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**High throughput *in silico* screening and *in vitro* testing of polypharmacological
bromodomain 4 and cancer associated kinase inhibitors**

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III. List of Abbreviations

-ΔG	change in free energy
•OH	hydroxyl
ACD	Asymmetric cell division
APC	apical junction complex
APC/C	anaphase promoting complex/cyclosome
ATP	adenosine triphosphate
AUC	area under the curve
AURK	aurora kinase
AURKA	aurora kinase A
AURKB	aurora kinase B
B-ALL	B cell acute lymphoblastic leukaemia
BCR	B-cell receptor
BD	bromodomain

BET	bromodomain and extra-terminal proteins
BID	basic residue-enriched interaction domain
BMI	body mass index
BRCA1	breast cancer type 1 susceptibility protein
BRD4	Bromodomain containing protein 4
BRD4-L	BRD4 long isoform
BRD4-S	BRD4 short isoform
CAK	CDK-activating kinases
CCM	complete culture media
CDK	cyclin dependant kinase
CHPC	Centre for high performance computing
Cip/Kip	CDK interacting protein/Kinase inhibitory protein
c-Myc	cellular myelocytomatosis
coifilin-PI3K	coifilin-phosphoinositide-3-kinase
CPC	chromosomal passenger complex
CPS	C-terminal cluster of phosphorylation sites
CRLF2	Cytokine receptor-like factor 2
CSC	Cancer stem cells
CSIR	Council for scientific and industrial research
CTM	C-terminal interaction motif
CV	crystal violet
DAD	N-terminal A-box/D-box activating domain
D-box	C-terminal located destruction box

DHE	dihydroethidium
DLBCL	diffuse large B cell lymphoma
DMEM	Dulbecco's modified Eagle's medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DUD-E	directory of useful decoys-enhanced
EGFR	epidermal growth factor receptor
ELISA	enzyme-linked immunosorbent assay
EMT	epithelial-mesenchymal transition
eRNA	enhancer ribonucleic acid
ER α	oestrogen receptor α
ER α	oestrogen receptor α
ET	extra terminal domain
FCS	foetal calf serum
FOS	AP-1 transcription factor subunit
FOXA1	forkhead box protein A1
G	gap phase
G ₀	gap zero phase
GLTSCR1	glioma tumour suppressor candidate region gene 1
GPX	glutathione peroxidase
GS	glutathione reductase
GSK3- β	glycogen synthase kinase 3 beta
H	hydrogen

H ₂ O ₂	hydrogen peroxide
H3	histone 3
H4	Histone 4
hCDH1	fizzy related protein homolog
HER2	human epidermal growth factor 2
HIF-1α	hypoxia-inducible factor 1-α
HTVS	high-throughput virtual screening
<i>IL7R</i>	Interleukin 7 receptor gene
INCEP	inner centromere protein
INK	inhibitors of CDK4 and CDK6
JAK/STAT	Janus kinase/signal transducer and activator of transcription
JMJD6	arginine demethylase and lysyl-hydroxylase
KEN	lysine (K), glutamic acid (E), asparagine (N) sequence
M	mitotic phase
MaSC	mammary stem cell
MET	mesenchymal-epithelial transition
MMTV-PyMT	mouse mammary tumour virus-polyoma middle tumour-antigen
mTOR	mammalian target of Rapamycin
MTT	2,5- dimethyl thiazolyl diphenyl tetrazolium bromide
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
NMC	Nut-midline carcinoma
NOC	nocodazole
NPS	N-terminal cluster of phosphorylation sites

x

NSD3	Nuclear Receptor Binding SET Domain Protein 3
O ₂ ⁻	superoxide
OD	optical density
OPLS-AA	all-atom optimized potentials for liquid simulations
P53	protein 53
PDB	Protein Data Bank
PI	propidium iodide
PLK1	polo-like kinase
P-TEFb	positive transcription elongation factor b
QSAR	quantitative structure-activity relationships
R ²	coefficient of determination
Rb	retinoblastoma
RELA	REL associated protein
RNA Pol II	RNA polymerase II
ROC	receiver operating characteristic
ROS	reactive oxygen species
R-point	restriction point
S	Synthesis phase
SBVS	Structure based virtual screening
SDC	Symmetrically divided stem cells
SEED	Ser (S)/Glu (E)/ Asp (D)
SMILES	simplified molecular-input line-entry system
SOD	superoxide dismutase

SP	standard precision
TK	tyrosine-kinase
TNBC	Triple negative breast cancer
v/v	volume/volume
VC	vehicle control
VSW	Virtual Screening Workflow
w/v	weight/volume

Abstract

Polypharmacology has recently become an intense subject of research, specifically in the development and design of anti-cancer compounds. Drugs that have more than one target, potentially have higher efficacy in the treatment of triple negative breast cancer and prevent the development of drug resistance. Bromodomain containing protein 4 (BRD4) is a known dysregulated enzyme in triple negative breast cancer, together with other kinases such as aurora kinase A and B (AURKA and AURKB), cyclin dependant kinases 4 and 6 (CDK4/6), polo-like kinase (PLK1) and epidermal growth factor receptor (EGFR). The research set out to identify a poly-inhibitors of BRD4 and cancer associated kinases, using virtual high throughput screening. The Biofocus™ library of compounds, supplied by the South African council for scientific and industrial research (CSIR), was docked into the crystal structures of BRD4 using the Maestro interface suite from which a list of promising compounds with docking scores lower than -9, indicating good enzyme/inhibitor interaction, was identified. This list of compounds was then docked into the crystal structures of AURKA and B, PLK1 and EGFR individually generating a second list of 10 compounds (docking score $\leq$ -9). The 10 promising compounds have either imidazo[1,2-*b*]pyridazine or 1*H*-imidazo[4,5-*c*]pyridine backbones in their chemical structure. The 10 poly-inhibitors underwent *in vitro* screening from which one compound was identified as being active. Further *in vitro* assays were performed to elucidate its effect on TNBCs *in vitro*.

1. Chapter 1: Introduction

Breast cancer, the leading cancer type among women globally, accounts for about 11.6% of all female cancers¹. There seems to be a recent decrease in death from breast cancer in developed countries, and a substantial increase in death due to breast cancer is seen in developing nations¹⁻². Additionally, an alarming amount of younger women present with cases of breast cancer¹. For cancer to develop, a complex and coordinated change in cellular signalling and metabolism must take place³. The mechanisms underpinning said changes are known as the hallmarks of cancer and include: sustained proliferative signalling, initiating replicative immortality, inducing invasion and metastasis, activating angiogenesis, evasion of growth suppression and eventual cell death⁴. Recently, due to extensive increases in research output, four additional hallmarks have been identified for inclusion into this list, namely; the ability of cells to regress from a specialized functional state, epigenetic changes and their function in altered gene expression, the effect of microorganisms and neuronal signalling⁵.

1.1. Triple negative breast cancer

Triple negative breast cancer (TNBC) refers to the phenotype of breast cancer that lack expression of the oestrogen and progesterone receptor as well as lacking human epidermal growth factor 2 (HER2) overexpression⁶. Between 10 and 15 % of breast carcinomas have this phenotype, constituting approximately 80 % of all basal-like-tumours⁶. Some risk factors predisposing women to TNBC include being young at the time of diagnosis, experiencing early onset of puberty, giving birth multiple times, but not breastfeed and a high body mass index (BMI)⁶. The majority of tumours that express the breast cancer type 1 susceptibility protein (BRCA1) are TNBC⁶. TNBC is associated with earlier relapse and worse prognosis compared to other breast cancer types⁶. It however shows increased chemosensitivity compared to other breast cancer subtypes⁶. TNBC is exceptionally heterogenous, making drug discovery for specific targets very difficult⁷. This heterogeneity is largely due, it seems, to breast cancer stem cells, underpinning the ability of TNBC to develop resistance to treatment, recur and metastasize⁷. Currently, there are multiple agents against specific TNBC targets being studied such as EGFR⁶.

1.2. Asymmetric cell division and cancer

Asymmetric cell division (ACD) is a property possessed by stem cells, where they divide in an asymmetrical fashion by unequally distributing cellular constituents as a result of unstable spindle orientation during cell division⁸. The distribution of the cellular constituents between the different daughter cells decides their individual fates of either becoming a rapid dividing progenitor cell or a self-renewing stem cell⁸⁻⁹. Asymmetric cell division sustains a consistent number of stem cells in addition to the production of progenitor cells that undergoes distinct differentiation following clonal expansion⁸. Due to the relatively long lifespans of adult mammary stem cells (MaSCs) and their ability to renew themselves, they are at increased risk of undergoing transformation events potentially leading to cancer⁹. Cancer stem cells (CSC) like stem cells divide both symmetrically (SCD) and asymmetrically⁸⁻¹¹. SCD leads to increased numbers of the CSC population and ACD maintains CSC whilst at the same time causing progressive tumour growth and metastasis⁹⁻¹¹. In breast cancer, breast CSC's progressively favour ACD above SCD in mammosphere (three dimensional cell culture of a heterogeneous population of cells growing in clumps that contain aggregates of CSC's) growth⁸.

1.3. Epithelial to mesenchymal transition in cancer

Epithelial cells provide cell-to-cell cohesion, which is essential for the maintenance of the human body, providing a physical barrier, that is paramount to the establishment and regulation of an internal environment¹². Mesenchymal cells, in contrast to epithelial cells, are motile and extremely invasive and act as supporting tissue, producing the extracellular matrix important for the support and structure of epithelial cells¹². Epithelial and mesenchymal phenotypes are very dynamic depending on the environment and specific cell type¹². The ability of epithelial and mesenchymal cells to transition between the phenotypes is known as epithelial-mesenchymal transition (EMT). The reverse of EMT is mesenchymal-epithelial transition (MET) which play pivotal roles in the wound healing and embryogenesis¹². EMT is characterized by a radical change in expression patterns and cytoskeleton remodelling which leads to the mobility and extensive invasiveness of mesenchymal cells¹³. EMT is strongly exploited

by tumours through the utilization of EMT transcription factors that enhance their mesenchymal phenotype, which in turn strengthens their invasiveness and resistance to treatment⁸. In addition to increasing the invasiveness of tumours, EMT can also aid in the acquisition of a CSC phenotype aiding metastasis⁸. The cancer stem cell phenotype is characterized by the ability to form mammospheres and tumorigenesis in mice¹⁴. There is some controversy regarding the role of EMT in cancer, because *in vitro* models are not indicative of the complete picture *in situ* and it is not possible to view this *in vivo* mainly because EMT is transient and reversible¹⁵.

1.4. The cell cycle

Most of the cells of the human body are not actively going through the cell cycle, some cells are terminally differentiated and are irreversibly withdrawn from the cell cycle, other cells are reversibly withdrawn from the cell cycle, known as quiescent cells or gap zero phase cells (G_0)¹⁶. The cell cycle has four sequential phases, the synthesis (S) phase and the mitotic (M) phase, these two phases are separated from each other by the gap (G) phases, namely G_1 and G_2 ¹⁶. The G_1 -phase follows the M-phase and G_2 follows on from the S-phase¹⁶. The G_1 -phase, is the point where the cell integrates mitogenic and growth inhibitory signals to decide between proceeding, pausing, or exiting the cell cycle¹⁷. The G_1 phase also contains a very important check-point known as the restriction point¹⁷. The S-phase is defined as the phase where deoxyribonucleic acid (DNA) synthesis occurs¹⁷. The G_2 -phase describes the time between the S-and M-phase where the cell prepares for division¹⁷. During the M-phase the replicated chromosomes are separated to opposite poles and cytokinesis occurs to produce 2 daughter cells¹⁷. The regulation of the cell cycle is carried out by cyclins (16 different types) and CDKs (nine different types)¹⁷.

When G_0 cells are stimulated to enter the cell cycle, the D-family of cyclins are induced. The D-cyclins do not oscillate during the cell cycle but are rather controlled by the presence of growth factors¹⁷. The D-cyclins activate and associate with CDK4 and CDK6¹⁷. The D-cyclins also target the retinoblastoma (Rb) protein, which plays a role in the restriction checkpoint activation, for phosphorylation¹⁷⁻¹⁸. The Rb protein also binds to E2F transcription factor family, that regulate the expression of many genes important in the regulation of cell cycle progression, inhibiting their function¹⁷. Thus

phosphorylation of Rb allows the E2F family of proteins to perform their function, more specifically the induction of cyclin E¹⁷. The cyclin E/CDK2 complex is important for the transition of G₁ to S-phase¹⁷. Cyclin A is regulated by the E2F proteins, and accumulates at the G₁/S transition and persists through S phase, where it initially associates with CDK2 but later in S-phase it associates with CDK1¹⁶⁻¹⁷. Cyclin A is important in the G₁/S progression, S-phase progression, and entry into M-phase¹⁷. The G₂-phase contains a checkpoint for DNA damage that prevent progression to M-phase if any damage is detected¹⁷. Aurora kinase A together with AURKB plays a critical role in the regulation of the progression of G₂-phase to M-phase¹⁹. The M-phase is regulated by CDK1 in association with cyclin A, B1 and B2¹⁷. For cells to exit M-phase and re-enter the G₁-phase, cyclin A and B must be degraded¹⁷.

1.5. CDK4/6

The CDKs are a conserved family of proline directed serine/threonine kinases that perform critical roles in the stepwise progression of eukaryotic cells through the cell cycle²⁰. All CDK activity is regulated by other kinases such as CDK-activating kinases (CAK), but most importantly by their interaction with cyclins²⁰. Cyclin/CDK complexes are in turn inhibited by the irreversible binding of CDK inhibitors from the CDK interacting protein/Kinase inhibitory protein (Cip/Kip) family and inhibitors of CDK4 and CDK6 (INK) family and through the cyclical degradation of cyclins throughout the cell cycle²⁰.

CDK4/6 regulate the G₁/S transition together with cyclin-D, INK4, and Rb²¹. Progression of cells through the G₁/S phase requires the phosphorylation of Rb by CDK4 or the highly homologous CDK6 in complex with cyclins-D1,D2, and D3²². During the G₁ phase of the cell cycle, a watershed is reached known as the restriction point (R-point) during which the cell either continues into S-phase or enters G₀ quiescence²². The impetus for the continuation with the cell cycle or quiescence is provided by antiproliferative signals²².

Insensitivity to the mentioned antiproliferative signals is a hallmark of cancer⁴. Hypophosphorylated Rb leads to sequestration and repression of E2F leads to a decrease in expression of genes important in G₁/S progression²². Hyperphosphorylation of Rb, however, impairs its repressive ability of E2F, leading to

an increase in the expression of genes important in DNA replication²². TNBC has been shown to overexpress CDK4, which act as a cancer stem cell regulator, and is associated with poor relapse free survival, high tumour grade and overall poor prognostic features²³.

CDK4/6 inhibition in TNBC prevents cancer stem cell self-renewal²³. Studies have shown that treatment with a CDK4/6 inhibitor in Rb proficient TNBC tumours leads to cell cycle arrest and tumour death, there is however a caveat, the expression of Rb in heterogenous TNBC is not well documented²⁴. CDK4/6 inhibition also leads to cellular myelocytomatosis (c-Myc) accumulation, which leads to increased glutamine metabolism, activation of the mammalian target of Rapamycin (mTOR) pathway and the blunting of the hypoxia-inducible factor 1- α (HIF-1 α) mediated response to hypoxia²⁵. Nut-midline carcinoma (NMC) models treated with a BRD4 inhibitor that overexpressed cyclin D1 or mutant cyclin D3 or that lost the function of Rb resisted the BRD4 inhibitor induced cell cycle arrest²⁶. NMC cells treated with a BRD4 and a CDK4/6 inhibitor showed synergistic effects, revealing the integral role that cell cycle regulation and specifically CDK4/6 regulation play in the efficacy of BRD4 inhibition in cancer²⁶.

1.6. Bromodomain and extra-terminal proteins

The bromodomain and extra-terminal proteins (BET) contain four members: BRD2, BRD3, BRD4 and BRDT that bind to acetylated lysine residues of histones and other nuclear protein targets by means of their two amino-terminal bromodomains²⁷. Bromodomain extra-terminal proteins are commonly associated with enhancers, their carboxy-terminals bind to positive transcription elongation factor b (P-TEFb) which contain Cyclin D and the CDK9, leading to the release of promoter-proximal bound RNA polymerase to start RNA elongation²⁷⁻²⁸. In the setting of cancer the BET bromodomains promote the progression of cells into the M-phase and G1-phase of the cell cycle²⁸.

1.7. Bromodomain containing protein 4

Bromodomain containing protein 4 is a cellular chromatin binding factor, regulating transcription elongation and epigenetic modification²⁹⁻³⁰. It has histone acetyltransferase binding abilities as well as kinase activity³¹. It forms fusion proteins with other nuclear proteins, an indication of its vital role in cancer progression³⁰. The overexpression of BRD4 have been associated with a poor outcome in cancer patients³⁰. Ectopic expression of BRD4 in oestrogen positive breast carcinoma results in an expression signature that predicts a negative prognosis in patients³². Recently BRD4 as a target for inhibition in TNBC has shown great promise³³.

The two bromodomains (BD1 and BD2) of BRD4 are in tandem within the amino-terminus, and facilitate binding to chromatin by means of their interaction with the acetylated lysine of histone 3 (H3) and 4 (H4)³⁴. Although the bromodomains of BRD4 largely bind to acetylated lysine residues of H3 and H4, BD2 is also able to bind to acetylated lysines of other proteins such as the REL associated protein (RELA), subunit of nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B) and the cyclin T1 subunit of P-TEFb³⁵. The structures of BD1 and BD2 consists of two interconnected loops, ZA and BC from their amino-and-carboxy terminals, that consist of the four α -helices A, B, C and Z³⁵. These 2 loops form the pocket that recognises the acetylated lysines, while the four helices form a lefthanded hydrophobic core³⁵.

The amino-terminal domains of BRD4 regulate its interaction with acetylated lysine residues, the carboxy-terminal however governs the interactions with the proteins for which BRD4 is a scaffold³⁵. The extra terminal domain (ET) of BRD4 specific binding partners include, glioma tumour suppressor candidate region gene 1 (GLTSCR1), arginine demethylase and lysyl-hydroxylase (JMJD6), and Nuclear Receptor Binding SET Domain Protein 3 (NSD3), all of which are important for transcription activation³². NSD3 specifically play an important role in aiding the ET mediation of P-TEFb independent transcriptional activation³². It has been shown that the basic residue-enriched interaction domain (BID) together with the N-terminal cluster of phosphorylation sites (NPS) have a phospho switch like mechanism that controls the binding of BRD4 with chromatin as well as its inter- and intra-molecular interactions with protein 53 (p53)³⁶. The Ser (S)/Glu (E)/ Asp (D) (SEED) motif, which forms part

of the C-terminal cluster of phosphorylation sites (CPS), form the site of phosphorylation of CK2, triggering conformational changes in BRD4, affecting its functions³⁵. The P-TEFb reaction site in BRD4 is located in the C-terminal interaction motif (CTM)³⁵.

In addition to their function as transcription factor scaffolds, BRD4 also have enzymatic functions³⁵. BRD4 functions as an atypical kinase phosphorylating serine 2 of the carboxy-terminal domain of RNA polymerase II (RNA Pol II CTD Ser2), this happens independently of P-TEFb, a known Pol II CTD Ser2 kinase³⁷. BRD4 also have histone acetyl-transferase activity, acetylating lysines on the tails of H3 and H4³⁸. BRD4 is contiguous, genome-wide, with H3K122ac and its HAT activity in H3 leads to nuclear expansion through nucleosomal disassembly, leading to chromosome de-compaction³⁸. Nucleosome occupancy is preferentially reduced by BRD4 around genes that it binds and regulate such as *MYC*, AP-1 transcription factor subunit (*FOS*) and *AURKB*³⁸.

BRD4 has recently been receiving a lot of attention as a potential anticancer target due to its role in the expression of the oncogene c-Myc (Figure 1.1)³⁵. The c-MYC protein is a transcription factor that affects cell proliferation, differentiation, cell-cell competition, replicative potential, metabolism and apoptosis³⁹. In the chromosome 8q24 deregulated subtype of TNBC there is a marked increase seen in *c-Myc* expression, which is due to enhancers and super-enhancers located in this region that strongly upregulates its expression⁴⁰. BRD4 plays a pivotal role in this over expression, reacting with the acetyl groups of transcription factors within RNA polymerase II complexes⁴⁰. When BRD4 is inhibited in this instance by a small molecule inhibitor such as JQ1, there is a marked decrease in *c-Myc* expression (Figure 1.1)⁴⁰. MYC expression regulation is not the only function of BRD4, it also plays a role in the regulation of MYC independent pathways, illustrated by the inhibition of growth in diffuse large B cell lymphoma (DLBCL) when treated with a BRD4 inhibitor, but when c-Myc is ectopically expressed, cell growth is not restored, pointing to other pathways under BRD4 control³⁵. The B-cell receptor (BCR) signalling pathway and the target genes of elongation E2F are affected by the inhibition of BRD4³⁵.

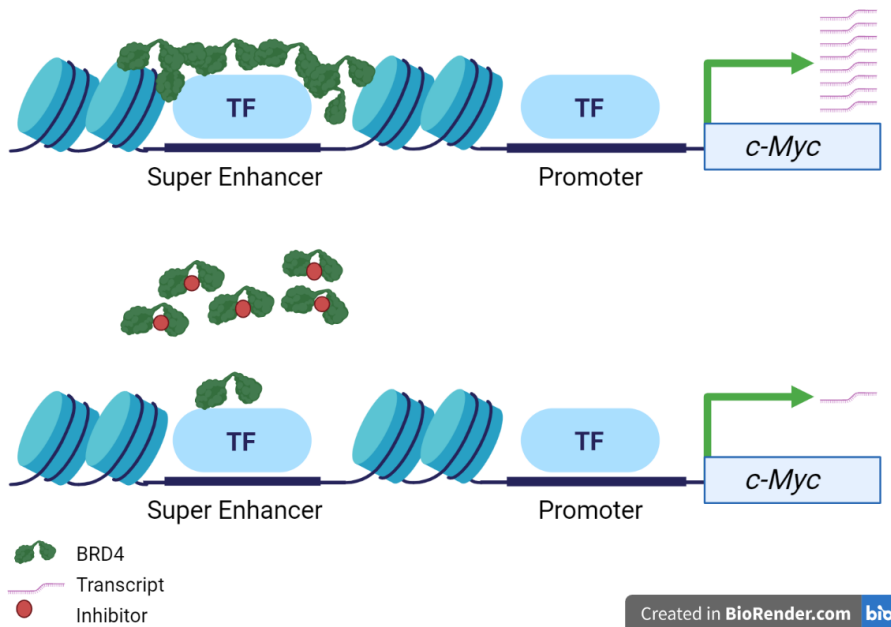


Figure 1. 1 Bromodomain (BD) inhibition displaces BRD4 from super enhancers inhibiting the expression of oncogenes such as *c-Myc*^{30,35}.

BRD4 enhances oestrogen induced transcriptional activity in oestrogen-positive breast cancer⁴¹. BRD4 co-occupies enhancer ribonucleic acid (eRNA) producing enhancer sequences together with oestrogen receptor α (ER α), forkhead box protein A1 (FOXA1) and H3K27ac⁴¹. It also regulates the recruitment of RNA Pol II to ER α bound enhancers⁴¹.

Two isoforms of BRD4 exist, the long isoform (BRD4-L) and the short isoform (BRD4-S)³³. The two isoforms have some common targets, as well as isoform selective targets³³. When BRD4-S is depleted in TNBC-derived xenografts it decreases the progression and growth of the cancer³³. Ectopic expression of BRD4-S increases the primary tumour growth in mouse mammary tumour virus-polyoma middle tumour-antigen (MMTV-PyMT) breast cancer model, as well as increasing the incidence of lung metastasis³³. Conversely the BRD4-L isoform seems to suppress tumour growth and show organ specific metastasis inhibition³³.

The Janus kinase/signal transducer and activator of transcription (JAK/STAT) pathway is a key player in the regulation of proliferation, migration and apoptosis³⁰. This

pathway is severely dysregulated in some cancers³⁰. Inhibition of BRD4 leads to a decrease in the binding of BRD4 to the Interleukin 7 receptor gene (*IL7R*), a key mediator together with Cytokine receptor-like factor 2 (CRLF2) in the proliferation of B cell acute lymphoblastic leukaemia (B-ALL) through the JAK/STAT pathway³⁰. The NF- κ B protein is another important player in the initiation and progression of cancer and is also suppressed by BRD4 inhibitors, blocking the binding of BRD4 to its acetylated lysine 310, inducing its ubiquitin-mediated degradation³⁰. BRD4 inhibition also leads to the inhibition of p53 acetylation, leading to caspase-dependant apoptosis in leukaemia cells with wild type p53³⁰. Triple negative breast cancer is a clinically aggressive disease for which there is no targeted therapy⁴². Triple negative breast cancer has preferential sensitivity *in vitro* (MDA MB-231 cells) and *in vivo* to BRD4 inhibition⁴². BRD4 seems to regulate cell motility (and by extension EMT) by regulating Jagged1/Notch1 signalling and through its interaction with EMT transcription factors such as Twist and Snail⁴³. BRD4 inhibition drastically decreases the expression of Snail which is a powerful EMT transcription factor, by decreasing the stability of the protein and the transcription thereof⁴³.

1.8. Aurora kinase A

The AURK family consists of 3 members in humans, AURKA, AURKB, and AURKC¹⁹. AURKA is a serine threonine kinase that regulates cell cycle (G2) progression and mitosis¹⁹. AURKA is overexpressed in many cancers including breast cancer where this overexpression is an indicator of a poor prognosis⁴⁴. AURKA overexpression is present in 90% of breast carcinomas⁴⁵. AURKA is associated with centrosomes and adjacent spindle fibres, where it plays a role in the regulation of protein function and localisation at the centrosome as well as regulating the assembly, stability and function of the mitotic spindle^{44,46}. It ushers the cell out of the G2/M checkpoint by activating the cyclin dependant kinase1/CyclinB complex, ushering the cell into the M-phase of the cell cycle⁸. It also prepares the cell to divide by its promotion of centrosome maturation and spindle formation⁸. When it is over expressed in cancer it is associated with a basal-like phenotype and poor prognosis⁴⁶.

The *AURKA* gene encodes for a 403 amino acid long protein⁴⁷. The protein is characterized by a C-terminal catalytic domain containing an activation loop⁴⁷. The

activation loop of AURKA contains a signature which requires the autophosphorylation of Thr288 for the activation of the kinase domain⁴⁷. The C-terminal located destruction box (D-box) together with the N-terminal A-box/D-box activating domain (DAD), play an important role in the degradation of AURKA by the anaphase promoting complex/cyclosome (APC/C) ubiquitin proteasome pathway⁴⁷. AURKA degradation occurs in M/G1 phase, when the D-box is targeted by fizzy related proteins that interact with APC and is dependent on the fizzy related protein homolog (hCDH1)⁴⁷. The N-terminal contains the lysine (K), glutamic acid (E), asparagine (N) sequence known as the KEN domain, which serves as a targeting signal for hCDH1-APC facilitated degradation⁴⁷.

Overexpression of AURKA leads to genomic instability, aneuploidy and malignant transformation⁴⁸. An increase in the activity of AURKA abrogates the mitotic spindle checkpoint leading to resistance against anti-mitotic drugs⁴⁵. The activity of microtubule inhibitors are increased by AURKA inhibition⁴⁵. AURKA plays a role in ACD, through its activity on microtubule dynamics that ensure cell polarity⁸. Inhibition of AURKA prevents microtubule shrinkage, growth rate and nucleation during interphase of HeLa cells⁸. The epithelial cell junction which is formed by the apical junction complex (AJC), is dependent upon the polarity of the cells⁸. The loss of polarity, frequently observed in cancer, leads to EMT, enabling cancer cell migration and thus metastasis⁸. AURKA overexpression induces breast cancer metastasis through reorganization of the actin cytoskeleton via the coifilin-phosphoinositide-3-kinase (coifilin-PI3K) pathway⁴⁹.

The basic helix loop protein 37 (N-Myc) is stabilized by AURKA, preventing its ubiquitin-mediated degradation, as well as inducing its expression by downregulating the expression of glycogen synthase kinase 3 beta (GSK3- β) (which binds to, phosphorylates and inactivate N-Myc) and upregulation of β -catenin and its downstream targets⁴⁸. AURKA also regulates c-Myc expression, consequently, AURKA overexpression is accompanied by c-Myc over expression which has been suggested as a mediator of AURKA oncogenic activity⁵⁰. Myc overexpression also in turn upregulates the expression of AURKA⁵¹.

Targeting AURKA in TNBC cells (MDA-MB-231) has shown potent antiproliferative effects pointing to a promising target in TNBC treatment⁵². Dual use of BRD4 and

AURKA inhibitors in the treatment of four high risk glioblastoma models have shown decreased viability and proliferation *in vitro*, this is ascribed to the ability of BRD4 inhibition to mitigate reflexive expression of AURKA, c-Myc and N-Myc in response to AURKA inhibition⁵³.

1.9. Aurora kinase B

AURKB is a chromosomal passenger complex (CPC) protein that plays a central role in chromosome segregation, cytokinesis and histone H3 phosphorylation⁵⁴.

AURKB is composed of 345 amino acids (Figure 1.2)⁴⁷. Like AURKA, AURKB has a catalytic domain, a C-terminal D-box and a N-terminal A-box/DAD domain (Figure 4)⁴⁷. The D-box domain of AURKB, even though it is similar to that of AURKA, does not undergo degradation by the same ubiquitin ligase, instead AURKB undergoes degradation following its binding to the human α -proteasome C8 subunit, in a proteasome dependent manner⁴⁷.

AURKB mRNA and protein levels peak at G2/M phase, with maximum kinase activity from metaphase until the end of mitosis⁴⁷. AURKB exerts its functions in concert with three other proteins with which it associates in the CPC⁴⁷. These three proteins are the inner centromere protein (INCEP), survivin and borealin/Dasra-B⁴⁷. The CPC is present on chromosome condensing arms in early prophase, where it displaces the heterochromatin protein-1 from the DNA as well as taking part in the recruitment of condensin proteins⁴⁷. It phosphorylates histone H3 from early G2 phase to prophase⁴⁷. The CPC localizes to the inner centromere from late prophase to metaphase, playing a role in the formation and stabilization of the bipolar mitotic spindle, kinetochore assembly, correction of non-bipolar chromosome-spindle attachment and control of the spindle checkpoint⁴⁷. At the beginning of anaphase, CPC relocates to the midzone of the mitotic spindle remaining evident in the midbody of telophasic cells where it modulates the activity of proteins active in spindle dynamics, cleavage furrow formation and cytokinesis⁴⁷.

Both overexpression and inhibition of AURKB lead to defects in the spindle assembly, centrosome function, chromosome alignment and cytokinesis in various cancer cells leading to mitotic catastrophe and multinucleation⁵⁴. Endoduplication following

AURKB inhibition in cancer, leads to cell death⁵⁵. Treatment of cells that overexpress c-Myc with an AURKB inhibitor leads to destabilization of the CPC, leading to cell death by means of apoptosis and lethal autophagy, sparing cells that do not overexpress c-Myc *in vitro*⁵⁶. Single-nucleotide polymorphisms in *AURKB* play a role in mediating taxane resistance, in TNBC, and is associated with a negative outcome⁵⁷.

1.10. Polo like kinase 1

Polo like kinases are a family of threonine kinases that play a key role in the regulation of several cellular processes, including DNA replication⁵⁸. PLK1 involves a highly conserved N-terminal catalytic kinase domain a C-terminal polo box domain and a connection region between the N-and C-terminal⁵⁹. The kinase domain of PLK1 is a serine/threonine kinase with a T-loop, whose phosphorylation is directly connected to the kinase activity of PLK1⁵⁹. The C-terminal polo box domain is a notable feature of the PLK family, consisting of two polo box structures with a flexible structure in the middle⁵⁹. The crystal structure of the PLK1 polo box domain indicates a structure analogous to two clips with the phosphopeptide clamped in the middle⁵⁹.

It plays an integral role in the initiation and progression of the cell cycle and the completion of mitosis⁵⁹. PLK1 controls mitotic entry, the G₂/M checkpoint, it coordinates centrosome and spindle assembly, plays an important role in abscission, DNA replication, meiosis and cytokinesis⁵⁹. It also plays an essential role in the exact regulation of the cell cycle, the DNA damage response, spindle assembly and genomic stability during mitosis⁵⁹.

Oncogenic transformation relies on both the activation of proto-oncogenes and the deactivation of tumour suppressor genes, both of which are related to cell cycle regulation⁵⁹. Since PLK1 is a regulator of the cell cycle, it has been found to be over expressed in various different cancers including breast cancer⁵⁹. PLK1 expression, however, is low in surrounding, normal, non-dividing tissue⁶⁰. Inhibiting PLK1 significantly reduces the rate of DNA synthesis in cancer cells, reducing growth and proliferation as well as inducing apoptosis⁵⁸. In TNBC, tumours that are Rb deficient, high amounts of PLK1 is expressed⁶¹. These Rb deficient triple negative tumours show increased sensitivity to PLK1 inhibition⁶¹. Treating with both a BRD4 inhibitor and a PLK1 inhibitor in a prostate cancer model shows synergism, leading to G₂/M arrest,

inhibition of growth, a massive increase in apoptosis and a drop in glycolysis⁶⁰. This can be ascribed to the upregulation, due to PLK1 inhibition of β -catenin, leading to its translocation to the nucleus preventing its cytoplasmic binding and degradation by GSK3- β which leads to c-Myc activation, while the inhibition of BRD4 directly downregulates c-Myc expression⁶⁰.

1.11. EGFR Kinase

Epidermal growth factor receptor kinase is a receptor tyrosine kinase frequently mutated and overexpressed in cancer⁶². The function of EGFR is to regulate tissue development and homeostasis of the epithelium⁶².

The EGFR protein encompasses a large extracellular domain, a transmembrane domain an intracellular juxtamembrane region, a tyrosine kinase domain and a regulatory C-terminal region (Figure 1.2)⁶³. The extracellular domain of EGFR consists of two homologous ligand binding domains, domain I and III, and the two cytosine rich domains, domain II and IV (Figure 1.2)⁶³. When an EGFR activator binds to the EGFR ligand binding site specific tyrosine residues of the C-terminal kinase domain is autophosphorylated, activating the kinase domain⁶⁴. Activation of EGFR leads to a signalling cascade in the cell, causing expression of genes that play roles in cell survival, proliferation and differentiation^{62,64}. Activation of EGFR isolated kinase domain proceeds via asymmetric dimerization of the C-terminal of the activator to the N-terminal of the receiver⁶⁴. Oncogenic activity of mutated EGFR have been ascribed to the facilitation of dimerization (and thus activation) due to a point mutation⁶⁴

The EGFR also functions in the regulation of autophagy and metabolism⁶². These functions are mainly performed under cellular and environmental stress⁶². The metabolic modulation function of EGFR, such as those important in cancer cell proliferation (pyrimidine and fatty acid synthesis and glucose metabolism) acts by either directly phosphorylating rate limiting enzymes or indirectly by activating c-Myc⁶².

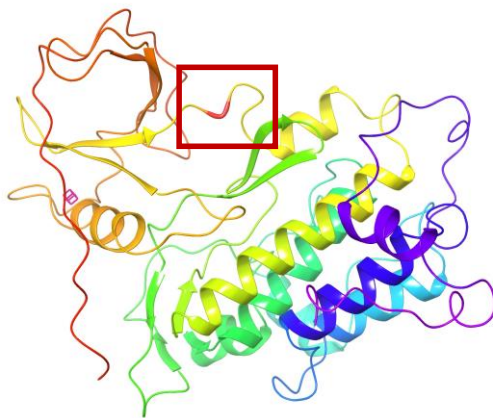


Figure 1. 2: A ribbon animation of the structure of EGFR. The red box indicates the hinge region where the important Met-963 residue can be found. Met-963 plays a crucial role in the binding of ligands and inhibitors to EGFR-TK.

In over 40% of TNBC patients EGFR is over expressed, and thus inhibiting EGFR could be a potentially effective treatment avenue in TNBC treatment⁶⁵⁻⁶⁶. In cancer cells treated with a BRD4 inhibitor, the upregulation of EGFR protected the cells from apoptosis⁶⁷. Consequently, knockdown of the EGFR gene led to increased sensitivity of the cells to the BRD4 inhibitor, leading to decreased viability and proliferation and increased apoptosis⁶⁷.

1.12. Polypharmacology

The advent of systems pharmacology has provided the platform for advocating the move away from single-target drug, towards multi-target drug design and discovery⁶⁸. Most drugs bind to multiple targets and affect multiple biological processes, from this, the term polypharmacology was coined to describe the binding of a drug to more than one molecular target, affecting multiple biological systems⁶⁸. The term pharmacology has historically referred to a single drug affecting multiple pathways, the current meaning of the term has however shifted towards exploiting this multitarget function in drug design and development, to target not just one desirable pathway, but multiple⁶⁸. The comprehensive study of cancer genetics and biology over the past few years have highlighted its evolution, adaptation and ability to develop drug resistance⁶⁹. Polypharmacology seeks to negate the high potential of cancer adaptation and

resistance to single target drugs⁶⁹. From this the rationale for this study arose. BRD4 is a prime target for drug discovery, but due to high rates of adaptation in cancer, the potential for drug resistance to develop is high. Therefore, the development of an inhibitor that targets BRD4 and/or AURKA, AURKB, PLK1 and EGFR described here, could potentially increase the efficacy of a single drug, by launching a multiple target approach against cancer.

1.13. Aim and Objectives

This study aimed to first identify BRD4/Kinase poly-inhibitors from a library of compounds by means of high-throughput virtual screening (HTVS). Next the identified poly-inhibitors were tested *in vitro* to assess their effectivity at inhibiting cell growth and induction of cell death.

The study design, materials, methods, statistical analysis, and ethics are described in detail in the next chapter, and are summarized in the schematic, presented in Figure 2.1.

The objectives of this study were:

- 1) identify poly-inhibitors of BRD4 and dysregulated cancer associated kinases, using HTVS approaches.
- 2) screen the identified compounds using crystal violet assay and select most effective compound/s
- 3) test the efficacy of selected compound/s on cellular metabolism, oxidative stress, cell cycle and apoptosis on triple negative breast cancer cells and a non-cancerous cell line.
- 4) elucidate the mechanisms underpinning their effectiveness on BRD4 and AURKA using ELISAs.

2. Chapter 2: Materials and methods

2.1. Study design

The study aimed to identify polypharmacological BRD4, and/or AURKA, AURKB and EGFR inhibitors through high-throughput virtual screening of the Biofocus library of 20 000 compounds. Next it was the aim to determine if these *in silico* identified inhibitors do in fact have an effect on breast cancer models *in vitro*, to elucidate if they are effective at inhibiting growth and inducing cell death.

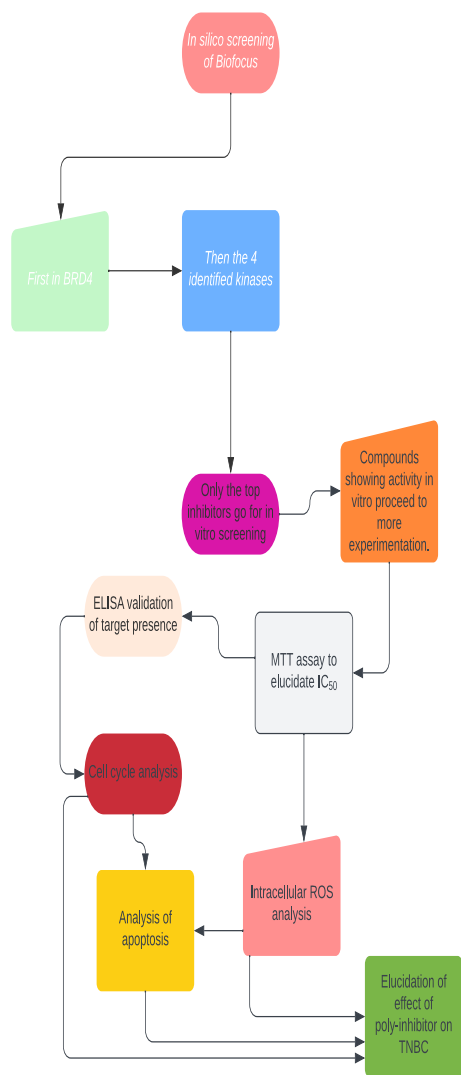


Figure 2. 1: Flow diagram depicting the study design. Starting with *in silico* experiments, progressing to initial *in vitro* screening. After initial screening the study progressed to *in vitro* to further elucidate the mechanism(s) of action of lead compounds.

2.2. Ethics

The study was granted ethics approval by the Faculty of Health Sciences Research Ethics Committee on the 25th of June 2019, and the approval was renewed on the 10th of June 2022 (Ethics Reference No. 252/2019). See back of this manuscript for the certificate.

2.3. High-throughput virtual screening

HTVS has become a common step in the drug discovery process, enabling rapid screening of large libraries of compounds, that would otherwise be impossible using traditional *in vitro* screening methods⁷⁰. HTVS techniques fall into two general groups, ligand based or structure based screening⁷¹. The ligand based techniques utilizes two modelling techniques, quantitative structure-activity relationships (QSAR) or ligand similarity models⁷¹. QSAR models the structural properties of known ligands to biological activity, that is, structural variations in compounds corresponds to different biological activities⁷². Thus the basis of QSAR is to model structural physicochemical properties that equates to biological activity and screen ligands based these properties⁷². Ligand similarity models utilizes two-dimensional fingerprints of known active compounds to screen for active compounds⁷³. Ligand based virtual screening is thus limited by the assumption that molecules similar to a known ligand possess the same activity, which invariably prevents the discovery of novel chemical classes of ligands⁷⁴.

2.3.1. Structure based virtual screening

Structure based virtual screening (SBVS) models the protein-ligand interaction, applying a docking score as a metric of the binding free energy⁷¹. SBVS employs molecular docking programs to dock a ligand into a known three-dimensional binding pocket of a known target molecule (usually a protein, but some programs can also dock a ligand into nucleic acids and ribozymes)^{71,75-76}. In SBVS the focus is on the steric and energetic activity at play in the protein-ligand interaction, which enables it to more accurately model the potential activity of very diverse sets of ligands⁷¹. SBVS takes into account the interactions between the protein and ligand in three-dimensional

space, leveraging molecular interactions, induced fit of the ligand in the binding pocket and the binding energies as recognition events in screening.

2.3.2. Docking

Molecular docking of the compound library was performed using Glide standard precision (Glide SP), with a maximum receptor grid cube size of 17 Å. Docking jobs were submitted to the Centre for high performance computing (CHPC) Lengau cluster (CHPC, Centre for Scientific and Industrial Research) to run on a single node, in parallel utilizing 24 processors.

Molecular docking aims to predict the optimal binding pose of a potentially active ligand within the binding pocket of the target protein and in so doing predict the binding affinity of the ligand to the protein. The docking software (Glide, Schrödinger Release 2019-1: Schrödinger, LLC, New York, NY, 2019) varies both the orientations of the ligand and the protein binding pocket. Each ligand docked into the protein generates a docking score relating to the free energy of binding, which ranks the ligands according to the highest binding affinity (the most negative docking scores, correlates to the best protein-ligand binding)⁷¹. Here, the Glide docking program (Schrödinger Release 2019-1: Schrödinger, LLC, New York, NY, 2019) suite was used. Glide analyses the docked ligand using an exhaustive conformational-, positional space, and orientational -search algorithm, which identifies an initial rough positional and scoring phase of the ligand, which is followed by analysis of the torsional energy of different minima using an all-atom optimized potentials for liquid simulations (OPLS-AA) force field⁷⁷. From these, the high energy poses are eliminated by imposing a cut-off of the allowed total conformational energy, compared to the lowest-energy state, these cut-offs are not rigid, but rather tailored for each ligand based on conformational energy minima⁷⁷. These cut-offs prevent the inclusions of conformations that are not suitable for receptor binding, such as long-range internal hydrogen bonds. Next the rigid core of the ligand is identified and the different poses of the rotamer groups are analysed⁷⁷. Each of the ligand poses are evaluated using a hierarchical scoring scheme that employs an algorithm with a “greedy” scoring function⁷⁷. The “greedy” scoring function, scores each atom of the ligand on its position with respect to the receptor as well scoring it based on the score it could get were it to move ± 1 Å in the X, Y or Z,

directions⁷⁷. At this point the ligand as a whole is allowed to move rigidly at $\pm 1 \text{ \AA}$ within the binding pocket in a refinement procedure which constitutes about ~5000 poses⁷⁷. Of these ~5000 only about ~400 of the best refined poses are further minimized (removal of those poses that cause steric clashes) on the OPLS-AA van der Waals and electrostatic grids for the binding pocket⁷⁷. After the minimization the Glide software selects six to three of the lowest-energy binding poses to establish the orientation of peripheral groups of the molecule by subjecting them to Monte Carlo simulations to find the global energy minimum⁷⁷⁻⁷⁸. Finally, the refined poses are scored using GlideScore and ranked according to a combination of the binding affinity according to the GlideScore, the energy-grid score, and the internal strain energy that directed the conformational-search⁷⁷. The GlideScore scoring function (figure 2.2) takes into account the lipophilic interactions between the ligand-atom and receptor-atom pairs taking into consideration the distances between these pairs (denoted by r_{lr})⁷⁷. Secondly, all ligand-receptor hydrogen interactions including the weighting of the hydrogen bond interactions based on if the donors and acceptors are each either neutral or charged⁷⁷. Metal-ligand interactions are also included, but only if the acceptor atoms are anionic, and only incorporates it into the score if the net charge is positive⁷⁷. Additionally polar non-hydrogen bonding interactions in hydrophobic regions are rewarded⁷⁷. Finally van der Waals and Coulomb (electrostatic) interaction energies between the ligand and receptor are also accounted for⁷⁷.

$$\begin{aligned} \Delta G_{bind} = & \Delta G_{lipo} \sum f(r_{lr}) + \Delta G_{hbond-neut-neut} \sum g(\Delta r)h(\Delta \alpha) \\ & + \Delta G_{hbond-neut-charged} \sum g(\Delta r)h(\Delta \alpha) \\ & + \Delta G_{hbond-charged-charged} \sum g(\Delta r)h(\Delta \alpha) + \Delta G_{metal} \sum f(r_{lm}) \\ & + \Delta G_{roth} + H_{roth} + \Delta G_{polar-phob} V_{polar-phob} + \Delta G_{coul} V_{coul} + \Delta G_{vdw} V_{vdw} \\ & + Solvation \end{aligned}$$

Figure 2. 2: The GlideScore function. The function considers lipophilic interactions between ligand-atom and receptor-atom pairs as a function of their distances between each other (r_{lr}). Weighted ligand-receptor hydrogen interactions (line 2) are considered as well as metal-ligand interactions (line 3). The polar non-hydrogen interactions are also considered as well as the electrostatic interactions (line 4 and 5).

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2.3.3. Protein preparation

The first step in docking is selection of suitable crystallized protein structures. A suitable protein will have a ligand bound, that is to ensure the protein is already configured into a bound state with regards to its folding. To this end, the Protein Data Bank (PDB) was searched for proteins (BRD4, AURKA, AURKB, EGFR, and PLK1) complexed with known inhibitors. Proteins were then prepared using the Protein Preparation Wizard (Schrödinger Release 2019-1: Maestro, Schrödinger, LLC, New York, NY, 2019). Some of the protein preparation performed includes hydrogen (H) bond optimization, by sampling 180° flips of the terminal chi angles of histidine, asparagine, and glutamine side chains⁷⁹. This appreciably changes the capabilities of the side chains to form H-bonds, without significantly changing the electron density. As well as restrained minimization, freely minimizing hydrogen atoms and at the same time allowing for the movement of heavy atoms, which relaxes strained bonds⁷⁹. Furthermore, any missing side were added with Prime (Schrödinger Release 2019-1: Maestro, Schrödinger, LLC, New York, NY, 2019). With regards to water, all co-crystallized water molecules were kept in the crystal structure during the hydrogen optimization and minimization steps, this is to prevent the protein from collapsing in on itself, and because water occupancy also plays a role in hydrogen bonding networks that are critical for ligand binding⁷⁹⁻⁸⁰. The minimized proteins were then superimposed on each other (all BRD4 proteins were superimposed on each other, the same was performed with AURKA, AURKB, EGFR and PLK1) and all water molecules, except those in the binding pocket and those within 5 Å of the binding pocket were removed. Additionally, superimposition of the proteins enables the identification of conserved water molecules that potentially play a role in the formation of ligand-protein water bridges⁸¹. Table 2.1 lists all the proteins used for the docking, additionally the resolution of each crystal structure is indicated if the proteins had any non-conserved waters within their binding sites.

Table 2. 1: List of PDB crystal structures used for enrichment testing and validation of protein crystal structures using decoys.

PDB ID	Resolution	Complexed with ligand	PDB ID	Resolution	Complexed with ligand
BRD4			AURKB		
3MXF	1.60 Å	Yes	4C2V	1.49 Å	Yes

PDB ID	Resolution	Complexed with ligand	PDB ID	Resolution	Complexed with ligand
4BJX	1.59 Å	Yes	5EYK	1.93 Å	Yes
4CL9	1.40 Å	Yes	5K3Y	1.60 Å	Yes
4F3I	1.40 Å	Yes	PLK1		
4OGI	1.73 Å	Yes	3P34	1.40 Å	Yes
5ACY	2.01 Å	Yes	3P36	1.59 Å	Yes
5F60	1.35 Å	Yes	3Q1I	1.40 Å	Yes
5F61	1.45 Å	Yes	4DFW	1.55 Å	Yes
5F63	1.45 Å	Yes	4LKL	1.58 Å	Yes
AURKA			4RCP	1.60 Å	Yes
2BMC	2.60 Å	Yes	EGFR		
2J50	3.00 Å	Yes	3UG2	2.50 Å	Yes
3D15	2.30 Å	Yes	4G5J	2.80 Å	Yes
3E5A	2.30 Å	Yes	4G5P	3.17 Å	Yes
3FDN	1.90 Å	Yes	4HJO	2.75 Å	Yes
3P9J	2.80 Å	Yes	4I22	1.71 Å	Yes
4JBQ	2.30 Å	Yes	4WKQ	1.85 Å	Yes
4ZS0	3.00 Å	Yes	4ZAU	2.80 Å	Yes
4ZTR	2.85 Å	Yes	5X2K	3.20 Å	Yes
4ZTS	2.90 Å	Yes	5XDK	2.35 Å	Yes
5EW9	2.18 Å	Yes	5XDL	2.70 Å	Yes
AURKB			5ZWJ	2.90 Å	Yes
2BFY	1.80 Å	Yes			
2BFY	1.80 Å	Yes			
4AF3	2.75 Å	Yes			

The next step was to test the hypothesis that when docking ligands into the selected proteins it would have high enrichment, that is only true ligands will bind to it. To test this, a set of known inhibitors needs to be docked into the proteins together with a set of decoy molecules⁸². These decoys have physicochemical properties which are similar to the known inhibitors, differing only in their 2D topology, and are thus

expected not to bind to the target proteins⁸². If there is a large amount of hits (that is, the decoys are docked effectively) then the protein is not suitable⁸². However, if the decoys do not dock successfully, then the protein is suitably sensitive⁸². This process is known as enrichment optimization⁸²⁻⁸³. It must be noted that it would seldomly happen that none of the decoys are docked successfully, there will almost always be some incorrect binding. However, the incorrect binding must be to a lesser extent relative to the known inhibitors⁸³. Consequently, a set of at least 5 decoys for each known inhibitor of the target proteins was obtained from the directory of useful decoys-enhanced (DUD-E) database and docked together with the known inhibitors into the proteins. Metrics of enrichment were obtained from the generated receiver operating characteristic (ROC) curve by calculating the area under the curve (AUC) for each individual protein⁸³. Figure 2.3 excellently illustrates how differing models of enrichment are represented in a ROC curve.

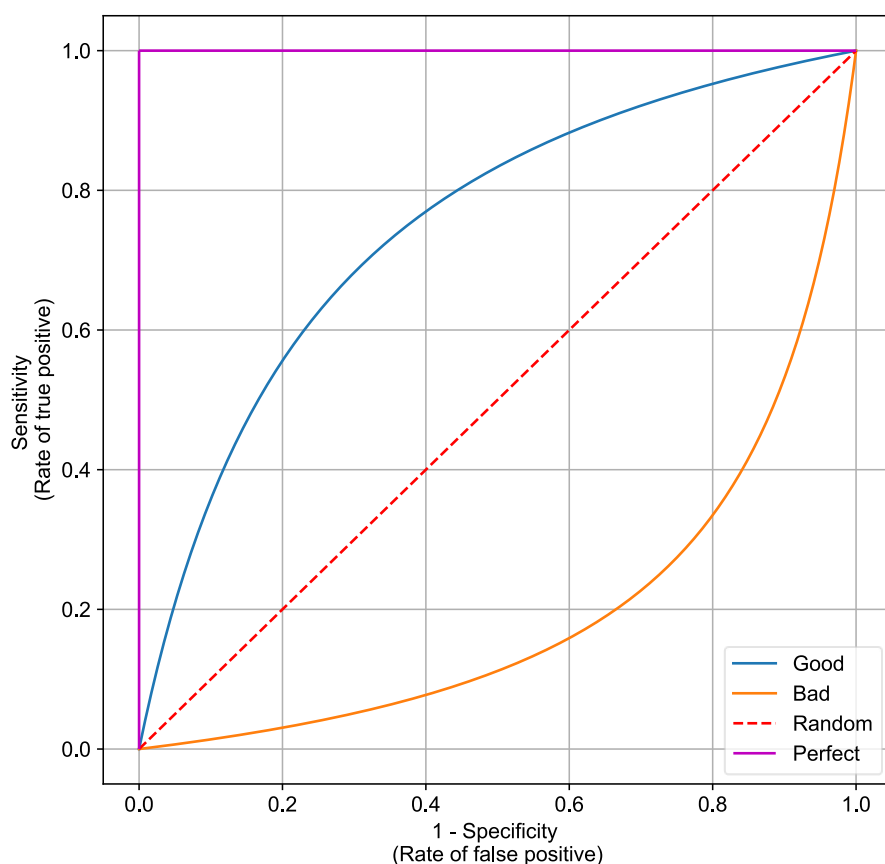


Figure 2. 3: Example ROC curves indicating good enrichment (blue line) random enrichment (red dotted line), very bad enrichment (orange line), and perfect enrichment (purple line).

An AUC of 1.0 equating to 100% correct predictions (i.e., it perfectly discriminates between actives and decoys). A perfect AUC is however near impossible, so a rule of thumb system exists to guide the researcher⁸⁴. An AUC score of 0.5 equates to no discrimination between ligands and decoys, a score between 0.5 and 0.7 is considered poor discrimination, a score between 0.7 and 0.8 is seen as having acceptable discrimination and a score between 0.8 and 0.9 is seen as having excellent discrimination⁸⁴. Thus, only proteins with AUC scores between 0.7 and 1.0 were selected. Most of the selected proteins have excellent discrimination. Additionally,

multiple crystal structures of each protein were selected for docking, which is known as ensemble docking⁸⁵. This is to account for the flexibility of the receptor/ligand interface which is not taken into account when docking into a single rigid protein structure⁸⁵.

2.3.4. Ligand preparation

The Biofocus library of compounds were supplied as simplified molecular-input line-entry system (SMILES) strings, containing no 3D coordinates. The smiles were then imported into the Maestro interface suite (Schrödinger Release 2019-1: Maestro, Schrödinger, LLC, New York, NY, 2019.) to generate 3D structures from the 2D smiles structures. LigPrep (Schrödinger Release 2019-1: Maestro, Schrödinger, LLC, New York, NY, 2019) was then used to prepare the 3D ligands for docking. Ligand prepping is performed using the OPLS_2005 force field⁸⁶. Firstly, the ionization and protonation states of each ligand is enumerated at a target pH 7,4 (LigPrep uses Epik or Empirical pKa prediction software for this)⁸⁷. Next the software creates different tautomeric states, ring conformations, and stereoisomers for each ligand⁸⁶⁻⁸⁷. Finally, LigPrep also corrects Lewis structures to generate the final energy minimized 3D structures⁸⁶. The preparation of the 20 000 Biofocus compounds yielded 48 000 structures due to the enumeration of stereoisomers, tautomers and different protonation states by Epik.

2.3.4.1. Docking into BRD4

Docking was performed by employing the Virtual Screening Workflow (VSW) software in Maestro. VSW was used to run Glide docking jobs at two accuracy levels, HTVS which has the lowest accuracy and standard precision (SP) docking. This enables the user to first run a low accuracy docking job, and from there submit the results of the first docking job to a second higher precision docking job. Due to the relatively small size of the Biofocus library, all the HTVS results were submitted to the SP job, thus the entire prepared library of compounds were docked at both accuracy levels.

The prepared Biofocus library of compounds was submitted for docking into the N-terminal Bromodomain of the BRD4 protein of those crystal structures with acceptable

enrichment ($7 < \text{AUC} \leq 1$, the list of protein crystal structures can be viewed in table 2.2). The VSW job was submitted to the Lengau cluster of the CHPC.

From the docking results those ligands with the lowest docking scores with a cut-off of -9, was selected for further docking into the different kinases, this amounted to 79 compounds.

2.3.5. Docking into kinases

The Biofocus ligands identified from the BRD4 docking job (79) with the lowest docking scores (docking score ≤ 9) were submitted for docking into the different kinases, following the same procedure mentioned above using VSW. A list of the PDB crystal structures of the different kinases with acceptable enrichment, $7 < \text{AUC} \leq 1$, can be viewed in table 2.2. The compounds that showed the highest affinity (docking score ≤ 9) for the kinase proteins were obtained from the CSIR Biofocus library and tested *in vitro*.

2.4. Cell culture protocol for *In vitro* studies

The MDA-MB-231 cells (American Type Culture Collection, ATCC), a HER2, oestrogen receptor, and progesterone receptor negative mammary gland adenocarcinoma epithelial cells, served as the TNBC model⁸⁸. The MDA-MB-231 cell line is an established TNBC model of late stage disease⁸⁸. The EA.hy926 cell line (ATCC), is a somatic cell hybrid with endothelial cell morphology with expression profiles relating to haemostasis⁸⁹. EA.hy926 cells are an established model for anticancer compound screening, to test endothelial cytotoxicity and thus served as a reference cell line⁸⁹. The cell lines were seeded in either 25cm² or 75cm² flasks containing complete culture media (CCM). The CCM for the MDA-MB-231 cells consisted of Dulbecco's modified Eagle's medium (DMEM), 10% foetal calf serum (FCS) and 1% L-glutamine. For the EA.hy926 cells the CCM consisted of Iscove's modified Dulbecco's medium, 10% FCS, and 1% L-glutamine. Once the cells reached 95% confluency they were used in different experiments, each of which were done in 3 biological repeats, and 3 technical repeats. All reagents, unless otherwise stated, were purchased from Sigma Aldrich (St. Louis, Missouri, USA).

Commented [A2]: Please provide more information about the cell lines e.g where they were purchased, their characteristics etc.

2.5. *In vitro* screening of compound D2-D11 with the crystal violet viability assay

In order to test for activity of the BRD4/Kinase poly-inhibitors, compounds D2-D11, identified by HTVS a crystal violet (CV) screening assay was performed in MDA-MB-231 cells. The assay tests the relative density of adherent cells in treatment groups, relative to an untreated control (or vehicle control). This is done by staining the DNA of cells with CV, washing the cells to remove excess dye (and in the process removing non-adherent or dead cells) and quantifying the amount of dye taken up by the monolayer using a plate reader⁹⁰. For the screening of D2-D11, a set concentration range was used across all compounds, 5 μ M, 2.5 μ M, 1.25 μ M, 0.625 μ M, 0.3125 μ M, 0.15625 μ M, 0.078125 μ M, and 0.0390625 μ M. Treatment time was set at 48 h, this is because the doubling time of MDA-MB-231 cells is between 38 and 42 hours, thus allowing ample time for the compounds to exert a cytotoxic effect on the cells⁹¹⁻⁹². The relatively low concentration range was used for two reasons, the first being practical, there was limited amount of each compound available (only 15 μ l at 10 mM was received for screening purposes from the CSIR). The second reason being the intent to screen for compounds that have a cytotoxic effect at lower concentrations, with the hope of finding a compound with nanomolar potency. Reference controls were also used in the form of medium only control and vehicle control at 0.1 % DMSO v/v in CCM. Each treatment condition was performed in triplicate and 3 biological repeats.

MDA-MB-231 cells were seeded in 96-well plates (a total of 5 96-well plates were seeded for each biological repeat) at a density of 5000 cells/well in 200 μ l of CCM and left overnight in a humidified incubator at 37 °C and 5 % CO₂ to adhere to the wells. After allowing the cells to attach the medium was aspirated and replaced with 200 μ l of CCM containing the concentration ranges explained above for compound D2-D11, as well as a vehicle control and CCM only control. The treated cells were then incubated for 48 hours in a humidified incubator at 37 °C and 5 % CO₂. After the 48-hour period the supernatant was removed and replaced with 100 μ l of 1 % glutaraldehyde (v/v in ddH₂O) and the plate was left to incubate at room temperature (21° C) for 15 minutes. Next the glutaraldehyde was removed and 100 μ l/well of 0.1 % crystal violet (w/v, prepared in ddH₂O) was added and left to incubate for 30 min at

room temperature, after which the crystal violet solution was discarded, and the wells washed in water for 2 minutes to remove any residual dye. The washed plates were then left on a paper towel away from direct light to dry for 24 hours. Once dry, 200 µl/well of 0.2 % Triton X (v/v in ddH₂O) were added to the wells to solubilise the dye and left to incubate for 1 hour. Then 100 µl of the Triton X supernatant was pipetted into a new 96-well plate and the absorbance measured at 570 nm using an ELx800 universal microplate reader (Bio-Tek µQuant, Bio-Tek Instruments Inc. Vermont, USA). The optical density (OD) from three technical replicates and three biological repeats were used to determine an average for each treatment group comparing each treatment to the vehicle control using the following equation and plotted into a curve using GraphPad Prism 8.1.

$$\% \text{ Viability} = (OD \text{ of Treatment} / OD \text{ of Vehicle Control}) \times 100$$

Only compounds identified as active after the screening, will be used for further experiments.

2.6. 2,5- dimethyl thiazolyl diphenyl tetrazolium bromide assay

The crystal violet assay is an effective tool in the screening process, however when looking to elucidate an IC₅₀ the gold standard has for many years been the 2,5-dimethyl thiazolyl diphenyl tetrazolium bromide (MTT) assay⁹³. The MTT assay tests cell viability and metabolic activity⁹³. The yellow MTT salt is reduced by NAD(P)H-dependent cellular oxidoreductases in viable and metabolically active cells to formazan, which is insoluble and purple in colour⁹³. Absorbance of the solubilised formazan are then measured and used as an indicator of cellular metabolic activity. All treatment conditions were performed in triplicate and 3 biological repeats.

MDA-MB-231 were seeded into 96-well plates at a density of 5000 cells/well with 200 µl of CCM, and left overnight to attach in a humidified incubator at 37 °C and 5 % CO₂. Next the cells were treated with a concentration range (5 µM, 2.5 µM, 1.25 µM, 0.625 µM, 0.3125 µM, 0.15625 µM, 0.078125 µM, and 0.0390625 µM) of the active compound(s) in CCM including a vehicle control of 0.1 % DMSO (v/v in CCM) and a CCM only control to a volume of 200 µl/ well, and left to incubate for 48 hours in a humidified incubator at 37 °C and 5 % CO₂. After incubation the supernatant was

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discarded and the cells incubated with 100 µl of CCM containing 5 mg/ml MTT for 4 hours at 37 °C and 5 % CO₂, covered in foil to protect it from light. Next, the supernatant was aspirated and DMSO added at 100 µl/well and incubated for 1 hour, to dissolve the formazan dye produced inside the cells. A spectrophotometric plate reader (Bio-Tek µQuant, Bio-Tek Instruments Inc. Vermont, USA) was used to measure the optical density at 570 nm at a reference wavelength of 690 nm. From these results the viability of each treatment condition was calculated using the % viability equation explained previously. Next the % viability of each treatment condition was plotted against its corresponding log. The graph was then fitted with a straight line and the IC₅₀ calculated using the formula $IC_{50} = \frac{0.5-b}{a}$ extrapolated from the formula of a straight line. This was all performed with the help of GraphPad prism version 8.1. Next, the EA.hy926 cells were treated with the IC₅₀ of the active compound(s) calculated for the MDA-MB-231 cells together with a vehicle control for reference. This was done in triplicate and with three biological repeats. The reasoning behind treatment of the EA.hy926 cells with the D2 IC₅₀ of the MDA-MB-231 cells is to test the effect of this inhibitory concentration, calculated in a TNBC model, on a somatic reference cell line.

2.7. *In vitro* analysis of the effect of the active compound(s) on MDA-MB-231 cells

The use of viability assays is not sufficient to draw conclusions on the effectiveness of a novel inhibitor. Thus, more specialised investigation is required to elucidate the mechanism by which the active compound(s) exhibits its effect.

2.7.1. Anti-BRD4 and anti-AURKA ELISA

The proposed targets for the screening were BRD4, AURKA, AURKB, PLK1 and EGFR. However, their presence and thus their overexpression could vary from immortalised cell line to immortalised cell line. This is due to the high rates of mutations and inherent genetic instability of cancer cells. Thus, it was necessary to elucidate if these target proteins were in fact present in the cell lines to be tested. An enzyme-linked immunosorbent assay (ELISA) was used to detect the presence of some target

proteins. However, due to the relative cost of ELISAs, it was decided to only test for two of the target proteins BRD4, and AURKA, due to the kit availability and the fact that these two proteins are known to be (over) expressed in MDA-MB-231 cells⁹⁴⁻⁹⁷. In addition to testing for the presence of BRD4 and AURKA in the cell lines, the expression of the proteins following treatment with the active compound(s) was also tested, to see if inhibition had any effect on the target protein concentration.

The ELISA is a powerful tool with which to quantify water soluble substances such as peptides, proteins, hormones, glycoproteins, and antibodies, to name a few⁹⁸. The ELISA is based on the principle of antibody-antigen binding. Here an antibody specific for a known antigen, BRD4 (both the long and short isoform) and AURKA was pre-coated on the surface of wells, then the sample containing the target antigens was added, if present the antigens will bind to the antibody⁹⁸. Next a biotinylated antibody specific to the target antigens was added to the wells that will also bind to the antigens, and streptavidin-HRP (horseradish peroxidase) was added to the wells⁹⁸. The Streptavidin has a high affinity for binding to biotin and will thus bind to the biotinylated antibodies⁹⁸. After this step the plate was washed to remove any unbound streptavidin-HRP and antibodies⁹⁸. Next a substrate was added that the HRP can enzymatically react with to produce a colour signal that was quantified using a spectrophotometric plate reader⁹⁸.

MDA-MB-231 and EA.hy926 cells were seeded into T75 flasks at a density of 700 000 cells/ flask, after which they were left to attach for 24 h in a humidified incubator at 37 °C and 5 % CO₂. After 24 h the medium from each flask was aspirated and replaced with medium containing a vehicle control (0.1 % DMSO v/v) or medium containing the IC₅₀ of the compound(s) as calculated using the MTT results. Next the flasks were incubated for 48 h in a humidified incubator at 37 °C and 5 % CO₂. After the 48 h incubation the treatment was aspirated and the cells were trypsinized and moved into correspondingly labelled 15 ml tubes with 5 % FBS (to inhibit further digestion) containing phosphate buffered saline (PBS) to dilute it to a concentration of approximately 1 million cells/ ml. Next a freeze thaw cycle was performed to lyse cells and liberate the proteins of interest (BRD4 and AURKA) by placing the tubes in a -20 °C freezer for 40 minutes to allow the cells to freeze, after which it was removed and allowed to defrost in a water bath at 25 °C. Next the thawed cell suspension was passed through a 32-gauge needle to further lyse the cells to form a homogenate.

After the cell lysing step the homogenate was centrifuged at 2500 x g for 20 minutes, after which the supernatant was aspirated and moved to new 15 ml tubes for analysis with ELISA. The experiment was performed in 3 technical repeats and 3 biological repeats for each treatment condition.

The protocols for BRD4 and AURKA detection was followed according to the manufacturer's guidelines of the Human Bromodomain-containing protein 4 ELISA kit, and Human Aurora Kinase A ELSA Kit (BT-Lab, Jiaxing, Zhejiang, China).

The wash buffer for both the BRD4 and AURKA ELISA kits were made diluted from 25 x to 1 x to form the working buffer solutions.

The AURKA and BRD4 ELISA standard concentrations were prepared as follows: 120 μ l of the standard solution was added to a 1 ml tube, together with 120 μ l of standard diluent and mixed thoroughly. This is the first standard and also the working standard (1200 ng/ l) for further serial dilutions to obtain the 4 other standards. In a series of serial dilutions, 120 μ l of the first standard was diluted with 120 μ l of standard diluent (and mixed) to form the second standard (600 ng/ l), 120 μ l was then taken from the second standard and added to 120 μ l of standard to form the third standard (300 ng/ l). This was repeated two more times to form the fourth (150 ng/ l) and fifth (75 ng/ l) standard. The standard solution alone served as the 0 ng/ l standard. Next 50 μ l of the standards was added to 6 wells, which served to generate a standard curve. Next, 40 μ l of the samples (MDA-MB-231 and EA.hy926 lysate supernatants) were added to the sample wells (standard and sample wells were noted and annotated on plate sealers and a notebook). To each sample well 10 μ l of anti-BRD4 or anti-AURKA antibody was added, and to both the sample and standard wells 50 μ l of streptavidin-HRP was added. The plate was then sealed and incubated for 60 minutes at 37 °C. After incubation the sealer was removed and the plate was washed 5 times with the 1 x wash buffer at 300 μ l/ well for 1 minute/ wash step. After washing the plate was blotted on absorbent paper and 50 μ l of substrate solution was added to each well and the plate sealed again and left to incubate in the dark at 37 °C for 10 minutes. Finally, after incubation, the stop solution was added to each well and the plate for each ELISA was read immediately using a spectrophotometric plate reader (Bio-Tek μ Quant, Bio-Tek Instruments Inc. Vermont, USA) at 450 nm wavelength.

Using the standards of each ELISA, a standard curve was plotted in GraphPad Prism 8, and the concentrations of the samples was interpolated from the standards by the program.

2.7.2. The effect of the active compound(s) on the cell cycle

Theoretically, D2, as a BRD4/Kinase poly-inhibitor, is expected to have large scale effects on the cell cycle by inhibiting AURKA and AURKB.

Flow cytometry is optimized to allow for the concomitant measurement of G1, S, G2 and M-phases⁹⁹. Flow cytometry has for the past few years played a significant role in the field of cancer research¹⁰⁰. A flow cytometer consists of a fluidics system and a laser and optics system for fluorescence detection¹⁰⁰. By labelling the DNA of cells with the fluorescent intercalating agent propidium iodide (PI) in the presence of RNase A it is possible to identify cells in the different phases of the cell cycle based on their fluorescent intensity¹⁰⁰⁻¹⁰¹. The rationale for the technique is that the PI dye binds proportionally to the amount of DNA present¹⁰¹. That is, cells in S-phase will have proportionally more DNA and thus fluoresce with more intensity¹⁰¹. From this, cells in G2 will have double the DNA of those in G1 and will thus with double the intensity¹⁰¹. By quantification of the fluorescence, it is possible to map the phases within a given sample of cells. For this experiment 6-well plates were seeded with 300 000 cells/well (MDA-MB-231 and EA.hy926 respectively), 3 wells were used as the vehicle control (0.1 % DMSO v/v in CCM), 3 wells contained the IC₅₀ of D2 and 3 wells contained 13.3 μ M nocodazole (NOC) as positive control (the concentration is based on IC₅₀ studies performed by another researcher in the same lab). The plates were then incubated for 48 hours in a humidified incubator at 37 °C and 5 % CO₂. After the 48 incubation the wells were cleared of all medium (which was added to labelled 15ml tubes corresponding to each well), washed with PBS (the PBS was also added to the labelled tubes) and the cells were trypsinized by the addition of 0.5 ml of trypsin to each well. The trypsin together with the cells was added to the labelled 15 ml tubes corresponding to each well. Next the tubes were centrifuged at 300 x g for 5 min, the supernatant stored in correspondingly labelled tubes and stored at -20° C. And the pellet is resuspended in 200 μ l of PBS, next the cells were counted using the trypan exclusion method and a haemocytometer. The cell concentration needed to be at least

750 000 cells/ml and no more than 1 million cells/ml. After counting the cells, the suspension was once again centrifuged at 300 x g for 5 min, after which the supernatant was discarded taking care to leave about 50 µl of PBS with the pellet to resuspend it. After resuspension of the cells in the residual PBS, it was added dropwise to 1 ml of ice cold 70% ethanol, whilst vortexing the tube actively. The cells needed to be fixed for at least 24 h, by storing them at -20° C.

To proceed to staining after the minimum of 24 h fixation, the fixed cells were centrifuged at 300 x g for 5 min at RT, after which the supernatant was removed and discarded. Next the pellet was re-suspended in 250 µl PBS, and centrifuged at 300 x g for 5 min, after which the supernatant was removed and discarded. Next 200 µL of Muse® Cell Cycle Reagent (Luminex Corporation, Austin, TX 78727, U.S.A.) was added to each tube, taking care to resuspend each pellet. The tubes containing the reagent was then left to incubate away from light, for 30 minutes. After the 30 minutes incubation the cells were analysed using the Muse flow cytometer (Luminex Corporation, Austin, TX 78727, U.S.A.) and the Muse® Cell Cycle analysis tool. Before the assay can be run, the gating and markers needs to be checked to make sure it is correct. The Muse makes use of forward scatter only, and it can only detect red and yellow fluorescence. Therefore, even though each Muse kit is completely optimised, the user is required to make sure that the gated population is correct (cells and not debris), this was done using the VC to optimize data acquisition. The Muse gives stepwise assistance in the experimental optimisation. There are 5000 events acquired per sample. The results for the Cell Cycle analysis assay is represented in a histogram with DNA content (ploidy) on the X-axis and percentage of cells on the Y-axis. Cells in G0/G1 will have the lowest ploidy (only 2 copies of each chromosomes) thus more to the left of the histogram. Cells in S will fluoresce more than G0/G1 cells due to synthesis of more chromosomal DNA, but G2/M cells will fluoresce with double the intensity of G0/G1 cells as they will eventually divide into two cells. The results were analysed using Excel and GraphPad Prism 8.1, gating graphs and histograms were acquired from the Muse software.

2.8. Investigation into the effect of D2 on reactive oxygen species levels and apoptosis

The healthy cell has a very complex relationship with reactive oxygen species (ROS)¹⁰². ROS, once viewed as just a by-product of cellular respiration, has since been found to play an important role in cellular function as important regulators in cellular signalling pathways¹⁰². ROS includes the free radicals, hydroxyl ($\cdot\text{OH}$), superoxide ($\text{O}_2^{\cdot-}$), as well as the non-radical molecules such as hydrogen peroxide (H_2O_2)¹⁰². Interestingly, H_2O_2 play an integral role as a second messenger in extracellular signal transduction and gene expression, collectively known as redox signalling¹⁰². The main sites of ROS production is the mitochondria in the electron transport chain where some 1-2% of O_2 is reduced to $\text{O}_2^{\cdot-}$, the endoplasmic reticulum in the process of protein oxidation, and peroxisomes during β -oxidation of fatty acids¹⁰². Additionally, some enzymatic reactions such as those involving xanthine oxidases, NADPH oxidases, cyclooxygenases, lipoxygenases, and the Fenton reaction where ferrous ions transform H_2O_2 into $\cdot\text{OH}$, produce ROS continually¹⁰². ROS is integral to cellular function, in moderate levels, contributing to proliferation and differentiation¹⁰². Regulation of ROS is contingent on the complex free radical scavenger system which include the superoxide dismutases (SODs), catalase, thioredoxin, glutathione reductase (GS), glutathione peroxidase (GPX), and peroxiredoxin, reducing ROS to water¹⁰²⁻¹⁰³.

A complex interconnection between ROS and cancer cells exists, whereby ROS production is increased (only slightly more compared to non-transformed cells), and in tandem so is scavenging, leveraging this slight increase in ROS in the process of initiation and progression¹⁰²⁻¹⁰³. This characteristic of cancer whereby it upregulates ROS to its advantage, whilst also upregulating the production of its quenching enzymes, leaves it in a precarious balancing act¹⁰²⁻¹⁰³. Slight increases in ROS as a result of exogenous interference with ROS upregulating drugs, can very easily tip this fine balance, overwhelming this redox arsenal of the cancer cells, rendering cells exposed to massive oxidative stress leading to apoptosis¹⁰².

2.8.1. Intracellular ROS assay

ROS is innocuous in cells, being a by-product of normal respiration. However when uncontrolled, say in the case of drug treatment to increase intracellular ROS, it can induce apoptosis. The Muse® Oxidative Stress Kit detects intracellular ROS by means of dihydroethidium (DHE) that reacts with superoxide radicals to form the fluorophore, ethidium bromide, that can intercalate with DNA and fluoresces red. DHE is readily cell permeable. Before beginning the Muse® Oxidative Stress reagents were allowed to warm up to RT away from light.

For this experiment 6-well plates were seeded with 300 000 cells/well (MDA-MB-231 and EA.hy926 respectively), 3 wells were used as the vehicle control (0.1 % DMSO v/v in CCM), 3 wells contained the IC50 of D2 and 3 wells contained 13.3 μ M nocodazole as positive control. The plates were then incubated for 48 hours in a humidified incubator at 37 °C and 5 % CO₂. After the 48 h incubation the plates were cleared of all medium (which was added to labelled 15 ml tubes corresponding to each well), washed with PBS (the PBS was also added to the labelled tubes) and the cells were trypsinized by the addition of 0.5 ml of trypsin to each well. The trypsin together with the cells was added to the labelled 15 ml tubes corresponding to each well. Next the tubes were centrifuged at 300 x g for 5 min, the supernatant stored in correspondingly labelled tubes and stored at -20° C. After which the pellet was resuspended in 60 μ l of the Muse® Oxidative Stress 1X assay buffer, and the cells were counted using the trypan exclusion method and a haemocytometer. There needed to be at least 750 000 cells/ml and no more than 10 million cells/ml. To stain, 10 μ l of the cell suspension was added into a 1.5 ml Eppendorf tube, and to that 190 μ l of the oxidative stress working solution was added, and mixed thoroughly by back pipetting, the samples were then left to incubate for 30 min at 37° C protected from light. After the 30 min incubation the cells were analysed using the Muse flow cytometer (Luminex Corporation, Austin, TX 78727, U.S.A.) and the Muse® Oxidative Stress analysis tool. Before the assay can be run, the gating and markers needs to be checked to make sure it is correct. The Muse makes use of forward scatter only, and it can only detect red and yellow fluorescence. Therefore, even though each Muse kit is completely optimised, the user is required to make sure that the gated population is correct (cells and not debris), this was done using the VC to optimize data acquisition. The Muse gives stepwise assistance in the experimental optimisation. The Muse

acquires 3000 events per sample, this is based on the optimizations for each assay. The results are displayed as a ROS profile histogram, similar to the Cell-cycle histogram. However, in this instance there are only 2 groups, ROS+ and ROS- and they should form clear individual peaks. The results were analysed using Excel and GraphPad Prism 8.1, gating and the ROS+ and ROS- histograms were acquired from the Muse software.

2.8.2. Apoptosis assay (Annexin V)

During apoptosis, distinct cellular changes take place, specifically membrane lipid scrambling, such as the phosphatidyl-serine flip. To distinguish between necrotic and early apoptotic cells, 7-aminoactinomycin-D can be used, because it will only bind to the DNA of dead or early apoptotic cells and not healthy cells. The Muse® Annexin V & Dead Cell Assay was used for the detection of live cells, dead cells and total (early + late) apoptotic cells.

For this experiment 6-well plates were seeded with 300 000 cells/well (MDA-MB-231 and EA.hy926 respectively), 3 wells were used as the vehicle control (0.1 % DMSO v/v in CCM), 3 wells contained the IC50 of D2 and 3 wells contained 13.3 μ M nocodazole as positive control. The plates were then incubated for 48 hours in a humidified incubator at 37 °C and 5 % CO₂. After the 48 hour incubation the wells were cleared of all medium (which was added to labelled 15 ml tubes corresponding to each well), washed with PBS (the PBS was also added to the labelled tubes) and the cells were trypsinized by the addition of 0.5 ml of trypsin to each well. The trypsin together with the cells was added to the labelled 15 ml tubes corresponding to each well. Next the tubes were centrifuged at 300 x g for 5 min, the supernatant stored in correspondingly labelled tubes and stored at -20° C. After which the pellet was resuspended in 100 μ l of PBS containing 15% FBS, and the cells were counted using the trypan exclusion method and a haemocytometer. There needed to be at least 750 000 cells/ml and no more than 10 million cells/ml. Before starting with the experiment, the reagents were left to reach RT. To stain the cells, 100 μ l of the Muse Annexin V & Dead Cell Reagent (Luminex Corporation, Austin, TX 78727, U.S.A.) was added to 100 μ l of cell suspension in each tube and mixed thoroughly by back pipetting. The tubes were then left to incubate at RT and protected from light, for 20

min. After the 20 minutes incubation the cells were analysed using the Muse flow cytometer (Luminex Corporation, Austin, TX 78727, U.S.A.) using the Annexin V & Dead Cell analysis tool. Before the assay can be run, the gating and markers needs to be checked to make sure it is correct. The Muse makes use of forward scatter only, and it can only detect red and yellow fluorescence. Therefore, even though each Muse kit is completely optimised, the user is required to make sure that the gated population is correct (cells and not debris), this was done using the VC to optimize data acquisition. The Muse gives stepwise assistance in the experimental optimisation. The Muse acquires 2000 events per sample, this is based on the optimizations for each assay. The apoptosis profile is divided into 4 quadrants. Quadrant 1 contains non-apoptotic/ live cells that are Annexin V and 7-AAD negative. The second quadrant contains the early apoptotic cells that are Annexin V positive but 7-AAD negative. Quadrant 3 contains the late stage apoptotic to dead cells, they are both Annexin V and 7-AAD positive. The fourth quadrant contain mostly nuclear debris that is Annexin V negative but 7-AAD positive. The results were analysed using Excel and GraphPad Prism 8.1, gating and apoptosis profile graphs were acquired from the Muse software.

2.9. Statistical analysis

For the determination of the IC_{50} of the compound at least three biological and three technical repeats for each biological repeat were performed with different concentration ranges of the compounds and relevant dimethyl sulfoxide (DMSO) vehicle controls. The coefficient of determination (R^2), should be $R^2 \geq 0.90$ for a linear regression model at 95% confidence interval and $\Delta R \geq 0.1$. For mechanistic studies treatment groups (vehicle control (VC) and compound(s)) were compared using unpaired, parametric Welch's-tests. The vehicle control was normalized to 100 % and the comparisons were made relative to the VC and presented in percentage form. All statistics were calculated using GraphPad Prism. Experiments performed individually are presented as standard deviations from the mean. Means are presented in bar charts and T-bars are used to indicate standard deviations. p-values less than 0.05 ($p \leq 0.05$) are considered statistically significant.

3. Results

The results of both the *in silico* screening and *in vitro* analysis follows here.

3.1. *In silico* screening results

The validation of the crystal structures for use in docking, using an enrichment protocol with known ligands and decoys, yielded results tabulated in table 3.1. Most of the crystal structures had proven sufficiently effective at discriminating between decoys and ligands, save for two AURKA crystal structures, namely 4ZS0 and 4ZTR. They had AUC values below 7 and were thus excluded from further experimentation. The ROC curves in Figure 3.1 of the BRD4 enrichment is an illustration of the effect of ensemble enrichment versus docking into a single crystal structure. By docking into more than one crystal structure, each with slight differences in the binding pocket due to varied inhibitors complexed with it, enables the docking software to mimic induced fit models of protein-ligand binding¹⁰⁴. It greatly increases the chances of identifying a potential hit, and at the same time it can greatly decrease the odds of identifying false positive hits¹⁰⁴. The intent was to select a diverse set of crystal structure for each protein based on different inhibitors complexed in the crystal structure as well as the resolution of the crystal structure. Based on the enrichment metrics this paid off.

Table 3. 1: Table of protein crystal structures with enrichment values (AUC).

PDB ID	AUC	7<AUC≤1	PDB ID	AUC	7<AUC≤1
BRD4			AURKB		
3MXF	0.842	Yes	4C2V	0.806	Yes
4BJX	0.783	Yes	5EYK	0.763	Yes
4CL9	0.816	Yes	5K3Y	0.774	Yes
4F3I	0.787	Yes	PLK1		
4OGI	0.838	Yes	3P34	0.787	Yes
5ACY	0.825	Yes	3P36	0.852	Yes
5F60	0.822	Yes	3Q1I	0.783	Yes
5F61	0.805	Yes	4DFW	0.770	Yes
5F63	0.814	Yes	4LKL	0.813	Yes
AURKA			4RCP	0.786	Yes

PDB ID	AUC	7<AUC≤1	PDB ID	AUC	7<AUC≤1
2BMC	0.812	Yes	EGFR		
2J50	0.800	Yes	3UG2	0.745	Yes
3D15	0.715	Yes	4G5J	0.744	Yes
3E5A	0.736	Yes	4G5P	0.802	Yes
3FDN	0.729	Yes	4HJO	0.826	Yes
3P9J	0.814	Yes	4I22	0.776	Yes
4JBQ	0.760	Yes	4WKQ	0.802	Yes
4ZS0	0.679	No	4ZAU	0.807	Yes
4ZTR	0.677	No	5X2K	0.757	Yes
4ZTS	0.792	Yes	5XDK	0.768	Yes
5EW9	0.840	Yes	5XDL	0.764	Yes
AURKB			5ZWJ	0.807	Yes
2BFY	0.740	Yes			
4AF3	0.793	Yes			
4B8M	0.803	Yes			

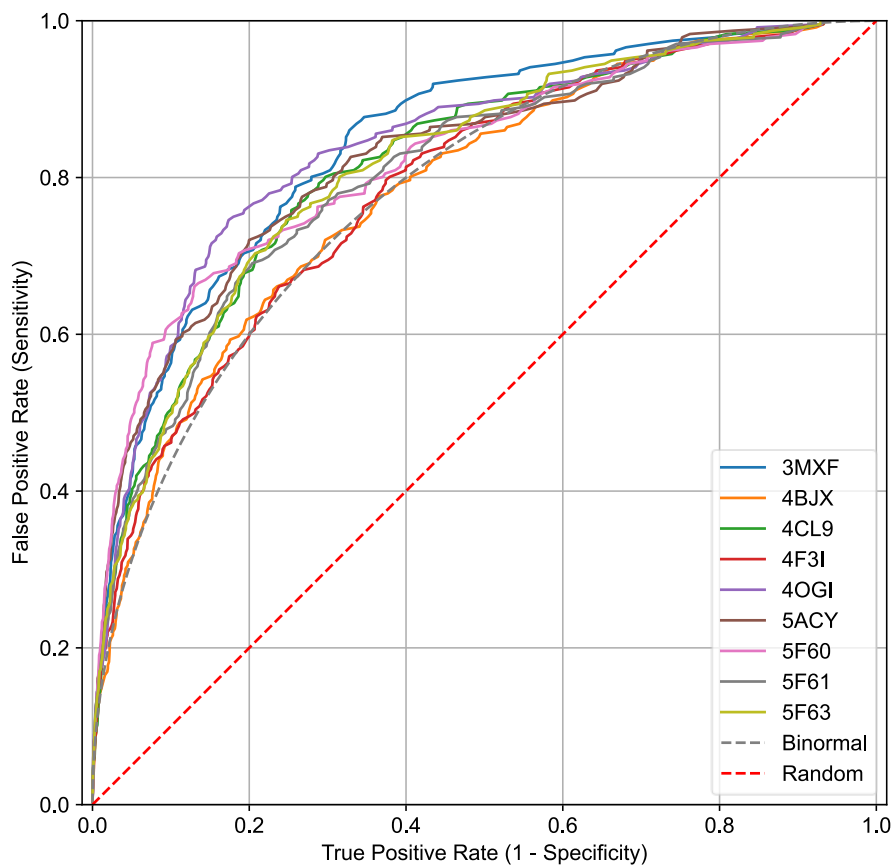
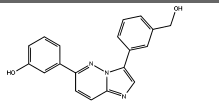
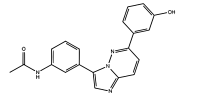
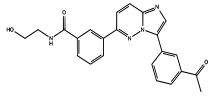
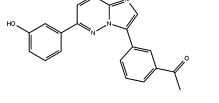
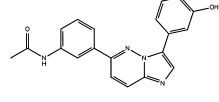
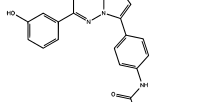
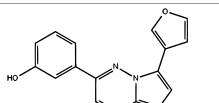
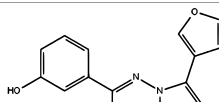
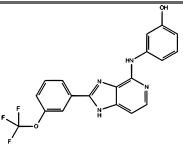
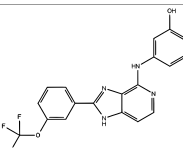


Figure 3. 1: A ranked overlay of all the ROC-curves generated for all the BRD4 protein crystal structures. The binormal curve serves as the predictive function of enrichment. Enrichment serves to normalize the ability of the model to discriminate between false and true positives.

Table 3. 2: Table of the lead compounds, with the compound names, their 2D structure and docking score in each target protein.

Compound	2D Structure	Docking Score			
		BRD4	AURKA	AURKB	EGFR
D2		-9.010	-9.964	-9.585	-10.631
D3		-9.305	-9.773	-9.320	-10.518
D4		-9.005	-10.406	-9.216	-10.256
D5		-9.125	-9.078	-9.354	-10.074
D6		-9.116	-9.590	-9.043	-9.821
D7		-9.357	-9.022	-10.511	-9.630
D8		-9.036	-9.134	-9.374	No binding
D9		-9.252	-9.294	No binding	No binding

Compound	2D Structure	Docking Score			
		BRD4	AURKA	AURKB	EGFR
D10		-9.511	-9.053	No binding	No binding
D11		-9.121	-9.041	No binding	No binding

The docking of the prepared Biofocus library (48 000 structures of which only 20 000 is unique, the rest are stereoisomers, tautomers and different protonation states of these unique ligands) of compounds into the ensemble of BRD4 crystal structures yielded 79 hits (with a docking score ≤ -9 , in other words the binding free energy is low, an indication of high binding affinity). Of these 79 compounds only 26 compounds were unique. Thus from 20 000 compounds, 26 potential hits were identified, meaning the BRD4 screening had a hit rate of 0.13%. Most of the compounds had imidazo[1,2-*b*]pyridazine, 1*H*-imidazo[4,5-*c*]pyridine, or 1*H*-indazole backbones. These 79 ligands were then docked into the different kinases, the results of which can be viewed in table 3.2. The naming of the compounds was arbitrarily based upon their location in the 96 well plate they were supplied in. Only ten compounds of the 26 unique ligands were identified as a 'hit' in one or more of the kinases (with a docking score ≤ -9). There were zero hits for PLK1, thus from here on there will be no referencing with regards to it. Ten compounds successfully docked into the AURKA crystal structures. Seven compounds successfully docked into the AURKB crystal structures (D2-D8). Six compounds successfully docked into the EGFR crystal structures (D2-D7). Compounds D2-D9 have imidazo[1,2-*b*]pyridazine backbones, whereas compounds D10-D11 have 1*H*-imidazo[4,5-*c*]pyridine backbones.

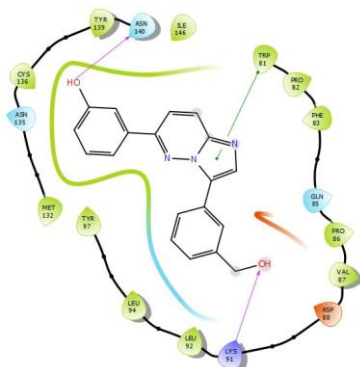
3.1.1. BRD4 protein-ligand interactions D2-D11

Hydrogen bond interactions are non-covalent electrostatic interactions between a proton of an electronegative atom (the hydrogen bond donor) and the lone pair of electrons on an electronegative atom (the hydrogen bond donor)¹⁰⁵. The strength of hydrogen bond interactions can vary greatly based upon the distance between the interacting atoms from 8.4 kJ/ mol to 84 kJ/ mol change in free energy ($-\Delta G$)¹⁰⁶. Another non-covalent interaction is known as π interactions. In the instance of π - π stacking interactions two aromatic rings, with π bonds, interact with each other¹⁰⁷. These stacking interactions can be grouped into edge-to-face (T-shaped) interactions, face-to-surface stacked interactions, and offset stacked interactions¹⁰⁷. The strength of π - π stacking interactions usually range between 1 kJ/ mol to 50 kJ/ mol¹⁰⁷. Another π interaction is the π -cation interaction, which are generally stronger than stacking interactions¹⁰⁸. The π -cation interaction is an electrostatic interaction whereby the electron cloud of a π system interacts with a positively charged cation¹⁰⁸. These interactions can be quite strong with some studies reporting binding energies of up to 117.2 kJ/mol¹⁰⁸.

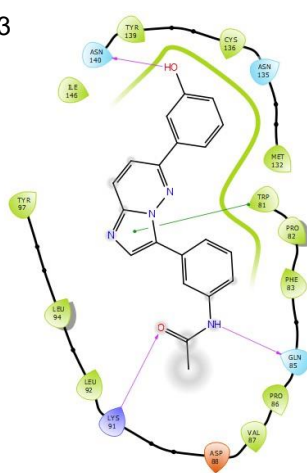
From the ligand-protein interaction diagrams of D2-D11 and BRD4 (Figure 3.2-3.4), a conserved hydrogen bond interaction between asparagine 140 (Asn-140) and hydrogen donors (as well as hydrogen acceptors) of D2-D11 can be observed. Asn-140 is part of the BC loop of the BD1 of BRD4, and plays a very important role in the recognition and hydrogen binding of the acetylated lysine tails of histones¹⁰⁹. Consequently Asn-140 also play a key role in the binding and stabilization of inhibitors in the binding pocket^{81,109}. Tryptophan-81 (Trp-81), which forms part of the ZA loop, also play an important role in the recognition and stabilization of acetylated histone tails inside the binding pocket¹⁰⁹. In addition to that it has been shown that it also plays a role in the stabilization of inhibitors in the binding pocket^{81,109}. Compounds D2, D3, D5, and D7 interacts with Trp-81 by π - π stacking, the interaction is between the aromatic side-group of Trp-81 (benzene) and the aromatic backbones of D2, D3, D5, and D7 (imidazole). This interaction is expected to stabilize the inhibitors along the WPF shelf, so named for the conserved Trp-81, proline-82 (Pro-82), and phenylalanine-83 (Phe-83) motif¹⁰⁹⁻¹¹⁰. Tyrosine-97 (Tyr-97) of the ZA loop, like Trp81, play an important role in histone as well as stabilization in the binding pocket¹⁰⁹.

Compounds D6-D9 form bridging hydrogen bonds via a water molecule in the ZA channel with Tyr-97. There is a hydrogen bond interaction between Lysine-91 (Lys-91) and D2, D3, D5, and D6. The positively charged aspartic acid-144 (Asp-144) which forms part of the BC loop, as well as Glycine-85 (Gln-85) seem to play a minor role in acetylated-lysine specificity and inhibitor binding affinities¹⁰⁹. Compound D4 forms a hydrogen bond with Asp-144, and D9 forms a salt bridge with Asp-144. A hydrogen bond interaction between Gln-85 and D3, D6, D7, D10, and D11¹⁰⁹⁻¹¹¹. Isoleucine-146 (Ile-146), in the α C helix of BD1 which encloses the hydrophobic WPF shelf, forms a hydrogen bond with D4, this however seems to have minimal overall effects on binding affinities¹⁰⁹. Pro-82, which forms part of the WPF shelf, has hydrogen bond interactions with compound D10 and D11.

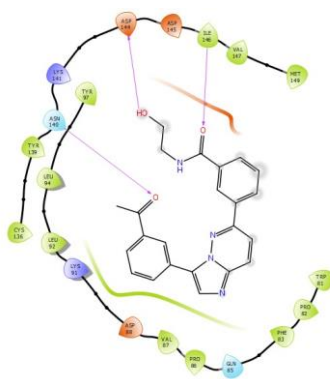
D2



D3



D4



D5

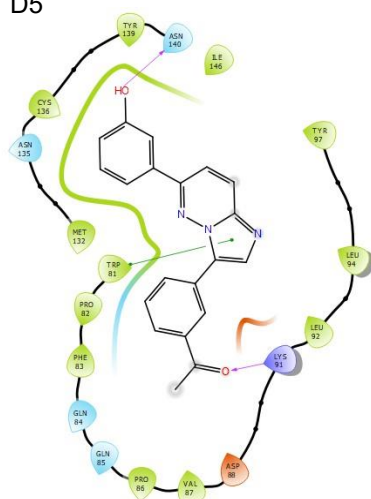
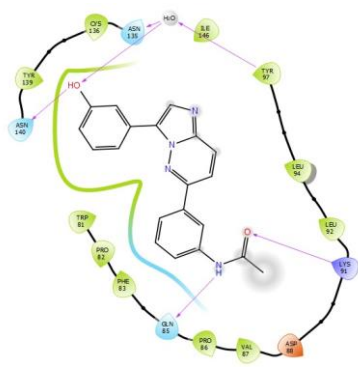
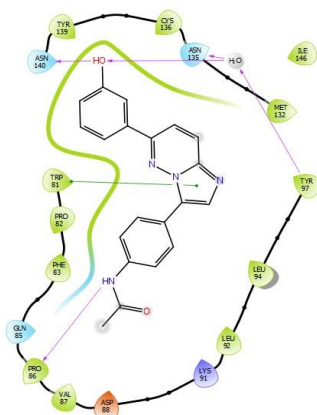


Figure 3. 2: Ligand-protein interaction diagram of D2-D5 complexed with BRD4. Note the hydrogen bond interaction between D2-11 with Asn-140, a conserved interaction that play a key role in effective inhibition. Compounds D2, D3, and D5 interacts with Trp-81 by π - π stacking. There is a hydrogen bond interaction between Lys-91 and D2, D3, and D5. D4 forms a hydrogen bond interaction with Asp 144 and Ile-146

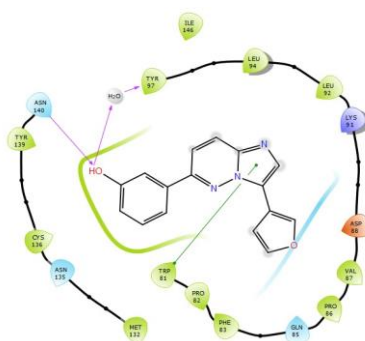
D6



D7



D8



D9

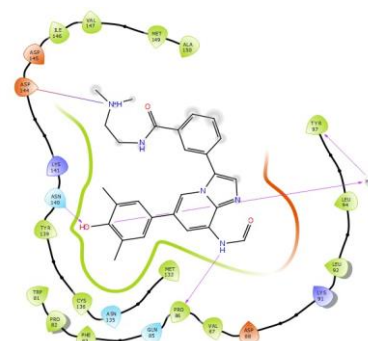


Figure 3. 3: Ligand-protein interaction diagram of D6-D9 complexed with BRD4. Note the hydrogen bond interaction between D6-9 with Asn-140, a conserved interaction that play a key role in effective inhibition. Compound D7 interacts with Trp-81 by π - π stacking. Compounds D6-D9 form bridging hydrogen bonds via a water molecule in the ZA channel with Tyr-97 and D6 and D7 form an additional interaction with Asn-135. D6 forms a hydrogen bond interaction with Lys-91 and Gln-85. D9 forms a salt-bridge interaction with Asp-144

D10

D11

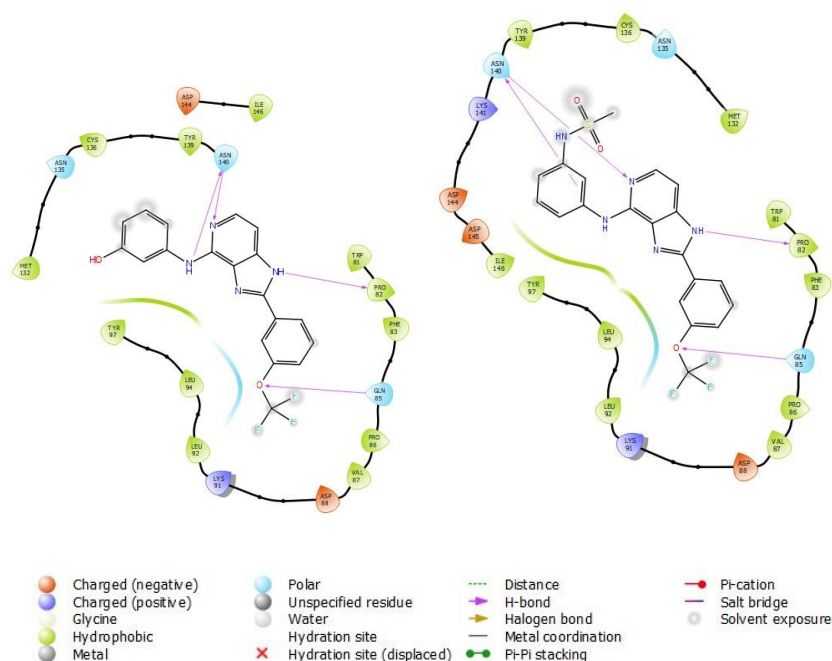


Figure 3. 4: Ligand-protein interaction diagram of D10-D11 complexed with BRD4. Note the hydrogen bond interaction between D10-11 with Asn-140, a conserved interaction that play a key role in effective inhibition. Also notice the hydrogen bond interaction between D10 and 11 with Gln-85 and Pro-82

3.1.2.AURKA protein-ligand interactions D2-D11

The ligand interaction diagrams of AURKA and compounds D2-D11 (Figures 3.5-3.7) show promising protein-ligand interactions.

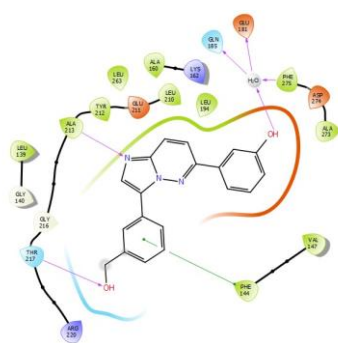
The residues number 210 to 216 in the crystal structure of AURKA, form the hinge region, which plays a significant role in the creation of the AURKA catalytic site within the adenosine triphosphate (ATP) binding pocket¹¹²⁻¹¹³. The highly conserved alanine-213 (Ala-213) forms part of this hinge region and forms the key interaction between the protein and the ligand¹¹²⁻¹¹³. Compounds D2, D4, D5, D7, D8, D9, D10, D11, all form hydrogen bond interactions with Ala-213. D2, D4, D7, D8, and D9 act as

hydrogen acceptors, whereas D5 act as a hydrogen donor. D10 and D11 act as both acceptor and donor, forming two hydrogen bonds with Ala-213.

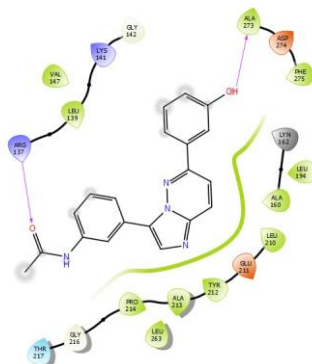
D3 and D5 form hydrogen bond interactions with Ala-273. Threonine-217 (Thr-217) interacts with D2 via a hydrogen bond. A water bridge is formed through binding of Phe-275 to a water molecule and the water molecule forming a hydrogen bond interaction with Gln-185 and Glu-188, and compounds D2 and D7 forms a hydrogen bond with the water molecule. D9 also forms a hydrogen bond with Gln-185 as well as Glutamic acid-211 (Glu-211), which forms part of the hinge region. This hydrogen bond interaction between Glu-211 and D9 is an interaction seen in other docking studies as well¹¹³⁻¹¹⁴.

D3 and D6 interacts with arginine-137 (Arg-137) via a hydrogen bond, a protein-ligand interaction previously reported on¹¹⁴. Whereas D10 and D11 form a π -cation interaction with Arg-137, whereby the π -system of the aromatic rings of D10 and D11 interacts with the cationic Arg-137 sidechain. A π - π stacking interaction occurs between D2 and D7 and Phe-144. A similar π - π stacking interaction occurs between D4 and D9, and Phe-275. A hydrogen bond interaction is formed by compound D6 and D9 and Lys-141 an interaction that has been seen in other docking studies as well¹¹³. Another interaction that has been seen in other docking studies is the hydrogen bond interaction formed between compound D8 and Lys-162¹¹³. A novel π -cation interaction is formed by D4 and D5 with Lys-162. Asp-274 acts as a donor in the hydrogen bonding interaction between it and compound D4 and D9, an interaction reported on previously in docking studies¹¹³. In an interaction seen before between an AURKA inhibitor and Leu-139, D8 forms a hydrogen bond with Leu-139¹¹³. Proline-214 which forms part of the hinge region interacts with D6, D10, and D11 through a hydrogen bond interaction. The interactions with the hinge region residues seem to play a role in the successful binding of inhibitors into the ATP catalytic site¹¹⁴.

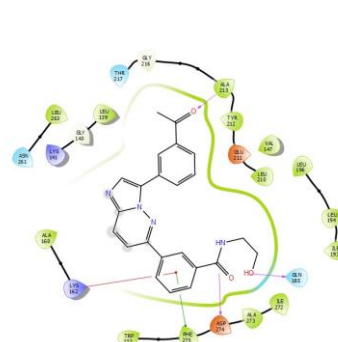
D2



D3



D4



D5

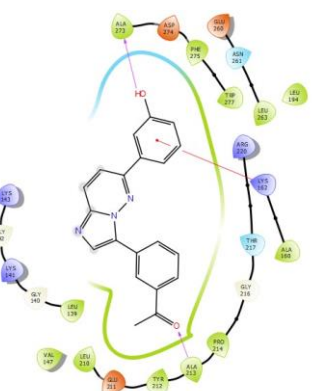
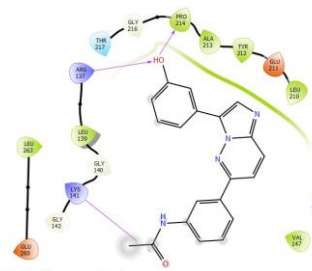


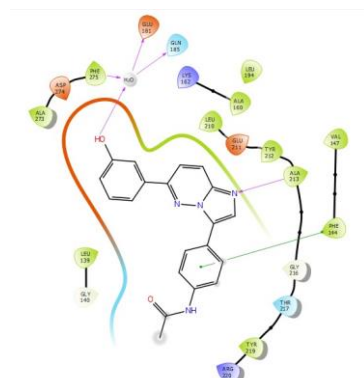
Figure 3. 5: Ligand-protein interaction diagram of D2-D5 complexed with AURKA. Compounds D2-D5 form hydrogen bond interactions with Ala-213. D3 and D5 form hydrogen bond interactions with Ala-273. Thr-217 forms a hydrogen bond with D2. A water bridge is formed between Phe-275, Gln-185, Glu-188, and compound D2. D3 forms a hydrogen bond interaction with Arg-137. A π - π stacking interaction occurs

between D2 and Phe-144. A similar π - π stacking interaction occurs between D4 and Phe-275. A hydrogen bond interaction is formed by compound D6 and Lys-141. A π -cation interaction is formed by D4 and D5 with Lys-162. Asp-274 forms a hydrogen bonding interaction with D4.

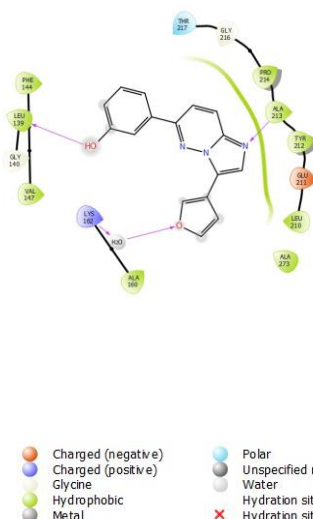
D6



D7



D8



D9

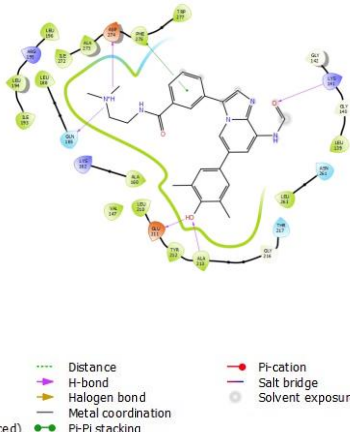


Figure 3. 6: Ligand-protein interaction diagram of D6-D9 complexed with AURKA. Compounds D7-D9 forms a hydrogen bond interaction with Ala-213. A water bridge is formed between Phe-275, Gln-185, Glu-188, and D7. D9 also forms a hydrogen bond with Gln-185 as well as Glu-211. D6 forms a hydrogen bond interaction with Arg-137. D7 forms a π - π stacking interaction with Phe-144. A similar π - π stacking interaction

occurs between D9 and Phe-275. A hydrogen bond interaction is formed by D9 and Lys-141. D8 forms a hydrogen bond interaction with Lys-162 and Leu-139. D9 forms a hydrogen bond interaction with Asp-274. D6 forms a hydrogen bond interaction with Pro-214

D10

D11

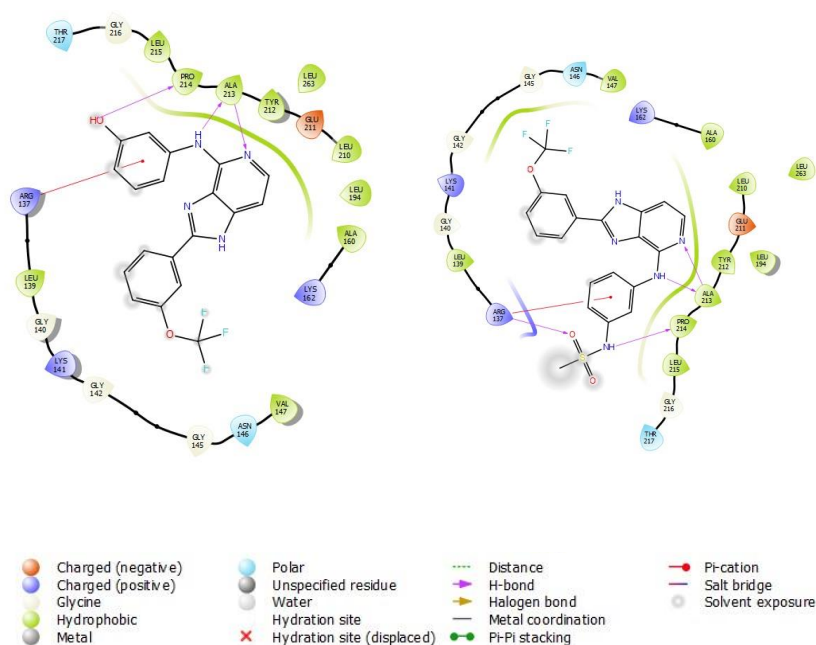


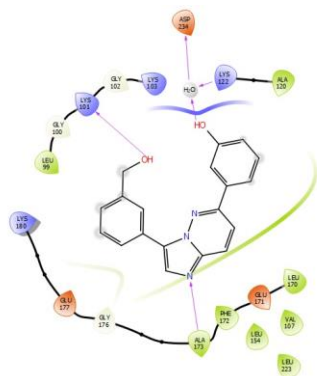
Figure 3. 7: Ligand-protein interaction diagram of D10-D11 complexed with AURKA. Compounds D10 and D1 forms a hydrogen bond interaction with Ala-213. D10 and D11 form a π -cation interaction with Arg-137. D10 and D11 forms a hydrogen bond interaction with Pro-214

3.1.3. AURKB protein-ligand interactions D2-D8

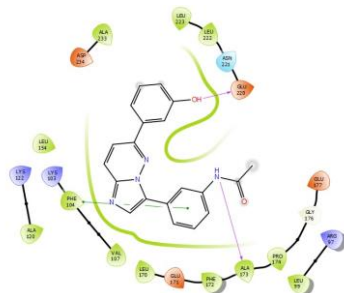
Like with AURKA, key residues within the AURKB hinge region play an important role in the binding of inhibitors into its catalytic site^{112,115}. The hinge region usually includes a hydrogen donor (Ala-213 in AURKA) flanked by two hydrogen acceptors^{112,115}. In AURKB the key residue is Ala-173, and hydrogen bonding interaction between this residue and an inhibitor seem to be the key indicator of its potential effectivity. From the ligand-protein interaction diagrams of compound D2-D8 with AURKB (Figure 3.8 and 3.9), compounds D2, D3, D4, D5, D7 and D8 forms hydrogen bond interactions with Ala-173. A water-bridge is formed between D2, Lys-122 and Asp-234, where D2 and Lys-122 acts as hydrogen bond donors, binding a water molecule water, and the water molecule acts as a hydrogen bond donor binding Asp-234. Hydrogen bond interactions between Lys-122 and AURKB inhibitors have previously been reported on, however a water-bridge hydrogen binding interaction of this kind seems to be a novel observation¹¹². Hydrogen bonding interactions with Lys-101 is an interaction known from other docking studies¹¹². Here D2 interact with Lys-101 via hydrogen bond interaction where the D2 hydroxyl group acts as a hydrogen bond donor. D2 and D6 forms a hydrogen bond with Asp-234 and Glu-177 forms hydrogen bond interactions with D7 and D8, interactions previously reported on with other inhibitors in docking studies¹¹².

D4 and a water molecule form a hydrogen bond with Lys-103, where the D4 carbonyl oxygen acts as a hydrogen acceptor and Lys-103 the donor, water acts as a hydrogen bond donor between its interaction with Lys-103. D5 also forms a hydrogen bond with Lys-103. D4 and D5 forms a hydrogen bond with Lys-180 and D7 forms a hydrogen bond with Gln-145. Additional interactions are hydrogen bonds formed by D3 and D4 with Glu-220, and π - π stacking interactions with Phe-104 and D3 and D6.

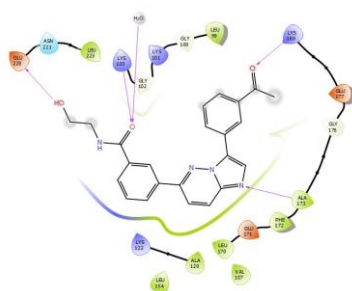
D2



D3



D4



D5

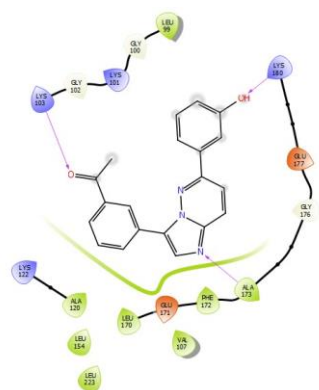
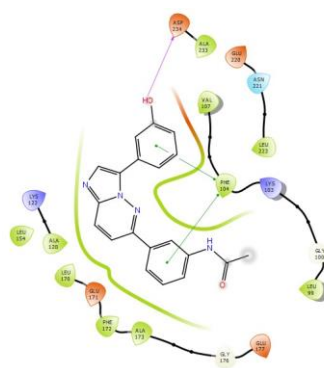


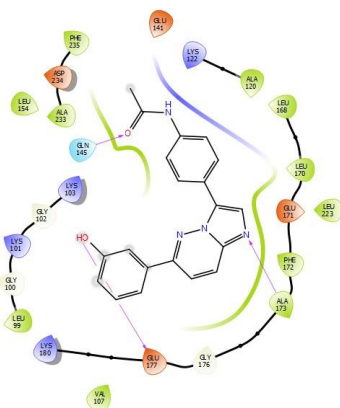
Figure 3. 8: Ligand-protein interaction diagram of D2-D5 complexed with AURKB. Compounds D2-D5 forms hydrogen bond interactions with Ala-173. A water-bridge is formed between D2, Lys-122 and Asp-234. D2 forms a hydrogen bond with Asp-234. D4 and a water molecule form a hydrogen bond with Lys-103. D5 also forms a hydrogen bond with Lys-103. D4 and D5 forms a hydrogen bond with Lys-180.

Additional interactions are hydrogen bonds formed by D3 and D4 with Glu-220, and π - π stacking interactions with Phe-104 and D3.

D6



D7



D8

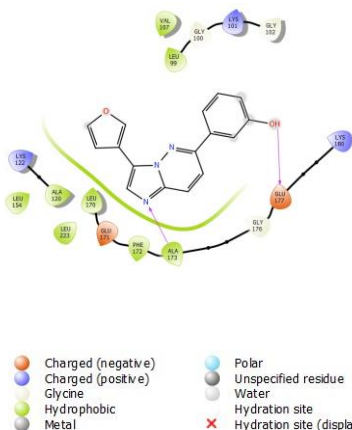
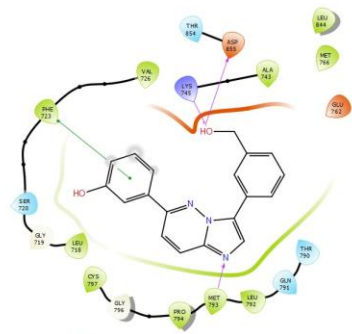


Figure 3. 9: Ligand-protein interaction diagram of D6-D8 complexed with AURKB. compounds D5, D7 and D8 forms hydrogen bond interactions with Ala-173. D6 forms a hydrogen bond with Asp-234 and Glu-177 forms hydrogen bond interactions with D7 and D8. D6 forms a π - π stacking interactions with Phe-104.

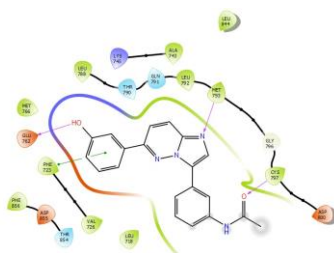
3.1.4. EGFR protein-ligand interactions D2-D7

Methionine-793 (Met-793) is an amino acid within the tyrosine-kinase (TK) domain of EGFR and seems to play an important role in the binding of EGFR-TK inhibitors, and its effectivity¹¹⁶⁻¹¹⁷. Met-793 play a role in molecular recognition of substrates in the TK binding pocket¹¹⁶⁻¹¹⁷. Another key residue within the TK domain of EGFR is Asp-800¹¹⁶⁻¹¹⁷. Compounds D2, D3, D4, D5, D6, and D7 all form hydrogen bond interactions with Met-793 (Figure 3.10 – 3.11). Compound D5 forms a hydrogen bond with the key Asp-800 residue. Phe-723, a residue found at the entrance of the TK active-site, interacts with D2-D4, D6 and D7 with via π - π stacking¹¹⁷. Compound D6 has two π - π stacking interactions between its aromatic side-groups and the aromatic sidechain of Phe-723. An important conserved interaction is the hydrogen bonding between D2 and Asp-855 and Lys-745¹¹⁷. Other hydrogen bond interactions observed are those of D3 and D7 with Glu-762, D3 with Cys-797 and D4 with Ser-720.

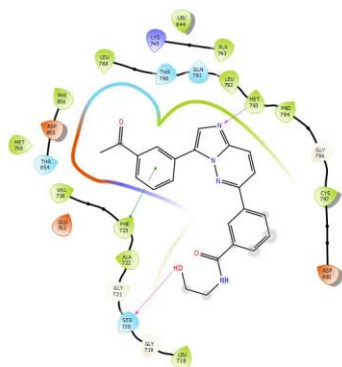
D2



D3



D4



D5

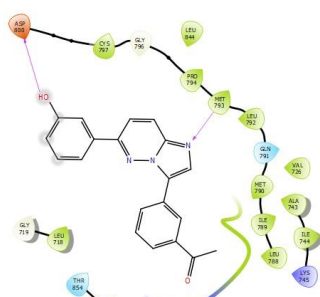


Figure 3. 10: Ligand-protein interaction diagram of D2-D5 complexed with EGFR. Compounds D2-D5 forms a hydrogen bond interactions with Met-793. Compound D5 forms a hydrogen bond with the key Asp-800 residue. Phe-723 interacts with D2-D4, via π - π stacking. D2 forms a hydrogen bond interaction with Asp-855 and Lys-745.

Other hydrogen bond interactions observed are those of D3 with Glu-762 and Cys-797 and D4 with Ser-720.

D6

D7

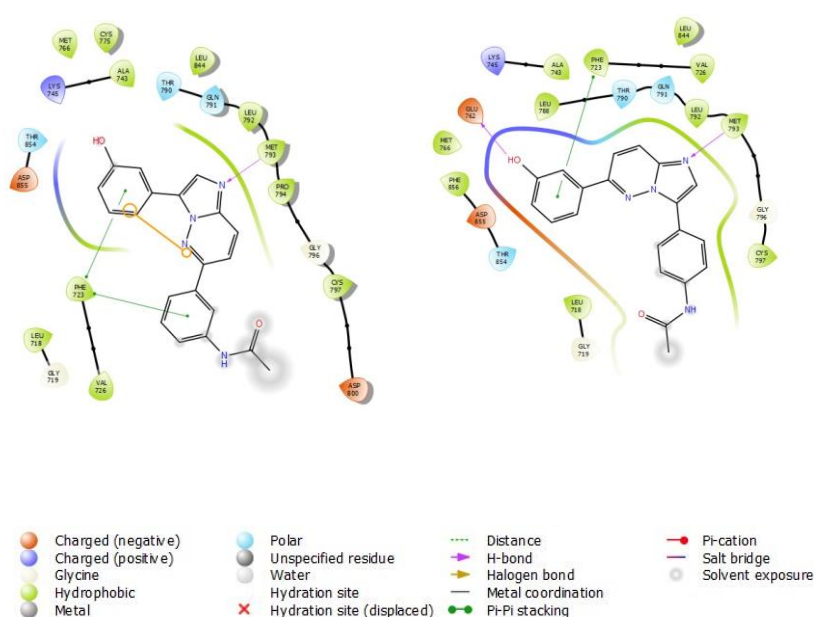


Figure 3. 11: Ligand-protein interaction diagram of D6-D7 complexed with EGFR. Compounds D6 and D7 form hydrogen bond interactions with Met-793. Phe-723 interacts with D6 and D7 with via π - π stacking. Compound D6 has two π - π stacking interactions with Phe-723. D7 forms a hydrogen bond interaction with Glu-762.

3.2. Results of the In vitro screening of compound D2-D11 with the CV viability assay

Screening of the lead compounds (D2-D11) in MDA-MB-231 cells using the CV assay showed that compounds D3- D11 had very little to no activity at the concentrations

they were tested at (Figure 3.12). It wasn't possible to calculate IC_{50} s for compound D3-D11, because the viability of the cells never went below 80%.

CV Lead Compounds D2-D11 Screening in MDA-MB-231

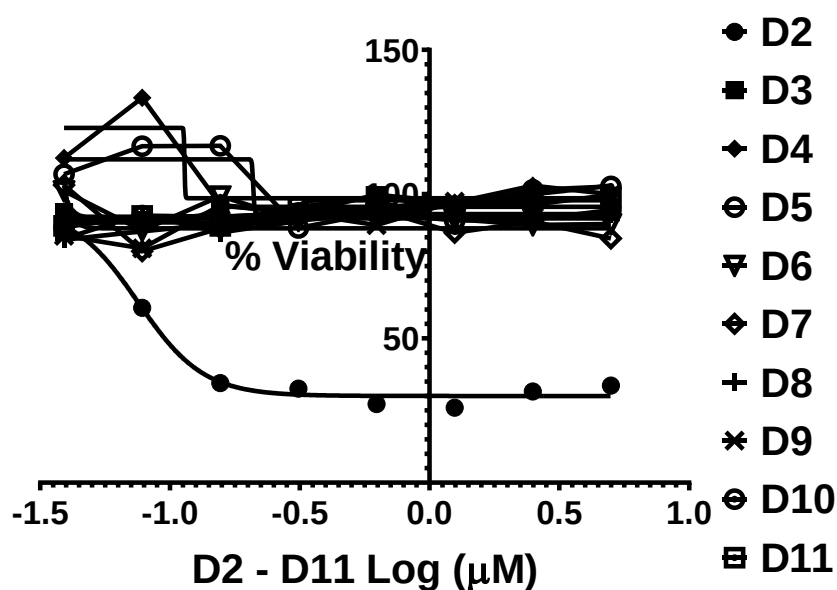


Figure 3. 12: Dose-response curve of compounds D2-D11 tested in MDA-MB-231 cells. The right-hand legend indicates which line style represents which compound.

Activity has however been observed in compound D2, from the dose-response curve it is clear that an increase in the concentration leads to a decrease in viability of the MDA-MB-231 cells. An IC_{50} of D2 was calculated from the screening results and it is in the nanomolar range (table 3.3).

Table 3. 3: A list of the screening results of compound D2-D11. It was not possible to calculate IC_{50} values for D3-D11.

Compound	IC_{50} μM
D2	0.1739
D3	~> 5

D4	~> 5
D5	~> 5
D6	~> 5
D7	~> 5
D8	~> 5
D9	~> 5
D10	~> 5
D11	~> 5

3.3. MTT results

The single active compound, D2, from the list of 10 hit compounds from the HTVS was tested on MDA-MB-231 cells to calculate its IC_{50} . From the dose-response curve, as was the case with the CV screening, a clear dose dependant decrease in cell viability can be seen (Figure 3.13). The IC_{50} is calculated as 0.1968 μ M which means it falls within the nanomolar range. The coefficient of determination (R^2), for the IC_{50} was calculated as $R^2 = 0.9911$, that is the $R^2 \geq 0.90$ and thus falls within accepted criteria for a linear regression model at 95% confidence interval and $\Delta R \geq 0.1^{118}$. The R^2 value indicates that the data falls within the model (the fitted regression line) 99.11% of the time.

MTT Dose Response Curve of D2 Treated MDA-MB-231

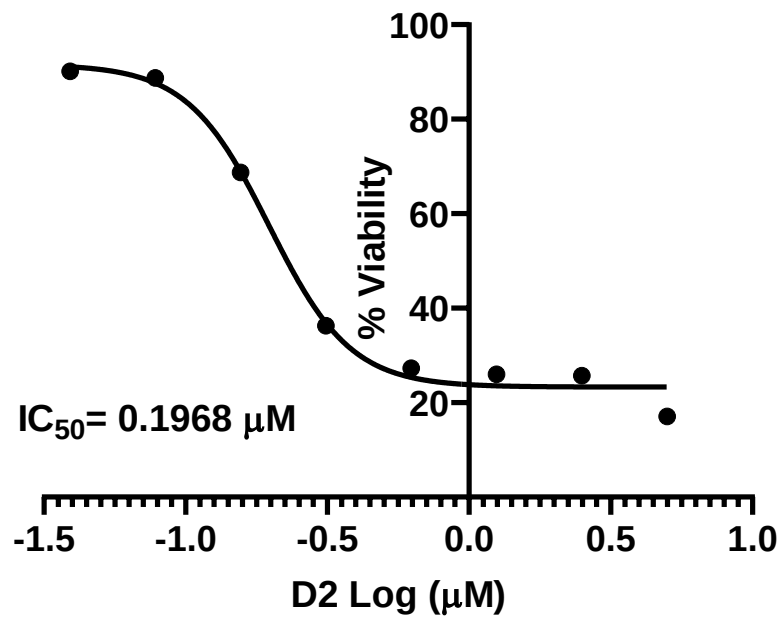


Figure 3. 13: The dose response curve of the MDA-MB-231 cells after 48 h treatment with D2, determined with the MTT assay. The IC₅₀ of 0.1968 μM is indicated on the graph. A non-linear regression curve fit was performed to estimate the IC₅₀. This experiment was performed in triplicate with three biological repeats.

Based on the results of the MTT assay, there is no significant difference in viability between the vehicle control (0.1% DMSO v/v in CCM), (1.158 OD ± 0.1292 OD) and the CCM only (1.174 OD ± 0.1044) controls (Figure 3.14), based upon the Welch's t-test ($p > 0.9999$). Based on this, any future experimentation can be performed with only a vehicle control and not both a vehicle and CCM control.

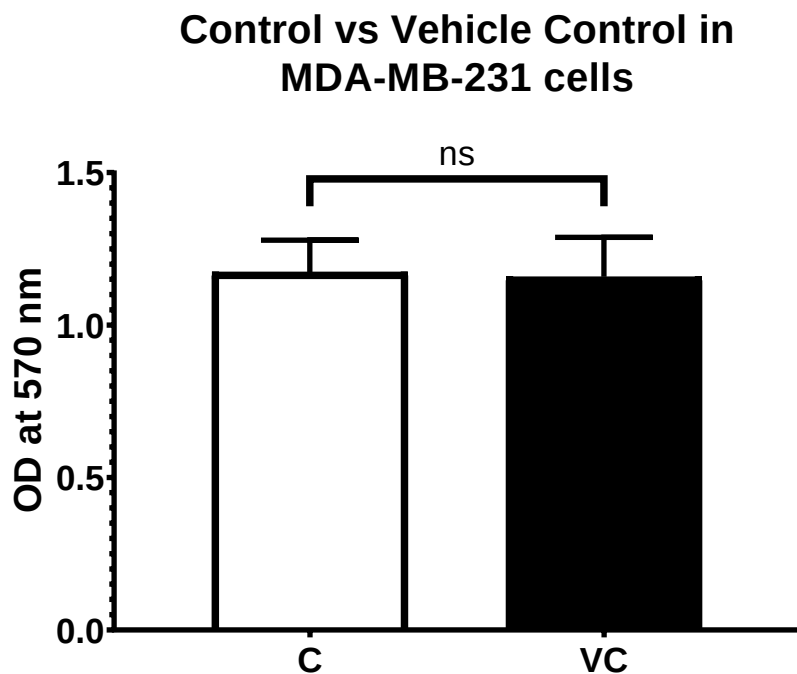


Figure 3. 14: A bar chart comparing the MTT assay ODs of the control (C) and vehicle control (VC). Error bars indicate the standard deviation from the mean (Std of C= 0.1044; Std of VC= 0.1292). No significant difference was found between C and VC cells (Welch's t-test $p > 0.9999$). OD: optical density at 570 nm. This experiment was performed in triplicate with three biological repeats.

Next, the EA.hy926 cells were treated with the IC_{50} of D2 calculated from the MTT assay of the MDA-MB-231 cells. The EA.hy926 treatment followed the same basic procedure of 5000 cells per well, but instead of a treatment range, cells were only treated with the IC_{50} and a vehicle control was also included (and not a CCM only control). From Figure 3.15 it is clear that D2 treatment with the MDA-MB-231 IC_{50} does not have a significant effect ($p=0.9862$) on EA.hy926 viability ($1.616 \text{ OD} \pm 0.1751 \text{ OD}$) relative to the VC ($1.583 \text{ OD} \pm 0.3561 \text{ OD}$).

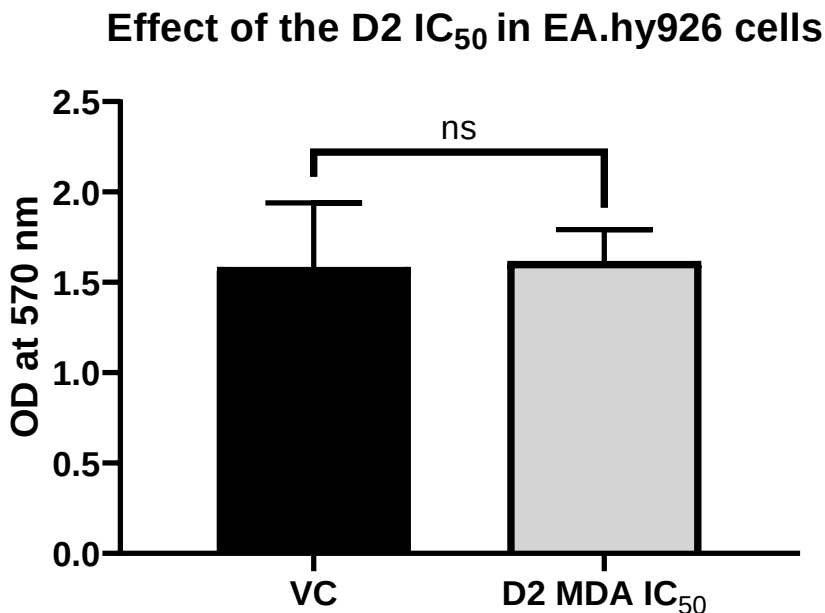


Figure 3. 15: Bar graph comparing the effect of the MDA-MB-231 IC₅₀ of D2 on EA.hy926 cells. Error bars indicate standard deviation from the mean (Std VC= 0.3561; Std D2 IC₅₀= 0.1751). No significance was found between VC and D2 MDA IC₅₀ treated EA.hy926 cells (Welch's t-test p= 0,9862). OD: optical density at 570 nm. This experiment was performed in triplicate with three biological repeats.

3.4. Results of the analysis of the *in vitro* effect of D2

The *in silico* results and cytotoxic effect of D2 observed in the *in vitro* screening and the IC₅₀ calculated from the MTT, pointed to efficacy as an inhibitor, warranting further investigation, the results of which follows here.

3.4.1. BRD4 and AURKA ELISA

The anti-BRD4 ELISA results indicate that there is expression of BRD4 in both MDA-MB-231 and EA.hy926 cells, thus D2 should theoretically be able to bind to BRD4 and inhibit its function. The EA.hy926 cells seem to express more BRD4 than the MDA-MB-231 cells (Figure 3.16).

In the MDA-MB-231 VC cells, the mean concentration of BRD4, as interpolated from the standard curve, is 413.20 ng/l ± 35.33 ng/ l and the mean concentration of the D2-treated cells is 335.30 ng/l ± 53.80 ng/ l. The decrease in BRD4 expression in D2

treated cells compared to the VC is statistically significant ($p=0.0030$). The EA.hy926 cells on the other hand showed a significant increase ($p=0.0133$) in the concentration of BRD4 in the D2-treated cells ($532.50 \text{ ng/l} \pm 65.99 \text{ ng/l}$) compared to the VC ($464.04 \text{ ng/l} \pm 20.97 \text{ ng/l}$).

Interpolated concentrations of BRD4 in MDA-MB-231 and EA.hy926 cells

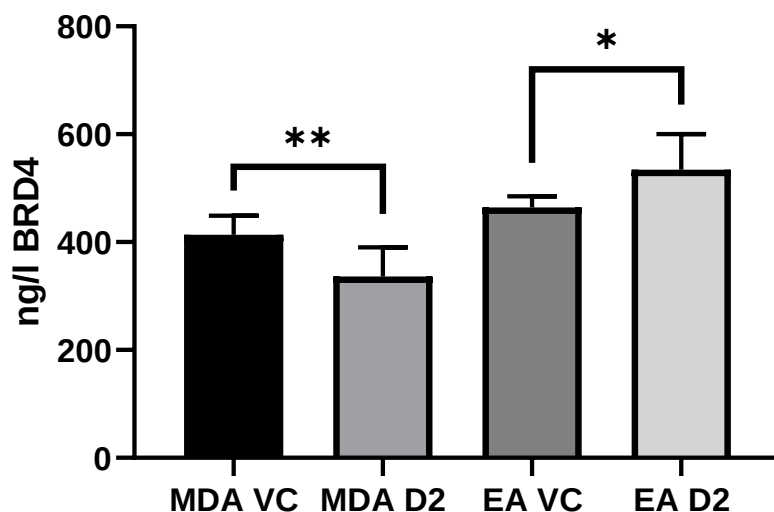


Figure 3. 16: Bar chart of the interpolated concentrations of BRD4 in the VC and D2 treated MDA-MB-231 and EA.hy926 cells. Error bars indicate the Std dev. Significance in differences between the VC vs D2 treatment in each cell line is indicated with asterisks (* $p<0.05$; ** $p<0.01$; *** $p<0.005$; **** $p<0.0001$). MDA VC vs MDA D2 ($p=0.0030$), EA VC vs EA D2 ($p=0.0133$). This experiment was performed in triplicate with three biological repeats.

From the anti-AURKA ELISA it is clear that both cell lines do express AURKA. Here MDA-MB-231 cells seem to express more AURKA than do the EA.hy926 cells.

The VC treated MDA-MB-231 cells (Figure 3.17) have a mean AURKA concentration of $3.79 \text{ ng/ml} \pm 0.2752 \text{ ng/ml}$, and the D2-treated cells that have an AURKA concentration ($3.41 \text{ ng/ml} \pm 0.4226 \text{ ng/ml}$) significantly lower than the VC ($p=0.0431$). The concentration of AURKA in the VC treated EA.hy926 cells ($2.96 \text{ ng/ml} \pm 0.3315$

ng/ ml) was not different than that of the D2 treated cells (mean concentration: 3.11 ng/ ml $\pm$ 0.6043 ng/ ml).

Interpolated concentrations of AURKA in MDA-MB-231 and EA.hy926 cells

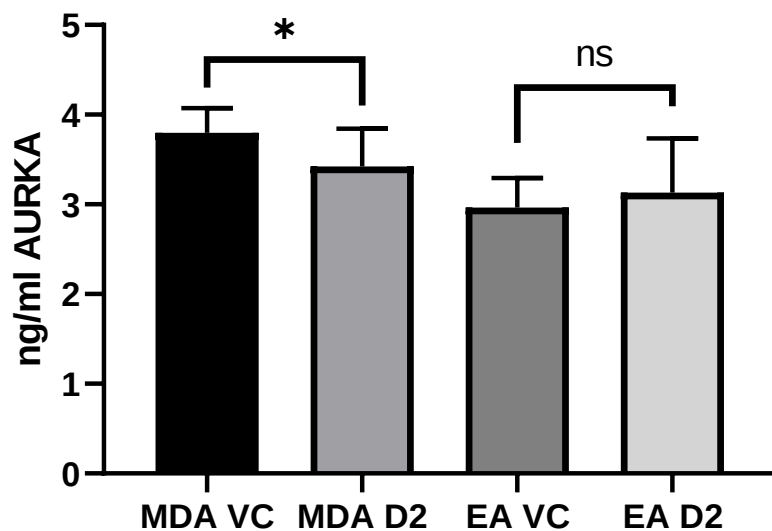


Figure 3. 17: Bar chart of the interpolated concentrations of AURKA in the VC and D2 treated MDA-MB-231 and EA.hy926 cells. Error bars indicate the Std dev. Significance in differences between the VC vs D2 treatment in each cell line is indicated with asterisks (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.005$; **** $p < 0.0001$). MDA VC vs MDA D2 ($p = 0.0431$), EA VC vs EA D2 ($p = 0.4821$). This experiment was performed in triplicate with three biological repeats.

3.4.2. The effect of D2 on the cell cycle

To analyse the effect of the D2 IC_{50} on the cell cycle, flow cytometry was employed.

Using the Muse specific Cell Cycle Analysis kit, the effect of D2 was elucidated. From the representative histograms (Figure 3.18), there is no difference in the number of MDA-MB-231 cells in the G0/G1 phase when treated with the D2 IC_{50} compared to the VC. There is however a decrease in the number of cells in S-phase indicating towards a potential block that prevents the cells from progressing to S-phase.

Statistical analysis of the results (Figure 3.20) indicated no significant difference between the amount of VC treated cells ($70.78 \% \pm 0.3663 \%$) and D2 treated ($72.69 \% \pm 2.381 \%$) cells in G0/G1-phase ($p=0.0587$). A significant difference in the number of cells in S-phase was noted between VC and D2 ($p=0.0093$), where the amount of D2 treated cells ($7.804 \% \pm 0.5766 \%$) in S-phase was decreased, relative to the VC ($8.903 \% \pm 0.4770 \%$). No significant difference ($p=0.5609$) was observed in the cell numbers in the G2/M-phase between VC treated cells ($19.76 \% \pm 1.030 \%$) and the D2 treated cells ($19.24 \% \pm 1.963 \%$). NOC had a significant effect on each phase of the cell cycle relative to the vehicle control ($p<0.05$). In G0/G1 were $47.08 \% \pm 2.226 \%$ of the cells. In S-phase it was $14.24 \% \pm 1.485 \%$ of the cells. And a significant accumulation of NOC treated cells in G2/M ($37.40 \% \pm 0.9823 \%$) is indicative of a G2/M block (a known effect of NOC).

The cell cycle analysis of the EA.hy926 cells treated with the IC_{50} of D2, as calculated in the MDA-MB-231 cells (Figure 3.19 and 3.21), showed no significant difference ($p=0.3685$) in the number of cells in G0/G1-phase between the D2 ($65.07 \% \pm 1.225 \%$) and VC-treated cells ($63.95 \% \pm 1.913 \%$). There was a significant difference between the cell number of VC and D2-treated cells in S-phase ($p=0.0003$), with the D2-treated cell number ($11.93 \% \pm 1.913 \%$) being higher than that of the VC ($8.443 \pm 0.3597 \%$). The difference in the number of cells in G2/M-phase between VC ($27.39 \% \pm 0.8161 \%$) and D2-treated cells ($22.76 \% \pm 1.736 \%$) were significant ($p=0.0072$). Analysis of the VC versus the NOC positive control showed no significant difference ($p=0.1301$) between cell numbers in G0/G1 ($61.98 \% \pm 0.8144 \%$). A significant difference ($p<0.0001$) in the cell numbers in S-phase between VC and NOC ($11.48 \% \pm 0.2381 \%$). The number of cells in G2/M-phase between VC and NOC ($25.40 \% \pm 0.9789 \%$) was significantly decreased ($p=0.0214$).

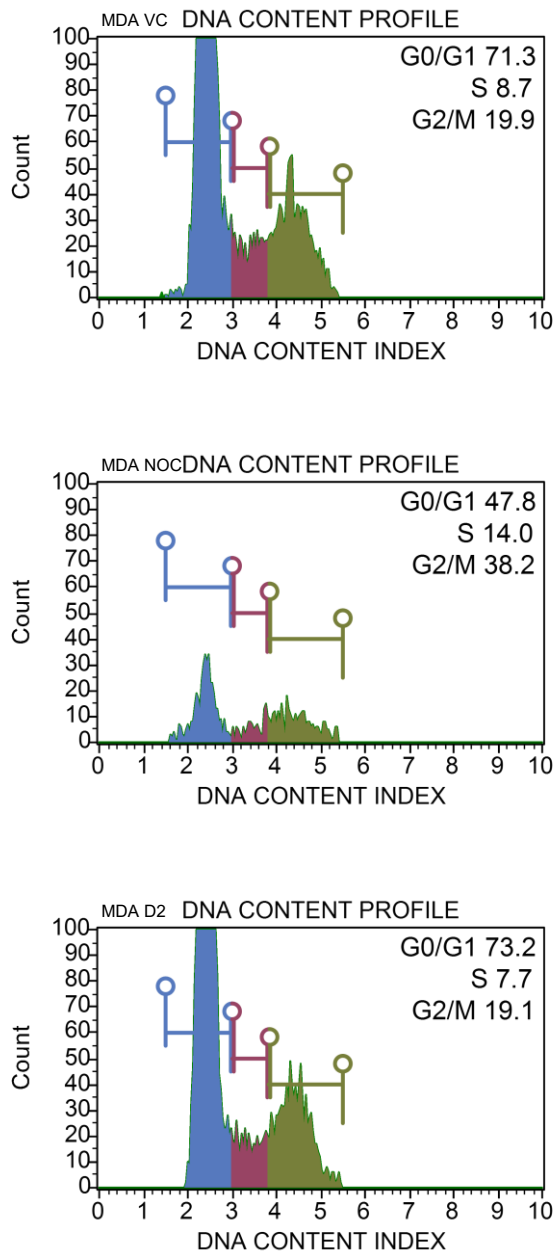


Figure 3. 18: Representative histograms of the DNA content vs the cell count percentage of MDA-MB-231 cells, chosen from three biological repeats to best represent the data. Graphs are marked as MDA VC, MDA NOC, and MDA D2 to indicate the treatment conditions. This experiment was performed in triplicate with three biological repeats.

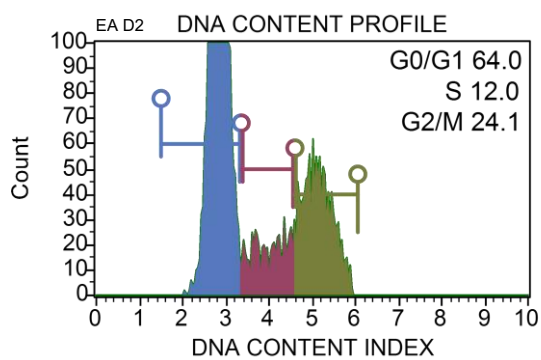
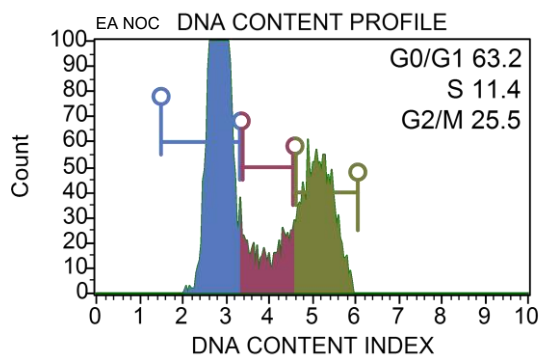
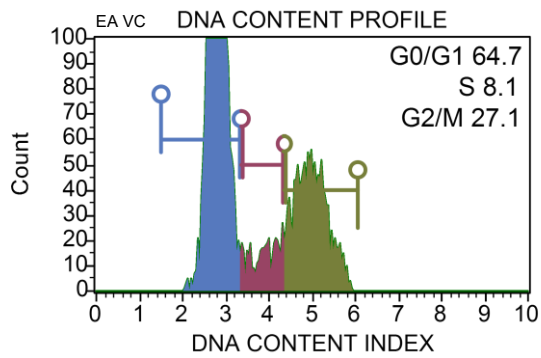


Figure 3. 19: Representative histograms of the DNA content vs the cell count percentage of EA.hy926 cells, chosen from three biological repeats to best represent the data. Graphs are marked as EA VC,

EA NOC, and EA D2 to indicate the treatment conditions. This experiment was performed in triplicate with three biological repeats.

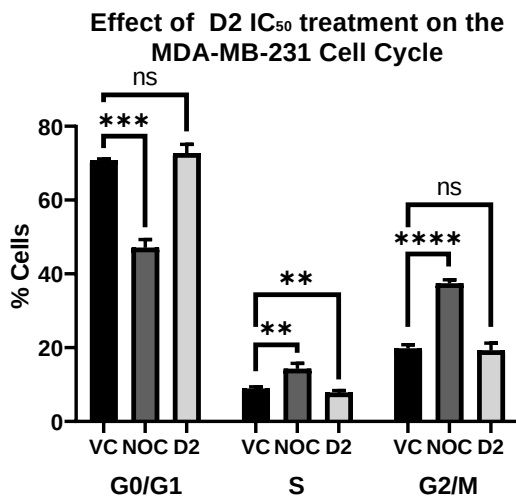


Figure 3. 20: Bar graph of MDA-MB-231 cells in the different cell cycles after treatment with the D2 IC₅₀. Each bar represents three technical repeats from three biological repeats. Each treatment within a cell cycle phase is contrasted with the VC. Error bars indicate the standard deviation from the mean. Welch's t-test was performed to compare each treatment condition with the VC. G0/G1: VC vs NOC ($p=0.0002$), VC vs D2 ($p=0.0587$); S: VC vs NOC ($p=0.0034$), VC vs D2 ($p=0.0093$), VC vs D2 ($p=0.0003$); G2/M: VC vs NOC ($p<0.0001$), VC vs D2 ($p=0.5609$). This experiment was performed in triplicate with three biological repeats.

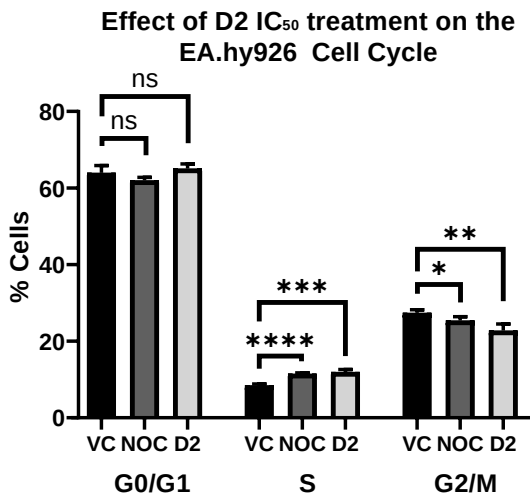


Figure 3. 21: Bar graph of EA.hy926 cells in the different cell cycles after treatment with the D2 IC₅₀ of the MDA-MB-231 cells. Each treatment within a cell cycle phase is contrasted with the VC. Error bars indicate the standard deviation from the mean. Welch's t-test was performed to compare each treatment condition with the VC. G0/G1: VC vs NOC ($p=0.1301$), VC vs D2 ($p=0.3685$); S: VC vs NOC ($p<0.0001$), VC vs D2 ($p=0.0003$); G2/M: VC vs NOC ($p=0.0214$), VC vs D2 ($p=0.0072$). This experiment was performed in triplicate with three biological repeats.

3.5. Results of the investigation into ROS and apoptosis in D2 treated cells

Analysis of BRD4 and AURKA expression, in conjunction with the analysis of the effect of D2 on the cell cycle shed some light on the activity of D2. But it did not adequately explain the cytotoxicity observed. Thus additional investigation into the effect of D2 in MDA-MB-231 and EA.hy926 cells followed.

3.5.1. Intracellular ROS

The MDA-MB-231 and EA.hy926 cells were treated with the D2 IC₅₀, with the VC as reference and NOC as positive control. DHE, a stain that is reduced to the fluorochrome EB by superoxide radicals and which intercalates into DNA serving as an indicator of intracellular ROS, were used to quantify oxidative burden of the treated cells.

From the histograms and statistical analysis (Figure 3.22 and 3.24) of the MDA-MB-231 a marked increase in ROS⁺ cells between the VC (17.94 % $\pm$ 2.676 %) and D2

treated cells ($57.70 \% \pm 2.294 \%$) can be observed. The ROS profiles of D2 indicates increased ROS even compared to the NOC positive control ($40.54\% \pm 0.9982 \%$). In terms of ROS- cells, there was a decrease in the amount of D2-treated cells ($41.92 \% \pm 2.222 \%$) relative to the VC ($81.33 \% \pm 2.519 \%$). A decrease in ROS- cells in the NOC treatment group ($59.00 \% \pm 0.8940 \%$) relative to the VC was also observed.

Statistical analysis of the ROS profiles of the MDA-MB-231 cells indicate significant increases in ROS+ ($p < 0.0001$) D2 treated cells relative to the VC (Figure 3.24). A significant decrease in ROS- cells were observed in the D2 treated cells relative to the VC ($p < 0.0001$). The NOC positive control had a significant decrease in ROS- cells ($p = 0.0001$) as well as a significant increase in ROS+ cells ($p = 0.0001$) relative to the VC.

The EA.hy926 cells treated with the D2 IC_{50} showed an increase ($24.04 \% \pm 1.580 \%$) in ROS+ cells relative to the VC cells ($16.29 \% \pm 2.379 \%$) as seen in Figure 3.23. The amount of ROS- cells in D2 ($75.92 \% \pm 1.567 \%$) was decreased relative to the VC ($83.61 \% \pm 2.353 \%$).

Statistical analysis (Figure 3.25) indicated that there was a significant decrease in ROS- cells in the D2 treatment group relative to the VC ($p = 0.0035$) and a significant increase in ROS+ cells of the D2 treatment group relative to the VC ($p = 0.0035$).

The amount of ROS+ cells in the NOC treatment group ($37.12 \% \pm 1.141 \%$) also increased significantly relative to the VC ($p < 0.0001$). And the amount of ROS- cells in the NOC treatment group ($62.93 \% \pm 1.085 \%$) also decreased significantly relative to the VC ($p = 0.0001$).

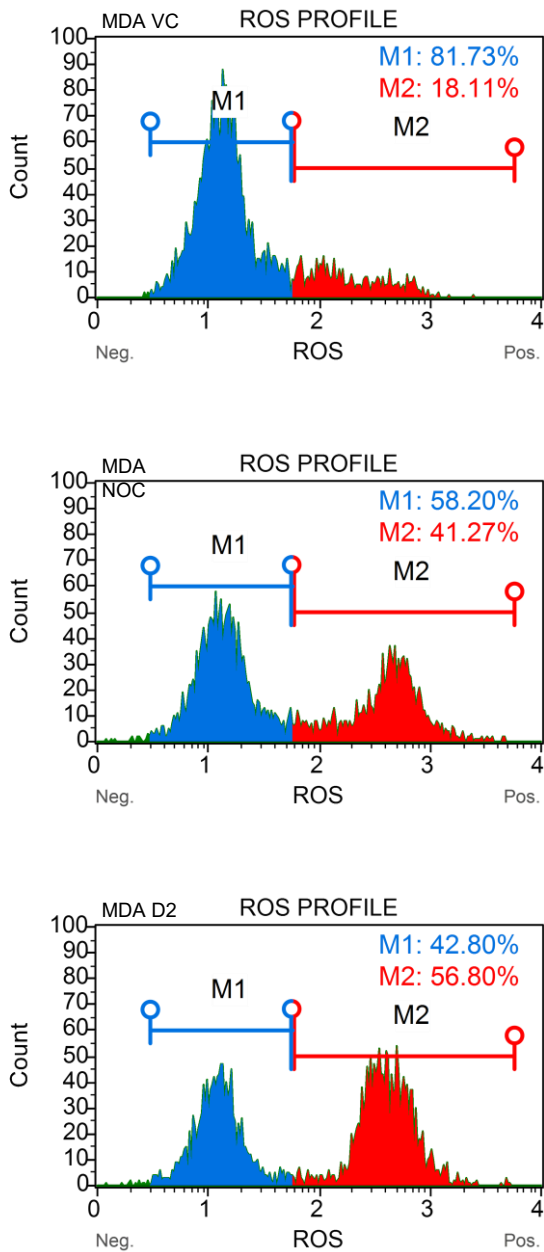


Figure 3. 22: Representative histograms of the different cell populations (M1: ROS- and M2: ROS+) within MDA-MB-231 cells after treatment with the D2 IC₅₀, chosen from three biological repeats to best represent the data. Graphs are marked as MDA VC, MDA NOC, and MDA D2 to indicate the treatment conditions. This experiment was performed in triplicate with three biological repeats.

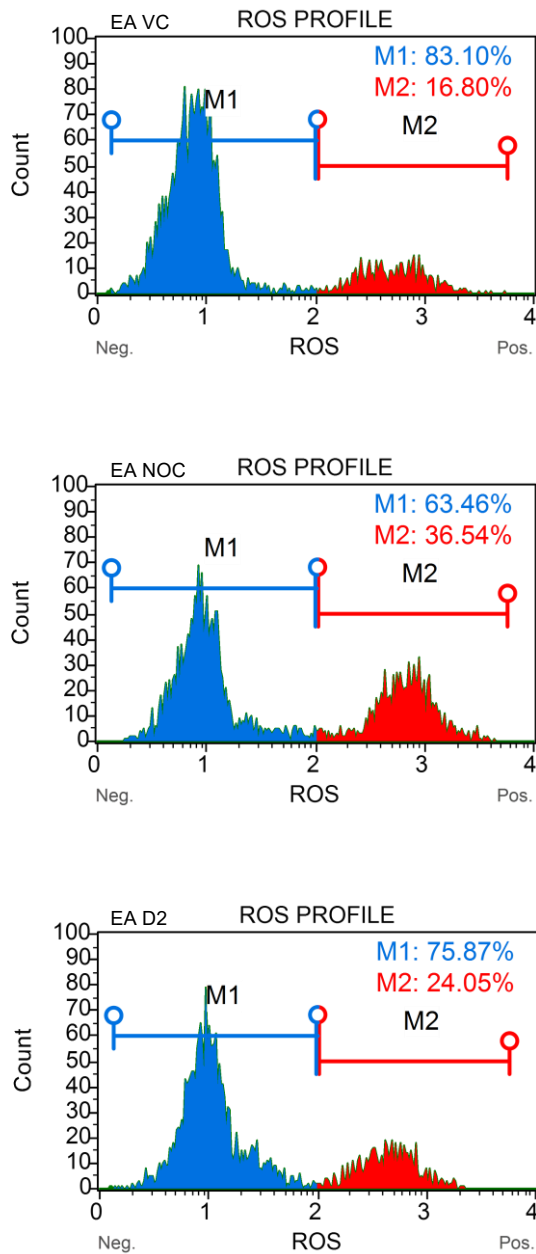


Figure 3. 23: Representative histograms of the different cell populations (M1: ROS- and M2: ROS+) within EA.hy926 cells after treatment with the D2 IC₅₀, chosen from three biological repeats to best represent the data. Graphs are marked as EA VC, EA NOC, and EA D2 to indicate the treatment conditions. This experiment was performed in triplicate with three biological repeats.

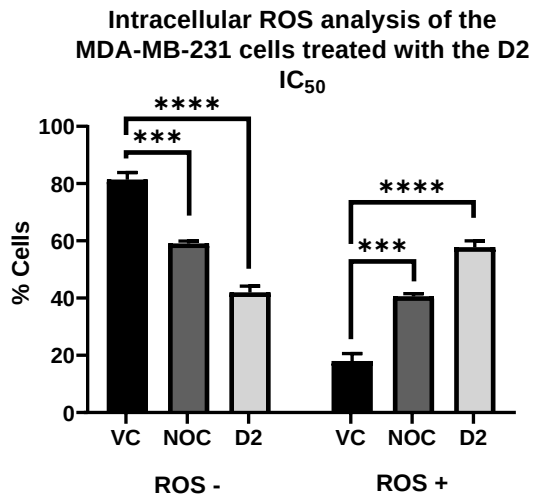


Figure 3. 24: Bar graph indicating the amount of ROS- and ROS+ cells in each treatment group, VC, NOC positive control, and D2 treated MDA-MB-231 cells. Error bars indicate the standard deviation from the mean. Welch's t-test was performed to compare each treatment condition with the VC. ROS-: VC vs. NOC $p=0.0001$; VC vs. D2 $p<0.0001$. ROS+: VC vs. NOC $p=0.0001$; VC vs. D2 $p<0.0001$. This experiment was performed in triplicate with three biological repeats.

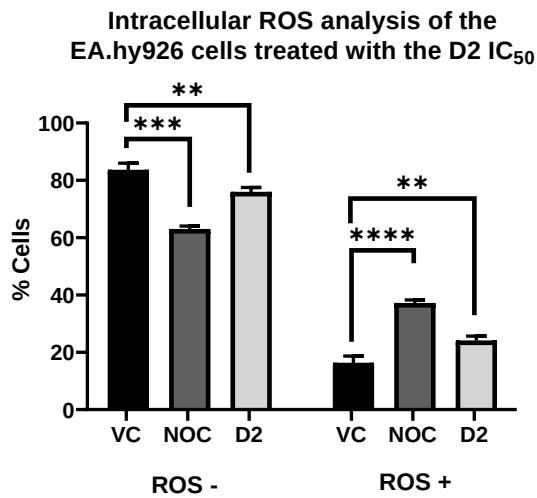
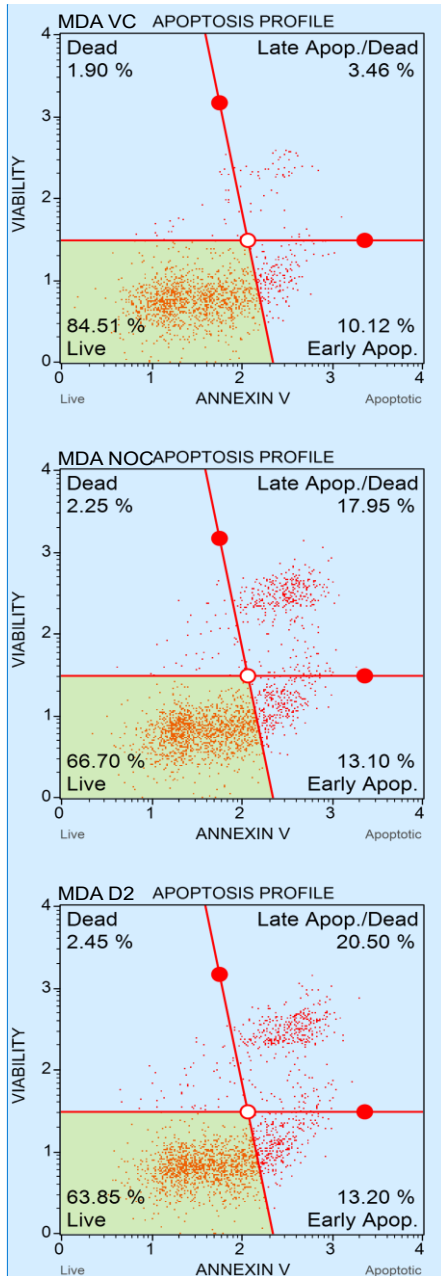


Figure 3. 25: Bar graph indicating the amount of ROS- and ROS+ cells in each treatment group, VC, NOC positive control, and D2 treated EA.hy926 cells. Error bars indicate the standard deviation from the mean. Welch's t-test was performed to compare each treatment condition with the VC. ROS-: VC vs. NOC $p=0.0001$; VC vs. D2 $p=0.0035$. ROS+: VC vs. NOC $p<0.0001$; VC vs. D2 $p=0.0035$. This experiment was performed in triplicate with three biological repeats.

3.5.2. Results of Annexin-V flow cytometry assay

From the apoptosis profiles (Figure 3.26) of the MDA-MB-231 cells under different treatment conditions (VC, NOC, and D2) a clear shift from living to apoptotic cells is visible. Analysis of the apoptotic profiles (Figure 3.28) confirms a significant decrease ($p < 0.0001$) in the number of living cells in the NOC treatment ($65.89 \% \pm 4.546 \%$), a significant increase ($p < 0.0001$) in apoptotic cells ($31.10 \% \pm 6.149 \%$), and, unexpectedly, no significant change ($p = 0.0568$) in the number of dead cells ($3.033 \% \pm 1.977 \%$) in the NOC treated control, relative to the VC. Looking at the D2 treated cells, the number of living cells ($63.36 \% \pm 0.7395 \%$) decreased significantly ($p < 0.0001$) relative to the VC ($84.06 \% \pm 2.809 \%$). The number of apoptotic cells ($34.82 \% \pm 0.9593 \%$) also increased significantly ($p < 0.0001$) versus the VC ($14.37 \% \pm 2.438 \%$), there however was no significant difference ($p = 0.6986$) in the number of dead cells ($1.833 \% \pm 0.3658 \%$) relative to the VC ($1.640 \% \pm 1.862 \%$).

Treatment of the EA.hy926 cells with the D2 IC_{50} caused a slight decrease (Figure 3.27) in the amount of living cells relative to the VC. Statistical analysis (Figure 3.29) indicated significant decrease ($p = 0.0380$) between the number of live cells in D2 ($83.63 \% \pm 0.9105 \%$) compared to the VC ($84.40 \% \pm 0.7089 \%$). There was no significant difference ($p = 0.0802$) in the number of apoptotic cells in D2 ($11.31 \% \pm 0.9078 \%$) compared to the VC ($11.89 \% \pm 0.5689 \%$). There was however a significant increase ($p < 0.0001$) in the number of dead cells in D2 ($5.092 \% \pm 0.8311 \%$) compared to the VC ($3.722 \% \pm 0.4868 \%$). The positive control, as expected, showed a significant ($p < 0.0001$) decline in live cells ($64.73 \% \pm 1.108 \%$), and a significant increase ($p < 0.0001$) in apoptotic cells ($28.96 \% \pm 1.503 \%$) as well as a significant increase ($p = 0.0003$) in dead cells ($6.356 \% \pm 1.397 \%$) relative to the VC.



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Figure 3. 26: Representative apoptosis profile of the MDA-MB-231 cells treated with IC_{50} of D2, chosen from three biological repeats to best represent the data. Treatment conditions are displayed at the top left of each graph (MDA VC, MDA NOC, MDA D2). This experiment was performed in triplicate with three biological repeats.

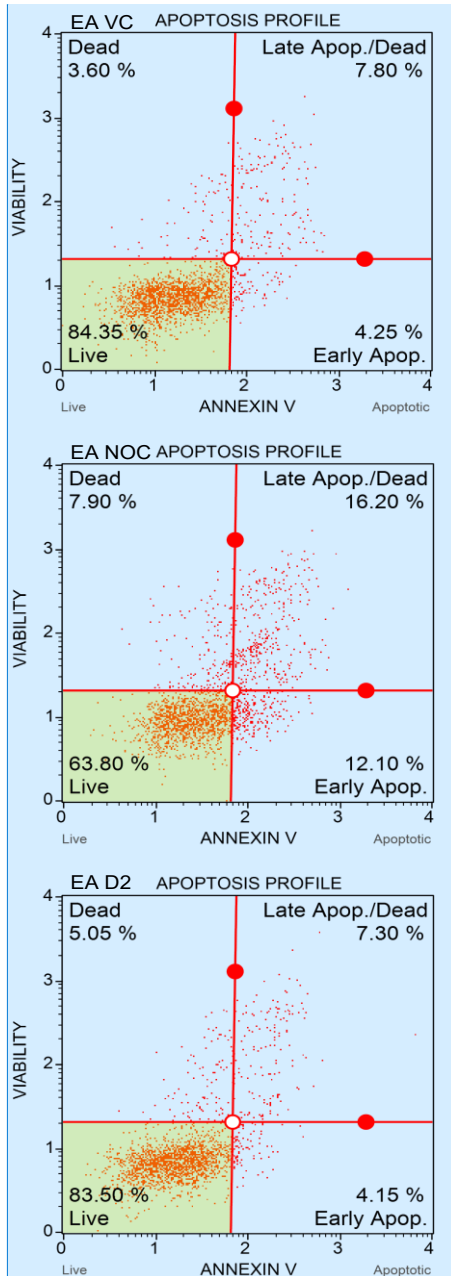


Figure 3. 27: Representative apoptosis profile of the EA.hy926 cells treated with IC_{50} of D2 chosen from three biological repeats to best represent the data. Treatment conditions are displayed at the top left of each graph (EA VC, EA NOC, EA D2). This experiment was performed in triplicate with three biological repeats.

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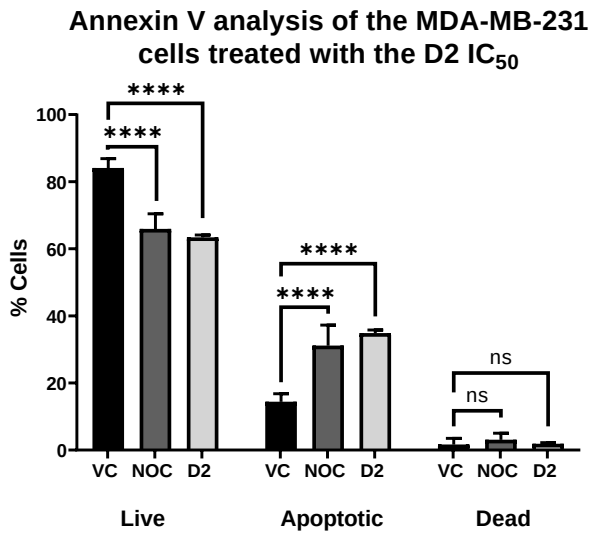


Figure 3. 28: Bar graph of the results of the Annexin-V flow cytometry of MDA-MB-231 cells. . Error bars indicate the standard deviation from the mean. Welch's t-test was performed to compare each treatment condition with the VC. Live cells: VC vs NOC $p < 0.0001$; VC vs D2 $p < 0.0001$. Apoptotic cells: VC vs NOC $p < 0.0001$; VC vs D2 $p < 0.0001$. Dead cells: VC vs NOC $p = 0.0568$; VC vs D2 $p = 0.6986$. This experiment was performed in triplicate with three biological repeats.

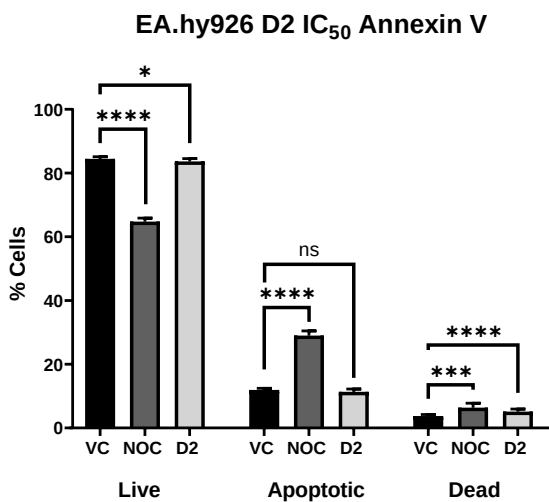


Figure 3. 29: Bar graph of the results of the Annexin-V flow cytometry of the EA.hy926 cells. . Error bars indicate the standard deviation from the mean. Welch's t-test was performed to compare each treatment condition with the VC. Live cells: VC vs NOC $p < 0.0001$; VC vs D2 $p = 0.0380$. Apoptotic cells: VC vs

NOC $p < 0.0001$; VC vs D2 $p = 0.0802$. Dead cells: VC vs NOC $p = 0.0003$; VC vs D2 $p < 0.0001$. This experiment was performed in triplicate with three biological repeats.

4. Discussion

The HTVS of the Biofocus library of compounds, containing 20 000 unique compounds, against BRD4, AURKA, AURKB, PLK1, and EGFR yielded some 10 hits for BRD4 and AURKA (D2-D11), 7 hits for AURKB (D2-D8), 6 hits for EGFR (D2-D7) and zero hits for PLK1. Interestingly all 10 of the compounds interacted favourably with the BD1 active site of BRD4. All 10 compounds formed hydrogen bond interactions with the highly conserved and very important Asn-140 residue of the BC loop. But from the *in vitro* screening only D2, also known as (3-{3-[3-(hydroxymethyl)phenyl]imidazo[1,2-b]pyridazin-6-yl}phenol) shows activity at relatively low concentrations. Compound D2 formed particularly favourable interactions with key residues of the BD1 BRD4. Notwithstanding the hydrogen bonding interaction of D2 with Asn-140, where the hydroxyl group of the phenol sidechain of D2 acts as the donor for the carboxamide oxygen of Asn-140. D2 interacts with 2 additional residues within the BD1 acetyl-lysine binding pocket, it forms a π - π stacking interaction with Trp-81 (part of the WPF shelf), forming a T-shaped π interaction, where the positively charged framework of the aromatic ring of Trp81 interacts with the π electron cloud of the aromatic imidazo-group of D2. Lys-91, a ZA loop residue, interacts with D2 to form a hydrogen bond by means of the hydroxyl group of the benzyl alcohol sidechain of D2 that act as the hydrogen bond acceptor and the protonated lysyl sidechain of Lys-91 acting as the hydrogen bond donor¹¹⁹⁻¹²⁰. The hydrogen bond between D2 and Asn-140 is a proven key interaction for BRD4 BD1 inhibitors^{109,121}. As well as the additional stabilizing interactions with the ZA loop residue Lys-91, and the WPF shelf residue Trp-81, identifies D2 as a strong candidate as a BRD4 inhibitor^{81,109,121-122}.

Perusing the docking results of AURKA, one would expect highly favourable screening results. But inspection of the protein-ligand interactions points to something else. The most important interaction to consider when screening for AURKA inhibitors of the catalytic site of the ATP binding pocket, is the interaction between the potential ligand and the Ala-213 residue^{112,114}. The ligand must act dominantly with Ala-213 via a

hydrogen bond interaction, otherwise it will not bind stably, and no inhibition will take place^{112,114}. Taking this into account it is clear that compounds D3 and D6 would definitely not have shown any activity with regards to AURKA inhibition due to their lack of Ala-213 ([Figure 3.2 and 3.3](#)) interaction. Compound D2 has a hydrogen bond interaction with Ala-213 where the amino group of Ala-213 donates a hydrogen to the nitrogen in position 9 of the imidazo[1,2-b]pyridazine backbone of D2. A T-shaped π - π stacking interaction between D2 and Phe-144, where the positively charged framework of the D2 aromatic ring interacts with the π electron cloud of the aromatic ring of Phe-144. This seems to serve as a stabilizing interaction. In another stabilizing interaction, D2 acts as a hydrogen acceptor at the hydroxyl group of its benzyl alcohol sidechain accepting the hydrogen donated by the amino backbone of Thr-217. The final stabilizing interaction is the formation of a water bridge between Phe-275 (donor) and a water molecule (acceptor), the hydroxyl group of the phenyl group acts as donator for the water molecule and the water molecule acts as donor for Gln-185 and Glu-181. The strong stabilizing interactions of D2 with AURKA predicts good protein-ligand interaction *in-vivo*¹¹²⁻¹¹⁴.

As is the case with BRD4 and AURKA, there is a watershed interaction between AURKB and its inhibitors. AURKB, like AURKA has a hinge region that plays an important role in the binding of inhibitors to the ATP catalytic site. The conserved hinge residue in AURKB is Ala-173^{112,115}. D2 interacts with Ala-173 as a hydrogen bond acceptor. The amino group of Ala-173 acts as hydrogen bond donor and nitrogen 9 of the imidazo[1,2-b]pyridazine backbone of D2. The rest of the interactions between AURKB and D2 are stabilizing interactions. A water bridge forms between D2, the hydrogen bond donor from the hydroxyl group of the phenol sidechain, the water molecule that acts as the acceptor for D2, as well as the hydrogen bond donated by the protonated lysyl side chain of Lys-122, and Asp-234 acts as the hydrogen bond acceptor from the water molecule. The hydroxyl group of the benzyl alcohol side chain of D2 donates a hydrogen bond to the carboxylic acid oxygen of Lys-101. These interactions seem to be indicators of success as inhibitors, Al-Sanea *et.al* previously reported on similar interactions in successful AURKB inhibitors *in silico*¹¹².

Compound D2 forms a key hydrogen bond interaction with the Met-793 residue of the EGFR-TK. The Met-793 forms part of the hinge region of the protein, connecting the C- and N-lobe, hydrogen bonding with this residue is a known interaction displayed by

the two known EGFR-TK inhibitors, gefitinib and erlotinib^{117,123-124}. The amino backbone of Met-793 donates a hydrogen bond to the nitrogen in spot nine of the imidazo[1,2-b]pyridazine backbone. This type of interaction, where the Met-793 backbone act as a hydrogen bond donor has previously been reported on¹¹⁷. Compound D2 has a hydrogen bond donor interaction between the hydroxyl group of the benzyl alcohol of D2 and the carbonic acid sidechain of Asp-855, and the hydrogen donor interaction between the protonated lysyl sidechain of Lys-745 (donor) and that same hydroxyl group of D2 (acceptor). This interaction with Asp-855 and Lys-745 seems like a conserved interaction, previously also reported in other studies^{117,123}. The interaction with Asp-855 could be an indicator of inhibitor effectivity¹¹⁷. Asp-855 is part of the DFG motif (so named due to the amino acids it is made up of, namely Asp-855 or D-855, Phe-856 or F-856, and Gly-857 or G-857), when the DFG motif is in the “in” motif, the EGFR protein is in active conformation state, if the DFG motif is in the “out” motif, the EGFR protein is in an inactive state^{115,125}. The “in” motif decreases the number of residues available for binding in the active site (only Asp-855 of the DFG), whereas the “out” conformation increases the availability of residues to bind (both Asp-855 and Phe-856 of the DFG can extend into the binding site)¹²⁵. This means that D2 can possibly effectively bind EGFR, in both the active and inactive state (only Asp-855 interacts with the binding site, thus DFG is in the “out” motif)¹²⁵. The π - π stacking interaction between Phe-723 is a very interesting interaction because Phe-723, which forms a part of the P-loop, faces the inside of the active site decreasing the accessibility of the active site to its substrates and inhibitors¹¹⁷. Thus, what would usually decrease effectivity, might now be playing a role in stabilization of the inhibitor within the TK binding pocket. The π - π stacking interaction is T-shaped, the positively charged framework of the Phe-723 aromatic ring interacts with the π electron cloud of the aromatic D2 phenol sidechain.

From the CV screening of compounds D2-D11 in MDA-MB-231 cells, it was possible to calculate a preliminary IC_{50} for D2 of 0.1739 μ M, this does not differ much from the IC_{50} calculated using the results of the MTT assay (0.1968 μ M), the gold standard in IC_{50} determination. The IC_{50} calculated from the MTT results is viewed as more reliable and was thus used in all further experiments¹²⁶. The higher concentration from the MTT assay, can be ascribed to the fact that the MTT assay makes use of metabolic

activity as an indicator of viability, as opposed to the CV assay that bases viability solely on adherence to the wells¹²⁶⁻¹²⁷.

No significant difference between the OD of control and vehicle control of MDA-MB-231 was noted, thus all other experiments only made use of a vehicle control. Treatment of EA.hy926 cells caused no significant decreases in viability relative to the vehicle control. From this it is assumed that the IC₅₀ of D2 for the MDA-MB-231 cells is significantly lower than what the IC₅₀ for the EA.hy926 cells would be. The reason no IC₅₀ for the EA.hy926 cells were calculated is because the focus is on TNBC models, and whatever concentration is cytotoxic to them should be tested against a reference cell line in order for it to be possible to truly compare the effect D2 has on a TNBC cell line vs a somatic reference cell line.

The ELISA results ([Figure 3.16 and 3.17](#)) confirmed that there is definite expression of two of the targets, namely BRD4 and AURKA. Additionally, quantification of the concentrations of both BRD4 and AURKA after D2 treatment produced interesting results. The concentration of BRD4 in D2 treated MDA-MB-231 cells relative to the VC, was significantly decreased. This correlates with some other studies of BRD4 inhibitors where the inhibitor seems to cause a decrease in BRD4 expression¹²⁸⁻¹³⁰. The VC treatment of the EA.hy926 cells had a higher concentration of BRD4 compared to the MDA-MB-231 cells, and contrary to what was observed in the MDA-MB-231 cells, the D2-treated EA.hy926 cells had a significantly higher concentration of BRD4 compared to the VC, this has also been observed by other researchers¹³¹.

The AURKA ELISA results ([Figure 3.17](#)) indicated a significant decrease in AURKA concentration in the D2 treated MDA-MB-231 cells, relative to the VC. The concentration of AURKA in the VC-treated EA.hy926 cells were not different compared to the D2-treated cells.

Flow cytometric analysis of the cell cycle in D2-treated MDA-MB-231 cells ([Figure 3.20](#)), highlighted a decrease in the percentage of cells in S-phase, a result consistent with BRD4 inhibition as well as AURKB inhibition^{129,132-134}. BRD4 plays multiple roles in the modulation of the cell cycle, especially entry of cells into the S-phase of the cell cycle by promoting the transcription of genes important in the progression from G1 to S¹³². BRD4 stimulates the binding of RNA Pol II and P-TEFb to the promoters of G1 genes, leading to transcriptional elongation and G1 progression¹³³. AURKB has a

canonical role in the CPC, regulating mitotic progression by means of the spindle assembly checkpoint, preventing progression if there are aberrant microtubule-kinetochore attachments¹³⁴. However, the results here, and those obtained by others suggest an additional role of AURKB, that of a modulator of G1-S progression¹³⁴⁻¹³⁶. AURKB can form a complex with mTOR and regulate specific epigenetic markers that play roles in the transition of G1 to S¹³⁴⁻¹³⁵. Additionally, AURKB has been shown to directly phosphorylate p53, leading to decreased expression of p53 target genes, and thus G1-S progression^{134,136}. The D2-treated EA.hy926 cells ([Figure 3.21](#)) showed a significant increase of cells in the S-phase and a significant decrease of cells in the G2/M phase. These results do not correlate with BRD4 inhibition, something that might be ascribed to the increase in BRD4 in D2 treated cells ([Figure 3.16](#)), where increased expression of BRD4 might overcome its inhibition¹³¹. The increase in the number of cell in S-phase correlates with EGFR inhibition, however it is more common to see significant increases in G2/M cell numbers due to arrest in the mitotic phase. The effect of D2 on the cell cycle of the EA.hy926 cells does not correlate with that of the MDA-MB-231 results. This might be explained by the fact that D2 is a poly-inhibitor and could thus be having an effect on multiple downstream effectors of the targets proteins. For instance, research has shown that p21 and p27 expression is upregulated when BRD4 is inhibited and p27 is upregulated when AURKA, and AURKB are inhibited^{133,137-139}. The upregulation of p21 and p27 then leads to inhibition of the cell cycle and could lead to cell cycle arrest, but only in G0/G1 and G2/M which does not explain the S-phase arrest^{133,138-139}. A possible explanation for the S-phase arrest in the D2 treated cells is the accumulation of double stranded breaks due to ROS¹⁴⁰⁻¹⁴¹. Increased ROS due to treatment with D2 could have led to an increase in double stranded breaks, which leads to activation of the checkpoint kinase 1 and 2, causing the cells to arrest in S-phase in order for the cellular machinery to repair the double stranded breaks¹⁴⁰⁻¹⁴¹. A potential reason for the lack of S-phase arrest in the D2 treated MDA-MB-231 cells is the fact that they are cancerous in nature, as opposed to the EA.hy926 cells which serve as a model of transformed (hybridized) umbilical vein endothelial cells, and they might have mutated machinery for double stranded break repair¹⁴². Heterogenicity in cancer is a favourable trait for cellular survival and proliferation, a trait which is the result of genomic instability¹⁴². This genomic instability can be induced by the cancer cells via dysregulation (and not complete silencing) of the double stranded break repair pathways¹⁴². Normally increased time spent in the

cell-phase where the double stranded break was identified (S-phase arrest for instance) increases the chances of correct repair by for instance homologous recombination, which could be the case in the D2-treated EA.hy926 cells¹⁴². But in cancerous cells such as MDA-MB-231 cells, it is probable that the repair process favours a “quick fix” with lower fidelity, such as non-homologous end joining (NEHJ), above homologous recombination which has high fidelity to ensure genomic stability¹⁴².

D2-treatment of the MDA-MB-231 cells caused a significant increase in ROS relative to the VC ([Figure 3.24](#)). An increase in ROS in the D2-treated EA.hy926 cells ([Figure 3.25](#)) was also observed, however the increase was not as steep as seen in the D2-treated MDA-MB-231 cells. This is a result consistent with EGFR and AURKA inhibition according to literature¹⁴³⁻¹⁴⁵. ROS in physiological normal quantities play a role in EGFR activity, specifically H₂O₂ which almost act as a second messenger molecule. EGFR phosphorylation is greatly enhanced by the presence of H₂O₂ by means of oxidation of specific residues, which increases downstream signalling. The EGFR half-life seems to be additionally increased by exposure to H₂O₂, from 8 h when activated by EGF to about 18 h following H₂O₂ activation¹⁴⁶. However, here the levels of ROS in the D2-treated cells, significantly exceed the levels found in the VC. Normally when EGF and/or EGFR is (over) expressed in cancer cells they perform a protective function, by inhibiting ROS over production and resultant toxicity¹⁴⁴. EGF actively blocks the ROS producing activity of transforming growth factor-beta (TGF- β)¹⁴⁴. TGF- β normally functions as a tumour suppressor cytokine, by inducing cell cycle arrest and apoptosis¹⁴⁴. However, cancer cells seem to acquire resistance to its signals, causing it to rather modulate invasion and metastasis¹⁴⁴. It does this in part by producing anti-apoptotic ligands that bind to EGFR, activating it, and in so doing, it protects the cancer cells from ROS¹⁴⁴. But once EGFR is inhibited, TGF- β can stimulate the production of ROS. It does so in two ways, by downregulating the genes of mitochondrial antioxidant genes, such as glutathione, and by activating NADPH-Oxidase¹⁴⁴. This upregulation of ROS production re-sensitizes the cancer cells to TGF- β induced-apoptosis. The increase in ROS also correlates to the effects observed when AURKA is knocked down in oral squamous cell carcinoma (OSCC) which leads to increased ROS production, beyond the threshold that the cancer cells can weather¹⁴⁵. In a study of AURKB inhibition it was observed that ROS production is

greatly upregulated causing downregulation of key molecules of differentiation and upregulating activated caspase 3¹⁴⁷. Inhibition of BRD4, contrastingly, seems to lead to a decrease in ROS by means of upregulated expression of the cytoprotective heme oxygenase-1 (HMOX1)¹⁴⁸.

Analysis of the apoptotic profiles of the D2-treated MDA-MB-231 cells ([Figure 3.28](#)), showed a significant increase in apoptosis relative to the VC. This correlates with marked increase in ROS, which could be due to the inhibition of EGFR and subsequent induction of TFG- β mediated-apoptosis¹⁴⁴. BRD4 inhibition has been shown to induce apoptosis in more than one way¹⁴⁹⁻¹⁵⁰. It can lead to the suppression of NF- κ B activation causing the activation of tumour necrosis factor-related apoptosis-inducing ligand (TRAIL) which binds to the death receptors-4 and -5, to form a trimer that recruits the Fas-associated death domain (FADD), that in turn recruits, activates and binds to caspase-8, triggering the induction of apoptosis¹⁴⁹. In another study BRD4 inhibition led to decreased levels of the anti-apoptotic B-cell lymphoma-2 (Bcl-2) protein and increased levels of Bcl-2-associated X (BAX) protein and activated caspase 3¹⁵⁰. BAX, because it is no longer inhibited by the anti-apoptotic Bcl-2, can translocate to the mitochondria where it oligomerizes with Bcl-2 homologous antagonist killer (BAK) forming pores, liberating cytochrome C¹⁵⁰. Cytochrome C activates the apoptotic protease activating factor-1 (Apaf-1) which eventually leads to the activation of caspase-3, committing the cell to apoptosis¹⁵⁰. Studies have shown that AURKA inhibition leads to increased ROS production as well as an increased expression of poly (ADP)-ribose polymerase (PARP) and an increase in activated caspase 3 leading to increased apoptotic events¹⁴⁵. Increased PARP leads to an increase in caspase-independent apoptosis¹⁴⁵. Inhibiting AURKB has been shown to increase the number of activated caspase 3, leading to increased apoptosis¹⁴⁷. The D2-treated EA.hy926 cells ([Figure 3.29](#)) showed no significant difference in the number of apoptotic cells, consistent with the relatively small increase in ROS. It did however show a small but significant increase in the amount of dead or necrotic cells relative to the VC, which could be due to the upregulation in BRD4 concentration observed from the ELISA, which means ROS is increased but pro-apoptotic molecules such as caspase 3, and BAX is inhibited, leading to necrotic cell death instead of apoptotic cell death¹⁵¹.

5. Conclusion

In conclusion, *in silico* HTVS of the Biofocus library of 20 000 compounds, firstly in BRD4, consequently yielding 79 hits with docking scores ≤ -9 . These 79 compounds were then docked into AURKA, AURKB, PLK1, and EGFR. This produced only ten compounds with docking scores ≤ -9 for the different kinases. Screening of the compounds *in vitro* using CV proved successful for only one compound, D2. From the HTVS results, D2 was assumed to be a poly-inhibitor of BRD4 via BD1, EGFR via the TK binding pocket, and AURKA and AURKB via the ATP catalytic site.

The IC_{50} of D2 was determined after 48 h of treatment, as 0.1968 μ M using the MTT cytotoxicity assay in MDA-MB-231. Consequent treatment of EA.hy926 with the MDA-MB-231 determined IC_{50} of D2 had no significant effect on viability based on MTT results. Compound D2 is thus a potential poly-inhibitor with nanomolar potency.

Elucidation of presence of two of the targets, BRD4 and AURKA, proved positive, with both MDA-MB-231 and EA.hy926 cells having sufficiently high concentrations of the two target protein. Cell cycle analysis of D2-treated MDA-MB-231 cells had results consistent with BRD4 and AURKB inhibition, namely decreased cells in S-phase. And the analysis of EA.hy926 cells had results consistent with ROS induced double stranded breaks and S-phase arrest. The significant increases in ROS in MDA-MB-231 cells and to a lesser extent in the EA.hy926 cells, following treatment with the D2 IC_{50} was consistent with the assumption that D2 inhibits AURKA, AURKB, and EGFR. This significant upregulation of ROS production together with the assumed expression, activation and modulation of pro-apoptotic molecules in response to BRD4, AURKA, AURKB, and EGFR inhibition led to a very significant increase in apoptosis in the MDA-MB-231 cells and to necrosis in the EA.hy926 cells.

Taking all of the results into account, it is assumed that D2 is in fact a poly-inhibitor of BRD4, AURKA, AURKB, and EGFR. This is a novel therapeutic approach to target these four specific proteins. Targeting multiple proteins of interest could potentially mean higher therapeutic outcomes coupled with decreased resistance. For instance dual BRD4 and AURKA inhibition prevents resistance to AURKA treatment alone⁵³. However these results do not prove this assumption in its entirety, it is necessary to

further investigate the mechanistic effect of D2. Expression profiles need to be tested, especially with regards to c-Myc. Additionally enzyme kinetics in the presence of D2 need to be tested. Additionally, to validate the HTVS results, X-ray crystallography of D2 bound to its target proteins could prove useful.

6. References

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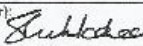
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Faculty of Health Sciences **Research Ethics Committee**

**Approval Certificate
Annual Renewal**

15 June 2022

Dear Mr DF Joubert,

Ethics Reference No.: 252/2019 – Line 4

Title: High throughput in silico screening and in vitro testing of polypharmacological bromodomain 4 and cancer associated kinase inhibitors

The **Annual Renewal** as supported by documents received between 2022-05-18 and 2022-06-15 for your research, was approved by the Faculty of Health Sciences Research Ethics Committee on 2022-06-15 as resolved by its quorate meeting.

Please note the following about your ethics approval:

- Renewal of ethics approval is valid for 1 year, subsequent annual renewal will become due on 2023-06-15.
- Please remember to use your protocol number (252/2019) on any documents or correspondence with the Research Ethics Committee regarding your research.
- Please note that the Research Ethics Committee may ask further questions, seek additional information, require further modification, monitor the conduct of your research, or suspend or withdraw ethics approval.

Ethics approval is subject to the following:

- The ethics approval is conditional on the research being conducted as stipulated by the details of all documents submitted to the Committee. In the event that a further need arises to change who the investigators are, the methods or any other aspect, such changes must be submitted as an Amendment for approval by the Committee.

We wish you the best with your research.

Yours sincerely

On behalf of the FHS REC, Dr R Sommers
MBChB, MMed (Int), MPharmMed, PhD
Deputy Chairperson of the Faculty of Health Sciences Research Ethics Committee, University of Pretoria

The Faculty of Health Sciences Research Ethics Committee complies with the SA National Act 61 of 2003 as it pertains to health research and the United States Code of Federal Regulations Title 46 and 48. This committee abides by the ethical norms and principles for research, established by the Declaration of Helsinki, the South African Medical Research Council Guidelines as well as the Guidelines for Ethical Research: Principles Structures and Processes, Second Edition 2016 (Department of Health)

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