Targeting Breast Cancer using Pimozide and its effect on Cell Proliferation, Invasion and Mitochondrial Metabolism

Submitted in partial fulfillment of the

requirements for the award of

Bachelor of Technology degree in Biotechnology

By

Deeksha R V (Reg.No – 39230010)

DEPARTMENT OF BIOTECHNOLOGY

SCHOOL OF BIO AND CHEMICAL ENGINEERING

SATHYABAMA

INSTITUTE OF SCIENCE AND TECHNOLOGY
(DEEMED TO BE UNIVERSITY)
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BONAFIDE CERTIFICATE

This is to certify that this Project Report is the bonafide work of **Deeksha R V (Reg.No 39230010)** who carried out her Project titled **"Targeting Breast Cancer using Pimozide and its effect on Cell Proliferation, Invasion and Mitochondrial metabolism"** under my supervision from January 2023 to May 2023.

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Submitted for Viva voce Examination held on _____

Internal Examiner **External Examiner**

DECLARATION

I, **Deeksha R V (Reg. No- 39230010),** hereby declare that the Project Report titled "**Targeting Breast Cancer using Pimozide and its effect on Cell Proliferation, Invasion and Mitochondrial Metabolism"** done by me under the guidance of **Dr. R.P Oviya, MS (Research). , Ph. D.** is submitted in partial fulfillment of the requirements for the award of Bachelor of Technology in **Biotechnology.**

DATE:

PLACE: Chennai **SIGNATURE OF THE CANDIDATE**

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ABSTRACT

Breast Cancer is a serious problem claiming the lives of 1 million people every year. Treatment methodologies have varied from chemotherapies to computational techniques on a wide chronological range. Targeted therapy continues to be an active research field; yet the biggest problem posed to this technique is the lack of information on specific signaling pathways of drug metabolism and delivery for some diseases. Over the last 2 decades, researchers have identified and hypothesized about several mechanisms and biochemical pathways connecting drug-cell interactions, to accelerate targeted therapies. This study aims to contribute toward the research on repurposed drugs for cancer treatment, by using the antipsychotic drug Pimozide to treat MMP-11 expressing MCF-7 Breast Cancer cells. To begin, MCF-7 Breast Cancer cell lines were cultured, and subjected to cytotoxicity assay. IC50 evaluation resulted in an optimized concentration of Pimozide that was made to interact with MCF-7 cells, to induce a better inhibitory effect on the cells. The success of Pimozide treatment was calculated by assessing the effect on several markers such as cell proliferation, invasion and mitochondrial metabolism markers, using Real Time Polymerase Chain Reaction (RT-PCR). Next, protein-protein interactions during Pimozide treatment were identified using in-silico tools to map the relevant biochemical signaling pathways.

CONTENTS

LIST OF ABBREVIATIONS

AKT	Ak strain Transforming
BC	Breast Cancer
CAD	Cationic Amphiphilic Drug
CCND1	Cyclin D1
CD	Cluster of Differentiation
CPZ	Chlorpromazine
CPTAC	Clinical Proteomic Tumor Analysis Consortium
CSC	Cancer Stem Cell
DPBP	Di Phenyl Butyl Piperidine
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl Sulfoxide
ECM	Extracellular Matrix
EMT	Epithelial Mesenchymal Transition
FBS	Fetal Bovine Serum
FDA	Food and Drug Administration
GTPase	Guanosine Triphosphate enzyme
GEPIA	Gene Expression Profiling Interactive Analysis
HER2+	Human Epidermal growth factor Receptor 2
IC50	Half-maximal Inhibitory Concentration
IGF-1	Insulin like Growth Factor – 1
JAK	Janus Kinase
LA-ICP-MS	Laser Ablation Inductively Coupled Plasma Mass Spectrometry
MALDI	Matrix Assisted Laser Absorption/Desorption Ionization
MCF	Michigan Cancer Foundation
MDA-MB-231	M.D. Anderson - Metastatic Breast 231
MM/PBSA	Molecular Mechanics Poisson–Boltzmann Surface Area
MMP-11	Matrix Metalloproteinase-11
PBMC	Peripheral Blood Mononuclear Cells
PBS	Phosphate Buffer Saline

PKM2	Pyruvate Kinase expression 2
RAN	Ras-related Nuclear protein
SMAD	Suppressor of Mothers Against Decapentaplegic
STAT	Signal Transducers and Activators of Transcription
TCGA	The Cancer Genome Atlas Program
TGF- β	Transforming Growth Factor – Beta
TIMP3	Tissue Inhibitors of Metalloproteinases
TNBC	Triple Negative Breast Cancer
UALCAN	The University of ALabama at Birmingham CANcer data analysis
USP1	Ubiquitin Specific Peptidase 1

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1 INTRODUCTION

The occurrence of Breast Cancer in humans has been recorded for over 3000 years. Despite rapid evolution of diagnosis and treatment techniques over time, Breast Cancer continues to be the second leading cause of death due to cancer globally, in today's world (Sung etal., 2021). According to the World Health Organization, around 12 million new cases of Breast Cancer are added every year. One of the significant contributing factors to Breast Cancer progression is the role of the Matrix Metalloproteinase enzymes, which degrade the Extracellular Matrix (ECM) thereby allowing metastasis and further invasion. MMPs are a family of endopeptidases whose role is to remodel the ECM and the surrounding tissue continuously in healthy cells. This process has important implications in several biological processes such as embryogenesis, morphogenesis, tissue repair and wound healing. They also regulate the release of bioactive molecules such as chemokines, cytokines, and growth factors. A typical MMP enzyme's structure comprises of an N-terminal zymogenic propeptide domain (about 80 amino acids), a metal-dependent catalytic domain (about 170 amino acids), a linker region (about 15 to 65 amino acids), and a C-terminal hemopexin-like domain (about 200 amino acids).

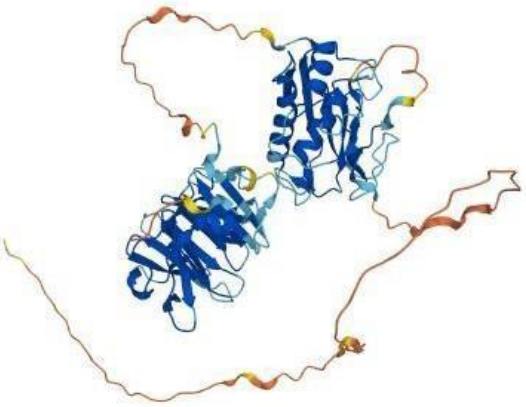


Fig 1.1: Structure of MMP-11, retrieved from Uniprot database

MMPs are generally produced as inactive proteins or “zymogens”, and this dormant state is controlled by the interaction between the Zn^{2+} ion and the catalytic domain. A loss of this interaction leads to partial activation of the MMP, while complete activation is achieved by autocatalysis. Their activation is generally inhibited once they complete their role in the ECM microenvironment, by the Tissue Inhibitors of Metalloproteinases or TIMPs.

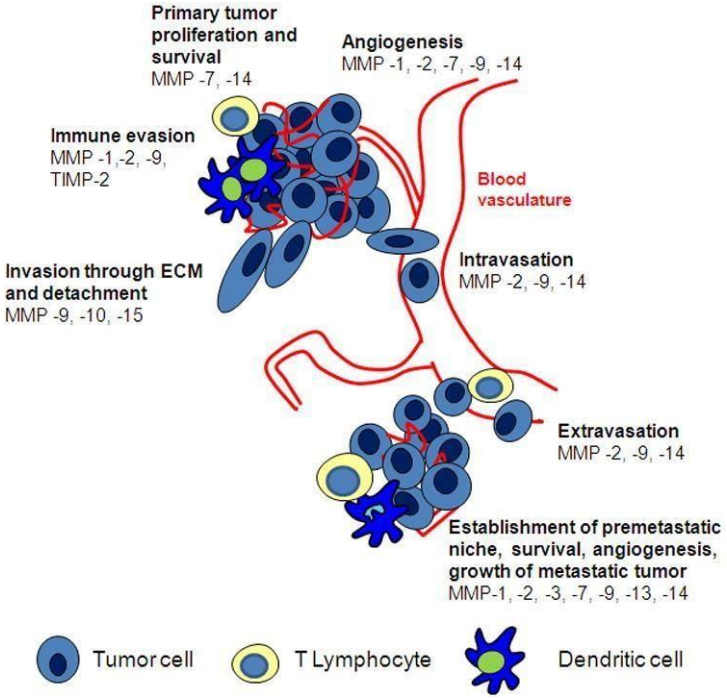


Fig 1.2: Role of several MMPs in cancer invasion. Credits (Winer et al., 2018)

The MMP family comprises of six subfamilies: (i) collagenases; (ii) gelatinases, (iii) stromelysins, (iv) matrilysins, (v) membrane-type, and (vi) other types. Of the several MMP enzymes, MMP-11 (stromelysin) has proved to be an excellent biomarker for Breast Cancer prognosis, although its functional role is not fully deciphered (Johnson et al., 2022). The expression levels of MMP-11 by different cells have been documented, although it is expressed as the highest in stromal cells.

Each MMP member is responsible for digesting specific components of the ECM. Stromelysins 1-3 therefore target specific substrates including proteoglycans, laminin, gelatins, fibronectin, entactin and collagens (Cabral-Pacheco et al., 2020). Macrophage-expressed MMP-11 can potentially aid invasion by HER2+ breast cancer cells, according to (Kang et al., 2022).The mechanism of cancer progression aided by MMP-11 is better understood by analyzing some of the biochemical pathways manipulated during prognosis. For instance, MMP-11 can inhibit the ubiquitination of Smad2 protein through the TGF-B signaling pathway, thereby allowing excess cell proliferation. Similarly, the progression of malignant tumors can be caused by increased production of IGF-1 growth factor, which is caused by MMP-11 hindrance. Additionally, high MMP-11 expression can also potentially lead to death due to a drastic decrease in CD8+ T cells in Breast Cancer patients (Kim et al., 2021). Breast cancer cells may further reach the central nervous system by activation of the COX-2 pathway that increases the permeability of the blood-brain barrier. MMP-11 also contributes todistant metastasis in breast cancer. The paradoxical role of MMP-11 has been a matter of concern in recent years. MMPs are known to cause both tumor-inducing as well as tumor-inhibiting roles in cancer. Therefore, the synthesis of MMP inhibitors has not been very successful due to the lack of specificity of the drugs against tumor-inducing MMPs. Moreover, some MMP inhibitors were also shown to cause adverse side effects in patients due to their degrading effect on helpful tumor-inhibiting MMPs.

The drugs so far active in clinical trials have generally been either zinc chelators or monoclonal antibodies. These include Batimastat (BB-94), Marimastat (BB-2516) of the former category and Andecaliximab (GS-5745) of the latter category, forbreast cancer. The concept of repurposed drugs in cancer treatment has given rise an importance to a class of drugs called “antipsychotics”. These drugs were originally synthesized to treat psychosis, schizophrenia, and bipolar disorders. Antipsychotics that have undergone clinical trials for breast cancer treatment so far include Paliperidone, Haloperidol, Olanzapine and Iloperidone which have been administered to patients based on their prolactin levels.

Overdosage of antipsychotics especially in breast cancer patients may result in an increase of prolactin levels, which may further induce exponential cell proliferation. Hence, great care must be taken while formulating the drug and dosage of antipsychotics especially in breast cancer patients.

One documented drug that showed a promising binding affinity with MMP-11 is Pimozide. However, the underlying mechanism of Pimozide treatment to target MMP-11 in Breast Cancer is yet to be investigated (Sathyanarayanan et al., 2020). Successful docking of Pimozide against MMP-11 from previous research has enabled further probing into the mechanism of Pimozide treatment, which has not yet reached satisfactory research. Pimozide is a first-generation antipsychotic drugbelonging to the Diphenybutylpiperidine group (DPBP) that is used to control involuntary motor tics caused by Tourette's disorder.

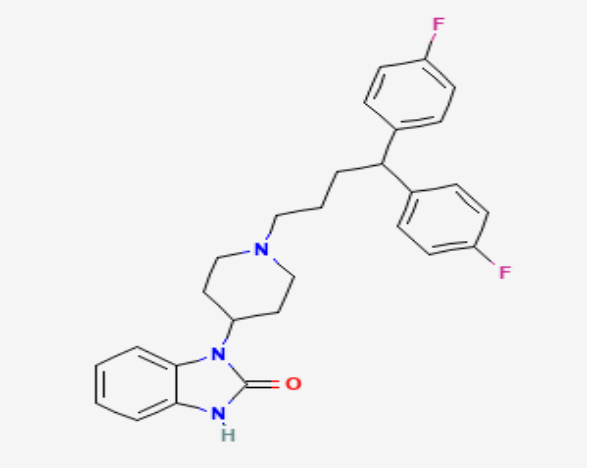


Fig 1.3: Pimozide structure, retrieved from Pubchem database

It was recently repurposed as an efficient drug to cure breast cancer, on observation that Schizophrenic patients treated with Pimozide were less prone to developing various cancers (Elmaci et al. 2018). More so, Pimozide gained its relevance as an efficient Breast Cancer target quite recently. It is known to suppress cell proliferation, promote apoptosis, inhibit epithelial mesenchymal transition, and downregulate the expression of MMP enzymes.

Moreover, Pimozide also eliminates the Warburg effect, which results in excess glucose uptake by cancer cells to aid the increased cell proliferation (Li et al., 2022), and inhibits the STAT-3 pathway activated in cancer stem cells (de Vega et al., 2018; Gonçalves et al., 2019). The promising features of Pimozide make it an interesting therapeutic agent for conducting further experimental studies to decipher several signaling mechanisms by which it inhibits breast cancer.

2 REVIEW OF LITERATURE

2.1 MMP11 AND ITS ROLE IN BREAST CANCER

2.1.1 Invention of new technologies for MMP-11 detection and Breast Cancer diagnosis

There is substantial evidence at this point that MMP-11 aids in the progression of cancer through several pathways. (de Vega et al., 2018) emphasized the need to utilize bio-image technology to better understand biological systems and to derive elaborate information on biomolecules. They used Matrix-Assisted Laser Desorption/Ionization Mass Spectrometry Imaging (MALDIMSI) for the first time to investigate the distribution of MMP-11 in human breast cancer tissues. As a result, they were able to find higher expression of MMP11 in breast cancer cells than normal breast cells; thereby indicating that this enzyme could be a potential biomarker for Breast Cancer prognosis. In another direction, (Eiro et al., 2020) found that, little information was available about the phenotype of peripheral blood mononuclear cells (PBMCs) from Breast Cancer patients. They analyzed the change in MMP11 expression in PBMCs pre and post interaction with cancer cells. Their research concluded with the notion that PBMCs affected by tumor infiltration only aid the tumor to become more aggressive. (Ren et al., 2020) emphasized the use of Color Doppler ultrasound, a high-resolution technique as a common method for diagnosis and detection of Breast Cancer, by assessing shape, direction and structure of cells. They used this technology combined with MMP11 detection, to accelerate the rate of diagnosis for Breast Cancer, and concluded that while both techniques are individually reliable, their combined technique makes up for the shortcomings of the individual techniques.

Despite its importance, scientists were quick to identify a rather disturbingly conflicting role of MMP-11, whereby its significance was reflected as both a positive and a negative impact. (B. Ma et al., 2021) assessed the conflicting roles played by MMP-11 and other MMPs during normal conditions and different phases of tumor progression. They elaborated on how scientists have recently found that MMP-11 may also play the role of a tumor suppressor, contrary to original theory that MMP11 aids tumor metastasis and invasion by degrading components of the ECM. A further improvement in technology was made by (Johnson et al., 2022), who presented methods for quantitative and multiplexed imaging of MMP-11 and CD45 in breast cancer tissues and investigated their potential for improved cancer characterization and patient stratification. They developed an immunohistochemistry-assisted laser ablation–inductively coupled plasma–mass spectrometry (LA–ICP–MS) method and optimized it using lanthanide-tagged monoclonal antibodies as proxies to determine spatial distributions and concentrations of the two breast cancer biomarkers. They found no significant correlation between CD45 and metastasis but found that MMP-11 was significantly up-regulated in metastatic samples compared to non-metastatic ones.

2.1.2 Disturbances in biochemical pathway signaling

The IGF-1 pathway has been a subject of interest for decades now, due to its vitally essential roles in biochemical mechanisms in the body. All pathways invariably aid a cancer's progression involuntarily, as they continuously accept messages to and fro between cells. (Ianza et al., 2021) reviewed the importance of Insulin-like Growth Factor – 1 as a therapeutic target in Breast Cancer. IGF-1 is known to promote resistance of Breast Cancer cells against BC therapy. Additionally, the IGF-1 pathway can also be activated by increased MMP11 expression, making this hormone an important target. In a similar fashion, (Zhuang et al., 2021) found another important pathway using Smad2 and Smad3 linked to MMP-11.

They hypothesized that through the TGF-β signaling pathway, MMP11 could significantly regulate the protein expression levels of Smad2 and Smad3 and inhibit the degradation of Smad2 through the ubiquitin proteasome pathway, thereby leading to excessive cell proliferation due to uncontrolled cell division. This study emphasized the resulting stability of Smad2 protein due to increased MMP11 expression and the overall Breast Cancer progression. During the time, they were yet to confirm a direct correlation between MMP11 and Smad3 protein. On another note, T cells are an important factor that decide the overall health of the immune system. Thereby, a decrease in T cell proliferation may lead to lack of reinforcements for the immune system to stop tumorigenesis. (Kim et al., 2021) analyzed the downregulation of genes associated with important cancer-eliminating factors such as CD4+ and CD8+ T cells, which was associated with high MMP11 expression. Additionally, B cell population was also found to be affected in the same manner. They hypothesized that the degradation of ECM by MMP-11 might have secondary effects on the immune system. Another vitally important cell, the macrophage, is also compromised for aiding cancer metastasis. (Kang et al., 2022). performed immunohistochemical analysis of breast cancer tissues to demonstrate that MMP11 expression in mononuclear inflammatory cells (predominantly macrophages) is an independent negative prognostic factor in breast cancer, whereas MMP11 expression in tumor cells and fibroblasts is not associated with patient survival. They additionally found that MMP11-overexpressing macrophages enhanced the migration of HER2-positive (HER2+) breast cancer cells.

2.2 REPURPOSING ANTIPSYCHOTICS FOR CANCER TREATMENT

Antipsychotics are drugs that help reduce involuntary muscle spasms, also called “tics” which are evident especially in Schizophrenic patients. Their role in cancer treatment was realized by a gradual trend that arose amongst drug consumers; schizophrenic patients were somehow found to be resistant to cancer attack. This brought an advent of methodologies to test the action of antipsychotics against cancer, and the results were thankfully positive. The same results were found for Breast Cancer patients.

Moreover, the use of repurposed drugs became important due to the significant amount of time and energy spent in synthesizing new drugs and getting FDA approval. (Yang et al., 2019) discussed the problem of Cancer Stem Cells (CSCs) during therapy, which are aided in growth by yes-associated proteins (YAP). Their solution to this problem was the treatment with chlorpromazine or CPZ, which suppressed stemness properties including mammosphere formation, aldehyde dehydrogenase (ALDH) activity, and stemness-related gene expressions in breast cancer cells and CSCs. Additionally, they also found that CPZ increased the susceptibility of breast cancer cells and drug-resistant cells towards chemotherapies. (Xu et al., 2019) discussed how Antipsychotic drugs show good bioavailability in the brain, which is essential because triple negative breast cancer (TNBC) patients have a high risk of brain metastases. Fluphenazine exhibited good anti-metastatic potential in a mouse brain metastasis model with an inhibition rate of 85%. In addition, Flu showed a strong inhibitory effect on spontaneous lung metastasis with the added benefit of lack of side effects. (Goyette et al., 2019) used the antipsychotics Thioridazine, Fluphenazine and Trifluoperazine to test their efficacy against TNBC. They were able to reduce tumor growth and metastatic burden. Collectively, the results suggest that these antipsychotics display anti-tumor and anti-metastatic activity and that they could potentially be repurposed, in combination with standard chemotherapy, for the treatment of TNBC. (Badran et al., 2020) discussed the major advantages of repurposed drugs; that their pharmacokinetic, pharmacodynamic, and toxicity profiles are well documented. They elaborated on Aripiprazole drug which showed a cytotoxicity (IC50 = 12.1 ± 0.40 μM) to MCF-7 cells, the best result compared to all other standard drugs tested. Additionally, they found that Aripiprazole significantly inhibited the cell cycle progression at subG0G1 phase, and enhanced apoptosis in MCF-7 breast cancer cells. (Shaw et al., 2021) reviewed the use of the diphenylbutylpiperidine (DPBP) class of antipsychotics including Fluspirilene, Penfluridol, and Pimozide. They discussed at length about Pimozide and how it derives its antipsychotic efficacy through dopamine D2 receptor inhibition. Some studies have shown that dopamine receptor activation is correlated with poor cancer prognoses.

They elaborated further on the role of Pimozide in Antiproliferative effects, STAT5 inhibition, pro-apoptotic effects, and anti-invasion effects. (Vlachos et al., 2021) did a similar review on antipsychotic drugs including Haloperidol, Trifluoperazine, Chlorpromazine, Pimozide, Fluspirilene, Penfluridol, Thioridazine, atypical antipsychotics such as Quetiapine, Olanzapine, Risperidone, Aripiprazole, and Clozapine. (Lianos et al., 2022) reviewed the change in trends in antipsychotics usage for cancer treatment over the years. They discussed futuristic approaches towards well-designed clinical and prospective trials are needed in order for reaching safe conclusions. They explained that the major goal is to potentially understand the responsible mechanisms of antipsychotics anticancer effect, and how metabolic disease is frequently associated with second-generation antipsychotics and impacts patient quality of life. They concluded that understanding the mechanisms of antipsychotic anticancer effects could open a critical crossroad for the development of novel drugs or drug combinations in order to improve the survival rates of cancer patients worldwide. (Leung et al., 2022) reviewed efficacy of antipsychotic treatment for BC. They concluded that antipsychotic use is moderately associated with breast cancer, possibly mediated by prolactin-elevating properties of certain medications, and that this risk should be weighed against the potential treatment effects for a balanced prescription decision. (Zhang et al., 2018) wrote about the high risk involved in using first-generation antipsychotics for cancer treatment, especially in the elderly, and how second-generation drugs exhibit lower mortality rates than their predecessors. Their study revealed promising anticancer potentials of Sertindole against breast cancers through autophagy-associated apoptosis, with probable applications for breast-to-brain metastasis.

2.2.1 The downsides of Antipsychotics in Breast Cancer treatment

(Johnston et al., 2018) brought out a different side of antipsychotics; elaborating on the controversies involved in using antipsychotics for BC treatment. Their paper detailed the effect of hyperprolactinemia-inducing antipsychotics which cause pre-cancerous lesions using STAT5 gene.

They also concluded the research with treatment of such affected patients with Ruxolitinib, a JAK1/2 inhibitor, to inhibit STAT5 activation, thereby protecting the patient's system from cancerous lesions. Similarly, (Rahman et al., 2022) conducted an observational study of US women aging between 18 – 64 years. In the largest study of antipsychotics taken by US women, a higher risk between antipsychotic drug use and increased risk for breast cancer was observed, with a differential higher association with antipsychotic categories that elevate prolactin. Their study confirmed other recent observational studies of increased breast cancer risk with antipsychotics that elevate prolactin.

2.3 PIMOZIDE'S CONTRIBUTION TO BREAST CANCER TREATMENT

(Elmaci & Altinoz, 2018) reported additional information on Pimozide, shedding light on current usage in clinic as a neuroleptic, which exerts versatile biological actions. They mentioned that Pimozide is a cationic amphiphilic drug (CAD); which blocks the synthesis of neutral lipids, impair cholesterol homeostasis of cancer cells and increases accumulation of diacylglycerol-3-phosphate. Pimozide was reported to exert tumoricidal activity which was first shown for melanoma and neuroblastoma via proposed anti- dopaminergic effects. (Mapes et al., 2018) inferred that selective blockade of STAT5 phosphorylation by Pimozide, a small-molecule inhibitor, markedly reduced the production of the EGF family growth factors and inhibited PRL-induced tumor cell proliferation in vitro. Pimozide administration to mice also suppressed CUZD1-driven mammary tumorigenesis in vivo. They concluded that blockade of the STAT5 signaling pathway downstream of CUZD1 may offer a therapeutic strategy for managing these breast tumors. (Dakir et al., 2018) found that Pimozide inhibited cell proliferation in a dose- and time-dependent manner in MDA-MB-231 breast cancer cells. Pimozide downregulated RAN GTPase and AKT at both protein and mRNA levels and inhibited the AKT signaling pathway in MDA-MB-231 breast cancer cells. Pimozide also inhibited the epithelial mesenchymal transition and cell migration and downregulated the expression of MMPs.

They concluded that Pimozide might inhibit tumor development by suppressing angiogenesis and by paracrine stimulation provided by host reactive stromal cells.

(A. Ma et al., 2019) demonstrated that hyper activation of the Deubiquitinase USP1 contributes to breast cancer metastasis. Upregulated USP1 expression in primary breast cancer specimens correlates with metastatic progression and poor prognosis in breast cancer patients. They inferred that pharmacological intervention of USP1 function by Pimozide significantly represses breast cancer metastasis in mice, suggesting a rationale for using USP1 inhibitors for treatment of patients with breast cancer. They concluded that USP1 as a promoter of breast cancer metastasis provides evidence for the potential practice of USP1 targeting in the treatment of breast cancer. (Gonçalves et al., 2019) reviewed the anti-neoplastic role of Pimozide during tumorigenesis and its potential in revert the process of undifferentiation and proliferation of CSC. They discussed that Pimozide: 1) blocks CSC features, as epithelial-to-mesenchymal transition (EMT), through inhibition of Wntβ/catenin signaling; 2) acts as an inhibitor of Signal Transducer and Activator of Transcription (STAT-3 and 5), pathway which is activated and upregulated in CSCs; 3) inhibits ubiquitin specific protease (USP1) and WD repeat containing protein 48 (WDR48), that are proteins responsible to inhibit the differentiation and to maintain the cell in an undifferentiated state. (Ranjan et al., 2020) reviewed that recent studies have suggested STAT3 signaling to be directly associated with the progression of several tumors such as brain cancer, breast cancer, prostate cancer, and lung cancer. They also discussed that STAT3 up-regulation has been associated with the poor prognosis of patients. (Dees et al., 2020) discussed that phosphorylated STAT3 (Tyr705) inhibition resulting from Pimozide treatment caused a downregulation of downstream transcriptional targets such as matrix metalloproteinase9 (MMP-9), both implicated in invasion and migration. They inferred that the identification of biomarkers of response to TNBC treatments is an active area of research in the field of precision medicine. Their results propose phosphorylated STAT3 (Tyr705) as a novel biomarker to guide Pimozide treatment as an inhibitor of invasion and migration. (Li et al., 2022) recalled that Pimozide's inhibitory effect on aerobic glycolysis has not been elucidated yet.

In their study, Pimozide inhibited the Warburg effect of breast cancer cells by hindering glucose uptake, ATP level and lactate production, suppressing the expression of PKM2, a rate-limiting enzyme in glycolysis. Pimozide was significantly involved in reprogramming glucose metabolism in breast cancer cells through a p53-dependent manner. They demonstrated Pimozide increased the expression of p53 through inhibition of the PI3K/Akt/MDM2 signaling pathway, which in turn downregulated the expression of PKM2.

Their results suggested that Pimozide mediates the p53 signaling pathway through PI3K/AKT/MDM2 to inhibit the Warburg effect and breast cancer growth, and it may be a potential aerobic glycolysis inhibitor for the treatment of breast cancer. Similarly other scientists from Philadelphia noted that TNBC is a challenging subtype of BC that does not respond to the targeted therapy. In their study, the FDA-approved drugs, Pimozide, Fluspirilene, Haloperidol, Lumateperone, Iloperidone, and Droperidol, showed variant effects on cell proliferation. Besides Pimozide exhibited a significant decrease in cell proliferation in both cell lines.

2.3.1 In-silico analysis

(Kandasamy et al., 2022) focused on the interconnected functional network of altered metabolism and EMT (epithelial to mesenchymal transition) signaling in breast cancer. They interlinked the metabolic and EMT signaling circuits. Using molecular docking, molecular dynamics simulation, and MMPBSA binding free energy studies, Capmatinib, Ponatinib, Naldemedine, and Pimozide were identified as potential repurposed drugs to block the function of all three target proteins.

2.4 EFFECTS OF TARGETING MMP-11 USING PIMOZIDE

(Sathyanarayanan et al., 2020) aimed to comprehend the prognostic and therapeutic value of matrix metalloproteinase (MMP11) in breast cancer using computational analyses. Tumors with high MMP11 expression showed poor overall survival and distant metastasis free survival. Further, protein-protein interactome network was constructed and molecular pathway analysis using Reactome database identified its putative role in diverse cellular processes. Their study concluded that Pimozide has a high binding affinity with MMP11 in Breast Cancer cells, although the exact mechanism was yet to be investigated, which is the basis of our present study.

3 AIM AND SCOPE

3.1 AIM

To analyze the potential of MMP-11 as a therapeutic target in breast cancer. To also analyze the potential of small molecular inhibitors against MMP-11 in breast cancer and identify the relevant cell signaling pathways including cell proliferation markers along with their important protein-protein interactions.

3.2 SCOPE

This study aims to identify the potential of targeting MMP-11 in ER+ breast cancer. This study intends to perform *in vitro* analysis of targeting MMP-11 and identify the importance of cell signaling pathways including IGF-1, CCND1, AKT, SMAD2, SMAD3, p65, TIMP3 and Beta Actin. Further protein-protein interactions play a major role in inducing signaling networks, especially in cancer. Hence identifying MMP-11's protein networks will pave the way for deciphering breast cancer metabolism.

4 MATERIALS AND METHODS

4.1 IN-SILICO EXPRESSION STUDIES

4.1.1 Pan-cancer study of MMP11 mRNA expression

The expression levels of MMP11 were analyzed using The Cancer Genome Atlas (TCGA) samples obtained from UALCAN (RRID SCR_015827). UALCAN is an interactive online tool aiding the in-depth analysis of TCGA gene expression data. The portal allows the exploration of relative expression of one or more genes, across tumor (including different stages, grades, and subtypes) and normal samples; the effect of the expression levels on the OS; and identify the most up and down-regulated genes in individual cancers. The number of MMP11 transcripts per million was plotted on the y-axis and compared for normal versus tumor samples, through several boxplots. A p-value <0.05 was considered significant.

4.1.2 Validation of MMP11 mRNA expression study in pan-cancer

The results from TCGA studies were validated using Gene Expression Profiling Interactive Analysis (GEPIA, 2017). Key interactive and programmable features offered by GEPIA include differential expression analysis, profiling charting, correlation analysis, patient survival analysis, related gene detection, and dimension reduction analysis. GEPIA bridges the gap between large data generated by cancer genomics and the transmission of integrated information to end users. All data was set to a default p-value of 0.01. The y-axis is set to the logarithmic value of the number of transcripts per million produced. Normal and tumor samples are separately color coded by red and grey boxes respectively.

4.1.3 Pan-cancer study of MMP11 protein expression

MMP11 protein expression levels were studied for pan-cancer using the Clinical Proteomic Tumor Analysis Consortium (CPTAC) dataset provided by UALCAN. This valuable dataset provides large-scale proteome and genome analysis to accelerate studies on the molecular basis of cancer progression. The z-values were plotted on the y-axis and compared for normal and tumor samples as boxplots. A p-value <0.05 was considered statistically significant.

4.2 IN-VITRO STUDIES

4.2.1 Mammalian cell culture

Human derived cell lines (Table 2.1) were maintained in T-25 cm^2 flasks (Hi Media, India), in Dulbecco's Modified Eagle Medium (DMEM) (HI Media, India) supplemented with 10% Fetal bovine serum (FBS) (Hi Media, India) containing 100 mg/L penicillin and 100 mg/L streptomycin (Hi Media, India) at 37^{o}C and 5% CO_2 in a humidified incubator. For sub-culturing, 80 to 90% confluent cells were washed in sterile Dulbecco's phosphate-buffered saline (PBS) (Hi Media, India) and then 0.5 to 2 mL of 1x Trypsin-EDTA solution dissolved in PBS [0.25% *w/v* Trypsin and 0.02% EDTA] (Hi Media, India) was added. Then the cells were centrifuged at 5000 rpm for 2 minutes and the pellet was suspended in a growth medium containing 10% FBS (Hi Media, India). 10 µL of cell suspension was stained with 10 µL of 0.4% trypan-blue solution and the cell numbers were estimated using Hemocytometer (ThermoFisher Scientific, USA). Cell line stocks were frozen in liquid nitrogen using a freezing mixture of 70% FBS, 20% DMEM, and 10% dimethylsulfoxide.

Table 4.1 List of cell lines used

Type of Cell line	Cell line	Medium used	Source of cell line
Control cells	HEK293	DMEM with 10% FBS	Adyar Cancer Institute
Breast cancer cells	MCF7	DMEM with 10% FBS	Adyar Cancer Institute

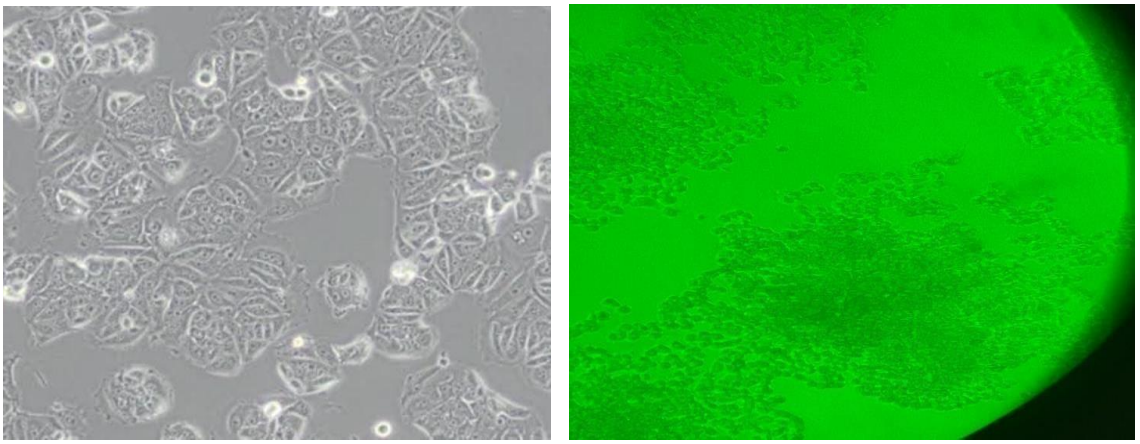


Fig 4.1: HEK293 and MCF7 cell lines

4.2.2 Stock preparation of Pimozide

50 mg of Pimozide (Cayman, USA) was added to 4 mL DMSO solution as per manufacturer's recommendation. The suspension was rapidly vortexed and incubated in a hot water bath at 60 ^{o}C for 20 min intervals till it completely dissolved. 30 µL of the resulting solution was aliquoted into 10 centrifuge tubes each, and further diluted with 666 µL DMSO. The tubes were stored in -20 ^{o}C for furtheruse.

4.2.3 IC50 evaluation of Pimozide

1 µL of HEK293 (control) cells and MCF7 (test) cells were seeded into 2 96-well plates respectively. The wells were split accordingly for blank seeding as well, to serve as an internal control. 27 mM of 50 mg Pimozide was seeded as1.7 µL, 2.0 µL, 2.3 µL and 2.7 µL respectively into 5 columns of wells containing the cells. The plates were incubated at 37 ^{o}C with 5% $CO_{2,}$. The absorbance of the cells was measured at 24 h, 48 h, 72 h and 96 h, with a wavelength of 570 nm.

4.2.4 Statistical Analysis of absorbance

The GraphPad Prism (version 7, USA) was used to perform statistical analysis, unpaired t-test. The *p*-value of less than 0.05 was considered to be significant. The values were represented as mean ± standard deviation (SD).

4.2.5 Colony forming Assay

2.5 µL of HEK293 (control) and MCF7 (test) cells (containing approximately 800 cells per µL) were seeded into 2 12-well plates respectively. 4 mL DMEM medium was added into both plates and incubated at 37^{o}C and 5% $CO_{2.}$ The medium was removed after 48 h incubation and the cells were washed with 1x PBS. The cells were then fixed with methanol and acetic acid at a ratio of 3:1 and incubated at room temperature for 5 min, after which it was removed. The cells were stained with 0.5% crystal violet for 15 minutes and washed off. The images of cells were clicked using Gel Doc XR+ Gel Documentation System (Biorad, USA) using epi-white filter and white light. The images were exported to ImageJ software for cell counting.

4.2.6 Total RNA Isolation

Total RNA was isolated using trizol reagent (Invitrogen, USA). Cells suspended in 1 mL trizol were added to 200 µL chloroform, mixed gently, and centrifuged at 10,000 rpm for 15 min at 4^{o}C. The aqueous phase was pipetted out without disturbing the contents and transferred to a fresh 1.5 mL tube and placed on ice.

Then, isopropanol was added to the aqueous phase and centrifuged at 10,000 rpm for 20 min at 4ºC. The white pellet thus formed was washed twice with 70% ethanol. Then the pellet was air-dried and dissolved in 20 µL of sterile nuclease-free water.

4.2.7 Agarose Gel Electrophoresis

To check the integrity of RNA, agarose gel electrophoresis was performed. 1% agarose in 100 mL of 1x Tris/Boric acid/EDTA (TBE) buffer (0.07 M Tris, 0.06 M Boric acid and 2 mM EDTA, pH 7.6) was prepared. The agarose was heated in the microwave oven until it completely dissolved, and poured into the gel tray after which the comb was placed. The agarose was allowed to solidify for 15 minutes and then the gel tray was immersed in the gel tank containing 1x TBE buffer. 10 µL of total RNA was mixed with 6 µL of 6x gel loading dye and loaded onto the agarose gel, and run at 100 V for 30 min. The gel was visualized and documented using the Gel Doc XR+ Gel Documentation System (Biorad, USA). under UV absorbance.

4.2.8 cDNA Conversion

1 µg of total RNA was converted to cDNA, using GeNei™ RT-PCR Kit according to the manufacturer's protocol in a two-step procedure. 12 µL of total RNA was mixed with 12 µL of gDNA wipeout buffer to eliminate genomic DNA contamination. The mixture's volume was adjusted to 84 µL with sterile nuclease-free water. The master mix was then prepared using 13 µL reverse transcriptase, 52 µL 5x RT buffer, 13 µL RT primer mix, and 14 µL of gDNA eliminated template RNA. The resulting mix was aliquoted into 4 tubes with 23 µL each. The tubes were incubated at 42ºC for 15 min, and then at 95ºC for 3 min to inactivate reverse transcriptase. The tubes were stored at -20ºC for further use.

4.2.9 Designing Real-Time quantitative Polymerase Chain Reaction Primers

Primers for seven genes, oncogenes, and tumor suppressor genes are given in Table 2.2, which were designed using EGI Products and Services (Eurofins Genomics India Pvt. Ltd.) primer designing tool. The primers were validated by primer BLAST (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) and then given for synthesizing to Eurogen, Bangalore. The primers were dissolved in sterile nuclease-free water and diluted to a working concentration of 1 µM for further use.

Table 4.2 List of primers used for qRT-PCR analysis

Gene	Primers for qRT-PCR
IGF1	FP: 5'CTCCTCGCATCTCTTCTACC 3' RP: 5'GAAATAAAAGCCCCTGTCTCC3'
AKT1	FP: 5'GCCCAACACCTTCATCATC3' RP: 5'AAGTCCATCTCCTCCTCCTC3'
CCND1	FP: 5'GCATCTACACCGACAACTCC3' RP: 5'TCTGTTTGTTCTCCTCCGCC3'
P65 RELa	FP: 5'ACAGATACCACCAAGACCC3' RP: 5'AGCCTCATAGAAGCCATCC3'
SMAD2	FP: 5'GTCGTCCATCTTGCCATTC3' RP: 5'TTCTCACACCACTTTTCTTCC3'
SMAD3	FP: 5'TGACCACCAGATGAACCAC3' RP: 5'AGGAGATGGAGCACCAGAAG3'
TIMP3	FP: 5'TGGAGGTCAACAAGTACCAG3' RP: 5'AAGTCACAAAGCAAGGCAG3'
MMP11	FP: 5'CGACTATGATGAGACCTGGAC3' RP: 5'GGGTAGCGAAAGGTGTAGAAG3'
BETA ACTIN	FP:5'ACTCTTCCAGCCTTCCTTC3' RP:5'TCTCCTTCTGCATCCTGTC3'

4.2.10 Polymerase Chain Reaction (PCR)

PCR was performed with gene-specific primers for nine genes (IGF1, AKT1, CCND1, P65, SMAD2, SMAD3, TIMP3, MMP11, and Beta Actin) using the GeNei PCR Master Mix Kit (2x) according to manufacturer's protocol. 12.5 µL of 2x master mix, 0.4 µL forward primer, 0.4 µL reverse primer and 1 µL of cDNA was added and the volume was made upto 25 µL using sterile nuclease-free water. The annealing temperatures were standardized by adding 3 to the lower temperature of both primers of a gene. PCR amplification was performed with Mastercycler Nexus Gradient Thermal Cycler (Eppendorf, Germany) for 30 cycles. The thermal cycling conditions included 72^{o}C for 30 sec, 94^{o}C for 30 sec, 59^{o}C for 30 sec, 72^{o}C for 30 sec, 72^{o}C for 2 min and 4^{o}C. The resulting PCR products run using Agarose Gel Electrophoresis and were checked for single band patterns using Gel Doc XR+ Gel Documentation System (Biorad, USA). under UV absorbance.

4.2.11 Quantitative Real-Time-Polymerase Chain Reaction (qRT-PCR)

qRT-PCR was performed with gene-specific primers for nine genes (IGF1, AKT1, CCND1, P65, SMAD2, SMAD3, TIMP3, MMP11) and BETA ACTIN as the normalizing control gene, using Hi-SYBR Master Mix Kit (Hi Media, India) according to the manufacturer's protocol in duplicates. 10 µL of SYBR Green master mix, 0.8 µL of forward primer (10 µM), 0.8 µL of reverse primer (10 µM), 6.8 µL of cDNA template was added and the volume was made to 20 µL with nuclease-free water. PCR amplification was performed with QuantStudio 12K Flex Real-Time PCR System (ThermoFisher Scientific, USA). The thermal-cycling conditions included 95^{o}C for 2 min (polymerase activation), 95^{o}C for 5 sec (denaturation), 60 to 65^{o}C for 10 sec (annealing), and 72^{o}C for 15 sec (extension) for 40 cycles. The program was set up to calculate cycle threshold value (CT-value) for 40 cycles.

The relative expression levels between target and control were measured with 2^-ΔΔCT method. In this method, the relative gene expression is assumed to be 1 for the reference sample so that 2^0 will provide a value of 1. It assumes 100% efficiency and uniform PCR amplification across all the samples. The results were reported as expression fold change when compared to control. Fold change >1.5 was taken as upregulation and < 0.5 was considered as downregulation in the gene expression.

4.2.13 Statistical Analysis

The GraphPad Prism (version 7, USA) was used to perform statistical analysis, unpaired t-test. The *p*-value of less than 0.05 was considered to be significant. The values were represented as mean ± standard deviation (SD).

4.3 VALIDATION OF PPIS, PROTEIN NETWORK CONSTRUCTION, AND IDENTIFICATION OF THE PUTATIVE ROLE OF MMP11 IN CELLULAR PROCESSES

4.3.1 PPIs of MMP-11 from STRING database

The PPIs of MMP-11 were searched from the publicly available PPI database STRING version 11.5. A protein-protein interaction network was created using MMP11 *Homosapien* as the query protein. The query was customized to include all data from text mining, experiments, databases, co-expression, gene fusion, neighborhood, and co-occurrence. The number of interacting partners was set to the maximum limit of 50. The interaction confidence was set to a medium confidence of 0.400 to increase the scope of the interactions. An interactive png was visualized to analyze the proteins directly interacting with MMP11. Pathway enrichment analysis using Gene Ontology, KEGG, Reactome and STRING was also performed for the protein network. The statistical background was set to the whole genome. A p-value <0.05 was considered statistically significant.

4.3.2 Protein Network and Gene Ontology Analysis (GO)

Cytoscape version 3.7.2 was used to build general networks using the Advanced Network Analysis Tool (ANAT) plugin in the *Homosapien* protein database. The respective physical interactions of each protein decide how the high confidence protein network is linked. The gene nomenclature of the anchor proteins and the bait MMP-11 protein were entered, and the protein confidence interval was set to 95%. The best-fit network obtained was selected and the anchor proteins which did not fit into the general network were left undisturbed. The PPIs from HIPPIE v2.0 database were obtained for MMP-11. The resulting protein network was analyzed by Reactome plug-in, which revealed molecular pathways associated with the protein network with a False Discovery Rate (FDR) of 0.05.

5 RESULTS AND DISCUSSION

5.1 IN-SILICO ANALYSIS

5.1.1 Pan-cancer study of MMP11 mRNA expression

MMP-11 is differentially expressed in various cancers. A pan cancer analysis of gene expression was conducted using the TCGA dataset in UALCAN database. MMP-11 gene expression was found to be significantly upregulated in Breast cancer (BRCA) and Uterine carcinoma (UCEC), with more than 100 transcripts per million produced in these tumor samples. The same gene was moderately upregulated in Cervical cancer (CESC), Colon cancer (COAD), Esophageal cancer (ESCA), Head and Neck cancer (HNSC), Kidney cancers (KIRC and KIRP), Lung cancer (LUAD and LUSC), Sarcoma (SARC) and Stomach cancer (STAD) tumor samples where 20-50 transcripts per million were produced. MMP-11 gene was upregulated at low levels in Glioblastoma (GBM), Liver cancer (LIHC), Prostate cancer (PRAD) and Thyroid cancer (THCA) tumor samples with only around 5 transcripts per million produced. Notably, MMP-11 was significantly downregulated in Pancreatic cancer (PAAD), dropping from around 750 (in normal samples) to 250 (in tumor samples) transcripts per million. From this study, it was observed that MMP-11 gene was expressed as the highest in BRCA tumor samples (240 transcripts per million), thereby indicating its significance in breast cancer prognosis. It can also be drawn that MMP-11 may prove to be an excellent therapeutic marker in breast cancer.

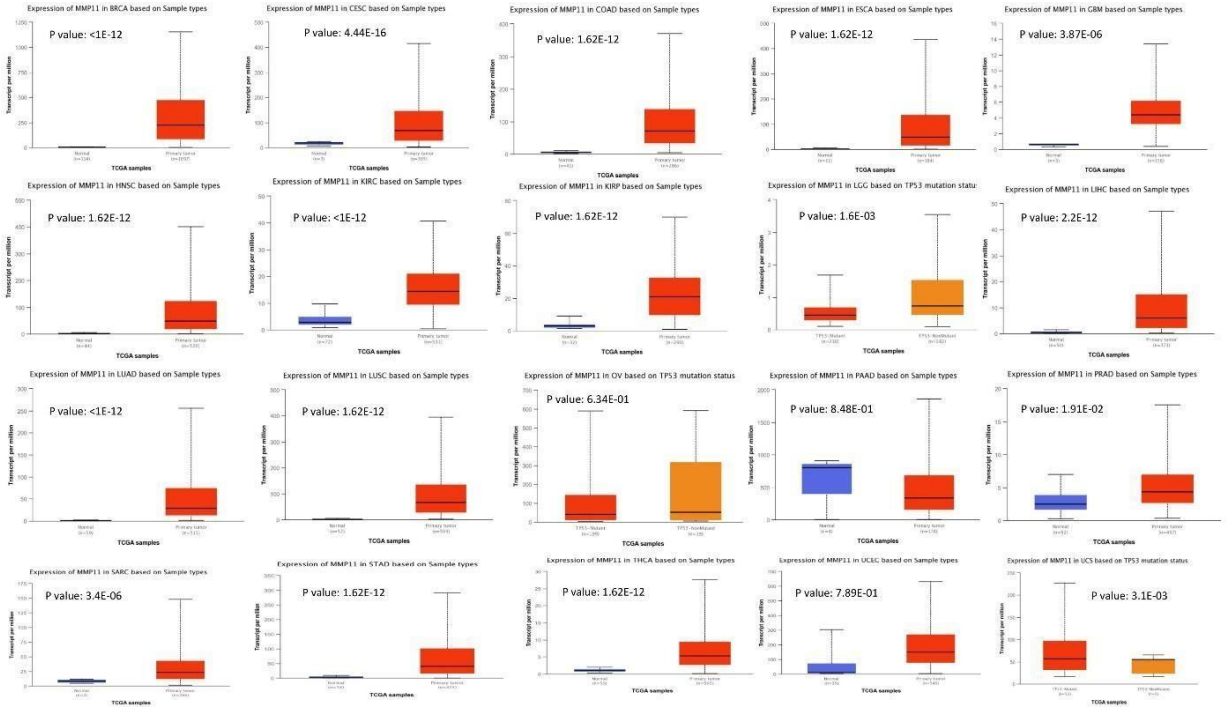


Fig 5.1: pan cancer analysis of MMP-11 gene expression from TCGA dataset

Table 5.1: p-values of TCGA gene expression analysis for various cancers

CANCER	p-value
BRCA	<1E-12
CESC	4.44E-16
COAD	1.62E-12
ESCA	1.62E-12
GBM	3.87E-06
HNSC	1.62E-12
KIRC	<1E-12
KIRP	1.62E-12
LGG	1.6E-03
LIHC	2.2E-12
LUAD	<1E-12
LUSC	1.62E-12
OV	6.34E-01
PAAD	8.48E-01
PRAD	1.91E-02
SARC	3.4E-06
STAD	1.62E-12
THCA	1.62E-12
UCEC	7.89E-01
UCS	3.1E-03

5.1.2 Validation of MMP11 mRNA expression study in pan-cancer

To confirm our findings, we searched through another database, namely Gene Expression Profiling Interactive Analysis (GEPIA, 2017), to study MMP-11 protein expression levels. All data was set to a default p-value of 0.01. Similar results were found for MMP-11 gene expression for the same set of cancers. In the given boxplots below [Fig 5.2], the y-axis is set to the logarithmic value of number of transcripts per million produced. The red boxes indicate tumor samples while grey boxes indicate normal samples. Once again, the highest MMP-11 gene expression was found in BRCA, while PRAD was upregulated the least, thereby matching the results from TCGA data analysis.

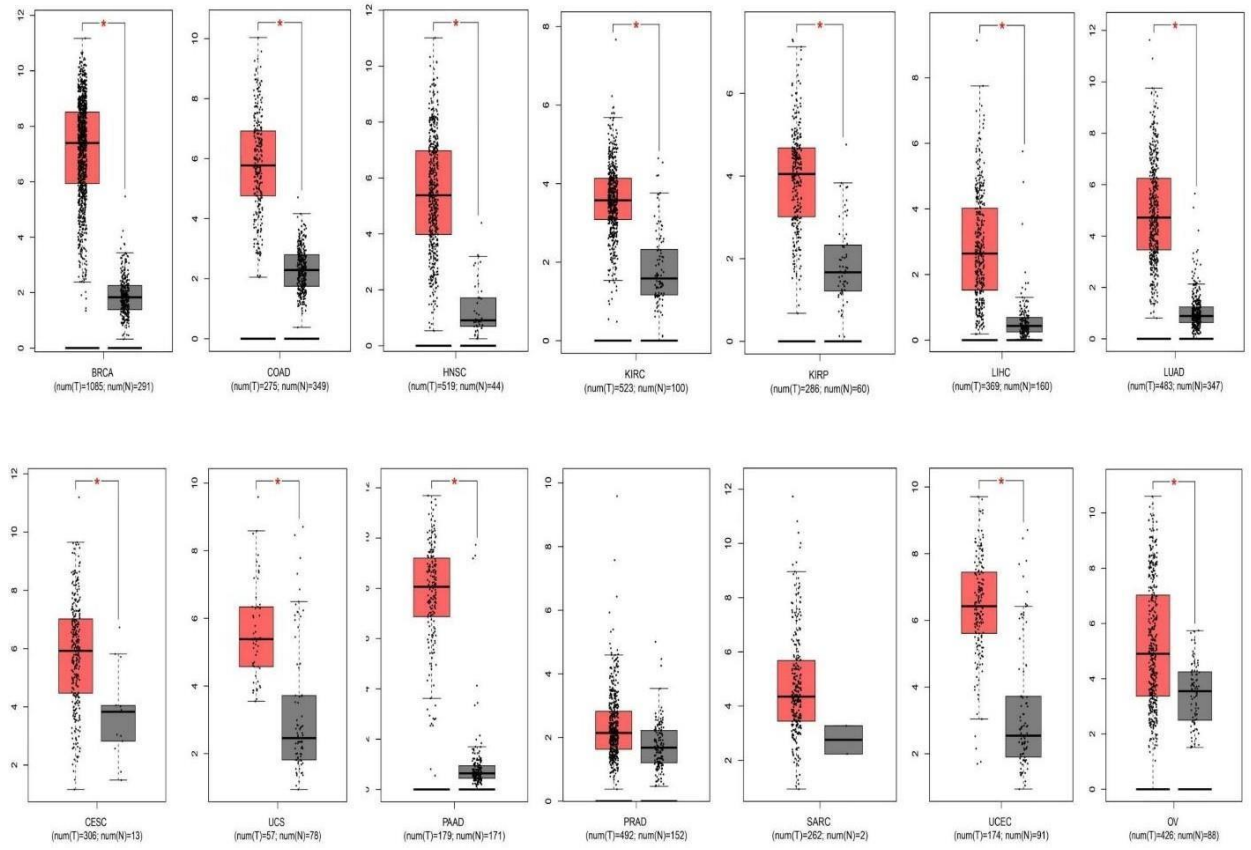


Fig 5.2: pan cancer analysis of MMP-11 gene expression from GEPIA database

5.1.3 Pan-cancer study of MMP11 protein expression

The protein expression levels of MMP-11 in pan cancer were studied using the CPTAC dataset in UALCAN database. Analysis showed that in BRCA, MMP-11 protein levels were distinctly downregulated in tumor samples. However, owing to a very high p-value of 0.848, this data could possibly be unreliable. Moreover, literature analysis from the recent past has shown that high MMP-11 protein expression is linked with poor prognosis in Breast cancer.

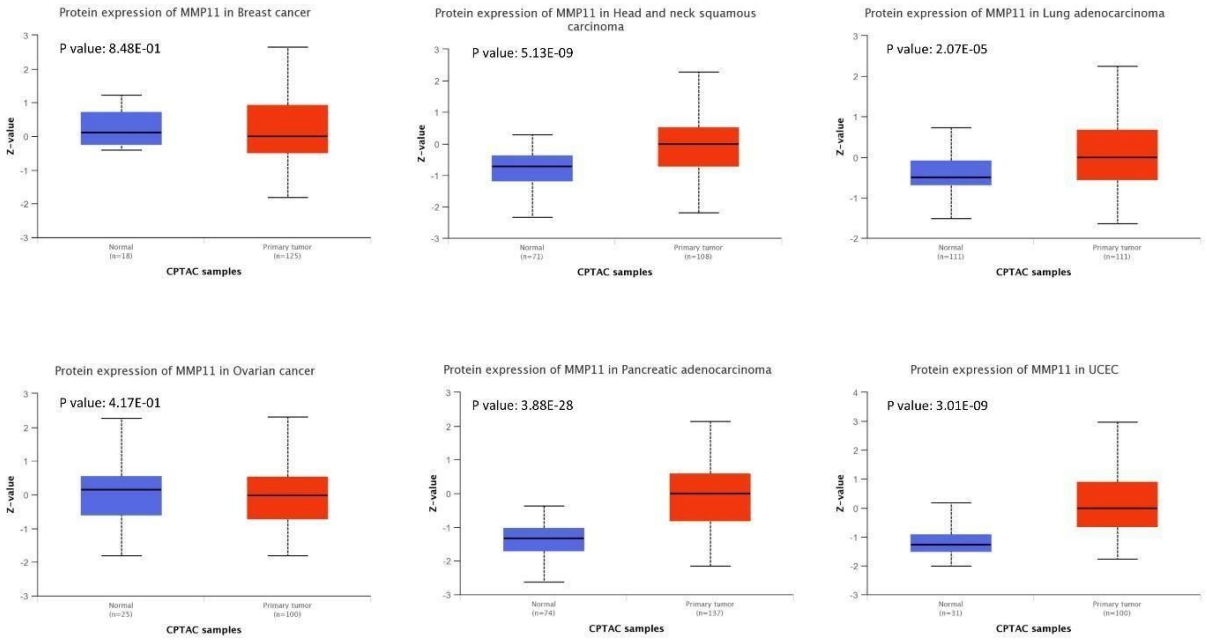


Fig 5.3: pan cancer analysis of MMP-11 protein expression from CPTAC dataset

Table 5.2: p-values of CPTAC protein expression analysis for various cancers

CANCER	p-value
BRCA	8.48E-01
HNSC	5.13E-09
LUAD	2.07E-05
OV	4.17E-01
PRAD	3.88E-28
UCEC	3.01E-09

5.2 IN-VITRO ANALYSIS

5.2.1 IC50 evaluation of Pimozide

The percentage cytotoxicity curves were constructed using absorbance data in the GraphPad prism V5.0 software (RRID SCR_015807). Graphical analysis revealed that a drug concentration of 17 µM resulted in no cytotoxicity in both HEK293 and MCF7 cells. A reportable cytotoxicity was found between concentrations of 20-30 µM in both cell lines. An overall higher cytotoxicity was found at 24 hr in MCF7 cells compared to HEK293. At 23 µM drug concentration, MCF7 cells showed a higher percentage toxicity than HEK293. The IC50 was found to be 25 µM in HEK293 comparing 48 hr and 72 hr, and 23.5 µM in MCF7 comparing 48 hr and 96 hr.

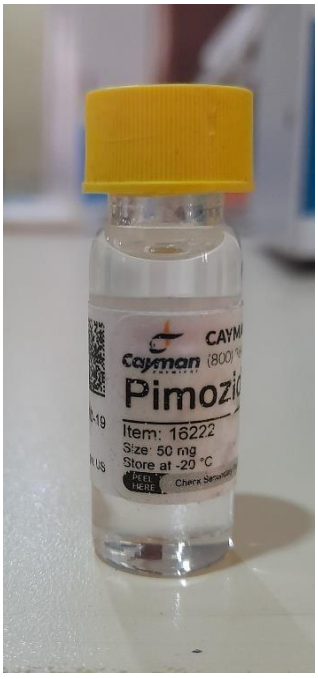


Fig 5.4: Pimozide drug

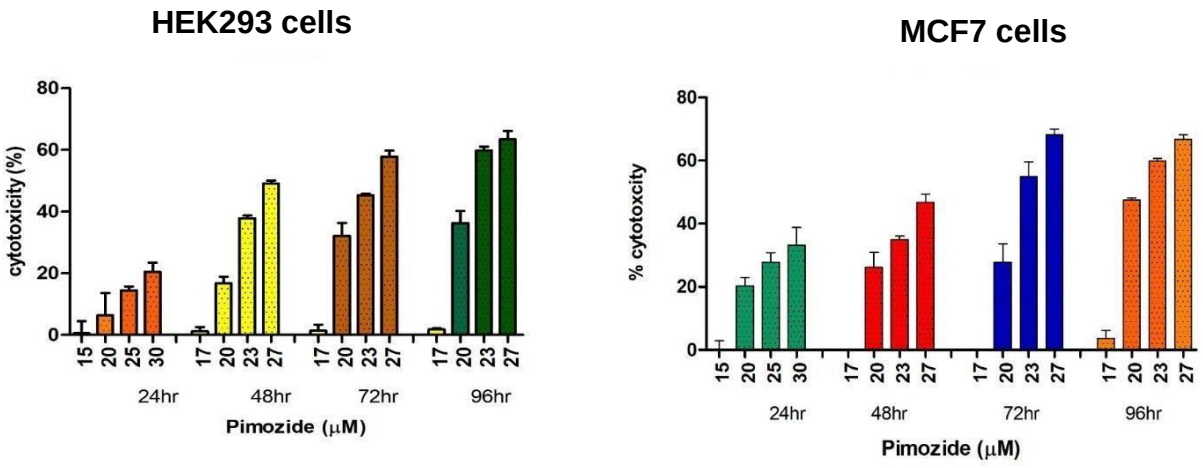


Fig 5.5: cytotoxicity studies for control cells (HEK293) and Breast cancer cells (MCF7)

5.2.2 UV Spectrophotometry studies

The absorbance of untreated and drug treated HEK293 and MCF7 cell lines was evaluated using Synergy H1 microplate reader (BioTek, United States) at 570 nm wavelength, at a temperature set to 23.6 °C for 24 hr, 48 hr, 72 hr and 96 hr durations. The curve was found to exponentially increase in both cell lines for untreated and treated cells. The curves of untreated HEK293 and MCF7 cell lines were also found to be approximately equal. However, the difference in absorbance between untreated and drug-treated cells was higher in MCF7 cells compared to HEK293 cells. While no reportable change in absorbance values from untreated to treated cells could be observed from HEK293 cell lines, the absorbance was found to significantly decrease in drug treated MCF7 cells at 48 hr, 72 hr and 96 hr durations, thereby inferring a decreased cell viability in the same. This proved that Pimozide resulted in an increased cytotoxicity in MCF7 cells compared to HEK293 cells.

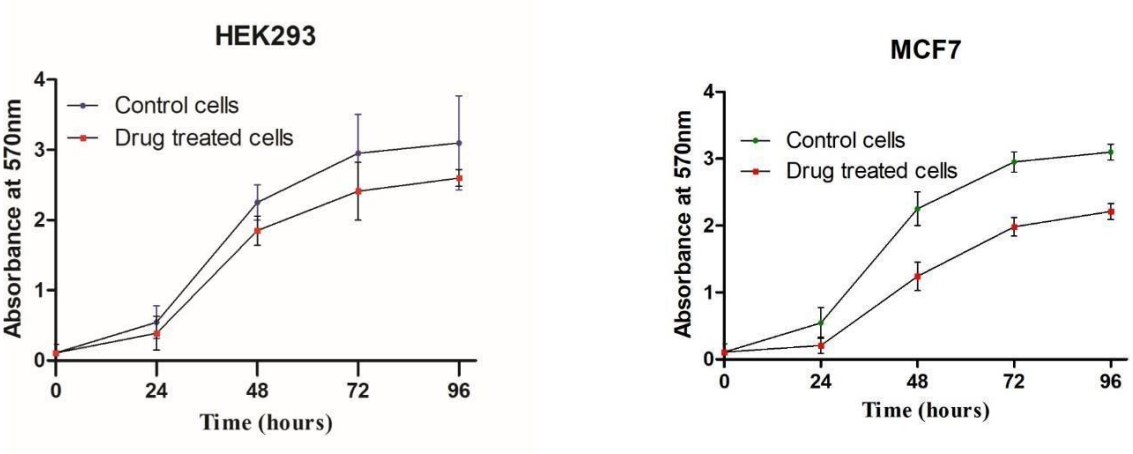


Fig 5.6: Absorbance graph for HEK293 and MCF7 cells

5.2.3 Colony Forming Assay studies

The results of crystal violet staining of HEK293 and MCF7 untreated and drug treated cell lines were visualized in Gel Doc XR+ Gel Documentation System (Biorad, USA) using epi-white filter and white light. The images were exported to ImageJ software for cell counting. A graphical representation of the number of colonies in HEK293 and MCF7 untreated and drug treated cells was obtained. Untreated HEK293 cells were found to have over 125 colonies, which reduced to approximately 90 colonies after drug treatment. Whereas in MCF7, the number of colonies reduced from 27 in untreated cells to 10 colonies in Pimozide treated cells.

HEK293

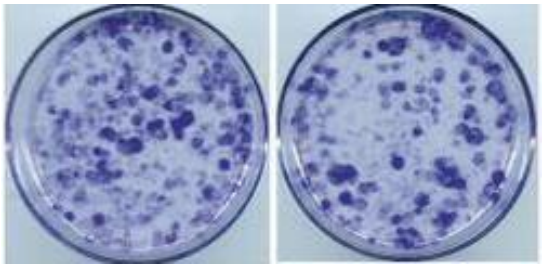


Fig 5.7: Control cells Drug treated cells

MCF7

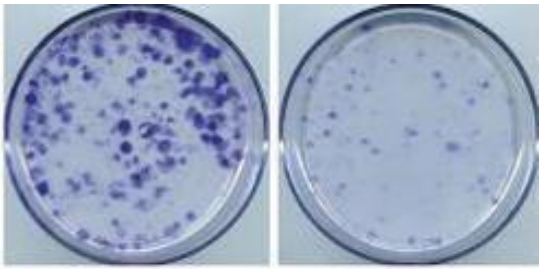


Fig 5.8: Control cells Drug treated cells

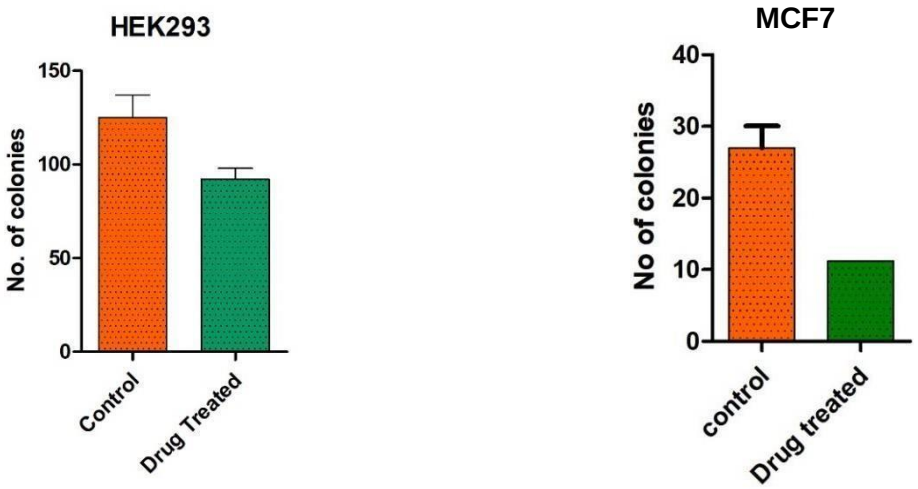


Fig 5.9: Bar graph depicting number of colonies in HEK293 and MCF7 cells

5.2.4 RNA isolation and Agarose Gel Electrophoresis

RNA isolated from drug treated MCF7 cells was obtained as a clear white pellet after precipitation and centrifugation with isopropanol. The pellet was air-dried at room temperature and made ready for Agarose Gel Electrophoresis to ensure that RNA was intact. Two wells of RNA samples were run and the resulting gel run was visualized under Gel Doc XR+ Gel Documentation System (Biorad, USA) using UV light. The bands appeared as 2 band patterns: the larger molecular weight band being mRNA and the smaller molecular weight band being t-RNA. This pattern thereby confirmed the integrity of the RNA samples.

Fig 5.10: separation of organic phase post centrifugation

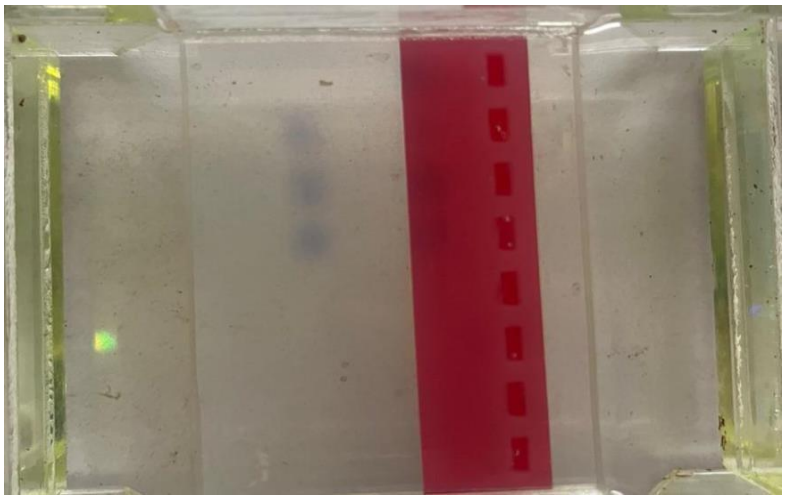


Fig 5.11: Agarose Gel Electrophoresis run for RNA isolated from Pimozide treated MCF-7 cells

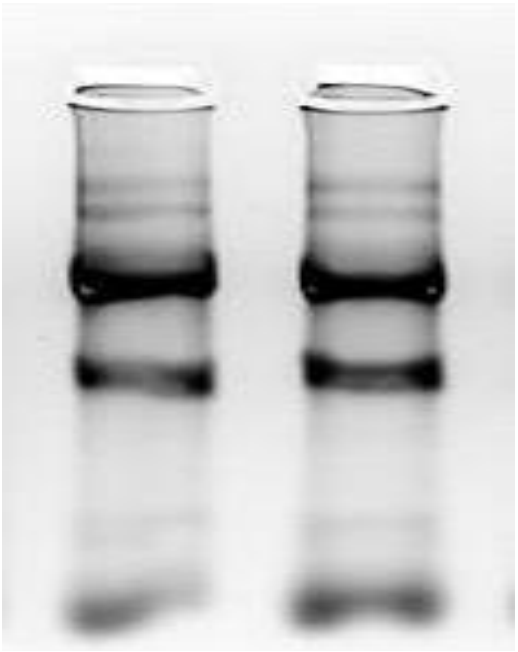


Fig 5.12: Gel Doc analysis of RNA isolated from drug treated MCF7 cells

5.2.5 Polymerase Chain Reaction studies (PCR)

PCR was performed for nine genes namely MMP11, IGF1, CCND1, AKT, p65, SMAD2, SMAD3, TIMP3 and BETA ACTIN. cDNA samples were obtained by reverse transcription of isolated RNA samples from drug treated MCF7 cells. The resulting PCR amplified products were run using Agarose Gel electrophoresis to confirm the amplification of the genes. The gel was visualized under Gel Doc XR+ Gel Documentation System (Biorad, USA). A single band pattern was obtained for all target genes which confirmed the good quality of the PCR run.

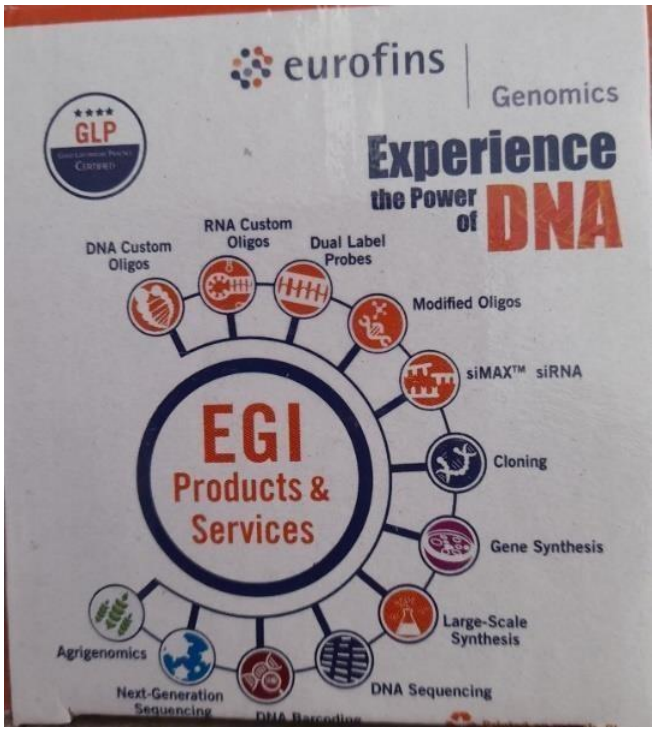


Fig 5.13: Primers package from Eurofins

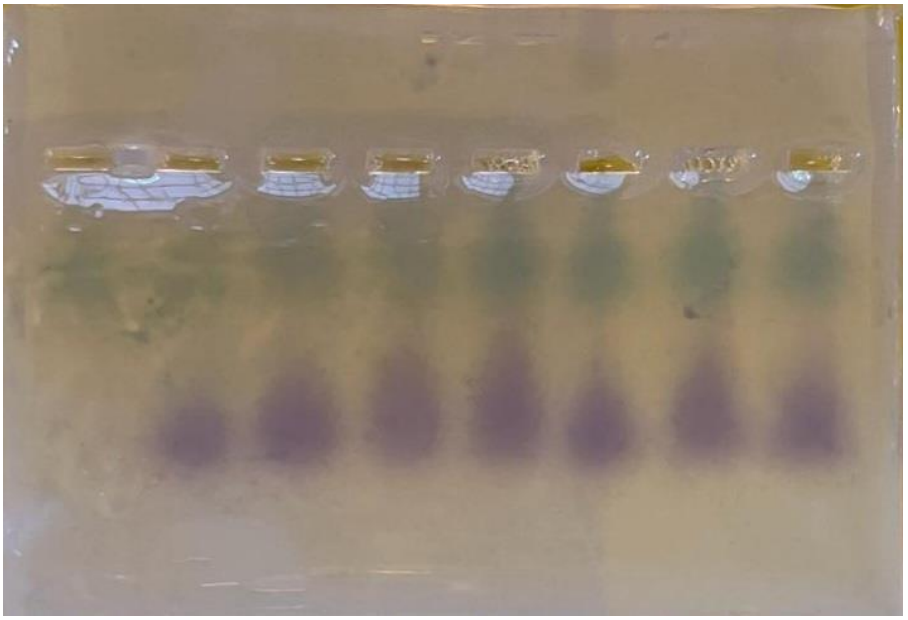


Fig 5.14: Agarose gel run for PCR products of target genes

Fig 5.15: Gel Doc analysis of PCR amplified products from MCF7 cells

5.2.6 Quantitative Real-Time-Polymerase Chain Reaction (qRT-PCR) studies

qRT-PCR results for 8 genes were analyzed using Beta Actin as the housekeeping gene. The resulting cyclic threshold values provided insights on the fold change for each gene before and after Pimozide treatment. The results showed that after Pimozide treatment of cells, there was a significant two-fold downregulation of several target genes in MCF7 cells: namely MMP-11 ($p = 0.003$), IGF1 ($p = 0.002$), CCND1 ($p = 0.01$), p65 ($p = 0.009$), SMAD3 ($p = 0.005$) and TIMP3 ($p = 0.0006$), while AKT ($p = 0.0004$) was found to be maximally downregulated. However, the tumor suppressor gene SMAD2 ($p = 0.0006$) was found to be distinctly upregulated which could infer in a renewed anti-tumor activity of SMAD2 after treatment.

Table 5.3: List of downregulated and upregulated genes post Pimozide treatment of MCF7 cells

Cancer	**Cell line**	**Downregulated Genes (Fold change)**	**Upregulated Genes (Fold change)**
Breast Cancer	MCF7	MMP-11 (0.63)	SMAD2 (1.39)
		IGF1 (0.50)	
		CCND1 (0.46)	
		AKT (0.21)	
		SMAD3 (0.50)	
		p65 (0.46)	
		TIMP3 (0.75)	

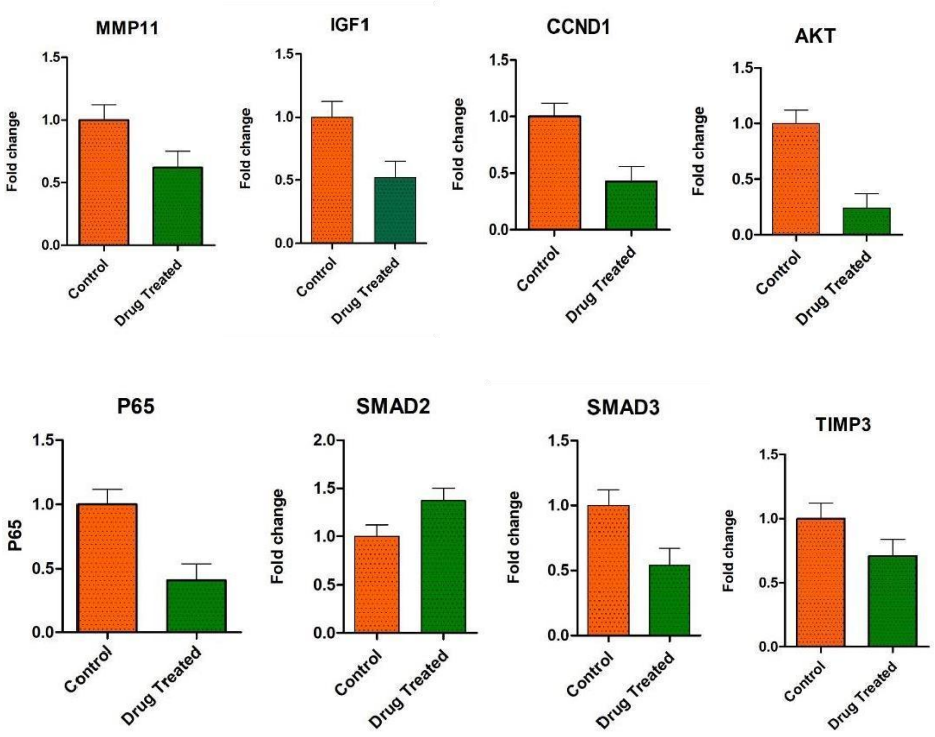


Fig 5.16: results of qRT-PCR studies

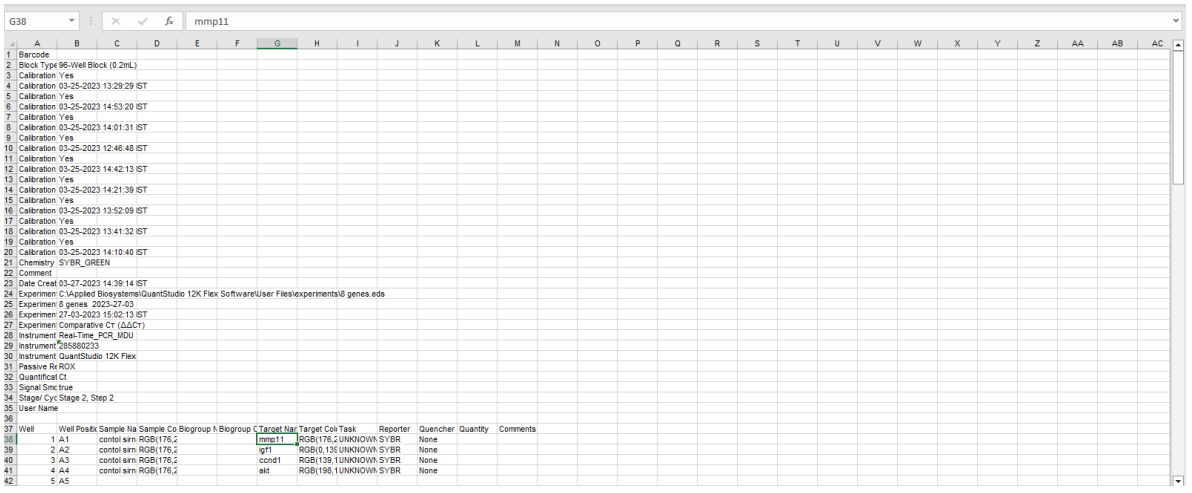
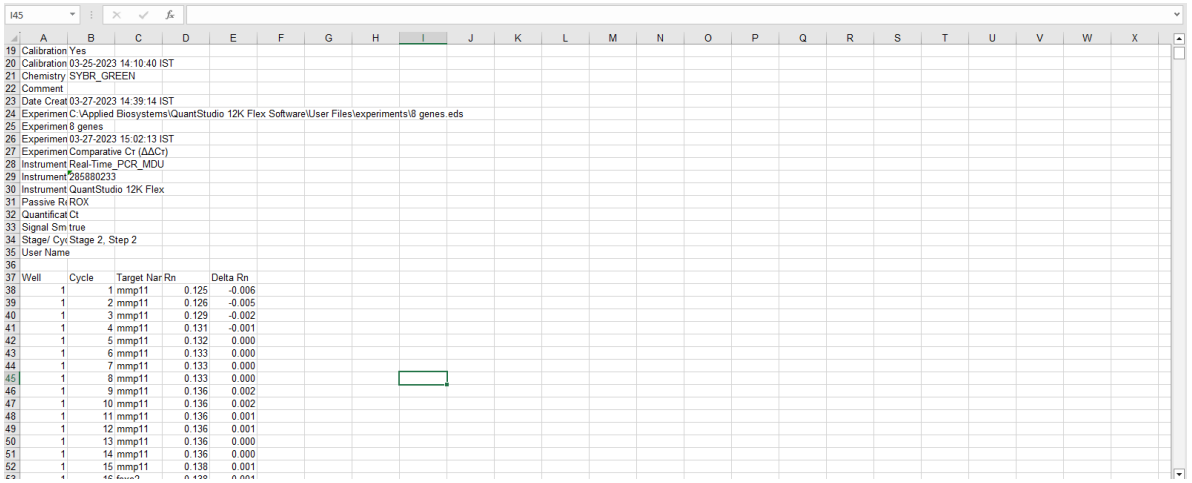


Fig 5.17: sample setup for qRT-PCR instrument



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29	Instrument 285880233					
30	Instrument QuantStudio 12K Flex					
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32	Quantificat Ct					
33	Signal Sm true					
34	Stage/ Cyc Stage 2, Step 2					
35	User Name					
36						
37	Well	Cycle	Target Nar	Rn	Delta Rn	
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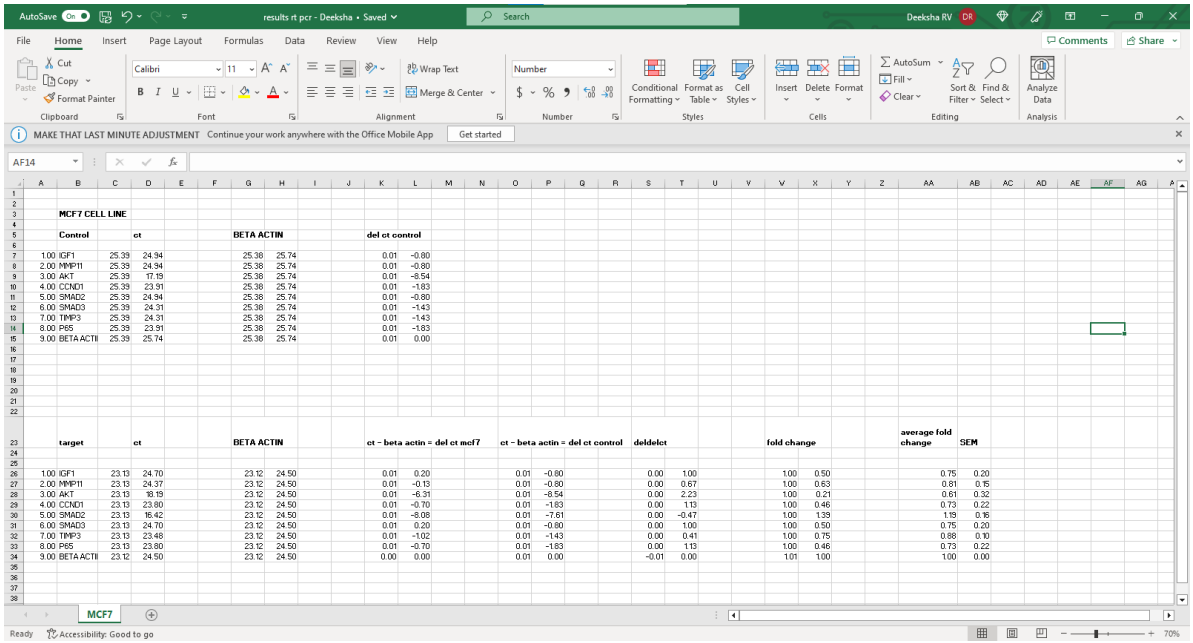
41	1	4	mmp11	0.131	-0.001	
42	1	5	mmp11	0.132	0.000	
43	1	6	mmp11	0.133	0.000	
44	1	7	mmp11	0.133	0.000	
45	1	8	mmp11	0.133	0.000	
46	1	9	mmp11	0.136	0.002	
47	1	10	mmp11	0.136	0.002	
48	1	11	mmp11	0.136	0.001	
49	1	12	mmp11	0.136	0.001	
50	1	13	mmp11	0.136	0.000	
51	1	14	mmp11	0.136	0.000	
52	1	15	mmp11	0.138	0.001	

Fig 5.18: amplification data from qRT-PCR instrument



MCF7 CELL LINE

	Control		ct		BETA ACTIN		del ct control	
1.00	IGF1	25.39	24.94		25.38	25.74	0.01	-0.80
2.00	MMP11	25.39	24.94		25.38	25.74	0.01	-0.80
3.00	AKT	25.39	17.19		25.38	25.74	0.01	-8.54
4.00	CCND1	25.39	23.91		25.38	25.74	0.01	-1.83
5.00	SMAD2	25.39	24.94		25.38	25.74	0.01	-0.80
6.00	SMAD3	25.39	24.31		25.38	25.74	0.01	-1.43
7.00	TIMP3	25.39	24.31		25.38	25.74	0.01	-1.43
8.00	P65	25.39	23.91		25.38	25.74	0.01	-1.83
9.00	BETA ACTI	25.39	25.14		25.38	25.74	0.01	0.00

	target		ct		BETA ACTIN		ct – beta actin = del ct mcf7		ct – beta actin = del ct control		deldelct		fold change		average fold change	SEM
1.00	IGF1	23.13	24.70		23.12	24.50	0.01	0.20	0.01	-0.80	0.00	1.00	1.00	0.50	0.75	0.20
2.00	MMP11	23.13	24.37		23.12	24.50	0.01	-0.13	0.01	-0.80	0.00	0.67	1.00	0.63	0.81	0.15
3.00	AKT	23.13	18.19		23.12	24.50	0.01	-6.31	0.01	-8.54	0.00	2.23	1.00	0.21	0.61	0.32
4.00	CCND1	23.13	23.80		23.12	24.50	0.01	-0.70	0.01	-1.83	0.00	1.13	1.00	0.46	0.73	0.22
5.00	SMAD2	23.13	16.42		23.12	24.50	0.01	-8.08	0.01	-7.61	0.00	-0.47	1.00	1.39	1.19	0.16
6.00	SMAD3	23.13	24.70		23.12	24.50	0.01	0.20	0.01	-0.80	0.00	1.00	1.00	0.50	0.75	0.20
7.00	TIMP3	23.13	23.48		23.12	24.50	0.01	-1.02	0.01	-1.43	0.00	0.41	1.00	0.75	0.88	0.10
8.00	P65	23.13	23.80		23.12	24.50	0.01	-0.70	0.01	-1.83	0.00	1.13	1.00	0.46	0.73	0.22
9.00	BETA ACTI	23.12	24.50		23.12	24.50	0.00	0.00	0.01	0.00	-0.01	0.00	1.01	1.00	1.00	0.00

Fig 5.19: calculation of fold change using CT values of control and drug-treated MCF7 cells

5.3 IDENTIFICATION OF PROTEIN-PROTEIN INTERACTIONS AND PROTEIN CONSTRUCTION NETWORK FOR TARGETING MMP-11

5.3.1 Protein-protein interactions and network construction using STRING database

A protein network was constructed for MMP-11 using 50 interacting partners. The network resulted in 211 nodes with a local clustering coefficient of 0.643, indicating a high density of the network. The network was adjusted to find the proteins that were directly associated with MMP-11, although it did not show the type of association. Twenty-four proteins in total were found to have a direct link with MMP-11, while fourteen of these were predicted by the software itself. Ten proteins were linked due to their known interactions from experimental studies. Functional enrichment of the network resulted in twenty-one clusters of interacting metalloproteases, with an overall p-value < 1.0e-16. Of these, eleven clusters showed an intervention of MMP-11. Downregulation of MMP-11 was controlled by TIMP3, NGF, TIMP1, SPOCK family, WFIKKN family among others, while its activation was regulated by several members of the MMP family, CRSS family, KLK family and FURIN among others. The TIMP family was found to interact with MMP-11 for both its activation and downregulation, suggesting a possible dual role in prognosis. While these networks do not directly imply specific possible interactions in breast cancer, some proteins from the network were screened from literature and identified to be potential partners in breast cancer prognosis. These include COX-2, FOX, ERBB family, IL6, IL10, WNT and AXIN2. Of these, FOX and YAP overexpression was claimed to result in good prognosis of ER+ breast cancer, while the overexpression of others was claimed to result in poor prognosis of the same.

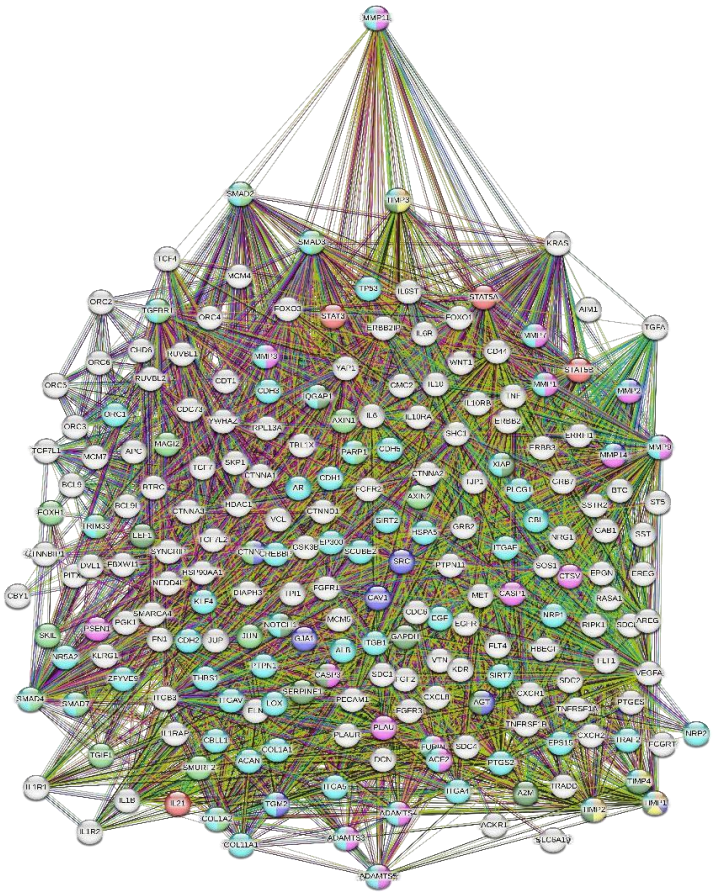


Fig 5.20: STRING network constructed for MMP-11 protein

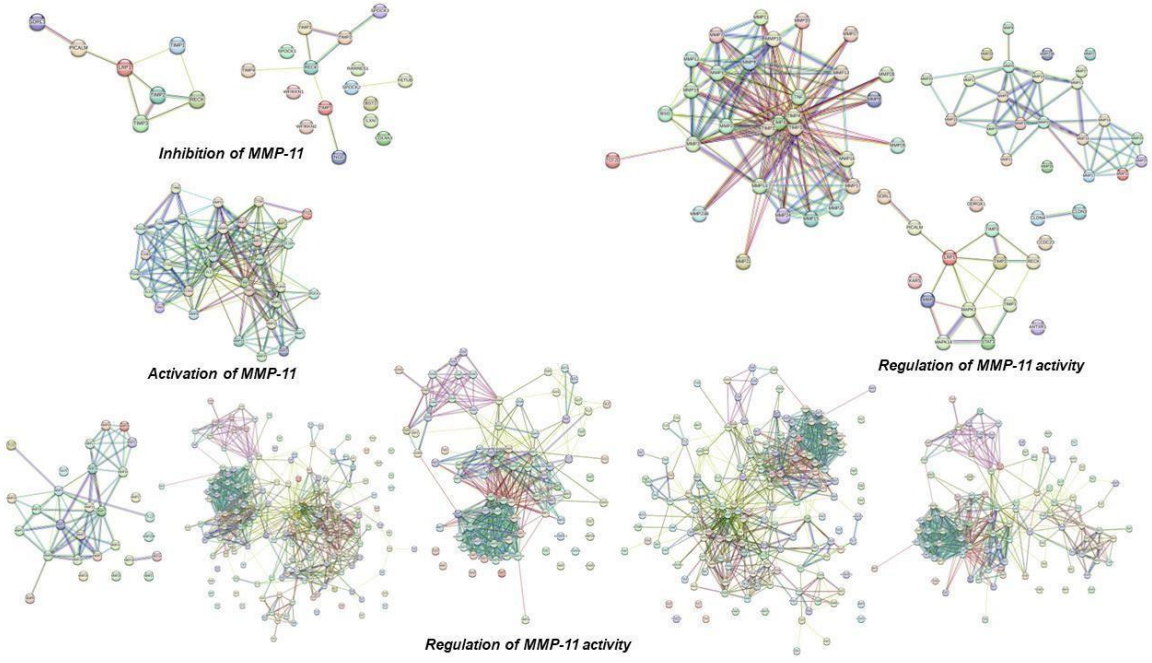


Fig 5.21: results of pathway enrichment analysis for MMP-11 protein

Table 5.4: list of interacting partners from STRING directly linked to MMP-11

Known interactions	**Predicted interactions**
SMAD2	TCF4
TIMP3	CD44
CDH1	STAT3
IL1B	TNF
ACAN	CMC2
SSTR2	MMP2
TIMP1	MMP1
A2M	MMP7
FURIN	AIM1
ALB	CHD6
	ORC6
	FN1
	ST5
	VEGF
	SDCBP

5.3.2 Validation of protein-protein interactions and network construction using Cytoscape

Results from Cytoscape revealed that MMP-11 was linked to several proteins of different families. Cytoscape deciphered an additional set of proteins linking MMP-11 through several networks, which were previously not shown by STRING. These include the MRPS family, TP53, TSPAN, RIF1, PDK1, FN1, TACC2, FBL, APP among others, while MMP-11 also showed a direct interaction with RNF181, HMCN1, XKR3, IGFALS, GXYLT1 and PLEKHM3.

Pathway enrichment analysis using the Reactome Plug-in revealed that MMP-11 was involved in a number of signaling pathways, several of them significant in breast cancer. These include EMT transition ($p<0.001$), Integrin-linked kinase signaling ($p = 0.009$), signaling events mediated by the VEGF family ($p<0.001$), plasma membrane estrogen receptor signaling ($p<0.001$), IL-3 mediated signaling events ($p<0.001$), integrin related cell surface interactions ($p<0.001$), Hepatocyte growth factor related cell signaling events ($p<0.001$) among many others.

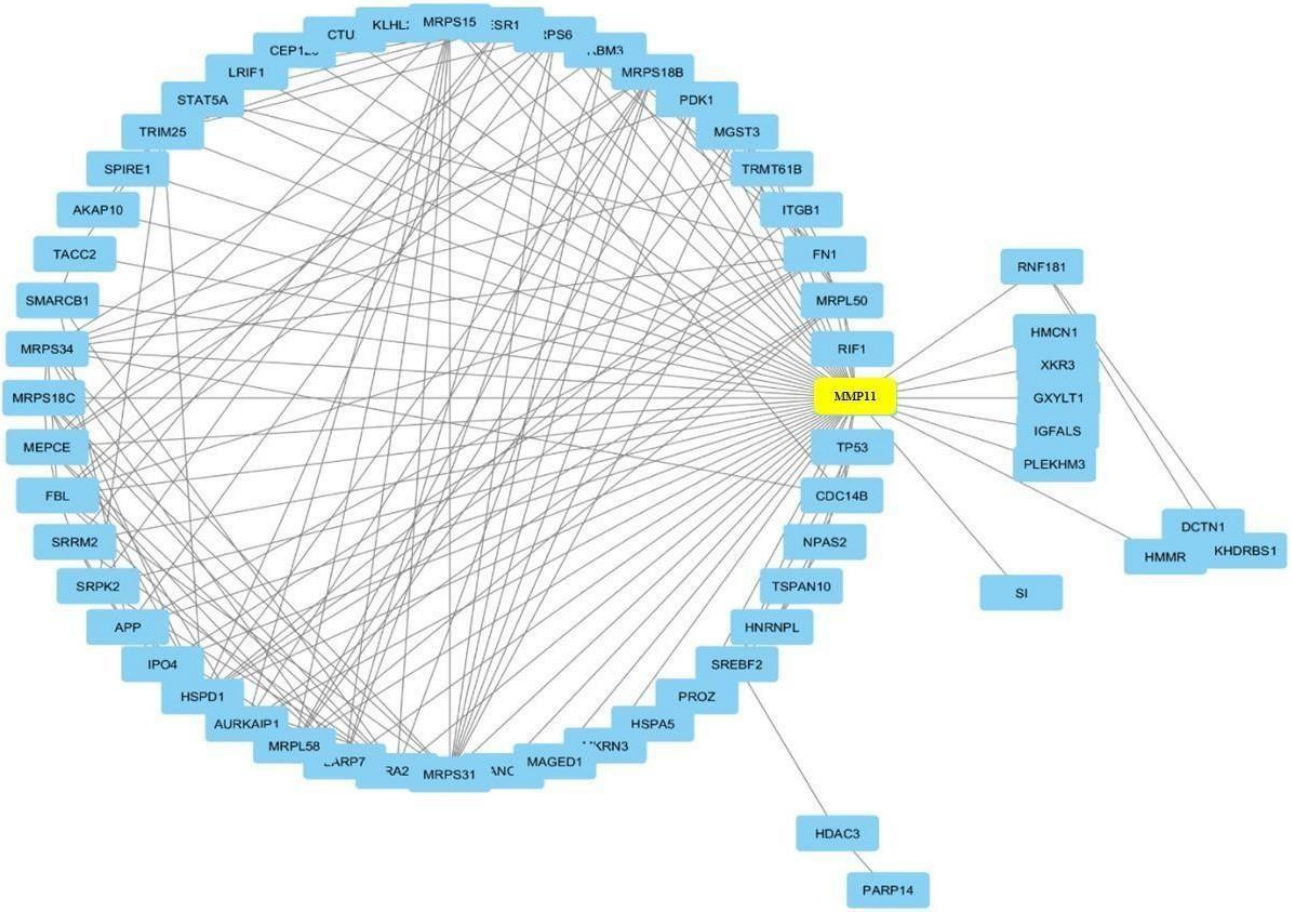


Fig 5.22: protein network constructed by Cytoscape

Molecular pathways involved
pathways related to cancer
PIP3/AKT pathway
estrogen signaling pathway
G2/M phase
MAPK signaling
Hedgehog signaling
Apoptosis
NF-kB pathway
oncogene induced senescence
Wnt signaling
P53 pathway
Circadian Rhythm

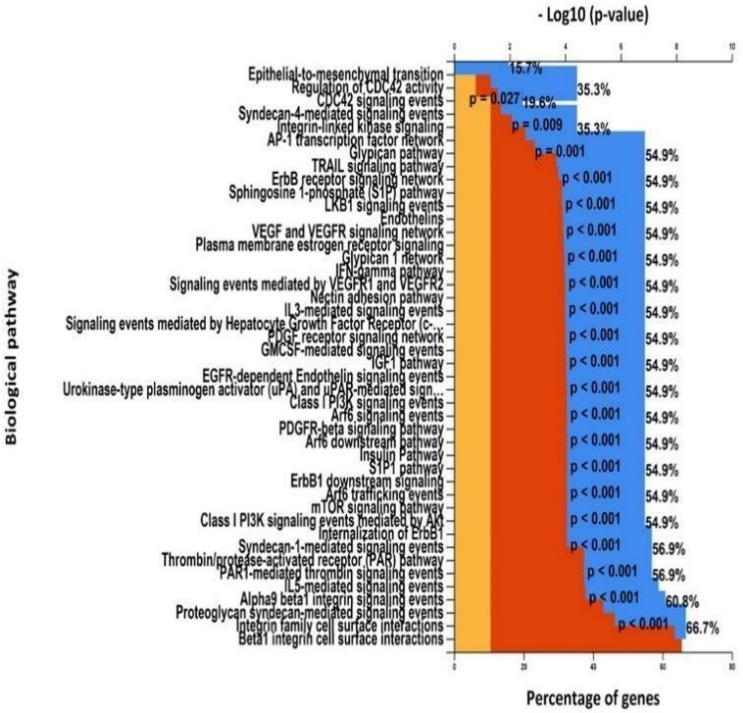


Fig 5.23: Results of pathway enrichment analysis by Cytoscape Reactome Plug-in

5.4 DISCUSSION

Breast cancer continues to be a great challenge for treatment due to the incomplete research on protein-protein interactions that make up several signaling pathways. Targeted drug delivery is an important and vital area that can be improved and modified only if the mechanisms and interacting partners are completely understood and verified. Moreover, due to the vast network of proteins that control several important biological processes at a cellular level, the inhibition of one target protein can potentially affect several other downstream pathways, thereby resulting in a desirable outcome. However, a complete understanding of the protein interactome is yet to be discovered. Hence, this study focused on deciphering various signaling pathways by which Pimozide, a known antipsychotic repurposed for cancer therapy, targeted MMP-11, an ECM remodeling endopeptidase that is growing importance for its prognostic value in Breast Cancer. This study mainly focused on Pimozide's effect on three mechanisms in MCF7 cells: namely cell proliferation, invasion, and mitochondrial metabolism. This study builds on previously published *in silico* research about the effective docking of Pimozide with MMP-11 as a targeted drug therapy for Breast Cancer. In our current study, ER+ breast cancer cell line, specifically MCF7 was used as the test subject. The HEK293 cell line was used as a control to observe the difference in Pimozide treatment in both cell lines. The cell lines were maintained in a CO_2 incubator and passaged at regular intervals when cells reached confluency. The resulting cells were subjected to different concentrations of Pimozide and IC50 evaluation was performed, which was found to be 25 µM in HEK293 comparing 48 hr and 72 hr, and 23.5 µM in MCF7 comparing 48 hr and 96 hr durations. UV spectrophotometry analysis revealed that Pimozide treatment resulted in a significant increase in cytotoxicity in MCF7 cells than in HEK293 cells, thereby inferring in a significant decrease in cell viability in the former than the latter. Colony forming assay further boosted the therapeutic value of Pimozide by showing a higher reduction of colonies in MCF7 than in HEK293.

The satisfying results further prompted PCR and RT-PCR studies targeting specific genes of interest to see their up- or down-regulation in MCF7 cells post Pimozide treatment. The results of RT-PCR proved that several cell proliferation markers such as IGF1, CCND1, AKT and P65, mitochondrial regulation marker TP53 and invasion markers MMP11, SMAD2 and SMAD3 were significantly affected. The in-vitro studies were further supported by in-silico studies of protein-protein interactions by constructing protein networks of MMP-11 using STRING and Cytoscape databases. A p-value < 0.05 was considered statistically significant for all experiments. The signaling pathways analysis gave a strong indication of the importance of MMP-11 as a therapeutic target for breast cancer treatment. The protein networks showed that targeting MMP-11 could have a potential impact on other proteins critical in ER+ breast cancer including ERBB family whose overexpression leads to endocrine resistance during therapy, Axin family whose overexpression is known to facilitate breast cancer invasion and metastasis, Wnt which is an important cell proliferation marker in BC, YAP AND FOX whose overexpression is correlated with good prognosis in BC, COX-2 whose overexpression is associated with aggressive BC, IL6 and IL10 that are important growth factors in BC. In the future, this study will be expanded further to decipher the role of Pimozide treatment to target MMP-11 in other cancers as well.

6 SUMMARY AND CONCLUSIONS

Breast Cancer is one of the oldest diseases studied globally. However, after innumerous research, this carcinoma continues to top the charts as the second leading cause of death worldwide, especially in women of Hispanic and Latin race. The treatment methodologies for BC have widely undergone transformation over the years, from direct chemotherapies using newly synthesized drugs to repurposed drugs that save time and energy for drug synthesis. Of the repurposed drugs, antipsychotics have paved the way to establish better treatment methodologies. Within antipsychotics, the DiPhenylButylpiperidine group, especially Pimozide has utilized various oncogenes and pathways, including JAK1/STAT3 and STAT5 pathways. Along with their seeming advantages, the use of antipsychotics for cancer treatment has been a subject of controversy. The role of MMP11 in poor BC prognosis has been a hot topic for decades. Recently, computational analysis revealed the successful binding of Pimozide against MMP11, although its mechanisms were still unclear. Hence this study compensates for this disparity by identifying specific pathways by which Pimozide affects Breast Cancer cells in proliferation, invasion and mitochondrial metabolism. Cell proliferation was found to be controlled by IGF1, AKT, CCND1 and p65, invasion was found to be controlled by MMP11, SMAD2 and SMAD3 while mitochondrial metabolism was found to be controlled by TIMP3. Pimozide treatment in MCF7 resulted in a direct downregulation of all these oncogenes while tumor suppressor gene SMAD2 was found to be upregulated. Bioinformatics analysis of protein networks linking MMP11 showed that besides these genes, the inhibition of MMP-11 could potentially affect several other downstream pathways linked to ERBB, FOX, COX-2 and Interleukins among others.

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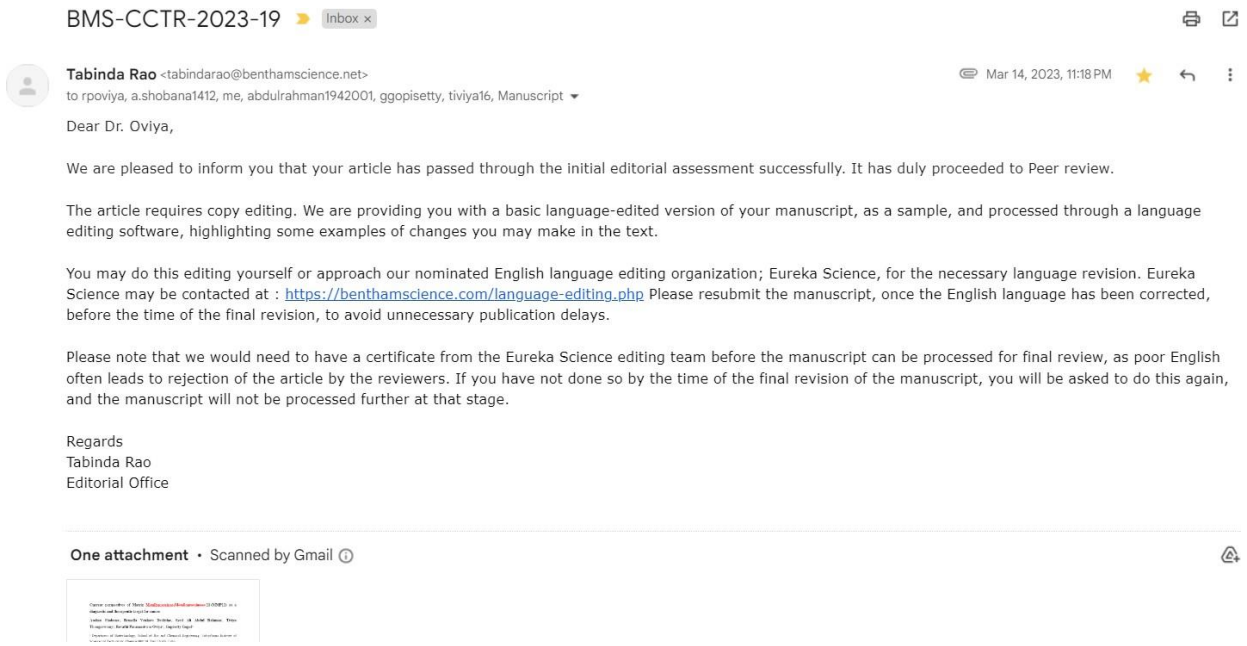
ANNEXURE

Submission and acceptance of review article titled *"*Current perspectives of Matrix Metalloproteinases as a diagnostic and therapeutic target for cancer*"* in **Current Cancer Therapy Reviews, Bentham Science Publishers**.



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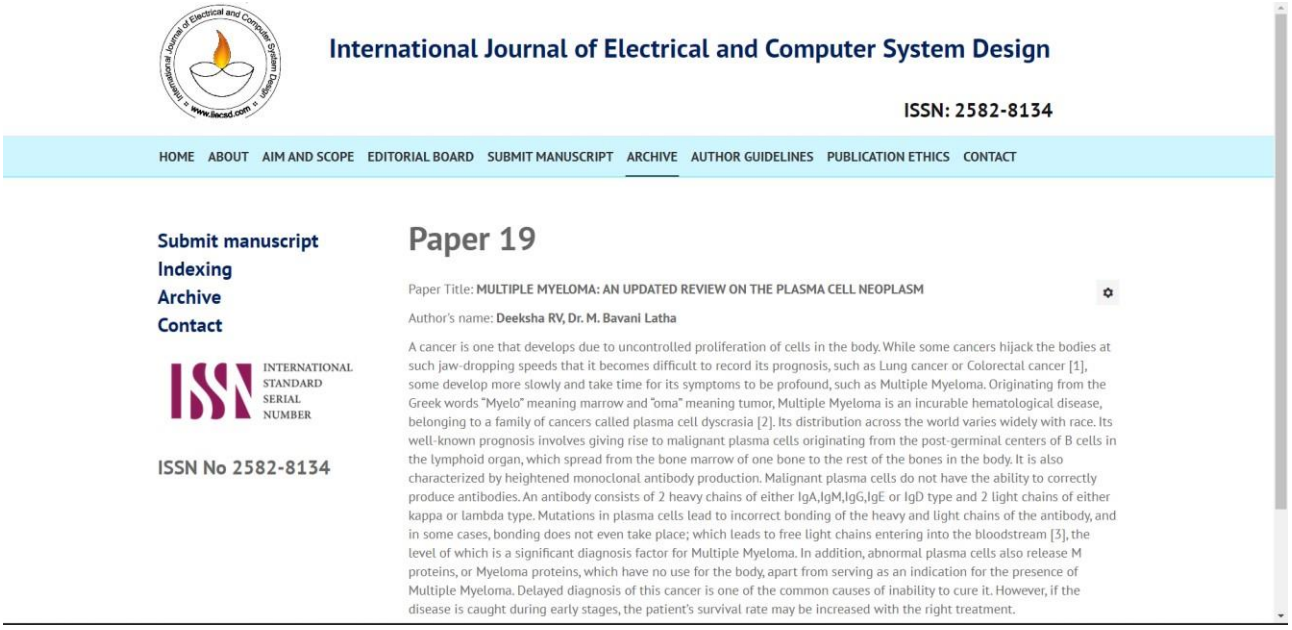

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Paper 19

Paper Title: **MULTIPLE MYELOMA: AN UPDATED REVIEW ON THE PLASMA CELL NEOPLASM**

Author's name: **Deeksha RV, Dr. M. Bavani Latha**

A cancer is one that develops due to uncontrolled proliferation of cells in the body. While some cancers hijack the bodies at such jaw-dropping speeds that it becomes difficult to record its prognosis, such as Lung cancer or Colorectal cancer [1], some develop more slowly and take time for its symptoms to be profound, such as Multiple Myeloma. Originating from the Greek words "Myelo" meaning marrow and "oma" meaning tumor, Multiple Myeloma is an incurable hematological disease, belonging to a family of cancers called plasma cell dyscrasia [2]. Its distribution across the world varies widely with race. Its well-known prognosis involves giving rise to malignant plasma cells originating from the post-germinal centers of B cells in the lymphoid organ, which spread from the bone marrow of one bone to the rest of the bones in the body. It is also characterized by heightened monoclonal antibody production. Malignant plasma cells do not have the ability to correctly produce antibodies. An antibody consists of 2 heavy chains of either IgA,IgM,IgG,IgE or IgD type and 2 light chains of either kappa or lambda type. Mutations in plasma cells lead to incorrect bonding of the heavy and light chains of the antibody, and in some cases, bonding does not even take place; which leads to free light chains entering into the bloodstream [3], the level of which is a significant diagnosis factor for Multiple Myeloma. In addition, abnormal plasma cells also release M proteins, or Myeloma proteins, which have no use for the body, apart from serving as an indication for the presence of Multiple Myeloma. Delayed diagnosis of this cancer is one of the common causes of inability to cure it. However, if the disease is caught during early stages, the patient's survival rate may be increased with the right treatment.

PATENTS FILING

Submission of new invention for patent filing to HumCen Global Pvt. Ltd on March 20, 2023.

1. Invention title: **Formulation of Protein-soup-mix â€“ a plant-based health supplement**

Inventors: Sathyabama Institute of Science and Technology, Deeksha RV, A. Shobana, Dr. R.P Oviya

TITLE : Formulation of Protein-soup-mix â€“ a plant-based health supplement

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2	R V Deeksha	Sathyabama Institute of Science and Technology, Chennai	
3	A. Shobana	Sathyabama Institute of Science and Technology, Chennai	
4	P.Oviya	Sathyabama Institute of Science and Technology, Chennai	

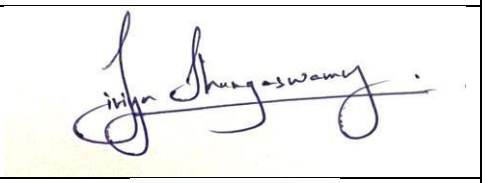
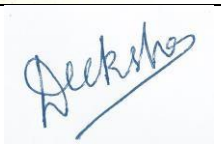
2. Invention title: **Development of anti-bacterial eye kajal using natural products**

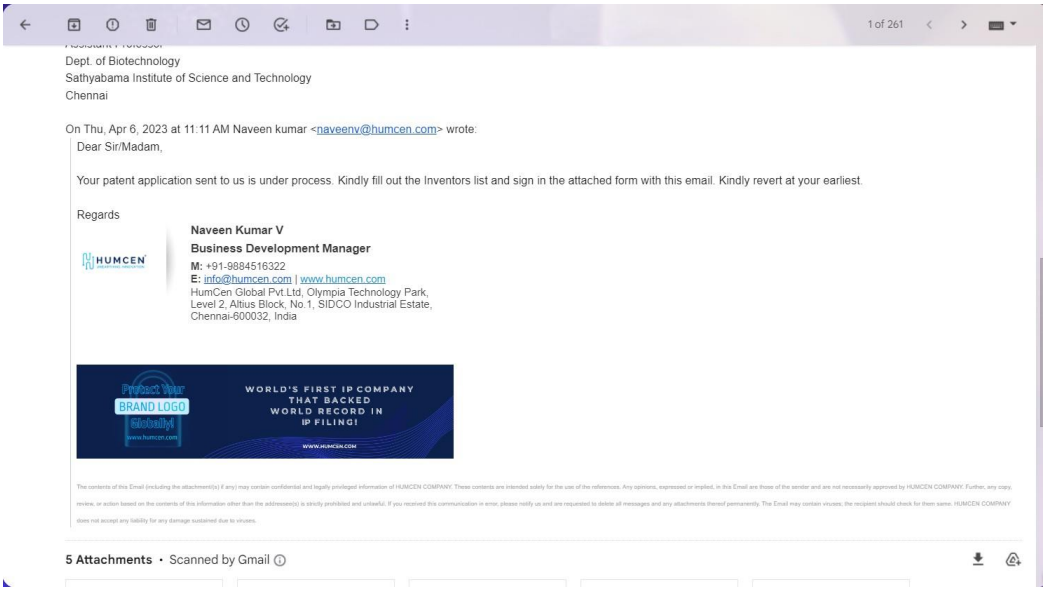
Inventors: Sathyabama Institute of Science and Technology, Deeksha RV, Tiviya Thangaswamy, Dr. R.P Oviya

TITLE : Development of anti-bacterial eye kajal using natural products

ALLOTED REF.NO – B3

WE NEED THE FOLLOWING DETAILS (MUST) FOR EVERY SINGLE APPLICATION

S.NO	INVENTOR NAME	AFFILIATION WITH FULL ADDRESS	SIGNATURE SOFTCOPY (USE BLUE PEN WITH WHITE BACKGROUND)
1	Sathyabama Institute of Science and Technology		
2	Tiviya Thangaswamy	Sathyabama Institute of Science and Technology, Chennai	
3	R V Deeksha	Sathyabama Institute of Science and Technology, Chennai	
4	P.Oviya	Sathyabama Institute of Science and Technology, Chennai	



1 of 261

Dept. of Biotechnology
Sathyabama Institute of Science and Technology
Chennai

On Thu, Apr 6, 2023 at 11:11 AM Naveen kumar <naveenv@humcen.com> wrote:

Dear Sir/Madam,

Your patent application sent to us is under process. Kindly fill out the Inventors list and sign in the attached form with this email. Kindly revert at your earliest.

Regards

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Level 2, Altius Block, No.1, SIDCO Industrial Estate,
Chennai-600032, India

5 Attachments • Scanned by Gmail

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