Natural Compounds Diosgenin and Panaxadiol Exhibit Anti-Cancer Activities, Potentially Through Targeting RORγ

By

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ABSTRACT

My study explores the potential anticancer properties of primarily two natural small molecule compounds, diosgenin and panaxadiol, in relation to Retinoic Acid Receptor-related Orphan Receptor Gamma (RORγ)-mediated cancer pathways. RORγ, a nuclear receptor, has been identified as a significant player in various cancers, and its inhibition has emerged as a promising therapeutic strategy. The compounds diosgenin, derived from fenugreek, and panaxadiol, isolated from the Panax genus, have shown promising anticancer activity in various pre-clinical models. However, their activities against RORγ in cancers remain unexplored. Through in-vitro experiments and RNA-seq-based functional analysis, my research investigated the effects of diosgenin and panaxadiol on RORγ-mediated signaling pathways and tumorigenesis. My findings suggest a significant inhibition of cancer cell proliferation in triple-negative breast cancer (TNBC), prostate and lung cancer cell models upon treatment with these compounds. Furthermore, RNA-seq analysis indicated that RORγ-mediated biochemical pathways such as cholesterol biosynthesis and metabolism are enriched in the differential gene expression profiles from the cells treated by the compounds. These preliminary results, therefore, suggest that diosgenin and panaxadiol could serve as novel therapeutic agents in the management of RORγ-related cancers. Further research is warranted to fully elucidate their anticancer mechanisms, potential synergies with existing therapies, and their efficacy in clinical trials. This research provides a critical step forward in the ongoing search for more effective and targeted therapies in the treatment of complex malignancies.

CHAPTER 1: INTRODUCTION

Cancer is a complex disease that can occur in any part of the body when cells in that area begin to grow uncontrollably and form a mass of abnormal cells called a tumor. There are several distinct varieties of cancer, each with its traits, causes, and available treatments.

Currently, cancer is one of the leading causes of death worldwide. Throughout the past several decades, however, breakthroughs in cancer prevention, early detection, and treatment have resulted from advancements in medical science and technology. For example, screening tests like mammography and colonoscopy can detect cancer in its early stages when it is more treatable, and new targeted therapies and immunotherapies along with surgical intervention, have been developed that can help fight certain types of cancer.

Research in cancer biology, genetics, and immunology continues to provide insights into the causes and mechanisms of cancer, which can lead to new treatment options and better outcomes for patients. Additionally, efforts to promote healthy lifestyles, such as eating a balanced diet, exercising regularly, and avoiding tobacco use, can help reduce the risk of developing certain types of cancer.

However, there are several hurdles in achieving to cure cancer, despite the significant progress that has been made in cancer treatment. Cancer is a complex disease that can develop in many different ways, and it can differ significantly from one person to another. This complexity makes it difficult to develop a universal cure that will work for patients. In some cases, cancer may not be detected until it has advanced to a later stage, which can make it more difficult to treat. This is why regular cancer screenings and early detection are important. Some cancer cells may develop resistance to certain types of

treatments, such as chemotherapy or radiation therapy. This can make it difficult to eradicate all cancer cells and may require the use of multiple treatment options.

Despite these limitations, researchers continue to work towards better understanding and treating cancer. New treatments and technologies are being developed to improve outcomes for patients.

1.1 Triple Negative Breast Cancer

Triple-negative breast cancer (TNBC) is a type of breast cancer that tests negative for estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2). TNBC is a particularly aggressive form of breast cancer, and it tends to have a poorer prognosis compared to other types of breast cancer. According to the American Cancer Society, TNBC accounts for approximately 10-15% of all breast cancers diagnosed. TNBC is more common in women with BRCA1 gene mutations. TNBC often has a poorer 5-year survival rate than other kinds of breast cancer. According to the American Cancer Society, the 5-year survival rate for localized TNBC is approximately 77%, compared to over 90% for other types of breast cancer. TNBC tends to be more aggressive and grow faster than other types of breast cancer, and it is more likely to recur after treatment. There are currently no targeted therapies approved specifically for TNBC, although researchers are actively studying new treatment options and biomarkers that may help guide treatment decisions for TNBC patients.

1.2 Prostate Cancer

Prostate cancer is a type of cancer that develops in the prostate gland, which is a part of the male reproductive system. Based on data from the American Cancer Society, prostate

cancer is the second most common cancer in men, after skin cancer. About 1 in 8 men will be diagnosed with prostate cancer at some point in their lifetime. Prostate cancer is typically slow-growing, and many men with early-stage prostate cancer may not experience any symptoms or require immediate treatment. The 5-year relative survival rate for all stages of prostate cancer is nearly 100%. The most common treatment options for prostate cancer include active surveillance, surgery, radiation therapy, hormone therapy, and chemotherapy, depending on the stage and severity of the cancer. Several factors can increase the risk of developing prostate cancer, including age, family history, and certain genetic mutations.

1.3 Lung Cancer

Lung cancer is a type of cancer that develops in the lungs. It is the leading cause of cancer-related deaths worldwide. According to the American Cancer Society, Lung cancer is the second most commonly diagnosed cancer in both men and women, after skin cancer. The 5-year relative survival rate for all stages of lung cancer combined is approximately 21%. The survival rate for lung cancer varies widely depending on the stage of the cancer at diagnosis. For example, the 5-year survival rate for localized lung cancer (cancer that has not spread beyond the lung) is approximately 61%, while the 5-year survival rate for metastatic lung cancer (cancer that has spread to other parts of the body) is only about 6%. Lung cancer is more common in older adults and in people who smoke or who have a history of smoking. There are several treatment options for lung cancer, including surgery, radiation therapy, chemotherapy, targeted therapy, and immunotherapy. The choice of treatment depends on the type and stage of the cancer, as well as other factors such as a person's overall health.

1.4 Nuclear Receptors

A class of proteins which bind directly to the DNA, thereby acting as transcription factors are known as nuclear receptors (NR). They regulate gene expression via their activation through the binding of ligands such as lipophilic molecules like steroids, endogenous and xenobiotic hormones, vitamins and other such molecules. NRs play important roles in modulating and maintaining embryonic development, adult homeostasis and metabolism in an organism. They are only found in animals (metazoans) while protists, algae, fungi and plants are devoid of these receptors. In humans, there are 48 nuclear receptors present in total, compared to mice and rats which have 49 and 47 of them, respectively. Structurally, a nuclear receptor is made of several domains which serve distinct functions. The A-B domain at the N-terminal acts as a regulatory domain that contains AF-1, the activation function 1, which works independently of the binding or presence of the ligand. Its sequence is highly variable among all the nuclear receptors. Along with the AF-2 which is present in the E-domain, the transcriptional activity of AF-1 becomes more powerful which on its own is usually quite poor. Then comes the C Domain, also known as the DNA-binding domain (DBD) which specifically binds to DNA sequences called the hormone response elements (HRE). It is an extremely structurally conserved domain with two zinc fingers that are involved in the direct binding of DNA. The ligand-binding domain (LBD) forms the E-domain which serves as the next most important domain to which the ligand binds in the ligand-binding cavity deep within the LBD. The action of AF-2 mentioned earlier depends on the ligand binding as opposed to AF-1. Together, they have a synergistic role in the modulation of gene expression. The DBD

and LBD are bridged by the hinge region which makes up the D-domain. Lastly, the last domain termed the F-Domain is present at the C-terminal.

Based on their mechanism of action, they are classified as Type I, II, III and IV nuclear receptors. Type I NR are located in the cytosol before ligand binding. Upon the diffusal of a ligand molecule into the cytoplasm, it binds to a type I NR which results in the dissociation of the heat shock protein (HSP) from the NR/HSP complex. That leads to the homo-dimerisation of NRs followed by translocation to the nucleus. Once inside the nucleus, the NR dimer binds to specific HREs. HREs can be made up of two half-sites separated by a variable amount of DNA and featuring an inverted repeat at the second half-site are recognized by type I nuclear receptors. This leads to the recruitment of transcription proteins which convert DNA to mRNA and eventually into a protein responsible for cellular function. In contrast to type I NR, type II NR are present within the nucleus and dimerise as hetero-dimers before binding to DNA. Additionally, the binding of a ligand to the NR causes the dissociation of corepressor proteins, with whom type II NR usually form complexes. Instead, it's replaced with the recruited coactivator proteins and RNA polymerase which transcribe DNA into mRNA. Nuclear type III receptors share a trait with type I receptors in that they both bind to DNA as homodimers. Nevertheless, type III nuclear receptors bind to direct repeat HREs rather than inverted repeat HREs, in contrast to type I nuclear receptors. Type IV nuclear receptors can bind as monomers or dimers, but only one of the receptor's DNA-binding domains can bind to a given half-site HRE.

Furthermore, NRs are also categorized into various subfamilies based on sequence homology. One of them is the Thyroid Hormone Receptor-like subfamily which includes the group of nuclear receptors known as the RAR-related orphan receptors (RORs) that

work as intracellular transcription factors. Among those, are three kinds of NRs - RORα, RORβ and RORγ and the corresponding genes RORA, RORB and RORC, respectively, are responsible for the encoding of each of them. They monomerically bind to HREs known as the ROR response elements (RORE) and do not require dimerisation. More light will be shed, particularly, on RORγ, its functions and why it's important to this study, in the following sections.

1.5 Retinoid Acid Receptor-related Orphan Receptor Gamma (RORγ)

The nuclear receptor ROR-gamma (RORγ) is a transcription factor that plays an important role in regulating immune cell differentiation and function. ROR-γ is primarily expressed in certain immune cells, such as Th17 cells, which are involved in immune defence against pathogens but also contribute to autoimmune diseases. ROR-gamma has been shown to regulate the differentiation of Th17 cells. Genetic studies have identified ROR-gamma as a key driver of autoimmune diseases such as multiple sclerosis, psoriasis, and inflammatory bowel disease (1) (Figure 1A). ROR-gamma is also involved in the regulation of other immune cells, such as natural killer (NK) cells and innate lymphoid cells (ILCs), and may play a role in tumor immunity (2). ROR-gamma has emerged as a promising therapeutic target for autoimmune and inflammatory diseases. Small molecule ROR-gamma inhibitors have been developed and are being evaluated in clinical trials for various indications. In addition to its role in immune cell function, ROR-gamma has also been implicated in the regulation of metabolism and circadian rhythms. The crystal structure of the ROR-gamma ligand-binding domain (LBD) has been solved (3), providing insights into the mechanism of ROR-gamma activation and potential drug design.

Overall, the current data suggest that ROR-gamma is an important regulator of immune cell function and a promising therapeutic target for autoimmune, inflammatory and metabolic disorders. However, further research is needed to fully understand the complex mechanisms of ROR-gamma regulation and its potential clinical applications.

1.6 Role of ROR-γ in Cancer and Tumorogenesis

Due to its above-mentioned implications in circadian rhythms, autoimmune and metabolic homeostasis, RORγ plays pivotal roles in cancer as those factors regulate cancer and tumour progression. Zou H. et al in Dr. Hongwu Chen's laboratory, conducted experiments using human TNBC cell lines like HCC70 and MDA-MB468 to demonstrate that RORγ can regulate the expression of a gene called SREBP2, which plays a critical role in regulating cholesterol metabolism. They found that RORγ functions as a positive regulator of SREBP2 gene expression, promoting the synthesis of cholesterol (4). The researchers have discovered that RORγ functions as a master regulator of cholesterol biosynthesis in breast cancer by directly activating the expression of key genes involved in cholesterol biosynthesis, including HMGCR, FDPS, and SQLE (5). These genes play essential roles in the synthesis of cholesterol in cancer cells. Increased cholesterol synthesis and accumulation have been shown to promote tumor growth and metastasis in various cancer subtypes. Therefore, targeting RORγ-mediated cholesterol biosynthesis may be a promising therapeutic strategy for TNBC.

Prostate cancer is one of the most common cancer in men. Androgen deprivation therapy (ADT) is one of the standard treatments for the advanced disease. However, ADT has several side effects, including dyslipidemia, which is characterized by an increase in low-

density lipoprotein cholesterol (LDL-C) and a decrease in high-density lipoprotein cholesterol (HDL-C) levels. Recent studies have suggested that RORγ may play a role in developing and progressing prostate cancer. RORγ has been shown to regulate cholesterol biosynthesis in prostate cancer cells. One study demonstrated that ROR-gamma activation in prostate cancer cells increased the expression of genes involved in cholesterol biosynthesis, such as HMGCR and LDLR, which increased intracellular cholesterol levels (5). Increased cholesterol synthesis and accumulation have been shown to promote tumor growth and metastasis in castration-resistant prostate cancers (CRPC). Moreover, some studies have also investigated the role of RORγ, involved in regulating lipid metabolism, in advanced prostate cancer. Yang N., et al found that RORγ expression was increased in advanced prostate cancer compared to normal prostate tissue and that RORγ activation led to increased expression of cholesterol biosynthesis genes (6-7) To further validate the role of RORγ in regulating cholesterol homeostasis in prostate cancer, the researchers conducted experiments in prostate cancer cell lines and found that RORγ inhibition led to decreased cholesterol biosynthesis and decreased cancer cell growth. Finally, the researchers examined the clinical relevance of their findings by analyzing RORγ expression in human prostate cancer samples. They found that high RORγ expression was associated with worse overall survival in patients with advanced prostate cancer. It was also found that overexpression of RORγ directly contributes to androgen receptor (AR) hyperactivity via its binding to an exonic RORE through the recruitment of NR coactivators SRC-1 and SRC-2 (6). Overall, the researchers concluded that dysregulation of cholesterol homeostasis in advanced prostate cancer is mediated by crosstalk between AR and liver X receptor (LXR) and that RORγ is a potential therapeutic target for the treatment of advanced prostate cancer.

Their findings suggest that targeting RORγ could potentially reduce cholesterol biosynthesis and cancer cell growth in advanced prostate cancer.

Cancer studies in lung cancer relating to RORγ are quite limited and infrequent and do call for further investigation. According to the few studies existing, in lung cancer, overexpression of RORγ2 has been shown to act as a prognostic factor in non-small cell lung cancer (NSCLC) (8). There were decreased expression levels of RORγt mRNA and protein in peripheral blood mononuclear cells (PBMCs) of NSCLC patients compared to healthy individuals. (9). On the other hand, there was an upregulation of RORγt mRNA in the blood of NSCLC patients as opposed to healthy individuals (10).

Taken together, these studies suggest that RORγ plays a critical role in regulating cholesterol biosynthesis and promoting tumor growth in TNBC and lung cancer. Therefore, targeting RORγ-mediated cholesterol biosynthesis may be a potential therapeutic strategy for these cancers.

1.7 RORγ Modulators

Targeted therapies against cancer are at a main front in today's day and age compared to traditional therapies like chemo- and radiation therapy due to their lack of selectivity. Targeted therapy includes antibody therapies and small-molecule modulators. The advantage that a small molecule has over an antibody is its wider range of binding targets due to its smaller size (< 500 Da) and higher membrane permeability. They find their way deep into the structure of a target protein and bind to its “pocket”. This leads

to the modulation of the particular protein's function and as a result, it affects the entire downstream biochemical pathway.

Many synthetic small molecules have been discovered and developed to target RORγ. A few of the widely approved/accepted synthetic small molecule inhibitors of RORγ are SR2211 (11), GSK805 (12) and XY018 (13). They have been shown to inhibit tumor proliferation and metastasis in castration-resistant prostate cancer and TNBC (4).

This study, however, focuses on two natural small-molecule modulators which have shown reasonable and scientific evidence to be investigated further in the context of modulation of RORγ.

1.7.1 Diosgenin: Diosgenin is a steroid saponin found in various plants, including wild yam and fenugreek. It is used commercially synthesising several steroids like cortisone, pregnenolone and progesterone. It acts as an apoptosis inducer, an antiviral agent, an antineoplastic agent and a metabolite. It has been studied for its potential anticancer activity, including its ability to modulate the activity of nuclear receptors such as ROR-gamma. Diosgenin acts as an inverse agonist for the NRs RORα and RORγ through direct binding, represses transcription and modifies target gene expression of RORα/RORγ in cell models (14). It has been shown to downregulate the target genes of RORα/RORγ, namely IL-17A and G6PC.

Overall, the current data suggest that diosgenin may have the potential as a therapeutic agent for cancer by targeting ROR-gamma and modulating immune responses. However, more research is needed to fully understand the mechanisms of diosgenin action and its potential clinical applications.

1.7.2 Panaxadiol: Panaxadiol is a triterpenoid saponin found in the roots of the Panax Ginseng plant and has been studied for its potential therapeutic effects. However, studies that specifically mention its interaction with RORγ or its role in cancer treatment are extremely limited. One study examined its role in inhibiting the RORγ/IL-17A pathway axis in STZ-induced type 1 diabetes (T1D). RORγ is a key transcription factor for proinflammatory IL-17A production. ROR deficiency was found to shield low-dose STZ-induced T1D mice models against STZ-induced T1D by reducing IL-17A production and enhancing islet β cell activity in the pancreas. Interestingly, it was found that panaxadiol from ginseng specifically inhibits ROR transcriptional activity, which reduces STZ-induced T1D by preventing the generation of IL-17A (15). It is important to emphasise that further research is needed to determine the full extent of panaxadiol's effects on RORγ and its potential as a therapeutic agent.

1.8 Hypothesis: Diosgenin and panaxadiol modulate cancer cell growth, survival and gene expression, potentially through targeting of ROR-γ in TNBC, prostate and lung cancer.

CHAPTER 2: MATERIALS AND METHODS

Cell lines: HCC70, C42B and PC9 were cultured in RPMI1640 supplemented with 10% FBS and 1% PenStrep. MDA-MB468 and A549 were cultured in DMEM, also, supplemented by 10% FBS and 1% PenStrep. Cells were grown at 37 °C in 5% CO_2 incubators. Cells were obtained from ATCC.

Chemicals: Diosgenin and panaxadiol were purchased from SelleckChem.

Microscopy: Human cancer cell lines were treated with varying concentrations of diosgenin and panaxadiol as indicated for 96 hours and then imaged at 4X magnification using a Bright Field (BF) filter under the microscope.

Cell viability, apoptosis, and colony formation: For cell viability, cells were seeded in 96-well plates at 1500–2500 cells per well in a total volume of 100 µl of media. After 4 days of incubation, Cell-Titer Glo reagents (Promega) were added, and luminescence was measured on a GLOMAX microplate luminometer (Pro- mega), according to the manufacturer's instructions. The estimated in vitro IC_{50} values were calculated by using GraphPad Prism 8 software. For cell growth, cells were seeded in 6-well plates at 2×10^5 per well and treated as indicated. Total viable cell numbers were counted using CountessTM II Automated Cell Counter (Invitrogen). For colony formation, 500–1000 cells were seeded in a well of 6-well plates and cultured for 14 days with the medium changing every 3 days. When the cell clone grew visible, the medium was removed. Then the plates

were washed with PBS two times, and the cell colonies were stained with 0.2% crystal violet (in 10% formalin) for 30 mins. Then, the plates were scanned for imaging.

RNA-seq alignment and analysis: HCC70, MDA-MB468 and A549 cells were treated as indicated before RNA extraction. RNA-seq libraries from 1 μg total RNA were prepared and validated as previously described (Yang et al., 2012). Sequencing was performed on an Illumina HiSeq 2000 Sequencer at BGI Tech (Hong Kong). The FASTQ-formatted sequence data were analyzed using a standard BWA-Bowtie-Cufflinks workflow. Sequence reads were aligned to the reference human genome assembly (hg38) with BWA and Bowtie software. Subsequently, the Cufflinks package (Trapnell et al., 2010) was applied for transcript assembly and quantification of gene expression. To avoid spurious fold levels due to low expression values, only subsets of genes that have expression value FPKM (fragments per kilobase of exon model per million mapped reads) above 1 for either the vehicle-treated cell or the compound-treated cells are included. GSEA was performed using the Java desktop software (http://www.broadinstitute.org/gsea) as described previously (Subramanian et al., 2005). Genes were ranked according to the shrunken limma log2 fold changes and the GSEA tool was used in 'pre-ranked' mode with all default parameters.

CHAPTER 3: RESULTS

3.1 Effects of two putative RORγ modulators on cancer cell growth

To start investigating the effects of diosgenin and panaxadiol, some preliminary imaging was done with TNBC cell lines like MDA-MB468 and HCC70 (Figure 3-4A). Post-treatment of 96 hours, the untreated control cells exhibited the standard round and irregularly spindle-shaped epithelial morphology of MDA-MB468 and HCC70, respectively. Both the cell lines retained their morphology at 2.5 μM treatment of diogenin as well as panaxadiol, but had a significantly decreased cell growth compared to the untreated cells. MDA-MB468 treated with higher drug concentrations displayed noticeable morphological changes and showed increased cytoplasmic extensions. There was an increasing reduction in cell growth starting from 10 to 20 μM. On the other hand, HCC70 cells underwent cell death starting at 10 μM and started to completely lose their original morphology. Following that, dose-dependent cell growth and the ability to divide unlimitedly were observed in MDA-MB468 and prostate cancer cell line C42B through the cell colony formation assay. Colonies formed by 5 μM diosgenin-treated MDA-MB468 cells decreased by approximately 50% versus the control cells (Figure 3B). No colonies were formed at higher doses. In contrast, C42B cell colonies grown after 5 μM diosgenin treatment were drastically less than the untreated cells. Again, no colonies were formed at higher doses. The opposite trend was seen when the cells were treated with panaxadiol at the same concentrations (Figure 4B). While the MDA-MB468 colonies steadily decreased from 5 to 20 μM, C42B colonies reached approximately 50% of the control colonies at 5 μM. They further decreased at 10 μM with zero colonies formed at 20 μM.

These results show that while the HCC70 cell line is sensitive to both panaxadiol and diosgenin, the MDA-MB468 and C42B cell line is more sensitive to diosgenin compared to panaxadiol. Although panaxadiol at lower concentrations was able to reduce cell growth in cancer cells, it did not have as much inhibitory effect on the cells' ability to form colonies and proliferate uncontrollably. Diosgenin, on the other hand, was successful in limiting cell growth as well as colony formation.

Next, to study the time-dependent effects in addition to dose-dependent cytotoxic effects, a cell proliferation assay was done. After three days of dose-dependent diosgenin treatment, it was able to decrease cell growth, albeit not significantly in TNBC cell lines: MDA-MB468, HCC70 (Figure 3C) and lung cancer cell line: PC9 (Figure 5A). Prostate cancer cell line: C42B showed a greater inhibition between the control group of cells and the treated cells. On the sixth day, the inhibition was more appreciable in the same group of cells. The cell death rate was greater than the cell growth rate at the highest concentration of 15 μM in C42B and lung cancer cell line: A549. However, A549 cells exhibited an abnormal trend where a lower concentration of 7.5 μM caused the same cell growth as the control cells and a greater concentration of 10 μM showed increased cell growth even more than the control cells (Figure 5A). The possible reasoning for that could be handling error while seeding the cells leading to an unequal number of plated cells, to begin with. Overall, diosgenin was able to exhibit an inhibitory effect on cell proliferation in MDA-MB468, HCC70, C42B and PC9 cells by the sixth day. Time- and dose-dependent treatment of panaxadiol also showed inhibitory effects in HCC70 and C42B (Figure 4C) as well as A549 and PC9 cell lines (Figure 5B). There was an increase in cell growth inhibition with an increase in drug concentration on the third day. On the sixth day of treatment, MDA-MB468 cells exhibited comparable cell growth at 7.5 and 10 μM compared to the untreated cells. However, there

was decreased cell growth at 15 μM. HCC70 and C42B cells showed inhibition in cell proliferation at 7.5 μM and increased cell death at higher doses of 10 and 15 μM. In PC9 cells, there was an overall there was an increase in cell death at all the given doses. Cell growth in A549 cells was inhibited at 7.5 and 10 μM and cell death increased at the highest concentration of 15 μM. In summary, HCC70, C42B, PC9 and A549 cell lines of different cancers were highly sensitive to panaxadiol at higher doses over time in 6 days and moderately sensitive at a low dose of 7.5 μM over the given time points except for PC9 cells which started showing cell death at 7.5 μM itself.

Thus, the findings suggest that TNBC (MDA-MB468 and HCC70), prostate cancer (C42B), and lung cancer line (PC9) are sensitive to diosgenin in a dose and time-dependent manner. In a similar manner, TNBC cells such as HCC70, prostate cancer (C42B) and lung cancer cell lines (PC9 and A549) are sensitive to panaxadiol and show inhibitory effects on cell growth and proliferation.

3.2 Analysis of the activities of two putative RORγ modulators in modulation of gene expression in cancer cells.

In the previous section, it was demonstrated that the cell growth of TNBC, prostate cancer and lung cancer cell lines are variably sensitive to diosgenin and panaxadiol in a time and dose-dependent manner. To investigate the biochemical pathways altered and differential gene expression by the activities of the two compounds, RNA-seq analysis of HCC70, MDA-468 and A549 was performed. Gene ontology (GO) analysis of the genes with expression down-regulated (1.5 fold) by diosgenin in HCC70 indicated that cholesterol biosynthesis and metabolism gene programs were significantly inhibited at 10 and 20 μM at 24hr

(Figure 6A). At 48hr, the pathways downregulated were mostly related to neurogenesis. Further examination by gene-set enrichment analysis (GSEA) also revealed that hallmarks of cholesterol biosynthesis were greatly enriched by diosgenin at 10 μM (24hr and 48hr) and 20 μM at 24hr (Figure 6B). Additionally, twenty-four hours of treatment of diosgenin resulted in a strong down-regulation of 25 out of the 26 cholesterol biosynthesis genes, including *MVK, LSS, HMGCS*, *HMGCR* and *SQLE* (Figure 6C). GO analysis of downregulated(1.5 fold) genes in HCC70 by panaxadiol treatment at a higher dose (20 μM) at 24hr also indicated cholesterol biosynthesis pathway to be slightly negatively impacted, but not at all at a lower dose i.e. 10 μM. Cholesterol biosynthesis gene program is controlled by RORγ through the regulation of the transcription factor SREBP2 (5). There could be a possibility that diosgenin and panaxadiol have the potential to act through RORγ in TNBC cell lines to downregulate the cholesterol biosynthesis program. However, there is insufficient evidence in this study to draw any direct linkage between the mechanism of action of the two compounds and RORγ. Nonetheless, the data does give a reasonable direction to investigate it further.

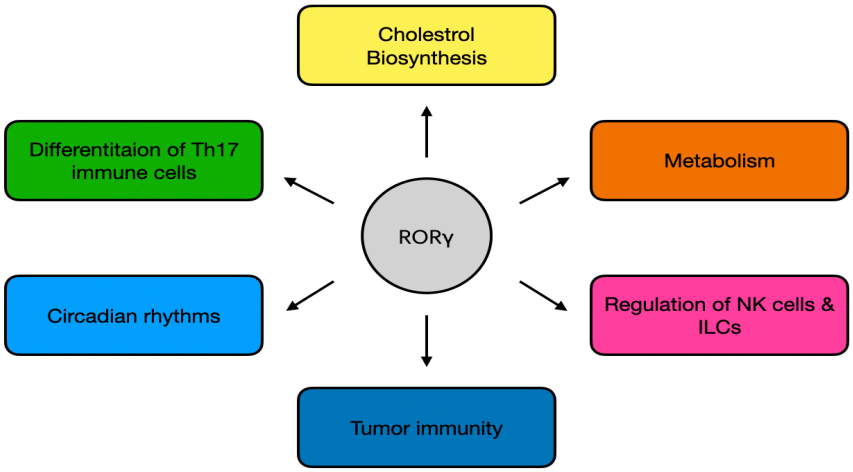
In MDA-MB468, GO analysis of downregulated genes by diosgenin (10 and 20 μM) also showed the cholesterol biosynthesis program to be majorly altered at 24hr (Figure 7A). GSEA analysis complemented the data from the GO analysis by revealing cholesterol biosynthesis hallmarks to be significantly enriched by diosgenin (20 μM) at 24hr (Figure 7B). Cholesterol biosynthesis was downregulated by panaxadiol (20 μM) at 24hr, according to GO analysis. Forty-eight-hour treatment of panaxadiol (20 μM) revealed similar results as 24hr. A lower concentration of panaxadiol (10 μM) altered other pathways such as development programs, neurogenesis, and protein-DNA packing complex at 24 and 48hr.

GO analysis of A549 cells treated with diosgenin for 24hr indicated cellular programs like cell differentiation, development, and neurogenesis were downregulated at a drug concentration of 10 μM and nuclear processes such as DNA replication and repair were impaired at a higher concentration (Figure 8A). At 48hr, there was a further downregulation of nuclear processes involving DNA binding and its replication and repair. According to GSEA analysis, 10uM and 20uM treatment of diosgenin significantly enriched cell cycle checkpoints and DNA repair gene programs, both at 24hr and 48hr (Figure 8B). Panaxadiol treatment (10 and 20 μM) of A549 cells exhibited only nerve development programs to be downregulated at 24hr, based on GO analysis.

CHAPTER 4: FIGURES

Fig. 1: ***A.*** *The known biological functions of RORγ. Th: T Helper, NK: Natural killer, ILC: Innate Lymphoid Cell;* ***B.*** *Schematic flowchart of this scientific study.*

A.



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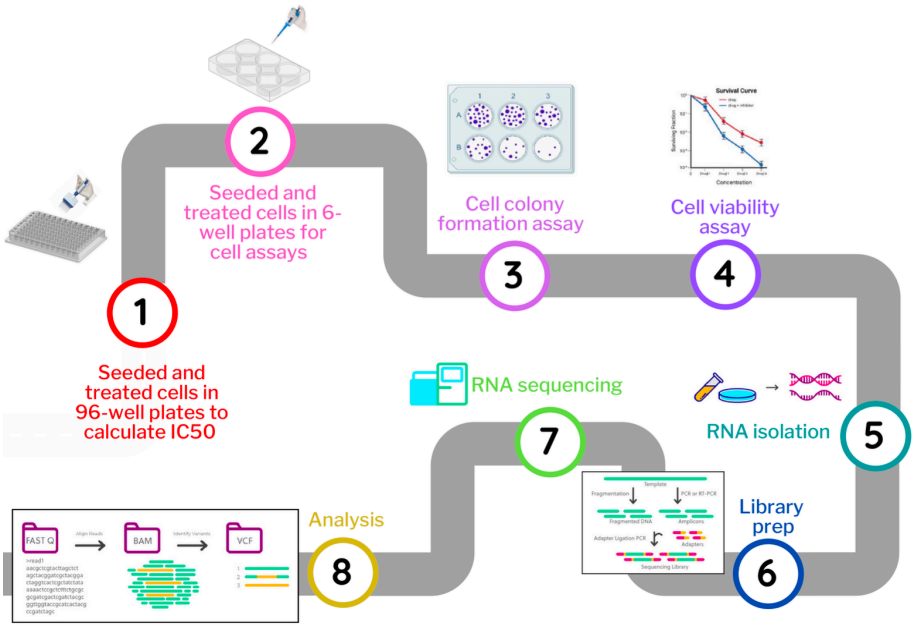
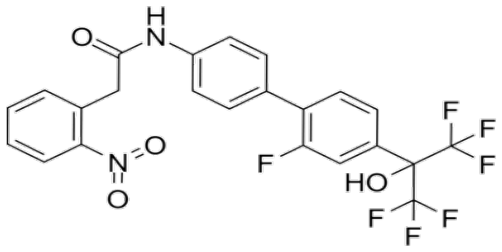
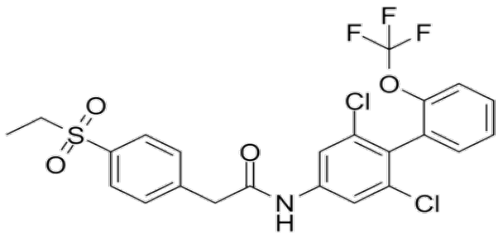


Fig. 2.: A. *Chemical structures of the three synthetic small-molecule inhibitors/antagonists of RORγ: XY018, GSK805 and SR2211;* ***B.*** *Chemical structures of the putative natural small-molecule modulators of RORγ: diosgenin and panaxadiol.*

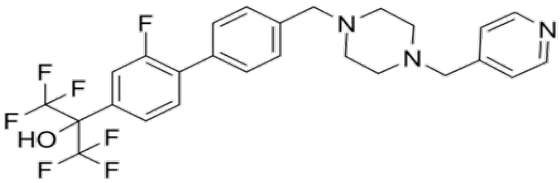
A.



XY018

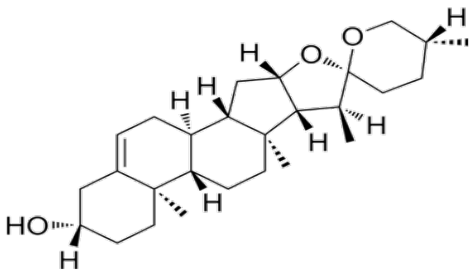


GSK805

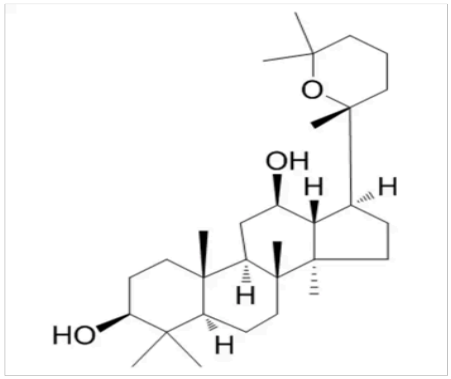


SR2211

B.



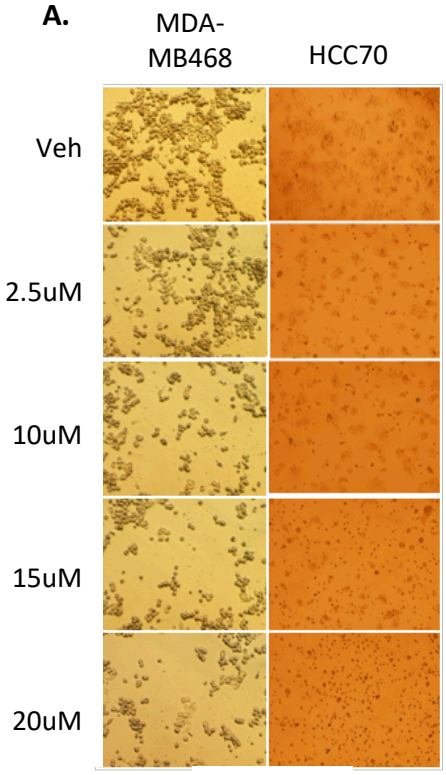
Diosgenin



Panaxadiol

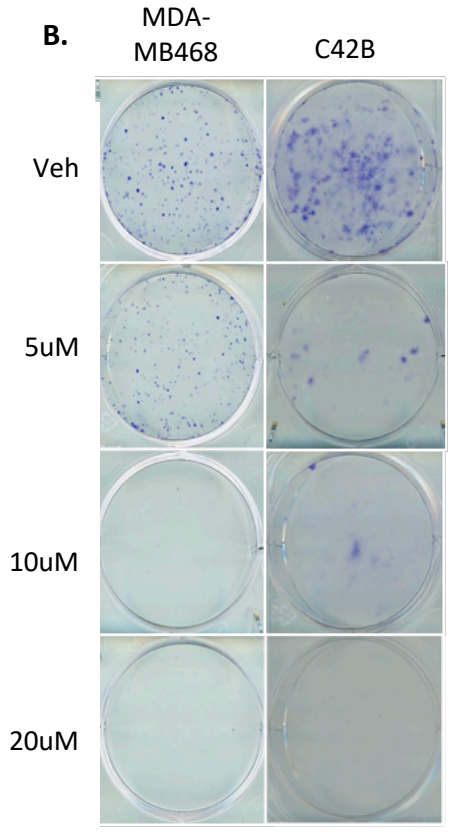
Fig. 3: ***A.*** *Microscopy imaging (4X BF) of MDA-MB-468 and HCC70 treated with diosgenin as indicated.* ***B.*** *MDA-MB468 & C42B cells were treated with diosgenin as indicated. Fourteen days later, representative images of colony formation were taken.* ***C.*** *MDA-MB468, HCC70 & C42B cells were treated with diosgenin as indicated. Three and six days later, viable cells were counted. Data are shown as mean ± SD. n = 2. Student's t-test. ** $p < 0.01$; * $p < 0.05$.*

A.



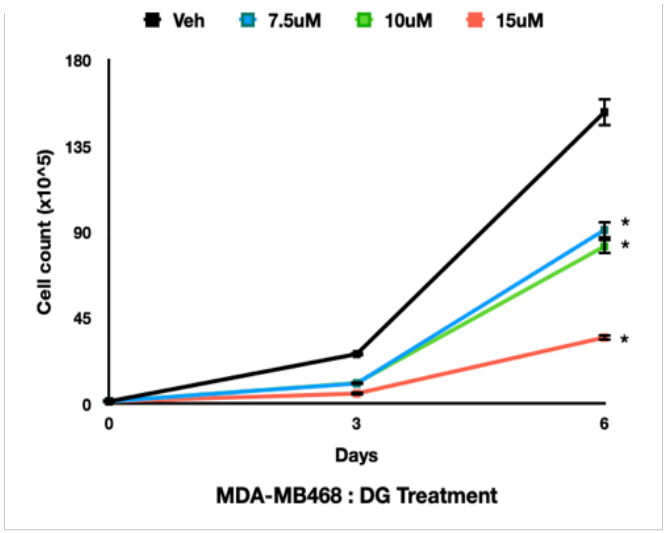
diosgenin

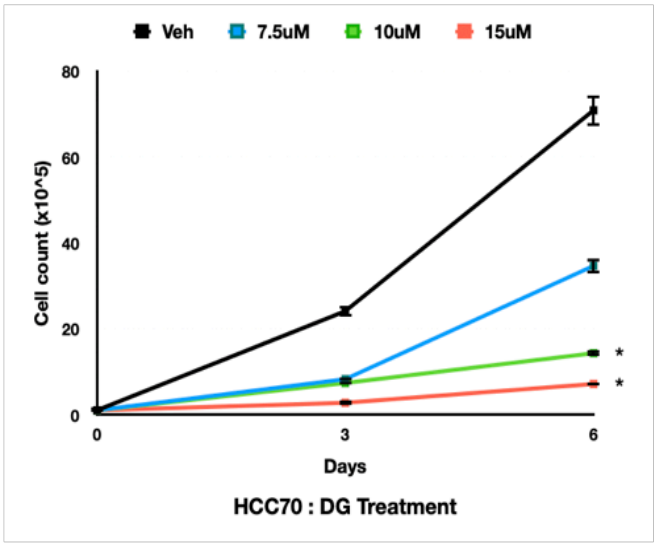
B.



diosgenin

C.





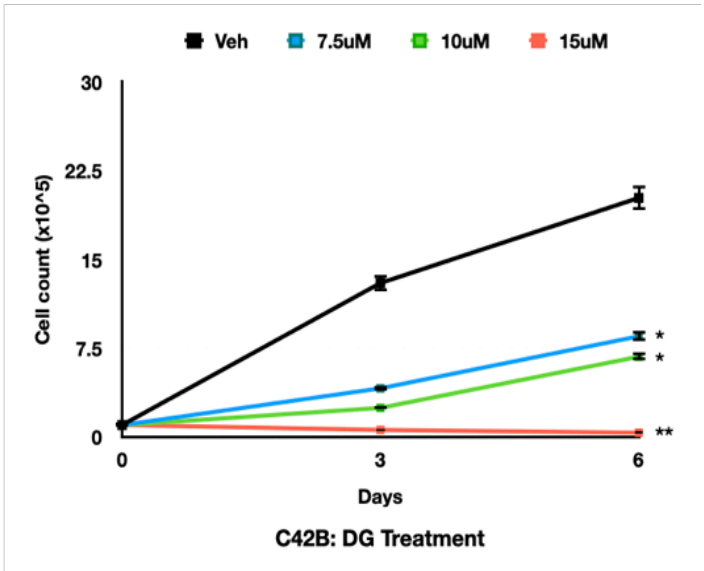
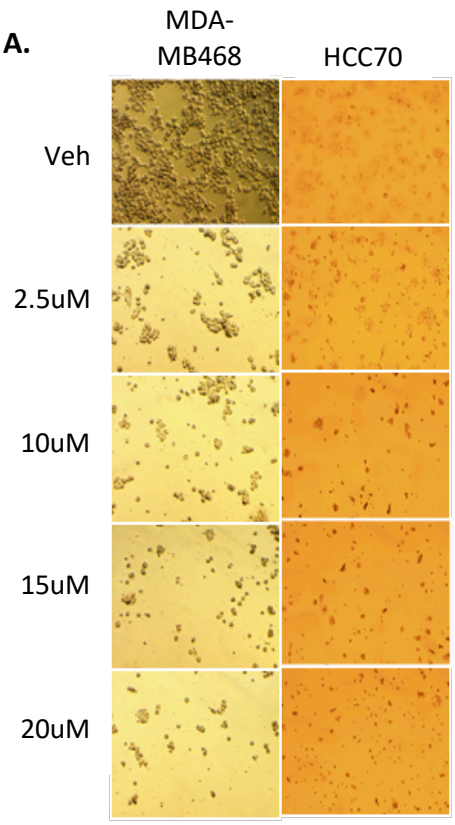
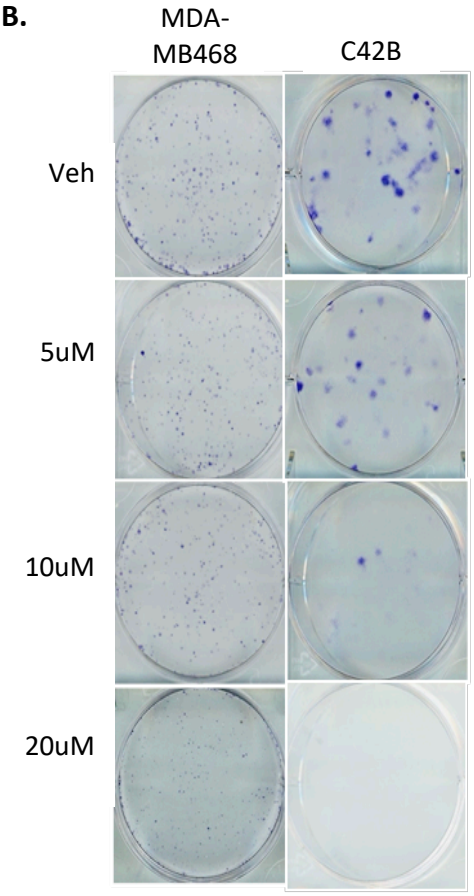


Fig. 4: ***A.*** *Microscopy imaging (4X BF) of MDA-MB-468 and HCC70 treated with diosgenin as indicated.* ***B.*** *MDA-MB468 & C42B cells were treated with panaxadiol as indicated. Fourteen days later, representative images of colony formation were taken.* ***C.*** *MDA-MB468, HCC70 & C42B cells were treated with panaxadiol as indicated. Three and six days later, viable cells were counted. Data are shown as mean ± SD. n = 2. Student's t-test. ** $p < 0.01$; * $p < 0.05$.*

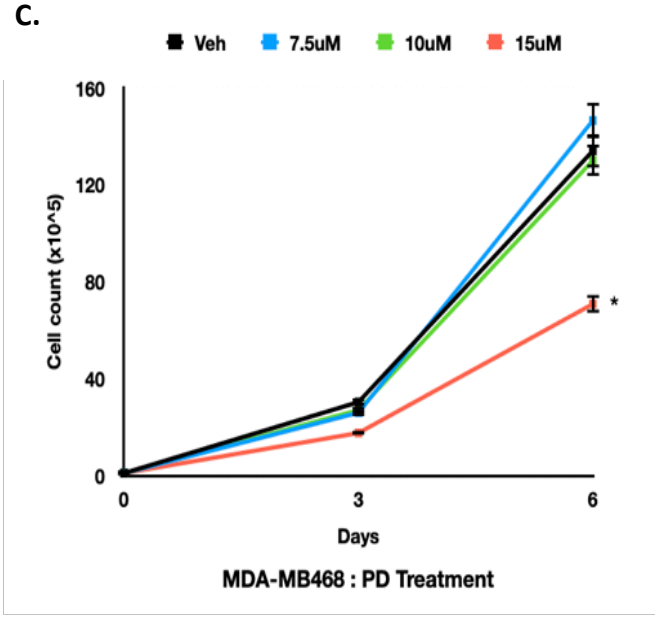
A.

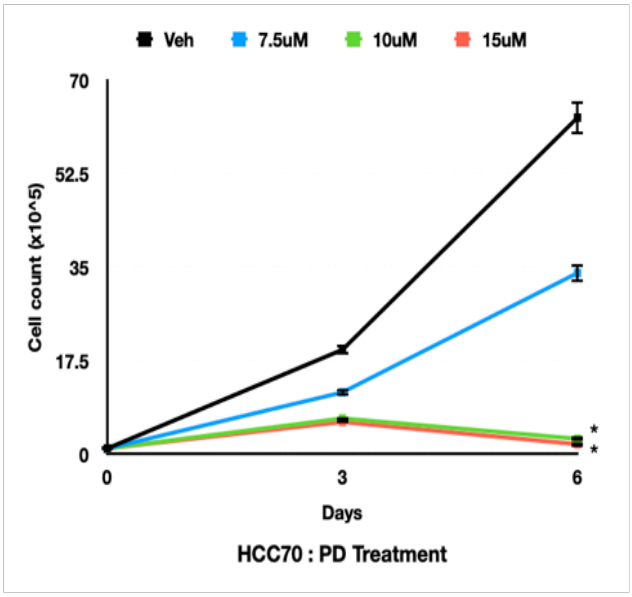

MDA-MB468
HCC70
Veh
2.5uM
10uM
15uM
20uM

B.


MDA-MB468
C42B
Veh
5uM
10uM
20uM

C.


Veh
7.5uM
10uM
15uM
Cell count (x10^5)
160
120
80
40
0
0
3
6
Days
*
MDA-MB468 : PD Treatment


Veh
7.5uM
10uM
15uM
Cell count (x10^5)
70
52.5
35
17.5
0
0
3
6
Days
*
*
HCC70 : PD Treatment

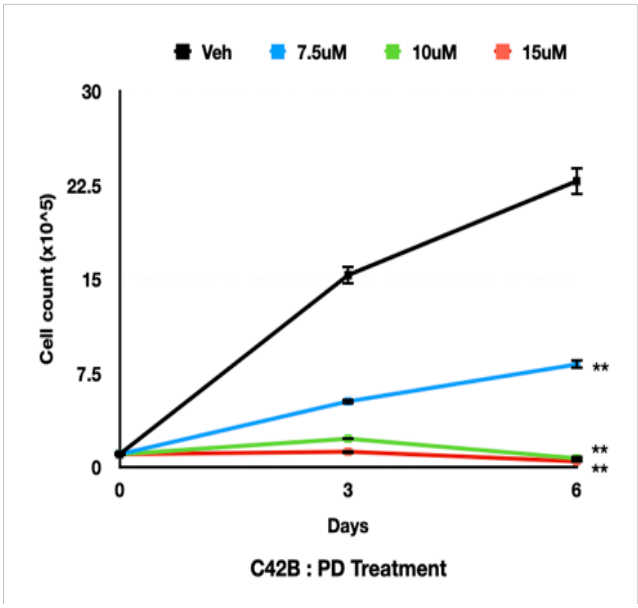
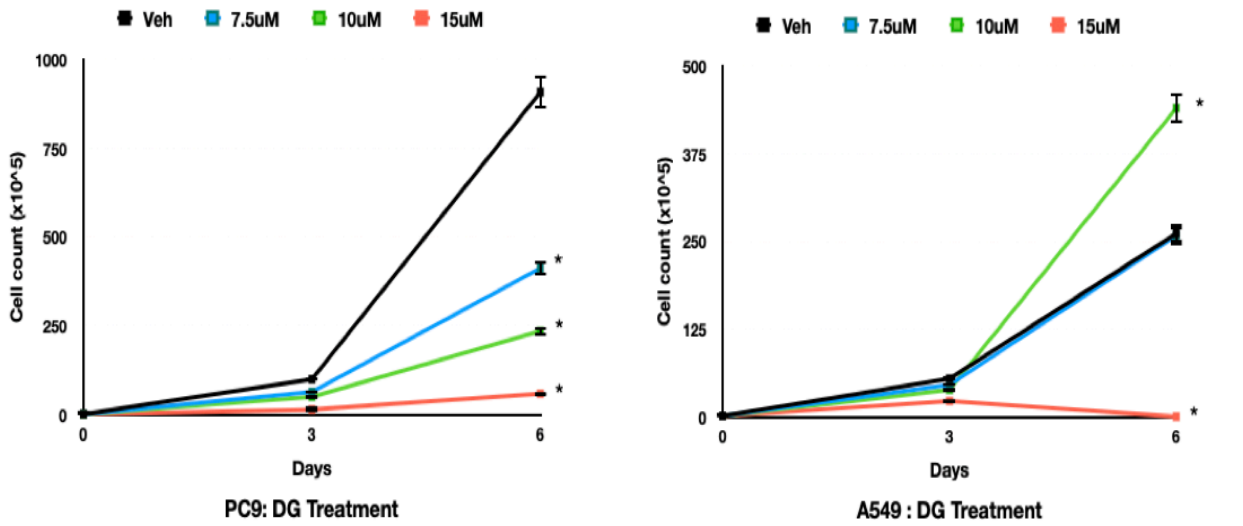

Veh
7.5uM
10uM
15uM
Cell count (x10^5)
30
22.5
15
7.5
0
0
3
6
Days
**
**
**
C42B : PD Treatment

Fig. 5: ***A.*** *PC9 and A459 cells were treated with diosgenin and* ***B.*** *panaxadiol as indicated. Three and six days later, viable cells were counted. Data are shown as mean ± SD. n = 2. Student's t-test. ** $p < 0.01$; * $p < 0.05$.*

A.



B.

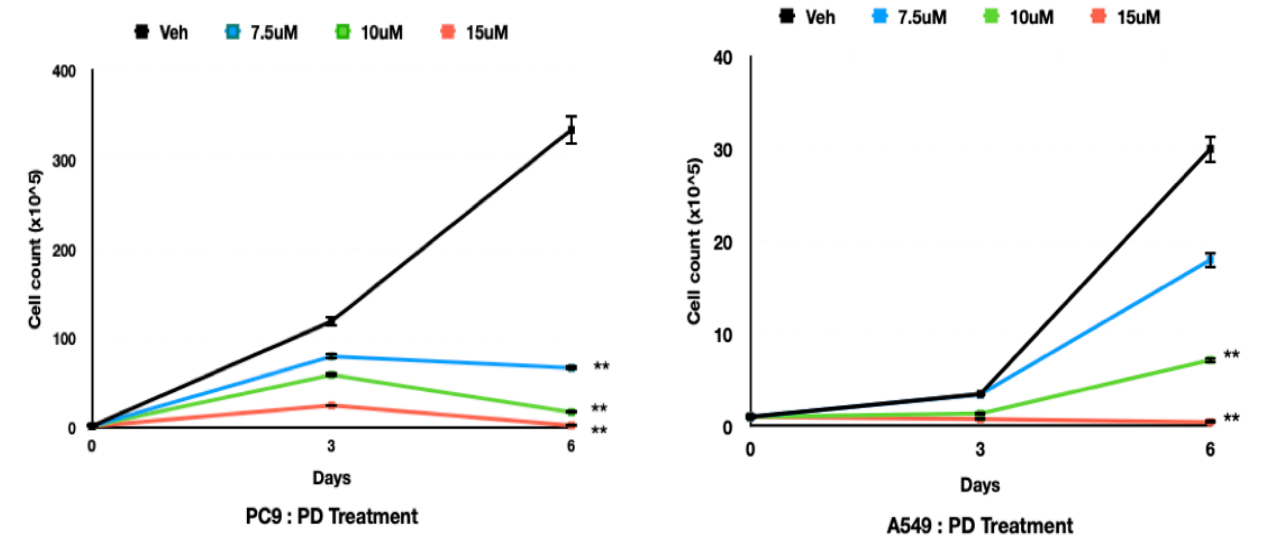


Fig. 6: *HCC70 RNA-seq data analysis.* ***A.*** *Gene ontology analysis of the downregulated gene expression (1.5 fold) in HCC70 cells treated with diosgenin an panaxadiol as indicated.* ***B.*** *GSEA plots depicting the enrichment of genes downregulated (1.5-fold) in HCC70 cells treated with diosgenin, as indicated for 24hr and 48hr. FDR false-discovery rate.* ***C.*** *Heat map display of fold changes (in log2) in mRNA expression of 26 cholesterol-biosynthesis genes analyzed by RNA-seq in HCC70 cells treated with different diosgenin as indicated.*

A.

10uM Diosgenin, 24hr

Description	logP
sterol biosynthetic/metabolic process	-15.58/-10.75
cholesterol biosynthetic/metabolic process	-13.38/-10.31
secondary alcohol biosynthetic/metabolic process	-13.38/-9.61
steroid biosynthetic/metabolic process	-12.26/-9.58

10uM Diosgenin, 48hr

Description	logP
regulation of cytosolic calcium ion concentration	-5.58
regulation of nervous system process	-5.13
nerve development	-5.09
calcium ion homeostasis	-5.07

20uM Diosgenin, 24hr

Description	logP
sterol biosynthetic process	-6.82
cholesterol biosynthetic process	-6.70
secondary alcohol biosynthetic process	-6.70
ribonucleotide metabolic/biosynthetic process	-5.54/-5.50

20uM Diosgenin, 48hr

Description	logP
Wnt signaling pathway	-4.66
cellular amino acid biosynthetic process	-4.42
regulation of neurogenesis	-4.05
regulation of nervous system development	-3.97

10uM Panaxadiol, 24hr

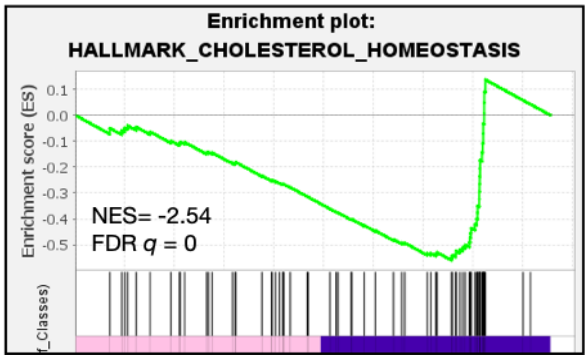
Description	logP
DNA replication-dependent nucleosome assembly	-8.68
protein-DNA complex	-5.82
regulation of Wnt signaling pathway	-1.34
histone H3-K27 trimethylation	-1.33

20uM Panaxadiol, 24hr

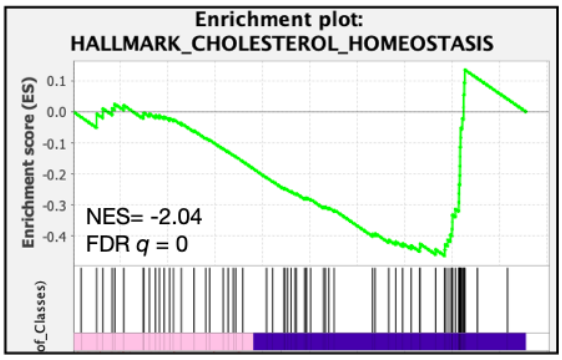
Description	logP
carboxylic acid biosynthetic process	-4.76
Steroid biosynthesis	-3.81
isoprenoid biosynthetic process	-3.06
cholesterol biosynthetic process	-2.66

B.

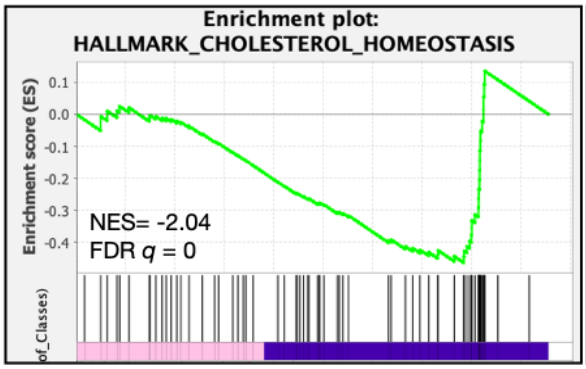
10uM Diosgenin, 24hr



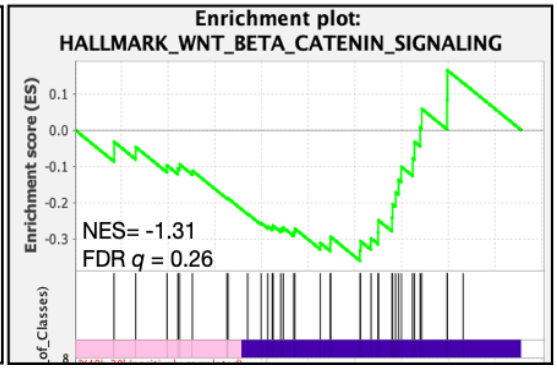
20uM Diosgenin, 24hr



20uM Diosgenin, 24hr



20uM Diosgenin, 48hr



C.

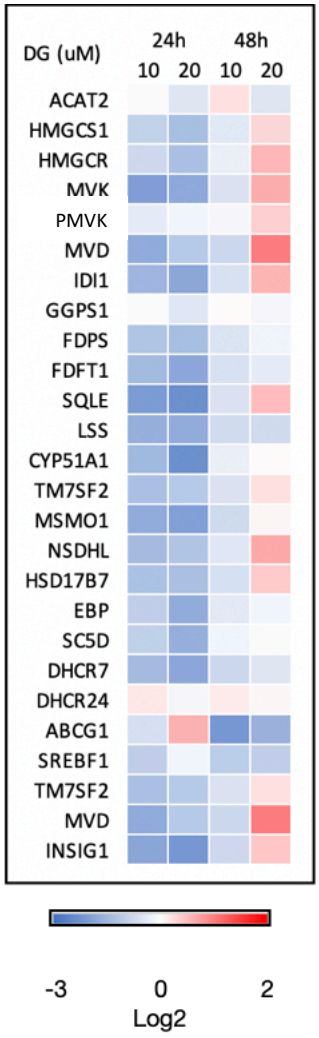

DG (uM)
24h
48h
10
20
10
20
ACAT2
HMGCS1
HMGCR
MVK
PMVK
MVD
IDI1
GGPS1
FDPS
FDFT1
SQLE
LSS
CYP51A1
TM7SF2
MSMO1
NSDHL
HSD17B7
EBP
SC5D
DHCR7
DHCR24
ABCG1
SREBF1
TM7SF2
MVD
INSIG1
-3
0
2
Log2

Fig. 7: *MDA-MB468 RNA-seq data analysis.* ***A.*** *Gene ontology analysis of the downregulated gene expression (1.5 fold) in MDA-MB468 cells treated with diosgenin an panaxadiol as indicated.* ***B.*** *GSEA plots depicting the enrichment of genes downregulated (1.5-fold) in MDA-MB468 cells treated with diosgenin and panaxadiol, as indicated for 24hr and 48hr. FDR false-discovery rate.*

A.

10uM Diosgenin, 24hr

Description	logP
sterol biosynthetic/metabolic process	-16.78/-14.05
cholesterol biosynthetic/metabolic process	-14.70/-13.03
secondary alcohol biosynthetic/metabolic process	-13.46/-13.49
steroid biosynthetic/metabolic process	-12.26/-9.58

20uM Diosgenin, 24hr

Description	logP
cholesterol biosynthetic process	-10.76/-8.33
secondary alcohol biosynthetic process	-8.6
isoprenoid biosynthetic process	-5.28
carboxylic acid biosynthetic process	-1.87

10uM Panaxadiol, 24hr

Description	logP
regulation of immune system process	-4.93
DNA packaging complex	-3.24
protein heterodimerization activity	-2.97

20uM Panaxadiol, 24hr

Description	logP
carboxylic acid biosynthetic process	-4.76
Steroid biosynthesis	-3.81
isoprenoid biosynthetic process	-3.06
cholesterol biosynthetic process	-2.66

10uM Panaxadiol, 48hr

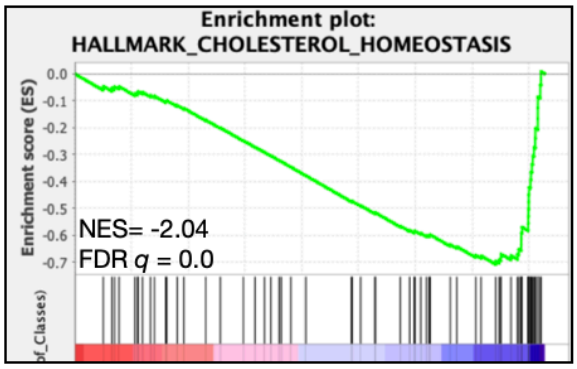
Description	logP
developmental process	-6.59
nervous system development	-4.10
protein-DNA complex assembly	-3.21
MAPK cascade	-2.59

20uM Panaxadiol, 48hr

Description	logP
protein-DNA complex assembly	-7.88
developmental process	-7.24
cell differentiation	-4.21
nervous system development	-2.79

B.

10uM Diosgenin, 24hr



20uM Diosgenin, 24hr

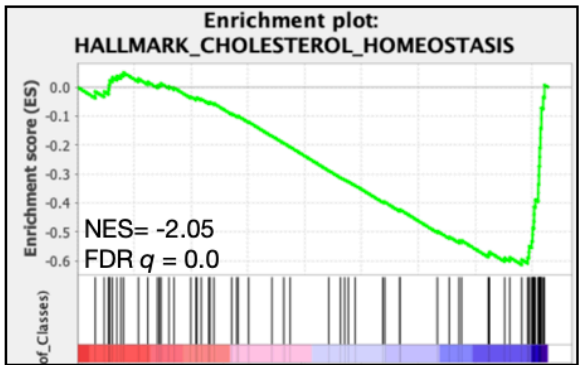


Fig. 8: *A549 RNA-seq data analysis.* ***A.*** *Gene ontology analysis of the downregulated gene expression (1.5 fold) in A549 cells treated with diosgenin an panaxadiol as indicated.* ***B.*** *GSEA plots depicting the enrichment of genes downregulated (1.5-fold) in A549 cells treated with diosgenin and panaxadiol, as indicated for 24hr and 48hr. FDR false-discovery rate.*

A.

10uM Diosgenin, 24hr

Description	logP
regulation of cell cycle	-11.44
cellular developmental process	-9.76
cell differentiation	-8.22
neurogenesis	-5.59

20uM Diosgenin, 24hr

Description	logP
DNA metabolic process	-62.05
DNA repair/replication	-45.67/-44.59
chromatin binding	-18.29

10uM Diosgenin, 48hr

Description	logP
nuclear division	-15.39
DNA metabolic process	-12.35
cellular response to DNA damage stimulus	-5.99
central nervous system neuron development	-1.06

20uM Diosgenin, 48hr

Description	logP
DNA metabolic process	-12.58
DNA repair	-11.88
nervous system development	-7.73
transmission of nerve impulse	-1.61

10uM Panaxadiol, 24hr

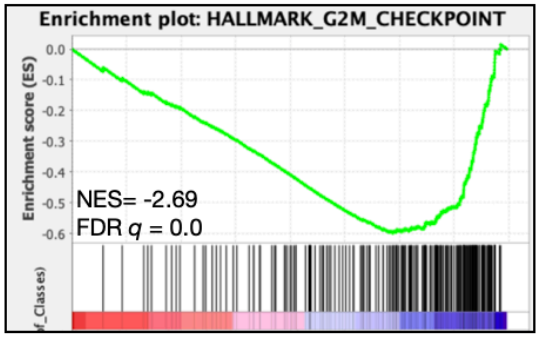
Description	logP
neuron projection guidance	-5.40
developmental process	-5.38
nervous system development	-4.85
axon development	-3.36

20uM Panaxadiol, 24hr

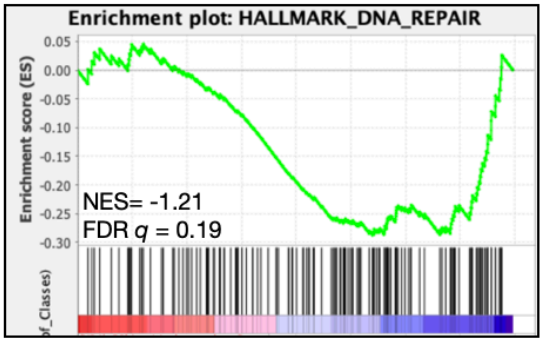
Description	logP
developmental process	-6.41
neurogenesis	-6.24
cell cycle checkpoint	-5.44
cell differentiation	-5.26

B.

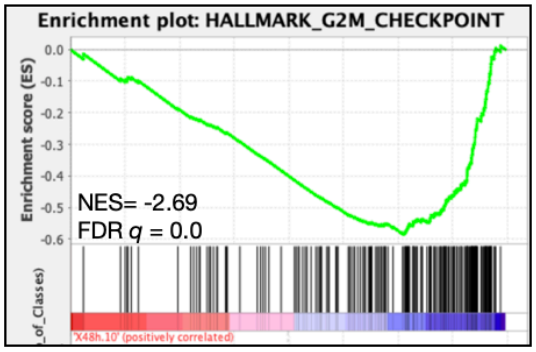
10uM Diosgenin, 24hr



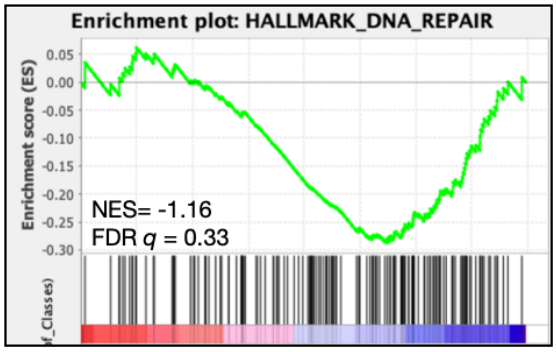
20uM Diosgenin, 24hr



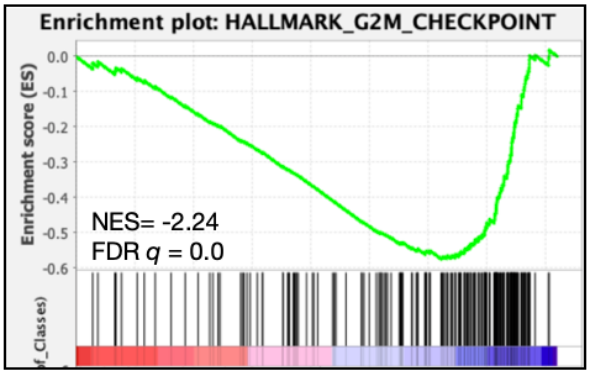
10uM Diosgenin, 48hr



20uM Diosgenin, 48hr



20uM Panaxadiol, 24hr



CHAPTER 5: DISCUSSION

My research presented here offers an initial investigation into the effects of diosgenin and panaxadiol on various cancer cell lines including TNBC (MDA-MB468, HCC70), prostate cancer (C42B), and lung cancer (PC9, A549). In my view, this study contributes significant insights into the pharmacological potential of these compounds, their dose-dependent cytotoxic effects, and the cellular pathways impacted by their activity.

The cell imaging results show that both diosgenin and panaxadiol inhibit cell growth in the TNBC lines MDA-MB468 and HCC70, even at a relatively low concentration (2.5 μM). Interestingly, while the cells retained their original morphology, their growth was significantly hindered. This suggests that the cytostatic effects of these compounds precede any cytotoxic or morphological changes. As the concentration increased, more pronounced effects were observed, including marked morphological changes in MDA-MB468 cells and the onset of cell death in HCC70 cells. This observation underscores the importance of dose in evaluating the efficacy of potential therapeutic agents.

The colony formation assays provided further insights into the inhibitory effects of diosgenin and panaxadiol. The remarkable decrease in colony formation in MDA-MB468 and C42B cell lines following diosgenin treatment highlights its potency in limiting not only cell growth but also cell survival. In contrast, panaxadiol showed a somewhat less inhibitory effect on the cells' ability to form colonies and proliferate uncontrollably. The

differences in response to the two compounds could point towards different mechanisms of action, possibly involving distinct molecular targets.

The time-dependent cell proliferation assay also revealed important dynamics of the effects of diosgenin and panaxadiol. diosgenin treatment led to a notable decrease in cell growth over time, although the A549 cell line showed an anomalous response. This could be due to experimental errors or a unique response mechanism in this particular cell line, warranting further investigation.

Both diosgenin and panaxadiol displayed significant time- and dose-dependent inhibitory effects on cell growth and proliferation in the various cancer cell lines. These compounds showed the most pronounced effects at higher concentrations and longer treatment durations, underscoring the importance of both dose and treatment duration in their therapeutic efficacy.

Further examination of the effects of diosgenin and panaxadiol on gene expression provided intriguing indications of their potential mechanisms of action. Both compounds seemed to downregulate genes associated with cholesterol biosynthesis and metabolism, a cellular process previously associated with cancer progression. More importantly, these findings suggest that the two natural compounds act through the inhibition of RORγ function in the cancer cells. This is because previous studies in our laboratory have established that cholesterol biosynthesis falls under the control of RORγ. However, additional studies are necessary to establish this reasoning definitively.

Notably, diosgenin appeared to have a more potent effect on cholesterol biosynthesis than panaxadiol, potentially due to variations in their chemical structures or modes of action. Furthermore, panaxadiol seemed to affect the Wnt signaling pathway in HCC70 cells at lower concentrations, suggesting a possible secondary mechanism of action that merits further exploration.

The role of RORγ in mediating cholesterol biosynthesis raises the possibility that both diosgenin and panaxadiol could act through this pathway. While the current study does not provide conclusive evidence for this, it certainly paves the way for future investigations into this potential mechanism of action.

Interestingly, different pathways were affected in different cell lines and at different treatment durations, pointing to a potential multi-targeted effect of these compounds. This could have therapeutic advantages as it could counteract the adaptability of cancer cells, a key challenge in cancer therapy.

In conclusion, my research offers valuable insights into the anticancer potential of diosgenin and panaxadiol, revealing their dose- and time-dependent cytotoxic effects, as well as the pathways they impact. Although these findings are promising, further studies are necessary to uncover their exact mechanisms of action and their therapeutic potential in vivo. As the compounds seem to have multi-targeted effects, future studies should also consider combinatorial therapies that could synergize with diosgenin or panaxadiol to provide a more effective cancer treatment.

CHAPTER 6: REFERENCES

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