

Drug Repurposing as a promising tool to target Breast Cancer

Submitted in partial fulfillment of the requirements for the award of
Bachelor of Technology degree in Biotechnology

By

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DEPARTMENT OF BIOTECHNOLOGY
SCHOOL OF BIO & CHEMICAL ENGINEERING

SATHYABAMA

**INSTITUTE OF SCIENCE AND TECHNOLOGY
(DEEMED TO BE UNIVERSITY)**

**Accredited with Grade “A” by NAAC
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BONAFIDE CERTIFICATE

This is to certify that this Project Report is the bonafide work of **Syed Ali Abdur Rahman (39230050)** who has done the Project work entitled "**Drug repurposing as a promising tool to target breast cancer**" under our supervision from **January 2023 to May 2023**.

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ABSTRACT

Breast cancer remains the most common cause of cancer-related deaths in women around the world, despite significant resources being spent on prevention and treatment. The therapy options are quite expensive and have negative side effects. Drug repurposing, or finding new uses for outdated medications, has become a cutting-edge strategy for developing new medications. Repositioning older, off patent non-cancer medications with clinical approval and known targets into newer indications is analogous to employing older weaponry in a more recent conflict. The repositioning strategy also provides better, safer, and more affordable medications with fewer or less noticeable adverse effects. This work employs the antipsychotic Pimozide drug and to study its effects on breast cancer cells that express MMP-11 in an effort to further the study the potential of repurposed drugs for the treatment of cancer. The study involved insilico analysis of MMP11 expression and its association with breast cancer patient survival. In vitro cytotoxicity tests employing colony formation, cell invasion, and colorimetric assays showed effective cytotoxicity against Breast cancer cells. The effectiveness of the treatment was determined by examining the impact on biochemical markers, including those that indicate invasion, cell proliferation, apoptosis, angiogenesis, and pro-survival using RT-PCR analysis. The analysis revealed downregulation of VEGF in pimozide treated cells and upregulation of TGF-beta in pimozide treated cells when compared to control cells. This showed that pimozide treatment can alter the gene expressions and modulate the cell signaling mechanism inhibiting angiogenesis thereby reducing cancer cell migration and invasion.

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LIST OF ABBREVIATIONS

AKT	Ak strain Transforming
BC	Breast Cancer
CAD	Cationic Amphiphilic Drug
CD	Cluster of Differentiation
CPZ	Chlorpromazine
CSC	Cancer Stem Cell
DPBP	Di Phenyl Butyl Piperidine
ECM	Extracellular Matrix
EMT	Epithelial Mesenchymal Transition
FDA	Food and Drug Administration
GTPase	Guanosine Triphosphate enzyme
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
MMP-11	Matrix Metalloproteinase-11
PBMC	Peripheral Blood Mononuclear Cells
SMAD	Suppressor of Mothers Against Decapentaplegic
STAT	Signal Transducers and Activators of Transcription
TGF- β	Transforming Growth Factor –

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1 INTRODUCTION

Breast cancer is the most common cancer and the second most common cancer in women worldwide. The leading cause of death from cancer in women. Despite advances in early detection, approximately 30% of patients with early-stage breast cancer still relapse with comprehensive breast cancer treatment (Gonzalez-Angulo *et al.*, 2007) Breast cancer is heterogeneous A disease characterized by distinct subtypes. clinically breast cancer It is classified into one of four major subtypes using three criteria. Immunohistochemical markers: estrogen receptor (ER), progesterone receptor (PR), Human epidermal growth factor receptor 2 (HER/Neu 2) to facilitate targeted therapy (Yuan *et al.*, 2015). In contrast to other MMPs, human MMP11 (stromelysin-3) is processed intracytoplasmically and released as an active enzyme in stromal cells of breast cancer, where it was first discovered (Basset, n.d.). One distinctive characteristic of MMP11 is the glutamine residue at position 215 (at 211 in human MMP11, P24347) of S1' deep pocket (Gall *et al.*, 2001). Breast cancers and stromal-fibroblast-like cells both expressed higher levels of MMP11, and both instances had a significantly lower overall survival (OS) rate (all p 0.05) (Min *et al.*, 2013a). According to (Devel *et al.*, 2010), MMP11- deficient fibroblasts were unable to aid in the implantation of cancer cells in mice, and spatial overexpression of MMP11 and collagen VI at the adipocyte-cancer cell interface promotes tumor evasion (Motrescu *et al.*, 2008). MMP-11 expression levels by various cells have been observed, however stromal cells have the greatest levels of MMP-11 expression. HER2+ breast cancer cells may be aided in invasion by macrophage-expressed MMP-11, according to (Kang *et al.*, 2022). By examining some of the metabolic pathways altered during prognosis, the process of cancer growth facilitated by MMP-11 is better understood.

For instance, MMP11 can block the TGF-B signaling pathway's ability to ubiquitinate the Smad2 protein, allowing excessive cell growth. Similar to how MMP-11 hindrance causes increased IGF1 growth factor synthesis, which in turn causes malignant tumor progression. Additionally, patients with breast cancer who have high MMP-11 expression may eventually die as a result of a sharp decline in CD8+ T cells (Kim *et al.*, 2021).

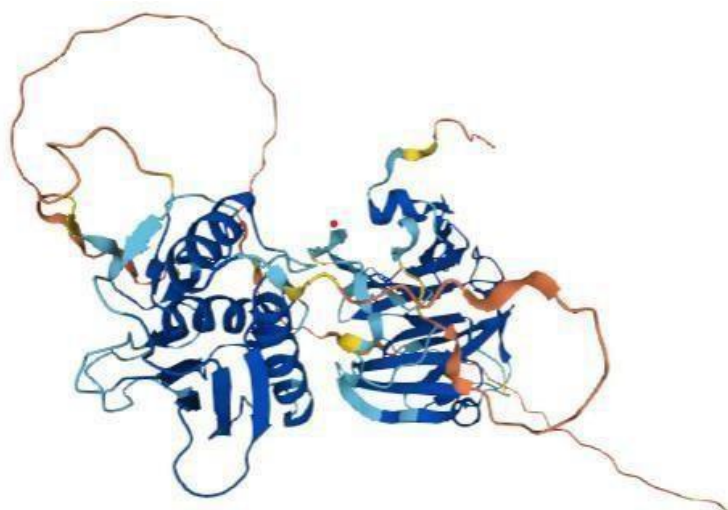


Fig 1.1, structure of MMP-11 protein from Uniprot database

The mechanism of Pimozide treatment has not yet been satisfactorily investigated, although previous study that successfully docked Pimozide against MMP-11 has allowed for future investigation. Pimozide, a first-generation antipsychotic medication from the Diphenylbutylpiperidine group (DPBP), is used to treat Tourette's disorder-related involuntary tics.

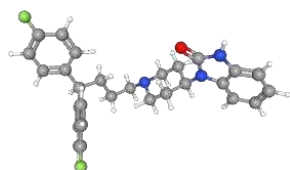


Fig 1.2: 3-D Pimozide structure, retrieved from Pubchem database

Given that individuals receiving Pimozide for schizophrenia were less likely to acquire other tumors, it was recently repurposed as an effective treatment for breast cancer (Elmaci & Altinoz, 2018).. In the past ten years, the use of antipsychotic medications to treat cancer has been extensively reported (Shaw *et al.*, 2021).

It has been shown to impede epithelial mesenchymal transition, increase apoptosis, decrease cell proliferation, and downregulate the production of MMP enzymes. Additionally, Pimozide also reverses the Warburg effect, which causes cancer cells to take up too much glucose in order to promote greater cell proliferation. (J. Li *et al.*, 2022), and inhibits the STAT-3 pathway activated in cancer stem cells (de Vega *et al.*, 2018; Gonçalves *et al.*, 2019).

A new research topic growing increasing interest parallelly is the advent of nanotechnology in targeted drug delivery. The concept of using a particle sized between 1 to 100 nm for drug delivery stems from a unique feature they offer; their ability to detect various molecular signals and biomarkers in real time. Moreover, their small size enables them to slip through the cell membrane in a manner that bigger macromolecules cannot. Metal NPs have so far been extensively used as carriers for antibodies, nucleic acids, small peptides, and chemotherapeutic drugs. Noble metal nanoparticles such as Platinum, Silver, and Gold are by far the most used for biomedical research purposes; specifically in drug delivery, radiotherapy enhancement and diagnostic assays, owing to their inert nature and ease of preparation protocols. Nanoparticles are synthesized by either of two methods: the top down approach and the bottom up approach. The top-down approach is based on breaking down raw materials into nano-sized particles using a number of techniques. This approach includes lithography, laser ablation and vapor deposition. Bottom-up approaches on the other hand deal with building the nanoparticles atom by atom. This approach includes sol-gel method, microemulsion and chemical vapor deposition. Metal NPs are known to increase bioavailability of drug and improve their stability and half-life as well. Moreover, they are non-toxic and do not intervene in the drug-cell interactions, making them extremely useful allies for therapy. Magnetic nanoparticles are the next set to join the list. These include Iron and Nickel NPs. Copper NPs have also been growing recognition due to their antibacterial and antifungal activity. Moreover, they are known to increase ROS production in cancer cells. They are also a cheaper alternative to other transition metals. Other upcoming metal nanoparticles used for cancer treatment include titanium dioxide and zinc oxide. Combinations of NPs such as Gold with silver, zinc and copper have also been documented.

Silver nanoparticle conjugated drugs have shown promising potential for the treatment of breast cancer. Silver nanoparticles have unique physical and chemical properties that make them attractive as drug delivery vehicles, including their small size, high surface area, and ability to target specific cells and tissues. Research and review papers have reported on the enhanced drug delivery capabilities of silver nanoparticles, which can improve the solubility, stability, and bioavailability of anticancer drugs. Furthermore, silver nanoparticles have been shown to possess strong antibacterial and anti-inflammatory properties, which could be beneficial for treating breast cancer, a disease that can be complicated by secondary infections and inflammation. Additionally, silver nanoparticle conjugated drugs can be targeted specifically to cancer cells by attaching them to targeting molecules such as antibodies or peptides. This targeted approach can improve efficacy while minimizing side effects associated with traditional cancer treatments. While the biocompatibility and biodegradability of silver nanoparticles have been demonstrated in various in vitro and in vivo studies, long-term safety implications of these nanoparticles still need to be fully understood. Despite manufacturing challenges, including difficulties in controlling the size, shape, and surface chemistry of nanoparticles, silver nanoparticle conjugated drugs have the potential to revolutionize breast cancer treatment, and more research is needed to further explore this promising therapy. Nickel NPs derived from Nickel oxide as a black powder, have been used to treat triple-negative breast cancer. Thermolysis is a common technique used to synthesize Nickel NPs. A unique feature of magnetic NPs such as Nickel is their ability to increase the temperature of a tumor to 40°C. This process is aided by a high-frequency external magnetic device.

According to recent studies, Nickel NPs take about 6 hours to enter the nucleus of MCF7 cells, and around 12 hours to cause a significant decline in cell viability. The conjugation of drugs to nanoparticles is an important step towards synthesizing nanoconjugates ready for targeted drug delivery. Essentially, this process involves incorporation of the drug while the nanoparticles are still being formulated. Polyethylene Glycol (PEG) is a commonly used polymer that acts as a binding agent for the mixture. The pH of the solution plays a significant role in deciding the efficiency of the nanocarrier-encapsulated drug. The circulation time of the nanoparticles *in vivo* also plays a major role in deciding the efficiency of the formulation.

Our drug of interest, Pimozide has been conjugated with zinc, copper and PLGA nanoparticles in previously published papers. However, Silver coated Pimozide has not been documented so far, and more so, for the treatment of breast cancer. Hence this study will steer towards an interesting direction to inhibit breast cancer cells, specifically, MCF7 cells.

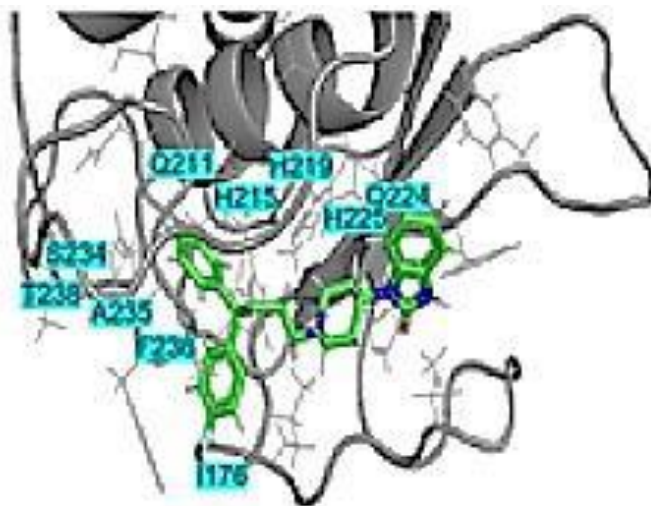


Fig 1.3 Docking of Pimozide with MMP-11

2 LITERATURE REVIEW

2.1 ROLE OF MMP11 IN VARIOUS CANCERS

2.1.1 Colorectal Cancer

Colorectal cancer (CRC), the world's third greatest cause of cancer mortality, is the third most prevalent adult cancer in men and the second most frequent in women (Biller & Schrag, 2021; Bray *et al.*, 2020; Dekker *et al.*, 2019; J. Li *et al.*, 2021). CRC has the greatest incidence of all malignancies in Taiwan, and it is responsible for the third highest cancer fatality (Chuang *et al.*, 2019). Many cancers, including oral squamous cell carcinoma (OSCC) (Lin *et al.*, 2015), hepatocellular carcinoma (HCC) (Saad *et al.*, 2020; Wang *et al.*, 2018), prostate cancer (Hsieh *et al.*, 2020), and urothelial cell carcinoma (UCC) (C.-C. Li *et al.*, 2020), have been linked to MMP-11 SNPs that increase the risk of cancer progression and development, metastasis, recurrence, or poor prognosis. However, the relationships and impacts of MMP-11 polymorphisms on CRC tumour growth and clinicopathologic features have not been studied. Its significance in breast cancer has been studied in the past (Min *et al.*, 2013c; Takeuchi *et al.*, 2011), but studies on its impact in colorectal cancer are scarce. A complicated process controls the production and activity of MMPs (Kessenbrock *et al.*, 2010), with CD147 playing a significant role in many of the signal pathways and associated molecules. The adhesion molecule CD147, commonly referred to as extracellular metalloproteinase inducer (EMMPRIN), belongs to the immunoglobulin superfamily. Through inducing tumor cells to release matrix metalloproteinases, it plays a crucial part in the growth and spread of tumors (MMPs). Numerous tumors have been researched to determine the connection between CD147 and MMP-9, MMP-2, and their relevance to patient prognosis and clinicopathological characteristics. However, there hasn't often been a link between the expression of CD147 and MMP-11. We examined

CD147 and MMP11 expression in colorectal cancer in the current study. We sought to clarify the level of expression of these two proteins in CRC tissue in comparison to normal mucosa, as well as their relationship to clinicopathological parameters and patient prognosis. This information could help to further our understanding of the disease's molecular mechanism and guide target therapy. We focused on five MMP-11 SNPs in this study: rs131451, rs738791, rs2267029, rs738792, and rs28382575, in order to uncover their associations with CRC susceptibility and clinicopathologic features.

2.1.2 Gastric Cancer

Despite recent declines in incidence and mortality rates worldwide, gastric cancer continues to rank third among all causes of cancer-related death (Ferlay *et al.*, 2021; Wong *et al.*, 2021). Early and advanced gastric cancer (AGC), which is defined as cancer that has invaded beyond the submucosal layer of the stomach wall, are two categories for gastric cancer based on the depth of tumor invasion (Coccolini *et al.*, 2016). Curative treatments include appropriate resections of primary gastric cancer lesions combined with regional lymph node dissections in AGC patients without distant metastases (Coccolini *et al.*, 2016).

Nevertheless, patients with non-metastatic AGC have dismal clinical outcomes, with a median overall survival of about 27 months (Desiderio *et al.*, 2021), even if they have undergone curative surgery. In order to predict patient outcomes and choose the best treatment options, numerous research have examined the prognostic features of gastric cancer (Jiang *et al.*, 2018; J. W. Lee *et al.*, 2020). Interleukin-6 (IL-6) and matrix metalloproteinase-11 (MMP-11), which are involved in the inflammatory response of the microenvironment and immune cells like T lymphocytes and macrophages, have been shown to have significant prognostic values in predicting clinical outcomes in patients with gastric cancer in earlier studies investigating the tumour microenvironment (E. Yang *et al.*, 2021; A. Z. Zhang *et al.*, 2021; X. Zhang *et al.*, 2016b). 2-deoxy-2-[18F] fluoro-D-glucose (FDG) PET/CT demonstrated therapeutic

efficacy in detecting unanticipated metastases on staging the work-up and recurrence of post-operative surveillance and predicting clinical outcomes in patients with gastric cancer (J. W. Lee *et al.*, 2020; E. Yang *et al.*, 2021; X. Zhang *et al.*, 2016b). Maximum FDG uptake and volumetric characteristics, such as metabolic tumour volume (MTV) and total lesion glycolysis (TLG), have typically been utilised as PET imaging parameters for gastric cancer in earlier research looking at the predictive importance of FDG PET/CT parameters (J. W. Lee *et al.*, 2020; G. Sun *et al.*, 2019). Along with these standard PET parameters, several studies have extracted a variety of quantitative textural features from PET images using textural analysis and other computational processing techniques, and they have assessed the clinical utility of the features in patients with gastric cancer (Liu *et al.*, 2021; Yoo *et al.*, 2021). The main idea behind the use of textural features is that they can represent biological properties of cancer tissues, such as patterns of genomic and proteomic expression, and hence provide a wealth of information that is distinct from conventional clinical criteria (Jiang *et al.*, 2018; J. W. Lee & Lee, 2018). The prognostic significance of textural features for metastases and clinical outcomes has shown encouraging results in earlier research (Liu *et al.*, 2021; Xue *et al.*, 2021; Yoo *et al.*, 2021).

2.1.3 Prostate Cancer

The second most prevalent cancer to be diagnosed worldwide is prostate cancer, which is also one of the leading causes of cancer death in males (Ferlay *et al.*, 2015; Siegel *et al.*, 2019). Even in Asian nations where the prevalence was previously reported to be low, prostate cancer incidence has recently grown significantly (Kimura & Egawa, 2018). MMP11 has relatively limited proteolytic potential when compared to other MMPs, yet there are still other possible explanations for its unique significance in the development of tumors. MMP11 is therefore processed intracellularly and secreted as an active enzyme, in contrast to the bulk of MMPs, which are released as proenzymes that need extracellular activation. This shows that this endopeptidase may play a special function in the development and spread of tumors (Brasse *et al.*, 2010; Peruzzi

et al., 2009b). It has also been demonstrated that carcinogenesis caused by MMP11 results from decreased cancer cell death through apoptosis and necrosis rather than increased cancer cell proliferation, demonstrating that MMP11's biological activity is favorable to cancer cell survival in the stromal environment (Boulay *et al.*, 2001). However, research suggests that MMP11 may change the stromal milieu of human carcinomas in order to promote tumor angiogenesis (Kanharat & Tuamsuk, 2015). We also analyzed the expression of stromal factors in NAFs or CAFs and fibroblasts from BPH specimens in the current investigation. Prostate cancer normally develops in the peripheral zone, whereas BPH is a benign enlargement of the prostate that typically affects older men and involves a change in the homeostasis of the prostate tissue. In comparison to NAFs and to matched CAFs, BPH fibroblasts expressed higher levels of FGF7, IL6, MMP2, and MMP11 than did NAFs. Our findings revealed some significant correlations between the CAFs molecular profile and age, pre-treatment PSA serum levels, pathologic stage, and Gleason score in patients with localized PCa who underwent radical prostatectomy, which also raises the possibility that CAFs play a role in tumor biology. We found a significant correlation between pre-treatment PSA blood levels, a known predictor of poor prognosis in PCa (Gasinska *et al.*, 2020), and CAF expression of the NF-B and FAP genes. According to studies, NF-B increases PCa cell survival, tumor invasion, metastasis, and chemoresistance (Verzella *et al.*, 2016).

2.1.4 Pancreatic Cancer

One of the most typical cancers still poses a serious threat to global public health. Pancreatic cancer. Pancreatic cancer is the fourth most common cause of cancer related deaths, while having a low prevalence (Miller *et al.*, 2016). The mRNA level of MMP-11 in pancreatic cancer tissues was considerably higher than that in healthy pancreatic tissues, according to RT-PCR research (Jones *et al.*, 2004). The overexpression of MMP-11 in stromal and epithelial cells during the development of pancreatic cancer may be related to the aggressive local growth and metastasis of

pancreatic cancer, as MMP-11 was found in epithelial cancer cells and their surrounding stromal cells in more than 80% of pancreatic cancer specimens, whereas MMP-11 was not detected in the stromal or epithelial cells of normal pancreatic tissue (von Marschall *et al.*, 1998). According to J. Lee *et al.*, 2019, pancreatic cancer patients' whole blood samples can particularly identify soluble MMP-11. Additionally, it was discovered that MMP-11 expression levels in pancreatic cancer tissues were noticeably higher than those in other tumor tissues and healthy tissues, indicating that MMP-11 may be a biomarker of pancreatic cancer. The MMP-11 protein's expression level in pancreatic cancer tissues is determined by its pattern of expression.

2.2 ROLE OF MMP11 IN BREAST CANCER

Breast cancer has had a significant negative impact on the patient's quality of life and psychological condition, similar to malignant tumors (Rebholz *et al.*, 2018). According to estimates, 30% of female cancer patients in the United States will have breast cancer by 2020, especially younger women between the ages of 20 and 59 (Siegel *et al.*, 2020). The elevated expression of MMP-11, also known as stromelysin-3, in stromal cells in invasive breast cancer was originally discovered (Basset, n.d.). While MMP-11 is processed in the cytoplasm and secreted in active enzyme form, the majority of MMPs are released as inactive proenzymes (X. Zhang *et al.*, 2016a). The unique property of MMP-11, which distinguishes it from other MMP members, suggests that it has a distinctive function in the initiation and growth of tumors. Previous research has demonstrated a link between high histologic grade, ER, HER2, big tumor size, lymph node involvement, overexpression of MMP-11, and poor prognosis in breast malignancies (Min *et al.*, 2013b; Naghshvar *et al.*, 2017). MMP-11 overexpression is significant because it has the ability to predict early diagnosis, prognosis, and adaptive treatment. MMPs have been suggested as potential targets for immunotherapy in a number of studies, with MMP2 and MMP7 being two examples (Yi *et al.*, 2007; Yokoyama *et al.*, 2008). MMP-11 may make an excellent candidate target for cancer

immunotherapy, according to recent findings (Peruzzi *et al.*, 2009a). The clinical outcomes of breast cancer have been linked to high MMP-11 expression, although little is known about the role of MMP-11 expression in breast cancer's anti-tumor immunity. According to a recent study, miR-1204's competitive endogenous RNA (ceRNA) circ-MMP11 (hsa circ 0062558) accelerated the development of breast cancer (Z. Li *et al.*, 2020).

However, little is understood regarding the role of circ-MMP11 in medication resistance and breast cancer development. Exosomes from tumors are associated with metastasis and medication resistance in many malignancies, as is becoming more and more obvious (Mashouri *et al.*, n.d.). According to several studies, exosomal circRNAs are extensively present, which raises the possibility that they have a clinically significant role in the detection and prognosis of human cancers (Fanale *et al.*, 2018).

In fact, several studies have shown that exosomal circRNAs were crucial for tumor development and medication resistance in human malignancies (Luo & Gui, 2020; Z. Sun *et al.*, 2018). To our understanding, it is currently unclear if circ-MMP11 may be transmitted by exosomes. Our findings in this study indicated that circ-MMP11 was elevated in the tissues and cells of lapatinib-resistant (LR) breast cancer. Additionally, these results revealed that circMMP11 may be transferred by exosomes and that it might increase lapatinib resistance in breast cancer through controlling the miR-153-3p/Anillin (ANLN) gene.

2.3 ROLE OF DRUG REPURPOSING IN CANCER

Repositioning a drug, also known as repurposing a drug, reprofiling a drug, The term "re-tasking" (also known as "drug redesigning," "drug resorting", "indication switching," and "therapeutic switching") refers to the a clinically proven medication has new applications with a reduced risk, time, and cost. Drug repositioning avoids the typical path of significantly increased rates, a slow pace, negative impacts, etc. (Ashburn & Thor, 2004; Paul *et al.*, 2010; Pushpakom *et al.*, 2019) It's evolving into an appealing

idea for the pharmaceutical industry. According to estimates, the Repositioning takes substantially less time while developing drugs (3-5 Years), money (\$0.3 billion), and at least one known and accepted side better chance of success; and positive impacts (Ashburn & Thor, 2004; Jadamba & Shin, 2016; Nosengo, 2016; Oprea *et al.*, 2011).

2.3.1 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. (C.-E. 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. 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. (W. 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. They elaborated on Aripiprazole drug which showed a cytotoxicity ($IC_{50} = 12.1 \pm 0.40 \mu M$) 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, proapoptotic 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 breast cancer treatment.

3 AIM AND SCOPE OF PRESENT INVESTIGATION

3.1 AIM:

To analyze of role of MMP11 and its association in breast cancer survival and to identify potential FDA-approved repurposed drugs for targeting breast cancer.

3.2 SCOPE:

This study is restricted to targeting MMP11 and its potential in Breast cancer treatment. The benefit of working with low-risk molecules is one advantage of repurposing medications to treat both common and unusual disorders. Repurposing medications for new indications provide an approach for discovering treatments for uncommon diseases that has benefits over conventional drug development due to cost effectiveness and a shortened timetable.

4 MATERIALS AND METHODS

4.1 IN-SILICO SURVIVAL STUDIES

4.1.1 Pan-cancer survival analysis of MMP-11 using TCGA

The survival analysis of MMP11 expressing patients was 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 survival analysis 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 up and down-regulated genes in individual cancers. The survival probability was plotted on the y-axis and compared for normal versus tumor samples, with the time in days plotted in the x-axis. A p-value <0.05 was considered significant.

4.1.2 Pan-cancer survival analysis of MMP-11 using KM

The Kaplan–Meier Plotter database (<https://kmplot.com/analysis/>), which is an online database including gene expression data and clinical data, was used for assessing different genes on survival among 15 cancer types. This database currently contains 6234 breast, 2190 ovarian, 3452 lung, and 1440 gastric cancer datasets. Importantly, the data sources of this database include not only GEO, but also European Genome-phenome Archive (EGA) and TCGA (The Cancer Genome Atlas). The prognostic significance of the mRNA expression of MMP-11 in 15 different human cancers was evaluated using the Kaplan–Meier plotter database, the hazard ratio with 95% confidence intervals and log rank P-value were also obtained.

4.1.3 Pan-cancer survival analysis of MMP-11 using TIMER

TIMER (<https://cistrome.shinyapps.io/timer/>) provides a user-friendly web interface to explore and visualize tumor immunologic and genomics data. The 'Gene' module was used to estimate the correlation between MMP-11 expression and immune infiltration level (the abundance of six TIIC subgroups: B cells, CD4+ T cells, CD8+ T cells, macrophages, neutrophils, and dendritic cells), as well as tumor purity, among 39 cancer types. The expression level of gene was adjusted by log2 TPM (transcripts per million).

4.1.4 Validation of MMP11 survival analysis study in pan-cancer

The results from above 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 was set to percentage of survival, which was plotted for normal and tumor samples, with the x-axis set to time in months. The subcellular localization for each protein was analyzed using FunRich software (version 3.1.3) by entering the list of the total number of interacting proteins which were used to construct a protein network.

4.2 IN-VITRO STUDIES

4.2.1 Mammalian cell culture

Human derived cell lines (Table 2.1) were maintained in T-25 cm² 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°C and 5% CO₂ 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. The analysis for contamination was assessed frequently using mycoplasma PCR ELISA kit (Roche, Switzerland).

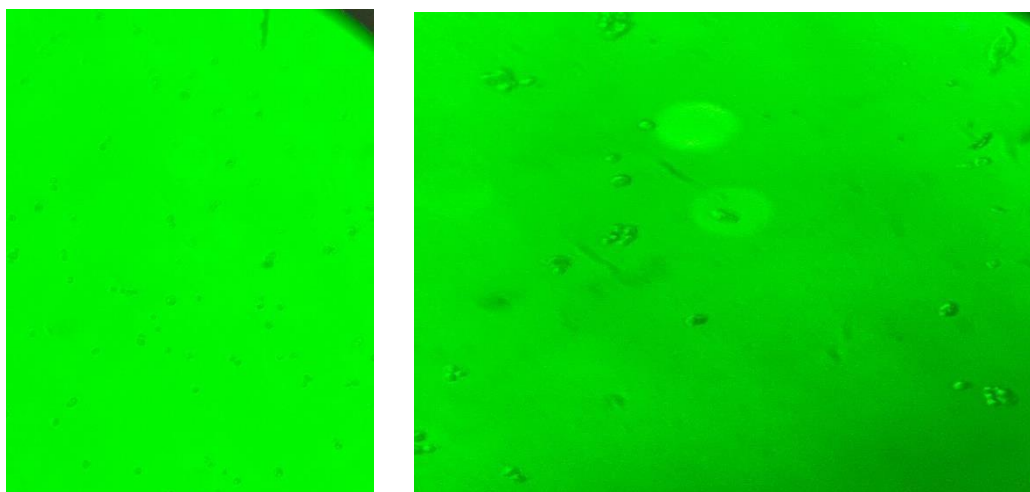


Fig 4.1: SK-BR-3 and MCF-7 cell lines

Table 4.1 List of cell lines used

Type of Cell line	Cell line	Medium used	Source of cell line
Control cells	SK-BR-3	DMEM with 10% FBS	Adyar Cancer Institute
Breast cancer cells	MCF7	DMEM with 10% FBS	Adyar Cancer Institute

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 oC 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 oC for further use.

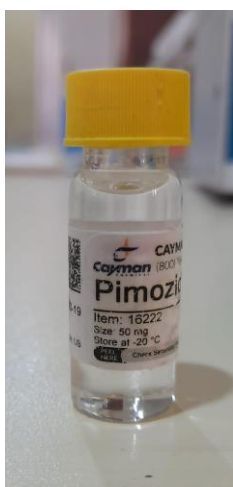


Fig 4.2: Pimozide drug

4.2.3 MTT assay

To prepare MTT solution, the MTT salt was used as a 5 mg/mL solution in PBS. The solution was mixed by vortexing, and filter sterilized after adding MTT. It was then stored at -20°C for further use. To perform the assay, the media from the cell cultures was discarded. Next, 50 µL of serum-free media and 50 µL of MTT solution was added to each well, and the plate was incubated at 37°C for 3 hours. After incubation, 150 µL of MTT solvent was added to each well, and the plate was wrapped in foil and shaken on an orbital shaker for 15 minutes. The solution was pipetted repeatedly to fully dissolve the MTT formazan. Finally, the absorbance was read at OD=570 nm.

4.2.4 NPs conjugation with Pimozide

Three nanoparticles namely Silver (Ag^+), Copper (Cu^{2+}) and Nickel (Ni^{2+}) were conjugated with Pimozide separately. To start with, 100 mg NP was dissolved in 5 mL sterile distilled water in 50 mL capacity centrifuge tubes by subjecting the mixture to a water bath sonicator for 30 min at regular intervals. For Nickel alone, the NPs were first ground well using mortar and pestle, before sonication, to improve the speed of dissolution. Sonication was repeated until complete dissolution occurred in all three tubes.

4.2.5 SEM analysis of NP conjugated Pimozide

Scanning Electron Microscope (SEM) analysis of the NPs conjugated with Pimozide was performed to assess the quality of conjugation and to validate the size of the NPs.

4.2.6 Cytotoxicity analysis of NP conjugated Pimozide on HEK293, MCF7 and SKBR3 cell lines

2000 cells were seeded in 96 well plates in triplicates and then the MCF7 cells were treated with 22 μ M of pimozide + 100 μ g/ml nanoparticle(silver, copper, nickel conjugated pimozide was prepared separately) for 24 hours. Then the cells were subjected to MTT assay. To perform the assay, the media from the cell cultures was discarded after 24 hours of treatment and then 50 μ L of serum-free media was added. 50 μ L of MTT solution was added to each well, and the plate was incubated at 37°C for 3 hours. After incubation, 150 μ L of MTT solvent was added to each well, and the plate was wrapped in foil and shaken on an orbital shaker for 15 minutes. The solution was pipetted repeatedly to fully dissolve the MTT formazan. Finally, the absorbance was read at OD=570 nm.

4.2.7 Total RNA Isolation

Total RNA was isolated using trizol reagent (Invitrogen, USA). MCF7 cells suspended in 1 mL trizol were added to 200 μ L chloroform, mixed gently, and centrifuged at 10,000 rpm for 15 min at 4°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 4 °C. The white pellet thus formed was washed twice with 70% ethanol. Then the pellet was air-dried and dissolved in 20 μ L of sterile nuclease-free water.

4.2.8 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 (0.13 M Tris and 2.5 mM EDTA, pH 7.6) was prepared. The agarose was heated at 60°C 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.

4.2.9 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 42°C for 15 min, and then at 95°C for 3 min to inactivate reverse transcriptase. The tubes were stored at -20°C for further use.

4.2.10 Designing of Real-Time quantitative Polymerase Chain Reaction Primers

Primers for three genes are given in Table 4.2, 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
VEGF	FP:5'TGCAGATTATGCGGATCAAACC3' RP:5'TGCATTACATTTGTTGTGCTGTAG3'
TGFB	FP:5'-CCCAGCATCTGCAAAGCTC-3' RP: 5'-GTCAATGTACAGCTGCCGCA-3'.
BETA ACTIN	FP:5'ACTCTTCCAGCCTTCCTTC3' RP:5'TCTCCTTCTGCATCCTGTC3'

4.2.11 Polymerase Chain Reaction (PCR)

PCR was performed with gene-specific primers for three genes (VEGF, TGFB and Beta Actin) from SK-BR-3 and MC-7 cell lines 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 up to 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°C for 30 sec, 94°C for 30 sec, 59°C for 30 sec, 72°C for 30 sec, 72°C for 2 min and 4°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.

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

qRT-PCR was performed with gene-specific primers for three genes (VEGF, TGFB) and BETA ACTIN as the normalizing control gene, using SensiFAST™ SYBR® Lo-ROX Kit (Bioline, UK) according to the manufacturer's protocol in triplicates. 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 (50 ng) was added and the volume was made to 20 µL with nuclease-free water. PCR amplification was performed with Mastercycler Nexus Gradient Thermal Cycler (Eppendorf, Germany). The thermal-cycling conditions included 95°C for 2 min (polymerase activation), 95°C for 5 sec, 60 to 65°C for 10 sec, 72°C for 15 sec 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 $2^{-\Delta\Delta CT}$ method. In this method, the relative gene expression is assumed to be 1 for the reference sample so that 20 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).

5 RESULTS AND DISCUSSION

5.1 IN-SILICO ANALYSIS

5.1.1 Pan-cancer survival analysis of MMP-11 using TCGA

As analyzed from the dataset, the probability of survival decreases over time for all expression levels of MMP11, indicating that MMP11 is a poor prognosis factor for all cancers displayed above. In all the cancers, patients expressing lower levels of MMP11 are observed to survive longer than patients with high expression levels. Exceptions to this observation include colon adenocarcinoma (COAD), brain lower grade glioma (LGG), kidney renal papillary cell carcinoma (KIRP) and uterine carcinosarcoma (UCS). Additionally, we observed that mortality rates of patients with different cancers varies widely with respect to their expression levels of MMP11. For example, while analysing low MMP11 expression patients with Glioblastoma multiforme (GBM) were found to survive for more than 5 years before death. Similarly, patients with Head and Neck squamous cell carcinoma (HNSC) were found to survive for more than 16 years before death. Patients with Ovarian serous cystadenocarcinoma (OV) and Lung squamous cell carcinoma (LUSC) were found to survive more than 13 years before death. Patients with Uterine Carcinosarcoma (UCS) were found to survive more than 8 years before death. The highest survival probability was found to be Thyroid carcinoma (THCA) patients.

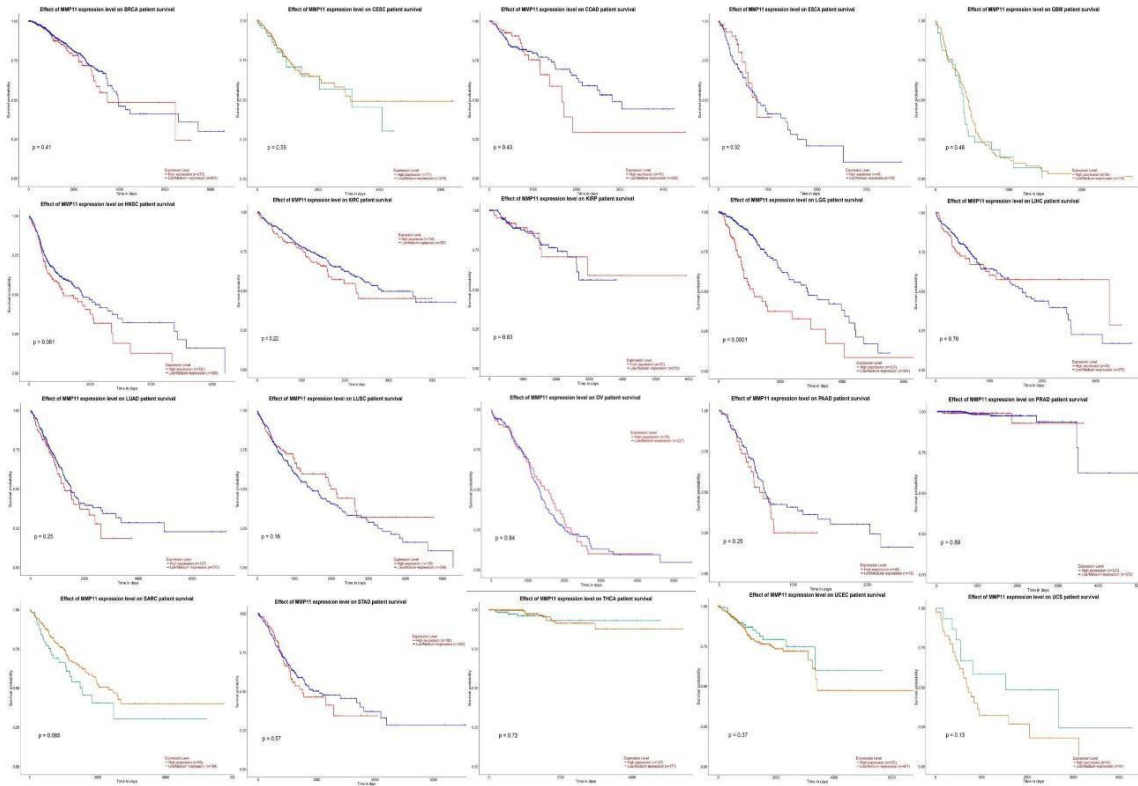


Fig 5.1: Pan-cancer survival analysis of MMP-11 using TCGA

5.1.2 Pan-cancer survival analysis of MMP-11 using KM

(a) The survival probability of patients for pan cancer based on MMP-11, was analyzed over time (months) using the KM plotter. The plots were categorized as red and black for high and low MMP-11 expression respectively. THCA ($p = 0.12$) patients stood out amongst other cancer patients, as the only ones to be healthy beyond 150 months of cancer development. All other cancers showed a sharp declining peak for survival, with low and high MMP-11 expression. ESCA ($p = 0.15$), HNSC ($p = 0.3$), OV ($p = 0.26$), LUSC ($p = 0.12$) and PAAD ($p = 0.002$) showed confirmed death of patients post 80 months. BRCA ($p = 0.02$), CESC ($p = 0.31$), KIRC ($p = 0.03$), LUAD ($p = 0.14$), KIRP ($p = 0.25$), SARC ($p = 0.007$), STAD ($p = 0.09$) and USBC ($p = 0.002$) showed a steady decline in survival until around 100 months, after which their chances of survival stabilized. In all cancers, the survival rate was found to be much lesser for patients with

high MMP-11 expression, compared to those with low MMP-11 expression. This analysis gives high MMP-11 expression a relevant significance as a therapeutic marker in cancer treatment. Considering the low p value for BRCA (0.02), this could indicate a stronger conviction of MMP-11's importance in breast cancer treatment.

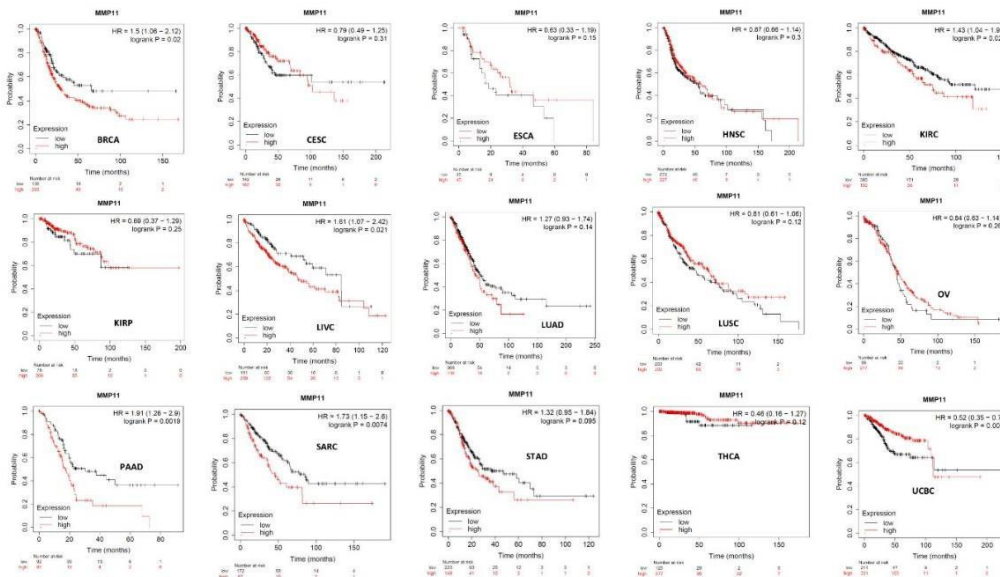


Fig 5.2: Survival analysis plot using KM Plotter

b) Survival analysis was performed for pan-cancer using the KM plotter. The graph below revealed hazard ratio values for pan-cancer. A hazard ratio (HR) is defined as the ratio of the hazard rate in the treated group to that in the control group. Generally, a higher hazard rate indicates a greater risk to survival in the treated group. All cancers listed in the graph showed variable HR values above 1, indicating a higher risk in treated patients. PAAD ($p = 0.2$) had the highest cutoff value of 20,000 with an HR value of 1.2. This was followed by BRCA ($p = 0.3$) with a cutoff value of 10,000 and a corresponding HR value of 1.2. An HR value of 1.2 could indicate that the treatment under test is showing a poor prognosis and could call for a change in therapy. On the lower level, LIRC ($p = 1$) had the lowest cutoff value of 500 with an HR value of 1, indicating no

survival rate difference between the control and treated groups. This was followed by KIRC (p = 0.09) which had an HR value of 1.4 at a corresponding cutoff value of 550.

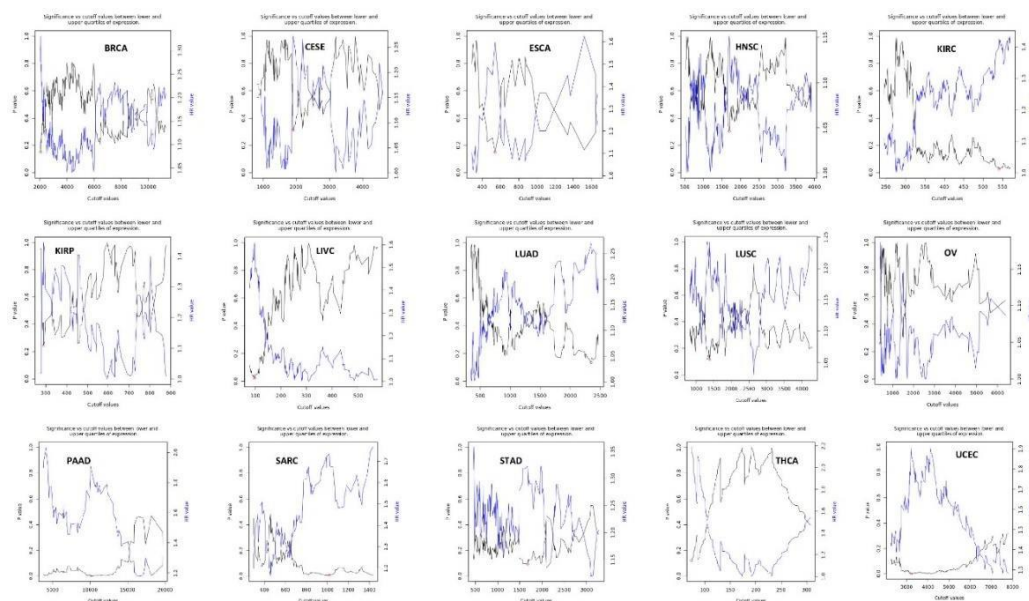


Fig 5.3: Hazard ratios vs cutoffs for various cancers using KM plotter

5.1.3 Pan-cancer survival analysis of MMP-11 using TIMER

A correlation heatmap was constructed for pan-cancer using data and tools from the TIMER database. A positive correlation was given by red blocks, while negative correlation was inferred by blue blocks. A p-value <0.05 was indicated by plain blocks while a p-value >0.05 was indicated by crossed blocks. Based on this legend, the cancers BRCA, KIRC, PAAD, SARC, STAD and UCEC showed a high positive correlation with p value <0.05, indicating a high statistical significance. TGCT and THCA showed a slightly negative correlation with p value <0.05 as well. Cancers with crossed blocks were not considered due to the high p-value inferred by them.

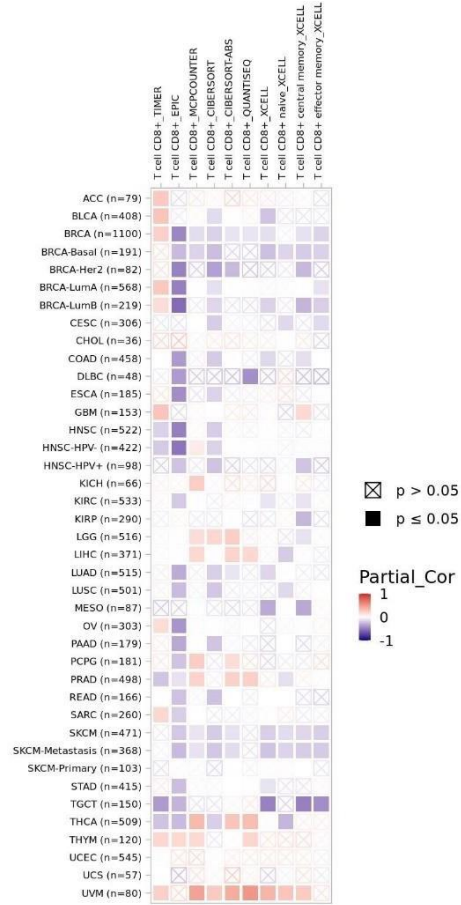


Fig 5.4: correlation plots from TIMER database

5.1.4 Validation of pan-cancer survival analysis of MMP-11 using GEPIA and FunRich Software

As analyzed from data provided by GEPIA database, Esophageal cancer (ESCA), Liver cancer (LIHC), Kidney cancer (KIRC), Lung cancer (LUSC), Ovarian cancer (OV) and Uterine cancer (UCS) show a lower probability of survival for low MMP11 expressions. Thyroid cancer (THCA) seems to be the least affected carcinoma by MMP-11 expression, as indicated by the high survival probability. This observation is drawn from both GEPIA and TCGA datasets. Uterine carcinoma (UCEC) and Sarcoma (SARC) show a clear distinction between survival probability rates for high and low MMP11 expression levels, indicating that a high expression level leads to a drastically low

survival probability. This distinction is lesser for other cancers. Some cancers show a very high impact of MMP11 expression levels on survival, bringing the probability close to 0 within 5 years itself. These include Glioblastoma multiforme (GBM), Pancreatic adenocarcinoma (PAAD) and Stomach adenocarcinoma (STAD). An important point to note here is that this data does not mention problem free survival, which is beyond the scope of this study. The overall analysis of data from GEPIA database coincides widely with that from other datasets.

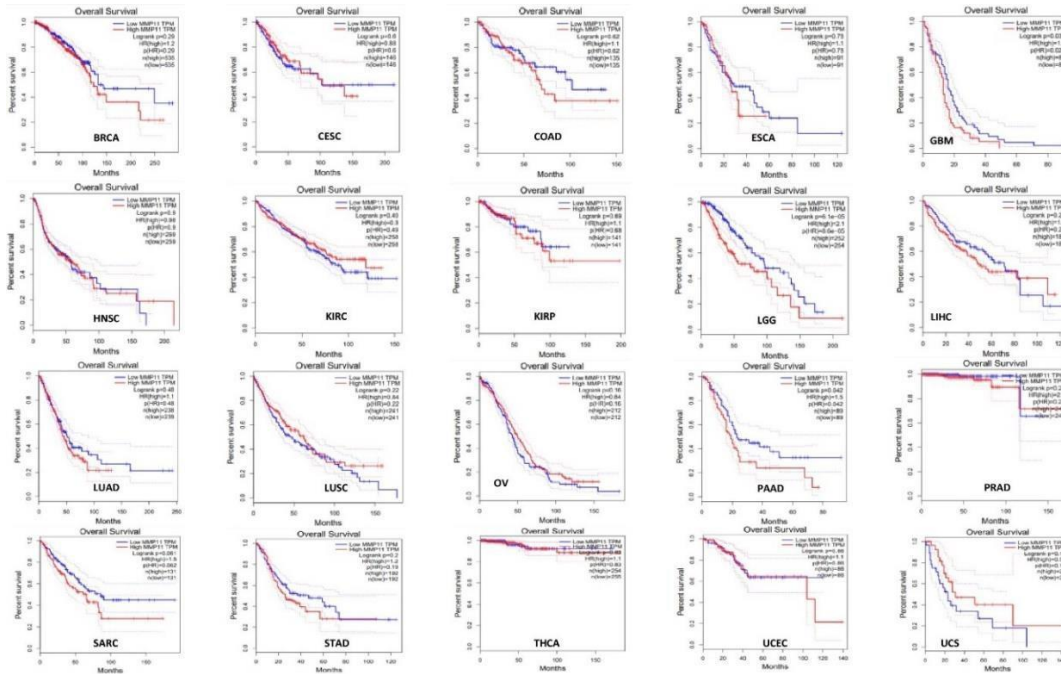


Fig 5.5: Validation of pan-cancer survival analysis of MMP-11 using GEPIA



Fig 5.7: conjugation of Ag^+ , Cu^{2+} and Ni^{2+} NPs with Pimozide separately

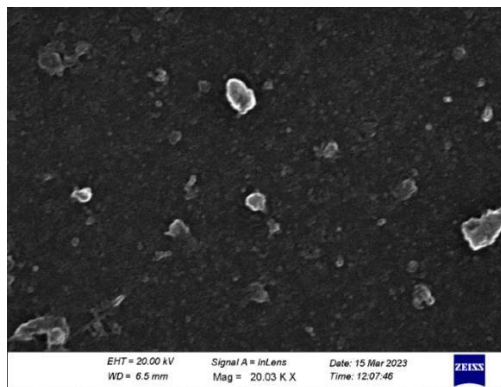
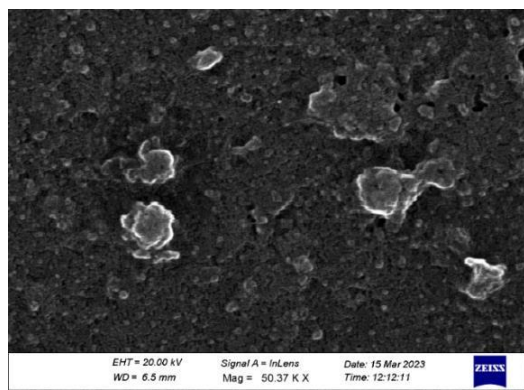
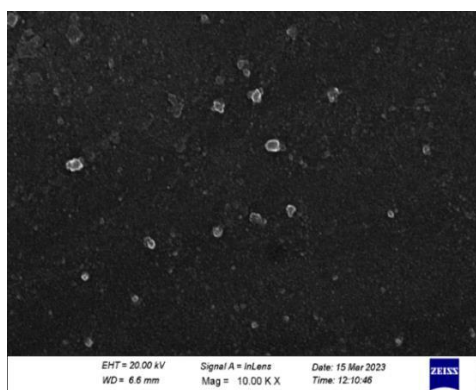


Fig 5.8: (Left, right and bottom) SEM images of Silver, Copper and Nickel conjugated Pimozide

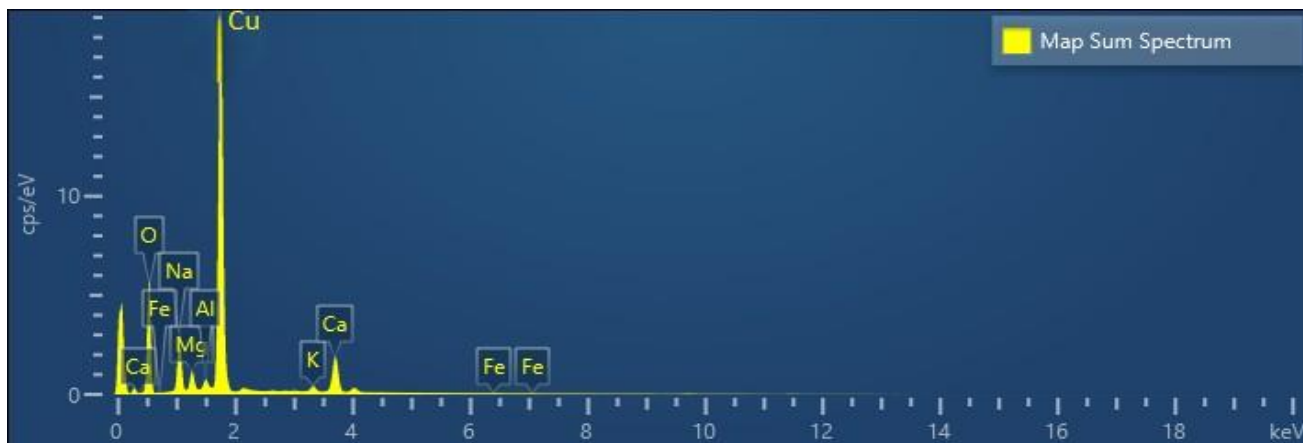


Fig 5.9: results from Map Sum Spectrum program

Table 5.1: Details of the EDX spectra vales measured in atomic% and weight%

Map Sum Spectrum				
Element	Line Type	Weight %	Weight % Sigma	Atomic %
O	K series	43.76	0.08	57.67
Na	K series	8.69	0.04	7.97
Mg	K series	2.35	0.02	2.04
Al	K series	0.97	0.02	0.76
Cu	K series	36.96	0.06	27.75
K	K series	0.85	0.02	0.46
Ca	K series	6.33	0.03	3.33
Fe	K series	0.10	0.02	0.04
Total		100.00		100.00

In order to confirms the formation of Cu nanoparticle with pimozide complex EDX analysis was performed. During the EDX measurements different areas were focused and corresponding peaks are shown in the figure.

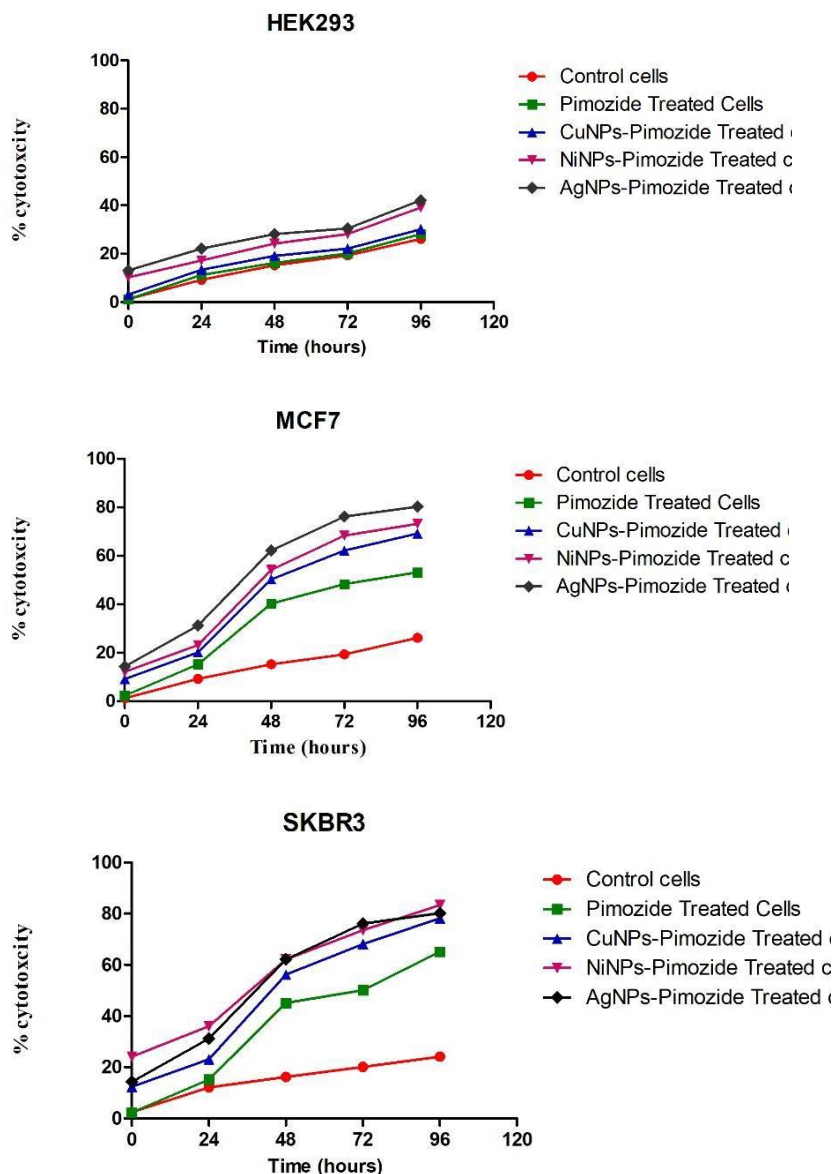


Fig 5.10: Cytotoxicity analysis of NP conjugated Pimozide on HEK293, MCF7 and SKBR3 cell lines

The difference in cytotoxicity of the three cell lines HEK293, MCF-7 and SK-BR-3 between control and drug treated cells was significantly high. Cytotoxicity analysis revealed that Pimozide treatment least affected HEK293 cells before NP conjugation. This could indicate a higher resistance of HEK293 cells against Pimozide, and thereby

suggest being an ineffective therapy for this cell line. However, the %cytotoxicity was found to be much higher for MCF7 and SK-BR-3 cell lines; the value being more significant in the former. Post NP conjugation with Pimozide and its treatment, the %cytotoxicity was found to moderately increase in HEK293, but rapidly increase in SK-BR-3 and MCF-7. The results were again most significant in MCF-7, suggesting a higher sensitivity of MCF-7 cells against Pimozide. Among the three nanoparticles used, Silver NPs were found to induce a much higher cytotoxic effect on the cell lines, compared to Copper and Nickel.

Table 5.2: Comparison of cytotoxicity in control vs test cells

	HEK 293 Cells		MCF7 Cells		SKBR3 Cells	
	Control	Drug-treated	Control	Drug-treated	Control	Drug-treated
%Cytotoxicity	Low	Low	Low	High	Low	High
Cell viability	High	High	High	Low	High	Low
Duration of high viability	24	24	24	24	24	24
Duration of low viability	96	96	96	96	96	96

Table 5.3: Comparison of cytotoxicity in NP treated cells

	HEK 293 Cells			MCF7 Cells			SKBR3 Cells		
	Cu	Ni	Ag	Cu	Ni	Ag	Cu	Ni	Ag
%Cytotoxicity	<i>High</i>	<i>High</i>	<i>Highest</i>	<i>High</i>	<i>High</i>	<i>Highest</i>	<i>High</i>	<i>High</i>	<i>Highest</i>
Cell viability	<i>Low</i>	<i>Low</i>	<i>Lowest</i>	<i>Low</i>	<i>Low</i>	<i>Lowest</i>	<i>Low</i>	<i>Low</i>	<i>Lowest</i>
Duration of high viability	24	24	24	24	24	24	24	24	24
Duration of low viability	96	96	96	96	96	96	96	96	96

5.2.2 RNA isolation and Agarose Gel Electrophoresis

RNA isolated from drug treated MCF7 and SKBR3 cells were obtained as clear white pellets after precipitation and centrifugation with isopropanol. The pellets were 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.

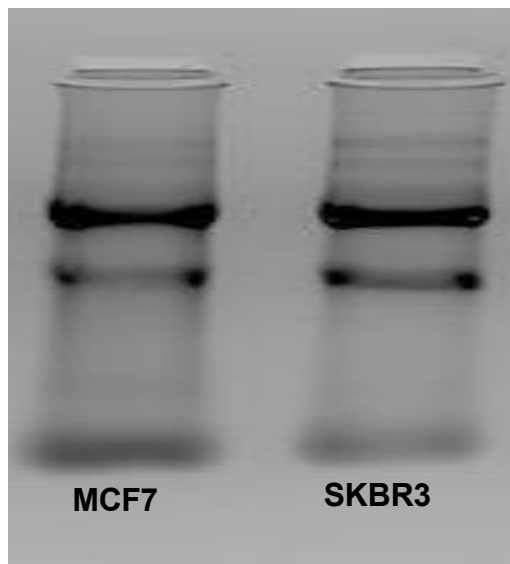


Fig 5.11(a) Gel Doc analysis of RNA isolated from drug treated MCF7 and SKBR3 cells (b)RNA isolation

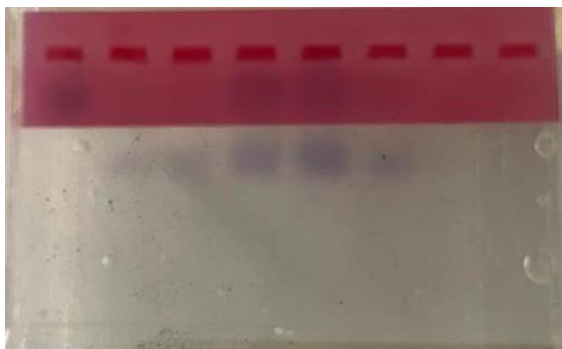


Fig 5.12: Agarose Gel Electrophoresis run for RNA isolated from Pimozide-treated MCF7 cells

5.2.3 Polymerase Chain Reaction studies (PCR)

PCR was performed for two genes namely VEGF and TGFB. cDNA samples obtained by reverse transcription of isolated RNA samples from drug treated MCF7 and SKBR3 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.

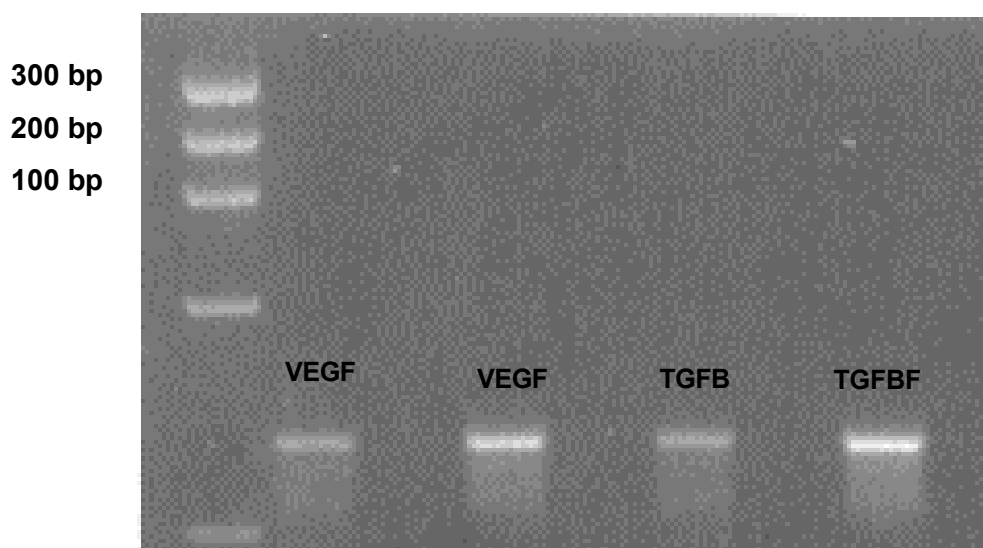


Fig 5.13: Gel Doc analysis of PCR amplified products from MCF7 and SKBR3 cells

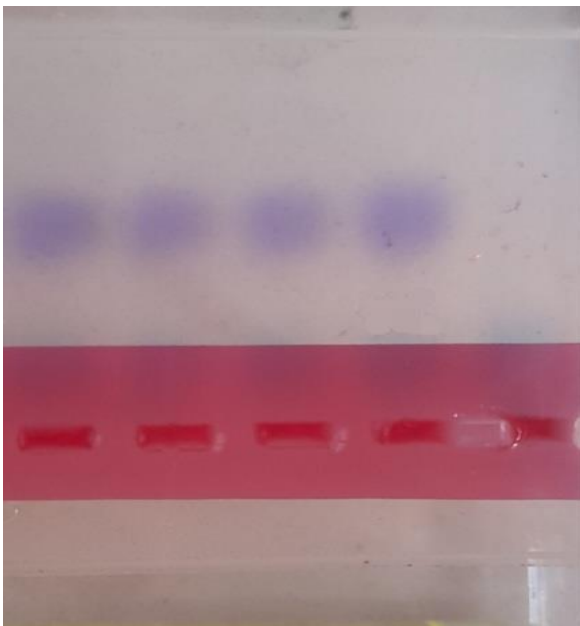


Fig 5.14: Agarose gel run for PCR products of target genes

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

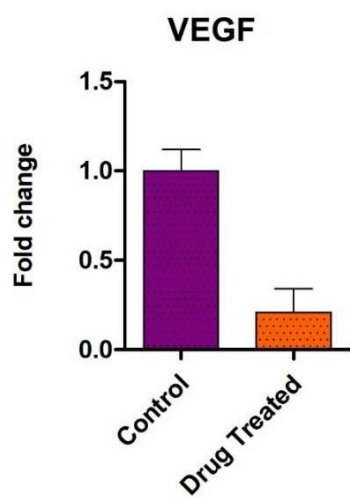
qRT-PCR results for 2 genes were analyzed using Beta Actin as the housekeeping gene. The resulting cyclic threshold values provided insights on the fold change for each gene before and after Pimozide treatment. After Pimozide treatment of cells, there was more than a two-fold downregulation of VEGF ($p=0.0006$) in both MCF7 and SKBR3 cells. However, the tumor suppressor gene TGFB ($p = 0.03$) was found to be distinctly upregulated.



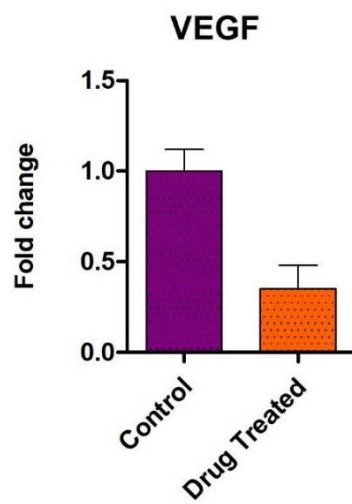
Fig 5.15: BETA ACTIN forward and reverse primers



Fig 5.16: Sterile nuclease-free water



MCF 7 (Estrogen +ve cell lines)



SKBR3 (Triple-ve cell line)

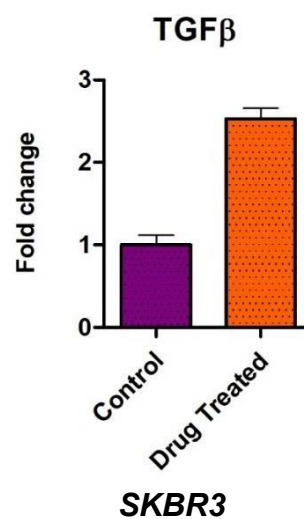
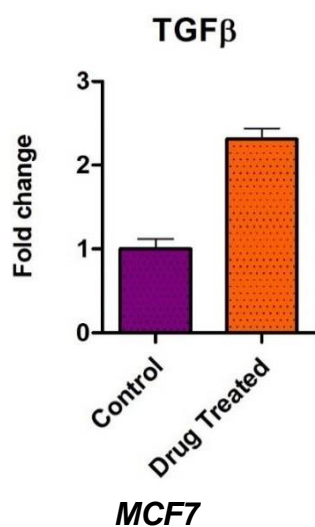


Fig 5.17: results of gene regulation from RT-PCR studies

[illegible]

Fig 5.18: Fold change calculation for genes of SKBR3 cell line

A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S	T	U	V	W	X
	MCF7																						
	control	ct				BETA ACTIN		del ct = ct - BETA ACTIN															
	1.00 BETA ACT1	28.17	27.74			28.17	27.74	0.00	0.00														
	2.00 VEGF - mc	22.21	20.03			28.17	27.74	-5.96	-7.71														
	3.00 TGFBR2 - r	19.35	21.19			28.17	27.74	-8.22	-6.55														
	target	ct				BETA ACTIN		del ct MCF7		del ct control		deldekt					fold change			average fold change	SEM		
	1.00 BETA ACT1	28.93	28.51			28.93	28.51	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00	1.00	1.00	1.00	0.00			
	2.00 VEGF - mc	22.97	22.91			28.93	28.51	-5.96	-5.60	-5.96	-7.71	0.00	2.11	1.00	0.23	1.00	0.23	1.00	0.62	0.31			
	3.00 TGFBR2 - r	20.71	20.68			28.93	28.51	-8.22	-7.83	-8.22	-6.55	0.00	-1.28	1.00	2.43	1.00	2.43	1.72	0.58				

Fig 5.19: Fold change calculation for genes of MCF7 cell line

DISCUSSION

According to Torre et al. (2017), BC is the most prevalent malignancy among women worldwide. Identification of prognostic markers is necessary for a better knowledge of the disease and to assist in determining the most effective treatment due to the heterogeneity of BC and its various subtypes. as well as aftercare procedures (Yersal and Barutca, 2014). A growing body of research shows how MMPs play a significant role in the development of tumours, are upregulated by BC's propensity for metastasis, and have a bad prognosis (Liu et al., 2012; Merdad et al., 2014; Zhang et al., 2008). MMP1, 9, 12, 14, and 15 were found to be strongly linked with poor OS in a group of 295 breast cancer patients (McGowan and Duffy, 2008). (Sathyanarayanan *et al.*, 2020), pimozone, an FDA-approved antipsychotic medication, was also identified as a promising MMP11 inhibitor. In-vitro and in-vivo studies of pimozone's cytotoxic effects on breast cancer have demonstrated that it modifies MMP1, 2, and 14, in part by inhibiting Ran GTPase (Dakir *et al.*, 2018). Through our in-silico investigation, we are the first group to report that pimozone interacts with the S1' binding pocket of MMP11, indicating that it may be more efficient and selective against MMP11 than other broad spectrum MMPis. Study provides comprehensive analysis of prognosis characteristics of MMP11 and its underlying genetic and epigenetic alteration profile in BC. However, experimental validation is required we conduct functional studies to elucidate the effects of MMP11 overexpression on clinical outcomes in BC. we also identified FDA approved pimozone targeting MMP11, along with 12 potent biological inhibitors, based on virtual

screening. Further studies in vitro also, in vivo models should be performed to assess efficacy these compounds in MMP11 inhibition. These powerful biopharmaceuticals acts as a specific, non-toxic and inexpensive alternative to existing MMP inhibitors. The activity of the pimozone was carried out experiment results, with that evidence the nanoparticles was added with the Pimozone. SEM were used to analyse the morphology of all nanoparticles conjugated pimozone. The figure shows the morphology of the silver, copper, nickel with the pimozone. All nanoparticle were integrated with Pimozone surface.

6 SUMMARY AND CONCLUSIONS

One of the first diseases to be examined on a global scale is breast cancer. However, despite much research, this carcinoma remains the second biggest cause of death globally, particularly among Indian women. In our survey of review of literature, we were able to understand the potential of repurposing through drugs the treatment of cancer, several repurposing drugs including metformin, antiparasitic drugs which are potential for the cancer treatment. We also investigated FDA approved drugs which provides potential to study using molecular docking for the MMP-11 for prognostic, diagnostic markers for repurposing and best to target MMP-11. MMP11's significance in poor BC prognosis has long been a contentious issue. Pimozide showed effective binding to MMP11 by computational study, albeit the processes underlying this were still unknown.

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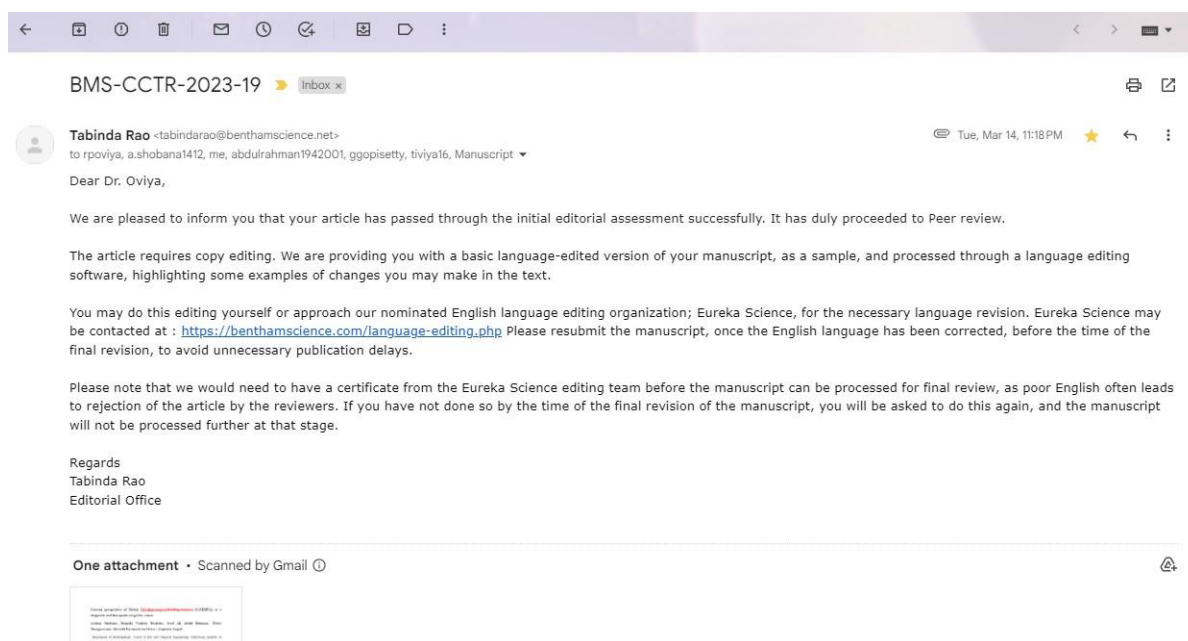
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ANNEXURE

Submission and acceptance of review paper titled “Current perspectives of Matrix Metalloproteinases 11 (MMP11) as a diagnostic and therapeutic target for cancer” to Current Cancer Therapy Reviews, Bentham Science Publishers.





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2.1 Targeting MMP11 (Matrix MetalloProteinase 11) in cancers MMP11 (Matrix Metalloproteinases) is a well known that immune cells can influence cancer cells to increase their proliferative and invasive properties. It is also known that Matrix metalloproteinases (MMPs) are crucial for remodelling the extracellular matrix and are also involved in the invasion of tumor cells. MMPs targeting may be effective as an additional therapeutic strategy in the treatment of cancer and there are 25 matrix metalloproteinases in humans, which are zinc-dependent endopeptidases with a powerful ability to break down extracellular matrix proteins¹ (Abdel-Hamid & Abass, 2021). Except for certain processes like tissue healing and remodelling, inflammation, and neoplasia, the extracellular matrix functions as a barrier to prevent cell movement (Wieczorek et al., 2015).

MMP-11, also known as stromelysin-3, is a member of the MMPs superfamily's stromelysin subgroup and has been found in stromal cells, nearby microenvironment, and cancer cells (B. Ma et al., 2021). When compared to cancer cells, stromal cells in tumor tissues are genetically more stable, but they differ from their counterparts in normal tissues in terms of the types and concentrations of specific proteins (Khare et al., 2021; Rundhaug, 2005). This trait implies that stromal antigens may serve as antitumoral targets. Currently, tumour endothelial cells, infiltrating macrophages/histiocytes, and fibroblasts linked to cancer are the targets of immune interventions (Kim et al., 2021).

Numerous studies have shown that MMPs are immune targets. MMP-11 is a perfect antigen target for immunotherapy because it is only expressed in the majority of primary solid cancer tissues and metastatic lesions. Several studies also showed that MMP-11 has therapeutic potential. In a colon adenocarcinoma mouse model, Peruzzi et al. found that a genetic vaccine against MMP-11 based on DNA electro-gene-transfer