 1% amphotericin B

2.1.2 Antibodies

Table 3. List of antibodies

Antigen	Label	Company	Clone	Cat #	Isotype	Con.
Actin	HRP	Santa Cruz	Polyclonal	F0711	Goat	1:200
BCL-xL	HRP	Santa Cruz	Monoclonal	Sc-8392	Mouse	1:200
BCL-2	HRP	Dako	Monoclonal	M0887	Mouse	1:1000
MCL-1	HRP	Santa Cruz	Monoclonal	Sc-12756	Mouse	1:200
cytochromec	FITC	BioLegend	6H2.B4	983502	IgG1	1:400
PARP	HRP	CST	Polyclonal	#9542	Rabbit	1:1000

2.1.3 Chemicals and drugs

Table 4. List of chemicals and drugs

Drug	Cat NO.	Dose	Company
A1331852	B2948-5	5mg	Biovision
ABT-199	T219	10mg	TargetMol
ABT263	Sc-20724	10mg	Santa Cruz

AZD1208	20235	5mg	Cayman chemical
AZD1480	10702	5mg	Cayman chemical
AZD4573	112088	10mg	MedChem Express
BSK-805	16714	5mg	Cayman chemical
CDK2-IN-4	117535	10mM×1ml	MedChem Express
GSK-650394	SML0773	5mg	Sigma
I-BET762	10676	1 mg	Cayman chemical
JQ1	1268524	10mg	Adoopbio-science
LDC4297	12653	10mg	MedChem Express
PD0332991	16273	10mg	Cayman chemical
S63845	S8383	1mg	Selleckchem
SGI-1776	16423	5mg	Cayman chemical

2.1.4 Chemicals and buffers

Table 5. List of chemicals and buffers

Solutions and buffers	**Company**
0.25%Trypsin-EDTA(1x)	Gibco
1.5 M Tris-Hcl (PH=6.8) solution	Sigma
1.5 M Tris-Hcl (PH=8.8) solution	Sigma
10% APS Solution	Sigma
10% SDS solution	Sigma
Acrylamide	Roth
Alamethicin	Enzo
Bovine serum albumin (BSA) (IgG Free)	VWR
Digitonin	Sigma
DPBS(1x)	Gibco
EDTA	Sigma

EDTA-free protease inhibitor	Merck
EGTA	Sigma
Formaldehyde solution	Sigma
HEPES Free acid	Sigma
KCL	Sigma
Mannitol	Sigma
Membrane blocking agent	GE healthcare UK
PBST solution	Sigma
Pierce Bovine Serum Albumin Standard Ampule	Thermo Fisher
Polaxamer 188	Fisher
Ponceau S staining reagent	Abcam
Precision Plus Protein western blotting Standards	Bio-Rad
Protein Extraction Reagent	Novagen
Polyvinylidene difluoride (PVDF) membrane	Bio-Rad
Re-blot plus strong solution	EMD Milipore Com
ß-mercaptoethanol	Merck
Succinic Acid	Sigma
Temed	Roth
Tween 20	VWR

2.1.5 BH3 profiling buffers

2.1.5.1 Mitochondrial buffers (MEB)

Table 6. Components of mitochondrial buffers (MEB)

MEB2-P25 for iBH3	MW	[Stock] M	Final Conc.	Final Vol (L)	Mass (g)
150 mM Mannitol	182	-	0.15	0.5	13.65
10 mM HEPES-KOH	238.3	-	0.01	0.5	1.19

150 mM KCl	74.55	-	0.15	0.5	5.59
1 mM EGTA	380.35	0.5	$1x10^{-3}$	0.5	1 mL
1 mM EDTA	292.24	0.5	$1x10^{-3}$	0.5	1 mL
0.1 % BSA	66463	100%	0.1	0.5	0.500
5 mM Succinate	118.09	-	0.005	0.5	0.295
Polaxamer 188	8400	-	2.5 g/L	0.5	1.25

Water was added to a beaker with the solids (Mannitol, HEPES, Succinic acid, BSA, KCl, EDTA and EGTA) dissolved them, the pH was adjusted to 7.5 +/- 0.1 with KOH, and water was added to the final volume, the mixture was then filtered through a 0.22-micron filter and kept at 4 °C.

2.1.5.2 10X Tween20 intracellular staining buffer (For cytochrome c antibody staining)

50 mL of PBS was used to dissolve 1 mL of Tween20 and 5g of BSA, the mixture was then filtered through a 0.22-micron filter and kept at 4 °C.

2.1.5.3 Peptide stock

Table 7. List of peptide stock

Peptide Name	**Sequence**		**Extinction Coeff. 280 nm**
	N-Term	**C-Term**	
hBIM	Acetyl-MRPEIWIAQELRRIGDEFNA-Amide		5500 cm^{-1} M^{-1}
Puma	Acetyl -EQWAREIGAQLRRMADDLNA- Amide		5500 cm^{-1} M^{-1}
HRKy[a]	Acetyl -SSAAQLTAARLKALGDELHQY- Amide		1490 cm^{-1} M^{-1}
MS1[b]	Acetyl-RPEIWMTQGLRRLGDEINAYYAR-Amide		8480 cm^{-1} M^{-1}
FS1[c]	Acetyl-QWVREIAAGLRLAADNVNAQLER-Amide		5500 cm^{-1} M^{-1}

a HRKy is a BCL-xL specific peptide.

b MS1 is a non-natural MCL-1specific peptide.

c FS1 is a non-natural BFL1/BCL2A1-specific peptide.

The Charité Institute of Biochemistry provides the required sequence of peptides. The concentration of each peptide was assessed using UV absorbance at 280 nm after it had been dissolved in DMSO.

2.1.6 Kits

Table 8. List of kits

Kit	**Company**
Cell Proliferation Kit I (MTT)	Sigma
PE Annexin V Apoptosis Detection Kit I	BD pharmingen
Zombie Aqua Fixable Viability Kit	BioLegend
Senescence-ß-Galactosidase Staining Kit	Sigma
Pierce BCA Protein Assay Reagent	Thermo Fisher Scientific
Immuno Cruz Western Blotting Luminol Reagent	Santa Cruz

2.1.7 Devices

Table 9. Devices

Devices	**Company**
Eppendorf centrifuge 5810R	Eppendorf
Eppendorf centrifuge 5417R	Eppendorf
Hoefer Semi Phor semi-dry transfer unit	Hoefer, Inc
Trans-blot SD semi-dry transfer cell	Bio-Rad
Mini protean Tetra cell	Bio-Rad
VersaMax microplate reader	Molecular Devices
Electrophoresis (PAC-200)	Bio-Rad
EMB 200-2	KERN & SOHN GmbH
IKA Magnetic stirrers	IKA
Eppendorf mixer 5436	Eppendorf

Microscope	Leica
HeRaeus Cell incubator	Thermo Fisher Scientific
Nanodrop 1000 spectrophotometer	VWR
CytoFlex Flow cytometer	Beckman Coulter GmbH

2.1.8 Miscellaneous materials

Table 10. List of material

Material	**Company**
0.2µM Sterile filter	Sarstedt AG & Co.KG
0.5,1.5-, and 2-ml reaction tubes	Sarstedt AG & Co.KG
0.2 µM filter	Sarstedt AG & Co.KG
6-,12-,24-,48-, and 96-wellplates	Greiner Bio-One
25cm^2, 50 cm^2,75 cm^2 Cell culture flask	Falcon
2 ml, 5 ml, 10 ml, 25 ml pipettes	Sarstedt AG & Co.KG
10 ml syringes	BD Biosciences
Counting chamber	Paul Marienfeld

2.1.9 Software

Table 11. Software

Software	**Company**
EndNote x7	Thomson Reuters
FACS® Diva	BD Biosciences
FlowJo 7.2.5 and V10	Tree Star.
GraphPad® Prism Version 6.0	Graph Pad Software
Illustrator CC	Adobe
Microsoft® Office 365 ProPlus	Microsoft
Photoshop CC	Adobe
R studio	RStudio, Inc.

2.2 **Methods**

2.2.1 Data mining

The gene expression data (HTSeq-FPKM and HTSeq-counts) with clinical information from the BRCA project were retrospectively collected from TCGA database (https://cancergenome.nih.gov/) (Tomczak et al., 2015), and level 3 HTSeq-FPKM data were converted to Transcripts Per Million (TPM) for the following analyses. The cancer cell line encyclopedia was used to download information about BRD4 mRNA expression in breast cancer cell lines (https://portals.broadinstitute.org/ccle) (Barretina et al., 2019, Barretina et al., 2012). The R Bioconductor package maftools was used to evaluate the gene mutation data that was download from TCGA (Mayakonda et al., 2018). The human protein atlas (https://www.proteinatlas.org) was used to download the BET protein expression data (Uhlen et al., 2015).

2.2.2 Cell culture

2.2.2.1 Cell lines

2.2.2.1.1 MDA-MB-231

The MDA-MB-231 cell line is an epithelial, human breast cancer cell line that was established from a pleural effusion of a 51-year-old Caucasian female with a metastatic mammary adenocarcinoma. It is TNBC cell line that lacks ER, PR and HER2 expression with the KRAS and BRAF mutation that is extremely aggressive, invasive and poorly differentiated (Hollestelle et al., 2010).

2.2.2.1.2 MDA-MB-436

The MDA-MB-436 cell line, an epithelial human breast cancer cell line, was created from a pleural effusion of a 43-year-old Caucasian female with a metastatic infiltrating ductal carcinoma. Moreover, it was a TNBC cell line lacking ER, PR and HER2 expression and had the RB1 and BRCA1 mutation (Elstrodt et al., 2006, Hollestelle et al., 2010).

2.2.2.2 Culture conditions

At 37 ^{o}C and 5% CO_2 cells were cultured in RPMI supplemented with 10% fetal calf serum, 1% penicillin, and streptomycin. Cells were passaged every 3-4 days.

2.2.2.3 Cell counting method

Cell suspensions were mixed (1:4 v/v) with 0.4% trypan blue solution (Sigma Aldrich). Trypan

blue cannot pass through intact living cell membranes, only dead and damaged cell membranes can be stained with trypan blue. A 10 µl cell / trypan blue mixture was transferred to a Neubauer chamber, after which the cells was counted in the four squares under the microscope. By multiplying the average number of cells per square, the dilution factor, and the chamber factor (10,000), the cell number per ml suspension was calculated.

2.2.3 Cells viability via 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium Bromide (MTT) assay

Cells were seeded in a 96-well plate in 6 replicates at the density of 2×10^3/well and incubated overnight, then treated with serial dilutions of JQ1 and I-BET762 at 37 °C for 72 hours to assess the dosage response to BET inhibitors. After 72 hours treatment, cells were incubated with 10 ul yellow MTT solution for 2-3 hours in the incubator. The 100 ul solubilization solution was then added and the plate was left in the incubator's humidified atmosphere for the duration of the next day. A microplate reader was used to measure the cells' spectrophotometry and the formazan product's absorbance wavelength was 490 nm.

2.2.4 Assessment of combination index via MTT assay

Cells were seeded in a 96-well plate in 3 replicas at the density of 2×10^3/well and adhered overnight to test the efficacy of combination therapy. Cells were treated with serial dilutions of JQ1 or second drug at 37 °C for 72 hours. After 72 hours treatment, cells were incubated with 10 ul yellow MTT solution for 2-3 hours in the incubator. The 100 ul solubilization solution was added then and the plate was incubated in the incubator with a humidified atmosphere for the duration of the next day. The spectrophotometry of the cells was measured by A microplate reader was used to measure the cells' spectrophotometry, and the wavelength which the formazan product was absorbed was 490 nm. The Combination Index (CI) was calculated via calcusyn software. CI < 1, $= 1$ and > 1 indicated synergism, additivity and antagonism, respectively.

2.2.5 Establishment of JQ1-resistant cell line

The JQ1-sensitive triple negative cell line MDA-MB-231 was used in order to create a cell line that is secondary resistant to JQ1. JQ1 resistant cell line is established by exposure to increasing concentration of JQ1. First, the MTT assay was used to calculate the IC50 (half maximal inhibitory

concentration) dose of JQ1 after 72 hours treatment. Next, the cells were passaged, left overnight for attachment and then replaced with media containing a 1/4 IC50 dose of JQ1. Passage the cells and kept media with the same dose of JQ1 in the presence of cells that had reached 70-80% confluence. After 2-3 times of passage, 1.5 times of the drug dose was increased. This protocol was repeated for a period of approximately 9 months, until the cells exhibited stable growth and proliferation in a culture medium with 5 μmol/L of JQ1. MDA-MB-231/JQ1 resistant (hereafter MDA-MB-231R) is the name of the resistant cell line (Figure 2).

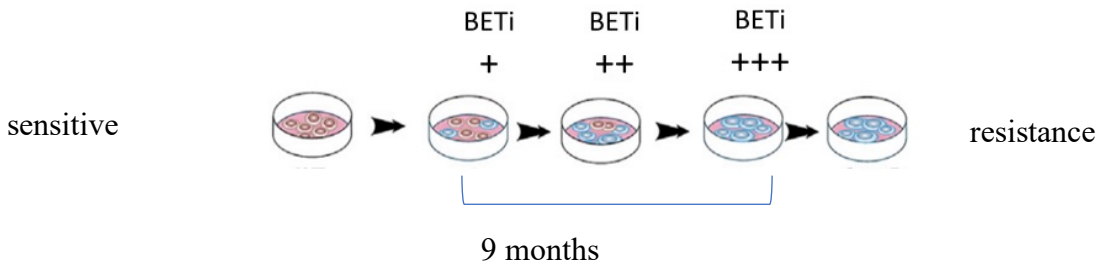


Figure 2. Workflow of establishment of resistant cell line.

2.2.6 Cell cycle via Propidium Iodide (PI) staining

Cells were plated in 6-well plates at a density of 1×10^5 cells/well and exposed to various concentrations of drug (the concentrations of JQ1 used in MDA-MB-231 is 0.1 μmol/L, 1 μmol/L, MDA-MB-231R and MDA-MB-436 is 0.5 μmol/L, 1 μmol/L for 48 hours). After drug treatment, cells were collected and then fixed in cold 70% ethanol and kept at 4°C overnight. After being stained with PI /RNase staining solution for 40 minutes at 37 °C and being rinsed with cold PBS, the cell cycle was analyzed using a flow cytometry and flowjo software.

2.2.7 Cell apoptosis via Annexin V/PI assay

Cells were seeded in 6-well plates overnight at a density of 1×10^5 cells/well and treated with JQ1 and/or AZD4573 for 48 hours. Cells were detached by 0.25% trypsin after 48 hours drug treatment, washed twice with 5 ml cold PBS and then resuspend in 1X Binding Buffer at a concentration of 1×10^6 cells/ml. A 5 ml FACS tube was filled with 100 μl of the solution (1×10^5 cells). The cells were gently vortexed after 1 ul of FITC Annexin V was added and incubated at room temperature (25 °C) for 15 minutes in the dark. 400 ul of 1X Binding Buffer and 2ul PI were added to each

tube. The cell apoptosis was analyzed using a flow cytometry and flowjo software. The apoptosis rate was calculated as the sum of proportion of early apoptotic and late apoptotic cells.

2.2.8 Cell senescence via Senescence-ß-Galactosidase staining assay

On 24-well plates, cells were plated overnight at a density of 1×10^4 cells/well and treated with various concentrations of JQ1. Cells were washed twice with 1 ml PBS per well after 72 hours treatment period. 300 ml per well of 1× Fixation Buffer was added and the plate was placed for 10 min at room temperature. The cells were washed 3 times with 1 ml PBS per well. 200ul of the staining mixture (prepared in advance according to the manufacturer's instructions) was added to each well and incubated at 37 °C without CO_2 until the cells were stained blue (2 hours to overnight, depending on the cells). To keep the plate from drying out, it was sealed with Parafilm and examined under a microscope.

2.2.9 BH3 profiling

BH3 profiling is a peptide-based technique for predicting the apoptosis of cancer cells by measuring the ability of different BH3 peptides to induce mitochondrial depolarization as a surrogate marker of cellular apoptotic response.

More than fifteen members of the BCL-2 family of proteins interact at the mitochondrial level to control the intrinsic pathway of apoptosis, which determines whether an individual will live-or-death decision. Apoptosis is triggered when the pro-apoptotic, pores-forming proteins BAK and/or BAK are activated by the pro-apoptosis activators, BIM or BID. Upon activation, BAX and BAK homo-oligomerize and cause Mitochondrial Outer Membrane Permeabilization (MOMP) which allows the cell to undergo irreversible apoptosis. The creation of the apoptosome and subsequent activation of the caspase cascade are caused by the release of cytochrome c into the cytosol when these pores in the outer mitochondrial membrane are produced (Fraser et al., 2019). The pro-apoptotic BH3 peptides (Figure 3), which mimic the activity of pro-apoptotic proteins from the BCL-2 family are used in the BH3 profiling test to determine the degree of cytochrome C release. A live cell is said to be "primed" for apoptosis if it expresses precisely the right amount of pro-survival proteins to block natural pro-apoptotic signals. In contrast, a cell is "unprimed" if it expresses an excess of pro-survival proteins that can act as a buffer against current and possibly

future pro-apoptotic molecules. Finally, cells that do not express sufficient levels of BAX and BAK to undergo MOMP and thus commit to apoptosis are “apoptosis refractory” (Figure 4). In order to identify whether the cells are primed or unprimed for apoptosis and which BCL-2 members of pro-survival molecules the cell is used for evasion of apoptosis, the BH3 profiling measures the amount of cellular cytochrome c that released in response to a board range of BH3 peptides.

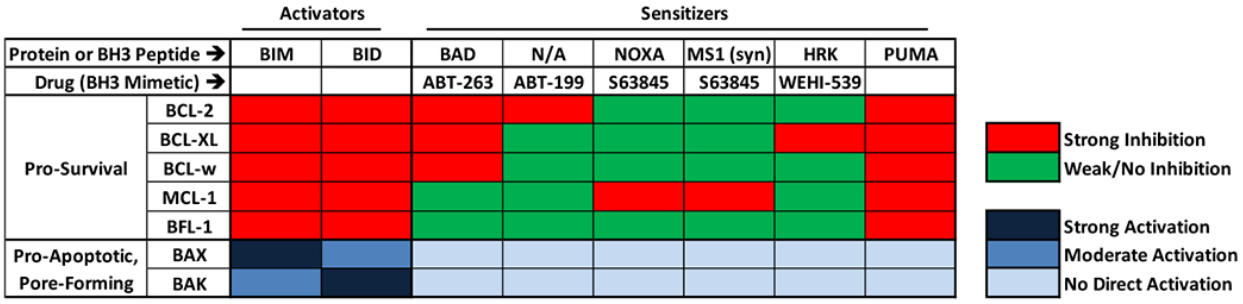


Figure 3. Binding pattern for BCL-2 family interactions.

Binding affinities for interactions between BH3 peptides generated from activator or sensitizer BH3-only proteins and their pro-survival and pro-apoptotic partners. Synthetic peptides are indicated by (syn) (Fraser et al., 2019).

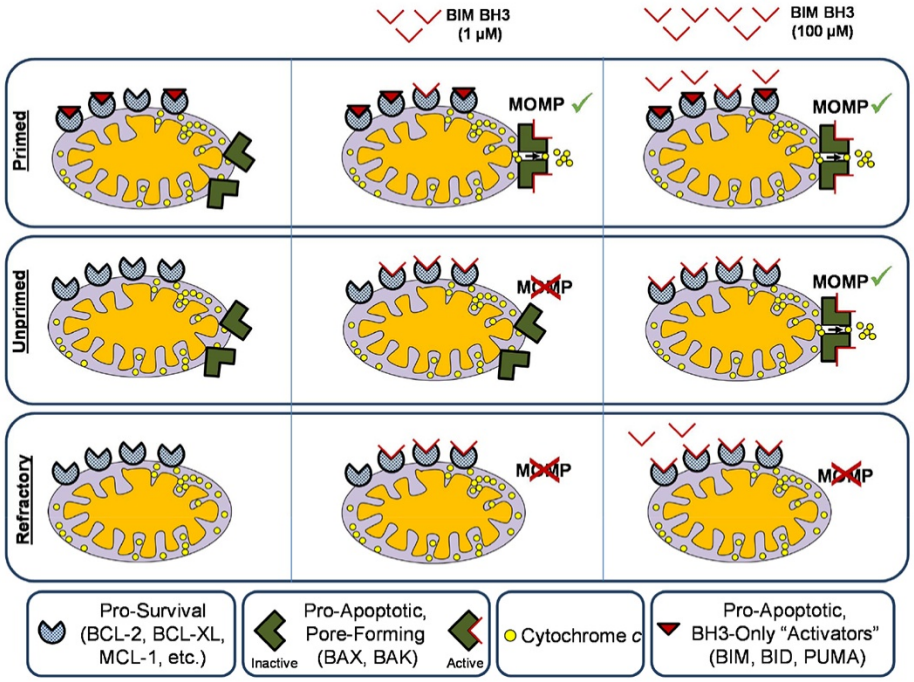


Figure 4. Response of three cell types (primed, unprimed and refractory) to pro-apoptotic BIM-BH3 induced apoptosis (Fraser et al., 2019).

2.2.9.1 Experimental procedure

Cells were plated in 6-well plates overnight at a density of 1×10^5 cells/well and treated with JQ1 and/or AZD4573 for 48 hours. After treatment, cells were separated using 0.25% trypsin, counted using the trypan blue exclusion assay and then washed twice with 5 ml cold PBS. The cells were then resuspended in 100 ul Zombie (1:500 in PBS) at room temperature (25 °C) in the dark for 25 min. After being spun down, the cells were cleaned in 1 ml PBS with 1% BSA. Then suspended at a density of 1×10^5 cells/ml in MEB buffer. Distribute 50ul cells to 96-well plate. Add 5ul 0.02% digit on in and various concentrations of peptide, make up the total liquid volume to 100 ul with MEB buffer. Alamethicin was used as a positive control for cytochrome c release and MEB buffer alone was used as the negative control. Wait 60 min at room temperature (25°C) in the dark. The cells were fixed by adding 40ul of 4% formaldehyde and terminated the peptide exposure and waiting for 12 minutes. Cells were washed twice to terminate the fixation using 1% PBS/BSA, and then add 90 µl 1% PBS/BSA to each well. Add 10 µl/well of the diluted anti-cytochrome c antibody (1:400) and stain at room temperature at 4 °C overnight. The results were analyzed by flow cytometry with the following approach.

2.2.9.2 FACS analysis for BH3 profiling.

The differences in cytochrome c negative cells can be examined between various situations/treatments can be investigated after selecting the population of interest. In our experiment, only zombie negative cells were included in the cytochrome c panel. The majority of the population in the negative control (DMSO) was cytochrome c positive. As a positive control, alamethicin, which results in the entire loss of cytochrome c, was used (Figure 5).

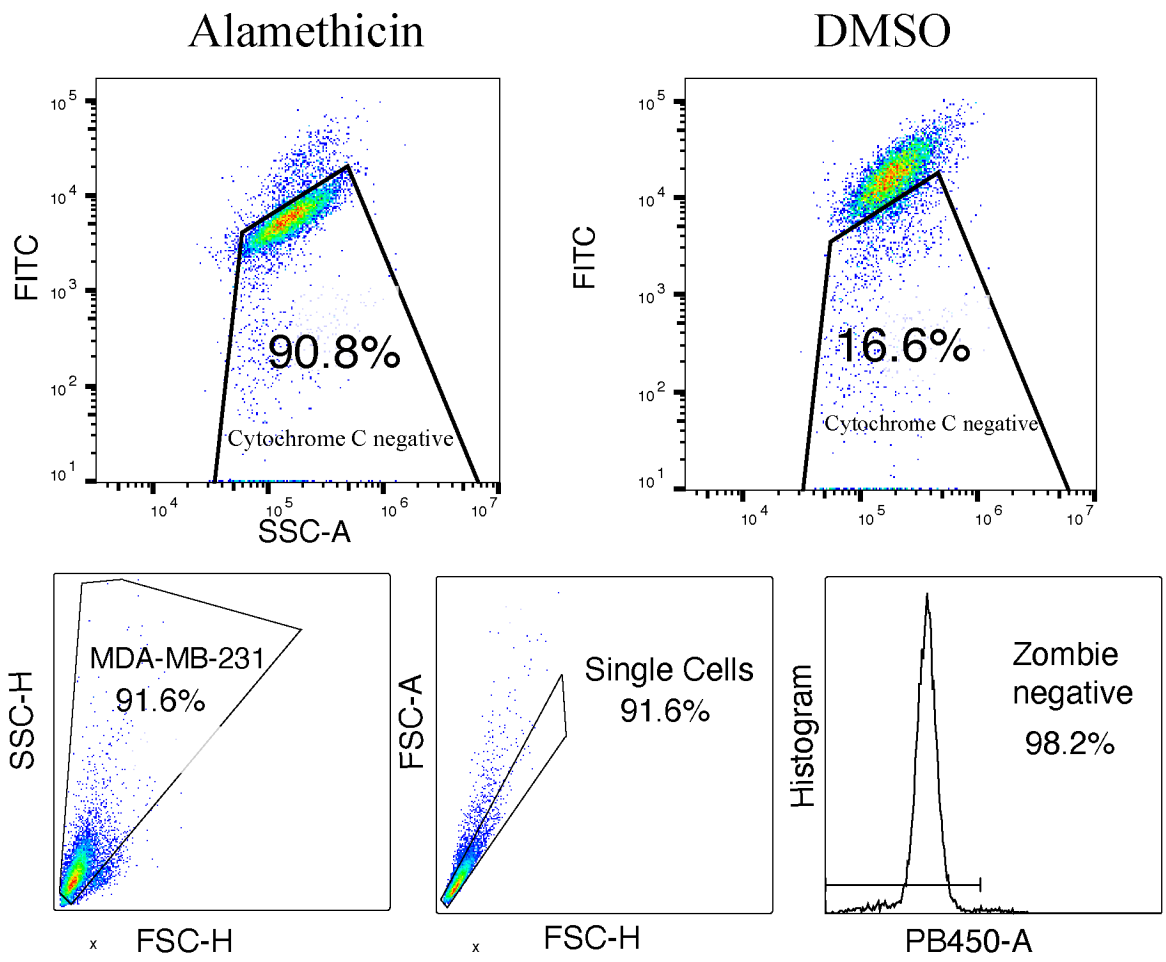


Figure 5. Workflow of FACS analysis for BH3 profiling.

The cell populations are selected by removing debris using the Forward Scatter (FSC) versus Side Scatter (SSC) method, multiple cells using the Forward Scatter Height (FSC-H) versus Forward Scatter Area (FSC-A) method, and non-viable cells (zombie positive cells) using the histogram method. Alamethicin-treated cells will not be stained positively for cytochrome c and be used to set a gate for cytochrome c negative cells. DMSO-treated cells will not loss cytochrome c and can be used to set a gate for cytochrome c positive cells. Setting gates in according with the positive control and negative control was the method used to quantify cytochrome c loss (Fraser et al., 2019).

2.2.10 Western blotting

Cells were placed in 10 cm culture dishes overnight at a density of 1×10^6 cells/well. Then treated with JQ1 and/or AZD4573 for 24 hours and 48 hours. After that, cells were transferred to 50 ml Falcon tubes using a cell scraper and washed twice with 5 ml cold PBS before transferring to 1.5 ml of Eppis. RIPA lysis buffer including protease inhibitor cocktail was added to the cell pellet, incubated on ice for at least 20 min then centrifuged at 13,000 rpm for 30 min and the supernatant

was stored at -20 °C and used for protein analysis. BCA test reagent was used to determine protein concentrations. In order to prepare for loading, 30-40 µg of each protein sample were taken, combined with SDS and loading buffer, denatured for 10 minutes at 95 °C, centrifuged at 4 °C and placed on ice. The markers and samples were loaded with a 50 µl syringe and two-dimensional electrophoresis was run from a low voltage of 50 to 100 volts, 25-30 mA for about 150 min until the running straits go down the bottom of the gel. The gel was retrieved and submerse into the transfer buffer for 30 minutes, and proteins were transferred using a semi-dry transfer method to a polyvinylidene difluoride (PVDF) membrane. The PVDF was activated with methanol for 1 min and then rinsed with transfer buffer before preparing the sandwich. Filter paper, Gel, PVDF, and then filter paper were used to create a transfer sandwich, ensuring there are no air bubbles between the gel and PVDF membrane, and squeezing out extra liquid. Membrane transfer condition: 15 voltages for 120 minutes. To check the transfer effect, the membrane was stained with Ponceau S staining reagent and blocked for 2 hours with 2% membrane blocking agent. Afterwards the membrane was shaken and incubated with primary antibody at 4 °C overnight. The next day the membrane was rinsed 3 times with PBST for 10 minutes each, the secondary antibody was added and incubated for 120 minutes at room temperature (25 °C) under gently shaking. The membrane was then rinsed 3 times with PBST for 10 minutes each and the results are detected by Immuno Cruz Western Blotting Luminol reagent, which illuminates the film after 2-60 minutes, depending on the antibody. Membranes were stripped with Re-blot reagent and rinsed 3 times with PBST for 10 minutes each. The transfer film was blocked with 2% film blocker for 30 minutes. The process was then repeated with another antibody.

2.2.10.1 Preparing the SDS Polyacrylamide-Gel (SDS-PAGE).

Table 12. The SDS Polyacrylamide-Gel (SDS-PAGE).

	Separating Gel 12%	**Separating Gel 8%**	**Stacking Gel 4%**
Distilled Deionized Water	4.3 ml	5,3 ml	3.2 ml
Acrylamide	3 ml	2.0 ml	0.5 ml
1.5M Tris-HCl (PH=8.8)	2.5 ml	2.5 ml	-
0.5M Tris-HCl (PH=6.8)	-	-	1.25 ml

10%SDS	100 µl	100 µl	50 µl
10% APS	100 µl	50 µl	50 µl
TEMED	10 µl	5 µl	10 µl
Volume (total)	10 ml	10 ml	5 ml

2.3 **Statistics**

Statistics were analyzed with R program (version 4.0) and GraphPad® Prism 9 software (GraphPad Software, La Jolla, USA). For normal distribution analysis, the Shapiro–Wilk test and Kolmogorov-Smirnov-Test were used. For two independent data, the Wilcoxon rank-sum test and unpaired t test (with Welch's correction in data without equal variances) were used; the paired t test or the Wilcoxon signed-rank test were used for two dependent data. For more than two independent samples, the Kurskal-Wallis test and one-way ANOVA (with Welch's correction in data without equal variances) were used. The half-maximal indices of IC50 were calculated using nonlinear regression analysis. P values <0.05 were assumed to be statistically significant. The following symbols were used: ns for non-significant, * for P<0.05, ** for P<0.01, *** for P<0.001 and **** for P<0.0001. Errors bars represent standard errors.

3 Results

3.1 Data analysis

3.1.1 BRD4 was over expressed in TNBC tissue and cell lines.

The TCGA database was used to evaluate the expression of the BET family genes in breast cancer tissues. And the results revealed that BRD4 mRNA expression was significantly greater in tumor tissues than in normal tissues, while BRD2, BRD3 and BRDT exhibited no statistically significant differences (Figure 6). Next, it was further analyzed the expression of BRD4 in various types of breast cancer tissues were then further examined. The expression of BRD4 mRNA was higher in TNBC than in other types of breast cancer tissues (Figure 7). The results were comparable in cell lines as well as at the tissue level (Figure 8).

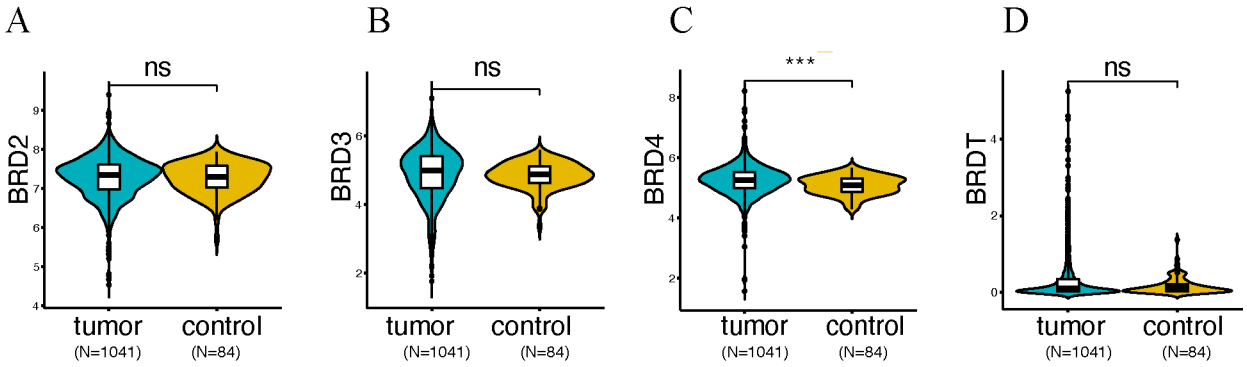


Figure 6. BET family gene expression in breast cancer tissues.

Breast cancer and normal tissues exhibit different levels of BRD2(A), BRD3(B), BRD4(C) and BRDT(D) mRNA expression. Ns for non-significant, *** for P<0.001. Errors bars represent standard errors.

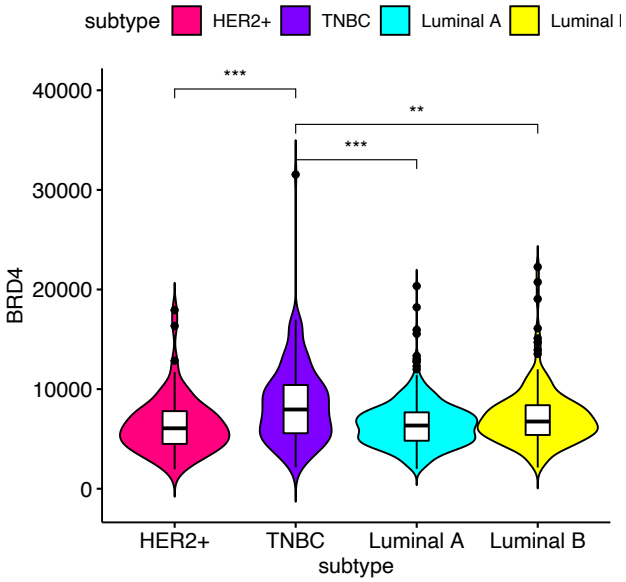


Figure 7. BRD4 mRNA expression in different subtype of breast cancer tissues.

TCGA samples' tumor subtype information is taken from the article (Berger et al., 2018). ** for P<0.01, and *** for P<0.001. Errors bars represent standard errors.

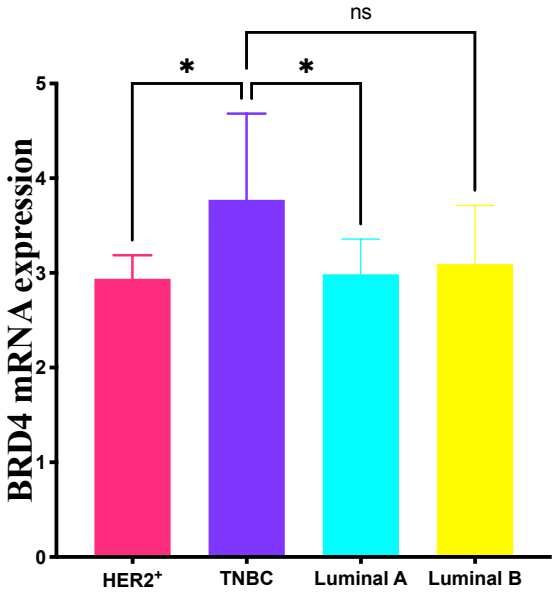


Figure 8. BRD4 mRNA expression in different breast cancer cell lines.

The classification of cell lines was based on the article (Dai et al., 2017). Ns for non-significant, * for P < 0.05, Errors bars represent standard errors.

3.1.2 BET family genes were barely mutated in breast cancer

Gene mutation is yet another issue with medication therapy. Mutations of the analysis of BET family gene mutations using TCGA data revealed that these genes were little mutated in comparison to the most mutated genes in breast cancer patients. BRD4 and BRDT mutations were

present in just 1% of patients (Figure 9).

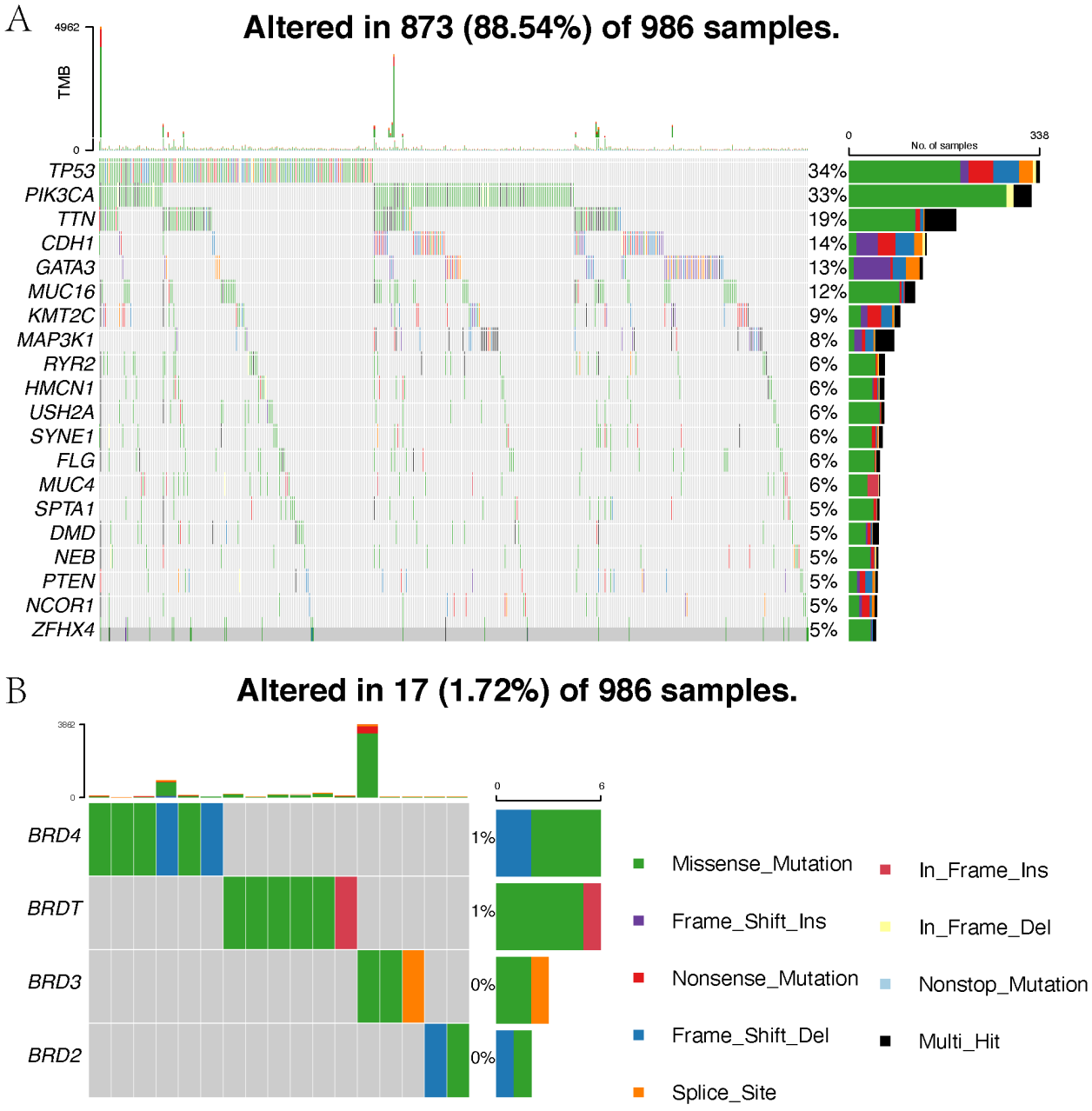


Figure 9. Gene mutation in TCGA breast cancer patients.

Genes from the BET family (B) were found in 17 samples, and the oncoplot of the top 20 most frequently mutated genes (A) were found in 873 samples. Each gene is arranged according to the total number of variants it contains, and the percentage next to each gene represents the ratio of tumor samples with its genetic alteration to the overall sample. Colored squares represent the presence and kind of gene mutation in the sample, while gray squares represent the absence of mutation. Genes that experience multiple mutations in the same sample are those with variants annotated as Multi_Hit.

3.1.3 BRD4 protein was highly expressed in breast cancer

The protein expression of BET family members in breast cancer tissues from HPA database was analyzed. Figure 10 showed the image of histological sections of BET family members. BRD4 was more commonly and more highly expressed than the other three members in the majority of patients (Table 13).

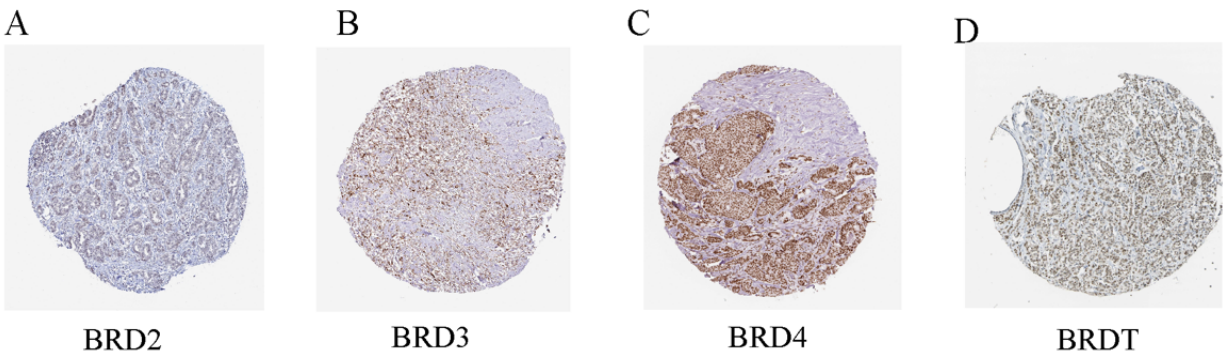


Figure 10. Protein expression of BET family gene BRD2 (A), BRD3 (B), BRD4 (C) and BRDT (D) in breast cancer patients.

The images of histological sections from cancer tissues obtained by immunohistochemistry. Samples were divided according to the level of antibody staining (high, medium, low and not detected), the intensity of staining (negative, weak, medium or strong) and the fraction of stained cells (< 25%, 25-75% or > 75%).

Table 13. Expression of BET family protein in breast cancer

	High	Medium	Low	not detected	Total patients
BRD2			2	9	11
BRD3		11		1	12
BRD4	8	3	0		11
BRDT	5	5	1		11

3.2 **BET inhibitors suppressed cell proliferation**

First, I started by researching the therapeutic effects of the BET inhibitors JQ1 and I-BET762 in breast cancer cell lines (Figure 11). The TNBC cell line, MDA-MB-231, is sensitive to BET inhibitors and the other TNBC cell line MDA-MB-436 is relatively resistant to BET inhibitors.

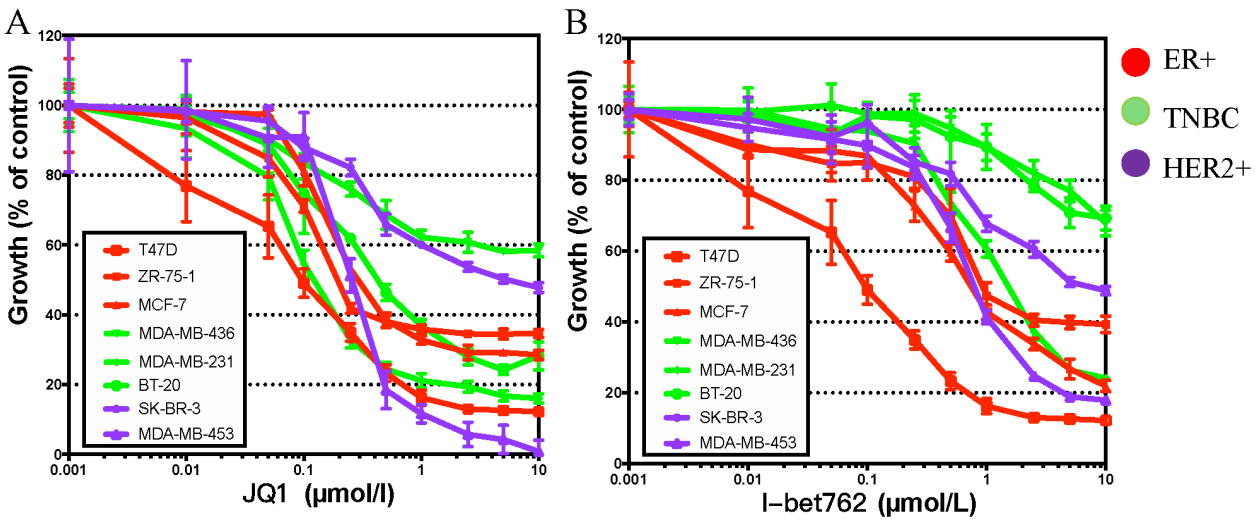


Figure 11. The different subtypes of breast cancer cells were treated with the BET inhibitor JQ1 (A) or I-BET762 (B) for 96 hours and cell viability was assessed by MTT assay. Errors bars represent standard errors.

3.2.1 JQ1 induced cell cycle G1 arrest in MDA-MB-231, but not in MDA-MB-436

In most cancer cell lines, BET inhibitor can cause cell cycle G1 arrest. The cell cycle of MDA-MB-231 and MDA-MB-436 treated with various concentrations of JQ1 for 48 hours was analyzed in our experiment using flow cytometry. In MDA-MB-231, G0-G1 populations were 59.2% (JQ1-0.1μmol/L) and 64.7% (JQ1-1.0μmol/L) as compared with 39.8% in controls. In MDA-MB-436, G0-G1 populations were 33.0%, 34.7% and 39.6% respectively. It was found that JQ1 enhanced the proportion of cells in G1 phase in sensitive MDA-MB-231cell line, but not significantly in main resistant MDA-MB-436 cell line (Figure 12).

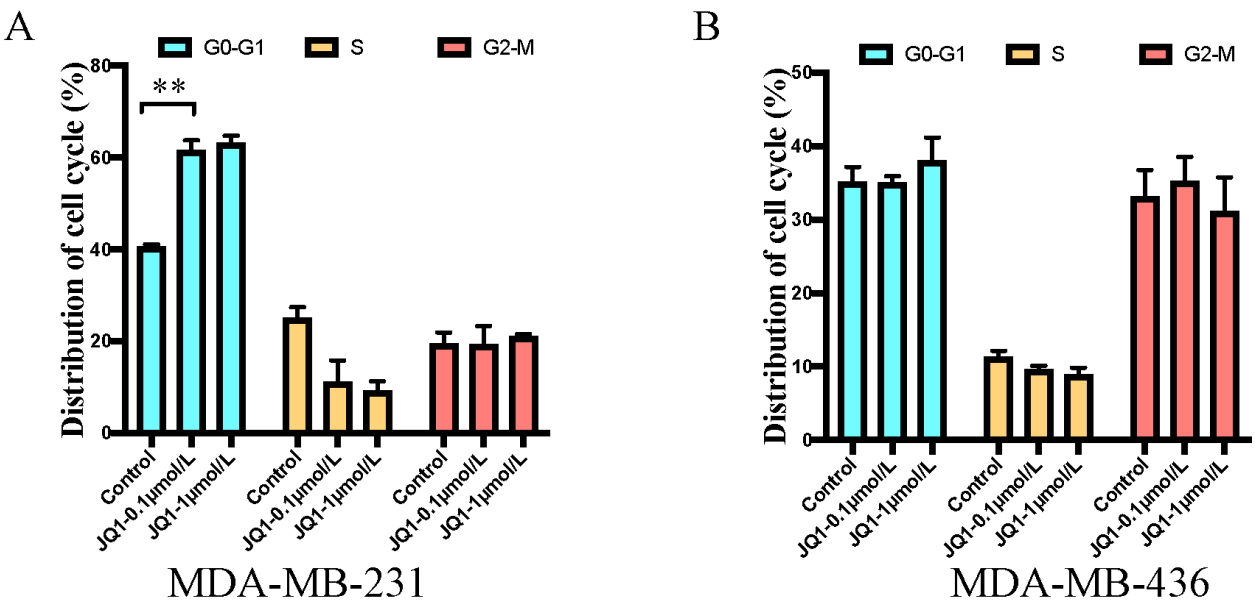


Figure 12. Analysis of cell cycle after treatment with different doses of JQ1 in MDA-MB-231 (A) or MDA-MB-436 (B).

A total of 1×10^5 cells were treated with indicated concentration of JQ1 for 48 hours. ** for $P < 0.01$. Errors bars represent standard errors.

3.2.2 JQ1 triggered cell senescence in both TNBC cell lines

MDA-MB-231 and MDA-MB-436cells were treated with varying concentrations of JQ1 for 72 hours, as shown in Figure 13, and the senescent cells were stained with senescence-associated beta-galactosidase. The results indicate that JQ1 could induce TNBC cells senescence.

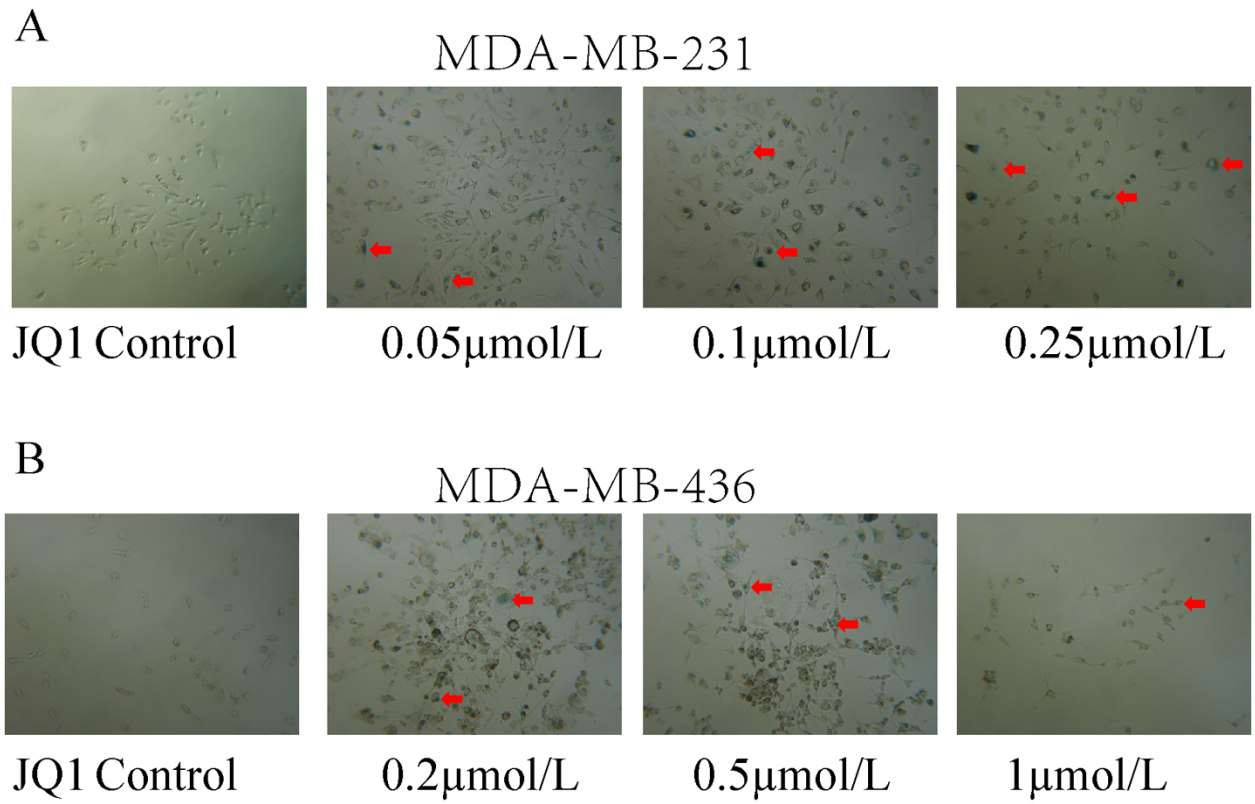


Figure 13. Analysis of cell senescence after treatment with different doses of JQ1 in MDA-MB-231 (A) or MDA-MB-436 (B) cells.

A total of 1×10^4cells were treated with indicated concentration of JQ1 for 72 hours, and cellular senescence was measured using Senescence-ß-Galactosidase Staining Kit. The senescent cells were indicated by red arrows.

3.2.3 Minor apoptosis inducted by JQ1 in both TNBC cell lines

The cells were incubated to JQ1 or DMSO for 48 hours, and Annexin V/PI staining test was used to track apoptosis. Figure 14 showed that in MDA-MB-231cells, high doses of JQ1 induced minimal cell apoptosis, while low doses of JQ1 did not cause any cell death. In MDA-MB-436 cells, no significant change in apoptosis was observed at either dose.

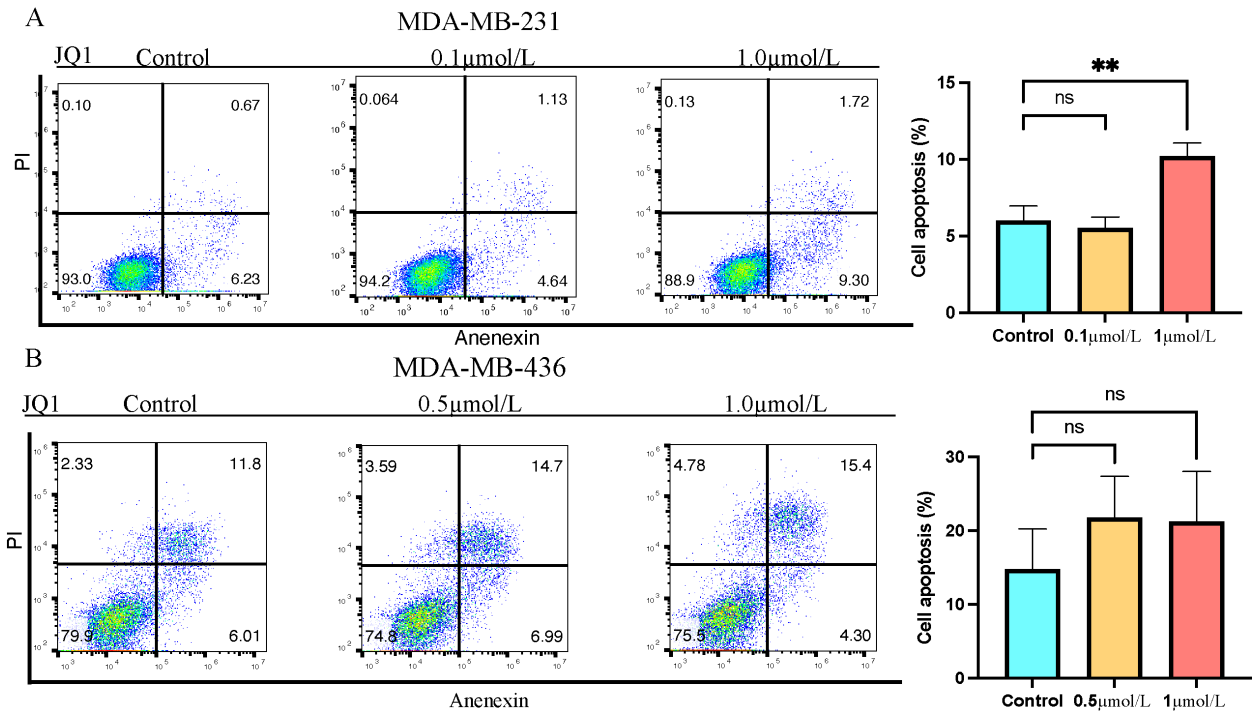


Figure 14. Analysis of cell apoptosis after treatment with different doses of JQ1 in MDA-MB-231 (A) or MDA-MB-436 (B) cells.

After 48 hours of treatment with DMSO or JQ1 (0.1 μmol/L, 1 mmol/L), apoptotic cells in the MDA-MB-231 and MDA-MB-436 cells were detected by Annexin V/PI staining and flow cytometry. Ns for non-significant, ** for $P < 0.01$. Errors bars represent standard errors.

3.3 **MDA-MB-231R was resistant to JQ1 and I-BET762**

Drug resistance index (DRI) was used to measure the impact of drug resistance. Drug resistance is measured by the IC50 ratio of drug-resistant cells to parental cells, which is referred to as DRI. Resistance is regarded as DRI > 5. The IC50 values of JQ1 for MDA-MB-231 and MDA-MB-231R were 0.24 μmol/L and 10.21 μmol/L, respectively, with a DRI of 42.54, as shown in Figure 15. I created a resistant cell line that does indeed resistant to JQ1. It's interesting, this cell line is also resistant to another BET inhibitorI-BET762.

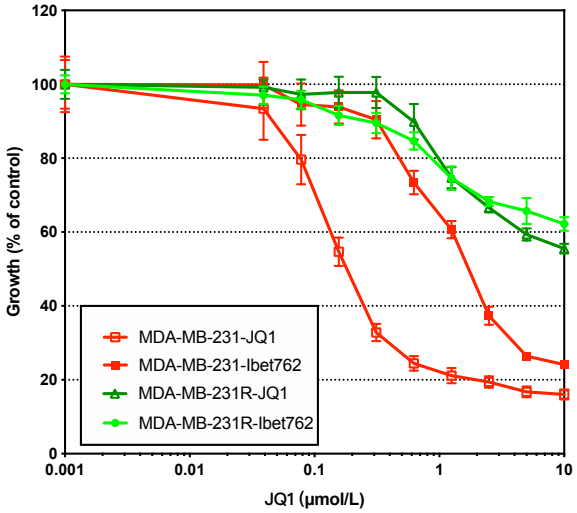


Figure 15. Cell viability of MDA-MB-231 and MDA-MB-231R after drugs treatment.

Cell viability was measured via MTT assay after 2×10^3 cells were treated with indicated concentrations of JQ1 or I-BET762 for 96 hours. Errors bars represent standard errors.

3.4 Drug screening suitable for combination therapy revealed the strongest efficacy of CDK9 and BET inhibitors

JQ1 was combined with a number of small molecular inhibitors to increase its anti-tumor effectiveness. Cells were treated with JQ1 and various small molecules for 72 hours and the drug responses of the cells were measured via MTT assay in MDA-MB-231 (Figure 16) and MDA-MB-436 (Figure 17) cells.

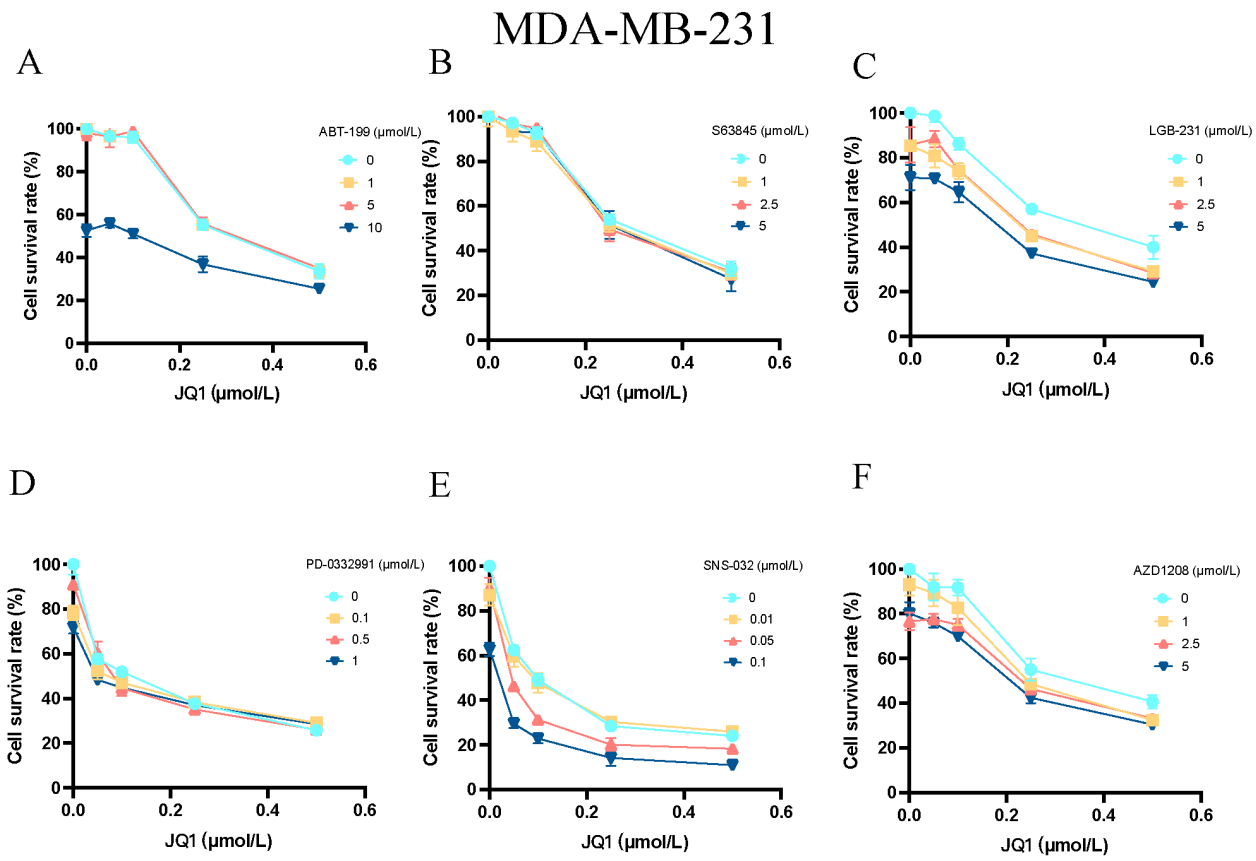


Figure 16. Cell viability of MDA-MB-231 after combined drugs treatment.

Cell viability was measured using MTT assay after 2×10^3 cells were treated for 96 hours with indicated concentrations of JQ1 and the other drug, including BCl-2 inhibitor ABT-199 (A), MCL-1 inhibitor S63845 (B), PIM inhibitor LGB231 (C), CD4/6 inhibitor PD0332991 (D), CD2/7/9 inhibitor SNS032 (E), and JAK2 inhibitor AZD1408(F). Errors bars represent standard errors.

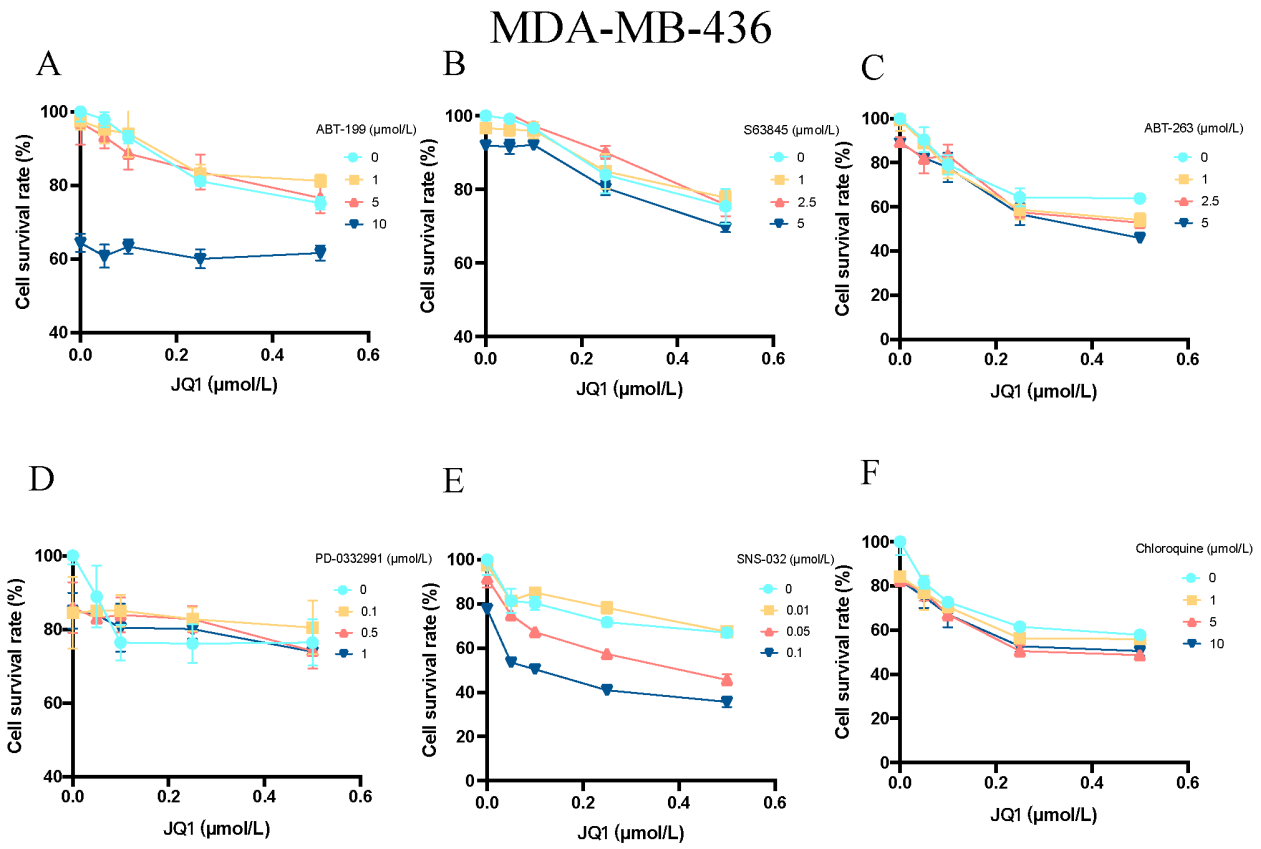


Figure 17. Cell viability of MDA-MB-436 cells after combined drugs treatment.

Cell viability was measured using MTT assay after 2×10^3 cells were treated for 96 hours with indicated concentrations of JQ1 and the other drug, including BCl-2 inhibitor ABT-199 (A), MCL-1 inhibitor S63845 (B), BCL-2/BCL-xL/BCL-w inhibitor ABT-263 (C), CD4/6 inhibitor PD0332991 (D), CD2/7/9 inhibitor SNS032 (E), and autophagy inhibitor chloroquine (F). Errors bars represent standard errors.

According to the results of several drug combinations, it was found that SNS-032, a CDK2/7/9 inhibitor, in combination with JQ1 had the strong combination effect in inhibiting the growth in both TNBC cell lines. More research was done into the effects of combining either a CDK2, CDK7, or CDK9 inhibitor with JQ1, respectively, and it was found that the CDK9 inhibitor AZD4573 combined with JQ1 had the strongest inhibitory impact (Figure 18).

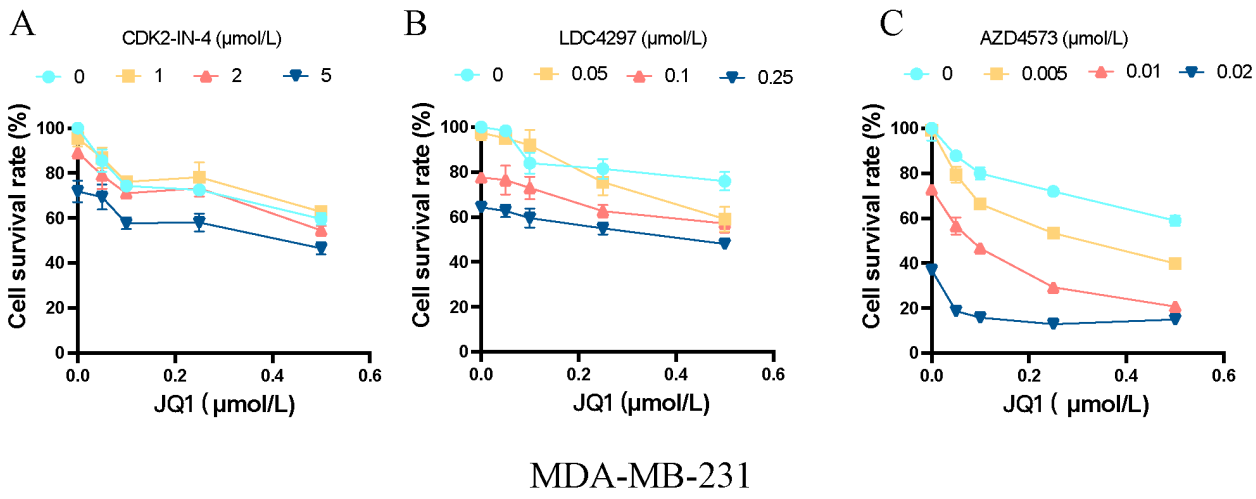


Figure 18. Cell viability of MDA-MB-231 after combined drugs treatment.

In TNBC cell lines MDA-MB-231 via MTT assay after 2 × 10^3 cells were treated with indicated concentrations of JQ1 and CDK2 inhibitor CDK2-IN-4 (A), CDK7 inhibitor LDC4297 (B) and CDK9 inhibitor AZD4573 (C). Errors bars represent standard errors.

The combination effect of CDK9 and BET inhibitors was evaluated using calcusyn software. The results showed that the CI calculated from the various drug dose combinations was less than 1, implying that the combination of these two drugs had a synergistic effect in not only sensitive MDA-MB-231 cells, but also primary resistant cell MDA-MB-436 and secondary resistant cells MDA-MB-231R (Figure 19).

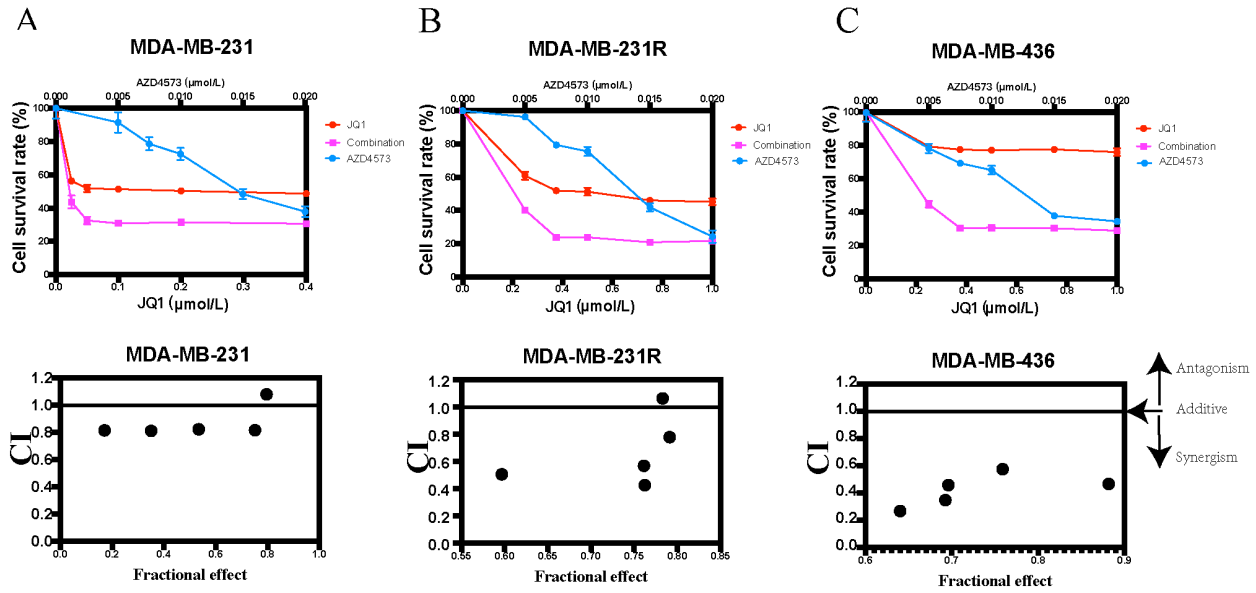


Figure 19. Analysis of drug combination effect in TNBC cell lines.

After being exposed to the prescribed dosages of JQ1 and/or CDK9 inhibitor AZD4573 in TNBC cell lines, a total of 2 x

10^3 cells were analyzed via MTT assay. The effect of combination therapy in MDA-MB-231(A), MDA-MB-231R (B) and MDA-MB-436 (C) was accessed by calcusyn software. CI defines Synergism (CI < 1), Additive Effect (CI = 1) and Antagonism (CI > 1). Errors bars represent standard errors.

3.4.1 JQ1 did not induce cell cycle G1 arrest in MDA-MB-231R cells

Initially, the cell cycle was measured between the sensitive and resistant cells of MDA-MB-231. Cell cycle was measured using PI staining assay after the cells were treated with JQ1 or DMSO for 48 hours. G1 populations were 44.4% (JQ1-0.1 μmol/L) and 49.5% (JQ1-1.0 μmol/L) compared with 35.9% in control in MDA-MB-231R. The result showed that JQ1 did not induce G1 cell cycle arrest in this resistant cell line, similar to primary resistant MDA-MB-436 cells （Figure 20）.

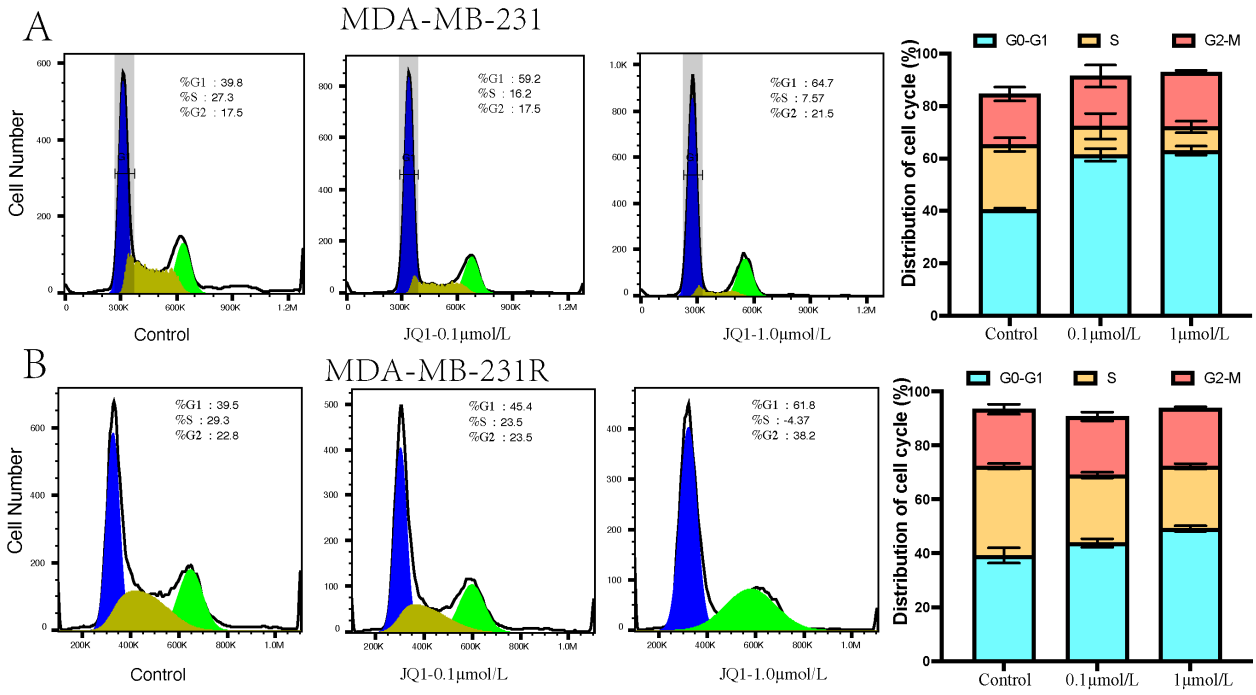


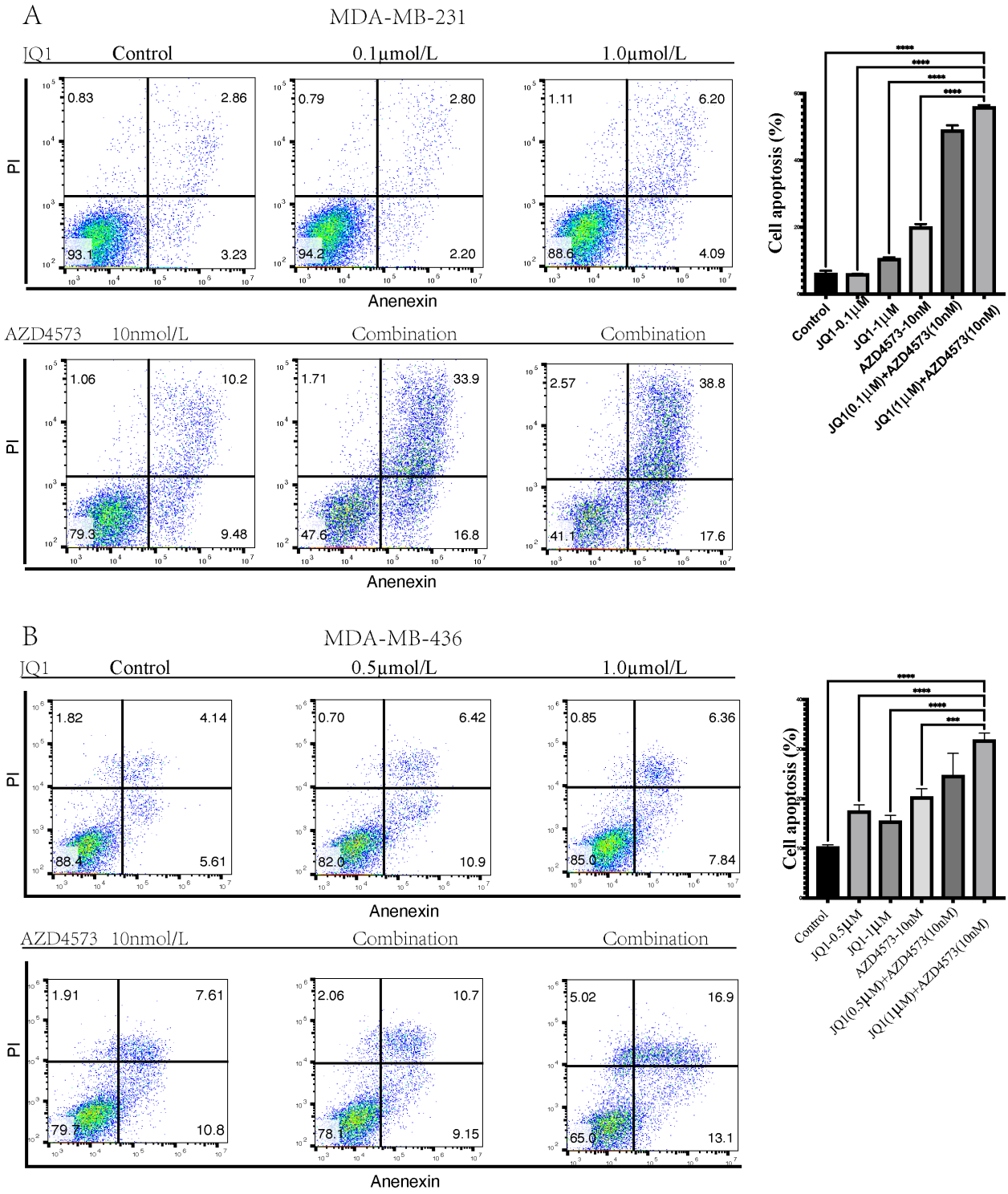
Figure 20. Cell cycle analysis via flow cytometer after treatment with different doses of JQ1 in MDA-MB-231 (A) or MDA-MB-231R (B).

A total of 1×10^5 cells were treated with indicated concentration of JQ1 for 48 hours. Errors bars represent standard errors.

3.4.2 Combination therapy significantly increased cell apoptosis

The cells were cultured with various drugs (both alone and in combination) for 48 hours, and cell apoptosis was measured using annexin-V/PI staining assay. In comparison with JQ1 treatment alone, which only induced minor apoptosis, the combination treatments with CDK9 inhibitor AZD4573 slightly raised cell apoptosis, not only in sensitive cell line but also in primary and

secondary resistant cell lines (Figure 21).



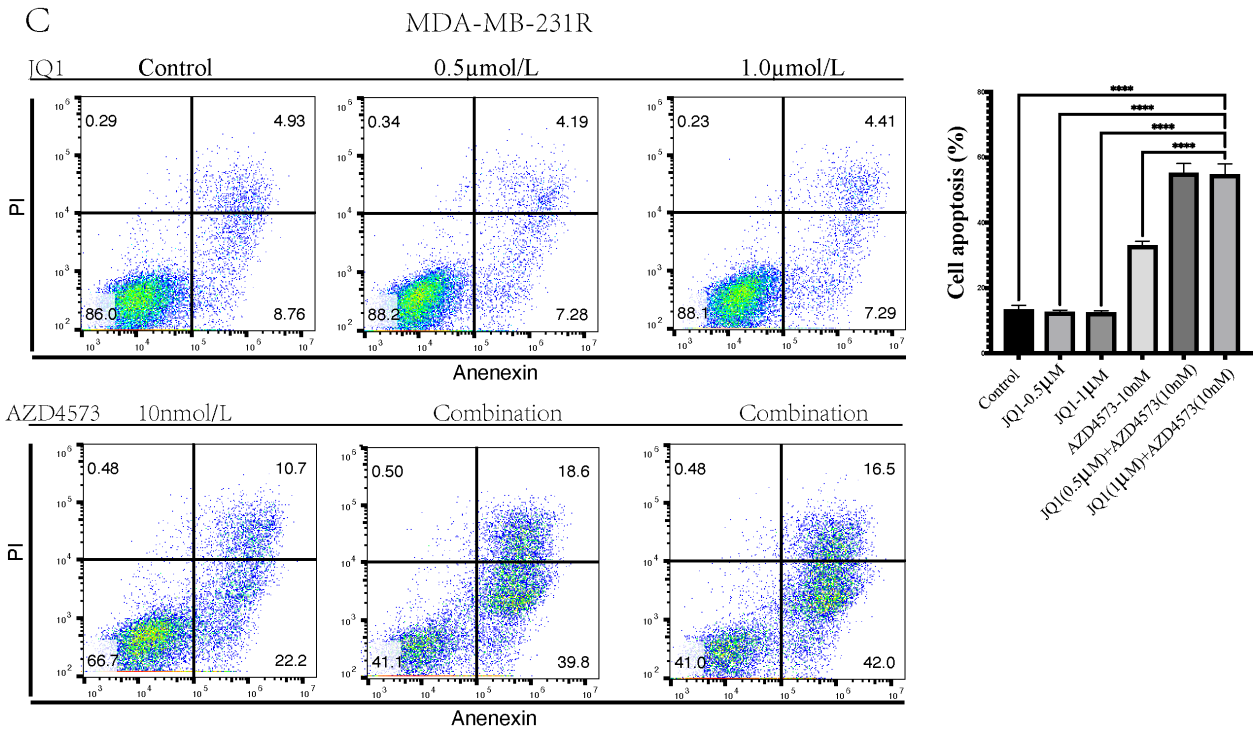


Figure 21. Cell apoptosis analysis via flow cytometer after treatment with different doses of drugs in MDA-MB-231 (A), MDA-MB-436 (B) and MDA-MB-231R (C) cells.

Cells were treated with the indicated concentration of JQ1 and/or AZD4573 for 48 hours. At the end of treatment, cells were washed with staining buffer and stained with Annexin V/PI. Using flow cytometry, apoptotic cells that included Annexin V^+/PI^- and Annexin V^+/PI^+ were identified. Cell apoptosis (%) in bar graph included early apoptosis (Annexin V^+/PI^-) and late apoptosis (Annexin V^+/PI^+). *** for $P < 0.001$, **** for $P < 0.0001$. Errors bars represent standard errors.

I also performed BH3 profiling analysis, another method to evaluate cell apoptosis *in vitro*. The cells were cultured with drugs (alone and in combination) for 48 hours, and cell apoptosis was measured using BH3 profiling staining technique. In each treatment group, the percentage of zombie-positive cells was determined. The percentage of cytochrome c loss in MDA-MB-231(A) was 9.34% (control group) and 26.1% (combination therapy group). In MDA-MB-231R (B), it was8.50% (control group) and 34.2% (combination therapy group), and MDA-MB-436, 12.2% (control group), 26.7% (combination therapy group). The proportion of Zombie-positive cells was significantly increased in the combination treatment group as compared with the control and monotherapy groups (Figure 22). In line with the earlier findings, the combo treatments enhanced cytochrome c loss, which implies increased cell apoptosis (Figure 23).

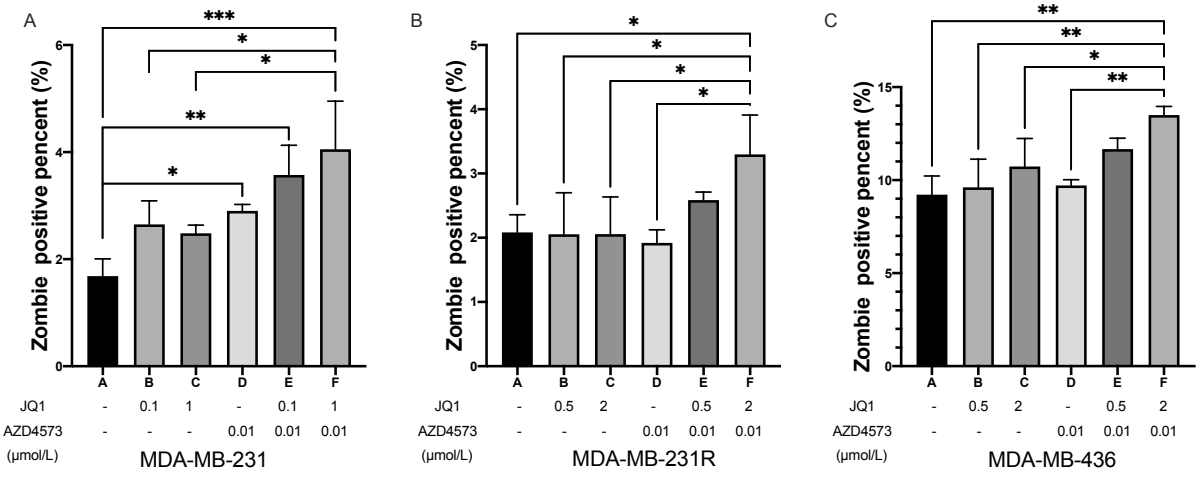
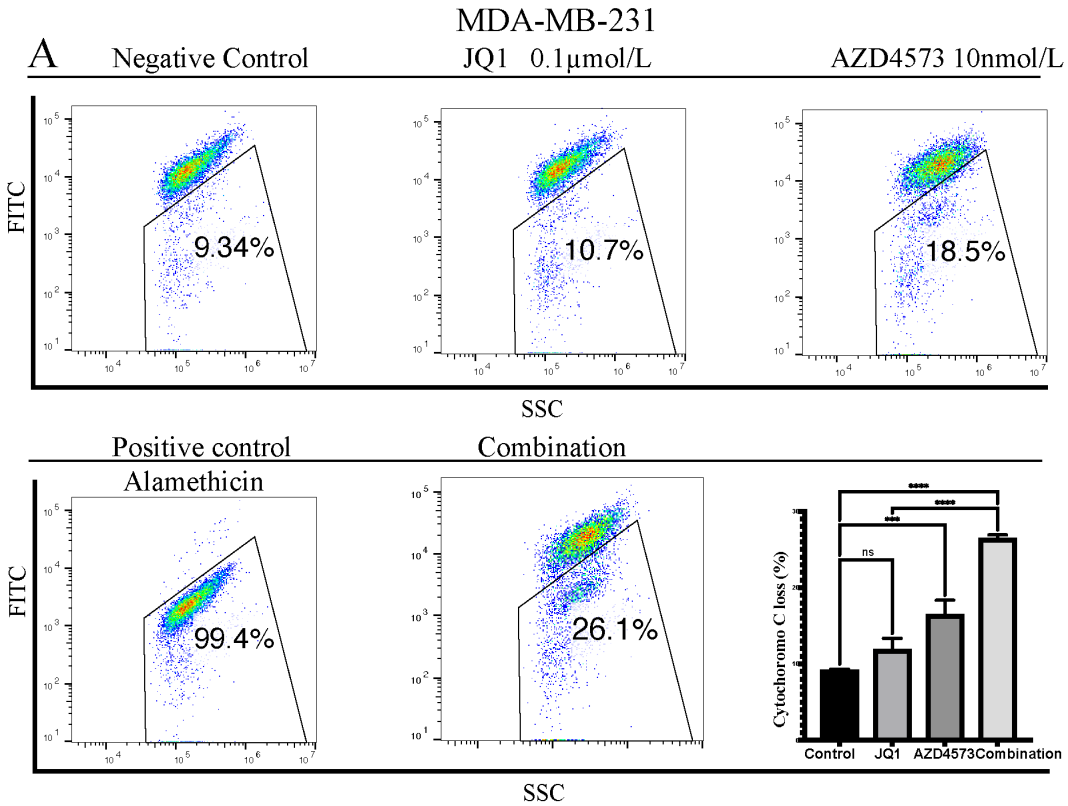


Figure 22. Cell death analysis by the proportion of zombie-positive cells after treatment with different doses of drugs in MDA-MB-231 (A), MDA-MB-231R (B) and MDA-MB-436 (C).

Cells were treated with the indicated concentrations of JQ1 and/or AZD4573 for 48 hours. At the end of treatment, cells were washed with staining buffer and stained with zombie antibody. Zombie positive cells were used as a marker of cell death in MDA-MB-231, MDA-MB-231R and MDA-MB-436 were shown. * for P<0.05, ** for P < 0.01, and *** for P<0.001. Errors bars represent standard errors.



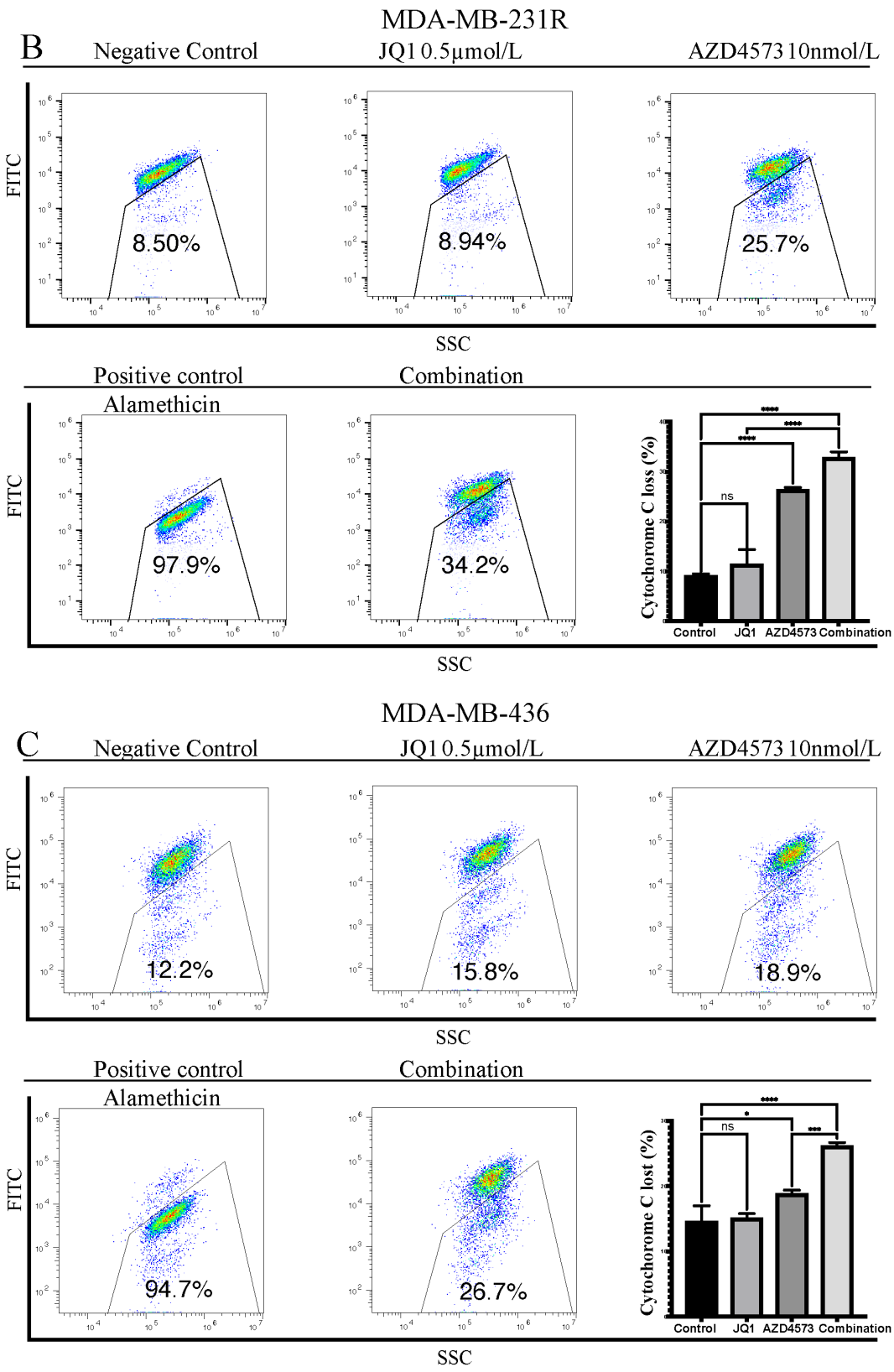


Figure 23. Cell apoptosis analysis by loss of cytochrome c after combination treatment in MDA-MB-231 (A), MDA-MB-231R (B) and MDA-MB-436 (C).

Cells were treated with the indicated concentrations of JQ1 and/or AZD4573 for 48 hours. At the end of treatment, cells

were washed with staining buffer and stained with cytochrome c antibody as described in methods. Using flow cytometry, it was possible to calculate the percentage of cytochrome c loss, which indicates apoptotic cells proportion. ns for non-significant, * for $P < 0.05$, ** for $P < 0.01$, *** for $P < 0.001$, and **** for $P < 0.0001$. Errors bars represent standard errors.

3.5 Three TNBC cells were primed for apoptosis

It was now interesting to learn the mechanism of apoptosis induction by combination therapy. According to the article, it is known BCL2 family proteins play key roles in cell apoptosis. Therefore, The BH3 profiling was used to analyze whether and which BCL2 family proteins were involved in apoptosis induced by the combination therapy.

First, I started by determining if the three TNBC cell lines were apoptotic primed, unprimed, or apoptosis refractory. After cells were washing and adding various concentrations of BIM or PUMA peptide for 1 hour, the depletion of cytochrome c in the cells was determined using BH3 profiling staining assay. Because they may bind to all anti-apoptotic proteins, the BIM and PUMA peptide can be utilized to assess the total level of priming in a cell. The difference between these two peptides is that the former activates BAX and/or BAK directly, while the later does not. The required dose to induce MOMP is inversely correlated with the level of priming of the cells (e.g., high dose of BIM required indicates that the cells have a low level of priming). The proportion of cytochrome c loss in this case was 9.62% (control) and 53.9% (1 μmol/L BIM) in MDA-MB-231, 5.33% (control) and 22.6% (1 μmol/L BIM) in MDA-MB-231R and 12.2% (control) and 23.6% (1 μmol/L BIM) in MDA-MB-436. After treatment with low doses of BIM or PUMA peptide, three TNBC cell lines had cytochrome c depletion, indicating that these three TNBC cells are ready to be primed for apoptosis. 1 μM of either PIM or PUMA peptides induced higher level of cytochrome c loss in MDA-MB231 cells than in MDA-MB-231R and MDA-MB-436 cells suggesting that MDA-MB-231 cells were more primed than the other 2 cell lines (Figure 24).

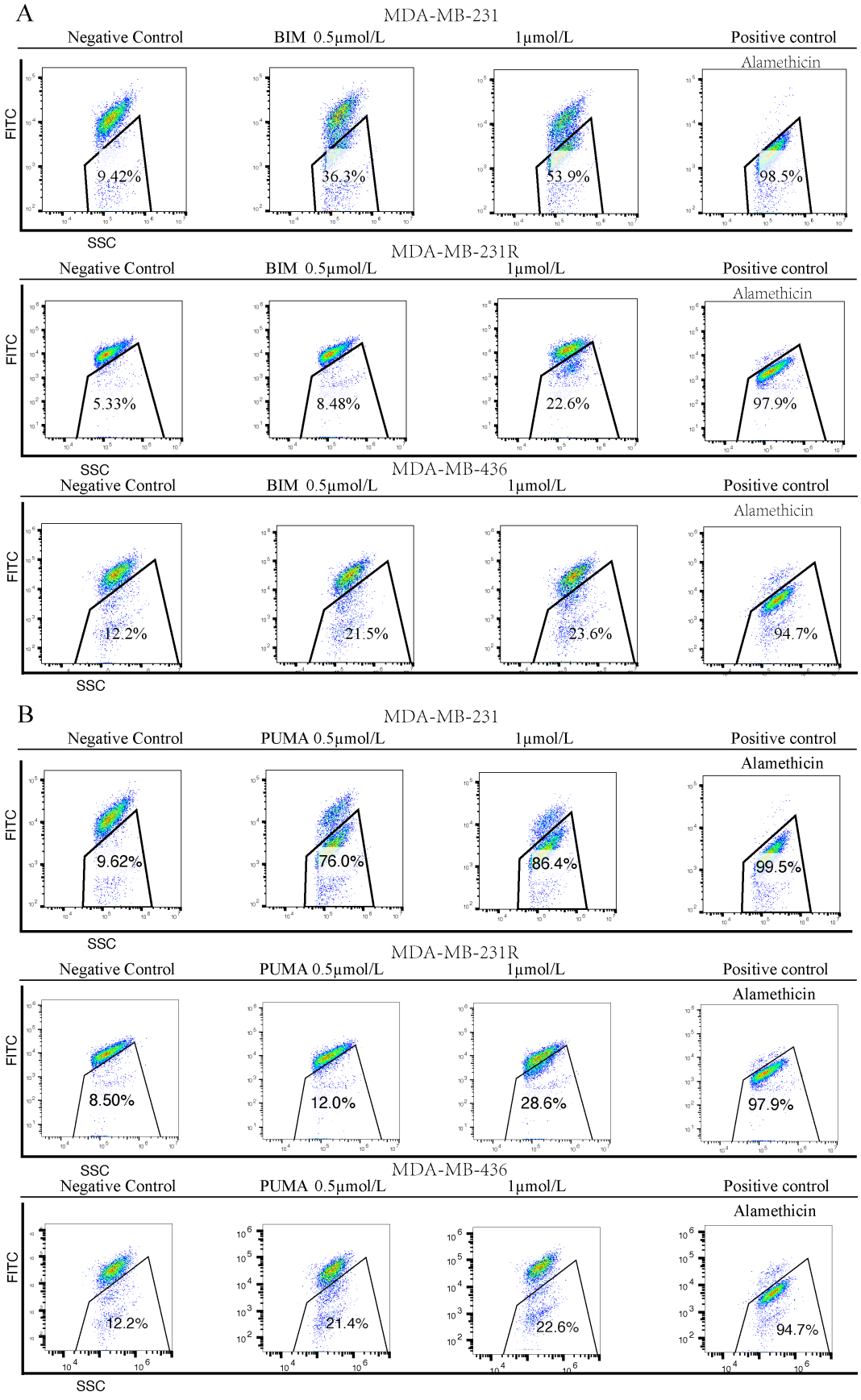


Figure 24. Cell apoptosis analysis via flow cytometer after treatment with different concentrations of BIM

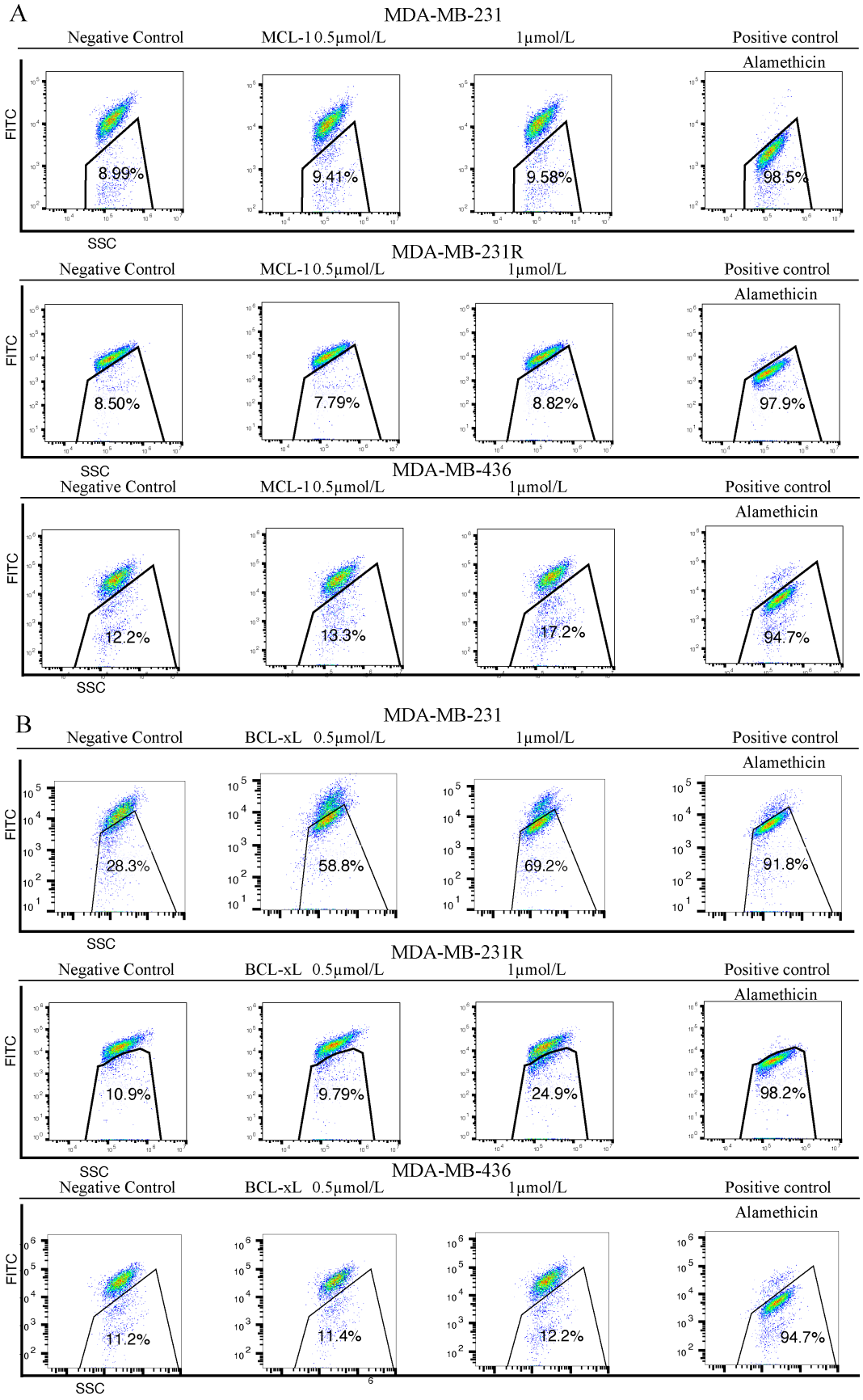
(A) or PUMA(B) peptide in MDA-MB-231, MDA-MB-231R and MDA-MB-436 cells.

Cells were washing and added various concentrations of BIM (A) or PUMA (B) peptide for 1 hour, then measured the cell cytochrome c loss using BH3 profiling staining assay.

3.6 **MDA-MB-231 and MDA-MB-231R were dependent on BCL-xL for survival**

Although certain anti-apoptotic proteins can be selectively inhibited by the BH3-only sensitizer proteins, BAX or BAK are not as strongly activated as BIM. Peptides that mimic these sensitizing proteins can be used to determine whether a cell is dependent on certain specific pro-survival proteins to survival.

Three TNBC cell lines were tested to see if they were dependent on MCL-1, BCL-xL and A1 for survival using the peptides MCL-1, BCL-xL, and A1. It was found low dose of BCL-xL peptide could induce increased cytochrome c loss in MDA-MB-231 and MDA-MB-231R cells, but not MCL-1or A1 peptides. The percentage of cytochrome c loss was 28.3% (control) and 69.2% (1 μmol/L BCL-xL) in MDA-MB-231, 10.9% (control) and 24.9% (1 μmol/L BCL-xL) in MDA-MB-231R, whereas all these three peptides did not cause cytochrome c loss in MDA-MB-436 cells (Figure 25). This suggested that MDA-MB-231 and MDA-MB-231R cells were dependent on BCL-xL for survival whereas MDA-MB-436 cells were not.


A
MDA-MB-231
Negative Control
MCL-1 0.5μmol/L
1μmol/L
Positive control
Alamethicin
FITC
SSC
8.99%
9.41%
9.58%
98.5%
MDA-MB-231R
Negative Control
MCL-1 0.5μmol/L
1μmol/L
Positive control
Alamethicin
FITC
SSC
8.50%
7.79%
8.82%
97.9%
MDA-MB-436
Negative Control
MCL-1 0.5μmol/L
1μmol/L
Positive control
Alamethicin
FITC
SSC
12.2%
13.3%
17.2%
94.7%
B
MDA-MB-231
Negative Control
BCL-xL 0.5μmol/L
1μmol/L
Positive control
Alamethicin
FITC
SSC
28.3%
58.8%
69.2%
91.8%
MDA-MB-231R
Negative Control
BCL-xL 0.5μmol/L
1μmol/L
Positive control
Alamethicin
FITC
SSC
10.9%
9.79%
24.9%
98.2%
MDA-MB-436
Negative Control
BCL-xL 0.5μmol/L
1μmol/L
Positive control
Alamethicin
FITC
SSC
11.2%
11.4%
12.2%
94.7%

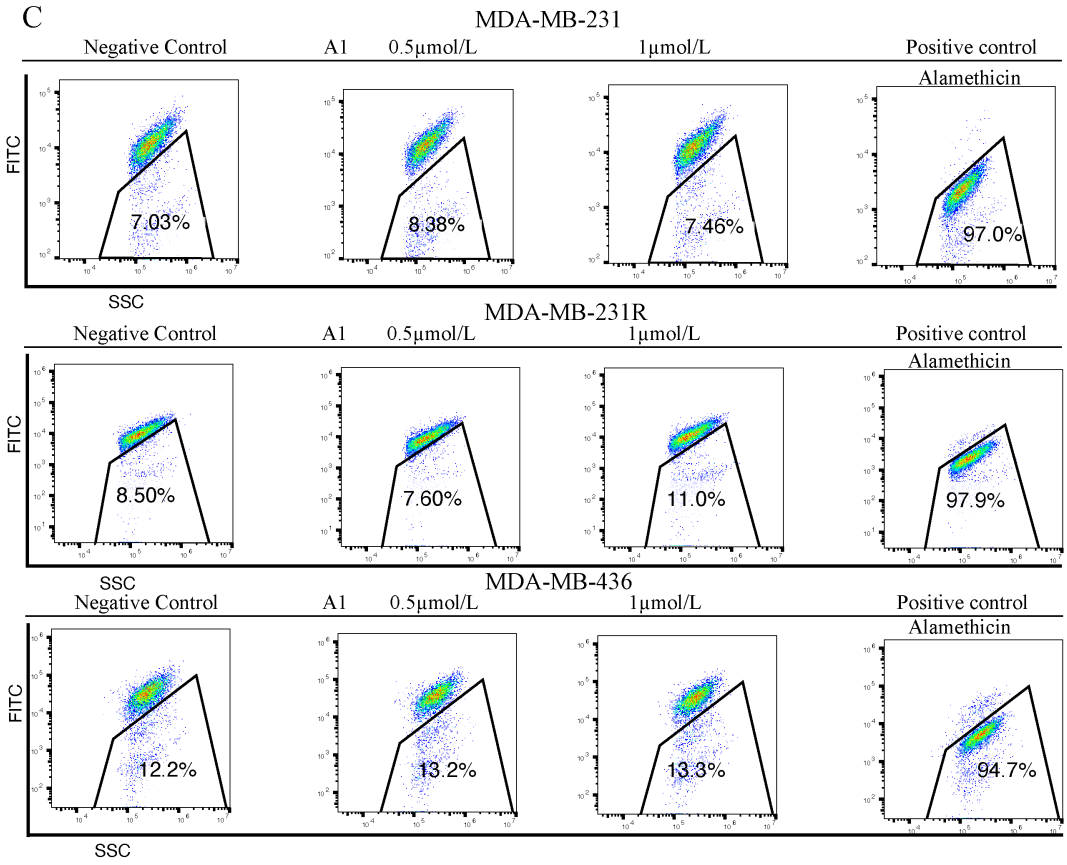


Figure 25. Cell apoptosis analysis via flow cytometer after treatment with different concentrations of MCL-1 (A), BCL-xL (B) and A1 (C) peptide in MDA-MB-231, MDA-MB-231R and MDA-MB-436 cells. Cells were washing and added various concentrations of MCL-1 (A), BCL-xL (B) and A1 (C) peptide for 1 hour, then measured the cell cytochrome c loss using BH3 profiling staining assay.

The findings were confirmed using BCL-2 family inhibitors via MTT assay. Here, the cells were treated with several tiny molecular inhibitors for 72 hours, then measured the cell viability via MTT assay. The result showed MDA-MB-231 was sensitive to A1331852 (BCL-xL inhibitor) but not to s63845 (MCL-1 inhibitor) and ABT199 (BCL2 inhibitor). MDA-MB-436 was relatively resistant to all three small molecular inhibitors (Figure 26).

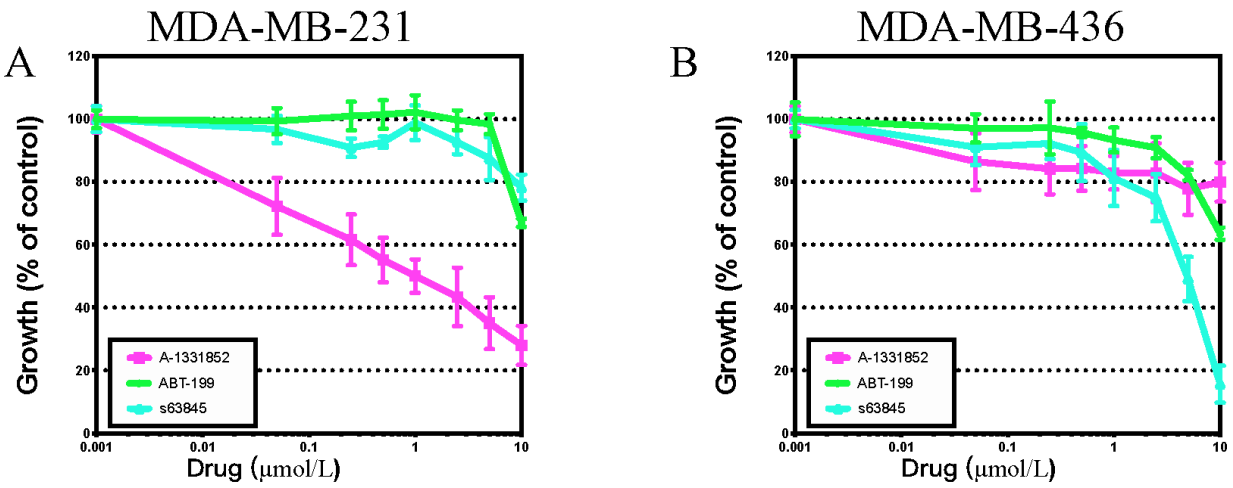


Figure 26. Cell viability of MDA-MB-231 (A) and MDA-MB-436 (B) cells after treatment of BCL-2 family inhibitors.

A total of 2×10^3 cells were treated with indicated concentrations of A1331852 (BCL-xL inhibitor), ABT199 (BCL-2 inhibitor) and s63845 (MCL-1 inhibitor) for 72 hours, then measured the cell viability via MTT assay. Error bars represent standard errors.

Also, the BH3 profiling analysis was carried out utilizing cells that pretreated with JQ1 and AZD4573, and the BCL-xL inhibitor A1331852 was utilized to replace the BCL-xL peptide. Before the BH3 profile assay, the cells were cultured with JQ1 and AZD4573 for 48 hours. The cells were then washed and the BH3 profiling was performed to measure the cytochrome c loss in response to the BCL-xL inhibitor A1331852. As shown in Figure 27, combination treatment caused cytochrome c loss after 48 hours, and this loss persisted when BCL-xL was further inhibited by A1331852 in MDA-MB-231 cells. MDA-MB-231R cells were not so sensitive to the 48 hours combination treatment but release of cytochrome c also increased if BCL-xL was inhibited by A1331852. These results not only strongly support the prior finding that MDA-MB-231 and MDA-MB-231R cells are dependent BCL-xL to survive, but also suggested that inhibition BCL-xL may be one of the mechanisms underlying the apoptosis induction by the combination treatment.

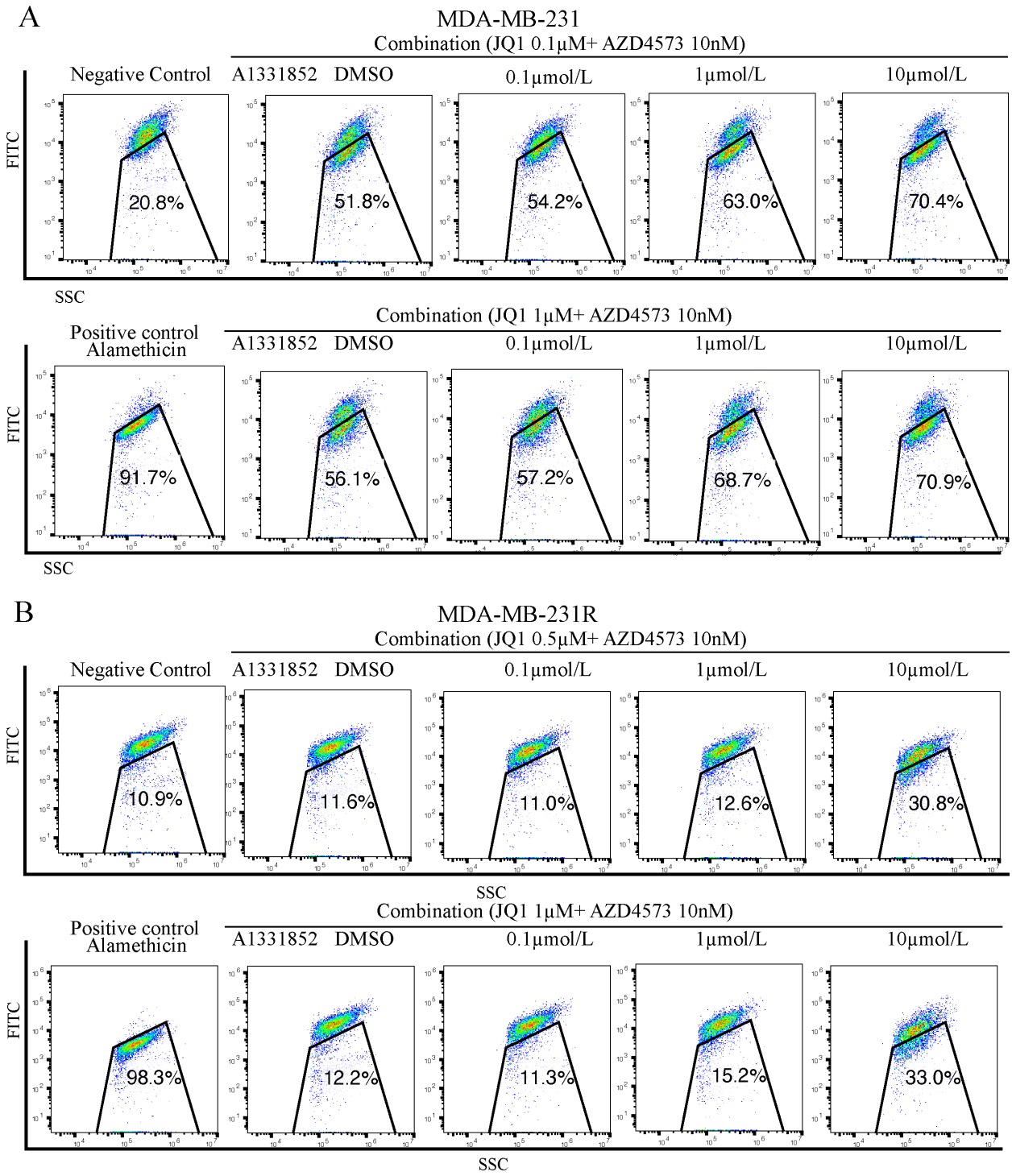


Figure 27. Response of MDA-MB-231 (A) and MDA-MB-231R (B) cells to A1331852-0 (BCL-xL inhibitor) induced apoptosis.

MDA-MB-231 (A) and MDA-MB-231R (B) cells were treated with JQ1 and AZD4573 for 48 hours, washed and various concentrations of A1331852 were added for 1 hour. After that, the cell cytochrome c release was measured using BH3 profiling staining assay.

To confirm the results of BH3 profiling assay, I combined JQ1 withA1331852 to treat MDA-MB-

231 and MDA-MB-231R and analyze the cytotoxicity used by MTT assay. As shown in Figure 28, all values of CI were less than 1. Combination treatment of MDA-MB-231 and MDA-MB-231R with JQ1 and A1331852 much more strongly inhibited cell proliferation than with each substance alone and this impact was highly synergistic as shown by Calcusyn software analysis.

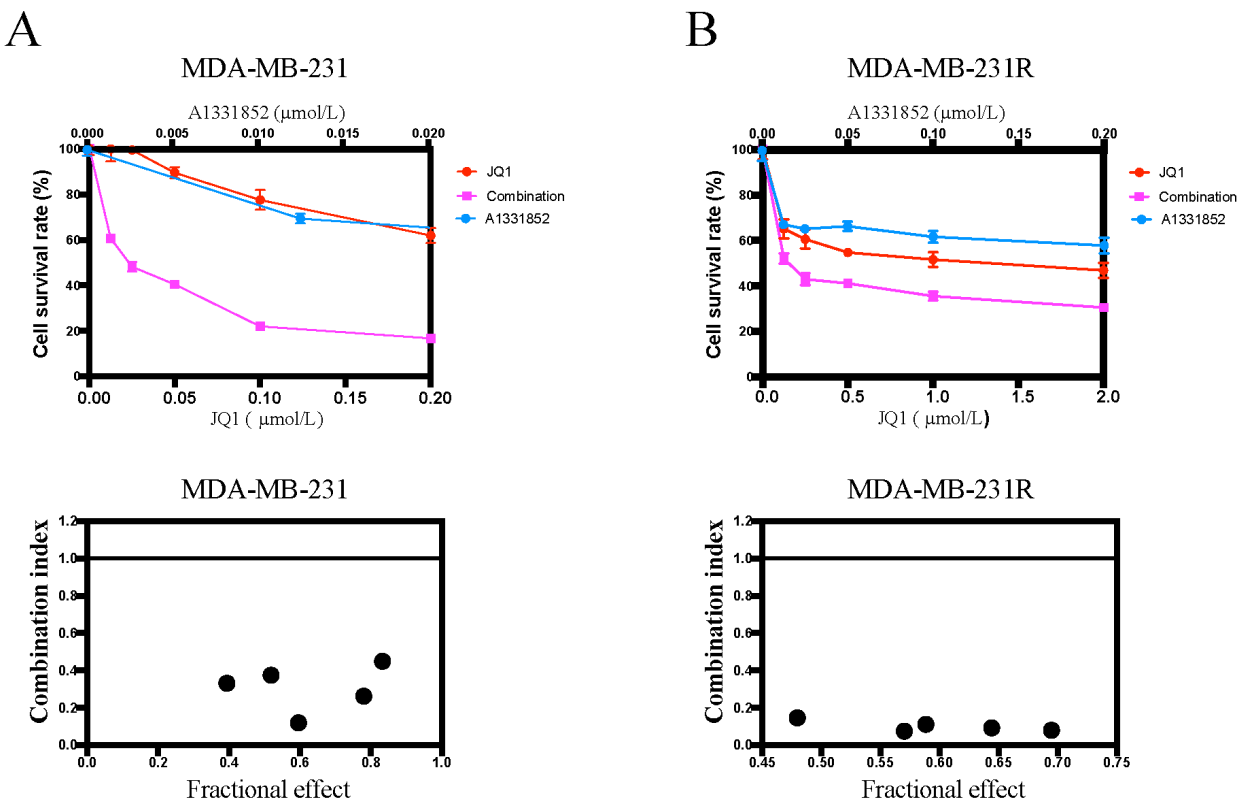


Figure 28. Analysis of the effects of JQ1 and A331852-0 drug combinations in MDA-MB-231 (A) and MDA-MB-231R (B).

A total of 2×10^3 cells were treated with indicated concentrations of JQ1 and A1331852 in MDA-MB-231 (A) and MDA-MB-231R (B) via MTT assay. Errors bars represent standard errors.

3.7 **MDA-MB-436was dependent on both BCL-xL and MCL-1 for survival**

The survival of MDA-MB-436 cells was not dependent solely on MCL-1, BCL-xL and A1, according to earlier findings. Further analysis of the effects of treatment with several BCL-2 family inhibitors in MDA-MB-436 revealed that only the MCL-1 inhibitor had a negligible impact at high concentrations (Figure 26). The MCL-1 inhibitor to further inhibit MCL-1 and checked whether this inhibition caused the cytochrome C release in cells that were pretreated with JQ1 and AZD4573. Unfortunately, cellular cytochrome c loss did not increase when treated with increasing

concentration of S63845 treatmentinMDA-MB-436 cells (Figure 29), indicating that cell apoptosis induced by combination treatment may not be dependent on MCL-1 inhibition alone.

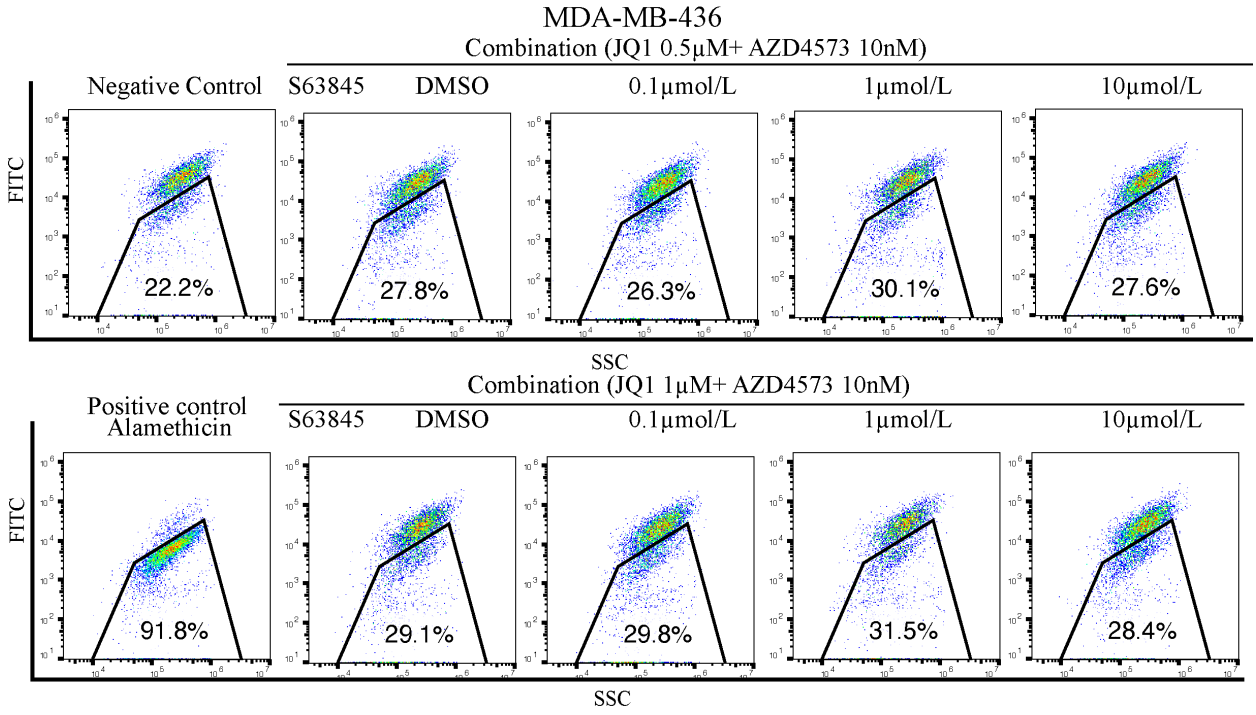


Figure 29. Cell apoptosis analysis via flow cytometer after treatment with different concentrations of S63845 (MCL-1 inhibitor) in MDA-MB-436 cells.

MDA-MB-436 was treated with JQ1 and AZD4573 for 48 hours, washed and various concentrations of S63845 were added for 1 hour. Thereafter, the cell cytochrome c loss was measured using BH3 profiling staining assay.

Therefore, it is considered that the survival of MDA-MB-436 does not depend on a singular BCL-2 family protein. The cells were treated with various combinations of BCL-2 inhibitors and found that the MCL-1 inhibitor and BCL-xL inhibitor could strongly inhibit cell viability. As shown in Figure 30, all CI values were less than 1, which means that the combination of these two drugs showed a strong synergistic effect.

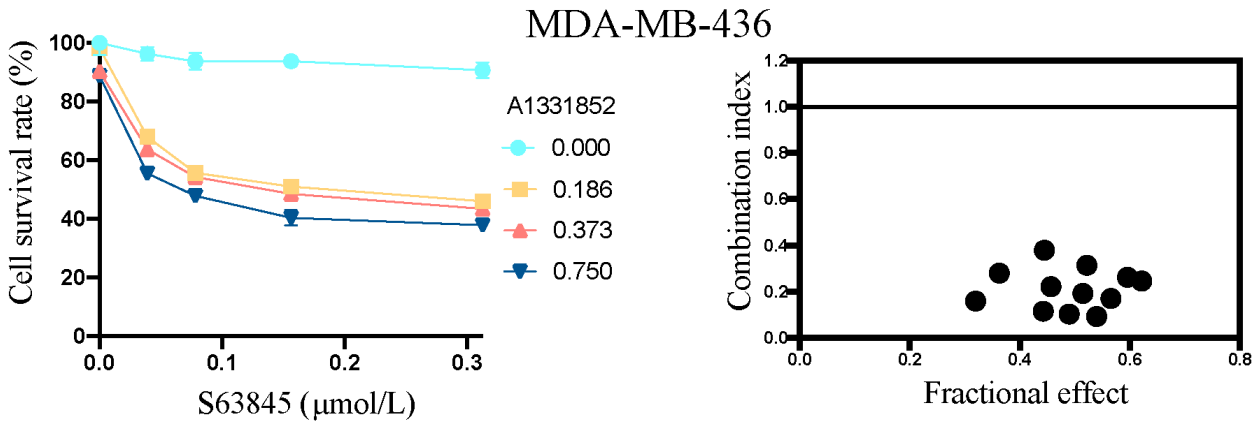


Figure 30. Analysis of combination effect of S63845 and A1331852 in MDA-MB-436 cells.

A total of 2×10^3cells were treated with indicated concentrations of S63845 and A1331852 via MTT assay (left). The combination indices of these two drugs were calculated using Calcusyn software (right). Errors bars represent standard errors.

3.8 Enhanced downregulation of BCL-xL and MCL-1 by combination of JQ1 and AZD4573

Our BH3 profiling and MTT assays strongly suggested that MDA-MB231 and MDA-MB231R cells rely on BCL-xL for survival and MDA-MB-436 cells rely on both BCL-xL and MCL-1. These results also further revealed that the greater inhibition of BCL-xL and/or MCL-1 by the combination treatment was the cause of the synergy between BET inhibitors and CDK9 inhibitors. Finally, western blotting proved the expression of BCL-2 family proteins. As shown in Figure 31, both the CDK9 inhibitor treatment group and in the combination treatment group were able to identify cleaved PARP, an indicator of apoptosis, was detected at 24 hours in MDA-MB-231 cells. In MDA-MB-231R cells, cleaved PARP was detected only in the combination treatment group, and in MDA-MB-436, cleaved PARP was detected only at 48 hours in the combination treatment. This result has strong correlation with the results of apoptosis detection, BH3 profiling and MTT assay that were previously discussed. Expression of BCL-xL was significantly down regulated by the combination treatment as compared to each substance alone after 48 hours in all three lines tested. Similarly, MCL-1 was also more significantly downregulated by the combination treatment in comparison with each substance alone after either 24- or 48-hours incubation. In contrast, it was only observed a downregulation of BCL-2 by JQ1 and combination of JQ1 and AZD4573 in

MDA-MB231cells after 48 hours.

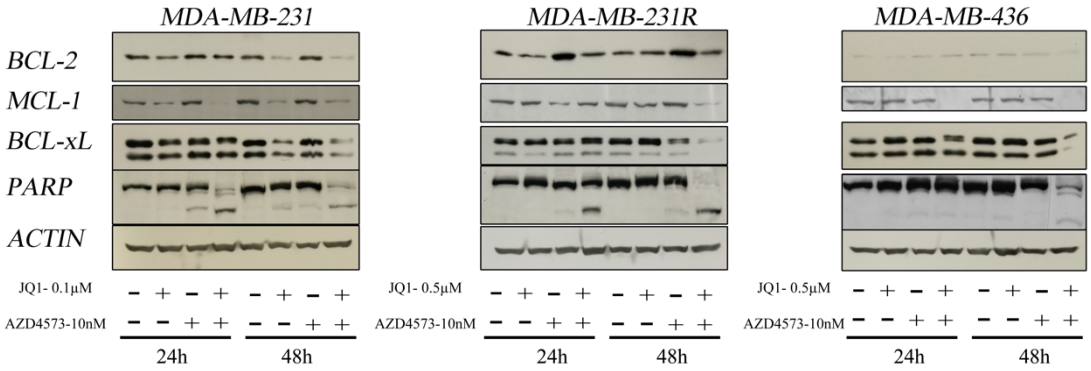


Figure 31. Protein expression after treatment of 24 hour and 48 hour in MDA-MB-231, MDA-MB-231R and MDA-MB-436 cells as analyzed by Western blots. Actin was used as a loading control.

4 Discussion

Triple-negative breast cancer (TNBC) is a highly aggressive and heterogeneous form of breast cancer. Although there has been a lot of research over the years, the outlook is still not great. Although recently the first antibody drug conjugate has been approved for the treatment of metastatic triple negative breast cancer (sacituzumab govitecan), systemic chemotherapy is still the main treatment option for these patients. Lacking other alternative treatment options is one of the main barriers for extension of patient survival.

4.1 BRD4 is one of the ideal therapeutic targets for TNBC

The most researched member of the BET family, BRD4 acts as genetic readers of histone acetyl-lysine residues to regulate gene transcription. It interacts with CDK9 and cyclin T1, the active form of P-TEFb, thereby phosphorylating RNA pol II and leading to the onset of transcriptional elongation. Because it is crucial to the initiation, elongation and regulation of transcription of many oncogenes, BRD4 has recently become a popular epigenetic target in oncology (Donati et al., 2018). It has been repeatedly shown that BRD4 and super-enhancers interact closely and have an impact on the transcriptional expression of oncogenes. According to numerous studies, BRD4 interacts with acetylated histones such as H3K9, H3K27, H4K5 and H4K8 (Jung et al., 2015) as well as non-histonic proteins such nuclear factor κB (NF-κB),TWIST, and GATA1 to regulate transcription (Yang et al., 2021). Moreover, it has been revealed that BRD4 plays a non-transcriptional role in regulating DNA replication and repair, telomere regulation (Donati et al., 2018).

A variety of cancers, including renal cell carcinoma (Sakaguchi et al., 2018), leukemia (Wedeh et al., 2015), non-small cell lung cancer (NSCLC) (Liao et al., 2016), head neck squamous cell carcinoma (HNSCC) (Wu et al., 2019) and also breast cancer (Lu et al., 2020), are known to have aberrant BRD4 function, which is frequently linked to a poor prognosis. BRD4 protein is significantly enriched in TNBC compared to non-TNBC tumors, according to Immunohistochemical (IHC) analysis of 67 breast cancer sections and the similar results were also found in cell lines (Verma et al., 2020) and is a favorable prognostic factor in breast cancer patients

(Suzuki et al., 2021). In TNBC, expression of phosphorylated BRD4 shows a strong prognostic value and is markedly associated with the activation status of the protein phosphatase 2A (PP2A) pathway (Sanz-Alvarez et al., 2021). In this data analysis, it was also found BRD4 is highly expressed in breast cancer in both mRNA and protein levels, especially in TNBC.

BRD4 has been shown to promote the expression of oncogenes by the possession of aberrant chromatin structure in various cancers including AML (Zuber et al., 2011), acute lymphoblastic leukemia (ALL) (Ott et al., 2012), NSCLC(Liao et al., 2016) and melanoma (Segura et al., 2013). The enhanced activity of BRD4 is associated with higher expression of oncogenes, such as MYC, NOTCH3 and NRG in cancer patients with amplified BRD4. The epithelial-mesenchymal transition, metastasis, genetic instability, and chemoresistance are all enhanced by these BRD4-driven oncogenes (Drumond-Bock and Bieniasz, 2021). Several crucial transcription factors and oncogenes are associated with super enhancers such as MYC, STAT3, EGFR, TAL1, PAK4 and INSEM1. BRD4 is preferentially bound to these enhancers and promote the expression of these transcription factors and oncogenes. As such, BRD4 is being pursued as intriguing fresh targets for cancer treatment. In breast cancer, inhibition of BRD4 suppresses the malignancy of breast cancer cells through controlling of snail (Lu et al., 2020). BRD4-knockdown could suppress Notch1 activity and prevent the migration and invasion of breast cancer (Andrieu et al., 2016).

Moreover, drug target failure and drug resistance are frequently brought on by genetic mutations. Genome-wide research have shown that BRD4 is widely distributed along the genome and that the BET family exhibits only few mutations. In a pan-cancer analysis of genomic alterations in BET genes across 20 common cancers from TCGA database, there were only 10 gene fusion events (8 from BRD4 and 2 from BRD3), a low frequency of recurrent BRD2 mutations in colon cancer (3.01%), and BRDT mutation in pancreatic cancer (0.55%) and endometrial cancer (8.12%) (Yang et al., 2017). The analysis of the TCGA breast cancer database revealed that missense mutation and frame shift deletion account for the majority of BRD4's less than 1% mutation rate.

Based on results of the data analysis and earlier research revealed that BRD4 expression is significantly higher in TNBC and that it is less likely to be mutated, and therefore it could be one of the best targets for TNBC.

4.2 **The role of BET inhibitor in TNBC**

Since the first BRD4 inhibitor JQ1 was developed in 2010, numerous BRD4 inhibitors have been discovered in the past few years(Filippakopoulos et al., 2010). Several distinct BET inhibitors showed the strong anticancer effectiveness in hematological cancer and c-myc overexpressed subtypes of solid tumors in preclinical and clinical research (Shorstova et al., 2021). The DNA-binding protein c-myc is involved in up to 70% of cancers, yet because to the fact that small molecule drugs cannot access the active portion of its structure, it is frequently regarded as non-pharmacological (Maxmen, 2012). JQ1 treatment down-regulates c-myc oncogene expression and is an ideal alternative to inhibit c-myc expression (Sengupta et al., 2015, Bihani et al., 2015). BET inhibitors not only regulate the oncogene c-myc, but also other genes associated with breast cancer, including the breast cancer amplification sequence 1 (BCAS1) gene and PDZ domain-containing 1 (PDZK1) (Perez-Salvia et al., 2017). According to numerous studies, JQ1 inhibits the development of ER positive breast cancer cells in a dose-dependent manner (Murakami et al., 2019, Sengupta et al., 2015, Nagarajan et al., 2014).

BET inhibitors induce cell cycle arrest in TNBC. BET proteins are the mitotic bookmarks and regulators of the cell cycle. BRD4 functions as a protein scaffold that recruits numerous proteins to chromatin and releases G1 cell cycle arrest (Ottinger et al., 2006). BRD4influences the transcriptional regulation of genes which is required to ensure cell cycle progression, including AURKB, RAN, KIF5B, RAD2 (Donati et al., 2018). BRD4 remains associated with the H4K5ac histone on chromatin during mitosis, causing the surrounding chromatin to decompress quickly and trigger post mitotic transcription (Devaiah and Singer, 2013). According to this study, it was confirmed that JQ1 and I-BET762 monotherapy could inhibit the cell growth dose dependently, which related with cell cycle G1 arrest in JQ1 sensitive MDA-MB-231 TNBC cells. This was possibly due to a significant inhibition of cell cycle-related proteins such as cyclinD1, and upregulation of P27 following JQ1 treatment or BRD4 silencing as shown by others (Wen et al., 2019, Nieto-Jimenez et al., 2017, Perez-Pena et al., 2016). In contrast, a change in cell cycle progression in JQ1 resistant MDA-MB-231R and MDA-MB-436 cells did not exhibit a change in cell cycle progression. Similar results were observed in other resistant cancer cell lines, such as

leukemia stem cells(Fong et al., 2015),or prostate cancer cells either (Pawar et al., 2018).In drug-resistant cell lines, chromatin-bound BRD4 is reduced but expression of important target genes is unaffected, pointing to possibility of alternative mechanisms of transcriptional regulation, which may account for the lack of cell cycle arrest (Fong et al., 2015) (Pawar et al., 2018).

This study demonstrated that JQ1 also induced cellular senescence in TNBC cells. This was consistent with earlier articles (Shu et al., 2016). In TNBC, inhibitor of BRD4 has been shown to induce senescence by down-regulating AURKA and AURKB expression, which are members of the aurora kinase subfamily of conserved serine/threonine kinases involved in cell mitosis (Xu et al., 2020). Moreover, senescence is brought on by the degradation of BET proteins via two distinct but linked processes, namely the weakening of non-homologous end-joining (NHEJ) and upregulation of autophagy gene expression (Wakita et al., 2020). Senescence, in one hand, can reduce tumor growth, but in other hand, cellular senescence also can accelerate drug-resistance and tumor progression (Gordon and Nelson, 2012). Senescent cells release a unique collection of substances known as the senescence-associated secretory phenotype (SASP), which has been postulated to carry pro-tumorigenic properties (Lau et al., 2019, Demaria et al., 2017, Sun et al., 2012). BRD4 is essential for SASP and downstream paracrine signaling, according to functional investigations and transcriptional profiling (Tasdemir et al., 2016). Genomic and transcriptomic analyses revealed that in BET inhibitor resistant cells G1-S and senescence-related genes are upregulated, while genes involved in cell cycle arrest are downregulated, which may mediate the escape from growth arrest (Ge et al., 2020) in TNBC. Combined treatment with BET inhibitors and ATR inhibitors resulted in elevated transcript levels of genes involved in senescence-associated secretory pathways in lymphoma (Muralidharan et al., 2016) and melanoma (Muralidharan et al., 2017).

The cellular responses to a variety of intrinsic and extrinsic signals include apoptosis and cellular senescence (Childs et al., 2014). There is debate regarding whether BET inhibitors induced apoptosis and may depend on the type of cell involved. In osteosarcoma (OS), all OS cells are sensitive to BET inhibition, but BET inhibition only induces apoptosis in primary OS, not in MG63 cell line (Baker et al., 2015). In rhabdomyosarcoma cells, JQ1 showed minimal response in both

cell lines, but synergistically induced apoptosis with the BCL-2 family inhibitors (Erdogdu et al., 2022). In breast cancer, JQ1 treatment induced apoptosis in a dose-responsive manner in MDA-MB-453, but minimal response in MDA-MB-468 and BT-20 (Park et al., 2019). Although all the TNBC cell lines studied in this work were determined by BH3 profiling to be primed for apoptosis, it was discovered that treatment with JQ1 scarcely caused cell apoptosis, especially in drug-resistant MDA-MB-436 and MDA-MB-231R cells. Results indicated that TNBC cells are not driven to apoptosis by BET inhibitors.

Following that, a combined strategy was used to try to enhance the effectiveness of BET inhibitors and reduce unspecific their cytotoxicities. Combination therapy is a combination of two or more therapeutic agents and is the cornerstone of cancer treatment. The combination of anticancer drugs improves efficacy compared to a single treatment because it targets important pathways in a typical synergistic or additive manner. This approach may reduce drug resistance while providing therapeutic anti-cancer benefits such as reducing tumor growth and metastasis potential, inhibiting mitosis active cells, reducing cancer stem cell populations, and inducing apoptosis (Bayat Mokhtari et al., 2017).

4.3 **Combination therapy to induce cell apoptosis**

As is well known, the ability to develop the apoptosis resistance is a characteristic of all types of cancer, and evasion of apoptosis is the third hallmark of cancer (Hanahan and Weinberg, 2011). Therefore, it was expected to identify methods to improve the drug efficacy and reduce resistance through induction of cell apoptosis since it was confirmed in preclinical research that the anti-cancer effect of BET inhibitors was determined by the apoptotic response (Conery et al., 2016).

In earlier research, most studies tried to enhance the ability of BET inhibitors to induce apoptosis through combination with other therapeutics such as BCL-2 family inhibitors (Cummin et al., 2020), CDK inhibitors (Baker et al., 2015) and other epigenetic regulator like HDAC inhibitors (Heinemann et al., 2015, Huan et al., 2020). In this investigation, BET inhibitors and BCL2 family inhibitors, such as ABT199 and S63845, were first combined in an attempt to increase the potential of the drug to induce apoptosis, but this did not achieve the desired efficacy. This is not consistent with what has been reported mostly in hematologic tumors (Fiskus et al., 2019, Cummin et al.,

2020, Carra et al., 2020, Esteve-Arenys et al., 2018) and some solid tumors (Lam et al., 2017, Tseng et al., 2020). This may be related to the different effect of BET inhibitor treatments on BCL-2 family protein expression in various tumor types. Secondly, CDK inhibitors are another class of drugs used in combination therapy with BET inhibitors. Combination treatment of BET inhibitors and pan-CDK inhibitors in OS cell(Baker et al., 2015), CDK2 inhibitors in MYC-driven medulloblastoma (Bolin et al., 2018), CDK4/6 inhibitors in TNBC (Ge et al., 2020), CDK7 inhibitors in leukemia (Guo et al., 2020), and CDK9 inhibitors in melanoma (Emran et al., 2021b)have been shown to possess very good synergistic therapeutic effects. In this study, several combinations of CDK inhibitors with BET inhibitors were screened and it was found that a CDK9 inhibitor had the strongest effect with BET inhibitors in the treatment of TNBC.

4.4 **CDK9 inhibitor in cancer**

CDK9 is a serine/threonine kinase that forms the catalytic core of P-TEFb, which is critical for the stabilization of the elongation process of RNA transcription(Romano and Giordano, 2008). CDK9 is broadly distributed in all types of human tissues, and cells that have undergone terminal differentiation express it most strongly (Bagella et al., 1998). In a large number of cancer types, including hematological diseases and solid tumors, CDK9 expression is associated with poor prognosis and resistance to anti-cancer treatment (Mandal et al., 2021). In breast cancer, high CDK9 expression is linked with low overall survival rate, and its expression predicts a potential prognostic biomarker role in patients who fail to achieve a complete response after neoadjuvant chemotherapy (Schlafstein et al., 2018). In TNBC, patients with high-CDK9 expression are also correlated with lower overall survival rate and inhibition of CDK9 show strong antineoplastic effects in TNBC cells (Brisard et al., 2018). The role of CDK9 in cancer pathogenesis is not fully established, but several studies have demonstrated its involvement in the activation of c-myc oncogenes and the over expression of MCL-1 and BCL-2 proteins (Phillips et al., 2020, Cidado et al., 2020, Luedtke et al., 2020, Lu et al., 2015, Boffo et al., 2018, Hashiguchi et al., 2019). In ER positive breast cancer cells, CDK9 inhibitor induce G2-M cell cycle arrest and down-regulate MYB target genes CCNB1 and CCNE1 (Mitra et al., 2016). A dual CDC7/CDK9 inhibitor, PHA767491, acts synergistically with EGFR-TKIs in TNBC cells to induce G2/M cell cycle arrest

and apoptosis and to overcome the resistance of TNBC to EGFR-TKIs (McLaughlin et al., 2019). The majority of studies demonstrate that CDK9 inhibitors could strongly induce apoptosis in tumor cells (Cassandri et al., 2020, Cidado et al., 2020, King et al., 2021).

Based on the crucial functions of CDK9 in cancer, several CDK9 inhibitors have been synthesized and are currently being studied in clinical trials. These include AZD-4573 (phase I, NCT04630756, NCT03263637, and phase II, NCT05140382), BAY-1143572 (phase I, NCT02345382 and NCT01938638), BAY-1251152 (phase I, NCT02745743 and NCT02635672), KB-0742 (phase I, NCT04718675), VIP152 (phase I, NCT04978779, NCT02635672 and NCT05371054), PRT2527 (phase I, NCT05159518) and GFH009 (phase I, NCT04588922). However, the U.S. Food and Drug Administration (FDA) has not approved CDK9 inhibitors due to their toxicity and dose-limiting side effects, which limit their clinical efficacy (Morales and Giordano, 2016). Reducing the doses and increasing the efficacies of the CDK9 inhibitors by using combination therapies with other therapeutics are likely one of the approaches to minimize adverse effects of CDK9 inhibitors.

4.5 **Combination of BET inhibitor with CDK9 inhibitor to induce cell apoptosis**

According to this study, CDK9 and BET inhibitors might achieve good synergistic effects at low dosages, not only in BET inhibitor sensitive cells, but also in primary and secondary resistant TNBC cells. In the few trials that have been published, the combination of the two drugs was effective in enhancing the antitumor effect in a subset of acute leukemia (AML) cells (McCalmont et al., 2020, Gerlach et al., 2018) and other solid tumors (Lu et al., 2015, Richter et al., 2020, Moreno et al., 2017, Emran et al., 2021b). According to additional research, the combination of the two-drug induced apoptosis by inhibiting pro-apoptotic proteins, such as BCL-2, MCL-1, BCL-xL (Emran et al., 2021a, Lam et al., 2017).

BCL-2 is essential for the survival of embryonic kidney progenitor cells, melanocyte progenitors, and mature B and T lymphocytes (Bouillet et al., 2001). Positive expression of BCL-2 was found in 73% of ER-positive breast cancers and was a prognostic marker independent of time in the early stages, but only 12.4% of TNBC expressed BCL-2 (Dawson et al., 2010). In aggressive B-cell lymphoma, JQ1 downregulates BCL-2 expression and is directly involved in the mitochondrial apoptotic pathway, whereas upregulation of BCL-2 is associated with intrinsic resistance to JQ1-

mediated death or resistance acquired after in vivo exposure (Hogg et al., 2016). JQ1 synergizes with the BCL-2 inhibitor ABT-263 in MYCN-amplified small cell lung cancer by disrupting the BIM/BCL-2 interaction (Wang et al., 2017). BET and BCL-2 inhibitors working together inhibit lymphoma in a synergistic manner (Elamin et al., 2022, Johnson-Farley et al., 2015, Kim et al., 2018). In this study, BCL-2 was downregulated by JQ1 in MDA-MB-231 at 48 hours but not significantly in MDA-MB-231R and MDA-MB-436 cells. Furthermore, the BCL-2 inhibitor ABT-199 alone and in combination with JQ1 did not achieve promising effect in MDA-MB-231 and MDA-MB-436 cells. As a result, in the cell lines employed, BCL-2 may not be an essential molecule for cell survival or a target for inducing death.

Another anti-apoptotic BCL-2 family member, MCL-1, is also a prognostic indicator in breast cancer. Its expression is higher in basal like breast cancers as compared to other subtypes (Campbell et al., 2018). MCL-1 and MYC cooperate in the maintenance of chemotherapy-resistant cancer stem cells (CSCs) in TNBC (Lee et al., 2017), and Wnt modulates MCL-1 to control cell survival in TNBC (Yang et al., 2014). BRD protein levels, particularly BRD4, correlated positively with sensitivity to BET inhibition and BET degraders can induced apoptosis in NSCLC by suppression of MCL-1 (Zong et al., 2020). In hepatocellular carcinoma cells (Zhang et al., 2018) and AML cells (Grundy et al., 2018), upregulation of MCL-1inhibits JQ1-triggered anticancer activity, whereas downregulation ofMCL-1 overcomes BET inhibitor resistance induced by low FBW7 expression in breast cancer (Wang et al., 2022). On the other hand, numerous studies of cell apoptosis induction by CDK9 inhibitors have revealed MCL-1 to play a major role (Gregory et al., 2015, Dey et al., 2017, Cidado et al., 2020). Thus, it is likely that the combination of a CDK9 inhibitor and a BET inhibitor can promote a stronger apoptosis by interfering with MCL-1additively or synergistically. Here, it was found that MCL-1 was downregulated in three TNBC cell lines following combination therapy, especially in MDA-MB-436 and a stronger apoptosis induced by the combination treatment was noticed. However, the expression of MCL-1 was not downregulated by JQ1 and combination of JQ1 and MCL-1 inhibitor did not induce apoptosis. Furthermore, BH3 profiling revealed that the survival of TNBC cancer cells was not dependent on MCL-1 alone. Therefore, it is unlikely that the stronger apoptosis induced by the combination of

JQ1 and AZD4573 are only due to the enhanced suppression of MCL-1.

BCL-xL is also essential for promoting the survival of cancer. It is frequently overexpressed in lung cancer, hepatocellular carcinoma and breast cancer (Bessou et al., 2020). The BCL-xL is qualitatively distinct from and ten times more efficient than BCL-2 in breast cancer cell lines (Fiebig et al., 2006). It promotes metastasis by induction of cytokines resistance (Fernandez et al., 2000) and mitochondria-dependent reactive oxygen species production (Bessou et al., 2020) in breast cancer cells. High expression levels of BCL-xL and CDK1 correlate with poor survival outcomes of TNBC patients (Castellanet et al., 2021). By inducing the death of senescent cells, targeting BCL-xL increases the effectiveness of BET inhibitors in treating TNBC (Gayle et al., 2019). The significance of BCL-xL for the survival of MDA-MB-231 parental and resistant cells was observed in this study. First, it was shown that all the TNBC cell lines are primed for apoptosis using BH3 profiling, and it was discovered that MDA-MB-231 parental and resistant cells were sensitive to BCL-xL mimic peptides while MDA-MB-436 not. This finding suggests that MDA-MB-231 parental and resistant cells are BCL-xL dependent for survival. This conclusion was supported by the results that MDA-MB-231 cells were sensitive to BCL-xL inhibitor A1331852 whereas MDA-MB-436 not. Moreover, a synergistic effect was observed when JQ1 and A1331852 were combined in the treatment of cells. This data is in agreement with results showing in SUM159JQ1 resistant cell line that the combined use of JQ1 and ABT263 (BCL-2/BCL-xL inhibitor) significantly reduced tumor weight in xenograft model (Shu et al., 2020). Finally, an enhanced downregulation of BCL-xL by the combination of JQ1 and AZD4573, and further inhibition of BCL-xL by A1331852 caused more apoptosis in cells that treated with JQ1 and AZD4573 were observed. All these data strongly support the conclusion that BCL-xL is the target of the combination therapy to induce apoptosis in MDA-MB-231 parental and resistant cells is clearly supported by all of these studies.

The combination of CDK9 and BET inhibitors induced apoptosis not only in parental and acquired resistant MDA-MB-231 cell lines, but also in primary resistant MDA-MB-436 cells. Although the MDA-MB-436 cells were primed for apoptosis, BH3 analysis did not reveal that the survival of MDA-MB-436 was only dependent on the anti-apoptotic proteins BCL-xL, MCL-1 or BFL-1 (A1),

respectively. As was previously mentioned, MCL-1 inhibition by itself did not cause the cells to undergo apoptosis. Furthermore, additional inhibition of MCL-1 by S63845 led to no further apoptosis in cells that treated with JQ1 and AZD4573. It's interesting to note when the MCL-1inhibitor with BCL-xL inhibitor was combined to treat the cells, a potent synergy between these two inhibitors were observed suggesting that MDA-MB-436 cells are dependent on both MCL-1 and BCL-xL for survival. The inhibition of one could make up for the other, and killing the cells with only one could not be sufficient. In this case, a simultaneous inhibition of both MCL-1 and BCL-xL in MDA-MB-436 cells treated with JQ1 and AZD4573 was observed. That might be the mechanism underlying the apoptosis induced by the combination in MDA-MB-436 cells. Inhibiting many anti-apoptotic proteins simultaneously is necessary to kill the cells has been reported before in melanoma cells (Emran et al., 2021b), and in AML (Gerlach et al., 2018).

In conclusion, BRD4 was a viable target for TNBC treatment and BET inhibitor effectively limited the growth of TNBC cells. When BET and CDK9 inhibitors are combined, TNBC cells are more likely to undergo cell apoptosis through the reduction of BCL-xL and MCL-1.

5 Limitation

In this study, the impact of BET inhibitors in combination with CDK9 inhibitors was explored in breast cancer cell lines sensitive or resistant cells to BET inhibitors. Limited by the long establishment time of tumor resistant cells, only one primary BETi-resistant cell line and one acquired BETi-resistant cell line were used in this study. Studies with more cell lines are necessary to access whether the conclusion reached in this study can extent to more cell lines. Moreover, only *in vitro* experiments were used in the ones that are presented here. Experiments using clinical samples and animal models are still needed to validate the conclusion. In any case, this study suggests that BET inhibitors combined with CDK9 inhibitors can effectively induce apoptosis in cells that are dependent on BCL-xL and/or MCL-1 proteins for survival and provide a hint for possible clinical applications of BET inhibitors in combination with CDK9 inhibitors for breast cancer patients.

6 References

AMBROSINI, G., DO, C., TYCKO, B., REALUBIT, R. B., KARAN, C., MUSI, E., CARVAJAL, R. D., CHUA, V., APLIN, A. E. & SCHWARTZ, G. K. 2019. Inhibition of NF-kappaB-Dependent Signaling Enhances Sensitivity and Overcomes Resistance to BET Inhibition in Uveal Melanoma. *Cancer Res,* 79**,** 2415-2425.

ANDRIEU, G., TRAN, A. H., STRISSEL, K. J. & DENIS, G. V. 2016. BRD4 Regulates Breast Cancer Dissemination through Jagged1/Notch1 Signaling. *Cancer Res,* 76**,** 6555-6567.

BAGELLA, L., MACLACHLAN, T. K., BUONO, R. J., PISANO, M. M., GIORDANO, A. & DE LUCA, A. 1998. Cloning of murine CDK9/PITALRE and its tissue-specific expression in development. *J Cell Physiol,* 177**,** 206-13.

BAKER, E. K., TAYLOR, S., GUPTE, A., SHARP, P. P., WALIA, M., WALSH, N. C., ZANNETTINO, A. C., CHALK, A. M., BURNS, C. J. & WALKLEY, C. R. 2015. BET inhibitors induce apoptosis through a MYC independent mechanism and synergise with CDK inhibitors to kill osteosarcoma cells. *Sci Rep,* 5**,** 10120.

BARRERO, M. J. 2017. Epigenetic Strategies to Boost Cancer Immunotherapies. *Int J Mol Sci,* 18.

BARRETINA, J., CAPONIGRO, G., STRANSKY, N., VENKATESAN, K., MARGOLIN, A. A., KIM, S., WILSON, C. J., LEHAR, J., KRYUKOV, G. V., SONKIN, D., REDDY, A., LIU, M., MURRAY, L., BERGER, M. F., MONAHAN, J. E., MORAIS, P., MELTZER, J., KOREJWA, A., JANE-VALBUENA, J., MAPA, F. A., THIBAULT, J., BRIC-FURLONG, E., RAMAN, P., SHIPWAY, A., ENGELS, I. H., CHENG, J., YU, G. K., YU, J., ASPESI, P., JR., DE SILVA, M., JAGTAP, K., JONES, M. D., WANG, L., HATTON, C., PALESCANDOLO, E., GUPTA, S., MAHAN, S., SOUGNEZ, C., ONOFRIO, R. C., LIEFELD, T., MACCONAILL, L., WINCKLER, W., REICH, M., LI, N., MESIROV, J. P., GABRIEL, S. B., GETZ, G., ARDLIE, K., CHAN, V., MYER, V. E., WEBER, B. L., PORTER, J., WARMUTH, M., FINAN, P., HARRIS, J. L., MEYERSON, M., GOLUB, T. R., MORRISSEY, M. P., SELLERS, W. R., SCHLEGEL, R. & GARRAWAY, L. A. 2012. The Cancer Cell Line Encyclopedia enables predictive modelling of anticancer drug sensitivity. *Nature,* 483**,** 603-7.

BARRETINA, J., CAPONIGRO, G., STRANSKY, N., VENKATESAN, K., MARGOLIN, A. A., KIM, S., WILSON, C. J., LEHAR, J., KRYUKOV, G. V., SONKIN, D., REDDY, A., LIU, M., MURRAY, L., BERGER, M. F., MONAHAN, J. E., MORAIS, P., MELTZER, J., KOREJWA, A., JANE-VALBUENA, J., MAPA, F. A., THIBAULT, J., BRIC-FURLONG, E., RAMAN, P., SHIPWAY, A., ENGELS, I. H., CHENG, J., YU, G. K., YU, J., ASPESI, P., JR., DE SILVA, M., JAGTAP, K., JONES, M. D., WANG, L., HATTON, C., PALESCANDOLO, E., GUPTA, S., MAHAN, S., SOUGNEZ, C., ONOFRIO, R. C., LIEFELD, T., MACCONAILL, L., WINCKLER, W., REICH, M., LI, N., MESIROV, J. P., GABRIEL, S. B., GETZ, G., ARDLIE, K., CHAN, V., MYER, V. E., WEBER, B. L., PORTER, J., WARMUTH, M., FINAN, P., HARRIS, J. L., MEYERSON, M., GOLUB, T. R., MORRISSEY, M. P., SELLERS, W. R., SCHLEGEL, R. & GARRAWAY, L. A. 2019. Addendum: The Cancer Cell Line Encyclopedia enables predictive modelling of anticancer drug sensitivity. *Nature,* 565**,** E5-E6.

BAUER, K., BERGER, D., ZIELINSKI, C. C., VALENT, P. & GRUNT, T. W. 2018. Hitting two

oncogenic machineries in cancer cells: cooperative effects of the multi-kinase inhibitor ponatinib and the BET bromodomain blockers JQ1 or dBET1 on human carcinoma cells. *Oncotarget,* 9**,** 26491-26506.
BAYAT MOKHTARI, R., HOMAYOUNI, T. S., BALUCH, N., MORGATSKAYA, E., KUMAR, S., DAS, B. & YEGER, H. 2017. Combination therapy in combating cancer. *Oncotarget,* 8**,** 38022-38043.
BERGER, A. C., KORKUT, A., KANCHI, R. S., HEGDE, A. M., LENOIR, W., LIU, W., LIU, Y., FAN, H., SHEN, H., RAVIKUMAR, V., RAO, A., SCHULTZ, A., LI, X., SUMAZIN, P., WILLIAMS, C., MESTDAGH, P., GUNARATNE, P. H., YAU, C., BOWLBY, R., ROBERTSON, A. G., TIEZZI, D. G., WANG, C., CHERNIACK, A. D., GODWIN, A. K., KUDERER, N. M., RADER, J. S., ZUNA, R. E., SOOD, A. K., LAZAR, A. J., OJESINA, A. I., ADEBAMOWO, C., ADEBAMOWO, S. N., BAGGERLY, K. A., CHEN, T. W., CHIU, H. S., LEFEVER, S., LIU, L., MACKENZIE, K., ORSULIC, S., ROSZIK, J., SHELLEY, C. S., SONG, Q., VELLANO, C. P., WENTZENSEN, N., CANCER GENOME ATLAS RESEARCH, N., WEINSTEIN, J. N., MILLS, G. B., LEVINE, D. A. & AKBANI, R. 2018. A Comprehensive Pan-Cancer Molecular Study of Gynecologic and Breast Cancers. *Cancer Cell,* 33**,** 690-705 e9.
BESSOU, M., LOPEZ, J., GADET, R., DEYGAS, M., POPGEORGIEV, N., PONCET, D., NOUGAREDE, A., BILLARD, P., MIKAELIAN, I., GONZALO, P., RIMOKH, R. & GILLET, G. 2020. The apoptosis inhibitor Bcl-xL controls breast cancer cell migration through mitochondria-dependent reactive oxygen species production. *Oncogene,* 39**,** 3056-3074.
BIANCHINI, G., BALKO, J. M., MAYER, I. A., SANDERS, M. E. & GIANNI, L. 2016. Triple-negative breast cancer: challenges and opportunities of a heterogeneous disease. *Nat Rev Clin Oncol,* 13**,** 674-690.
BIHANI, T., EZELL, S. A., LADD, B., GROSSKURTH, S. E., MAZZOLA, A. M., PIETRAS, M., REIMER, C., ZINDA, M., FAWELL, S. & D'CRUZ, C. M. 2015. Resistance to everolimus driven by epigenetic regulation of MYC in ER+ breast cancers. *Oncotarget,* 6**,** 2407-20.
BOFFO, S., DAMATO, A., ALFANO, L. & GIORDANO, A. 2018. CDK9 inhibitors in acute myeloid leukemia. *J Exp Clin Cancer Res,* 37**,** 36.
BOLIN, S., BORGENVIK, A., PERSSON, C. U., SUNDSTROM, A., QI, J., BRADNER, J. E., WEISS, W. A., CHO, Y. J., WEISHAUPT, H. & SWARTLING, F. J. 2018. Combined BET bromodomain and CDK2 inhibition in MYC-driven medulloblastoma. *Oncogene,* 37**,** 2850-2862.
BOUILLET, P., CORY, S., ZHANG, L. C., STRASSER, A. & ADAMS, J. M. 2001. Degenerative disorders caused by Bcl-2 deficiency prevented by loss of its BH3-only antagonist Bim. *Dev Cell,* 1**,** 645-53.
BRISARD, D., ECKERDT, F., MARSH, L. A., BLYTH, G. T., JAIN, S., CRISTOFANILLI, M., HORIUCHI, D. & PLATANIAS, L. C. 2018. Antineoplastic effects of selective CDK9 inhibition with atuveciclib on cancer stem-like cells in triple-negative breast cancer. *Oncotarget,* 9**,** 37305-37318.
CALDER, J., NAGELBERG, A., LUU, J., LU, D. & LOCKWOOD, W. W. 2021. Resistance to BET inhibitors in lung adenocarcinoma is mediated by casein kinase phosphorylation of BRD4. *Oncogenesis,* 10**,** 27.
CAMPBELL, K. J., DHAYADE, S., FERRARI, N., SIMS, A. H., JOHNSON, E., MASON, S. M., DICKSON, A., RYAN, K. M., KALNA, G., EDWARDS, J., TAIT, S. W. G. & BLYTH, K. 2018.

MCL-1 is a prognostic indicator and drug target in breast cancer. *Cell Death Dis,* 9**,** 19.
CARRA, G., NICOLI, P., LINGUA, M. F., MAFFEO, B., CARTELLA, A., CIRCOSTA, P., BRANCACCIO, M., PARVIS, G., GAIDANO, V., GUERRASIO, A., SAGLIO, G., TAULLI, R. & MOROTTI, A. 2020. Inhibition of bromodomain and extra-terminal proteins increases sensitivity to venetoclax in chronic lymphocytic leukaemia. *J Cell Mol Med,* 24**,** 1650-1657.
CASSANDRI, M., FIORAVANTI, R., POMELLA, S., VALENTE, S., ROTILI, D., DEL BALDO, G., DE ANGELIS, B., ROTA, R. & MAI, A. 2020. CDK9 as a Valuable Target in Cancer: From Natural Compounds Inhibitors to Current Treatment in Pediatric Soft Tissue Sarcomas. *Front Pharmacol,* 11**,** 1230.
CASTELLANET, O., AHMAD, F., VINIK, Y., MILLS, G. B., HABERMANN, B., BORG, J. P., LEV, S., LAMBALLE, F. & MAINA, F. 2021. BCL-XL blockage in TNBC models confers vulnerability to inhibition of specific cell cycle regulators. *Theranostics,* 11**,** 9180-9197.
CHILDS, B. G., BAKER, D. J., KIRKLAND, J. L., CAMPISI, J. & VAN DEURSEN, J. M. 2014. Senescence and apoptosis: dueling or complementary cell fates? *EMBO Rep,* 15**,** 1139-53.
CIDADO, J., BOIKO, S., PROIA, T., FERGUSON, D., CRISCIONE, S. W., SAN MARTIN, M., POP-DAMKOV, P., SU, N., ROAMIO FRANKLIN, V. N., SEKHAR REDDY CHILAMAKURI, C., D'SANTOS, C. S., SHAO, W., SAEH, J. C., KOCH, R., WEINSTOCK, D. M., ZINDA, M., FAWELL, S. E. & DREW, L. 2020. AZD4573 Is a Highly Selective CDK9 Inhibitor That Suppresses MCL-1 and Induces Apoptosis in Hematologic Cancer Cells. *Clin Cancer Res,* 26**,** 922-934.
CONERY, A. R., CENTORE, R. C., SPILLANE, K. L., FOLLMER, N. E., BOMMI-REDDY, A., HATTON, C., BRYANT, B. M., GRENINGER, P., AMZALLAG, A., BENES, C. H., MERTZ, J. A. & SIMS, R. J., 3RD 2016. Preclinical Anticancer Efficacy of BET Bromodomain Inhibitors Is Determined by the Apoptotic Response. *Cancer Res,* 76**,** 1313-9.
CUMMIN, T. E. C., COX, K. L., MURRAY, T. D., TURAJ, A. H., DUNNING, L., ENGLISH, V. L., FELL, R., PACKHAM, G., MA, Y., POWELL, B., JOHNSON, P. W. M., CRAGG, M. S. & CARTER, M. J. 2020. BET inhibitors synergize with venetoclax to induce apoptosis in MYC-driven lymphomas with high BCL-2 expression. *Blood Adv,* 4**,** 3316-3328.
DA MOTTA, L. L., LEDAKI, I., PURSHOUSE, K., HAIDER, S., DE BASTIANI, M. A., BABAN, D., MOROTTI, M., STEERS, G., WIGFIELD, S., BRIDGES, E., LI, J. L., KNAPP, S., EBNER, D., KLAMT, F., HARRIS, A. L. & MCINTYRE, A. 2017. The BET inhibitor JQ1 selectively impairs tumour response to hypoxia and downregulates CA9 and angiogenesis in triple negative breast cancer. *Oncogene,* 36**,** 122-132.
DA SILVA, J. L., CARDOSO NUNES, N. C., IZETTI, P., DE MESQUITA, G. G. & DE MELO, A. C. 2020. Triple negative breast cancer: A thorough review of biomarkers. *Crit Rev Oncol Hematol,* 145**,** 102855.
DAI, X., CHENG, H., BAI, Z. & LI, J. 2017. Breast Cancer Cell Line Classification and Its Relevance with Breast Tumor Subtyping. *J Cancer,* 8**,** 3131-3141.
DAWSON, S. J., MAKRETSOV, N., BLOWS, F. M., DRIVER, K. E., PROVENZANO, E., LE QUESNE, J., BAGLIETTO, L., SEVERI, G., GILES, G. G., MCLEAN, C. A., CALLAGY, G., GREEN, A. R., ELLIS, I., GELMON, K., TURASHVILI, G., LEUNG, S., APARICIO, S., HUNTSMAN, D., CALDAS, C. & PHAROAH, P. 2010. BCL2 in breast cancer: a favourable prognostic marker across molecular subtypes and independent of adjuvant therapy received. *Br J*

Cancer, 103**,** 668-75.
DELMORE, J. E., ISSA, G. C., LEMIEUX, M. E., RAHL, P. B., SHI, J., JACOBS, H. M., KASTRITIS, E., GILPATRICK, T., PARANAL, R. M., QI, J., CHESI, M., SCHINZEL, A. C., MCKEOWN, M. R., HEFFERNAN, T. P., VAKOC, C. R., BERGSAGEL, P. L., GHOBRIAL, I. M., RICHARDSON, P. G., YOUNG, R. A., HAHN, W. C., ANDERSON, K. C., KUNG, A. L., BRADNER, J. E. & MITSIADES, C. S. 2011. BET bromodomain inhibition as a therapeutic strategy to target c-Myc. *Cell,* 146**,** 904-17.
DEMARIA, M., O'LEARY, M. N., CHANG, J., SHAO, L., LIU, S., ALIMIRAH, F., KOENIG, K., LE, C., MITIN, N., DEAL, A. M., ALSTON, S., ACADEMIA, E. C., KILMARX, S., VALDOVINOS, A., WANG, B., DE BRUIN, A., KENNEDY, B. K., MELOV, S., ZHOU, D., SHARPLESS, N. E., MUSS, H. & CAMPISI, J. 2017. Cellular Senescence Promotes Adverse Effects of Chemotherapy and Cancer Relapse. *Cancer Discov,* 7**,** 165-176.
DEVAIAH, B. N. & SINGER, D. S. 2013. Two faces of brd4: mitotic bookmark and transcriptional lynchpin. *Transcription,* 4**,** 13-7.
DEY, J., DECKWERTH, T. L., KERWIN, W. S., CASALINI, J. R., MERRELL, A. J., GRENLEY, M. O., BURNS, C., DITZLER, S. H., DIXON, C. P., BEIRNE, E., GILLESPIE, K. C., KLEINMAN, E. F. & KLINGHOFFER, R. A. 2017. Voruciclib, a clinical stage oral CDK9 inhibitor, represses MCL-1 and sensitizes high-risk Diffuse Large B-cell Lymphoma to BCL2 inhibition. *Sci Rep,* 7**,** 18007.
DONATI, B., LORENZINI, E. & CIARROCCHI, A. 2018. BRD4 and Cancer: going beyond transcriptional regulation. *Mol Cancer,* 17**,** 164.
DRUMOND-BOCK, A. L. & BIENIASZ, M. 2021. The role of distinct BRD4 isoforms and their contribution to high-grade serous ovarian carcinoma pathogenesis. *Mol Cancer,* 20**,** 145.
ELAMIN, G., ALJOUNDI, A. & SOLIMAN, M. E. S. 2022. Co-Binding of JQ1 and Venetoclax Exhibited Synergetic Inhibitory Effect for Cancer Therapy; Potential Line of Treatment for the Waldenstrom Macroglobulinemia Lymphoma. *Chem Biodivers***,** e202100845.
ELSTRODT, F., HOLLESTELLE, A., NAGEL, J. H., GORIN, M., WASIELEWSKI, M., VAN DEN OUWELAND, A., MERAJVER, S. D., ETHIER, S. P. & SCHUTTE, M. 2006. BRCA1 mutation analysis of 41 human breast cancer cell lines reveals three new deleterious mutants. *Cancer Res,* 66**,** 41-5.
EMRAN, A. A., TSENG, H. Y., GUNATILAKE, D., COOK, S. J., AHMED, F., WANG, S., HERSEY, P., GALLAGHER, S. J. & TIFFEN, J. C. 2021a. A Combination of Epigenetic BET and CDK9 Inhibitors for Treatment of Human Melanoma. *J Invest Dermatol*.
EMRAN, A. A., TSENG, H. Y., GUNATILAKE, D., COOK, S. J., AHMED, F., WANG, S., HERSEY, P., GALLAGHER, S. J. & TIFFEN, J. C. 2021b. A Combination of Epigenetic BET and CDK9 Inhibitors for Treatment of Human Melanoma. *J Invest Dermatol,* 141**,** 2238-2249 e12.
ERDOGDU, U., DOLGIKH, N., LASZIG, S., SARCHEN, V., MEISTER, M. T., WANIOR, M., KNAPP, S. & BOEDICKER, C. 2022. Selective BH3 mimetics synergize with BET inhibition to induce mitochondrial apoptosis in rhabdomyosarcoma cells. *Neoplasia,* 24**,** 109-119.
ESTEVE-ARENYS, A., VALERO, J. G., CHAMORRO-JORGANES, A., GONZALEZ, D., RODRIGUEZ, V., DLOUHY, I., SALAVERRIA, I., CAMPO, E., COLOMER, D., MARTINEZ, A., RYMKIEWICZ, G., PEREZ-GALAN, P., LOPEZ-GUILLERMO, A. & ROUE, G. 2018. The BET bromodomain inhibitor CPI203 overcomes resistance to ABT-199 (venetoclax) by

downregulation of BFL-1/A1 in in vitro and in vivo models of MYC+/BCL2+ double hit lymphoma. *Oncogene,* 37**,** 1830-1844.
FENG, Q., ZHANG, Z., SHEA, M. J., CREIGHTON, C. J., COARFA, C., HILSENBECK, S. G., LANZ, R., HE, B., WANG, L., FU, X., NARDONE, A., SONG, Y., BRADNER, J., MITSIADES, N., MITSIADES, C. S., OSBORNE, C. K., SCHIFF, R. & O'MALLEY, B. W. 2014. An epigenomic approach to therapy for tamoxifen-resistant breast cancer. *Cell Res,* 24**,** 809-19.
FERNANDEZ, Y., ESPANA, L., MANAS, S., FABRA, A. & SIERRA, A. 2000. Bcl-xL promotes metastasis of breast cancer cells by induction of cytokines resistance. *Cell Death Differ,* 7**,** 350-9.
FIEBIG, A. A., ZHU, W., HOLLERBACH, C., LEBER, B. & ANDREWS, D. W. 2006. Bcl-XL is qualitatively different from and ten times more effective than Bcl-2 when expressed in a breast cancer cell line. *BMC Cancer,* 6**,** 213.
FILIPPAKOPOULOS, P., QI, J., PICAUD, S., SHEN, Y., SMITH, W. B., FEDOROV, O., MORSE, E. M., KEATES, T., HICKMAN, T. T., FELLETAR, I., PHILPOTT, M., MUNRO, S., MCKEOWN, M. R., WANG, Y., CHRISTIE, A. L., WEST, N., CAMERON, M. J., SCHWARTZ, B., HEIGHTMAN, T. D., LA THANGUE, N., FRENCH, C. A., WIEST, O., KUNG, A. L., KNAPP, S. & BRADNER, J. E. 2010. Selective inhibition of BET bromodomains. *Nature,* 468**,** 1067-73.
FISKUS, W., CAI, T., DINARDO, C. D., KORNBLAU, S. M., BORTHAKUR, G., KADIA, T. M., PEMMARAJU, N., BOSE, P., MASAROVA, L., RAJAPAKSHE, K., PERERA, D., COARFA, C., MILL, C. P., SAENZ, D. T., SAENZ, D. N., SUN, B., KHOURY, J. D., SHEN, Y., KONOPLEVA, M. & BHALLA, K. N. 2019. Superior efficacy of cotreatment with BET protein inhibitor and BCL2 or MCL1 inhibitor against AML blast progenitor cells. *Blood Cancer J,* 9**,** 4.
FISKUS, W., SHARMA, S., QI, J., SHAH, B., DEVARAJ, S. G., LEVEQUE, C., PORTIER, B. P., IYER, S., BRADNER, J. E. & BHALLA, K. N. 2014. BET protein antagonist JQ1 is synergistically lethal with FLT3 tyrosine kinase inhibitor (TKI) and overcomes resistance to FLT3-TKI in AML cells expressing FLT-ITD. *Mol Cancer Ther,* 13**,** 2315-27.
FONG, C. Y., GILAN, O., LAM, E. Y., RUBIN, A. F., FTOUNI, S., TYLER, D., STANLEY, K., SINHA, D., YEH, P., MORISON, J., GIOTOPOULOS, G., LUGO, D., JEFFREY, P., LEE, S. C., CARPENTER, C., GREGORY, R., RAMSAY, R. G., LANE, S. W., ABDEL-WAHAB, O., KOUZARIDES, T., JOHNSTONE, R. W., DAWSON, S. J., HUNTLY, B. J., PRINJHA, R. K., PAPENFUSS, A. T. & DAWSON, M. A. 2015. BET inhibitor resistance emerges from leukaemia stem cells. *Nature,* 525**,** 538-42.
FRASER, C., RYAN, J. & SAROSIEK, K. 2019. BH3 Profiling: A Functional Assay to Measure Apoptotic Priming and Dependencies. *Methods Mol Biol,* 1877**,** 61-76.
GAYLE, S. S., SAHNI, J. M., WEBB, B. M., WEBER-BONK, K. L., SHIVELY, M. S., SPINA, R., BAR, E. E., SUMMERS, M. K. & KERI, R. A. 2019. Targeting BCL-xL improves the efficacy of bromodomain and extra-terminal protein inhibitors in triple-negative breast cancer by eliciting the death of senescent cells. *J Biol Chem,* 294**,** 875-886.
GE, J. Y., SHU, S., KWON, M., JOVANOVIC, B., MURPHY, K., GULVADY, A., FASSL, A., TRINH, A., KUANG, Y., HEAVEY, G. A., LUOMA, A., PAWELETZ, C., THORNER, A. R., WUCHERPFENNIG, K. W., QI, J., BROWN, M., SICINSKI, P., MCDONALD, T. O., PELLMAN, D., MICHOR, F. & POLYAK, K. 2020. Acquired resistance to combined BET and CDK4/6 inhibition in triple-negative breast cancer. *Nat Commun,* 11**,** 2350.

GERLACH, D., TONTSCH-GRUNT, U., BAUM, A., POPOW, J., SCHARN, D., HOFMANN, M. H., ENGELHARDT, H., KAYA, O., BECK, J., SCHWEIFER, N., GERSTBERGER, T., ZUBER, J., SAVARESE, F. & KRAUT, N. 2018. The novel BET bromodomain inhibitor BI 894999 represses super-enhancer-associated transcription and synergizes with CDK9 inhibition in AML. *Oncogene,* 37**,** 2687-2701.
GORDON, R. R. & NELSON, P. S. 2012. Cellular senescence and cancer chemotherapy resistance. *Drug Resist Updat,* 15**,** 123-31.
GREGORY, G. P., HOGG, S. J., KATS, L. M., VIDACS, E., BAKER, A. J., GILAN, O., LEFEBURE, M., MARTIN, B. P., DAWSON, M. A., JOHNSTONE, R. W. & SHORTT, J. 2015. CDK9 inhibition by dinaciclib potently suppresses Mcl-1 to induce durable apoptotic responses in aggressive MYC-driven B-cell lymphoma in vivo. *Leukemia,* 29**,** 1437-41.
GRUNDY, M., SEEDHOUSE, C., JONES, T., ELMI, L., HALL, M., GRAHAM, A., RUSSELL, N. & PALLIS, M. 2018. Predicting effective pro-apoptotic anti-leukaemic drug combinations using co-operative dynamic BH3 profiling. *PLoS One,* 13**,** e0190682.
GUO, L., LI, J., ZENG, H., GUZMAN, A. G., LI, T., LEE, M., ZHOU, Y., GOODELL, M. A., STEPHAN, C., DAVIES, P. J. A., DAWSON, M. A., SUN, D. & HUANG, Y. 2020. A combination strategy targeting enhancer plasticity exerts synergistic lethality against BETi-resistant leukemia cells. *Nat Commun,* 11**,** 740.
HANAHAN, D. & WEINBERG, R. A. 2011. Hallmarks of cancer: the next generation. *Cell,* 144**,** 646-74.
HARBECK, N., PENAULT-LLORCA, F., CORTES, J., GNANT, M., HOUSSAMI, N., POORTMANS, P., RUDDY, K., TSANG, J. & CARDOSO, F. 2019. Breast cancer. *Nat Rev Dis Primers,* 5**,** 66.
HASHIGUCHI, T., BRUSS, N., BEST, S., LAM, V., DANILOVA, O., PAIVA, C. J., WOLF, J., GILBERT, E. W., OKADA, C. Y., KAUR, P., DREW, L., CIDADO, J., HURLIN, P. & DANILOV, A. V. 2019. Cyclin-Dependent Kinase-9 Is a Therapeutic Target in MYC-Expressing Diffuse Large B-Cell Lymphoma. *Mol Cancer Ther,* 18**,** 1520-1532.
HEINEMANN, A., CULLINANE, C., DE PAOLI-ISEPPI, R., WILMOTT, J. S., GUNATILAKE, D., MADORE, J., STRBENAC, D., YANG, J. Y., GOWRISHANKAR, K., TIFFEN, J. C., PRINJHA, R. K., SMITHERS, N., MCARTHUR, G. A., HERSEY, P. & GALLAGHER, S. J. 2015. Combining BET and HDAC inhibitors synergistically induces apoptosis of melanoma and suppresses AKT and YAP signaling. *Oncotarget,* 6**,** 21507-21.
HOGG, S. J., NEWBOLD, A., VERVOORT, S. J., CLUSE, L. A., MARTIN, B. P., GREGORY, G. P., LEFEBURE, M., VIDACS, E., TOTHILL, R. W., BRADNER, J. E., SHORTT, J. & JOHNSTONE, R. W. 2016. BET Inhibition Induces Apoptosis in Aggressive B-Cell Lymphoma via Epigenetic Regulation of BCL-2 Family Members. *Mol Cancer Ther,* 15**,** 2030-41.
HOLLESTELLE, A., NAGEL, J. H., SMID, M., LAM, S., ELSTRODT, F., WASIELEWSKI, M., NG, S. S., FRENCH, P. J., PEETERS, J. K., ROZENDAAL, M. J., RIAZ, M., KOOPMAN, D. G., TEN HAGEN, T. L., DE LEEUW, B. H., ZWARTHOFF, E. C., TEUNISSE, A., VAN DER SPEK, P. J., KLIJN, J. G., DINJENS, W. N., ETHIER, S. P., CLEVERS, H., JOCHEMSEN, A. G., DEN BAKKER, M. A., FOEKENS, J. A., MARTENS, J. W. & SCHUTTE, M. 2010. Distinct gene mutation profiles among luminal-type and basal-type breast cancer cell lines. *Breast Cancer Res Treat,* 121**,** 53-64.

HUAN, S., GUI, T., XU, Q., ZHUANG, S., LI, Z., SHI, Y., LIN, J., GONG, B., MIAO, G., TAM, M., ZHANG, H. T., ZHA, Z. & WU, C. 2020. Combination BET Family Protein and HDAC Inhibition Synergistically Elicits Chondrosarcoma Cell Apoptosis Through RAD51-Related DNA Damage Repair. *Cancer Manag Res,* 12**,** 4429-4439.
JANG, J. E., EOM, J. I., JEUNG, H. K., CHEONG, J. W., LEE, J. Y., KIM, J. S. & MIN, Y. H. 2017. Targeting AMPK-ULK1-mediated autophagy for combating BET inhibitor resistance in acute myeloid leukemia stem cells. *Autophagy,* 13**,** 761-762.
JIN, X., YAN, Y., WANG, D., DING, D., MA, T., YE, Z., JIMENEZ, R., WANG, L., WU, H. & HUANG, H. 2018. DUB3 Promotes BET Inhibitor Resistance and Cancer Progression by Deubiquitinating BRD4. *Mol Cell,* 71**,** 592-605 e4.
JOHNSON-FARLEY, N., VELIZ, J., BHAGAVATHI, S. & BERTINO, J. R. 2015. ABT-199, a BH3 mimetic that specifically targets Bcl-2, enhances the antitumor activity of chemotherapy, bortezomib and JQ1 in "double hit" lymphoma cells. *Leuk Lymphoma,* 56**,** 2146-52.
JUNG, M., GELATO, K. A., FERNANDEZ-MONTALVAN, A., SIEGEL, S. & HAENDLER, B. 2015. Targeting BET bromodomains for cancer treatment. *Epigenomics,* 7**,** 487-501.
KELLY, A. D. & ISSA, J. J. 2017. The promise of epigenetic therapy: reprogramming the cancer epigenome. *Curr Opin Genet Dev,* 42**,** 68-77.
KIM, S. R., LEWIS, J. M., CYRENNE, B. M., MONICO, P. F., MIRZA, F. N., CARLSON, K. R., FOSS, F. M. & GIRARDI, M. 2018. BET inhibition in advanced cutaneous T cell lymphoma is synergistically potentiated by BCL2 inhibition or HDAC inhibition. *Oncotarget,* 9**,** 29193-29207.
KING, H. M., RANA, S., KUBICA, S. P., MALLAREDDY, J. R., KIZHAKE, S., EZELL, E. L., ZAHID, M., NALDRETT, M. J., ALVAREZ, S., LAW, H. C., WOODS, N. T. & NATARAJAN, A. 2021. Aminopyrazole based CDK9 PROTAC sensitizes pancreatic cancer cells to venetoclax. *Bioorg Med Chem Lett,* 43**,** 128061.
KLAUBER-DEMORE, N., SCHULTE, B. A. & WANG, G. Y. 2018. Targeting MYC for triple-negative breast cancer treatment. *Oncoscience,* 5**,** 120-121.
LAM, L. T., LIN, X., FAIVRE, E. J., YANG, Z., HUANG, X., WILCOX, D. M., BELLIN, R. J., JIN, S., TAHIR, S. K., MITTEN, M., MAGOC, T., BHATHENA, A., KATI, W. M., ALBERT, D. H., SHEN, Y. & UZIEL, T. 2017. Vulnerability of Small-Cell Lung Cancer to Apoptosis Induced by the Combination of BET Bromodomain Proteins and BCL2 Inhibitors. *Mol Cancer Ther,* 16**,** 1511-1520.
LAU, L., PORCIUNCULA, A., YU, A., IWAKURA, Y. & DAVID, G. 2019. Uncoupling the Senescence-Associated Secretory Phenotype from Cell Cycle Exit via Interleukin-1 Inactivation Unveils Its Protumorigenic Role. *Mol Cell Biol,* 39.
LEE, D. H., QI, J., BRADNER, J. E., SAID, J. W., DOAN, N. B., FORSCHER, C., YANG, H. & KOEFFLER, H. P. 2015. Synergistic effect of JQ1 and rapamycin for treatment of human osteosarcoma. *Int J Cancer,* 136**,** 2055-64.
LEE, K. M., GILTNANE, J. M., BALKO, J. M., SCHWARZ, L. J., GUERRERO-ZOTANO, A. L., HUTCHINSON, K. E., NIXON, M. J., ESTRADA, M. V., SANCHEZ, V., SANDERS, M. E., LEE, T., GOMEZ, H., LLUCH, A., PEREZ-FIDALGO, J. A., WOLF, M. M., ANDREJEVA, G., RATHMELL, J. C., FESIK, S. W. & ARTEAGA, C. L. 2017. MYC and MCL1 Cooperatively Promote Chemotherapy-Resistant Breast Cancer Stem Cells via Regulation of Mitochondrial

Oxidative Phosphorylation. *Cell Metab,* 26**,** 633-647 e7.
LI, W., GUPTA, S. K., HAN, W., KUNDSON, R. A., NELSON, S., KNUTSON, D., GREIPP, P. T., ELSAWA, S. F., SOTOMAYOR, E. M. & GUPTA, M. 2019. Targeting MYC activity in double-hit lymphoma with MYC and BCL2 and/or BCL6 rearrangements with epigenetic bromodomain inhibitors. *J Hematol Oncol,* 12**,** 73.
LIAO, Y. F., WU, Y. B., LONG, X., ZHU, S. Q., JIN, C., XU, J. J. & DING, J. Y. 2016. High level of BRD4 promotes non-small cell lung cancer progression. *Oncotarget,* 7**,** 9491-500.
LU, H., XUE, Y., YU, G. K., ARIAS, C., LIN, J., FONG, S., FAURE, M., WEISBURD, B., JI, X., MERCIER, A., SUTTON, J., LUO, K., GAO, Z. & ZHOU, Q. 2015. Compensatory induction of MYC expression by sustained CDK9 inhibition via a BRD4-dependent mechanism. *Elife,* 4**,** e06535.
LU, L., CHEN, Z., LIN, X., TIAN, L., SU, Q., AN, P., LI, W., WU, Y., DU, J., SHAN, H., CHIANG, C. M. & WANG, H. 2020. Inhibition of BRD4 suppresses the malignancy of breast cancer cells via regulation of Snail. *Cell Death Differ,* 27**,** 255-268.
LUAN, W., PANG, Y., LI, R., WEI, X., JIAO, X., SHI, J., YU, J., MAO, H. & LIU, P. 2019. Akt/mTOR-Mediated Autophagy Confers Resistance To BET Inhibitor JQ1 In Ovarian Cancer. *Onco Targets Ther,* 12**,** 8063-8074.
LUEDTKE, D. A., SU, Y., MA, J., LI, X., BUCK, S. A., EDWARDS, H., POLIN, L., KUSHNER, J., DZINIC, S. H., WHITE, K., LIN, H., TAUB, J. W. & GE, Y. 2020. Inhibition of CDK9 by voruciclib synergistically enhances cell death induced by the Bcl-2 selective inhibitor venetoclax in preclinical models of acute myeloid leukemia. *Signal Transduct Target Ther,* 5**,** 17.
MAGGISANO, V., CELANO, M., MALIVINDI, R., BARONE, I., COSCO, D., MIO, C., MIGNOGNA, C., PANZA, S., DAMANTE, G., FRESTA, M., ANDO, S., RUSSO, D., CATALANO, S. & BULOTTA, S. 2019. Nanoparticles Loaded with the BET Inhibitor JQ1 Block the Growth of Triple Negative Breast Cancer Cells In Vitro and In Vivo. *Cancers (Basel),* 12.
MANDAL, R., BECKER, S. & STREBHARDT, K. 2021. Targeting CDK9 for Anti-Cancer Therapeutics. *Cancers (Basel),* 13.
MATKAR, S., SHARMA, P., GAO, S., GURUNG, B., KATONA, B. W., LIAO, J., MUHAMMAD, A. B., KONG, X. C., WANG, L., JIN, G., DANG, C. V. & HUA, X. 2015. An Epigenetic Pathway Regulates Sensitivity of Breast Cancer Cells to HER2 Inhibition via FOXO/c-Myc Axis. *Cancer Cell,* 28**,** 472-485.
MAXMEN, A. 2012. Cancer research: Open ambition. *Nature,* 488**,** 148-50.
MAYAKONDA, A., LIN, D. C., ASSENOV, Y., PLASS, C. & KOEFFLER, H. P. 2018. Maftools: efficient and comprehensive analysis of somatic variants in cancer. *Genome Res,* 28**,** 1747-1756.
MCCALMONT, H., LI, K. L., JONES, L., TOUBIA, J., BRAY, S. C., CASOLARI, D. A., MAYOH, C., SAMARAWEERA, S. E., LEWIS, I. D., PRINJHA, R. K., SMITHERS, N., WANG, S., LOCK, R. B. & D'ANDREA, R. J. 2020. Efficacy of combined CDK9/BET inhibition in preclinical models of MLL-rearranged acute leukemia. *Blood Adv,* 4**,** 296-300.
MCLAUGHLIN, R. P., HE, J., VAN DER NOORD, V. E., REDEL, J., FOEKENS, J. A., MARTENS, J. W. M., SMID, M., ZHANG, Y. & VAN DE WATER, B. 2019. A kinase inhibitor screen identifies a dual cdc7/CDK9 inhibitor to sensitise triple-negative breast cancer to EGFR-targeted therapy. *Breast Cancer Res,* 21**,** 77.
MERTZ, J. A., CONERY, A. R., BRYANT, B. M., SANDY, P., BALASUBRAMANIAN, S.,

MELE, D. A., BERGERON, L. & SIMS, R. J., 3RD 2011. Targeting MYC dependence in cancer by inhibiting BET bromodomains. *Proc Natl Acad Sci U S A,* 108**,** 16669-74.
MIO, C., GERRATANA, L., BOLIS, M., CAPONNETTO, F., ZANELLO, A., BARBINA, M., DI LORETO, C., GARATTINI, E., DAMANTE, G. & PUGLISI, F. 2019. BET proteins regulate homologous recombination-mediated DNA repair: BRCAness and implications for cancer therapy. *Int J Cancer,* 144**,** 755-766.
MITRA, P., YANG, R. M., SUTTON, J., RAMSAY, R. G. & GONDA, T. J. 2016. CDK9 inhibitors selectively target estrogen receptor-positive breast cancer cells through combined inhibition of MYB and MCL-1 expression. *Oncotarget,* 7**,** 9069-83.
MORALES, F. & GIORDANO, A. 2016. Overview of CDK9 as a target in cancer research. *Cell Cycle,* 15**,** 519-27.
MORENO, N., HOLSTEN, T., MERTINS, J., ZHOGBI, A., JOHANN, P., KOOL, M., MEISTERERNST, M. & KERL, K. 2017. Combined BRD4 and CDK9 inhibition as a new therapeutic approach in malignant rhabdoid tumors. *Oncotarget,* 8**,** 84986-84995.
MURAKAMI, S., LI, R., NAGARI, A., CHAE, M., CAMACHO, C. V. & KRAUS, W. L. 2019. Distinct Roles for BET Family Members in Estrogen Receptor alpha Enhancer Function and Gene Regulation in Breast Cancer Cells. *Mol Cancer Res,* 17**,** 2356-2368.
MURALIDHARAN, S. V., BHADURY, J., NILSSON, L. M., GREEN, L. C., MCLURE, K. G. & NILSSON, J. A. 2016. BET bromodomain inhibitors synergize with ATR inhibitors to induce DNA damage, apoptosis, senescence-associated secretory pathway and ER stress in Myc-induced lymphoma cells. *Oncogene,* 35**,** 4689-97.
MURALIDHARAN, S. V., EINARSDOTTIR, B. O., BHADURY, J., LINDBERG, M. F., WU, J., CAMPEAU, E., BAGGE, R. O., STIERNER, U., NY, L., NILSSON, L. M. & NILSSON, J. A. 2017. BET bromodomain inhibitors synergize with ATR inhibitors in melanoma. *Cell Death Dis,* 8**,** e2982.
NAGARAJAN, S., HOSSAN, T., ALAWI, M., NAJAFOVA, Z., INDENBIRKEN, D., BEDI, U., TAIPALEENMAKI, H., BEN-BATALLA, I., SCHELLER, M., LOGES, S., KNAPP, S., HESSE, E., CHIANG, C. M., GRUNDHOFF, A. & JOHNSEN, S. A. 2014. Bromodomain protein BRD4 is required for estrogen receptor-dependent enhancer activation and gene transcription. *Cell Rep,* 8**,** 460-9.
NIETO-JIMENEZ, C., ALCARAZ-SANABRIA, A., PEREZ-PENA, J., CORRALES-SANCHEZ, V., SERRANO-HERAS, G., GALAN-MOYA, E. M., SERRANO-OVIEDO, L., MONTERO, J. C., BURGOS, M., LLOPIS, J., PANDIELLA, A. & OCANA, A. 2017. Targeting basal-like breast tumors with bromodomain and extraterminal domain (BET) and polo-like kinase inhibitors. *Oncotarget,* 8**,** 19478-19490.
NIETO-JIMENEZ, C., GALAN-MOYA, E. M., CORRALES-SANCHEZ, V., NOBLEJAS-LOPEZ, M. D. M., BURGOS, M., DOMINGO, B., MONTERO, J. C., GOMEZ-JUAREZ, M., PICAZO-MARTINEZ, M. G., ESPARIS-OGANDO, A., PANDIELLA, A. & OCANA, A. 2020. Inhibition of the mitotic kinase PLK1 overcomes therapeutic resistance to BET inhibitors in triple negative breast cancer. *Cancer Lett,* 491**,** 50-59.
OTT, C. J., KOPP, N., BIRD, L., PARANAL, R. M., QI, J., BOWMAN, T., RODIG, S. J., KUNG, A. L., BRADNER, J. E. & WEINSTOCK, D. M. 2012. BET bromodomain inhibition targets both c-Myc and IL7R in high-risk acute lymphoblastic leukemia. *Blood,* 120**,** 2843-52.

OTTINGER, M., CHRISTALLA, T., NATHAN, K., BRINKMANN, M. M., VIEJO-BORBOLLA, A. & SCHULZ, T. F. 2006. Kaposi's sarcoma-associated herpesvirus LANA-1 interacts with the short variant of BRD4 and releases cells from a BRD4- and BRD2/RING3-induced G1 cell cycle arrest. *J Virol,* 80**,** 10772-86.
PAOLUZZI, L., HANNIFORD, D., SOKOLOVA, E., OSMAN, I., DARVISHIAN, F., WANG, J., BRADNER, J. E. & HERNANDO, E. 2016. BET and BRAF inhibitors act synergistically against BRAF-mutant melanoma. *Cancer Med,* 5**,** 1183-93.
PARK, I. H., YANG, H. N., JEON, S. Y., HWANG, J. A., KIM, M. K., KONG, S. Y., SHIM, S. H. & LEE, K. S. 2019. Anti-tumor activity of BET inhibitors in androgen-receptor-expressing triple-negative breast cancer. *Sci Rep,* 9**,** 13305.
PARK, J. W. & HAN, J. W. 2019. Targeting epigenetics for cancer therapy. *Arch Pharm Res,* 42**,** 159-170.
PAWAR, A., GOLLAVILLI, P. N., WANG, S. & ASANGANI, I. A. 2018. Resistance to BET Inhibitor Leads to Alternative Therapeutic Vulnerabilities in Castration-Resistant Prostate Cancer. *Cell Rep,* 22**,** 2236-2245.
PEREZ-PENA, J., SERRANO-HERAS, G., MONTERO, J. C., CORRALES-SANCHEZ, V., PANDIELLA, A. & OCANA, A. 2016. In Silico Analysis Guides Selection of BET Inhibitors for Triple-Negative Breast Cancer Treatment. *Mol Cancer Ther,* 15**,** 1823-33.
PEREZ-SALVIA, M. & ESTELLER, M. 2017. Bromodomain inhibitors and cancer therapy: From structures to applications. *Epigenetics,* 12**,** 323-339.
PEREZ-SALVIA, M., SIMO-RIUDALBAS, L., LLINAS-ARIAS, P., ROA, L., SETIEN, F., SOLER, M., DE MOURA, M. C., BRADNER, J. E., GONZALEZ-SUAREZ, E., MOUTINHO, C. & ESTELLER, M. 2017. Bromodomain inhibition shows antitumoral activity in mice and human luminal breast cancer. *Oncotarget,* 8**,** 51621-51629.
PHILLIPS, D. C., JIN, S., GREGORY, G. P., ZHANG, Q., XUE, J., ZHAO, X., CHEN, J., TONG, Y., ZHANG, H., SMITH, M., TAHIR, S. K., CLARK, R. F., PENNING, T. D., DEVLIN, J. R., SHORTT, J., HSI, E. D., ALBERT, D. H., KONOPLEVA, M., JOHNSTONE, R. W., LEVERSON, J. D. & SOUERS, A. J. 2020. A novel CDK9 inhibitor increases the efficacy of venetoclax (ABT-199) in multiple models of hematologic malignancies. *Leukemia,* 34**,** 1646-1657.
RICHTER, G. H. S., HENSEL, T., SCHMIDT, O., SARATOV, V., VON HEYKING, K., BECKER-DETTLING, F., PREXLER, C., YEN, H. Y., STEIGER, K., FULDA, S., DIRKSEN, U., WEICHERT, W., WANG, S., BURDACH, S. & SCHAFER, B. W. 2020. Combined Inhibition of Epigenetic Readers and Transcription Initiation Targets the EWS-ETS Transcriptional Program in Ewing Sarcoma. *Cancers (Basel),* 12.
ROMANO, G. & GIORDANO, A. 2008. Role of the cyclin-dependent kinase 9-related pathway in mammalian gene expression and human diseases. *Cell Cycle,* 7**,** 3664-8.
SAHNI, J. M., GAYLE, S. S., BONK, K. L., VITE, L. C., YORI, J. L., WEBB, B., RAMOS, E. K., SEACHRIST, D. D., LANDIS, M. D., CHANG, J. C., BRADNER, J. E. & KERI, R. A. 2016. Bromodomain and Extraterminal Protein Inhibition Blocks Growth of Triple-negative Breast Cancers through the Suppression of Aurora Kinases. *J Biol Chem,* 291**,** 23756-23768.
SAKAGUCHI, T., YOSHINO, H., SUGITA, S., MIYAMOTO, K., YONEMORI, M., OSAKO, Y., MEGURO-HORIKE, M., HORIKE, S. I., NAKAGAWA, M. & ENOKIDA, H. 2018. Bromodomain protein BRD4 inhibitor JQ1 regulates potential prognostic molecules in advanced

renal cell carcinoma. *Oncotarget,* 9**,** 23003-23017.
SANZ-ALVAREZ, M., CRISTOBAL, I., LUQUE, M., SANTOS, A., ZAZO, S., MADOZ-GURPIDE, J., CARAMES, C., CHIANG, C. M., GARCIA-FONCILLAS, J., EROLES, P., ALBANELL, J. & ROJO, F. 2021. Expression of Phosphorylated BRD4 Is Markedly Associated with the Activation Status of the PP2A Pathway and Shows a Strong Prognostic Value in Triple Negative Breast Cancer Patients. *Cancers (Basel),* 13.
SCHLAFSTEIN, A. J., WITHERS, A. E., RUDRA, S., DANELIA, D., SWITCHENKO, J. M., MISTER, D., HARARI, S., ZHANG, H., DADDACHA, W., EHDAIVAND, S., LI, X., TORRES, M. A. & YU, D. S. 2018. CDK9 Expression Shows Role as a Potential Prognostic Biomarker in Breast Cancer Patients Who Fail to Achieve Pathologic Complete Response after Neoadjuvant Chemotherapy. *Int J Breast Cancer,* 2018**,** 6945129.
SEGURA, M. F., FONTANALS-CIRERA, B., GAZIEL-SOVRAN, A., GUIJARRO, M. V., HANNIFORD, D., ZHANG, G., GONZALEZ-GOMEZ, P., MORANTE, M., JUBIERRE, L., ZHANG, W., DARVISHIAN, F., OHLMEYER, M., OSMAN, I., ZHOU, M. M. & HERNANDO, E. 2013. BRD4 sustains melanoma proliferation and represents a new target for epigenetic therapy. *Cancer Res,* 73**,** 6264-76.
SENGUPTA, S., BIARNES, M. C., CLARKE, R. & JORDAN, V. C. 2015. Inhibition of BET proteins impairs estrogen-mediated growth and transcription in breast cancers by pausing RNA polymerase advancement. *Breast Cancer Res Treat,* 150**,** 265-78.
SHAHBAZI, J., LIU, P. Y., ATMADIBRATA, B., BRADNER, J. E., MARSHALL, G. M., LOCK, R. B. & LIU, T. 2016. The Bromodomain Inhibitor JQ1 and the Histone Deacetylase Inhibitor Panobinostat Synergistically Reduce N-Myc Expression and Induce Anticancer Effects. *Clin Cancer Res,* 22**,** 2534-44.
SHORSTOVA, T., FOULKES, W. D. & WITCHER, M. 2021. Achieving clinical success with BET inhibitors as anti-cancer agents. *Br J Cancer,* 124**,** 1478-1490.
SHU, S., LIN, C. Y., HE, H. H., WITWICKI, R. M., TABASSUM, D. P., ROBERTS, J. M., JANISZEWSKA, M., HUH, S. J., LIANG, Y., RYAN, J., DOHERTY, E., MOHAMMED, H., GUO, H., STOVER, D. G., EKRAM, M. B., BROWN, J., D'SANTOS, C., KROP, I. E., DILLON, D., MCKEOWN, M., OTT, C., QI, J., NI, M., RAO, P. K., DUARTE, M., WU, S. Y., CHIANG, C. M., ANDERS, L., YOUNG, R. A., WINER, E., LETAI, A., BARRY, W. T., CARROLL, J. S., LONG, H., BROWN, M., LIU, X. S., MEYER, C. A., BRADNER, J. E. & POLYAK, K. 2016. Response and resistance to BET bromodomain inhibitors in triple-negative breast cancer. *Nature,* 529**,** 413-417.
SHU, S., WU, H. J., GE, J. Y., ZEID, R., HARRIS, I. S., JOVANOVIC, B., MURPHY, K., WANG, B., QIU, X., ENDRESS, J. E., REYES, J., LIM, K., FONT-TELLO, A., SYAMALA, S., XIAO, T., REDDY CHILAMAKURI, C. S., PAPACHRISTOU, E. K., D'SANTOS, C., ANAND, J., HINOHARA, K., LI, W., MCDONALD, T. O., LUOMA, A., MODISTE, R. J., NGUYEN, Q. D., MICHEL, B., CEJAS, P., KADOCH, C., JAFFE, J. D., WUCHERPFENNIG, K. W., QI, J., LIU, X. S., LONG, H., BROWN, M., CARROLL, J. S., BRUGGE, J. S., BRADNER, J., MICHOR, F. & POLYAK, K. 2020. Synthetic Lethal and Resistance Interactions with BET Bromodomain Inhibitors in Triple-Negative Breast Cancer. *Mol Cell,* 78**,** 1096-1113 e8.
STATHIS, A. & BERTONI, F. 2018. BET Proteins as Targets for Anticancer Treatment. *Cancer Discov,* 8**,** 24-36.

STRATIKOPOULOS, E. E., DENDY, M., SZABOLCS, M., KHAYKIN, A. J., LEFEBVRE, C., ZHOU, M. M. & PARSONS, R. 2015. Kinase and BET Inhibitors Together Clamp Inhibition of PI3K Signaling and Overcome Resistance to Therapy. *Cancer Cell,* 27**,** 837-51.
SUN, Y., CAMPISI, J., HIGANO, C., BEER, T. M., PORTER, P., COLEMAN, I., TRUE, L. & NELSON, P. S. 2012. Treatment-induced damage to the tumor microenvironment promotes prostate cancer therapy resistance through WNT16B. *Nat Med,* 18**,** 1359-68.
SUNG, H., FERLAY, J., SIEGEL, R. L., LAVERSANNE, M., SOERJOMATARAM, I., JEMAL, A. & BRAY, F. 2021. Global cancer statistics 2020: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA Cancer J Clin*.
SUZUKI, C., YAMADA, A., ADACHI, S., SHIMA, H., KIDA, K., OSHI, M., SUGAE, S., YAMAMOTO, S., NARUI, K., TANABE, M., TAKABE, K., ICHIKAWA, Y., ISHIKAWA, T. & ENDO, I. 2021. Bromodomain-containing Protein 4 Is a Favourable Prognostic Factor in Breast Cancer Patients. *Anticancer Res,* 41**,** 3597-3606.
TASDEMIR, N., BANITO, A., ROE, J. S., ALONSO-CURBELO, D., CAMIOLO, M., TSCHAHARGANEH, D. F., HUANG, C. H., AKSOY, O., BOLDEN, J. E., CHEN, C. C., FENNELL, M., THAPAR, V., CHICAS, A., VAKOC, C. R. & LOWE, S. W. 2016. BRD4 Connects Enhancer Remodeling to Senescence Immune Surveillance. *Cancer Discov,* 6**,** 612-29.
TOMCZAK, K., CZERWINSKA, P. & WIZNEROWICZ, M. 2015. The Cancer Genome Atlas (TCGA): an immeasurable source of knowledge. *Contemp Oncol (Pozn),* 19**,** A68-77.
TSENG, H. Y., DREYER, J., EMRAN, A. A., GUNATILAKE, D., PIROZYAN, M., CULLINANE, C., DUTTON-REGESTER, K., RIZOS, H., HAYWARD, N. K., MCARTHUR, G., HERSEY, P., TIFFEN, J. & GALLAGHER, S. 2020. Co-targeting bromodomain and extra-terminal proteins and MCL1 induces synergistic cell death in melanoma. *Int J Cancer,* 147**,** 2176-2189.
UHLEN, M., FAGERBERG, L., HALLSTROM, B. M., LINDSKOG, C., OKSVOLD, P., MARDINOGLU, A., SIVERTSSON, A., KAMPF, C., SJOSTEDT, E., ASPLUND, A., OLSSON, I., EDLUND, K., LUNDBERG, E., NAVANI, S., SZIGYARTO, C. A., ODEBERG, J., DJUREINOVIC, D., TAKANEN, J. O., HOBER, S., ALM, T., EDQVIST, P. H., BERLING, H., TEGEL, H., MULDER, J., ROCKBERG, J., NILSSON, P., SCHWENK, J. M., HAMSTEN, M., VON FEILITZEN, K., FORSBERG, M., PERSSON, L., JOHANSSON, F., ZWAHLEN, M., VON HEIJNE, G., NIELSEN, J. & PONTEN, F. 2015. Proteomics. Tissue-based map of the human proteome. *Science,* 347**,** 1260419.
VAZQUEZ, R., RIVEIRO, M. E., ASTORGUES-XERRI, L., ODORE, E., REZAI, K., ERBA, E., PANINI, N., RINALDI, A., KWEE, I., BELTRAME, L., BEKRADDA, M., CVITKOVIC, E., BERTONI, F., FRAPOLLI, R. & D'INCALCI, M. 2017. The bromodomain inhibitor OTX015 (MK-8628) exerts anti-tumor activity in triple-negative breast cancer models as single agent and in combination with everolimus. *Oncotarget,* 8**,** 7598-7613.
VERDIN, E. & OTT, M. 2015. 50 years of protein acetylation: from gene regulation to epigenetics, metabolism and beyond. *Nat Rev Mol Cell Biol,* 16**,** 258-64.
VERMA, N., VINIK, Y., SAROHA, A., NAIR, N. U., RUPPIN, E., MILLS, G., KARN, T., DUBEY, V., KHERA, L., RAJ, H., MAINA, F. & LEV, S. 2020. Synthetic lethal combination targeting BET uncovered intrinsic susceptibility of TNBC to ferroptosis. *Sci Adv,* 6.
WAKITA, M., TAKAHASHI, A., SANO, O., LOO, T. M., IMAI, Y., NARUKAWA, M., IWATA,

H., MATSUDAIRA, T., KAWAMOTO, S., OHTANI, N., YOSHIMORI, T. & HARA, E. 2020. A BET family protein degrader provokes senolysis by targeting NHEJ and autophagy in senescent cells. *Nat Commun,* 11**,** 1935.
WANG, H., HONG, B., LI, X., DENG, K., LI, H., YAN LUI, V. W. & LIN, W. 2017. JQ1 synergizes with the Bcl-2 inhibitor ABT-263 against MYCN-amplified small cell lung cancer. *Oncotarget,* 8**,** 86312-86324.
WANG, J., LIU, Z., WANG, Z., WANG, S., CHEN, Z., LI, Z., ZHANG, M., ZOU, J., DONG, B., GAO, J. & SHEN, L. 2018. Targeting c-Myc: JQ1 as a promising option for c-Myc-amplified esophageal squamous cell carcinoma. *Cancer Lett,* 419**,** 64-74.
WANG, X., WEI, X., CAO, Y. & XING, P. 2022. Mcl-1 inhibition overcomes BET inhibitor resistance induced by low FBW7 expression in breast cancer. *J Cell Mol Med,* 26**,** 1672-1683.
WEDEH, G., CERNY-REITERER, S., EISENWORT, G., HERRMANN, H., BLATT, K., HADZIJUSUFOVIC, E., SADOVNIK, I., MULLAUER, L., SCHWAAB, J., HOFFMANN, T., BRADNER, J. E., RADIA, D., SPERR, W. R., HOERMANN, G., REITER, A., HORNY, H. P., ZUBER, J., AROCK, M. & VALENT, P. 2015. Identification of bromodomain-containing protein-4 as a novel marker and epigenetic target in mast cell leukemia. *Leukemia,* 29**,** 2230-7.
WEN, N., GUO, B., ZHENG, H., XU, L., LIANG, H., WANG, Q., WANG, D., CHEN, X., ZHANG, S., LI, Y. & ZHANG, L. 2019. Bromodomain inhibitor jq1 induces cell cycle arrest and apoptosis of glioma stem cells through the VEGF/PI3K/AKT signaling pathway. *Int J Oncol,* 55**,** 879-895.
WHITE, M. E., FENGER, J. M. & CARSON, W. E., 3RD 2019. Emerging roles of and therapeutic strategies targeting BRD4 in cancer. *Cell Immunol,* 337**,** 48-53.
WOODCOCK, C. L. & GHOSH, R. P. 2010. Chromatin higher-order structure and dynamics. *Cold Spring Harb Perspect Biol,* 2**,** a000596.
WU, Y., WANG, Y., DIAO, P., ZHANG, W., LI, J., GE, H., SONG, Y., LI, Z., WANG, D., LIU, L., JIANG, H. & CHENG, J. 2019. Therapeutic Targeting of BRD4 in Head Neck Squamous Cell Carcinoma. *Theranostics,* 9**,** 1777-1793.
XU, J. L., YUAN, Y. J., LV, J., QI, D., WU, M. D., LAN, J., LIU, S. N., YANG, Y., ZHAI, J. & JIANG, H. M. 2020. Inhibition of BRD4 triggers cellular senescence through suppressing aurora kinases in oesophageal cancer cells. *J Cell Mol Med,* 24**,** 13036-13045.
YANG, H., WEI, L., XUN, Y., YANG, A. & YOU, H. 2021. BRD4: An emerging prospective therapeutic target in glioma. *Mol Ther Oncolytics,* 21**,** 1-14.
YANG, L., PEREZ, A. A., FUJIE, S., WARDEN, C., LI, J., WANG, Y., YUNG, B., CHEN, Y. R., LIU, X., ZHANG, H., ZHENG, S., LIU, Z., ANN, D. & YEN, Y. 2014. Wnt modulates MCL1 to control cell survival in triple negative breast cancer. *BMC Cancer,* 14**,** 124.
YANG, L., ZHANG, Y., SHAN, W., HU, Z., YUAN, J., PI, J., WANG, Y., FAN, L., TANG, Z., LI, C., HU, X., TANYI, J. L., FAN, Y., HUANG, Q., MONTONE, K., DANG, C. V. & ZHANG, L. 2017. Repression of BET activity sensitizes homologous recombination-proficient cancers to PARP inhibition. *Sci Transl Med,* 9.
YIN, L., DUAN, J. J., BIAN, X. W. & YU, S. C. 2020. Triple-negative breast cancer molecular subtyping and treatment progress. *Breast Cancer Res,* 22**,** 61.
ZAWISTOWSKI, J. S., BEVILL, S. M., GOULET, D. R., STUHLMILLER, T. J., BELTRAN, A. S., OLIVARES-QUINTERO, J. F., SINGH, D., SCIAKY, N., PARKER, J. S., RASHID, N. U.,

CHEN, X., DUNCAN, J. S., WHITTLE, M. C., ANGUS, S. P., VELARDE, S. H., GOLITZ, B. T., HE, X., SANTOS, C., DARR, D. B., GALLAGHER, K., GRAVES, L. M., PEROU, C. M., CAREY, L. A., EARP, H. S. & JOHNSON, G. L. 2017. Enhancer Remodeling during Adaptive Bypass to MEK Inhibition Is Attenuated by Pharmacologic Targeting of the P-TEFb Complex. *Cancer Discov,* 7**,** 302-321.

ZHANG, H. P., LI, G. Q., ZHANG, Y., GUO, W. Z., ZHANG, J. K., LI, J., LV, J. F. & ZHANG, S. J. 2018. Upregulation of Mcl-1 inhibits JQ1-triggered anticancer activity in hepatocellular carcinoma cells. *Biochem Biophys Res Commun,* 495**,** 2456-2461.

ZHANG, P., WANG, D., ZHAO, Y., REN, S., GAO, K., YE, Z., WANG, S., PAN, C. W., ZHU, Y., YAN, Y., YANG, Y., WU, D., HE, Y., ZHANG, J., LU, D., LIU, X., YU, L., ZHAO, S., LI, Y., LIN, D., WANG, Y., WANG, L., CHEN, Y., SUN, Y., WANG, C. & HUANG, H. 2017. Intrinsic BET inhibitor resistance in SPOP-mutated prostate cancer is mediated by BET protein stabilization and AKT-mTORC1 activation. *Nat Med,* 23**,** 1055-1062.

ZHANG, W., GE, H., JIANG, Y., HUANG, R., WU, Y., WANG, D., GUO, S., LI, S., WANG, Y., JIANG, H. & CHENG, J. 2020. Combinational therapeutic targeting of BRD4 and CDK7 synergistically induces anticancer effects in head and neck squamous cell carcinoma. *Cancer Lett,* 469**,** 510-523.

ZHAO, Y., YANG, C. Y. & WANG, S. 2013. The making of I-BET762, a BET bromodomain inhibitor now in clinical development. *J Med Chem,* 56**,** 7498-500.

ZONG, D., GU, J., CAVALCANTE, G. C., YAO, W., ZHANG, G., WANG, S., OWONIKOKO, T. K., HE, X. & SUN, S. Y. 2020. BRD4 Levels Determine the Response of Human Lung Cancer Cells to BET Degraders That Potently Induce Apoptosis through Suppression of Mcl-1. *Cancer Res,* 80**,** 2380-2393.

ZUBER, J., SHI, J., WANG, E., RAPPAPORT, A. R., HERRMANN, H., SISON, E. A., MAGOON, D., QI, J., BLATT, K., WUNDERLICH, M., TAYLOR, M. J., JOHNS, C., CHICAS, A., MULLOY, J. C., KOGAN, S. C., BROWN, P., VALENT, P., BRADNER, J. E., LOWE, S. W. & VAKOC, C. R. 2011. RNAi screen identifies Brd4 as a therapeutic target in acute myeloid leukaemia. *Nature,* 478**,** 524-8.

7 Statutory Declaration

"I, Yuanchun Ye, by personally signing this document in lieu of an oath, hereby affirm that I prepared the submitted dissertation on the topic [Response and Resistance to BET Inhibitors in Triple Negative Breast Cancer/Ansprechen auf und Resistenz gegen BET-Inhibitoren bei triple-negativem Brustkrebs], independently and without the support of third parties and that I used no other sources or aids than those stated.

All parts that are based on the publications or presentations of other authors, either in letter or spirit, are specified as such in accordance with the citing guidelines. The sections on methodology (in particular regarding practical work, laboratory regulations, and statistical processing) and results (in particular regarding figures, charts, and tables) are exclusively my responsibility.

Furthermore, I declare that I have correctly marked all of the data, the analyses, and the conclusions generated from data obtained in collaboration with other persons and that I have correctly marked my own contribution and the contributions of other persons (cf. the declaration of contribution). I have correctly marked all texts or parts of texts that were generated in collaboration with other individuals.

My contributions to any publications related to this dissertation correspond to those stated in the below joint declaration made together with my supervisor. All publications created within the scope of the dissertation comply with the guidelines on authorship of the ICMJE (International Committee of Medical Journal Editors; www.icmje.org). In addition, I declare that I shall comply with the regulations of Charité – Universitätsmedizin Berlin on ensuring good scientific practice.

I declare that I have not yet submitted this dissertation in an identical or similar form to another faculty member.

The significance of this statutory declaration and the consequences of a false statutory declaration under criminal law (Sections 156 and 161 of the German Criminal Code) are known to me."

Date: Signature

8 Curriculum Vitae

For data protection reasons, my CV will not be published in the electronic version of my work.

9 List of Publication

- Chen Y, Ye Y, Krauß PL, Löwe P, Pfeiffenberger M, Damerau A, Ehlers L, Buttgereit T, Hoff P, Buttgereit F, Gaber T. Age-related increase of mitochondrial content in human memory CD4+ T cells contributes to ROS-mediated increased expression of proinflammatory cytokines. Front Immunol. 2022 Jul 22; 13:911050. doi: 10.3389/fimmu.2022.911050. PMID: 35935995; PMCID: PMC9353942.
- Ye Y, Ge O, Zang C, Yu L, Eucker J, Chen Y. LINC01094 Predicts Poor Prognosis in Patients with Gastric Cancer and is Correlated With EMT and Macrophage Infiltration. Technol Cancer Res Treat. 2022 Jan-Dec; 21:15330338221080977. doi: 10.1177/15330338221080977. PMID: 35254147; PMCID: PMC8905065.
- Ge O, Huang A, Wang X, Chen Y, Ye Y, Schomburg L. PALB2 upregulation is associated with a poor prognosis in pancreatic ductal adenocarcinoma. Oncol Lett. 2021 Mar; 21(3):224. doi: 10.3892/ol.2021.12485. Epub 2021 Jan 24. PMID: 33613713; PMCID: PMC7856690.
- Yuling Chen, Yuanchun Ye, Hao Wu, Pierre-Louis Krauß, Pelle Löwe, Moritz Pfeiffenberger, Lisa Ehlers, Thomas Buttgereit, Paula Hoff, Frank Buttgereit and Timo Gaber. Metabolic Reprogramming in Memory CD4+T Cells Is Associated with Immune Cell Dysfunction During Aging. Arthritis & Rheumatology. 2020 72(10):1408.
- Fan, G., Zhu, S., Ye, M., Long, Y., Liu, Z., Ye, Y., Han, W., &Xie, Y. Three-dimensional-printed models in bladder radical cystectomy: a valuable tool for surgical training and education. Three-dimensional-printed models in bladder radical cystectomy: a valuable tool for surgical training and education. International journal of clinical and experimental medicine. 2019.12(8):10145-10150.

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My heart races as I finish the paper; how many respectable friends helped me from the beginning to the end of the subject thesis? Please accept my heartfelt gratitude!

11 Certificate of the Accredited Statistician

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Bescheinigung

Hiermit bescheinige ich, dass Herr *Yuanchun Ye* innerhalb der Service Unit Biometrie des Instituts für Biometrie und klinische Epidemiologie (iBikE) bei mir eine statistische Beratung zu einem Promotionsvorhaben wahrgenommen hat. Folgende Beratungstermine wurden wahrgenommen:

- Termin 1: *18.06.2021*

Folgende wesentliche Ratschläge hinsichtlich einer sinnvollen Auswertung und Interpretation der Daten wurden während der Beratung erteilt:

- Kruskal-Wallis Test und One-way ANOVA
- Graphische Darstellung
- T-test für abhängige Stichproben oder Wilcoxon-Test

Diese Bescheinigung garantiert nicht die richtige Umsetzung der in der Beratung gemachten Vorschläge, die korrekte Durchführung der empfohlenen statistischen Verfahren und die richtige Darstellung und Interpretation der Ergebnisse. Die Verantwortung hierfür obliegt allein dem Promovierenden. Das Institut für Biometrie und klinische Epidemiologie übernimmt hierfür keine Haftung.

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