Preliminary validation of a computer-based approach to find new lead molecules targeting the oncogenic Forkhead box M1 transcription factor

by

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Abstract:

The upregulation of the forkhead box M1 (FOXM1) transcription factor is directly correlated with cancer initiation, invasion, and drug resistance. Because the overexpression of FOXM1 is considered an important factor in carcinogenesis, this protein could be a relevant drug target in cancer treatment, and possibly, in cancer imaging. Literature reports describe the use of (a) siRNA, (b) proteasome inhibitors (which upregulate the expression of an endogenous negative regulator of FOXM1), and (c) other small-molecule drugs targeting FOXM1, as the three main strategies employed to decrease the transcriptional activity of FOXM1 *in vivo* and *in vitro*. Nevertheless, the first two approaches are either inconvenient because of the poor stability and the inefficient oligonucleotide intracellular delivery system (siRNA), or they potentially would exert significant side effects (proteasome inhibition). For this reason, developing new small-molecule drugs, that directly target the FOXM1 protein, represents an interesting and promising research opportunity in Medicinal Chemistry.

As a part of a long-term multidisciplinary research project aimed to validate the FOXM1 protein as a drug target, we recently conducted a molecular modeling (docking) approach in which we determined the theoretical binding energies of 3,323 drugs that are (or were) approved by FDA, using the reported FOXM1-DNA binding domain. The aim of this research work was to validate a computer-based (*in silico*) approach by correlating the calculated binding energies of hit molecules, with their ability to interfere with the transcriptional activity of FOXM1 *in vitro* using breast cancer (MDA-MB-231 and MCF-7) and non-cancer (MCF-10A) cells, screening for potency and selectivity, respectively. In this regard, we carried out (A) MTT colorimetric and (B) western blot assays to evaluate

the effect exerted by selected drugs (troglitazone, β-estradiol-3-benzoate gliquidone, dehydrocholic acid and metocurine, using thiostrepton as a positive control) on cell viability and protein expression. To determine the ability of the test drugs to block the binding interaction between FOXM1 and DNA, we used the electrophoretic mobility shift assay (EMSA).

The MTT assay results illustrated that troglitazone, β-estradiol-3-benzoate and the control drug thiostrepton are cytostatic to MDA-MB-231 cancer cells but they are not selective as they also inhibited the proliferation of normal MCF-10A cells. However, these drugs did not inhibit MCF-7 cells proliferation. Western blotting assay results showed that troglitazone significantly inhibited FOXM1 protein expression at a concentration lower than its IC_{50} value, suggesting that troglitazone cytostatic effect is FOXM1-dependent. Rather than decreasing FOXM1 expression, β-estradiol-3-benzoate increased protein expression as it might act by FOXM1-independent pathways. In addition, it is a hormone-like drug and is likely to be an unsuitable scaffold to design new FOXM1 inhibitors. Gliquidone gradually inhibited FOXM1 protein expression, but this inhibitory effect did not correlate with its effects on cell viability (MDA-MB-231 cancer cells). Finally, dehydrocholic acid and metocurine did not show any effect on cell viability or protein expression, which rules them out as scaffolds to design new FOXM1 inhibitors. Regarding the EMSA assay, we could not modify this assay to work with our recombinant FOXM1 DBD-DNA complex; accordingly, we could not validate the *in-silico* model to determine the mechanism of drugs on cell proliferation and protein expression.

In summary, we identified the drug troglitazone as the most promising lead molecule to be used in future drug design programs. On this regard, our group is currently

developing troglitazone-based FOXM1 inhibitors capable of exerting binding interactions via a π-sulfur interaction involving troglitazone's thiazolidinedione ring and a His287 residue, within the FOXM1 – DNA binding pocket. This binding interaction seems to be the main driving force guiding the inhibitory effects of several types of FOXM1 inhibitors, and it constitutes a novel mechanism of action for future FOXM1 inhibitors.

Preface:

This thesis is an original work by Asya S Egweda Elshaikh. The research project, of which this thesis is a part, received research support from the Faculty of pharmacy and pharmaceutical sciences, through the Noujaim Fund for strategic initiatives, Project Name “Preliminary validation of a computer-based approach to find new lead molecules targeting the oncogenic Forkhead box M1 transcription factor”.

Dedication:

I dedicate my thesis to number of people without whom this thesis might not have been written, and to whom I am greatly indebted.

To my father “Salem” and mother “Aziza”, who have been a source of encouragement and inspiration to me throughout my life. To my beloved husband “Thaha”, very special thanks for your emotional support as I added the roles of being wife and then a mother.

To my brothers: Egweda, Majid and Mohammed; and sisters: Nawal, Salha, Sara, Halima, Ferdous and Hend. I could not have done this work without your support along the way and I will always appreciate all you have done, especially my brother “Majid” for helping me to be in this level of education.

I also want to remember my brother” Adel “whose life was cut short by cancer. May you find peace and happiness in the Paradise.

Last but not least, I dedicate this work and give special thanks to my little wonderful daughter “Somow” for being the hope of my life.

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List of Abbreviations

AKT	Protein kinase B
ANOVA	Analysis of variance
ATM	Ataxia-telangiectasia mutated
APAF1	Apoptotic protease activating factor 1
BAX	Apoptosis regulator BAX
BIM	Bcl-2-like protein 11
CCNB1	Cyclin B1
Cdc25	Cell division cycle 25
Cdk	Cyclin-dependent kinase
CDKI	Cyclin-dependent kinase inhibitor
CENP	Centromere protein
ChT-L	chymotrypsin-like
Cks	Cyclin-dependent kinase subunit
DBD	DNA-binding domain
DMEM	Dulbecco's Modified Eagle's Medium
DMSO-d6	Deuterated Dimethyl Sulfoxide
dsDNA	Double stranded DNA
DUSP1	Dual specificity protein phosphatase 1
EC_{50}	Half maximal effective concentration
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal Growth Factor
EGFR	Epidermal Growth Factor Receptor
EMSA	Electrophoretic mobility shift assay
EMT	Epithelial-to-mesenchymal Transition
ErbB2	Receptor Tyrosine-protein Kinase
ERK	Extracellular-signal-regulated Kinase
ERα	Estrogen Receptor alpha

ERβ	Estrogen Receptor beta
EXO1	Exonuclease 1
FADD	Fas-Associated protein with Death Domain
FBS	Fetal Bovine Serum
FDA	Food and Drug Administration
FOXM1	Forkhead Box M1
GM	Growth medium
GSH	Glutathione
HCC	Hepatocellular Carcinoma
HER	Human Epidermal Growth Factor Receptor
HER2	Human Epidermal growth factor receptor type 2
HIF-1α	Hypoxia-inducible Factor 1-alpha
H-NMR	Hydrogen-Nuclear Magnetic Resonance
IC_{50}	Half Maximal Inhibitory Concentration
IL-6	Interleukin 6
INCENP	Inner centromere protein
IκB	Nuclear factor kappa B
JNK	c-Jun N-terminal kinases
LC_{50}	Median lethal dose
LOX	Lysyl Oxidase
LOXL2	Lysyl Oxidase-like 2
MAPK	Mitogen-activated Protein Kinase
Mcl-1	Induced myeloid leukemia cell differentiation protein
MMP	Matrix Metalloprotease
Nek2	NINA-Related kinase 2
NF-κB	Nuclear factor kappa B
NIDDM	Noninsulin-dependent diabetes mellitus
NRD	N-terminal Auto-repressor domain
$p21^{CIP}$	Cyclin-dependent kinase inhibitor 1

$p27^{KIP}$	Cyclin-dependent kinase inhibitor 1B
p53	Tumor suppressor protein
PARP	Poly (ADP-ribose) polymerase
PBS	Phosphate Buffered Saline
PI3K	Phosphoinositide 3-kinase
Plk	Polo-like Kinase
POLE2	DNA polymerase epsilon subunit 2
PPAR	Peroxisome proliferator-activated receptors
pRB	Retinoblastoma Tumor suppressor protein
PRDX3	Peroxiredoxin 3
PUMA	p53 upregulated modulator of apoptosis
RFC4	Replication factor C subunit 4
RIPA	Radioimmunoprecipitation assay buffer
ROS	Reactive Oxygen Species
SCF	SKP, F-box containing complex
SDS	Sodium dodecyl sulfate
SFN	Stratifin
siRNA	Small interference RNA
SKP2	S-phase kinase associated protein 2
Snail1	Zinc finger protein SNAI1
SOD	Superoxide Dismutase
Sp1	specificity protein 1
SQRT	Square Roots
STAT3	Signal transducer and activator of transcription 3
STDEV	standard deviation
TBE	Tris, Boric acid, EDTA
TCAG	the Center of Applied Genomics
TEMED	Tetramethylethylenediamine
TGFβ	Transforming Growth Factor beta

TKI	Tyrosine Kinase Inhibitors
TNBC	Triple negative breast cancer
TNF-α	tumor necrosis factor alpha
TZD	Thiazolidinedione
VEGF	Vascular endothelial growth factor

Chapter 1: Introduction

1.0. Introduction:

1.1. Cancer:

Cancer is one of the leading causes of death affecting millions of people around the world every year. Cancer is defined as the uncontrolled growth of abnormal cells, which differentiate from normal cells essentially by being less specialized and going through an uncontrolled continued cell division (1). Cancer cells can affect distant organs via the circulatory system if the disease is not diagnosed in early stages (2).

Canadian Cancer Statistics estimated that, in 2015 about 197,000 Canadians were expected to develop cancer, and nearly 78,000 cancer patients were likely to die due to this disease (3). According to this publication, the estimated lung cancer-related deaths is accounted for the highest among cancer deaths, 26.6% in males and 27% in females. The second type of cancer-related deaths is colorectal cancer (12.4%), followed by breast cancer (13.6%), then colorectal cancer (11.5% in females) (3), as represented in Table 1 below.

Table 1: represents the estimated incidence, mortality rate, and percentage of cancer deaths in Canadian males and females in 2015 (3).

Cancer type	Estimated new cases			Estimated mortality rates			Percentage of estimated cancer deaths	
	Total	Males	Females	Total	Males	Females	Males	Females
Lung	26,600	13,600	13,000	20,900	10,900	10,000	26.6%	27%
Breast	25,200	200	25,000	5,100	100	5,000	0.1%	13.6%
Colorectal	25,100	14,000	11,100	9,300	5,100	4,200	12.4%	11.5%
Prostate	24,000	24,000	—	4,100	4,100	—	10.1%	—

Many factors can increase the chance of developing cancer; some of them are external including tobacco use, cancer-causing pathogens, obesity and insufficient physical activities. Some of these factors can be avoided such as smoking; however, the others are internal factors such as individual genetic variation, immune conditions, and hormones which cannot be prevented (1,4).

Although most cancers respond initially to chemotherapy, over time some cancer patients develop resistance to pharmacological treatment and become unresponsive to anti-cancer agents. A variety of mechanisms are involved in drug resistance including: drug efflux or alteration of drug transport across plasma membranes, inhibition of programed cell death, DNA-damage repair, drug inactivation, and alteration (mutation) in drug targets, and the effect of growth factors (5,6).

Different types of treatment can be used either as standalone therapies or in combination depending on the type of cancer and the stage of the corresponding disease. Surgery is the treatment of choice in early stages along with radiotherapy which is also used in treating well localized "solid" cancers. Chemotherapeutic agents that exert significant cytotoxicity are used in developed or metastatic stages (5).

1.2. FOX family:

The Forkhead box (FOX) is a family of more than 100 transcription proteins. Mutation, upregulation or downregulation of FOX proteins have been associated with variety of diseases and deaths. These proteins subdivided into subfamilies going from FOXA to FOXS (7) and all of them have three main parts:

1. *N*-terminal auto-repressor domain (NRD).

2. Winged helix / DNA-binding domain (DBD).

3. C-terminal domain (8).

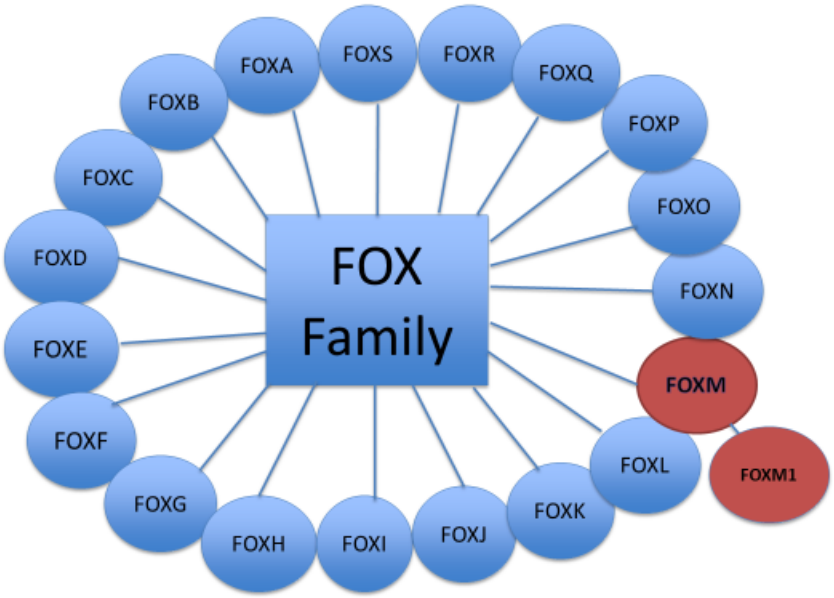


Figure 1: The phylogenetic tree of forkhead box transcription factor family. According to the phylogenetic classification, nomenclature of the subclasses is based on similarities in DNA binding domain of the transcriptional proteins.

DBD in all FOX members consists of about 100 amino acids and binds to the DNA by the winged helix formed from 3 alpha helices and two wing-like loops. Each member identifies different DNA sequence close to a core sequence (9). Variation in amino acid content between FOX proteins reflects a diversity in the biological functions including proliferation, stress resistance, apoptosis, reproduction, development, and metabolism (9).

Some of these proteins including FOXD1 which is necessary for kidney development. knocking this protein down causes death in 24 hours in mice according to Levinson, *et al*. (10). The single exon gene/ FOXL2 is responsible for female sex

determination and is necessary for development of eyelid, ovary and pituitary (11,12). FOXP2 plays a crucial role in language and speech development such as the ability of making sentences and grammatical skills (13). FOXN1 and FOXQ1 are essential for the development of mammalian hair follicle while FOXP3 is required for normal immune function and its mutation leads to immune dysregulation (14). FOXO subfamily is subdivided into 4 subclasses named FOXO1, FOXO3, FOXO4, and FOXO6 which were found to be regulators of biological pathways involving metabolism, apoptosis, cell cycle arrest, DNA damage repair and stress resistance (15). FOXO3a acts as a tumor suppressor via different mechanisms such as upregulating the expression of cyclin-dependent kinase inhibitor (CDKI) proteins $p27^{kip}$ which induces the cell cycle arrest, and elevating the apoptotic activator BIM expression leading to apoptosis in breast cancer. Together FOXO4, FOXO3a stop the cell cycle via the downregulation of cyclin D by $p27^{kip}$ independent mechanism (16).

FOXM1 is normally expressed in proliferating cells such as embryonic tissues, but it is not significantly expressed in differentiated and quiescent cells. FOXM1 controls important biological processes including cell division, differentiation, migration, angiogenesis, and DNA-repair. Overexpression of FOXM1 in terminally differentiated cells has been correlated with cancer initiation, angiogenesis, invasion, metastasis, and drug resistance (17).

Recently, many studies have proved the significant role of FOXM1 in tumorigenesis and drug resistance. Wang *et al*. suggested that the inhibition of the signaling pathway of FOXM1 could have a crucial impact on cancer therapy. In addition, using

FOXM1 inhibitors, in combination treatment, may decrease cancer resistance to chemotherapeutic agents (18).

1.3. FOXM1 structure:

FOXM1 compasses 10 exons and is mapped to 12p13-3 chromosome (19,20). As mentioned before, FOXM1 consists of three main parts, NRD, DBD, and C-terminal domain. In details, NRD, also known as the inhibitory domain because of its regulatory effect on the transcriptional activities of FOXM1; this domain contains 232 amino acids. DBD which is located in between NRD at amino acid number 233 and C-terminal domain at amino acid number 332. C-terminal/transactivation domain includes amino acid residues between 333 and 748; this part has a phosphorylation site (cyclin-Cdk) which is essential for the translocation of the protein from the cytoplasm to nucleus to start its transcriptional activity (21).

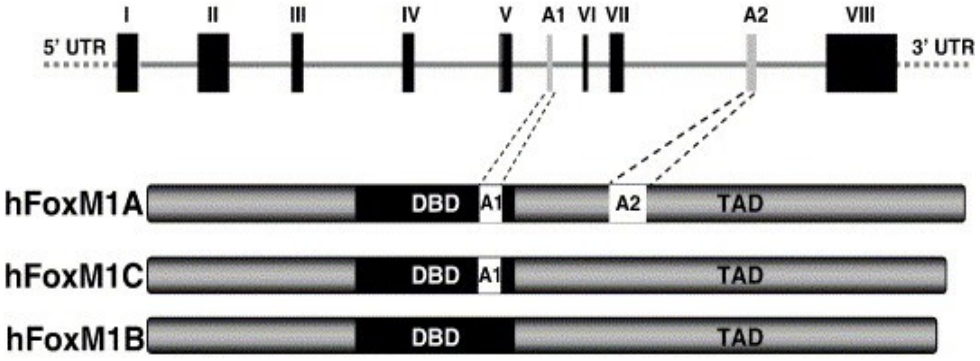


Figure 2: The three spliced isoforms of human FOXM1 transcription protein are spliced with two exons Va and VIIa named A1 and A2 respectively. Exon A1 consists of 15 amino acid sequences and inserted within the DNA binding domain of FOXM1a and FOXM1c. Exon A2 consists of 38 amino acid sequences and inserted within the C-terminal transactivation domain of FOXM1a only. FOXM1b is not spliced with any of the exons (22), with permission, license number 4143280401551.

Natural splicing of two exons (Va/ A1 and VIIa/ A2) results in three different isoforms, namely FOXM1a, FOXM1b and FOXM1c. FOXM1a has the two splicing exons (A1, A2) and it is transcriptionally inactive as a result of the disruption of the C-terminal domain via VIIa. FOXM1b has neither Va nor VIIa, while FOXM1c contains the alternative splicing exon Va (A1) only and both of them are capable of performing the transcriptional activity of the protein (23,24).

1.4. Activation of FOXM1 transcriptional activity:

As mentioned above, FOXM1 is expressed at high levels in embryonic, proliferating mesenchymal and epithelial tissues and at different levels in testis, thymus, lung and intestine (22,25,26). Large-scale gene expression analysis has shown the upregulation of FOXM1 in most human carcinomas including breast, ovary, prostate, kidney, bladder, liver, pancreas, stomach, and colon (22,27).

FOXM1 at mRNA and protein levels are almost absent in differentiated cells, once the quiescent cells in resting/ G0 phase are stimulated to re-enter the cell cycle by mitogens, the protein expression increases by the end of G1 phase and the onset of S phase reaching the maximum at G2 and M phase (22,26,28). Inactivation of pRB by phosphorylation via Cyclin D/Cdk4, 6 is necessary for the cells to proceed to S phase and to mitigate its inhibitory effect on FOXM1, as well (22). Dissociation of pRB from E2F up on its phosphorylation is also necessary to alleviate the inhibitory effect on cyclin E (28,29). Cyclin E/Cdk2 complex then carries out the second round of phosphorylation of pRB thus promoting the cell cycle (29).

By the end of S phase, further phosphorylation of the C-terminal of FOXM1 via Cyclin A/Cdk2 is mediated by the regulatory mitogen-activated protein kinases (MAPK) pathway leading to the translocation of the protein from the cytoplasm to nucleus (22,30). This step is required for the disruption of intramolecular auto inhibitory interaction between the NRD and C-terminal domain resulting the transcription of number of G2/M-specific genes which are necessary for the regulation of mitosis and chromosomal segregation during G2 phase (31–33).

The final phosphorylation of C-terminal of the protein is carried out by the kinase Polo like kinase 1(Plk1), kinases Cyclin A/Cdk1, 2 and Cyclin B/Cdk1 allowing the initiation of FOXM1 transcriptional activity required for mitotic progression (33,34).

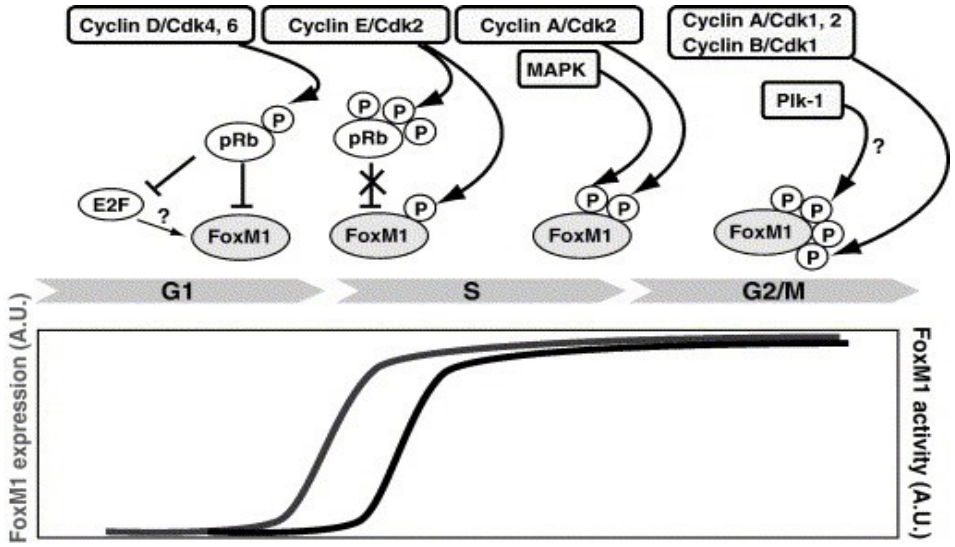


Figure 3: Regulation of FOXM1 expression during G1, S, G2 and M phases of the cell cycle. C-myc and /or E2F initiate FOXM1 expression at mRNA and protein levels by the end of G1-phase. In response to a mitogen, Cyclin D/Cdk4, 6 and Cyclin E/Cdk2 phosphorylate/ inactivate pRb to promote the cell cycle progression and alleviate the inhibitory effect of pRb on FOXM1. Multiple phosphorylation of FOXM1 via cyclin/cdk complexes starting from the late-G1 until the entry to M-phase is necessary to induce its transcriptional activity during G2/M. Mitogen-activated protein kinase (MAPK) cascade and Cyclin A/Cdk2 mediate FOXM1 phosphorylation during S-phase. This event required for the next important phosphorylation steps during G2/M. Further phosphorylation is mediated by Cyclin A/Cdk1, 2, Cyclin B/Cdk1 and Plk-1 during G2/M resulting in complete activation of FOXM1 (22), with permission, license number 4143280401551.

1.5. FOXM1 and cell cycle progression:

FOXM1 is a major regulator of cell cycle progression especially in G1/S and G2/M transitions (35,36). Wouter *et al*. proved the knocking down of Trident (FOXM1) in mice models causes postnatal death due to failure of cardiac muscles (37).

1.5.1. FOXM1 and G1/S:

FOXM1 regulates the expression of the dual specificity Cdc25A phosphatase which is necessary for the dephosphorylation and activation of Cdk2 kinase protein (38).

FOXM1 plays a crucial role in the expression of SCF ubiquitin ligase members (SKP2 and Cks1) which are responsible for the ubiquitination of phosphorylated cyclin-dependent kinase inhibitor (CDKI) proteins, $p27^{KIP1}$ and $p21^{Cip1}$, followed by proteasomal degradation of these cell cycle inhibitory proteins (29,39–41). Depletion of the CDKI from the nucleus is important for the activation and cooperation of Cdk2-cyclin E complex with Cdk4/6-cyclin D to inhibit pRB and activate the transcriptional activity of E2F which is necessary for the progression of S phase (28,42).

1.5.2. FOXM1 and G2/M:

FOXM1 regulates the expression of Cdc25B phosphatase which is necessary for the dephosphorylation and activation of Cdk1 kinase protein (43). Activated Cdk1 combines with cyclin B to initiate G2/M transition (43,44).

FOXM1 coordinates the transcription of PLK1 which is necessary for the phosphorylation and activation of Cdc25c phosphatase during G2-phase. Activation of Cdc25c phosphatase is critical for the activation of Cdk-cyclin complexes which are required for G2/M transition (43,45).

FOXM1 modulates the expression of kinetochore binding protein/ CENP-F gene that is essential for promoting the chromosomal segregation. Additionally, FOXM1 activates Aurora B kinase, and survivin and they form a complex with INCENP, this complex is required for Aurora B kinase activity (46). By direct binding to the proteins' promoters, FOM1 was found to coordinate the transcription of CENP-A and CENP-B which are essential for assembly of the kinetochore (47–49).

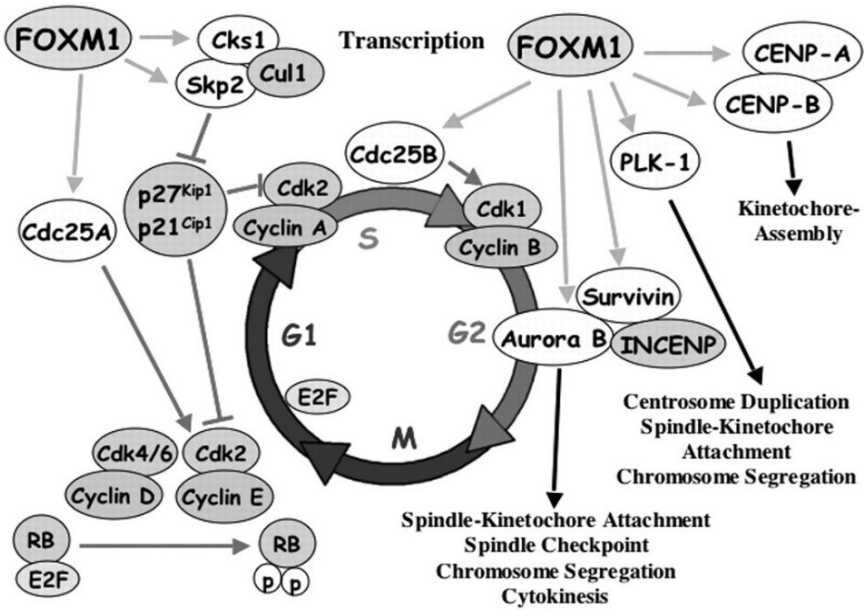


Figure 4: FOXM1 target genes during the cell cycle progression. (A) G1/S transition: FOXM1 induces Cdc25A phosphatase expression that is necessary for dephosphorylation/ activation of Cdk2 kinase. FOXM1 regulates the expression of SCF ubiquitin ligase members (Skp2 and Cks1). Skp2 and Cks1 are necessary to recognise the phosphorylated cyclin-dependent kinase inhibitor (CDKI) proteins, $p27^{Kip1}$ and $p21^{Cip1}$ leading to ubiquitin-mediated proteasome degradation of these cell cycle inhibitory proteins. Nuclear depletion of CDKI proteins $p27^{Kip1}$ and $p21^{Cip1}$ activates Cdk2-cyclin E complex leading to their cooperation with Cdk4/6-cyclin D to phosphorylate the pRB. Phosphorylated pRB dissociates from E2F which promotes S-phase genes expression. (B) G2/M transition: FOXM1 activates the transcriptional activity of Cdc25B phosphatase which dephosphorylates/ activates Cdk1 kinase. FOXM1 mediates PLK1 transcription and activates aurora B kinase and survivin to cooperate with INCENP. Additionally, FOXM1 induces the expression of CENPA and CENPB which are required for kinetochore assembly (29), with permission.

1.6. Modulation of FOXM1:

The expression of FOXM1 is regulated by number of genes and receptors, these proteins either upregulate or downregulate the transcriptional activity of FOXM1.

1.6.1. Positive regulators:

1.6.1.1. TNFα/ROS/HIF-1α:

Under the normal conditions, reactive oxygen species (ROS) including superoxide radical (O^{2-}), hydroxyl radical ($^{\cdot}OH$) and hydrogen peroxide (H_2O_2) are produced as cellular byproducts of mitochondrial aerobic respiration (50). Excessive ROS production is correlated with age-related diseases, nuclear or mitochondrial DNA damage and cancers (50,51). Superoxide dismutase (SOD), catalase, glutathione (GSH), peroxidases and peroxiredoxin ($PRDX_3$) detoxifying enzymes are necessary to keep the intracellular balance between ROS production and detoxification to protect the cells from the damaging effect of the reactive species (52,53).

Tumor necrosis factor alpha (TNF-α) is a cytokine that is produced by tumor inflammatory cells and was found to control variety of tumorigenesis processes including cell proliferation, survival, migration and angiogenesis. TNFα induces the overexpression of FOXM1 in hepatoma cells in presence of ROS (54). The transcription factor/ hypoxia-inducible factor (HIF-1) that plays a crucial role in cell proliferation is directly activated by ROS under hypoxic conditions. Consequently, activated HIF-1α upregulates the expression of FOXM1 at mRNA and protein levels resulting in resistance to apoptosis (54,55). Xia *et al*. illustrated that TNF-α activates TNF-α/ROS/HIF-1α pathway which induce the expression of FoxM1 (54).

1.6.1.2. Raf/MEK/MAPK:

As mentioned above, MAPK, which has an important role in cancer angiogenesis, invasion, and metastasis (30), is necessary for FOXM1 activation. However, complete activation of Raf/MEK/MAPK pathway is required for the translocation of FOXM1 from the cytoplasm to the nucleus because blocking of only MAPK pathway was found to downregulate the transcriptional activity of FOXM1. Recently, targeting this pathway using small-molecule inhibitors has been introduced into clinical trials (56).

1.6.1.3. Cyclin D/CDK4/CDK6/RB:

In response to a mitogen or proliferated factor, activated CDK4/6 complexes to cyclin D to initiate the phosphorylation and dissociation pRB from E2F which is necessary for the DNA synthesis (57,58). The inhibitory effect of pRB is required to prevent the cell cycle transition in quiescent, senescent and differentiated cells. It has been found that CDK4/6 signaling pathway suppresses the cellular senescence through the upregulation of FOXM1(58,59).

1.6.1.4. Estrogen receptor alpha:

Estrogen receptor alpha (ERα) is the master regulator of the growth and differentiation of normal and carcinogenic mammary epithelial cells and its expression is relatively low in non-proliferating cells. ERα was found to be upregulated in more than 60% of all breast cancer cell lines (60–62). FOXM1 expression at mRNA and protein levels was found to be upregulated in ERα+ breast cancer patients in response to the mitogenic estrogen hormone (63,64). However, Madureira *et al*. found that inhibiting FOXM1 expression in MCF-7, ER+ breast cancer cells, using small interference RNA (siRNA) or

U0126 (MEK inhibitor) caused the termination of the receptor expression (63) suggesting that there is positive feedback between the two proteins.

1.6.1.5. EGFR:

Epidermal growth factor receptor (EGFR) is a family of proteins that are involved in vital biological processes including cells proliferation and survival. Human epidermal growth factor receptor-2 (HER-2) which is a member of EGFR family has been found to be overexpressed in different tumors (65,66). Elevating or inhibiting of HER2 expression was found to be associated with equivalent changes in FOXM1 expression at mRNA level in 11 different breast cancer cell lines and this correlation was supported by in *vivo* study using mice models according to Francis *et al*. (67).

1.6.1.6. ERK:

The MAPK family members which are named extracellular signal-regulated protein kinases (ERK) regulate different biological functions including cell proliferation and apoptosis by being a part of Ras/Raf/MEK/ERK signaling pathway but the exact mechanism of ERK still not clear (68). Different studies established that the activation of Ras/MAPK pathway is crucial for the induction of cell cycle progression in hepatocellular carcinoma (HTCC) cells where ERK was found to be upregulated. FOXM1 is one of the downstream targets of ERK and found to be upregulated in different human cancers. However, FOXM1 was found to protect the ERK activity via the activation of SKP2 and Cks1 which degrade DUSP1/ ERK inhibitor indicating the positive feedback between the two proteins (69).

1.6.1.7. NF-κB:

Nuclear factor kappa-B (NF-kB) is a transcription factor and the key promoter of many proteins such as cytokines (TNFα, IL6), cell adhesion molecules (E-selectin), inducible pro-inflammatory enzymes which are involved in cancer progression and invasion. Furthermore, NF-kB is a regulator of epithelial-mesenchymal transformation (EMT) in breast cancers (70–72). Inactivation of NF-κB in different triple negative breast cancer cell lines using fungal secondary metabolite panepoxydone (PP) was found to downregulate FOXM1. Additionally, silencing NF-κB decreased FOXM1 expression in the same manner indicating that FOXM1 is a downstream effector of NF-κB (72).

1.6.2. Negative regulators:

1.6.2.1. p53:

Phosphoprotein p53 transcription factor is the main tumor suppressor that is activated by different oncogenic signals to prevent the tumor development (73). The tumor suppressor p53 induces cellular growth arrest by upregulating the transcription of p21 (74,75) and SFN (inhibitor of G2/M transition). p53 promotes apoptosis via increasing the expression of the apoptotic factors such as PUMA (76), BAX (77) and APAF1 (78). Additionally, p53 downregulates the transcription of PIK1 (79). p53 was found to be either mutated or inactivated in most of human cancers (74). Pandit *et al*. demonstrated that FOXM1 is one of downregulated target genes of the tumor suppressor p53 as its activity was increased upon p53 depletion in different cancer cell lines (78).

1.6.2.2. FOXO3a:

FOXO3a is a subclass of FOX proteins superfamily and involved in variety of normal biological function including cell proliferation and differentiation (80). FOXO3a phosphorylation at specific amino acid residues prevents it from binding to the DNA and translocate it to the cytoplasm (81). He *et al*. proved that FOXM1 is downstream target of FOXO3a because he found that FOXO3a dephosphorylation by using casticin in a concentration-dependent manner inhibited FOXM1 expression and induced cell cycle arrest (82). However, the exact mechanism by which FOXO3a controls FOXM1 is not clear.

1.6.2.3. $p19^{ARF}$:

$p19^{ARF}$ is a tumor suppressor mediates cell cycle arrest via stabilizing the tumor suppressor p53 (83). As proved by Kalinichenko *et al*., treating the osteosarcoma U2OS cells with $p19^{ARF}$ inhibits FOXM1 activity through binding to the alternative reading frame of FOXM1 which is contained between 26 and 44 residues and responsible for the inhibitory interaction with the oncogenic protein (84).

1.7. FOXM1 and tumorigenesis:

According to large-scale gene expression analysis, FOXM1 is on of the highly upregulated genes in wide variety of human tumors originated from different tissues including breast, prostate, lung, ovary, colon, stomach, pancreas, liver, bladder and kidney (27). As mentioned above, FOXM1 is important for embryonic development and injured tissue repair. FOXM1 is the key regulator of vital biological functions including cell proliferation, cell cycle progression, cell renewal, differentiation, DNA damage repair, cell

survival, tissue homeostasis, cell migration, angiogenesis, and apoptosis (17,85). Upregulated expression of FOXM1 protein in human adult tissues has been regarded as a hallmark of carcinogenesis (22).

There are at least three different mechanisms by which FOXM1 participates in cell transformation:

1. FOXM1 inhibits cellular senescence by antagonizing p53 and $p16^{INKP4}$-independent senescence resulting in downregulation of $p27^{Kip1}$ expression (86).
2. High FOXM1 level induces the expression of anti-oxidant enzymes such as SOD2 and PRDX3 to scavenge the reactive oxygen species (ROS); and consequently, makes cancer cells resistant to the effect of oxidative stress (51). Under the normal conditions, the accumulation of ROS damages the DNA leading to apoptosis (18,51).
3. Inducing stem cell-like properties such as proliferation, immortality, self-renewal and differentiation (87).

1.8. FOXM1 and cancer progression:

In addition to tumor initiation, FOXM1 has been involved in tumor cells proliferation, migration, invasion and angiogenesis. Consequently, FOXM1 has been regarded as an indicator of the early stages of tumorigenesis. Several studies provided evidences for such implications including:

1. Ahmed *et al.* studied the biological consequences of FOXM1 regulation in MDA-MB-231 and SUM149 cancer cells using siRNA. They found that down-regulation of the FOXM1 protein reduced the proliferation of breast cancer cells by

decreasing the expression of E2F1 and CDK2 and increasing the level of p21 and p27 expression (88). Furthermore, in a different report, other research group reported the induction of colorectal cancer tumor tissue in a mice model using Rosa26-FOXM1b transgenic mice that express human Foxm1b complementary DNA, or colonic FOXM1 fl/fl allele deleted mice. They found an increase in the size and number of colorectal cancer cells in Foxm1b transgenic mice with a significant reduction in cancer growth in FOXM1 fl/fl allele deleted mice. This effect was associated with a decrease in the expression of survivin and cyclin A2, B1. In addition, they used siRNA–depleted human DLD1 and mouse CT26 colon cancer cell lines and they found a reduction in DNA replication and anchorage-independent cell growth (89).

2. Inhibition of FOXM1 reduced the migration, invasion and angiogenesis ability of the pancreatic cancer cells by suppressing the expression of vascular endothelial growth factor (VEGF) and matrix metalloproteinases (MMP-2 and MMP-9) which are found to be upregulated directly and indirectly by FOXM1 in different human cancers (90).
3. FOXM1 induces epithelial-mesenchymal transition (EMT) by activating AKT-Snail1 pathway. Additionally, FOXM1 promotes the motility and invasiveness of cancer cells via upregulating the expression of lysyl oxidase collagen cross-linking proteins LOX and LOX2 leading to a pre-metastatic niche formation in distant organs (91).

4. FOXM1 downregulation inhibited cell-cell contact which is necessary for the proliferation, differentiation and motility of cancer cells by downregulating cyclin A and PIK1 (92).

1.9. FOXM1 and the resistance to chemotherapy:

Even though some of cancer patients initially respond to the anti-cancer drugs, by the time the effectiveness of the chemotherapy is significantly decreased due to the development of acquired drug resistance (5). Several studies have reported that FOXM1 plays an essential role in acquired drug resistance development (80). The overall mechanisms by which FOXM1 mediates the drug resistance are not fully established; however, the involvement of FOXO has been reported. Most of the chemotherapeutic drugs including cisplatin (93), doxorubicin (94), gefitinib (95) and paclitaxel (96), exert their anti-cancer effect via upregulating FOXO3a.

The upregulated PI3K-AKT signaling pathway in cancer cells downregulates FOXO proteins (96). The inhibitory effect of PI3K-AKT pathway on FOXO upregulates FOXM1 which is a downstream target of FOXO3a in particular (95). This evidence revealed that FOXM1 is a downstream effector of the PI3K-AKT-FOXO pathway which is essential to understand cancer drug resistance.

Plenty of evidences have proved the implication of FOXM1 in the development of chemoresistance to different classes of the chemotherapy including taxanes, platinum-based drugs, anthracyclines and endocrine therapy. The chemoresistance of these drugs will be discussed in detail later.

1.10. FOXM1 expression in breast cancers:

As mentioned above, Pilarsky *et al*. reported that FOXM1 is highly expressed in 10 out of 11 human cancers including breast, prostate, lung, ovary, colon, stomach, pancreas, liver, bladder and kidney (27).

Breast cancer is one of the most prevalent cancers around the world, with the highest mortality rates among malignancies in women (97–99). FOXM1 was reported to be differently expressed in different types of breast cancers. One study was carried out by Diane *et al*. showed that, stage II ductal carcinoma cells exhibited 4 to 9-fold increase in FOXM1 expression while stage III showed an increase of 76 to 116-fold in comparison to non-cancer breast epithelial cell lines (MCF-10A and MCF-12A) (49).

The second form of the breast cancers is basal-like breast cancer also known as triple negative breast cancer because of lacking PR, ER, and HER2 (100). Triple negative cancer accounts for about 15% of all breast cancer and FOXM1 was found to be highly expressed in 75% of the triple negative cancer patients (101). FOXM1 is overexpressed in about 20% of ER+ breast cancer and advanced stages of most breast cancers.

Variety of evidences have proved the implication of FOXM1 in breast cancers promotion, progression, invasiveness, and resistance to chemotherapy including:

1. Breast cancer promotion via VEGF-FOXO3a-FOXM1 pathway: FOXO3a downregulates VEGF expression by direct protein interaction to *VEGF* promoter. However, both of FOXM1 and FOXO3a competitively bind to the forkhead response element (FHRE) of the *VEGF* promoter. Karadedou *et al.* showed that activated FOXM1 displaces FOXO3a from the FHRE to induce the

expression of VEGF in four human breast cancer cell lines (SKBR3, MCF-7, BT474 and MDA-MB-231). VEGF leads to the activation of Raf-Ras-MAPK and PI3K-AKTpathways which are crucial for longevity, angiogenesis, survival and migration of the cancer cells (102).

2. Upregulation of ERα: As mentioned above, there is a positive feedback between FOXM1 and ERα proteins. FOXM1 downregulates ERβ and induces ERα expression (103). According to Madureira *et al*., FOXM1 upregulates ERα in MCF-7 and ZR-75-30, ER+ breast cancer cells, by direct protein binding interaction to FHREs of the ER promoter. Even though there is strong evidence suggesting that FOXM1 cooperates with FOXO3a to regulate ER gene expression, the exact mechanism is not clear (63).
3. Induction of breast cancer resistance: Millour *et al*. found that FOXM1 expression in epirubicin resistant MCF-7-EPI^{R} cells is higher comparing to sensitive MCF-7 cells due to mutation of the tumor suppressor p53. The anti-cancer agent, epirubicin, acts by upregulating p53, which is important for the drug sensitivity, and pRB tumor suppressor proteins to downregulate E2F; these effects result in FOXM1 downregulation. They also found that epirubicin in a negative loop can activate ATM which promotes E2F transcriptional activity and FOXM1 expression. Silencing ATM in MCF-7-EPIR cells using siRNA or suppression of ATM in U2OS cells re-sensitize these cells to epirubicin. (104). One more evidence, protein- protein binding interaction of FOXM1 and NF-κB induces doxorubicin resistance in triple negative breast cancer cells (MDA-MB-231) via upregulating DNA repair genes, RFC4, POLE2, EXO1 and PLK4

which protect the breast cancer cells from damaging the DNA double-strand (105).

4. Induction of epithelial-to-mesenchymal transition (EMT): In response to a growth factor such as EGF and TGFβ, FOXM1 expression is upregulated indirectly via inducting the MAPK in EGF pathway. Consequently, FOXM1 activates Slug by direct binding interaction to the Slug activator. Activated Slug inhibits the expression of an epithelial marker, E-cadherin and activates mesenchymal transcriptomes. These steps are necessary for EMT which is the main feature of invasiveness and metastasis of the breast cancers (106).

1.11. FOXM1 and cancer treatment:

FOXM1 protein has been attracting the researches as a therapeutic target because of its implication in the different features of cancer including tumour progression, invasion, angiogenesis and metastasis (90,107,108). Numerous evidence has supported this idea such as FOXM1 depletion using RNA interference induces mitotic catastrophe in breast cancer cells as reported by Diane *et al*. (49). Similarly, knockdown of FOXM1 via siRNA significantly reduces lung and prostate cancer cells proliferation and prevents the anchorage-independent cell growth (109,110).

In addition, many studies have proved that FOXM1 is an essential regulator of the acquired drug resistance in cancer cell, suggesting that targeting the oncogenic protein FOXM1 could be a promising strategy in cancer prevention and treatment (111). However, Gartel *et al.* described FOXM1 transcription factor as “undruggable” (112). In addition, additional evidence suggested that FOXM1 modulation would not be “safe”. To illustrate

this, Laoukili *et al.* reported that genetic depletion or inhibition of FOXM1 in mouse embryonic fibroblasts is toxic and mutagenic. FOXM1 controls the expression of G2-specific genes and it is essential for chromosome stability, consequently, loss of FOXM1 protein causes multiple cell cycle defects such as delay in G2, chromosomal mis-segregation and failure of cytokinesis (113). Furthermore, FOXM1 is important for tissue repair in adults. Kalinichenko, *et al.* established that premature FoxM1 expression in regenerating transgenic mice lungs caused early activation of cyclins A, E, B, F and Cdk1; additionally, reduced the pulmonary level of p21. These effects lead to stimulation of cell proliferation of different lung cell types following lung injury (114).

Drugs modulation of FOXM1 transcriptional activity:

Even though modulating FOXM1 via pharmacological interventions is a complicated strategy, accumulating evidence suggesting that targeting this oncogenic protein using modulators (49,112) can be a promising tool to prevent cancer resistance to wide range of chemotherapies such as tamoxifen (64), cisplatin (115), doxorubicin (105), and gefitinib (116).

1.11.1. siRNA:

Small interference ribonucleic acid (siRNA) is the most common gene therapy that needs specific oligonucleotide drug delivery system to modulate a specific protein. This strategy has been applied to downregulate FOXM1 expression *in vitro* in different studies including:

a. As reported by Jiang *et al.* inhibiting FOXM1 transcriptional activity using siRNA inhibited nasopharyngeal carcinoma (NPC) cells proliferation via the

suppression of cyclin D1 and cyclin E1. Furthermore, siRNA downregulation of FOXM1 caused apoptosis by different pathways; one of which was by the activation of p53 and Bax, and down-regulation of Bcl-2 resulting in losing the potential of the mitochondrial membrane. The other mechanism involved the upregulation of cytochrome c and increasing its release into the cytosol, and activation of cleaved capsase-3, caspase-9 and cleaved PARP. Transfection of siRNA into NPC resulted in the activation of FADD and cleaved caspase-8 which also induced apoptosis of NPC. Moreover, transfection of siRNA was found to downregulate the expression of VEGF but did not affect HIF-1α in comparison with thiostrepton (117).

b. Silencing of FOXM1 using siRNA decreased the expression of ERα and repressed the proliferative effect of E2 in MCF-7 cells and sensitized the resistant cells to the effect of tamoxifen (64).

c. *In vitro* study on human osteosarcoma cells (U2OS) and mouse embryonic fibroblasts proved that the depletion of FOXM1 using siRNA prevented the mitotic progression and accumulated nuclear levels of $p27^{Kip1}$and $p21^{Cip1}$ causing the death of the cancer cells (29). Xue *et al.* proved that silencing of FOXM1 via siRNA repressed the proliferation of clear cell renal cell carcinoma (ccRCC) via downregulation of cyclin B1, Cdk2 and cyclin D1, and the increased the expression of $p21^{Cip1}$ and $p27^{Kip1}$ (118).

Despite the effectiveness of this strategy of treatment, the clinical application has been challenging because of the need to develop accurate nanoparticle carriers to deliver siRNA to the tumor. However, recently in a study that carried out by Wang *et al*., they used

intratumoral injections of polyethylimine (PEI)-encapsulated anti-FOXM1 siRNA and found that the treatment was retained within the tumor and able to specifically suppress FOXM1 and its downstream target genes; suggesting that this approach could be a potential anti- cancer therapy (36).

1.11.2. Proteasome inhibitors:

The large complex proteasome proteins are the main component of the ubiquitin proteasome pathway (UPP). UPP plays an essential role in the regulation of the cell function and maintenance of homeostasis by degradation of about 80% of the intracellular proteins (119). This pathway controls the expression of variety of genes that are essential for cell cycle progression, proliferation and apoptosis such as NF-κB, c-Myc, p53, Bcl-2, p21, p27 and HIFl (120); however, disturbance of this pathway is associated with diseases, particularly cancer (119,120).

Many studies have established the proteasome inhibitors as potential anti-cancer agents because of their ability to inhibit the proliferation and induce the apoptosis to a wide verity of solid tumors (121–123). The preclinical studies have showed that the cytotoxic effect of the proteasome inhibitors is higher on the cancer cells because of the high proliferation rate comparing to the quiescent cells (124).

One of the main targets of the proteasome inhibitor in the malignant cells is NF-κB. NF-κB is highly elevated in carcinoma cells and plays an essential role in caner progression by the induction of proliferation, angiogenesis, migration and inhibition of apoptosis. Proteasomal degradation of NF-κB inhibitor (IκB) activates NF-κB which is then translocated to the nucleus. The potential inhibitors prevent the activation and nuclear translocation of NFκB via suppressing the proteasomal degradation of IκB (125).

Proteasome inhibitors prevent G1/S transition by preventing the degradation of CDK inhibitor p27 which inhibits cyclin D and cyclin E. Proteasome inhibitors also activate p53-dependent apoptosis by inhibiting the proteasomal degradation of the tumor suppressor p53 resulting in the activation of pro-apoptotic genes such as p21, PUMA and Bax (120).

The mechanism by which proteasome inhibitors inhibit FOXM1 is not well understood, but it is believed that all of the inhibitors downregulate FOXM1 by stabilizing the negative regulators of FOXM1.

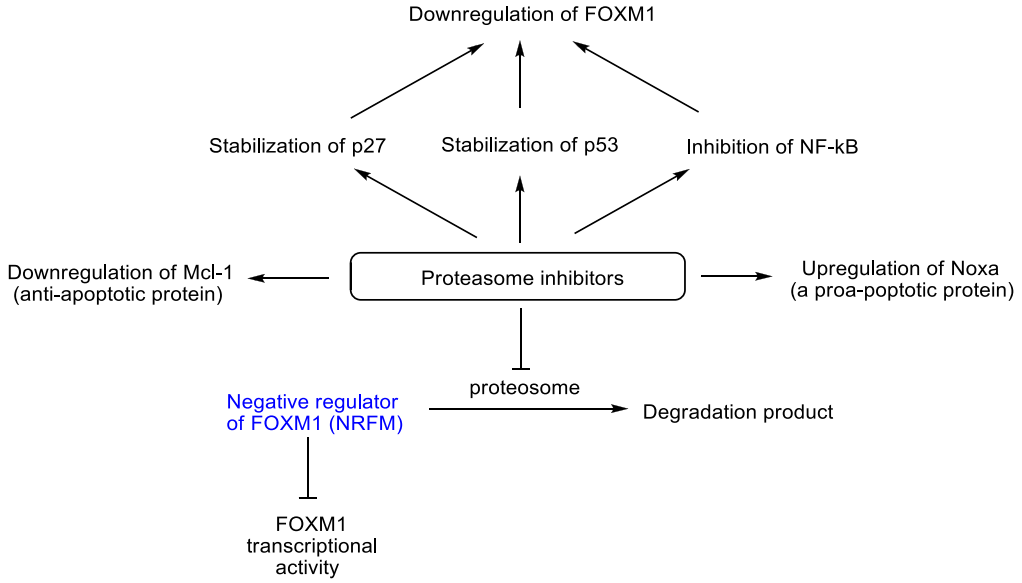


Figure 5: indirect inhibition of FOXM1 by the proteasome inhibitors via the stabilization of FOXM1 negative regulators (120).

1.11.2.1. Thiostrepton:

The thiazole antibiotic thiostrepton is a potent proteasome inhibitor and was found to downregulate the expression and transcriptional activity of FOXM1 *in vitro* in different human cancer cell lines (126–129). For the first time, *in vivo* study was carried out by Wang *et al*. established that micelle-encapsulated thiostrepton inhibited the growth and

induced the death of MDA-MB-231 and HepG2 cancer xenografts. The proteasome inhibitor thiostrepton was found to induce its cytotoxic effect by the suppression of FOXM1 and upregulating the expression of cleaved caspase-3 expression (130). Kwok *et al.* demonstrated that thiostrepton selectively promotes the cell death in breast cancer MCF-7 cells due to the suppression of FOXM1 expression comparing to non-transformed breast epithelial cells (127).

However, thiostrepton is a well-known inhibitor of 20S proteasome and downregulates FOXM1 by stabilizing a negative regulator of the protein (NRFM), as mentioned above (127). Additionally, Gormally *et al*. reported that thiostrepton is significantly toxic at very low concentration, while higher concentration needed to induce FOXM1 inhibition, indicating that thiostrepton acts by FOXM1-independent pathways. These off-target activities of the drug confound its use as a direct inhibitor of FOXM1 (131).

1.11.2.2. Siomycin A:

The thiazole antibiotic siomycin A is a thiazole antibiotic and was identified a suppressor of FOXM1 transcriptional activity. Using high-throughput screening study, Radhakrishnan *et al.* found that siomycin A, out of 2000 compounds obtained from NCI database, inhibited the transcriptional activity of FOXM1in a specifically designed cell line which highly expressed FOXM1. The thiazole antibiotic/ siomycin A suppresses FOXM1 activity by two different ways, one of them is by inhibiting FOXM1 phosphorylation by CDK 1/2 which is necessary for the activation of FOXM1. The second mechanism is via inhibiting FOXM1 at mRNA and protein levels (107). Other study carried out by Bhat *et*

al. demonstrated that siomycin A induced apoptosis through downregulation FOXM1 and the antiapoptotic Mcl-1 protein in melanoma cells (126).

1.11.3. Platinum-based chemotherapeutics:

The platinum-based drugs (cisplatin, oxaliplatin and carboplatin) are well-known anti-cancer agents and have been used worldwide for treatment of different cancers (132). The main mechanism of action of the drugs is by covalent binding interaction to DNA to recognize the DNA-damage and activate DNA repair pathways resulting in the induction of programmed cell death (apoptosis). Additionally, the drugs were found to induce the cytotoxic effect by binding to non-DNA target genes such as ubiquitin and cytoskeletal proteins (133).

As mentioned before, elevated FOXM1 expression due to the inhibitory effect of PI3K-AKT pathway on FOXO3a mediates the acquired drug resistance against the different chemotherapies including platinum-based drugs (100). In this regard, inhibiting FOXM1 by small-molecule inhibitors could sensitize the tumor cells to the cytotoxic and cytostatic effect of the approved chemotherapeutic agents. Lin *et al*. found that inhibiting FOXM1 by the small molecule inhibitor thiostrepton sensitize the highly metastatic and invasive Daoy medulloblastoma (MB) cells to the antitumor effect of cisplatin (115).

1.11.4. Anthracyclines:

The most well-known anthracycline drugs are doxorubicin, epirubicin and daunorubicin which have been used for the treatment of a wide range of cancers such as breast, ovary and lung carcinomas and leukemia (134,135). Anthracyclines work as DNA intercalators leading to blockade of DNA and RNA synthesis and induction of cell death (104).

It has been reported that the levels of FOXM1 expression are the main determinant of cellular sensitivity to genotoxic anthracyclines. One piece of evidence showing that upregulated FOXM1 expression in MCF-7 cells promotes epirubicin resistance while silencing FOXM1 via siRNA sensitizes the resistant MCF-7-EPI^{R} to epirubicin (104).

Anthracyclines control FOXM1 expression by different mechanisms including, a) activation of the tumor suppressors p53, p21^{Cip1}, Rb (136) leading to suppression of E2F-dependent expression of FOXM1. Some studies showed that losing of p53 function and/or an increasing ATM expression and activity develop epirubicin resistance (104), b) induction of FOXO3a expression (94,137) resulting in FOXM1 suppression and induction of cell death (80).

In addition, the genotoxic anthracyclines promote the accumulation of ROS which upregulates- FOXM1 expression in a negative feedback loop leading to the activation of the detoxifying enzymes such as SOD, PRDX3 and catalase. This negative feedback activation of FOXM1 antagonizes the cytotoxic effect of the chemotherapeutic agents and induce survival of the cancer cells (51).

1.11.5. Taxanes:

Taxanes are natural products produced by Yews plants in the genus *Taxus* but now taxanes are synthesized artificially (138). Paclitaxel (Taxol) and docetaxel (Taxotere) belong to the family of taxanes and both have therapeutic indications for solid tumors, including ovarian, breast, lung, and gastric cancers. The main mechanism of action of taxanes is by disruption of microtubule function and prevention of formation of mitotic spindle leading to the mitotic arrest. Consequently, the cells either die during mitotic arrest or exit the mitotic phase without cell division (138–140). Additionally, taxanes induce

apoptosis via increasing the production of TNF-α through direct binding interaction to TNF receptor (TNFR1) (141).

Taxanes also can induce their cytotoxic effect via upregulating FOXO3a which competitively downregulates the transcriptional activity of FOXM1 (142). Taxanes activateFOXO3a via stress-activated MAPK JNK which was found to promote FOXO3a by at least three different mechanisms, a) inhibiting PI3K-AKT pathway-dependent phosphorylation/ deactivation of FOXO3a (96), b) direct phosphorylation/ activation of FOXO proteins (143) and, c) phosphorylation/ inactivation of FOXO-sequestering chaperone protein 14-3-3 (144).

FOXM1 mediates the acquired drug resistance against taxanes via stabilization and regulation of microtubules and other transcriptional proteins involved in G2 and M phases (87,145). Silencing FOXM1 by siRNA was found to sensitize breast cancer cells to the cytotoxic effect of paclitaxel (145).

1.11.6. EGFR/ HER2 inhibitors:

Epidermal Growth Factor Receptor (EGFR) superfamily consists of transmembrane receptors including HER-1/EGFR, HER-2/ErbB-2, HER-3/ ErbB-3, and HER-4/ErbB-4 (18,95,146). EGF peptides including transforming growth factor-alpha (TGF-a), EGF, amphiregulin and heparin-binding EGF (HB- EGF) are the main activators of the EGFR leading to their dimerization (homo-dimerization or hetero-dimerization with other EGFR members) (18,146). EGFR is then auto-or-trans-phosphorylated for its activation at certain tyrosine residues resulting in the induction of various downstream signaling pathways such as Ras/Raf/mitogen-activated protein kinase and PI3K/AKT (18,87,147). The activation of these cascades leading the upregulation of Cyclin-Cdk and

PLK1 (147) which, ultimately, activate the phosphorylation/ activation of FOXM1 (18,67,87,147).

Several studies have established that HER2/ErbB2 as an upstream regulator of FOXO3a and FOXM1 (18,67,87,147). There is a strong correlation between HER2 and FOXM1 expression in breast cancers, upregulation or suppression of HER2 correlates with the protein and mRNA levels of FOXM1 in breast cancer cells *in vitro* and *in vivo* (67).

The main EGFR/ HER2 inhibitors, namely lapatinib, herceptin and gefitinib, act by deactivating PI3K-AKT signaling cascade leading to the upregulation of FOXO3a which consequently downregulates FOXM1 (87,95). Herceptin acts by disrupting the interaction between HER2 and HER3 resulting in upregulation of the Cdk inhibitor p27 which induces cell cycle arrest followed by cell death (147,148). The other two inhibitors (Lapatinib and gefitinib) are tyrosine kinase inhibitors (TKI) (149,150) that competitively inhibit the ATP-binding domain of the EGFR leading to cancer cells death (150).

It has been reported that FOXM1 upregulation mediates the inherent and acquired resistance to the drugs in breast carcinoma cells (147). Depletion of FOXO3a by using siRNA protect drug-sensitive breast cancer cells from the cell cycle arrest and FOXM1 downregulation effects of gefitinib while reintroducing FOXO3a resentisizes the drug-resistance EGFR upregulated breast carcinoma cells to the cytotoxic effect of the drug (95).

1.11.7. CDK inhibitors:

Cyclin-dependent Kinase inhibitors mainly act by inhibiting phosphorylation/ activation of FOXM1. Roscovitine was found to induce cell cycle arrest and apoptosis via inhibiting Bcl-2 expression and upregulating p53 (151). The CDK inhibitor, Novartis,

works by inhibiting CyclinD1 and CDK4 causing FOXM1 reduction at mRNA and protein levels 6 hours after the treatment of sensitive neuroblastoma cells with the drug while novartis- resistant neuroblastoma cells showed no reduction at the mRNA level of the protein (152).

1.11.8. NF-κB inhibitors:

As mentioned above, the transcription factor NF-κB regulates the expression of proteins that are key promoters of cancer invasion and progression such as cytokines, inducible pro-inflammatory enzymes, E-selectin, and chemokines, in addition to activation of EMT (72). Over expression of the protein is associated with the aggressiveness of wide range of cancers. Furthermore, NF-κB upregulates FOXM1 but the exact mechanism is not clear. It was reported that silencing NF-κB decreased FOXM1 expression in the same manner suggesting that FOXM1 is a downstream effector of NF-κB (153).

Panepoxydone is an NF-κB inhibitor that downregulates the transcription protein through inhibiting the phosphorylation of IκB causing cytoplasmic accumulation of NF-κB and prevention of its nuclear translocation. Reduced expression of NF-κB in the nucleus by panepoxydone ultimately causes the downregulation of FOXM1, cyclin D1, slug protein, and survivin and upregulation of Bax and cleaved PARP (72).

1.11.9. Endocrine therapy:

Deregulation of hormone receptors has been associated with wide variety of human malignancies. ERα is found to be upregulated in about 60% of human breast cancers and linked with FOXM1 upregulation at mRNA and protein levels (60–63).

Tamoxifen and fulvestrant are the most commonly used chemotherapies that regulate ERα in ERα-positive breast cancer patients (148). These drugs are usually used in combination with other anti-cancer treatment such as paclitaxel that is able to inactive PI3K-AKT cascade to activate FOXO3a activity (96).

1.11.10. Forkhead domain inhibitory compound-6 (FDI-6):

Gormally *et al*. identified several small molecule inhibitors that block the interaction between FOXM1-DBD and its target DNA sequence. Authors found these drugs by using a high-throughput screening assay with a library of 54,211 small molecules. Only the top three compounds, namely FDI-6, FDI-10 and FDI-11 were described as potent direct FXOM1 inhibitors. Using the EMSA (electrophoretic mobility shift assay), FDI-6 showed the highest inhibitory effect on the FOXM1 DBD–DNA complex (131).

In comparison with thiostrepton, FDI-6 is not a proteasome inhibitor and which shows a convenient correlation between the concentration required for growth inhibition(IC_{50}) and disruption of the FOXM1 DBD–DNA binding interaction in EMSA (18.0 ± 3.0, and 22.5 ± 12.3, μM respectively) (131).

1.12.0. Defining the binding site for drugs within the FOXM1/DNA domain:

As part of an interdisciplinary research project aimed to validate the FOXM1 transcription factor as a drug target, we carried out a series of molecular dynamic and molecular modeling (docking) procedures. We determined, for the first time, the binding energies of 3,323 FDA-approved drugs in what we think might constitute a binding site for drugs in the FOXM1/DNA domain (unpublished). This part of the research project was carried out by a Ph. D. student (Rodrigo Aguayo, Department of Pharmacy, Faculty of Chemistry, National Autonomous University of Mexico), as part of a collaborative research work. This modeling was done before I started my research work and, consequently, was the basis on which this thesis is based.

1.12.1. Characterization of the FOXM1c/DNA-binding domain:

We carried out a serial of molecular dynamics (MD) simulations to characterize the FOXM1c/DNA-binding domain to identify a region where small molecule inhibitors could potentially exert binding interactions within the FOXM1c/DNA complex.

The end result of these simulations is presented as a figure (figure 6), where we propose a region containing specific aminoacids that could be involved in drug binding sites. In this regard, we have identified the following aminoacid residues: Arg236, Tyr241, Lys278, Arg286, His287, Ser290, Arg297, Ser306, Trp308, and Asn283. As a result of these MD simulations, we identified two potential binding sites for small molecule inhibitors within the FOXM1/DNA-binding domain. Particularly, one of two binding sites exhibited more amino acids that could be involved in the interaction and this specific region was chosen to carry out the screening (docking) study.

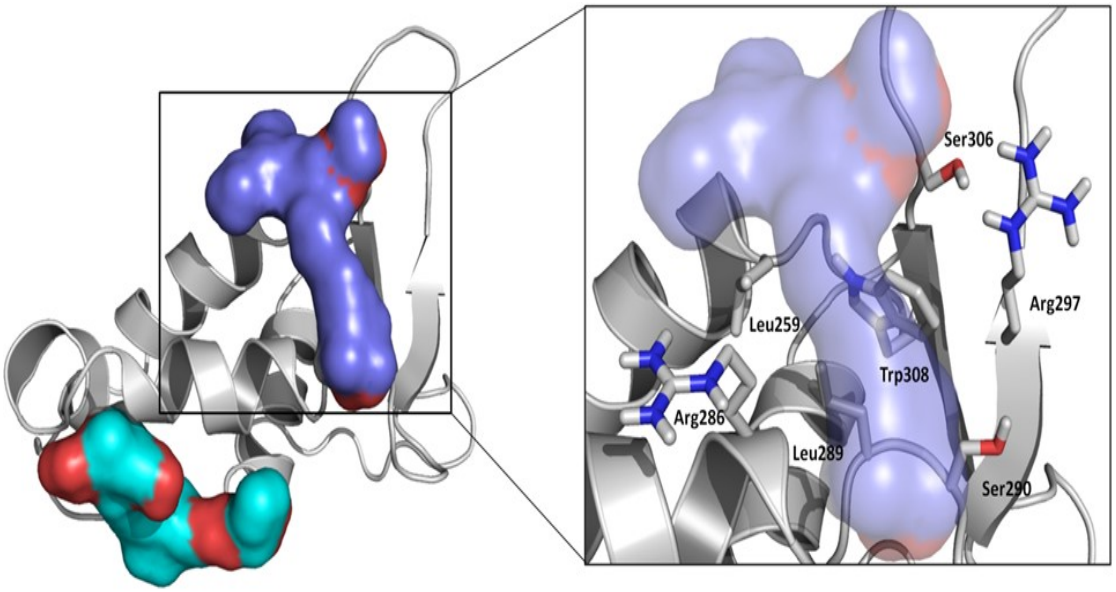


Figure 6: Potential binding sites for small molecule drugs within the FOXM1/DNA domain. These binding sites are represented (1) in light green/red, and (2) purple/red. We zoomed in the second binding site, which showed a higher number of "available" amino acid residues than the first binding site.

1.12.2. Docking study:

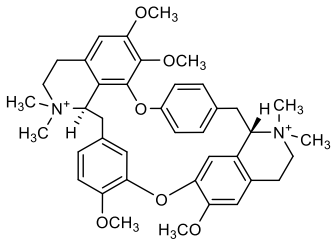
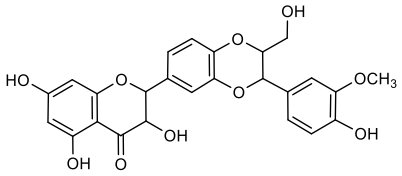
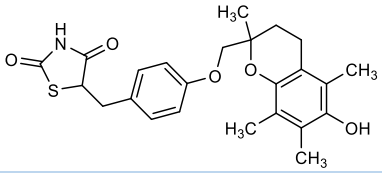
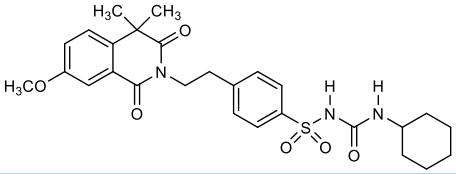
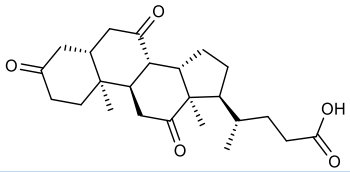
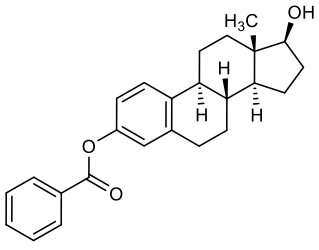
Once the appropriate cluster in the FOXM1c/DNA binding domain was defined, we performed a molecular modeling (docking) study on 3,323 FDA-approved drugs contained in the ZINC database. At the end of the docking protocol, we selected the top 10 molecules for which we recorded the lowest binding (free) energies (Table 2).

Table 2: Binding free energies calculated with the MM-PBSA method. The binding energy was calculated based on the total contribution of ΔEvdW (van der Waal), ΔEelec (electrostatic), ΔGpolar (polar) subtracted from the ΔGnonpolar (nonpolar) solvation energy.

Compd.	ΔE_{vdW} (kJ/mol)	ΔE_{elec} (kJ/mol)	ΔG_{polar} (kJ/mol)	$\Delta G_{nonpolar}$ (kJ/mol)	$\Delta G_{binding}$	
					(kJ/mol)	(kcal/mol)
ZN01	-96.5 ± 8.6	418.4 ± 21.0	31.8 ± 36.2	-11.6 ± 1.0	342.0 ± 33.9	81.8 ± 8.1
ZN02	-168.2 ± 21.7	-20.1 ± 17.5	119.7 ± 26.0	-17.2 ± 1.7	-85.8 ± 17.5	-20.5 ± 4.2
ZN03	-114.5 ± 12.2	-49.5 ± 10.3	115.3 ± 17.8	-13.1 ± 1.1	-61.8 ± 18.5	-14.8 ± 4.4
ZN04	-111.3 ± 15.1	-36.1 ± 14.1	92.7 ± 27.2	-12.6 ± 1.2	-67.3 ± 23.6	-16.1 ± 5.7
ZN05	-70.5 ± 25.1	-39.3 ± 32.1	78.6 ± 39.9	-8.2 ± 2.8	-39.3 ± 38.3	-9.4 ± 9.2
ZN06	-157.9 ± 19.5	-41.1 ± 21.3	131.1 ± 24.9	-17.4 ± 1.5	-85.3 ± 26.2	-20.4 ± 6.3
ZN07	-51.8 ± 11.3	-431.7 ± 17.9	322.3 ± 32.9	-10.0 ± 0.7	-171.2 ± 32.8	-41.0 ± 7.8
ZN08	-98.3 ± 13.3	-29.3 ± 15.2	66.6 ± 26.6	-11.4 ± 1.3	-72.3 ± 23.3	-17.3 ± 5.6
ZN09	-87.4 ± 9.1	-10.1 ± 6.2	41.0 ± 9.4	-10.5 ± 1.0	-67.0 ± 10.2	-16.0 ± 2.5
ZN10	-162.5 ± 10.6	-22.6 ± 11.3	115.7 ± 12.9	-17.5 ± 0.9	-86.9 ± 12.6	-20.8 ± 3.0

The docking protocol showed 10 molecules with different ID numbers in the ZINC database; nevertheless, a closer look at the chemical structures of these potential FOXM1 inhibitors revealed that some of them are, in fact, the same molecule classified using a different ZINC code because they refer to different salts. Therefore, table 3 presents the chemical structures and ZINC codes for all hit molecules and grouping with the same chemical entity.

Table 3: Chemical structures of the compounds identified by molecular dynamics and molecular modeling (docking) simulation. All these molecular formulas were obtained from the ZINC database.

ID	Drug name	Formula
ZN01	Metocurine	
ZN02, ZN06, ZN10	Silybin	
ZN03, ZN04	Troglitazone	
ZN05	Gliquidone	
ZN07	Dehydrocholic acid	
ZN08, ZN09	Estradiol	

The three different forms of silybin (codes ZN02, ZN06 and ZN10) showed the lowest RMSD values and the lowest binding free energies suggesting that the drug could be an interesting FOXM1 inhibitor. For dehydrocholic acid (code ZN07) the RMSD analysis showed a change from its initial binding mode, enabling a conformation with high affinity, therefore, this may represent an adaptable and flexible inhibitor within the binding site. On the other hand, compounds metocurine (code ZN01), gliquidone (code ZN05), and estradiol (codes ZN08 and ZN09) showed the highest RMSD values and the highest binding free energies, suggesting high inhibition of the FOXM1c transcriptional activity.

Interestingly, most of the selected drugs promoted a structural conformation modification of the target FOXM1c protein, between the amino acid residues 280-296. This conformational change is similar to that observed in the MD simulation with the DNA, suggesting that these molecules could in fact act as ligands and potential inhibitors of the FOXM1c transcriptional activity.

1.13.0. Description of the hit molecules found with the docking protocol.

Five out of the ten drugs were selected for testing and screening in vitro using thiostrepton as a control. The drugs to be tested are troglitazone (ZN03: -14.8 ± 4.4, ZN04: -16.1 ± 5.7 kcal/mol), β-Estradiol-3-benzoate (ZN08: -17.3 ± 5.6, ZN09: -16.0 ± 2.5 kcal/mol), gliquidone (ZN05: -9.4 ± 9.2 kcal/mol), dehydrocholic acid (ZN07: - 41.0 ± 7.8 kcal/mol) and metocurine (ZN01: 81.8 ± 8.1 kcal/mol).

1.13.1. Troglitazone:

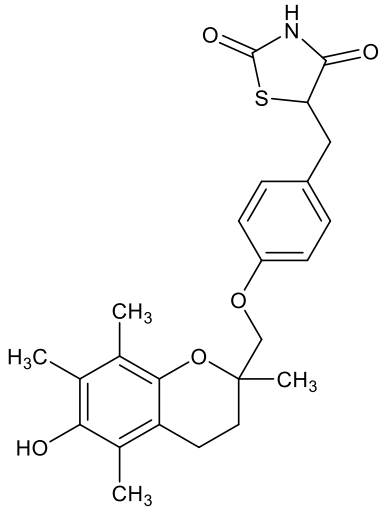


Figure 7: Chemical structure of troglitazone.

IUPAC Name: 5-({4-[(6-hydroxy-2,5,7,8-tetramethyl-3,4-dihydro-2H-1-benzopyran-2-yl) methoxy] phenyl} methyl)-1,3-thiazolidine-2,4-dione.

Chemical formula: $C_{24}H_{27}NO_5S$.

Troglitazone (Rezulin) is a thiazolidinedione (TZD) antihyperglycemic agent was used for treatment of noninsulin-dependent diabetes mellitus (NIDDM) which is known as type II diabetes (154). Troglitazone binds to peroxisome proliferator-activated alpha and gamma receptors (PPARα and PPARγ) to sensitize muscles and adipose tissues to insulin

(155). Additionally, troglitazone reduces inflammation by increasing IκB, leading to a decrease in NF-κB (156).

In the US, the antidiabetic agent was used alone or in combination with sulfonylureas or other antidiabetic agents three years before being withdrawn from the market in 2000 due to deaths from severe hepatotoxicity. FDA-approved dose was 400 to 800 mg once daily (157).

Recently, a few studies have established the potential anti-cancer effects exerted by troglitazone using different types of cancer cell lines. For example, the report by Yu *et al.,* who described the PPARγ-ligand troglitazone as cancer cell growth inhibitor, which also induced cell cycle arrest and apoptosis in MDA-MB-231 breast cancer cells in a dose-dependent manner. Troglitazone acted by decreasing the expression of pRb, cyclin D1, 2, 3, and Cdk2, 4, 6, and increasing the expression of p21 and p27, via a PPARγ-dependent pathway (158). However, in another study, Yu *et al*. found that the ERα "cross-talks" with the PPARγ pathway, leading to cell resistance to troglitazone in MCF-7 cancer cells. They observed that treating these cells with a combination of troglitazone and tamoxifen (an ER inhibitor), significantly inhibited MCF-7 cell proliferation, suggesting that troglitazone enhances the anti-proliferative effect of tamoxifen on ERα-positive breast cancer cells (159).

In a different study, Cheon *et al*. found that troglitazone and ciglitazone prevented stomach cancer cell replication via a PPAR-independent pathway, but the exact mechanism is not clear yet. The study showed that troglitazone significantly suppressed ERK phosphorylation and upregulated p21, resulting in significant inhibition of stomach cancer

cells growth *in vitro*. Furthermore, troglitazone inhibited the expression of genes important for DNA replication (160).

Another study carried out by Petrovic *et al*. it was reported that troglitazone, rosiglitazone and pioglitazone, suppressed FOXM1 expression, indirectly, through downregulation of Sp1 in liver cancer cells (HepG2), leading to inhibition of cancer cell replication (161). It has been reported that thiazolidinediones control the activity of Sp1 by both PPARγ-dependent and independent-pathways (161–163), in particular, by inducing the proteasomal degradation of Sp1. However, this mechanism still warrants further investigation (162).

Finally, Wang *et al.* reported that in addition to the activation of PPARγ, troglitazone acts as an inverse agonist to estrogen-related receptors (ERRα and ERRγ) as it interferes with the interaction between these receptors and their coactivator (GC-1α), leading to the inhibition of ERR signaling pathways in breast cancer cells (164). Inhibiting the ability of ERRα and ERRγ to bind to their coactivators also leads to reduction of mitochondrial mass and suppression of superoxide dismutase expression which causes induction of ROS production. Elevated ROS production consequently induces the expression of cell cycle inhibitor $p21^{WAF1}$. Additionally, troglitazone inhibits the expression of the coactivators PGC-1α and PGC-1β which play a crucial role in mitochondrial function regulation (164).

1.13.2. β-estradiol-3-benzoate:

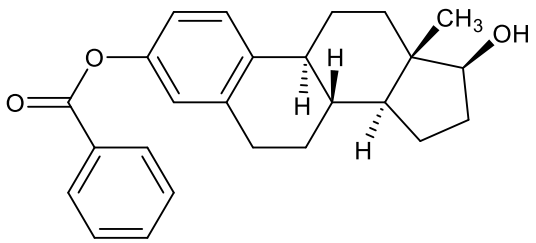


Figure 8: Chemical structure of beta estradiol-3-benzoate.

IUPAC Name: [(8R,9S,13S,14S,17S)-17-hydroxy-13-methyl-6,7,8,9,11,12,14,15,16,17-decahydrocyclopenta[a]phenanthren-3-yl] benzoate (165).

Chemical formula: $C_{25}H_{28}O_3$.

Estradiol (Vagifem) has been used for treatment of menopause symptoms, such as hot flashes; dryness, and vaginal irritation. Additionally estradiol is used as a replacement of natural estrogen in case of ovarian failure and for osteoporosis prevention in postmenopausal women (166).

FDA-approved doses for postmenopausal symptoms are 0.45 mg to 2 mg orally once a day, 1 to 5 mg of estradiol cypionate IM every 3 to 4 weeks or 10 to 20 mg of estradiol valerate IM every 4 weeks. 0.05 or 0.1 mg/day vaginal ring. 0.025 to 0.1 mg/day (transdermal film) applied topically 1 or 2 times a week (166).

Estrogen has been an interesting case because of its inhibitory role on the development of hepatocellular carcinoma (HCC) especially in males (167). β-estradiol has been studied for its anti-cancer activity in different cancer cell lines. For example, 17-β-estradiol (E2) inhibited the proliferation of anoikis resistant hepatocellular carcinoma cells

(AR HCC) which are resistant to doxorubicin, 5-florouracil, sorafenib and cisplatin. E2 acts by three main mechanisms:

a) inhibiting the production of intrleukin-6 (IL-6), which is essential for the modulation of inflammation-associated cancers via the activation of downstream target genes that promote cancer initiation, metastasis and invasion.
b) suppressing the phosphorylation of signal transducer and activator of transcription 3 (STAT3).
c) inducing the cell cycle arrest and apoptosis through increasing phosphorylation of JNK (167).

On the other hand, many reports have established the positive correlation between estrogen hormone and FOXM1. Estrogen hormone was described as a mitogen that induces FOXM1 expression at mRNA and protein levels indirectly through ERα (63,64) which was found to be upregulated in more than 60% of all breast cancer cell lines (60–62). Furthermore, suppressing FOXM1 expression in MCF-7 using siRNA inhibited the expression of ERα, indicating the positive loop between FOXM1 and the estrogen receptors (63).

Despite the effectiveness of the drug in the inhibition of cancer cell proliferation, the lack of selectivity between normal and cancer cells represents one of the main obstacles precluding the use of this hormone in long-term cancer treatment (167).

There is an increasing need to design chemotherapeutic agents with high selectivity toward cancer cells. In this regard, there is an interesting case using the drug chlorambucil, a nitrogen mustard cytostatic anti-cancer agent that alkylates DNA, this drug is not selective and it forms adducts with RNA, leading to systemic cytotoxicity, anemia and

bone marrow suppression (168). Gupta *et al.* synthesized a site-directed anticancer drug for the treatment of hormone-dependent breast cancer. This drug is a hybrid of estradiol and chlorambucil.

Estradiol Pt (II) hybrid showed a significant cytotoxic effect in hormone-dependent MCF-7 and hormone-independent MDA-MB-436/486 cancer cell lines without side effects (168).

1.13.3. Gliquidone:

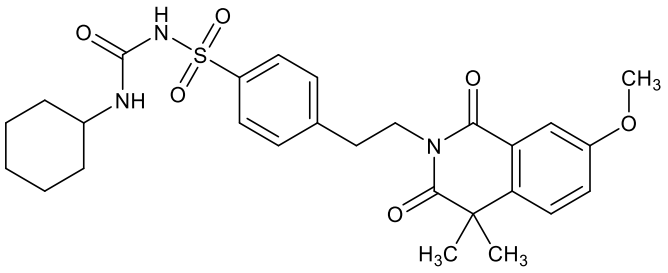


Figure 9: Chemical structure of gliquidone.

IUPAC Name: 1-cyclohexyl-3-{4-[2-(7-methoxy-4,4-dimethyl-1,3-dioxo-1,2,3,4-tetra-hydroisoquinolin -2-yl)ethyl]benzenesulfonyl} urea.

Chemical Formula: $C_{27}H_{33}N_3O_6S$.

Gliquidone (Glurenorm) is a second-generation antihyperglycemic sulfonylurea. Gliquidone is used for the treatment of NIDDM/ type II diabetes in adults. The drug has PPARγ agonist activity and acts by lowering blood glucose levels by inducing the pancreas to produce and release insulin (169).

The FDA-approved dose of gliquidone is 15 mg once a day, up to 30 minutes prior to breakfast. This dose is then increased to 45 mg to 60 mg/ day divided into two doses (170).

1.13.4. Dehydrocholic acid:

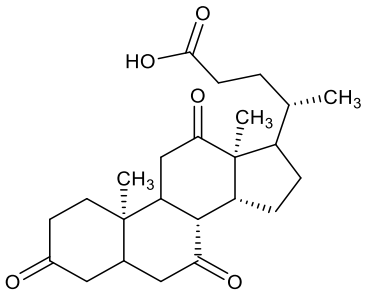


Figure 10: Chemical structure of dehydrocholic acid.

IUPAC name: (4R)-4-[(5S,8R,9S,10S,13R,14S,17R)-10,13-dimethyl-3,7,12-trioxo-1,2,4,5,6,8,9,11,14,15,16,17-dodecahydrocyclopenta[a]phenanthren-17-yl] pentanoic acid. Chemical formula: $C_{24}H_{34}O_5$.

Dehydrocholic acid (Dycholium) is a semisynthetic bile acid derived from cholic acid. It is used as a Gastrointestinal agent that promotes the bile flow to the duodenum (cholagogue) or induces the bile production by the liver (hydrocholeretic) and diuretic(171). The FDA- approved dose is 250 to 500 mg oral doses three times daily after meals (172).

1.13.5. Metocurine:

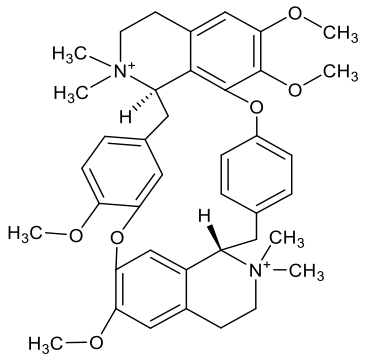


Figure 11: Chemical structure of metocurine.

IUPAC Name: (1S,16R)-9,10,21,25-tetramethoxy-15,15,30,30-tetramethyl-7,23-dioxa-15,30-diazaheptacycl0-[22.6.2.2^{3},^{6}.1^{8},^{12}.1^{18},^{22}.0^{27},^{31}.0^{16},^{34}] hexatriaconta-3,5,8 (34), 9,11,18 (33), 19,21,24,26,31,35-dodecaene-15,30-diium.

Chemical Formula: $C_{40}H_{48}N_2O_6$.

Metocurine is a muscle relaxant that acts by antagonizing the neurotransmitter action of acetylcholine via competitive binding with cholinergic receptors on the motor end-plate (173). In Canada, the drug was known as Metubine Iodide and administered intravenously (IV) as metubine Iodide Injection 2mg/mL. This drug was withdrawn from the market in 1998 (174).

1.13.6. Thiostrepton:

Thiostrepton (bryamycin) is a peptide antibiotic produced by the growth of *Streptomyces azureus* strains. It inhibits gram-positive bacteria (175). The drug is used topically in veterinarian antibacterial preparations and has a potency of more than 900 units/ mg (176).

A significant number of studies have established the anti-cancer activity of the thiazole antibiotic thiostrepton. The main mechanism of action exerted by thiostrepton is

by suppressing the expression and the transcriptional activity of FOXM1. Thiostrepton is currently used in veterinary medicine (177).

Zhang *et al.* established that targeting the FOXM1 pathway with thiostrepton in TP53 wild type and mutant ovarian, endometrial and lung cancer cells induces apoptosis and inhibits cell viability. Furthermore, thiostrepton was more potent than cisplatin in these cells and sensitized cancer cells to cisplatin *in vitro,* and carboplatin *in vivo* using a nude mice model (178).

As mentioned above, micelle-encapsulated thiostrepton inhibited the growth (and induced apoptosis) of both HepG2 and MDA-MB-231 cells in cancer xenografts via the inhibition of FOXM1 (130).

According to Kwok *et al*., thiostrepton selectively induces cell death of MCF-7 breast cancer cells over non-transformed breast epithelial MCF-10A cells, and he explained this by the fact that breast cancer cells highly express FOXM1 which make them more susceptible to the repression of FOXM1 expression comparing to the non-transformed cells (127).

However, thiostrepton is a proteasome inhibitor that binds to 23S subunit of rRNA leading to the inhibition of translation and protein synthesis of all proteins and not only FOXM1 (179). It also acts on 20S proteasome which downregulates FOXM1 by stabilizing the negative regulators of the protein (NRFM) as mentioned in the introduction; inhibition of proteasome activity influences a wide range of physiological and pathological processes (180).

1.14. Hypothesis:

According to a computer-based drug screening protocol carried out by our group (details of which are not reported here), using the crystal structure reported for the DNA binding domain of the FOXM1 transcription factor, we hypothesize that it is possible to extrapolate theoretical binding energies with an in vitro downregulation of this protein, exerting a direct binding inhibition at the protein-DNA level.

If the test drugs are able to inhibit the transcriptional activity of FOXM1 this inhibition should be observed by a significant:

1. Shift in the band detected for the FOXM1 protein when recombinant protein (FOXM1-DBD) is incubated with increasing concentrations of the test drug, relative to the band detected for the recombinant protein in the absence of the drug using electrophoretic mobility shift assay (EMSA).
2. Decrease in the FOXM1 protein expression in cancer cells, as well as a reduction in the protein expression of selected FOXM1 downstream targets, determined by western blot analysis.

1.15. Objectives:

1. To determine the concentration-dependent effects exerted by the test drugs on triple negative breast cancer cells (MDA-MB-231), estrogen receptor positive (ERα+) breast cancer cells (MCF-7), and normal mammary epithelial (MCF-10A) cells viability.
2. To determine the concentration-dependent effects of the test drugs on the expression of FOXM1 protein.
3. To determine the shift in band of FOXM1 protein when FOXM1-DBD is incubated with increasing concentrations of the test drug, relative to the band detected for the protein-DBD in the absence of the drug.

Chapter 2: Materials and Methods

2.0. Materials and Methods:

2.1. Drugs:

Table 4: A list of the drugs to be tested and their sources, CAS number and % of purity.

Drug	Source (Company)	CAS number	Purity
Troglitazone	Toronto Research Chemicals (TRC)	97322-87-7	>98%
Gliquidone	TCI AMIRICA	33342-05-1	>98%
β-estradiol-3-benzoate	Sigma-Aldrich	50-50-0	>97%
Metocurine	Specs Natural Products	5152-30-7	Not reported
Dehydrocholic acid	Alinda Chemical Ltd	81-23-2	Not reported
Thiostrepton	Sigma-Aldrich	1393-48-2	>90%

To confirm the identity of the test drugs, we used ^{1}H-NMR. We prepared drug samples by dissolving about 10 mg of each drug in 1 mL dimethyl Sulfoxide-d6 (DMSO-d6, D,99.9%, Cambridge Isotope Laboratories, Inc), and sent them for analysis as solutions (600 MHz NMR spectrometer; Bruker Ascend).

2.2. Cell Lines

2.2.1. Human triple negative breast cancer cell line (MDA-MB-231):

MDA-MB-231 cell line was obtained from Dr. Afsaneh Lavasanifar's lab, Faculty of Pharmacy and Pharmaceutical Sciences, University of Alberta. The cells received were

grown in RPMI 1640 with L-glut & Hepes (Gibco by life technologies) supplemented with 10% FBS (Sigma), and 1% penicillin and streptomycin 100 IU/mL (Gibco by life technologies).

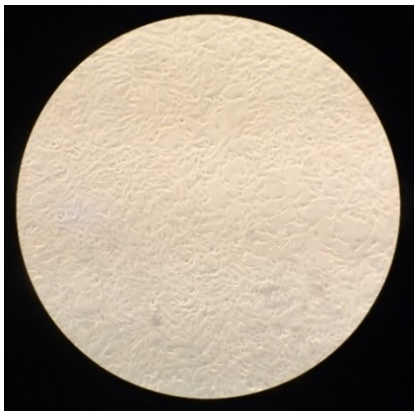


Figure 12: A photo of human triple negative breast cancer (MDA-MB-231) cells; 100X scale.

2.2.2. Human Michigan Cancer Foundation-7 (MCF-7) cell line:

The ERα+ breast cancer cell line was obtained from Dr. Frank Wuest's lab, department of Oncology, Cross Cancer Institute. The cells were grown in DMEM/ F12 (Gibco by Life technologies) supplemented with 10% FBS, and 1% penicillin and streptomycin 100 IU/mL.

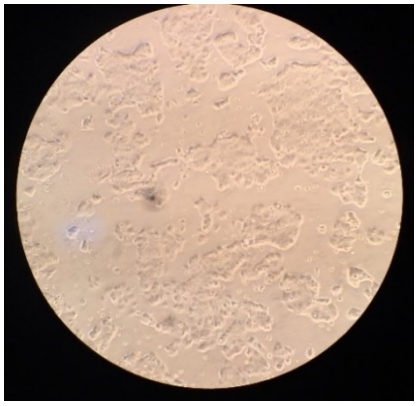


Figure 13: A photo of estrogen receptor alpha- positive human Michigan Cancer Foundation-7 (MCF-7) cells; 100X scale.

2.2.3. Human Michigan Cancer Foundation-10A (MCF-10A) cells:

Non-tumorigenic mammary epithelial cells. This cell line was obtained from from Dr. Frank Wuest's lab, department of Oncology, Cross Cancer Institute. The cells were grown in DMEM/ F12 supplemented with MEGM SingleQuot Kit Suppl. & Growth Factor (Lonza/Clonetics Corporation).

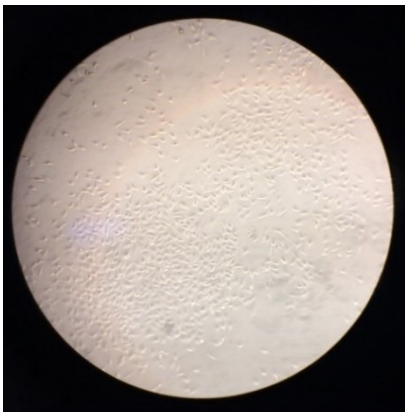


Figure 14: A photo of normal mammary epithelial human Michigan Cancer Foundation-10A (MCF-10A) cells; 100X scale.

2.3. MTT Colorimetric Assay:

The cell viability assay (MTT), which was proposed to determine the IC_{50} value for each drug was carried out three times in triplicate or quadruplicate. The MDA-MB-231, MCF-7, and MCF-10A cell lines' passages range between 13 and 16. 10 mM stock solutions were prepared for each compound; namely: troglitazone, β-estradiol-3-benzoate, gliquidone, dehydrocholic acid and metocurine, using thiostrepton as a control, by dissolving the drug in DMSO.

Briefly, when the cells were ~80% confluent in T 75 cm^3 culture flasks, we harvested the cells with 2 mL of 0.05 % Trypsin-EDTA (1X) solution and 4 mL of GM,

centrifuged the tubes at 1200 rpm for 6 min at the room temperature, and then re-suspended the cells in 1 mL of GM. Second, to count the cells, we mixed 25 µL of the cell suspension with 25 µL of trypan blue and 50 µL of PBS in a small bijou bottle. We counted the cells in 10 µL of the blue mixture under the microscope in the four quadrants on haemocytometer. The number of cells in 1 mL was calculated as following:

Cells/mL = Number of total cells (the 4 quadrants) x 4 x 10,000 / 4 = cells /mL

Third, we seeded the cells in 96 well plates [~ 4000 cells/well] 100 µL of cell suspension/well. Then, we incubated the seeded plates for 24 hours under standard conditions (37°C and 5% CO_2), until the confluence was ~ 70%.

The second day, we prepared serial dilutions of the compounds in 96 well plates. Next, we prepared a dosing intermediate 96 well plate containing 188 µL of GM in each well. We transferred 12 µL of the serial dilution from the stock compound plate to the corresponding wells in the dosing plate, mixed well and then transferred 20 µL of each well in the dosing intermediate plate to the corresponding wells in the seeded plates. The final concentrations of each compound in the wells were 100, 25, 6.25, 1.56, 0.39, and 0.098 µM, in addition to 1 % DMSO in media as a control. Finally, we incubated the treated plates for 48 h.

The fourth day, we prepared a 3 mg/mL solution of MTT in PBS and then added 30 µL of this reagent to each well; we incubated this mixture for 3 hours at 37°C. We tapped the media and the reagent from the plates and left them to dry inside the hood. Finally, we added 100 µL of DMSO to dissolve the purple formazan crystals and then read absorbance at 570 nm using spectrophotometer.

2.4. Western Blotting:

The immuno blotting assay, which was proposed to determine the effect of each drug (troglitazone, β-estradiol-3-benzoate, gliquidone, dehydrocholic acid and metocurine, using thiostrepton as a control) on FOXM1 protein expression, was carried out three times. The cell lines' passages ranged between 12 and 18.

We cultured MDA-MB-231 cells in 6 well plates for 24 hours until reaching 80-90% confluence. We treated the cells with three different drug concentrations using DMSO as a control. We prepared the drug dilutions previously by diluting the stock solution in DMSO to get the following concentrations: 10, 20, and 40 μM, except for troglitazone, for which the concentrations were 2.5, 5, and 10 μM. The final concentration of DMSO in the media was not higher than 1%.

24 hours after the treatment we harvested the cells with 100 μL of 0.05 % Trypsin-EDTA (1X) solution and 1 mL of GM, centrifuged the tubes at 12000 xg for 6 min at the room temperature and washed the pellets twice with PBS. We stored the cells pellets at -80 C^o after sucking out the washing buffer.

In the second day, we prepared the samples by adding 50 μL of 2 % SDS in RIPA Buffer (Sigma) followed by sonication for 30 seconds.

2.4.1. Lowry protein assay for protein estimation:

For protein estimation, we prepared serial dilutions of bovine serum albumin (BSA), ranging between 1.35 and 0 μg /mL, and then we diluted the samples 25X (2 μL in 48 μL of dd H2O). We added 5 μL of each sample and 5 μL of the standard to 96 well plate in duplicate. We added 25 μL of reagent A mixed with reagent S (Bio Rad) to each well;

and then, 200 µL of reagent B (Bio Rad) and incubated the plate for 15 minutes at the room temperature. We read the samples absorbance using iMark microplate reader (Bio Rad) at 570 nm.

2.4.2. Immuno blotting and protein quantification:

We loaded 20 µL of the sample containing 30 µg of cell's protein from different treatments in the stacking gel before being resolved by 8% SDS-PAGE, at 150 V, for 1 hour and 20 minutes in the same electrophoretic tank. After running, we transferred the proteins at 25 V for 30 minutes to PVDF membrane for immunoblotting. We incubated the membranes in blocking buffer, 5% fat free milk in TTBS, for 2 hours at the room temperature before being incubated with 1:1000 of FOXM1 antibody (A-11) mouse monoclonal IgG_2 (Santa Cruz Biotechnology, Inc.) for 24 hours with rocking at 4°C. Next day, we washed the membranes four times with TTBS 10 minutes each and then incubated with goat anti-mouse HRP-conjugated secondary antibodies (Santa Cruz Biotechnology) at 1:2000 for 1hour at room temperature. We added 600 µL of Chemiluminescence ECL Prime Western Blotting solutions (Amersham) to the membranes for development for 5 minutes in dark; finally, we used Quantity One software (Imaging system VersaDoc MP5000 Bio-Rad) for protein quantification by dividing the densitometric value obtained of FOXM1 by densitometric value of the β-actin in the same sample.

2.5. Electrophoretic mobility shift assay (EMSA):

We applied EMSA assay to determine the ability of the drug molecules to inhibit the binding interaction between FOXM1-DBD and DNA. In this experiment, we tested only the drugs that affected cell proliferation and had an effect on FOXM1 protein

expression; these drugs are troglitazone, β-estradiol-3-benzoate, gliquidone, using thiostrepton as a control.

First, we prepared 6% DNA retardation gel consisting of 19.5 mL DNAse, RNAse water, 4.5 mL of 40 % acrylamide (Bio-Rad), 6 mL 5X TBE, 150 µL 10 % ammonium persulfate (APS) and 23 µL of tetramethylethylenediamine (TEMED). Then, we prepared the samples by incubating 4 µL of recombinant FOXM1-DBD (expression region from 235 to 327 amino acids, E-coli, purchased from My Biosource Inc.) with 2 µL of our potential inhibitors (50 µM) for 10 min on ice. After 10 min, we added 1 µL of 17 nM double stranded DNA (dsDNA) sequence (5′-FAM-AAACAAACAAACAATC-3′), (IDT) and 7 µL of binding buffer contained 50 mM Tris PH = 7.5, 150 mM KCl, 1mM DTT, 1 mM MgSO4 and 10% Glycerol to the FOXM1-DBD and drug mixture and incubated the tubes for 20 min in dark at room temperature before loading the samples to the gels. We performed electrophoresis for about 1 h at 100 V on ice, then read the gels using Typhoon Trio in the lab of Dr. Mark Glover, department of Biochemistry.

2.6. Cell line authentication:

To confirm the identity of the cell lines, we submitted DNA of the three cell samples (MCF-10A, MCF-7, MDA-MB-231) to the Center of Applied Genomics (TCAG) Genetic Analysis Facility in Toronto for STR profiling using Promega's GenePrint 10 System (part B9510).

We extracted DNA from the cells as directed by DNeasy blood and tissue kit instructions (Qiagen) as following:

We allowed the frozen cells to thaw for 3-5 min in the incubator and the centrifuged at 300 x g for 5 min then suspended in 200 µL of PBS. We added 20 µL of proteinase K to the suspension followed by adding 200 µL of AL buffer, mixed thoroughly and incubated the tubes at 56^{o} C for 10 min. Then, added 200 µL of ethanol (96-100%) to the mixture and vortexed. We transferred the mixture into DNeasy mini spin columns placed in 2 mL collection tubes and centrifuged at 6000 xg for 1 min. after transferring the mini spin columns into new 2 mL collection tubes, we added 500 µL of buffer AW1 and centrifuged once again at 6000 xg for 1 min. In new 2 mL tubes, we added 500 µL of buffer AW2 and centrifuged at 20000 xg for 3 min before transferring the spin columns into 1.5 mL microcentrifuge tubes. Finally, we eluted the DNA by adding 200 µL of buffer AE to the center of the spin column membrane and the centrifuged the tubes for 1 min at 6000 xg.

Finally, we prepared 10 ng/µL sample concentrations, labeled well and shipped to the center in Toronto.

2.7. Statistical Analysis:

We used Graph Pad Prism 5.01 (Graph Pad Software, San Diego, CA, USA) to perform the statistical analysis. To determine the significance between treated and untreated (control) groups, we used a one-way analysis of variance (ANOVA) followed by Tukey's post-test.

2.7.1. MTT Colorimetric Assay:

To eliminate the vehicle effect in MTT colorimetric assay, we normalized the cell viability of the treated cells, to that of cells treated with 1% DMSO in media, considering the viability of DMSO treated cells as 100 %.

% cells viability = (drug treated cells/ DMSO treated cells) x100

We used nonlinear regression analysis to measure the relative IC_{50} of each treated group to get the mean of each repetition. Using Microsoft excel, we calculated the STDEV of the means and divided them by the SQRT of the number of repetitions (n = 3), to get the SEM. We displayed the results (IC_{50} values) as mean ± SEM.

2.7.2. Western Blot:

To eliminate the vehicle effect in Western Blot, we considered the protein expression of the control sample (cells treated with DMSO only) as 100 % protein expression. We performed one-way ANOVA followed by Tukey's multiple comparison test to measure the difference in the inhibitory effect of the different concentrations of each potential inhibitor. We represented the significance of the effect as ''***'' for $P < 0.005$, ''**'' for $P < 0.01$ & ''*'' for $P < 0.05$.

Chapter 3: Results &Discussion

3. Results and Discussion:

3.1. ^{1}H NMR Spectroscopy:

Before conducting the in vitro experiments, we confirmed the identity of all drugs using 600MHz ^{1}H NMR Spectroscopy.

3.1.1. Troglitazone:

^{1}H-NMR (DMSO-d6, 600MHz): δ = 1.3 (s, 3H, a), 1.816 (m, 1H, b), 1.9685 (m, 3H, c), 2.025 (s, 3H, d), 2.043 (s, 3H e), 2.568 (t, J = 7.2 Hz, 2H, f), 3.055 (dd, J = 13.8 Hz, 1H, g), 3.296 (dd, J = 14.4 Hz, 1H, g), 3.9405 (m, 2H, h), 4.863 (dd, J = 9 Hz, 1H, i), 6.907 (d, J = 8.4 Hz, 2H, j), 7.13 (m, 2H, k), 7.436 (d, J = 7.8 Hz, 1H, l), 12.028 (s, 1H, m) (Appendix Image 1).

3.1.2. β-Estradiol-3-benzoate:

^{1}H-NMR (DMSO-d6, 600MHz): δ = 0.7 (s, 3H, methyl group, a), 1.209 – 1.420 (m, 7H, alkyl groups, b), 1.605 (m, 1H. c), 1.817 – 1.914 (m, 3H, alkyl groups, d), 2.203 (m, 1H, e), 2.328 (m, 1H, f), 2.823 (t, J = 4.8 Hz, 2H, g), 3.542 (m, 1H, h), 4.519 (d, J = 4.8 Hz, 1H, i) 6.950 (s, 1H, j), 7.003 (dd, J = 8.4 Hz, 1H, k), 7.351 (d, J = 8.4 Hz, 1H, l) 7.603 (t, J = 7.8 Hz, 2H, m), 7.743 (t, J = 7.8 Hz, 1H, n), 8.111 (d, J = 7.2 Hz, 2H, o) (Appendix Image 2).

3.1.3. Gliquidone:

^{1}H-NMR (DMSO-d6, 600MHz): δ = 1.083 – 1.649 (m, 10H, cyclohexane, a-f), 1.404 (s, 6H, g), 2.935 (t, J = 7.2 Hz, 2H, h), 3.261 (t, J = 7.2 Hz, 1H, i), 3.836 (s, 3H, j), 4.154 (t, J = 7.2 Hz, 2H, k), 6.299 (d, J = 7.2 Hz, 1H, l), 7.293 (dd, J = 8.4 Hz, 1H, m), 7.424 (d, J = 8.4 Hz, 2H, n), 7.528 (d, J = 2.4 Hz, 1H, o), 7.583 (d, J = 9 Hz, 1H, p), 7.786 (d, J = 9 Hz, 2H, q) (Appendix Image 3).

3.1.4. Dehydrocholic acid:

^{1}H-NMR (DMSO-d6, 600MHz): δ = 0.765 (d, J = 6.6 Hz, 3H, a), 1.009 (s, 3H, b), 1.229 – 1.476 (m, 7H, alkyl groups, c, d), 1.5005, (td, J = 15 Hz, 2H, e), 1.731 (m, 1H, f), 1.785 –2.312 (m, 14H, alkyl groups), 2.486 (td, J = 18.6 Hz, 1H, h), 2.837 (t, J = 13.2 Hz, 1H, i), 2.983 (dd, J = 13.2 Hz, 1H, j), 3.047 (t, J = 12 Hz, 1H, k), 11.974 (s, 1H, l) (Appendix Image 4).

3.1.5. Metocurine:

^{1}H-NMR (DMSO-d6, 600MHz): δ = 2.776 – 2.859 (m, 2H, alkyl groups, a), 2.929 (dd, J = 10.8 Hz, 6H, b), 3.105 – 3.220 (m, 8H, methyl groups, c), 3.434 – 3.652 (m,10H, methyl groups, d), 3.866 (d, J = 5.4 Hz, 6H, e), 3.957 (m, 6H, f), 4.246-4.396 (m, 2H, g), 4.761 (s, 1H, i), 5.260--6.146 (2 dd, J = 8.4 Hz, 2H, h), 6.696-6.7.267 (m, 8H, aromatic) (Appendix Image 5).

3.2. MTT Assay:

We evaluated the viability of the treated cells using the MTT colorimetric assay as described in methods and materials. The results showed that each cell line responded differently to the given drugs.

3.2.1. Human triple negative cancer cell line (MDA-MB-231):

As it is shown in table 5, the triple-negative breast cancer MDA-MB-231 cells responded to the inhibitory effect exerted by thiostrepton, troglitazone and β-estradiol-3-benzoate; whereas gliquidone, dehydrocholic acid and metocurine exhibited no inhibitory effect on cell viability.

Table 5: Concentrations (μM) of the potential inhibitors required to inhibit cell proliferation of MBA-MB-231 breast cancer cells by 50 % (IC_{50}) using the MTT colorimetric assay. IC_{50} values are represented as the mean ± standard error of the mean (SEM); experiments were performed in triplicate (n = 3). IC_{50} values were generated using GraphPad v5.01 Prism software.

Drug	**IC_{50} against MDA-MB-231 (μM)**
Thiostrepton	3.1 ± 1.2 μM
Troglitazone	63.4 ± 17.2 μM
β-Estradiol-3-benzoate	40 ± 22 μM
Gliquidone	> 100 μM
Dehydrocholic acid	> 100 μM
Metocurine	> 100 μM

Our control, thiostrepton, significantly inhibited the viability of MDA-MB-231 (IC_{50} = 3.1 ± 1.2 μM; figure 15). This result is in agreement with literature reports. In this regard, Halasi *et al*. reported that MDA-MB-231 cells are susceptible to the inhibitory effect exerted by thiostrepton (IC_{50} = 3.5 ± 0.4 μM, after 48 h of incubation) with different concentrations of the thiazole antibiotic (181), even though they used the cell counter to count the viable cells instead of measuring the absorbance using MTT assay as we did.

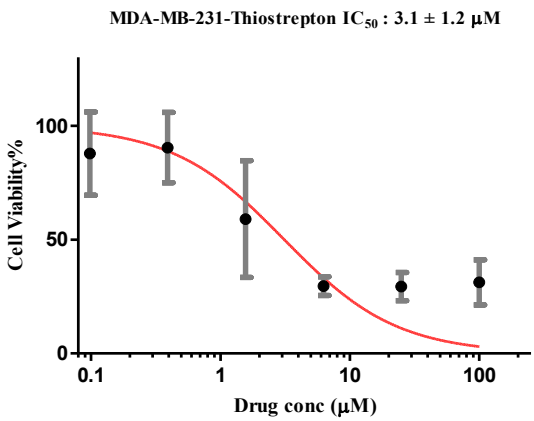


Figure 15: MTT result represented by the relationship between % cell viability and thiostrepton drug concentrations. The drug concentrations for MTT assay were 100 µM, 25 µM, 6.25 µM, 1.56 µM, 0.39 µM, 0.098 µM and 0 µM (containing 1% of DMSO in RPMI), which was considered in the statistics as the 100% cell viability. Incubation time: 48 h; the experiments were performed three times (n = 3). The result was expressed as the mean values ± SEM; the IC_{50} value was determined using GraphPad v5.01 Prism software.

On the other hand, the former antidiabetic drug troglitazone showed an inhibitory effect on the viability of MDA-MB-231 cells (IC_{50} = 63.4 ± 17.2 µM; figure 16).

Some studies have established the effect of troglitazone on the viability of breast cancer cells; for example, Malaviya *et al*. reported that treatment with 3.2–50 µM troglitazone for a 4-day period significantly inhibited MDA-MB-231 cell growth in a dose-dependent manner (182). Another study carried out by Yu *et al*., authors established the anti-proliferative effect of troglitazone on the triple negative breast cancer (MDA-MB-231 cells). In the same study, Yu *et al*. reported that incubating MDA-MB-231 cancer cells with 25–75 µM troglitazone for 48 h significantly inhibited cell growth in a dose-dependent manner (158) using MTT assay.

Other studies have tested the effect of the drug on cancer cells from different origin. For example, Saha *et al*. evaluated the toxic effect of troglitazone on human hepatocytes

(THLE-2 cells). After 72 h of incubation with different concentrations of the drug using MTT assay, the cytotoxicity of troglitazone (IC_{50} = 41.12 ± 4.3 µM) (183). In addition, Keil *et al*. conducted cell proliferation assay (MTS) to measure the lethal concentration (LC_{50}) of troglitazone that kills 50% of HepG2 liver cancer cells after the incubation for 48 h with different concentrations of the drug. Troglitazone was very toxic at low concentration (LC_{50} = 20.6 ± 0.6 µM) (184).

Even though the conditions of the experiments were different from what we applied in this project, all evidence support that the drug is toxic to different cancer cell lines, which may respond differently to the drug.

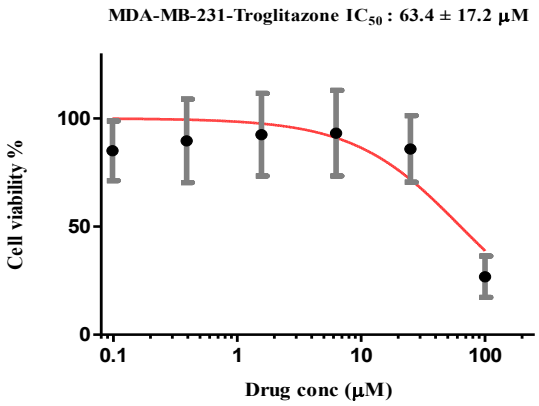


Figure 16: MTT assay represented by the relationship between % cell viability and troglitazone drug concentrations. The drug concentrations for MTT assay were 100 µM, 25 µM, 6.25 µM, 1.56 µM, 0.39 µM, 0.098 µM and 0 µM (containing 1% of DMSO in RPMI), which was considered as the 100% cell viability. Time period = 48 h; the experiments were performed three times (n = 3); the result is expressed as the mean values ± SEM; the IC_{50} value was determined using GraphPad v5.01 Prism software.

The drug β-estradiol-3-benzoate was very active and inhibited cell proliferation significantly at a low concentration (IC_{50} = 40 ± 22 µM; figure 17). In the literature, some

studies have evaluated the anti-cancer activity of this drug for example: a) Seulki Lee *et al*. demonstrated that 17-β-estradiol has antiproliferative effect on HCC cells at high concentration (~ 80 µM) after 48h incubation period using MTS assay (167); b) Hsu *et al*. reported that treatment of LoVo colorectal cancer cells with 0.01 µM 17-β-estradiol for 48 h inhibited the cell viability by 28.0% (185); c) another study was carried out by Schaufelberger *et al*. demonstrated that 0.01 µM 17-β-estradiol reduced the viable cell number of microglial (BV2) cells by 20.6% after 24 h of incubation using MTT assay (186).

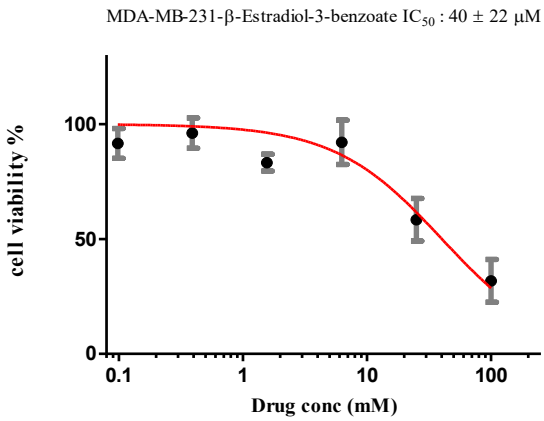


Figure 17: MTT result represented by the relationship between % cell viability and β-estradiol-3-benzoate drug concentrations. The drug concentrations for MTT assay were 100 µM, 25 µM, 6.25 µM, 1.56 µM, 0.39 µM, 0.098 µM and 0 µM (containing 1% of DMSO in RPMI which was considered as the 100% cell viability. Time period = 48 h; the experiments were performed three times (n = 3); the result is expressed as the mean values ± SEM; the IC_{50} value was determined using GraphPad v5.01 Prism software.

Regarding to gliquidone, dehydrocholic acid and metocurine, there are no previous studies describing their cancer cell proliferation inhibition effect. In our study, these drugs did not show any antiproliferative effect on the MDA-MB-231 compared to the control (thiostrepton).

3.2.2. Human ERα + breast cancer cell line (MCF-7):

As shown in table 6, MCF-7 cancer cells did not respond to any of the test drugs, or even the positive control drug, thiostrepton.

Thiostrepton has been reported as potent inhibitor on different types of triple negative breast cancer cells (TNBC). Interestingly, Yang *et al*. carried out an MTT assay to examine whether the inhibitory effect of thiostrepton is different among the different breast cancer subtypes. First, they treated three TNBC cell lines (MDA-MB-231, SUM149 and BT549), and two ER+ breast cancer cell lines (MCF-7 and T47D), using increasing concentrations of thiostrepton (0 – 20 μM) incubating for 24 h. They found that the TNBC cells are more susceptible to the inhibitory effect of the drug at about 10 μM. Interestingly, considering that ER+ breast cancer cells grow slowly, they extended the treatment up to 7 days. After 7 days of treatment, they found that the difference in the IC_{50} values between TNBC and ER+ breast cancer cells was very low (187). Consequently, despite the apparent lack of activity of thiostrepton in our initial experiment, this evidence supports (somehow) our findings on MCF-7 cells, which were not affected by thiostrepton.

In this regard, we needed to measure the level of FOXM1 protein expression in both breast cancer cell lines to correlate between FOXM1 protein level and cancer cell rate of growth and susceptibility to the drugs.

Table 6: Concentrations (μM) of the potential inhibitors required to inhibit cell proliferation of MCF-7 breast cancer cells by 50 % (IC_{50}) using the MTT colorimetric assay. IC_{50} values are represented as the mean ± standard error of the mean (SEM) experiments were performed in triplicate (n=3). IC_{50} values were generated using GraphPad v5.01 Prism software.

Drug	IC $_{50}$ against MCF-7 (μM)
Thiostrepton	> 100 μM
Troglitazone	> 100 μM
β-Estradiol-3-benzoate	>100 μM
Gliquidone	> 100 μM
Dehydrocholic acid	> 100 μM
Metocurine	> 100 μM

3.2.3. Human normal mammary epithelial cells (MCF-10A):

To determine the relative selectivity of the test drugs on cancer vs normal cells, we examined their inhibitory effect on untransformed mammary epithelial cells (MCF-10A), using the MTT assay under the same experimental conditions. Four out of six drugs (thiostrepton, troglitazone, β-estradiol-3-benzoate and gliquidone) inhibited the cell viability of MCF-10A cells, with different IC_{50} values (22.7 ± 12.3, 5.0 ± 2.0, 33.8 ± 2.1 and 44.12 ± 14.7 μM respectively), as shown in table 7. The other two drugs (dehydrocholic acid and metocurine) did not affect cell viability of normal cells.

Table 7: Concentrations (μM) of the potential inhibitors required to inhibit cell proliferation of MCF-10A breast normal epithelial breast cells by 50 % (IC_{50}) using the MTT colorimetric assay. IC_{50} values are represented as the mean ± standard error of the mean (SEM) experiments were performed in triplicate (n=3). IC_{50} values were generated using GraphPad v5.01 Prism software.

Drug	IC_{50} against MCF-10Acells (μM)
Thiostrepton	22.7 ± 12.3 μM
Troglitazone	5.0 ± 2.0 μM
β-Estradiol-3-benzoate	33.8 ± 2.1 μM
Gliquidone	44.12 ± 14.7 μM
Dehydrocholic acid	> 100 μM
Metocurine	> 100 μM

Some of these findings are not in agreement with literature reports. In this regard, Jimmy *et al*. reported that thiostrepton selectively inhibited the proliferation of MCF-7 with no effect on the viability of MCF-10A cells, after treatment with 10 μM thiostrepton for 72 h using flow cytometry. Cell cycle analysis showed that thiostrepton induced MCF-7 cells accumulation at G1 phase followed by cell death; However, MCF-10A cells showed cell cycle progression with the majority of the cells being at G1 and G2-M phases (127).

On the other hand, as mentioned in the introduction, Gormally *et al*. reported thiostrepton has a cytotoxic effect at very low concentration (IC_{50} = 2.7 μM) and this concentration is significantly below the concentration required to inhibit FOXM1 expression (45.0 ± 16.4 μM) (131). Even though the IC_{50} value reported by Gormally *et al*. is different than that obtained in our study (IC_{50} = 22.7 ± 12.3 μM; figure 18), this evidence supports our observation that thiostrepton is toxic and non-selective.

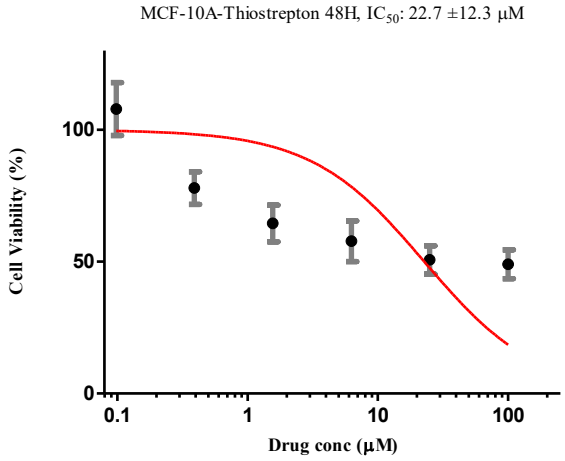


Figure 18: MTT result represented by the relationship between % cell viability and thiostrepton drug concentrations. The drug concentrations for MTT assay were 100 µM, 25 µM, 6.25 µM, 1.56 µM, 0.39 µM, 0.098 µM and 0 µM (containing 1% of DMSO in RPMI RPMI) which was considered as the 100% cell viability. Time period = 48 h; the experiments were performed three times (n = 3); the result is expressed as the mean values ± SEM; the IC_{50} value was determined using GraphPad v5.01 Prism software.

Despite the fact that troglitazone has anticancer activity, as reported in the literature, as it inhibited the proliferation of different cancer cells, none of the studies showed whether the drug is selective to cancer cells over the untransformed cells, or not (160–162,164). Our results suggest that troglitazone is toxic enough to normal breast cells MCF-10A at low concentration (IC_{50} = 5.0 ± 2.0 µM; figure 19).

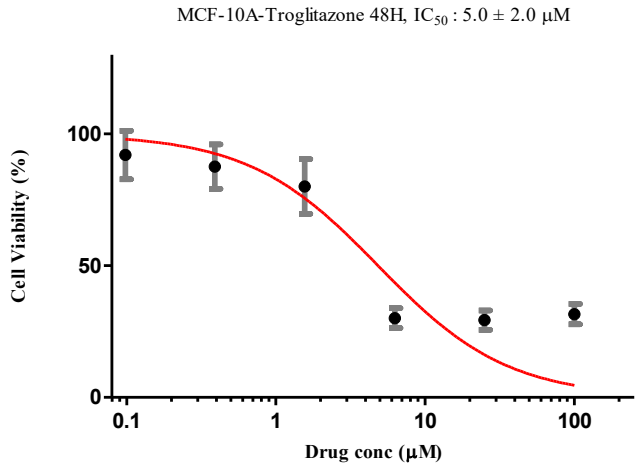


Figure 19: MTT result represented by the relationship between % cell viability and troglitazone drug concentrations. The drug concentrations for MTT assay were 100 µM, 25 µM, 6.25 µM, 1.56 µM, 0.39 µM, 0.098 µM and 0 µM (containing 1% of DMSO in RPMI), which was considered as the 100% cell viability. Time period = 48 h; the experiments were performed three times (n = 3); the result is expressed as the mean values ± SEM; the IC_{50} value was determined using GraphPad v5.01 Prism software.

As mentioned above, β-estradiol 3-benzoate has anticancer activity against HCC, additionally; we found that the drug is active against MDA-MB-231. However, the drug also affected the untransformed MCF-10A cells (IC_{50} = 33.8 ± 2.1 µM; figure 20). In the literature, Lee *et al*. reported that the drug acts as a hormone and it is not selective to tumor cells. The side effects of the drug appeared as physiological and physical changes *in vivo* (167).

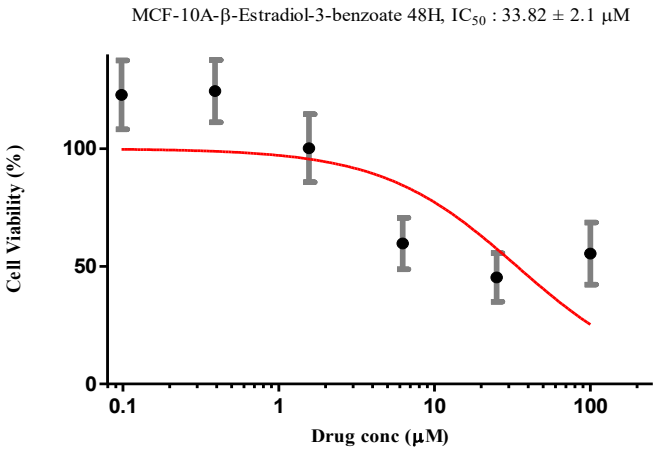


Figure 20: MTT result represented by the relationship between % cell viability and β-estradiol-3-benzoate drug concentrations. The drug concentrations for MTT assay were 100 µM, 25 µM, 6.25 µM, 1.56 µM, 0.39 µM, 0.098 µM and 0 µM (containing 1% of DMSO in RPMI), which was considered as the 100% cell viability. Time period = 48 h; the experiments were performed three times (n = 3); the result is expressed as the mean values ± SEM; the IC_{50} value was determined using GraphPad v5.01 Prism software.

We tested the anti-cancer activity of the antidiabetic drug gliquidone for the first time, and there is no previous study to which we could compare our results. The drug did not show any effect on the viability of the cancer cells (MDA-MB-231 and MCF-7), however, it inhibited the proliferation of the normal MCF-10A cells (IC_{50} = 44.12 ± 14.7 µM; figure 21).

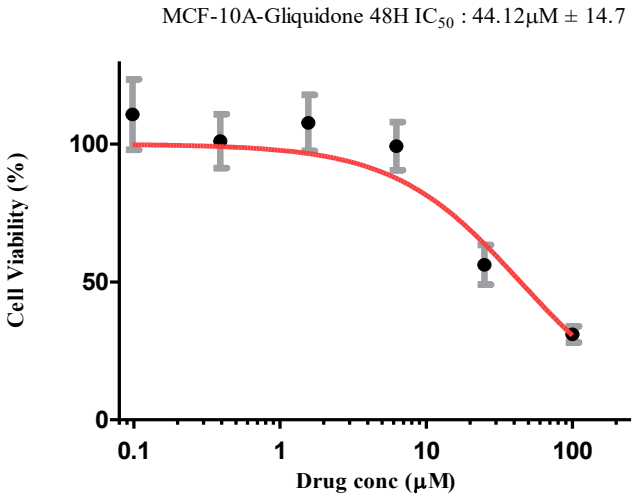


Figure 21: MTT result represented by the relationship between % cell viability and gliquidone drug concentrations. The drug concentrations for MTT assay were 100 µM, 25 µM, 6.25 µM, 1.56 µM, 0.39 µM, 0.098 µM and 0 µM (containing 1% of DMSO in RPMI), which was considered as the 100% cell viability. Time period = 48 h; the experiments were performed three times (n = 3); the result is expressed as the mean values ± SEM; the IC_{50} value was determined using GraphPad v5.01 Prism software.

3.3. Immuno blotting:

Triple negative cancer cell line (MDA-MB-231):

In the lab, one of my lab mates carried out western blotting on the three cell lines without treating them to evaluate the level of FOXM1 protein expression in these cells. The results showed that the MDA-MB-231 significantly expressed FOXM1, and this value was considered the maximum (100 %). On the other hand, the same experiment using the ER+ MCF-7 cells showed about 60% protein expression compared to that observed in TNBC cells, and the protein expression levels in MCF-10A cells (figure 22) was negligible, as determined using Fused H3 as the house keeping gene. In this regard, Yang *et al*. reported that the level of FOXM1 protein expression in MCF-7 is lower than that of MDA-MB-231 cells because of the statistical difference in the rate of the cell proliferation. Since MCF-7 are slower growing cells, they showed lower levels of FOXM1 protein compared to other breast cancer cell lines (188).

Based on this result, we cannot correlate between the level FOXM1 protein expression and the effect of the drugs on the cell viability, because four out of the selected drugs suppressed the viability of MCF-10A cells which showed the lowest level of the protein expression while they did not affect the proliferation of MCF-7 cells which had about 60 % FOXM1 protein level comparing to MDA-MB-231 cancer cells. The result also suggests that MCF-7 cells are drug resistance, and the drugs act by different mechanisms which could involve blocking FOXM1-DBD in MDA-MB-231 cells.

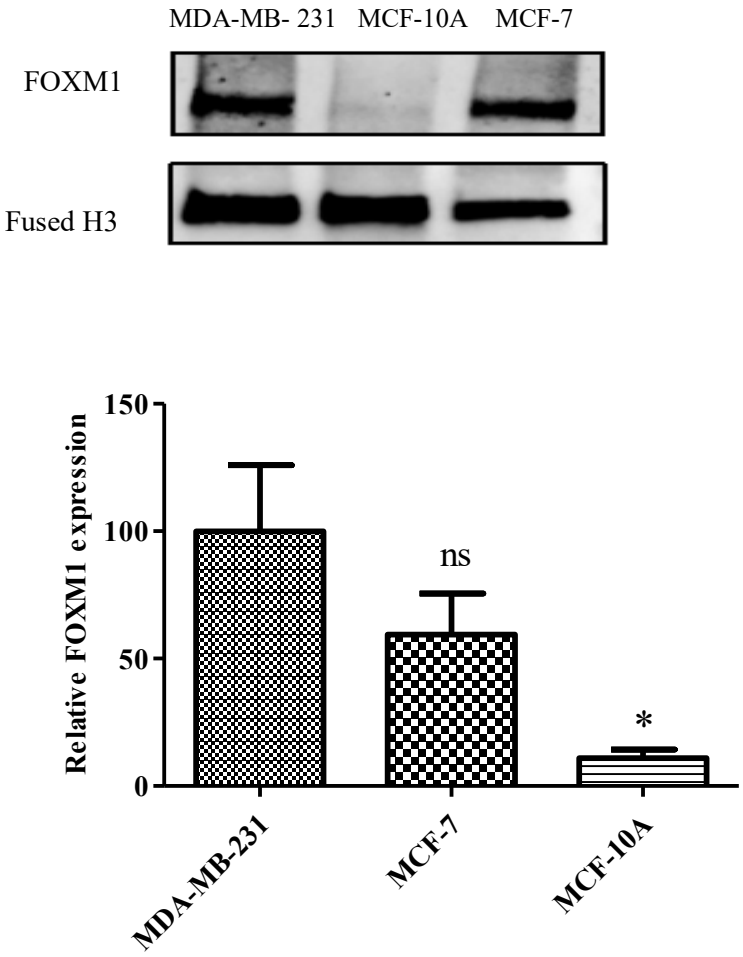


Figure 22: FOXM1 protein was separated by gel electrophoresis and detected using the enhanced chemiluminescence method. The graph represents the relative amount of FOXM1 proteinnormalized to Fused H3 in MDA-MB-231 triple negative and MCF-7 ERα+ breast cancer cells; and MCF-10A normal mammary epithelial cells. Results are expressed as the means ± SEM, N=3. Statistical analysis was performed by using one-way ANOVA followed by Tukey test. (* for $p < 0.05$); $P < 0.05$ MCF-10A compared to MDA-MB-231. ns means not significant.

Thiostrepton significantly inhibited cell viability of MDA-MB-231 cells (IC_{50} = 3.1 ± 1.2 µM, 48 h of treatment), and at the same time, it exerted inhibition of FOXM1 expression at 20 µM ($p < 0.005$), after 24 h treatment (figure 23). In the literature, Halasi *et al*. reported that after 48 h treatment with 5 µM thiostrepton, the expression of FOXM1 in MDA-MB-231 cells was significantly decreased (181). The 4-fold difference in

concentration needed to inhibit FOXM1 expression, between their results and ours could be due to different incubation times (24 vs 48 hours).

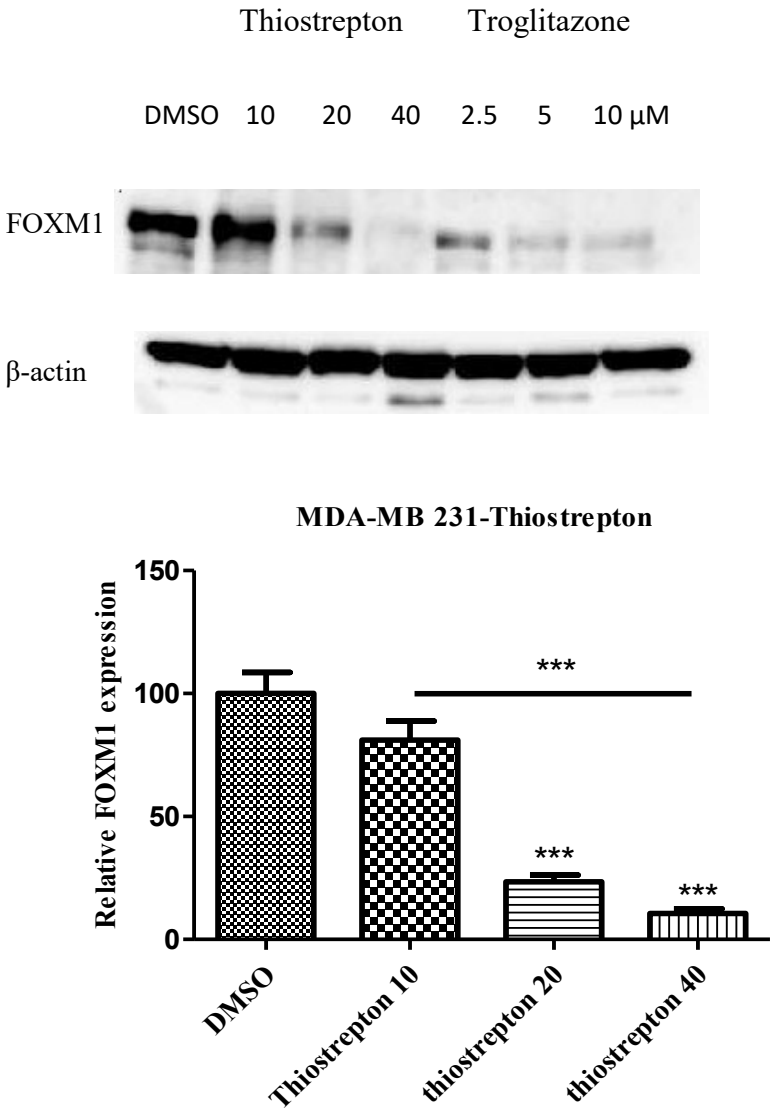


Figure 23: FOXM1 protein was separated by gel electrophoresis and detected using the enhanced chemiluminescence method. The graph represents the relative amount of FOXM1 protein normalized to β-actin in MDA-MB-231 triple negative breast cancer cells under different concentrations (DMSO, 10, 20, 40 µM) of our control, thiostrepton, after 24 h of incubation. Results are expressed as the means ± SEM, N=3. Statistical analysis was performed by using one-way ANOVA followed by Tukey test. (*** for $p < 0.005$). $P < 0.005$ thiostrepton 40 µM compared to DMSO, $p < 0.005$ thiostrepton 20 µM compared to DMSO. $P < 0.005$ thiostrepton 20, 40 µM compared to thiostrepton 10 µM.

Troglitazone inhibited the proliferation of MDA-MB-231 cells at a higher concentration (IC_{50} = 63.4 ± 17.2 µM, 48 h of treatment) comparing to thiostrepton, but it inhibited FOXM1 expression significantly at a very low concentration (2.5 µM; figure 24). In the literature, Petrovic *et al*. demonstrated that 50 µM troglitazone treatment of HepG2 cells induces the downregulation of FOXM1 after only a 12 h incubation period (161).

For comparison purposes, no previous studies described the effect of the drug on FOXM1 protein expression in breast cancer cells. Consequently, the difference in the cellular response to the suppressive effect of the drug on the protein expression could be explained by the variations of the cancer cells' origin and incubation time.

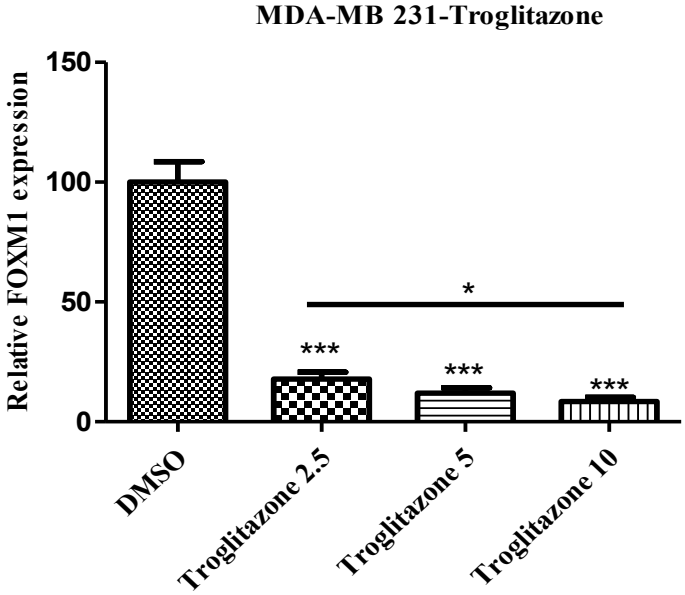
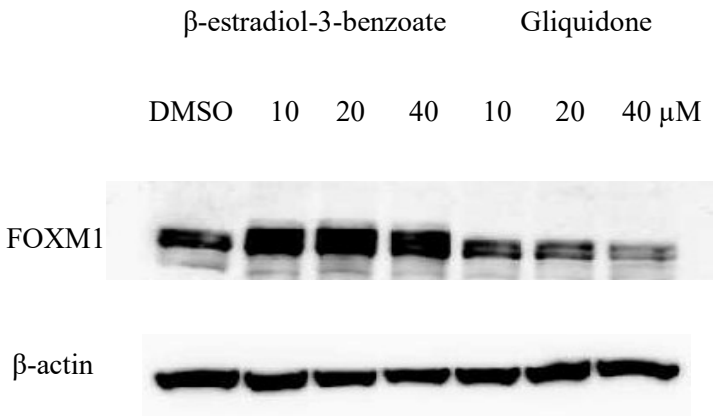


Figure 24: FOXM1 protein was separated by gel electrophoresis and detected using the enhanced chemiluminescence method. The graph represents the relative amount of FOXM1 protein normalized to β-actin in MDA-MB-231 triple negative breast cancer cells under different concentrations (DMSO, 2.5, 5, 10 µM) of troglitazone, after 24 h of incubation. Results are expressed as the means ± SEM, N=3. Statistical analysis was performed by using one-way ANOVA followed by Tukey test. (*** for $p < 0.005$, * for $p < 0.05$). $P < 0.005$ troglitazone 2.5, 5, 10 µM compared to DMSO. $P < 0.05$ troglitazone 10 µM compared to 2.5 µM.

Even though β-estradiol-3-benzoate inhibited the cell viability of MDA-MB-231 at low concentration (IC_{50} = 40 ± 22 μM), the western blot assay showed no significant reduction on FOXM1 expression. Instead, our results suggest that this hormone-like drug induced protein expression (up to about 200 % at 20 μM; Figure 25) instead of decreasing its expression. This result provides essential insights for future drug development programs, in the sense that hormone-like drugs are not useful scaffolds to be considered in medicinal chemistry.



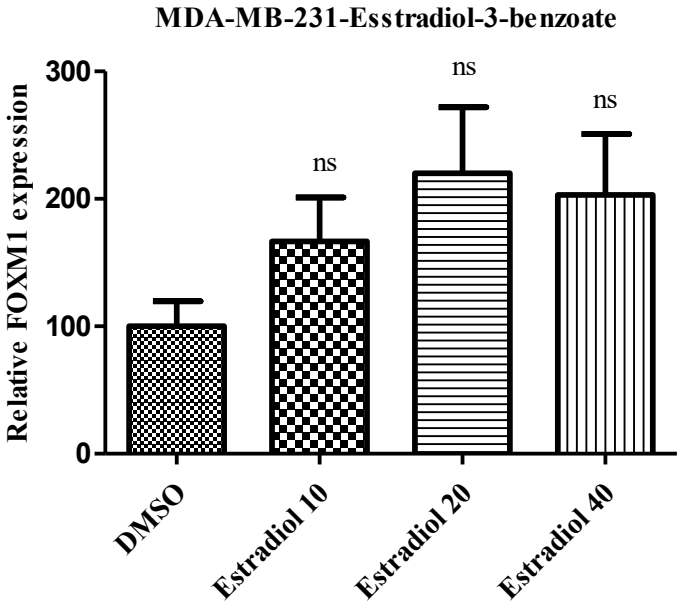


Figure 25: FOXM1 protein was separated by gel electrophoresis and detected using the enhanced chemiluminescence method. The graph represents the relative amount of FOXM1 protein normalized to β-actin in MDA-MB-231 triple negative breast cancer cells under different concentrations (DMSO, 10 μM, 20 μM, 40) of β-estradiol-3-benzoate after 24 h of incubation. Results are expressed as the means ± SEM, N=3. Statistical analysis was performed by using one-way ANOVA followed by Tukey test. ns means not significant.

Our results indicate that the drug gliquidone did not inhibit cell viability of MDA-MB-231 cells, but it decreased the proliferation of normal MCF-10A. On this regard, western blot analysis using this drug showed that gliquidone exerted a modest dose-dependent-inhibition of FOXM1 protein expression. ($p < 0.05$ at 40 μM; Figure 26), which makes this chemical structure an interesting alternative. Structural modifications on gliquidone may provide potentially useful new scaffolds in medicinal chemistry.

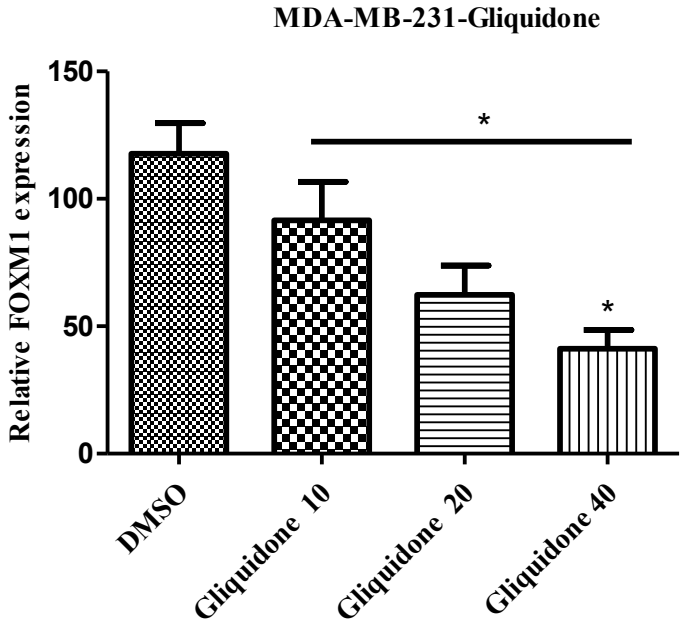
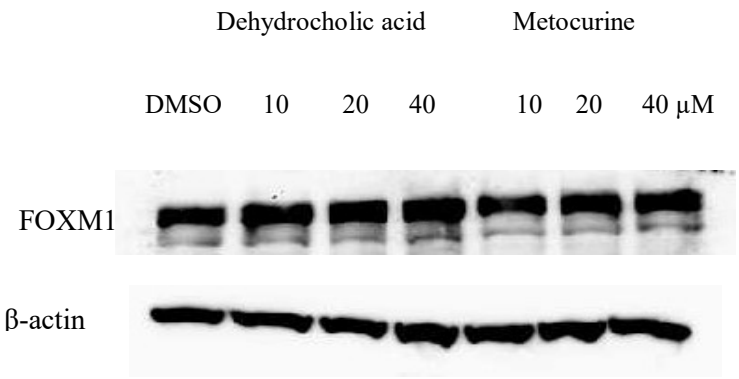


Figure 26 FOXM1 protein was separated by gel electrophoresis and detected using the enhanced chemiluminescence method. The graph represents the relative amount of FOXM1 protein normalized to β-actin in MDA-MB-231 triple negative breast cancer cells under different concentrations (DMSO, 10 μM, 20 μM, 40) of gliquidone after 24 h of incubation. Results are expressed as the means ± SEM, N=3. Statistical analysis was performed by using one-way ANOVA followed by Tukey test. (* for $p < 0.05$). $P < 0.05$ gliquidone 40μM compared to DMSO and gliquidone 10 μM.

Regarding the drugs dehydrocholic acid and metocurine, these drugs did not show any inhibitory effect on the cell viability of cell lines, and did not inhibit FOXM1 protein expression in MDA-MB-231 (figure 27). As described for the drug estradiol, dehydrocholic acid may also exert a hormone-like effect on cell proliferation, causing an increase of FOXM1 expression. This proved to be the case for metocurine as well, and consequently, these two drugs do not constitute an alternative in drug design efforts focused on FOXM1 inhibition.



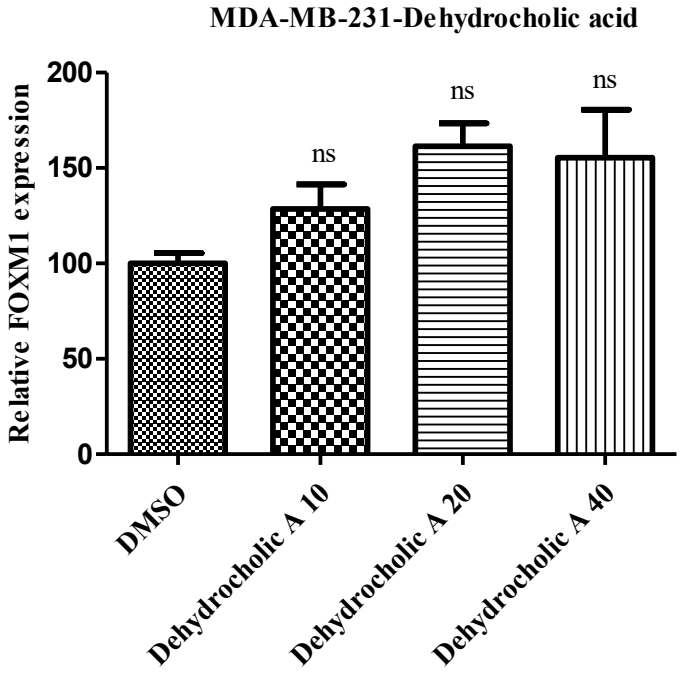


Figure 27: FOXM1 protein was separated by gel electrophoresis and detected using the enhanced chemiluminescence method. The graph represents the relative amount of FOXM1 protein normalizedto β-actin in MDA-MB-231 triple negative breast cancer cells under different concentrations (DMSO, 10 µM, 20 µM, 40) of dehydrocholic acid after 24 h of incubation. Results are expressed as the means ± SEM, N=3. Statistical analysis was performed by using one-way ANOVA followed by Tukey test. ns means not significant.

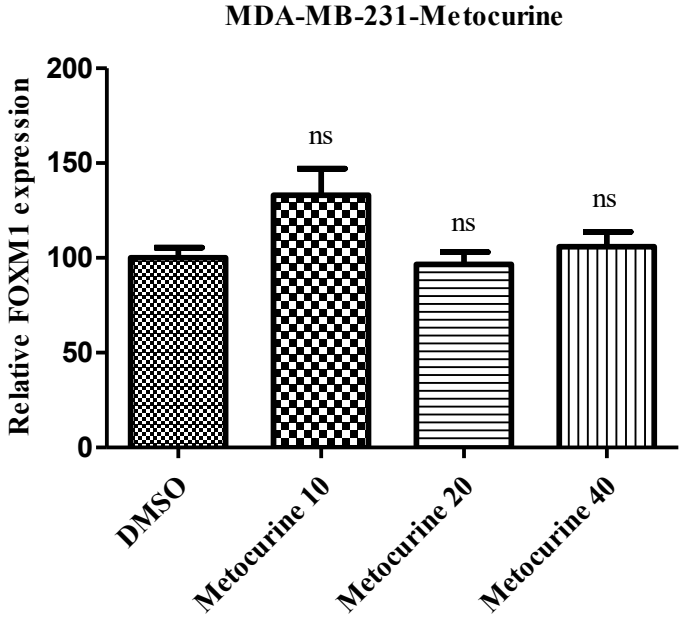


Figure 28: FOXM1 protein was separated by gel electrophoresis and detected using the enhanced chemiluminescence method. The graph represents the amount of FOXM1 protein normalizedto β-actin in MDA-MB-231 triple negative breast cancer cells under different concentrations (DMSO, 10 µM, 20 µM, 40) of metocurine after 24 h of incubation. Results are expressed as the means ± SEM, N=3. Statistical analysis was performed by using one-way ANOVA followed by Tukey test. ns means not significant.

3.4. EMSA assay:

This assay is probably the most important in terms of evaluating the ability of the test drugs to inhibit, directly, binding interactions between the FOXM1-DBD and DNA. We were looking for shifts in bands for the FOXM-DBD when incubated with increasing concentrations of the test drugs, relative to the band detected for the protein in the absence of the drug. Unfortunately, the assay did not work well as the FOXM1 protein-DNA complex apparently "got stuck" at the top of the gel and no shift was observed, even with the positive control (FDI-6). Consequently, despite several efforts aimed to correct this issue, we were unable to do so. These are some of the attempts we made:

- We changed the polyacrylamide gel concentration from 6% to 3% in order to make it easier for the complex to run into the gel. This change had no effect and the issue persisted.
- We changed the gel and buffer pH from 7.0 to 9.5 in order to change the isoelectric point of the protein, and make the protein/DNA complex more negative, trying to promote its entrance into the gel. This change did not produce any changes and we could not address the issue.
- We changed the protein concentration from 25 µg/µL to 100 ng/µL of the stock (2.5 mg/mL) because high protein/nucleic acid ratio did not allow DNA to migrate through the gel (figure 29); however; free DNA migrated normally while its mobility unchanged in presence of low protein concentration (100 ng/µL; figure 30).

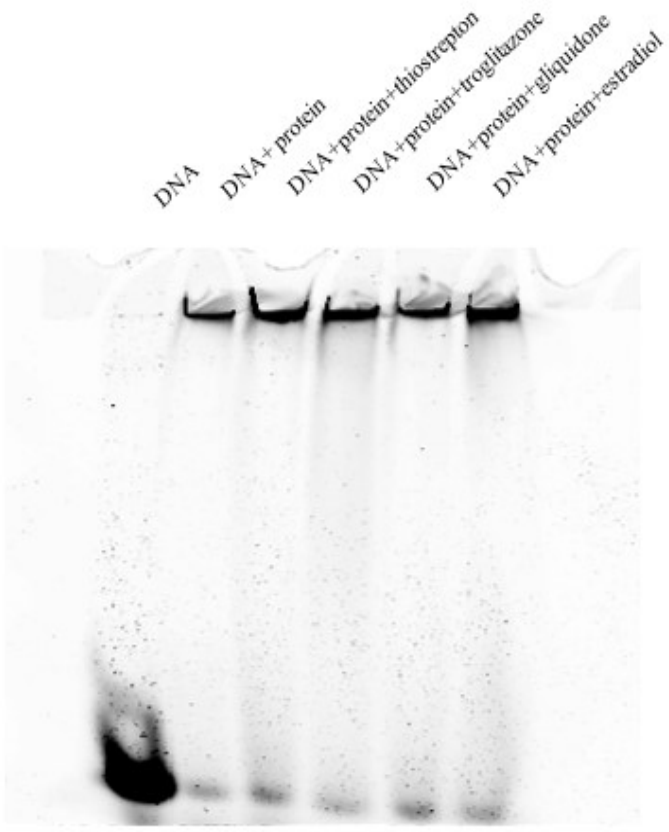


Figure 29: Representative EMSA image shows the association between 25 μM of the stock (2.5 mg/mL) FOXM1-DBD with its DNA sequence in presence of 50 μM of thiostrepton (positive control), troglitazone, gliquidone, and β-estradiol-3-benzoate, using DNA and DNA + protein as negative controls; 20 minutes' incubation period.

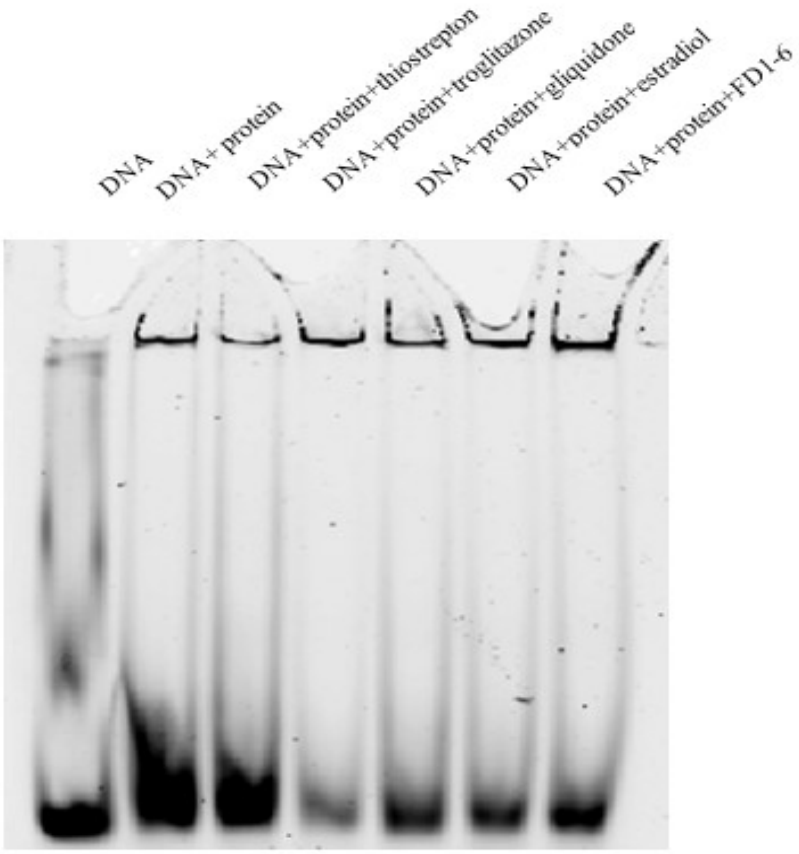


Figure 30: Representative EMSA image shows the association between 100 ng/µL of the stock (2.5 mg/mL) FOXM1-DBD with its DNA sequence in presence of 50 µM of thiostrepton (positive control), troglitazone, gliquidone, β-estradiol-3-benzoate and FDI-6 (second positive control), using DNA and DNA + protein as negative controls; 20 minutes' incubation period.

Nevertheless, as mentioned above, none of the changes resulted in a favorable outcome as we observed the FOXM1-DNA complex always on top of the gel (no run into the gel), which made our screening assay unreliable. Another possible explanation could be due to protein aggregation on top of the gel.

One possible solution to this problem may consist in extracting the nuclear protein after treating the cells with different drug concentrations instead of using the recombinant

FOXM1-DBD (only the DNA binding domain). Nevertheless, I was unable to explore this possibility due to time limitations, and this will have to be addressed by someone in Dr. Velazquez's group in the near future. In the meantime, the EMSA assay was deemed to be inconclusive at this point, and the screening assays based on cell proliferation inhibition, as well as western blotting are the only two screening techniques with some degree of validity to determine the effects of the test molecules. We acknowledge this as an important limitation to our study.

3.5. Cell line authentication:

Because of the unresponsiveness of MCF-7 to the drugs, we wanted to confirm the identity of these cells to rule out any mutations. We prepared the nuclear extracts of the three cell lines and sent them to the Center of Applied Genomics (TCAG) Genetic Analysis Facility in Toronto to check their genomic integrity.

The cell line authentication report showed that the MCF-7 and the MCF-10A cells matched the specifications of MCF7 (ATCC® HTB-22™) and MCF-10A (ATCC® CRL-10317™), respectively (100%), whereas the MDA-MB-231 showed only 83% overall match (only 15 out of 18 alleles compared to the profile for MDA-MB-231; ATCC® HTB-26™).

Table 8: Cell lines authentication report.

GP-VEL1501	**MCF-10A**	**GenePrint**	**D21S11**	**28**	**30**	**not tested**
GP-VEL1501	MCF-10A	GenePrint	D5S818	10	13	match
GP-VEL1501	MCF-10A	GenePrint	D7S820	10	11	match
GP-VEL1501	MCF-10A	GenePrint	TH01	8	9.3	match
GP-VEL1501	MCF-10A	GenePrint	TPOX	9	11	match
GP-VEL1501	MCF-10A	GenePrint	vWA	15	17	match
Overall match: 100%						
GP-VEL1501	MCF-7	GenePrint	AMEL	X	X	match
GP-VEL1501	MCF-7	GenePrint	CSF1PO	10	10	match
GP-VEL1501	MCF-7	GenePrint	D13S317	11	11	match
GP-VEL1501	MCF-7	GenePrint	D16S539	11	12	match
GP-VEL1501	MCF-7	GenePrint	D21S11	30	30	match
GP-VEL1501	MCF-7	GenePrint	D5S818	11	12	not tested
GP-VEL1501	MCF-7	GenePrint	D7S820	8	9	match
GP-VEL1501	MCF-7	GenePrint	TH01	6	6	match
GP-VEL1501	MCF-7	GenePrint	TPOX	9	12	match
GP-VEL1501	MCF-7	GenePrint	vWA	14	15	match
Overall match: 100%						
GP-VEL1501	MDA-MB-231	GenePrint	AMEL	X	X	match
GP-VEL1501	MDA-MB-231	GenePrint	CSF1PO	12	13	match
GP-VEL1501	MDA-MB-231	GenePrint	D13S317	13	13	match
GP-VEL1501	MDA-MB-231	GenePrint	D16S539	12	12	match
GP-VEL1501	MDA-MB-231	GenePrint	D21S11	30	33.2	not tested
GP-VEL1501	MDA-MB-231	GenePrint	D5S818	12	12	match
GP-VEL1501	MDA-MB-231	GenePrint	D7S820	8	8	1 allele shared
GP-VEL1501	MDA-MB-231	GenePrint	TH01	7	9.3	match
GP-VEL1501	MDA-MB-231	GenePrint	TPOX	9	9	1 allele shared
GP-VEL1501	MDA-MB-231	GenePrint	vWA	15	15	1 allele shared
Overall match: 83%						

Taken together, these results suggest that:

- Thiostrepton acts as a potent anticancer agent that supresses the proliferation of different breast cancer cells (127). Nagaratna *et al*. established that thiostrepton selectively supresses the growth of cancer cells by direct interaction to FOXM1 transcription protein leading to downregulation of important downstream target genes, that play essential roles in cell cycle progression including CDC25B, CCNB1 and MYC, without affecting the untransformed MCF-10A cells (189). However, our results revealed that the drug is not selective and it is toxic to normal breast cells, as it also inhibited the proliferation of untransformed MCF-10A cells.

  Thiostrepton is also a proteasome inhibitor that downregulates FOXM1 expression by hindering the proteasomal degradation of its negative regulators (NRFM) as reported by Gartel *et al*. (112). Inhibiting the proteasome activity also affects their crucial pathways leading to disruption of a wide range of physiological and pathological processes (180). Additionally, Gormally *et al*. illustrated that thiostrepton is cytotoxic at a concentration significantly below the concentration required to inhibit FOXM1 protein expression, indicating that the cytotoxic effect exerted by thiostrepton involves additional FOXM1-independent pathways (131).

- As reported in previous studies, troglitazone is a relatively potent inhibitor of cancer cells proliferation. Our results showed that troglitazone inhibited the proliferation of MDA-MB-231 breast cancer cells and significantly inhibited protein expression at low concentrations, suggesting that protein expression is critical for inhibiting breast cancer cell viability. Nevertheless, troglitazone also inhibited the viability of MCF-10A cells, indicating that the drug is non-selective, and is likely to exert FOXM1-

independent cell proliferation inhibition. In this regard, it is well established that troglitazone acts as anti-cancer agent via different mechanisms including: a) suppressing ERK phosphorylation and upregulating p21 leading the inhibition of stomach cancer cells growth (160); b) suppressing FOXM1 expression via downregulating sp1 activities by PPARγ-independent pathways (161–163); and c) elevating ROS level via reducing the mitochondrial mass and suppressing superoxide dismutase expression resulting in induced expression of cell cycle inhibitor $p21^{WAF1}$ (164).

In a study carried out by Shiau *et al.* they devoid troglitazone from its PPARγ-agonist effect by introducing a double bond surrounding the thiazolidinedione ring. This study showed that the PPARγ-inactive analogues of troglitazone, ciglitazone induce apoptosis in prostate cancer cells via inhibiting the antiapoptotic Bcl-xL and Bcl-2 (190).

Direct binding interaction between the drug and FOXM1-DBD could be another anti-cancer mechanism of action of this particular drug based on our results, to prove that we need to perform EMSA assay to determine if there is shift in band when the recombinant protein incubated with increasing concentrations of troglitazone, relative to the band detected for the recombinant protein in the absence of the drug.

Since we identified that the anti-diabetic drug troglitazone as the most promising inhibitor (among those we tested) of triple negative cancer cell viability, as well as inhibition of FOXM1 protein expression, our group is taking this lead forward by designing a series of troglitazone derivatives. The main idea behind this new series of compounds seem to inhibit FOXM1 transcriptional activity through a π-sulfur

interaction involving troglitazone's thiazolidinedione ring and a His287 residue in the FOXM1 – DBD. In addition to blocking PPARγ-agonist effect of troglitazone based on the study carried out by Shiau *et al*., further modification of the chemical structure of troglitazone derivatives could block off-target activities of the drug in term of medicinal chemistry.

- β-estradiol-3-benzoate showed a potent inhibitory effect on the proliferation of both cancer and non-cancer cells except for the slow growing MCF-7. However, results in the western blot screening assay showed an increase in FOXM1 expression, rather than a decrease.

  Even though the drug possesses anti-cancer activity as described by Lee *et al*., he also found that the lack of selectivity of this hormone (between cancer and normal cells) precludes its use as an anticancer drug (167).
- The antihyperglycemic drug gliquidone decreased the proliferation of normal breast cells only, and did not show any effect on the viability of cancer cells, which appear to be resistant to this drug under our experimental conditions. Interestingly, the drug gradually inhibited FOXM1 expression in a concentration-dependent fashion. However, further investigation about whether the drug inhibited protein expression by a direct or an indirect mechanism is still needed.
- The last two drugs, dehydrocholic acid and metocurine did not show any inhibitory activities on cell proliferation or FOXM1 protein expression.
- In this project, we could not prove or disprove the screening protocol, because MTT and WB assays we applied are indirect indicators to the mechanism of action and cannot determine whether the inhibition of cell viability and protein expression is due

to direct or indirect binding interaction between the drugs and FOXM1-DBD. However, their results suggested that the selected drugs act by FOXM1-independent pathways as they inhibited the proliferation of non-cancer MCF-10A cells which showed the lowest level of FOXM1 protein expression.

Additionally, β-estradiol and dehydrocholic acid which had low free binding energy values (-17.3 ± 5.6 and - 41.0 ± 7,8 kcal/mol respectively), were proposed to have higher affinity and blocking effect on FOXM1-DBD; however, WB results illustrated that the drugs induced the protein expression instead of inhibiting it and it is suggested that the drugs have off-target effects as they seemed to induce the upstream upregulators of FOXM1 or may have promoting effect on FOXM1 protein itself; however, the exact mechanisms of action are not clear.

The only way to correlate between the docking study and direct binding of the drugs to FOXM1-DBD and the subsequent effects on the cell proliferation and protein expression is by achieving EMSA or one of its alternatives in the future.

Conclusion:

FOXM1 is a subfamily of transcription proteins which are responsible for the expression of various proteins essential for DNA replication and mitosis. Large-scale gene expression analysis has identified FOXM1 as one of commonly upregulated genes in the early stages of carcinogenesis, therefore, upregulation of FOXM1 expression has been regarded as a hallmark of different types of human carcinogenesis (22,27). The aim of this project was to determine if a computer-based drug design approach on FDA-approved drugs could provide interesting scaffolds which could be used in the future to design new FOXM1 inhibitors. We first identified compounds that could inhibit the transcriptional activity of FOXM1 directly *in silico* and then examined their ability to interfere with transcriptional activity of FOXM1 *in vitro.*

Our results demonstrate that thiostrepton, troglitazone and β-estradiol-3-benzoate are cytostatic to breast cancer cells, but they are not selective. We identified the drug troglitazone as a potent lead inhibitor of breast cancer cell viability and FOXM1 protein expression at low concentrations (MDA-MB-231 cancer cells). Gliquidone is non-toxic to breast cancer cells but supressed FOXM1 protein expression to some extent, which makes this drug an interesting scaffold to be explored in the future. Dehydrocholic acid and metocurine are not direct or indirect inhibitors of FOXM1 protein expression, because we did not observe any effect on breast cancer cell viability or protein levels. However, we need to perform EMSA or other alternative assay to correlate between the *in-silico* model results and studies conducted to test the drugs on cell lines.

Based on our findings, our research group will work on derivatives of troglitazone that exert binding interactions involving a His287 residue, present in the FOXM1 – DNA

binding pocket. This binding interaction seems to be the main driving force guiding FOXM1 inhibition.

Future Directions:

1. Gliquidone and troglitazone were the only drugs that showed inhibitory effect on the protein expression which make them interesting scaffolds for further exploration in the future. To determine whether the downregulation of FOXM1 protein is due to the inhibition of FOXM1 transcription, we will need to measure the mRNA level using quantitative Real Time-PCR (qRT-PCR).
2. To validate the *in-silico* model results, which showed the direct binding between FOXM1-DBD and the test drugs, we will need to address the issues associated with the EMSA assay or perform other alternatives such as cellular thermal shift assay (CETSA) or pull-down assay. Without one of these essential screening assays, we do not know at this point if the protein expression suppression exerted by the active drugs is due to a direct or an indirect mechanism. By applying one of these assays we should see a decrease in the binding interaction between FOXM1-DBD and its DNA target sequence.
3. Upon the confirmation of validation process, we can procced with derivatization of the dugs which have inhibitory effect on the protein expression including troglitazone and/or gliquidone to narrow down their targets and increase their selectivity toward FOXM1 protein in cancer cells.
4. To determine whether the troglitazone effect is FoxM1-mediated or PPARγ-mediated, different approches could be done. First, we can compare FOXM1 protein level in MDA-MB-231 cells treated with other PPARγ-agonist such as rosiglitazone. If rosiglitazone did not supress FOXM1 expression that will indicate that troglitazne acts by FoxM1-mediated effect. Second, we can use PPARγ-inactive analogues of

troglitazone and test its effect on FOXM1 protein expression level. However, if FOXM1 expression was suppressed can mean troglitazone FoxM1-mediated effect.

5. Since in this project we testsd only the effect of the drugs on cell viability, future work could involve determining whether the apoptosis is the mechnism of the cytotoxicity of the active drugs on cancer cells. Microscopy or flow cytometry could be applied to count the apoptotic cancer cells after treatment with the test drugs.

References:

1. Cancer Facts and Figures 2015. American Cancer Society. 2015.

2. Chambers AF, Groom AC, MacDonald IC. Metastasis: Dissemination and growth of cancer cells in metastatic sites. Nat Rev Cancer. 2002 Aug;2(8):563–72.

3. Canadian Cancer Society. Canadian Cancer Statistics Special topic : Predictions of the future burden of cancer in Canada. Public Heal Agency Canada. 2015;1–151.

4. Morris CR, Epstein J, Nassere K, Hofer BM, Rico J, Bates JH SK. Trends in Cancer Incidence, Mortality, Risk Factors, Health Behaviors in California. Sacramento, CA: California Department of Public Health, Cancer Surveillance Section. 2010.

5. Chorawala MR, Oza PM, Shah GB. Mechanisms of Anticancer Drugs Resistance : An Overview. Int J Pharm Sci Drug Res. 2012;4(1):1–9.

6. Housman G, Byler S, Heerboth S, Lapinska K, Longacre M, Snyder N, et al. Drug resistance in cancer: an overview. Cancers (Basel). 2014;6(3):1769–92.

7. Thackray VG. Molecular and Cellular Endocrinology Fox tales : Regulation of gonadotropin gene expression by forkhead transcription factors. Mol Cell Endocrinol. Elsevier Ireland Ltd; 2014;385(1–2):62–70.

8. Mencalha L, Binato R, Ferreira GM, Rocher B Du, Abdelhay E. Forkhead Box M1 (FoxM1) Gene Is a New STAT3 Transcriptional Factor Target and Is Essential for Proliferation , Survival and DNA Repair of K562 Cell Line. PLoS One. 2012;7(10):1–10.

9. Wijchers PJEC, Burbach JPH, Smidt MP. In control of biology : of mice , men and Foxes. Biochem. 2006;246(397):233–46.

10. Levinson RS, Batourina E, Choi C, Vorontchikhina M, Kitajewski J, Mendelsohn CL. Foxd1-dependent signals control cellularity in the renal capsule , a structure required for normal renal development. Development. 2005;132(3):529–39.

11. Crisponi L, Deiana M, Loi A, Chiappe F, Uda M, Amati P, et al. The putative forkhead transcription factor FOXL2 is mutated in blepharophimosis / ptosis / epicanthus inversus syndrome. Nat Genet. 2001;27(2):159–66.

12. Uhlenhaut NH, Treier M. Forkhead transcription factors in ovarian function. Reproduction. 2011;142(4):489–95.

13. Lai CSL, Fisher SE, Hurst JA, Vargha-khadem F. A forkhead-domain gene is mutated in a severe speech and language disorder. Nature. 2001;413(6855):519–23.

14. Wildin RS, Ramsdell F, Faravelli F, Buist N, Levy- E, Mazzella M, et al. The

immune dysregulation , polyendocrinopathy , enteropathy , X-linked syndrome (IPEX) is caused by mutations of FOXP3. Nat Genet. 2001;27(1):20–1.

15. Accili D, Arden KC. FoxOs at the Crossroads of Cellular Review Metabolism , Differentiation , and Transformation. Cell. 2004;117(4):421–6.

16. Birkenkamp KU, Coffer PJ. Regulation of cell survival and proliferation by the FOXO (Forkhead box , class O) subfamily of Forkhead transcription factors. Biochem Soc Trans. 2003;31(Pt 1):292–7.

17. Zona S, Bella L, Burton MJ, Nestal de Moraes G, Lam EW-F. FOXM1: An emerging master regulator of DNA damage response and genotoxic agent resistance. Biochim Biophys Acta - Gene Regul Mech. Elsevier B.V.; 2014;1839(11):1316–22.

18. Wang Z, Ahmad A, Li Y, Banerjee S, Kong D, Sarkar FH. Forkhead box M1 transcription factor : A novel target for cancer therapy. Cancer Treat Rev. Elsevier Ltd; 2010;36(2):151–6.

19. Korver W, Roose J, Heinen K, Weghuis O, Bruijn D De, Kessel AG Van, et al. The Human TRIDENT / HFH-11 / FKHL16 Gene : Structure , Localization , and Promoter Characterization. Genomics. 1997;442(46):435–42.

20. Katoh M, Igarashi M, Fukuda H, Nakagama H, Katoh M. Cancer genetics and genomics of human FOX family genes. Cancer Lett. Elsevier Ireland Ltd; 2013;328(2):198–206.

21. Park HJ, Wang Z, Costa RH, Tyner A, Lau LF, Raychaudhuri P. An N-terminal inhibitory domain modulates activity of FoxM1 during cell cycle. Oncogene. 2008;27(12):1696–704.

22. Laoukili J, Stahl M, Medema RH. FoxM1: At the crossroads of ageing and cancer. Biochim Biophys Acta - Rev Cancer. 2007;1775(1):92–102.

23. Helma D.C. Schwenen, L.J.M. MSCW and EW-FL. FOXM1 Homo sapiens forkhead box M1. Transcr Factor Encycl p 4. 2009;

24. Kim YH, Choi MH, Kim J, Lim IK, Park TJ. C-terminus-deleted FoxM1 is expressed in cancer cell lines and induces chromosome instability. Carcinogenesis. 2013;34(8):1907–17.

25. Ye H, Kelly TF, Samadani U, Lim L, Rubio S, Overdier DG, et al. Hepatocyte Nuclear Factor 3 / fork head Homolog 11 Is Expressed in Proliferating Epithelial and Mesenchymal Cells of Embryonic and Adult Tissues. Mol Cell Biol. 1997;17(3):1626–41.

26. Korver W, Roose J, Clevers H. The winged-helix transcription factor Trident is expressed in cycling cells. Nucleic Acids Res. 1997;25(9):1715–9.

27. Pilarsky C, Wenzig M, Specht T, Saeger HD, Gru R. Identification and Validation of Commonly Overexpressed Genes in Solid Tumors by Comparison of Microarray Data. Neoplasia. 2004;6(6):744–50.

28. Sloan M, Cancer K. G1 cell-cycle control and cancer. Nat Publ Gr. 2004;432(7015):298–306.

29. Wang I, Chen Y, Hughes D, Petrovic V, Major ML, Park HJ, et al. Forkhead Box M1 Regulates the Transcriptional Network of Genes Essential for Mitotic Progression and Genes Encoding the SCF (Skp2-Cks1) Ubiquitin Ligase. Mol Cell Biol. 2005;25(24):10875–94.

30. Ma RYM, Tong THK, Cheung AMS, Tsang ACC, Leung WY, Yao K. Raf / MEK / MAPK signaling stimulates the nuclear translocation and transactivating activity of FOXM1c. Cell Sci. 2005;118(4):795–806.

31. Laoukili J, Alvarez M, Meijer LAT, Stahl M, Mohammed S, Kleij L, et al. Activation of FoxM1 during G 2 Requires Cyclin A / Cdk-Dependent Relief of Autorepression by the FoxM1 N-Terminal Domain. Mol Cell Biol. 2008;28(9):3076–87.

32. Chen Y, Dominguez-brauer C, Wang Z, Asara JM, Costa RH, Tyner AL, et al. A Conserved Phosphorylation Site within the Forkhead Domain of FoxM1B Is Required for Its Activation by Cyclin-CDK1. Biol Chem. 2009;284(44):30695–707.

33. Stein A, Hibbert RG, Heidebrecht T. Structure of the FoxM1 DNA-recognition domain bound to a promoter sequence. Nucleic Acids Res. 2010;38(13):4527–38.

34. Fu Z, Malureanu L, Huang J, Wang W, Li H, Deursen JM Van, et al. Plk1-dependent phosphorylation of FoxM1 regulates a transcriptional programme required for mitotic progression. Nat Cell Biol. 2008;10(9):1076–82.

35. Leung TWC, Lin SSW, Tsang ACC, Tong CSW, Ching JCY, Ying W, et al. Over-expression of FoxM1 stimulates cyclin B1 expression. FEBS. 2001;507(1):59–66.

36. Wang M, Gartel AL. The suppression of FOXM1 and its targets in breast cancer xenograft tumors by siRNA. Oncotarget. 2011;2(12):1218–26.

37. Korver W, Schilham MW, Moerer P, Den MJ Van, Dam K, Lamers WH, et al. Uncoupling of S phase and mitosis in cardiomyocytes and hepatocytes lacking the winged-helix transcription factor Trident. Curr Biol. 1998;8(24):1327–30.

38. Sullivan C, Liu Y, Shen J, Curtis A, Newman C, Hock JM. Novel Interactions between FOXM1 and CDC25A Regulate the Cell Cycle. PLoS One. 2012;7(12):1–13.

39. Bornstein G, Bloom J, Sitry-shevah D, Nakayama K, Pagano M, Hershko A. Role of the SCF Skp2 Ubiquitin Ligase in the Degradation of p21 Cip1 in S Phase *. Biol

Chem. 2003;278(28):25752–7.

40. Carrano AC, Eytan E, Hershko A, Pagano M. SKP2 is required for ubiquitin-mediated degradation of the CDK inhibitor p27. Nat Cell Biol. 1999;1(4):193–9.

41. Ganoth D, Bornstein G, Ko TK, Larsen B, Tyers M, Pagano M, et al. The cell-cycle regulatory protein Cks1 is required for SCF Skp2 -mediated ubiquitinylation of p27. Nat Cell Biol. 2001;3(3):321–4.

42. Sherr CJ, Roberts JM. PERSPECTIVE CDK inhibitors : positive and negative regulators of G 1 -phase progression. GENES Dev. 1999;13(901):1501–12.

43. Wang X, Kiyokawa H, Dennewitz MB, Costa RH. The Forkhead Box m1b transcription factor is essential for hepatocyte DNA replication and mitosis during mouse liver regeneration. PNAS. 2002;99(26):16881–6.

44. Lammer C, Wagerer S, Saffrich R, Mertens D, Ansorge W, Hoffmann I, et al. The cdc25B phosphatase is essential for the G 2 / M phase transition in human cells. Cell Sci. 1998;111(Pt 16):2445–53.

45. Barr FA, Silljé HHW, Nigg EA, Martinsried D-. POLO-LIKE KINASES AND THE ORCHESTRATION OF CELL DIVISION. Nat Publ Gr. 2004;5(6):429–40.

46. Meraldi P, Honda R, Nigg EA. Aurora kinases link chromosome segregation and cell division to cancer susceptibility. Curr Opin Genet Dev. 2004 Feb;14(1):29–36.

47. Lens, Susanne M.A. Medema RH. The Survivin / Aurora B Complex: Its Role in Coordinating Tension and Attachment. Cell Cycle. 2003;2(6):507–10.

48. Amor DJ, Kalitsis P, Sumer H, Choo KHA. Building the centromere : from foundation proteins to 3D organization. Trends Cell Biol. 2004;14(7):359–68.

49. Wonsey DR, Follettie MT. Loss of the forkhead transcription factor FoxM1 causes centrosome amplification and mitotic catastrophe. Cancer Res. 2005;65(12):5181–9.

50. Benz CC, Yau C. Ageing, oxidative stress and cancer: paradigms in parallax. Nat Rev Cancer. 2008;8(11):875–80.

51. Park HJ, Carr JR, Wang Z, Nogueira V, Hay N, Tyner AL, et al. FoxM1 , a critical regulator of oxidative stress during oncogenesis. EMBO J. Nature Publishing Group; 2009;28(19):2908–18.

52. Dro W, Immunochemistry D, Krebsforschungszentrum D. Free Radicals in the Physiological Control of Cell Function. Physiol Rev. 2002;82(1):47–95.

53. Gutteridge JMC, Halliwell B. Free Radicals and Antioxidants in the Year 2000 A Historical Look to the Future. Ann N Y Acad Sci. 2000;899:136–47.

54. Wu Y, Zhou BP. TNF- a / NF- k B / Snail pathway in cancer cell migration and invasion. Br J Cancer. 2010;102(4):639–44.

55. Xia L, Huang W, Wang B, Liu M, Zhang Q, Yan W, et al. Transcriptional Up-Regulation of FoxM1 in Response to Hypoxia Is Mediated by HIF-1. J Cell Biochem. 2009;106(2):247–56.

56. Sebolt-leopold JS, Herrera R. TARGETING THE MITOGEN- ACTIVATED PROTEIN KINASE CASCADE TO TREAT CANCER. Nat Rev CANCER. 2004;4(12):937–47.

57. Musgrove EA, Caldon CE, Barraclough J, Stone A, Sutherland RL. Cyclin D as a therapeutic target in cancer. Nat Rev Cancer. 2011;11(8):558–72.

58. Rader J, Russell MR, Hart LS, Nakazawa MS, Belcastro LT, Martinez D, et al. Dual CDK4 / CDK6 Inhibition Induces Cell-Cycle Arrest and Senescence in Neuroblastoma. Clin Cancer Res. 2013;19(22):6173–82.

59. Anders L, Ke N, Hydbring P, Choi YJ, Widlund HR, Chick JM, et al. A Systematic Screen for CDK4 / 6 Substrates Links FOXM1 Phosphorylation to Senescence Suppression in Cancer Cells. Cancer Cell. Elsevier Inc.; 2011;20(5):620–34.

60. Hospital ST. Breast cancer : further metabolic-endocrine risk markers ? Br J Cancer. 1997;76(12):1652–4.

61. Khan SA, Mary AM, Khurana KK, Meguid MM, Numann PJ. Estrogen Receptor Expression in Benign Breast Epithelium and Breast Cancer Risk. Natl Cancer Inst. 1998;90(1):37–42.

62. Saba R, Alsayed A, Zacny JP, Dudek AZ. The Role of Forkhead Box Protein M1 in Breast Cancer Progression and Resistance to Therapy. Int J Breast Cancer. 2016;2016:1–8.

63. Madureira PA, Varshochi R, Constantinidou D, Francis RE, Coombes RC, Yao K, et al. The Forkhead Box M1 Protein Regulates the Transcription of the Estrogen Receptor alpha in Breast Cancer Cells * □. Biol Chem. 2006;281(35):25167–76.

64. Millour J, Constantinidou D, Stavropoulou A V, Myatt SS, Kwok J. FOXM1 is a transcriptional target of ER a and has a critical role in breast cancer endocrine sensitivity and resistance. Oncogene. Nature Publishing Group; 2010;29(20):2983–95.

65. Yarden Y, Sliwkowski MX. UNTANGLING THE ErbB SIGNALLING NETWORK. Nat Rev Mol CELL Biol. 2001;2(2):127–37.

66. Zhang H, Berezov A, Wang Q, Zhang G, Drebin J, Murali R, et al. ErbB receptors : from oncogenes to targeted cancer therapies. Clin Investig (Lond). 2007;117(8):2051–8.

67. Francis RE, Myatt SS, Krol J, Hartman J, Peck B, Mcgovern UB, et al. FoxM1 is a downstream target and marker of HER2 overexpression in breast cancer. Int J Oncol. 2011;35(1):57–68.

68. Manuscript A, Death C. How ERK1/2 Activation Controls Cell Proliferation and Cell Death Is Subcellular Localization the Answer? Cell Cycle. 2009;8(8):1168–75.

69. Calvisi DF, Pinna F, Ladu S, Pellegrino R, Simile MM, Frau M, et al. Forkhead box M1B is a determinant of rat susceptibility to hepatocarcinogenesis and sustains ERK activity in human HCC. Gut. 2009;58(5):679–87.

70. Huber MA, Azoitei N, Baumann B, Grünert S, Sommer A, Pehamberger H, et al. NF- κ B is essential for epithelial- mesenchymal transition and metastasis in a model of breast cancer progression. Clin Investig (Lond). 2004;114(4):569–81.

71. Marianna Penzo, Paul E. Massa, Eleonora Olivotto, Francesca Bianchi RM, Borzi, Adedayo Hanidu, Xiang Li, Jun Li6 and KBM. Sustained NF-κB activation produces a short-term cell proliferation block in conjunction with repressing effectors of cell cycle progression controlled by E2F or FoxM1. Cell Physiol. 2009;218(1):215–27.

72. Arora R, Yates C, Gary BD, Mcclellan S, Tan M, Xi Y, et al. Panepoxydone Targets NF-kB and FOXM1 to Inhibit Proliferation , Induce Apoptosis and Reverse Epithelial to Mesenchymal Transition in Breast Cancer. PLoS One. 2014;9(6):1–12.

73. Vousden KH, Prives C. Blinded by the Light : The Growing Complexity of p53. Cell. 2009;137(3):413–31.

74. Pandit B, Halasi M, Gartel AL, Pandit B, Halasi M, Gartel AL. p53 negatively regulates expression of FoxM1. Cell Cycle. 2009;8(20):3425–7.

75. Gomes NP. Gene-specific repression of the p53 target gene PUMA via intragenic CTCF – Cohesin binding. GENES Dev. 2010;24(10):1022–34.

76. Yu J, Wang Z, Kinzler KW, Vogelstein B, Zhang L. PUMA mediates the apoptotic response to p53 in colorectal cancer cells. PNAS. 2003;100(4):1931–6.

77. Miyashita T, Reed JC. Tumor Suppressor p53 Is a Direct Transcriptional Activator of the Human bax Gene. Cell. 1995;80(2):293–9.

78. Gomes NP. Gene-specific repression of the p53 target gene PUMA via intragenic CTCF – Cohesin binding. GENES Dev. 2010;24(10):1022–34.

79. Sur S, Pagliarini R, Bunz F, Rago C, Diaz LA, Kinzler KW, et al. A panel of isogenic human cancer cells suggests a therapeutic approach for cancers with inactivated p53. PNAS. 2009;106(10):3964–9.

80. Myatt SS, Lam EWF. The emerging roles of forkhead box (Fox) proteins in cancer.

Nat Rev Cancer. 2007;7:847–59.

81. Nakamura N, Ramaswamy S, Vazquez F, Signoretti S, Loda M, Sellers WR. Forkhead Transcription Factors Are Critical Effectors of Cell Death and Cell Cycle Arrest Downstream of PTEN. Mol Cell Biol. 2000;20(23):8969–82.

82. He L, Yang X, Cao X, Liu FEI, Quan M, Cao J. Casticin induces growth suppression and cell cycle arrest through activation of FOXO3a in hepatocellular carcinoma. Oncol Rep. 2013;29(1):103–8.

83. Clark PA, Stott F, Ludwig RL, Vousden KH. p19ARF links the tumour suppressor p53 to Ras. Nature. 1998;395(6698):125.

84. Kalinichenko V V, Major ML, Wang X, Petrovic V, Kuechle J, Yoder HM, et al. Foxm1b transcription factor is essential for development of hepatocellular carcinomas and is negatively regulated by the p19 ARF tumor suppressor. GENES Dev. 2004;18(7):830–50.

85. Bella L, Zona S, Moraes GN De, Lam EW. Seminars in Cancer Biology FOXM1 : A key oncofoetal transcription factor in health and disease. Semin Cancer Biol. Elsevier Ltd; 2014;29:32–9.

86. Zeng J, Wang L, Li Q, Li W, Björkholm M, Jia J XD. FoxM1 is up-regulated in gastric cancer and its inhibition leads to cellular senescence , partially dependent on p27 kip1. Pathology. 2009;218(4):419–27.

87. Koo C-Y, Muir KW, Lam EW-F. FOXM1: From cancer initiation to progression and treatment. Biochim Biophys Acta. Elsevier B.V.; 2012;1819(1):28–37.

88. Ahmad, Aamir, Zhiwei Wang, Dejuan Kong, Shadan Ali, Yiwei Li, Sanjeev Banerjee, Raza Ali FHS. FoxM1 down-regulation leads to inhibition of proliferation, migration and invasion of breast cancer cells through the modulation of extra-cellular matrix degrading factors. Breast Cancer Res Treat. 2010;122(2):337–46.

89. Yoshida Y, Wang IC, Yoder HM, Davidson NO, Costa RH. The Forkhead Box M1 Transcription Factor Contributes to the Development and Growth of Mouse Colorectal Cancer. Gastroenterology. 2007;132(4):1420–31.

90. Wang Z, Banerjee S, Kong D, Li Y, Sarkar FH. Down-regulation of Forkhead Box M1 Transcription Factor Leads to the Inhibition of Invasion and Angiogenesis of Pancreatic Cancer Cells. Cancer Res. 2007;67(17):8293–300.

91. Jung H, Gusarova G, Wang Z, Carr JR, Li J, Kim K, et al. Deregulation of FoxM1b leads to tumour metastasis. EMBO Mol Med. 2011;3(1):21–34.

92. Faust D, Al-butmeh F, Linz B, Dietrich C. Involvement of the transcription factor FoxM1 in contact inhibition. Biochem Biophys Res Commun. Elsevier Inc.;

2012;426(4):659–63.

93. Manuscript A. FOXO3a mediates the cytotoxic effects of cisplatin in colon cancer cells. Mol Cancer Ther. 2008;7(10):3237–46.

94. Hui RC, Francis RE, Guest SK, Costa JR, Gomes AR, Myatt SS, et al. Doxorubicin activates FOXO3a to induce the expression of multidrug resistance gene ABCB1 (MDR1) in K562 leukemic cells. MolCancer Ther. 2008;7(3):670–8.

95. Mcgovern UB, Francis RE, Peck B, Guest SK, Wang J, Myatt SS, et al. Gefitinib (Iressa) represses FOXM1 expression via FOXO3a in breast cancer. Mol Cancer Ther. 2009;8(3):582–91.

96. Sunters A, Madureira PA, Pomeranz KM, Aubert M, Brosens JJ, Cook SJ, et al. Paclitaxel-Induced Nuclear Translocation of FOXO3a in Breast Cancer Cells Is Mediated by c-Jun NH 2 -Terminal Kinase and Akt. Cancer Res. 2006;66(1):212–20.

97. Bao L, Haque A, Jackson K, Hazari S, Moroz K, Jetly R, et al. Increased Expression of P-Glycoprotein Is Associated with Doxorubicin Chemoresistance in the Metastatic 4T1 Breast Cancer Model. Am J Pathol. Elsevier Inc.; 2011;178(2):838–52.

98. Wang YC, Tsai EM. Role of Estrogen and Xenoestrogen in Chemoresistance of Breast Cancer. J Exp Clin Med. Elsevier Taiwan LLC; 2012;4(6):320–4.

99. Wei Q, He W, Yao J, Guo L, Lu Y, Cao X. Identification and characterization of microRNAs expressed in human breast cancer T-47D cells in response to prolactin treatment by Solexa deep-sequencing technology. Biochem Biophys Res Commun. Elsevier Masson SAS; 2013;432(3):480–7.

100. Nielsen TO, Hsu FD, Jensen K, Cheang M, Karaca G, Hu Z, et al. Immunohistochemical and Clinical Characterization of the Basal- Like Subtype of Invasive Breast Carcinoma. Clin Cancer Res. 2004;10(919):5367–74.

101. Bergamaschi A, Madak-Erdogan Z, Kim Y, Choi Y-L, Lu H, Katzenellenbogen BS. The forkhead transcription factor FOXM1 promotes endocrine resistance and invasiveness in estrogen receptor–positive breast cancer by expansion of stem-like cancer cells. Breast Cancer Res. 2014;16(5):1–18.

102. Karadedou CT, Gomes AR, Chen J, Petkovic M, Ho K, Zwolinska AK, et al. FOXO3a represses VEGF expression through FOXM1-dependent and -independent mechanisms in breast cancer. Oncogene. 2012;31(14):1845–58.

103. Horimoto Y, Hartman J, Millour J, Pollock S, Olmos Y, Ho K, et al. ERb1 Represses FOXM1 Expression through Targeting ER alpha to Control Cell Proliferation in Breast Cancer. Am J Pathol. 2011;179(3):1148–56.

104. Millour J, Olano N De, Horimoto Y, Monteiro LJ, Langer JK, Aligue R, et al. ATM and p53 regulate FOXM1 expression via E2F in breast cancer epirubicin treatment and resistance. Mol Cancer Ther. 2016;10(6):1046–58.

105. Park Y, Jung SY, Jennings NB, Rodriguez-aguayo C, Peng G, Lee S, et al. FOXM1 mediates Dox resistance in breast cancer by enhancing DNA repair. Carcinogenesis. 2012;33(10):1843–53.

106. Yang C, Chen H, Tan G, Gao W, Cheng L, Jiang X, et al. FOXM1 promotes the epithelial to mesenchymal transition by stimulating the transcription of Slug in human breast cancer. Cancer Lett. 2013;340(1):104–12.

107. Radhakrishnan SK, Bhat UG, Hughes DE, Wang I, Costa RH, Gartel AL. Identification of a Chemical Inhibitor of the Oncogenic Transcription Factor Forkhead Box M1. Cancer Res. 2006;66(19):9731–5.

108. Radhakrishnan SK, Gartel AL. FOXM1 : The Achilles ' heel of cancer ? Nat Genet. 2008;8(3):1.

109. Kim I, Ackerson T, Ramakrishna S, Tretiakova M, Wang I, Kalin T V, et al. The Forkhead Box m1 Transcription Factor Stimulates the Proliferation of Tumor Cells during Development of Lung Cancer. Cancer Res. 2006;66(21):2153–61.

110. Kalin T V, Wang I, Ackerson TJ, Major ML, Carol J, Kalinichenko V V, et al. Increased Levels of the FoxM1 Transcription Factor Accelerate Development and Progression of Prostate Carcinomas in both TRAMP and LADY Transgenic Mice. Cancer Res. 2006;66(3):1712–20.

111. Myatt SS, Lam EW. Targeting FOXM1. Nat Rev Cancer. 2007;8(3):204.

112. Gartel AL, Gartel AL. The oncogenic transcription factor FOXM1 and anticancer therapy. Cancer Cell. 2012;11(18):3341–2.

113. Laoukili J, Kooistra MRH, Brás A, Kauw J, Kerkhoven RM, Morrison A, et al. FoxM1 is required for execution of the mitotic programme and chromosome stability. Nat Cell Biol [Internet]. 2005;7(2):126–36. Available from: http://www.nature.com/doifinder/10.1038/ncb1217

114. Kalinichenko V V., Gusarova GA, Tan Y, Wang IC, Major ML, Wang X, et al. Ubiquitous expression of the Forkhead Box M1B transgene accelerates proliferation of distinct pulmonary cell types following lung injury. J Biol Chem. 2003;278(39):37888–94.

115. Lin J, Zheng Y, Chen KUN, Huang Z. Inhibition of FOXM1 by thiostrepton sensitizes medulloblastoma to the effects of chemotherapy. Oncol Rep. 2013;30(4):1739–44.

116. Xu N, Zhang X, Wang X, Ge H, Wang X, Garfield D, et al. FoxM1 mediated

resistance to gefitinib in non-small-cell lung cancer cells. Acta Pharmacol Sin. Nature Publishing Group; 2012;33(5):675–81.

117. Jiang L, Wang P, Chen L, Chen H. Down-regulation of FoxM1 by thiostrepton or small interfering RNA inhibits proliferation , transformation ability and angiogenesis , and induces apoptosis of nasopharyngeal carcinoma cells. Int J Clin Exp Pathol. 2014;7(9):5450–60.

118. Xue Y, Xiao R, Long D, Zou X, Wang X, Zhang G, et al. Overexpression of FoxM1 is associated with tumor progression in patients with clear cell renal cell carcinoma. Transl Med. 2012;10(1):1–13.

119. JB Almond and GM Cohen. The proteasome: a novel target for cancer chemotherapy. Leukemia. 2002;16(4):433–43.

120. Crawford LJ, Walker B, Irvine AE. Proteasome inhibitors in cancer therapy. J Cell Commun Signal. 2011;5(2):101–10.

121. Shinobu Imajoh-Ohmi, Tomoko Kawaguchi, Shinji Sugiyama, Keiji Tanaka satoshi O and HK. LACTACYSTIN, A SPECIFIC INHIBITOR OF THE PROTEASOME, INDUCES APOPTOSIS IN HUMAN MONOBLAST U937 CELLS. Biochem Biophys Res Commun. 1995;217(3):1070–7.

122. Ito H, Kawashima S. Apoptosis induction resulting from proteasome inhibition. Biochem. 1996;317(Pt 2):385–8.

123. Drexler HCA. Activation of the cell death program by inhibition of proteasome function. Natl Acad Sci United States Am. 1997;94(3):855–60.

124. Nawrocki ST, Carew JS, Maclean KH, Courage JF, Huang P, Houghton JA, et al. Myc regulates aggresome formation , the induction of Noxa , and apoptosis in response to the combination of bortezomib and SAHA. Blood. 2008;112(7):2917–27.

125. Luqman S, Pezzuto JM. NF κ B : A Promising Target for Natural Products in Cancer Chemoprevention. Phytother Res. 2010;24(7):949–63.

126. Bhat UG, Zipfel PA, Tyler DS, Gartel AL, Bhat UG, Zipfel PA, et al. Novel anticancer compounds induce apoptosis in melanoma cells. Cell Cycle. 2008;7(12):1851–5.

127. Kwok JM, Myatt SS, Marson CM, Coombes RC, Constantinidou D, Lam EW. Thiostrepton selectively targets breast cancer cells through inhibition of forkhead box M1 expression. Mol Cancer Ther. 2008;7(7):2022–32.

128. Bhat UG, Halasi M, Gartel AL. Thiazole Antibiotics Target FoxM1 and Induce Apoptosis in Human Cancer Cells. PLoS One. 2009;4(5):e5592.

129. Pandit B, Bhat U, Gartel AL. Proteasome inhibitory activity of thiazole antibiotics. Cancer Biol Ther. 2011;11(1):43–7.

130. Gartel MW and AL. Micelle-encapsulated thiostrepton as an effective nanomedicine for inhibiting tumor growth and for suppressing FOXM1 in human xenografts. Mol Cancer Ther. 2011;10(12):2287–97.

131. Gormally M V, Dexheimer TS, Marsico G, Sanders DA, Michael S, Jadhav A, et al. Suppression of the FOXM1 transcriptional programme via novel small molecule inhibition. Nat Commun. 2014;5(5165):1–11.

132. Kelland L. The resurgence of platinum-based cancer chemotherapy. Nat Rev Cancer. 2007;7(8):573–85.

133. Hato S V, Khong A, Vries IJM De, Lesterhuis WJ. Molecular Pathways : The Immunogenic Effects of Platinum- Based Chemotherapeutics. Clin Cancer Res. 2014;20(11):2831–7.

134. Hale JP, Lewis IJ. Anthracyclines : cardiotoxicity and its prevention. Arch Dis Child. 1994;71(5):457–62.

135. Millour J, Olano N De, Horimoto Y, Monteiro LJ, Langer JK, Aligue R, et al. ATM and p53 regulate FOXM1 expression via E2F in breast cancer epirubicin treatment and resistance. Mol Cancer Ther. 2011;10(6):1046–58.

136. AM Barsotti and C Prives. Pro-proliferative FoxM1 is a target of p53-mediated repression. Oncogene. 2009;28(48):4295–305.

137. Hui RC, Gomes AR, Constantinidou D, Costa JR, Karadedou CT, Mattos SF De, et al. The Forkhead Transcription Factor FOXO3a Increases Phosphoinositide-3 Kinase / Akt Activity in Drug-Resistant Leukemic Cells through Induction of PIK3CA Expression. Mol Cell Biol. 2008;28(19):5886–98.

138. Kang BW, Kwon O, Chung HY, Yu W. Taxanes in the Treatment of Advanced Gastric Cancer. Molecules. 2016;21(5):1–10.

139. Demidenko ZN, Kalurupalle S, Hanko C, Lim C, Broude E, Blagosklonny M V. Mechanism of G1-like arrest by low concentrations of paclitaxel : next cell cycle p53-dependent arrest with sub G1 DNA content mediated by prolonged mitosis. Oncogene. 2008;27(32):4402–10.

140. Wang H, Vo T, Hajar A, Li S, Chen X, Parissenti AM, et al. Multiple mechanisms underlying acquired resistance to taxanes in selected docetaxel- resistant MCF-7 breast cancer cells. BMC Cancer. 2014;14(37):1–15.

141. Sprowl JA, Reed K, Armstrong SR, Lanner C, Guo B, Kalatskaya I, et al. Alterations in tumor necrosis factor signaling pathways are associated with cytotoxicity and resistance to taxanes : a study in isogenic resistant tumor cells. Breast Cancer Res.

BioMed Central Ltd; 2012;14(1):1–18.

142. Gomes AR, Zhao F, Lam EWF. Role and regulation of the forkhead transcription factors FOXO3a and FOXM1 in carcinogenesis and drug resistance. Chin J Cancer. 2013;32(7):365–70.

143. Essers MAG, Weijzen S, Vries-smits AMM De, Ruiter ND De, Johannes L. FOXO transcription factor activation by oxidative stress mediated by the small GTPase Ral and JNK. Eur Mol Biol Organ. 2004;23(24):4802–12.

144. Sunayama J, Tsuruta F, Masuyama N, Gotoh Y, Mori Y, Hattori S, et al. JNK antagonizes Akt-mediated survival signals by phosphorylating 14-3-3. cell Biol. 2005;170(2):295–304.

145. Janai R. Carr, Hyun Jung Park, Zebin Wang, Megan M. Kiefer and PR. FoxM1 Mediates Resistance to Herceptin and Paclitaxel Janai. Cancer Res. 2011;70(12):5054–63.

146. Sharma S V, Bell DW, Settleman J, Haber DA. Epidermal growth factor receptor mutations in lung cancer. Nat Rev Cancer. 2007;7(3):169–81.

147. Carr JR, Park HJ, Wang Z, Kiefer MM, Raychaudhuri P. FoxM1 Mediates Resistance to Herceptin and Paclitaxel. Cancer Res. 2010;70(12):5054–63.

148. Halasi M, Gartel AL. Targeting FOXM1 in cancer. Biochem Pharmacol. Elsevier Inc.; 2013;85(5):644–52.

149. Erp NP Van, Gelderblom H, Guchelaar H. Clinical pharmacokinetics of tyrosine kinase inhibitors. Cancer Treat Rev. Elsevier Ltd; 2009;35(8):692–706.

150. Carter CA, Kelly RJ, Mdphd GG, Carter CA, Kelly RJ, Mdphd GG, et al. Small-molecule inhibitors of the human epidermal receptor family. Expert Opin Investig Drugs. 2009;18(12):1829–42.

151. Gurkan AC, Arisan ED, Obakan P, Ozfiliz P. Roscovitine-treated HeLa cells finalize autophagy later than apoptosis by downregulating Bcl-2. Mol Med Rep. 2015;11(3):1968–74.

152. Martinez D, Li Y, Carpenter EL, Attiyeh EF, Diskin SJ, Kim S, et al. Dual CDK4/CDK6 Inhibition Induces Cell Cycle Arrest and Senescence in Neuroblastoma. Clin Cancer Res. 2013;19(22):1–16.

153. Jiang L, Wang P, Deng B, Huang C. Overexpression of Forkhead Box M1 transcription factor and nuclear factor – kB in laryngeal squamous cell carcinoma : a potential indicator for poor prognosis. Hum Pathol. Elsevier B.V.; 2011;42(8):1185–93.

154. Troglitazone [Internet]. Drugbank. [cited 2017 Feb 17]. Available from:

https://www.drugbank.ca/drugs/DB00197

155. Parulkar AA, Pendergrass ML, Granda-Ayala R, Lee TR FV. Nonhypoglycemic Effects of Thiazolidinediones. Ann Intern Med. 2001;134(1):61–71.

156. Hamouda W, Assian E, Dandona P. Nuclear Factor- kB Suppressive and Inhibitor-kB Stimulatory Effects of Troglitazone in Obese Patients with Type 2 Diabetes : Evidence of an Antiinflammatory Action ?*. Clin Endocrinol Metab. 2001;86(7):3250–6.

157. Troglitazone [Internet]. [cited 2017 Feb 7]. Available from: https://livertox.nlm.nih.gov//Troglitazone.htm

158. Yu H, Lee Y, Noh E, Lee K, Kim J, Song E, et al. Induction of G 1 phase arrest and apoptosis in MDA-MB-231 breast cancer cells by troglitazone , a synthetic peroxisome proliferator-activated receptor g (PPAR g) ligand. Cell Biol Int. 2008;32(8):906–12.

159. Yu H, Noh E, Lee Y, Roh S, Song E, Han M, et al. Troglitazone enhances tamoxifen-induced growth inhibitory activity of MCF-7 cells. Biochem Biophys Res Commun. Elsevier Inc.; 2008;377(1):242–7.

160. Cheon CW, Kim DH, Kim DH, Cho YH, Kim JH, Cheon CW, et al. Effects of ciglitazone and troglitazone on the proliferation of human stomach cancer cells. World J Gastroenterol. 2009;15(3):310–20.

161. Petrovic V, Costa RH, Lau LF, Raychaudhuri P, Angela L. Negative regulation of the oncogenic transcription factor Foxm1 by thiazolidinediones and mithramycin. Cancer Biol Ther. 2010;9(12):1008–16.

162. Yang C, Wang Y, Wei S, Lin L, Chen C, Lee C, et al. Peroxisome Proliferator-Activated Receptor gamma– Independent Suppression of Androgen Receptor Expression by Troglitazone Mechanism and Pharmacologic Exploitation. Cancer Res. 2007;67(7):3229–38.

163. Necela BM, Su W, Thompson EA. Peroxisome Proliferator-activated Receptor gamma Down-regulates Follistatin in Intestinal Epithelial Cells through SP1. Biol Chem. 2008;283(44):29784–94.

164. Wang Y, Fang F, Wong C. Troglitazone is an estrogen-related receptor alpha and gamma inverse agonist. Biochem Pharmacol. Elsevier Inc.; 2010;80(1):80–5.

165. B-estradiol-3-benzoate [Internet]. Pubchem. [cited 2017 Feb 17]. Available from: https://pubchem.ncbi.nlm.nih.gov/compound/estradiol_benzoate

166. Estradiol Information from Drugs.com [Internet]. Drugs.com. [cited 2017 Feb 7]. Available from: https://www.drugs.com/estradiol.html

167. Lee S, Lee M, Bin J, Jo A, Ju E, Jong S, et al. 17b-estradiol exerts anticancer effects in anoikis-resistant hepatocellular carcinoma cell lines by targeting IL-6 / STAT3 signaling. Biochem Biophys Res Commun. Elsevier Ltd; 2016;473(4):1247–54.

168. Gupta A, Saha P, Descôteaux C, Leblanc V, Asselin É, Bérubé G. Design , synthesis and biological evaluation of estradiol – chlorambucil hybrids as anticancer agents. Bioorg Med Chem Lett. Elsevier Ltd; 2010;20(5):1614–8.

169. Gliquidone [Internet]. National Center for Biotechnology Information. PubChem Compound Database CID=91610. [cited 2017 Feb 7]. Available from: https://pubchem.ncbi.nlm.nih.gov/compound/91610

170. Gliquidone [Internet]. MedicinesComplete. [cited 2017 Apr 7]. Available from: https://www-medicinescomplete-com.login.ezproxy.library.ualberta.ca

171. Dehydrocholic acid [Internet]. National Center for Biotechnology Information. PubChem Compound Database; CID=6674. [cited 2017 Feb 12]. Available from: https://pubchem.ncbi.nlm.nih.gov/compound/6674

172. Dehydrocholic acid [Internet]. MedicinesComplete. Available from: https://www-medicinescomplete-com.login.ezproxy.library.ualberta.ca

173. Liu M, Dilger JP. Site Selectivity of Competitive Antagonists for the Mouse Adult Muscle Nicotinic Acetylcholine Receptor. Mol Pharmacol. 2009;75(1):166–73.

174. Metocurine Iodide [Internet]. Drugbank. [cited 2017 Feb 12]. Available from: https://www.drugbank.ca/drugs/DB00416

175. WEISBLUM B, DEMOHN V. Thiostrepton, an Inhibitor of 50S Ribosome Subunit Function. BACrERIOLOGY. 1970;101(3):1073–5.

176. Thiostrepton [Internet]. London: Pharmaceutical Press. [cited 2017 Jul 5]. Available from: https://www-medicinescomplete-com.login.ezproxy.library.ualberta.ca

177. Chiu W-T, Huang Y-F, Tsai H-Y, Chen C-C, Chang C-H, Huang S-C, et al. FOXM1 confers to epithelial-mesenchymal transition, stemness and chemoresistance in epithelial ovarian carcinoma cells. Oncotarget. 2015;6(4):2349–65.

178. Zhang X, Cheng L, Minn K, Madan R, Godwin AK, Shridhar V, et al. Targeting of mutant p53-induced FoxM1 with thiostrepton induces cytotoxicity and enhances carboplatin sensitivity in cancer cells. Oncotarget. 2014;5(22):11365–80.

179. Jonker HRA, Ilin S, Grimm SK, Wöhnert J, Schwalbe H. L11 domain rearrangement upon binding to RNA and thiostrepton studied by NMR spectroscopy. Nucleic Acids Res. 2007;35(2):441–54.

180. Pandit B, Gartel AL. Proteasome inhibitors suppress expression of NPM and ARF proteins. Cell Cycle. 2011;10(22):3827–9.

181. Halasi M, Zhao H, Dahari H, Bhat UG, Gonzalez EB, Lyubimov A V., et al. Thiazole antibiotics against breast cancer. Cell Cycle. 2010;9(6):1214–7.

182. Malaviya A, Sylvester PW. Mechanisms Mediating the Effects of γ -Tocotrienol When Used in Combination with PPAR γ Agonists or Antagonists on MCF-7 and MDA-MB-231 Breast Cancer Cells. Int J Breast Cancer. 2013;2013:1–17.

183. Saha S, New LS, Ho HK, Chui WK, Chun E, Chan Y. Direct toxicity effects of sulfo-conjugated troglitazone on human hepatocytes. Toxicol Lett. Elsevier Ireland Ltd; 2010;195(2–3):135–41.

184. Keil AM, Frederick DM, Jacinto EY, Kennedy EL, Zauhar RJ, West NM, et al. Cytotoxicity of thiazolidinedione- , oxazolidinedione- and pyrrolidinedione-ring containing compounds in HepG2 cells. Toxicol Vitr. Elsevier Ltd; 2015;29(7):1887–96.

185. Hsu HH, Kuo WW, Ju DT, Yeh YL, Tu CC, Tsai YL, et al. Estradiol agonists inhibit human lovo colorectal-cancer cell proliferation and migration through p53. World J Gastroenterol. 2014;20(44):16665–73.

186. Schaufelberger SA, Rosselli M, Barchiesi F, Gillespie DG, Jackson EK, Dubey RK. 2-Methoxyestradiol, an endogenous 17β-estradiol metabolite, inhibits microglial proliferation and activation via an estrogen receptor-independent mechanism. Am J Physiol - Endocrinol Metab. 2016;310(5):E313–22.

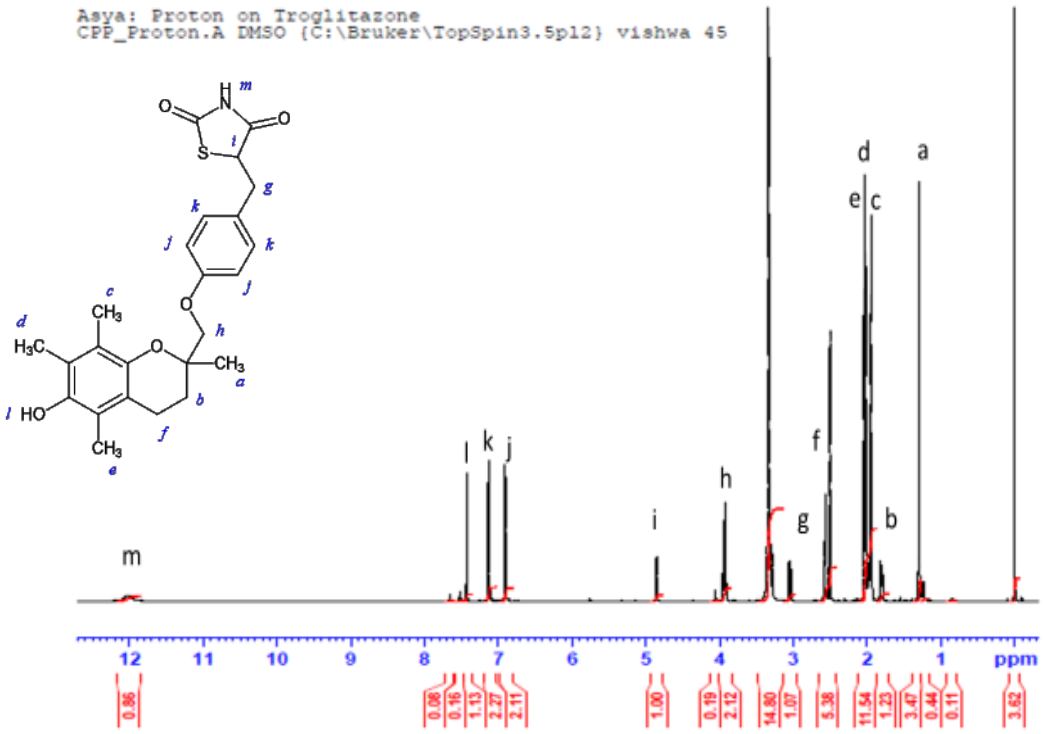
187. Yang N, Zhou T-C, Lei X, Wang C, Yan M, Wang Z-F, et al. Inhibition of Sonic Hedgehog Signaling Pathway by Thiazole Antibiotic Thiostrepton Attenuates the CD44 + / CD24- Stem-Like Population and Sphere-Forming Capacity in Triple-Negative Breast Cancer. Cell Physiol Biochem. 2016;38(3):1157–70.

188. Yang C, Chen H, Yu L, Shan L, Xie L, Hu J, et al. Inhibition of FOXM1 transcription factor suppresses cell proliferation and tumor growth of breast cancer. Cancer Gene Ther. 2013;20(2):117–24.

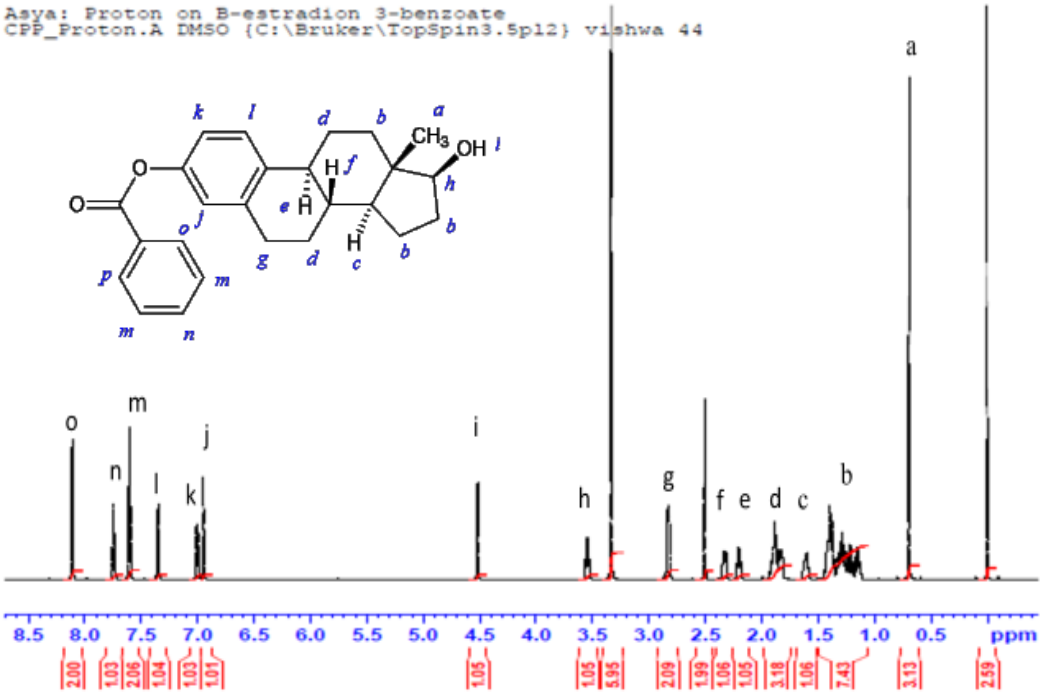
189. Hegde NS, Sanders DA, Rodriguez R, Balasubramanian S. The transcription factor FOXM1 is a cellular target of the natural product thiostrepton. Nat Chem. 2011;3(9):725–31.

190. Shiau C, Yang C, Kulp SK, Chen K, Chen C, Huang J, et al. Thiazolidenediones Mediate Apoptosis in Prostate Cancer Cells in Part through Inhibition of Bcl-xL / Bcl-2 Functions Independently of PPAR γ. Cancer Res. 2005;65(4):1561–9.

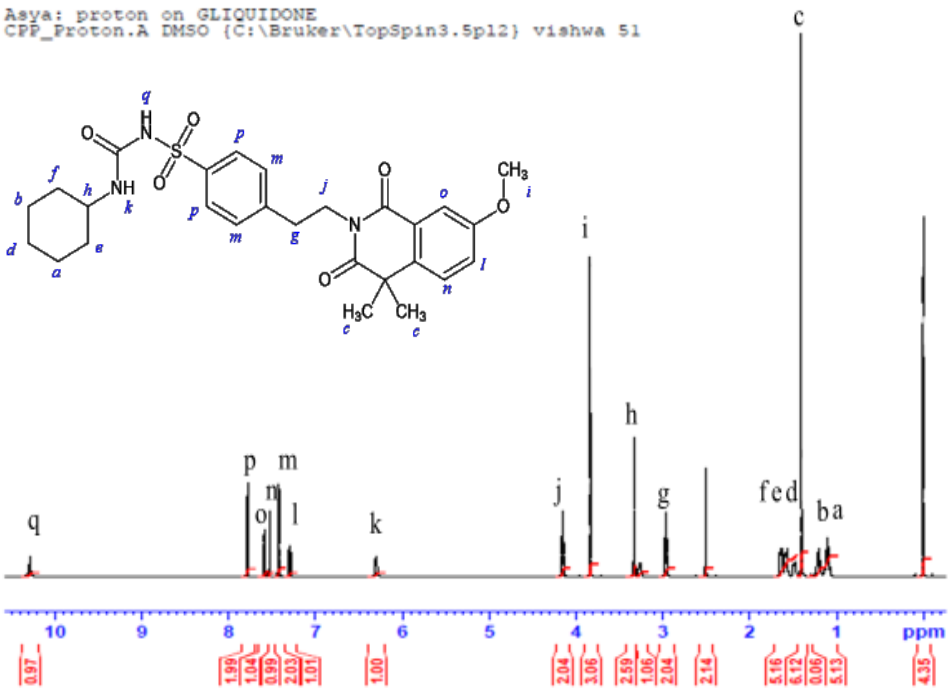
Appendix:



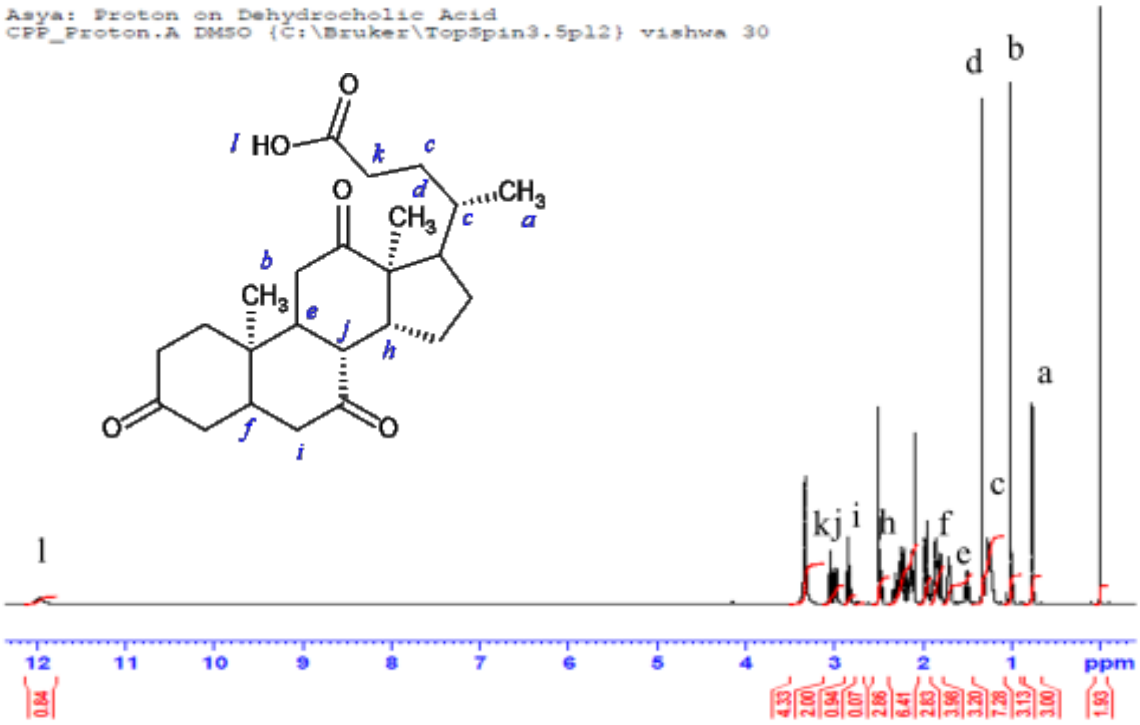
1. ^{1}H NMR of troglitazone



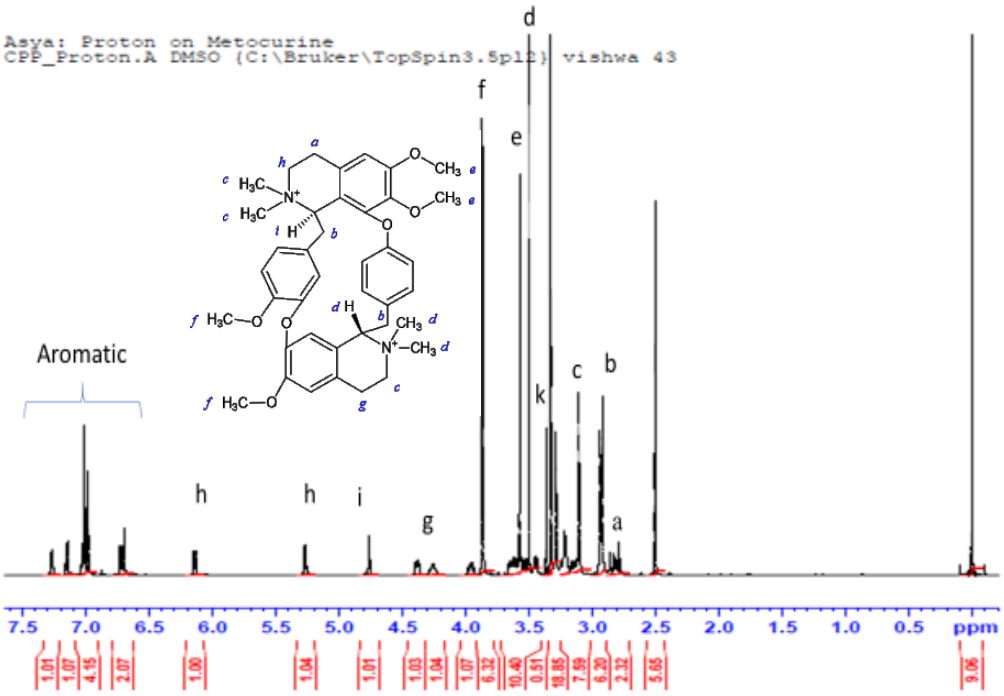
2. ^{1}H NMR of β-estradiol-3-benzoate



3. ^{1}H NMR of gliquidone



4. ^{1}H NMR of dehydrocholic acid



5. ^{1}H NMR of metocurine