# “COMPARISON OF EFFECT OF BONE MORPHOGENETIC PROTEIN (BMP) AGONIST SB4 & ITS COMBINATION WITH STANDARD CARE DRUGS ON PROSTATE CANCER CELL LINES - AN IN-VITRO STUDY”

By
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# ABSTRACT

**Introduction:** Prostate cancer is the second most common cancer among men throughout the world. Metastasis of the disease poses a difficult challenge, this arises from the resistance to chemotherapy and radiotherapy developed by a subpopulation of cancer cells. One approach to overcome this, dictates that these resistant cells could be converted into more sensitive cells by inducing differentiation by utilizing the BMP signaling pathway. Although, not many studies have been carried out considering BMPs as therapeutic agents due to its high cost. Recently, one small molecule compound known as Sb4 has shown its effectiveness as a BMP agonist. This study aims to assess the effects of Sb4 on DU 145 prostate cancer cell line.

**Methods:** This study conducted MTT based cytotoxicity assays and Migration assay to assess the cytotoxic concentrations of Sb4 and its combination with Docetaxel on DU 145 cells as well as determining whether Sb4 treatment has any effect on the cellular motility and migratory capability of DU 145 cells.

**Results:** The results showed that the cells treated with Sb4 (1μM) followed by Docetaxel (10.78 nM) showed 35.44% cell viability compared to 45.97% viability when treated with Docetaxel (10.78 nM) alone, but the cells treated with Sb4 (100nM) showed increased cell viability (54.17%) ($F=12.63$, p-value= $<0.01$). The migration assay showed that when treated with Sb4, the wound closure was 73.62% compared to 96.58% in negative control ($F=3.28$, p-value=0.021).

**Conclusion:** In conclusion, the effect of high concentration Sb4 is tumor suppressive and low concentration is tumor promoting. Further studies with Sb4 need to be carried out to understand

the intricacies of the mechanisms as well as gather evidence on its mechanism of action in prostate cancer.

# Introduction

Prostate cancer is the second most common cancer among men throughout the world. It comprises 13.3% of all new cancers diagnosed among men.[1] This high incidence rate could be due to a legitimate increase in disease incidence or it could reflect more widespread awareness among the population and better screening protocols regarding prostate cancer in asymptomatic men in recent years. Asian countries, including India, have comparatively lower incidence rates for prostate cancer. This incidence has been rising in the past decade.[2-3] Prostate cancer comes as the third most common cancer site among men, following lung and oral cancer in India.[4]

In the past, it was believed that cancer was a homogeneous mass of rapidly proliferating cells, and therapies were created to eradicate these cells. Recent research, however, has revealed that the differentiation and proliferation rate of cancer cells vary. This phenomenon, described as the presence of cell subpopulations with distinct phenotypes and genotypes that may display different biological behaviors, is called tumor heterogeneity. Cancer stem cells (CSCs), a small percentage of cells with the capacity to initiate and sustain cancer growth, are a major contributor to the heterogeneity of a tumor.[5]

Much like Adult stem cells, CSCs exhibit asymmetrical division through this, they show the ability to self-renewal and to give rise to proliferating cells as well as differentiated cells. Despite being a small population, CSCs are known to be very important in the development of many types of cancers. Additionally, they are extremely resistant to conventional chemotherapy and radiotherapy, which results in recurrence of the tumor. Furthermore, CSCs are thought to be a cause of local invasion and metastasis, leading to tumor recurrence and the difficulty of obtaining a full recovery.[6]

Once it was discovered that patients with metastatic Prostate Cancer (mPC) experienced considerable tumor regression following surgical castration, androgen deprivation therapy (ADT) became the standard treatment for mPC patients. Most patients responded well to ADT, still, castration-resistant PCs (CRPCs) frequently occur. The median overall survival of mCRPC was shown to be extended by almost three months with Docetaxel-based treatment.[7] The presence of surface markers such as CD133 and CD44 on cells can be used to identify and isolate the CSCs from the rest of the cancer population. These CSCs with stem cell like properties have been identified in various cancers, there is also evidence for their presence in prostate cancer. The overwhelming body of evidence points to the critical role that CSCs play in the initiation, metastasis, and resistance to treatment of prostate cancer.[8]

Docetaxel is the standard care treatment which can be used in case of castration-resistant prostate cancer. Docetaxel is a second-generation chemotherapeutic agent. It is a member of the taxane family with paciltaxel. The primary mechanism of action of Docetaxel is to bind with β-tubulin which is a component of the cytoskeleton. This stops the cell cycle during G2/M by preventing microtubules from properly assembling into the mitotic spindle. Additionally, Docetaxel reduces the expression of the anti-apoptotic gene BCL2, which is frequently overexpressed by cancer cells.[9] There are many possible mechanisms through which a cancer stem cell may achieve resistance to many chemotherapeutic agents including Docetaxel, these mechanisms include ATP-Binding cassette transporters which are also known as drug efflux pumps which act to reduce the drug concentration in the intracellular environment, alteration in substrate structure like microtubules (β-tubulin), upregulation of BCL2 gene expression and other anti-apoptotic pathways.[9-10]

Due to their tumorigenic and treatment resistant nature, CSCs are believed to be a promising target for new therapies intended for eradication of cancer. Apart from substances that specifically target CSCs elimination, medications that cause these cells to undergo terminal differentiation or that cause them to differentiate into non stem cells that are susceptible to traditional anticancer therapies is known as “Differentiation therapy”. Targeting the CSCs by this approach to differentiate them into more sensitive types of cancer cells which can then be targeted by standard chemotherapy drugs has a higher chance of bringing about complete remission.[11]

There are many known differentiation inducing compounds such as retinoic acid, dimethyl sulfoxide, cAMP, TGF-β and sodium butyrate. Most of these compounds act by activating the signaling pathways necessary for expression of genes related to regulation of cell cycle and differentiation. In addition to being a potentially effective treatment, differentiation-inducing therapy may also help pinpoint the underlying cause of tumor heterogeneity. Early studies have shown the promise of differentiation in cancer treatment and have discovered a number of differentiation-related targets and markers, providing a basis for developing a number of differentiation-inducing therapies.[12]

Retinoic acid is a derivative of vitamin A, it is widely recognized for its potential for inducing differentiation in normal stem cells. The transcription of genes which regulate differentiation is triggered by the interaction between retinoic acid and the retinoic acid receptor (RAR), which forms a heterodimer with retinoid X receptor (RXR) and binds to the retinoic acid response element (RAREs) on DNA. RA plays a critical role in intercellular communication throughout the early stages of vertebrate organogenesis.[6] Patients with acute promyelocytic

leukemia (APL) currently receive differentiation treatment with tretinoin (All-trans Retinoic Acid), which is a pharmaceutical version of RA.[13] All-trans Retinoic Acid (ATRA) has also proven to be effective in inducing differentiation in-vitro studies against glioma,[14] non-small cell lung adenocarcinoma,[15] breast cancer,[16] cervical cancer,[17] and prostate cancer.[18] In addition to inducing differentiation, at high concentrations, retinoids including All-trans retinoic acid have direct cytotoxic effects as well.[19] The efficacy in human clinical trials for prostate cancer has not yielded positive results.[20]

The most potent differentiation inducer is Bone Morphogenetic Protein (BMP) signaling. BMPs are members of transforming growth factor beta (TGF-β) superfamily. The majority of genes that BMP signaling either activates or suppresses are terminal differentiation regulators. BMP signaling stimulates neural stem cell proliferation early in embryonic development. Later on, BMP signaling causes neural precursors to differentiate and stops their proliferation. In the case of malignancies, either tumorigenesis in the original tumor sites or cancer cell colonization in the metastatic locations is promoted by compromised BMP signaling or overexpression of BMP antagonists.[6]

However it is observed that the effect of BMPs on both proliferation and differentiation can cause proliferation of tumor cells but it can also cause differentiation of CSCs which leads to tumor suppression, the effect of this phenomena varies amongst different cancer types.[22] Studies in the past have shown that BMPs have effects like tumor-proliferative effect on hepato-cellular carcinoma[23] and breast cancer[24], but tumor-suppressive effect on glioma[25] and prostate cancer.[26] However, the use of BMP signaling molecules is not economically feasible for therapeutic use.

In the context of kidney development, the significance of BMP signaling and its downstream signaling pathways in the kidneys was well known. In the mature kidney, BMP-7 has shown to play a critical role in regulating the cellular and biological responses to renal injury. Pathophysiology of chronic kidney disease (CKD) is also significantly impacted due to these functions. Recombinant BMP-7 has been shown in many studies to sustain kidney function in an acute ischemia injury model while inhibiting the pathophysiology of renal injury.[27]

Recently, for the purposes of harnessing the usefulness of BMP signaling in CKD, a strategy was proposed to use alternate small molecules which are easier to synthesise and more cost effective. A high-throughput screening was done to assess which compound activated BMP signaling and was able to increase phosphorylation of the secondary messengers of the cascade and expression of the downstream genes of BMP signaling as a result. 2-[[(4-Bromophenyl)methyl]thio]benzoxazole also known as Sb4 was found to be the most promising alternative for activation of the canonical BMP pathway and downstream gene expression.[28] In light of the effectiveness of Sb4, this study was done to determine whether a more cost effective compound is able to bring about the effects of BMP signaling like inducing differentiation and enhancing the effectiveness of chemotherapy in the setting of prostate cancer.

In order to test the compounds in an in-vitro setting, a continuous cell line of metastatic prostate cancer, DU 145 was selected. DU 145 is a cell line that originates from human prostate adenocarcinoma metastatic to the brain, it shows epithelial morphology, and it was derived from the brain of a 69-year-old prostate cancer patient. DU 145 requires a mixture of 90% Eagle's Minimum Essential Medium (EMEM) and 10% Fetal Bovine Serum (FBS), 2 mM L-glutamine, and 2.2 g/L sodium bicarbonate as growth medium. Environmental conditions for its growth are

5% $CO_2$ and 37℃ temperature. They have a population doubling time ranging from 30-40 hours.[29]

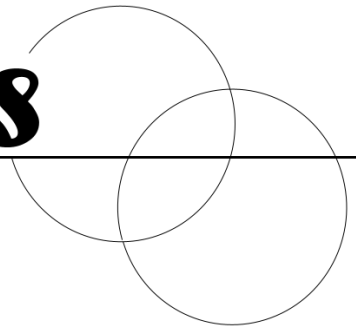
Cytotoxic effects of a drug on a cell line in-vitro can be assessed using a cytotoxicity assay like MTT assay. This assay utilizes reduction of MTT dye (3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide) to assess the viability of live cells. In live cells, MTT, a yellow water-soluble tetrazolium dye, is converted to a purple colored formazan product which is insoluble in water. Spectrophotometry can be used to measure the amount of MTT-formazan generated. The percentage of live cells directly correlates with the amount of Formazan generated. Thus, the number of surviving cells after treatment with a compound can be estimated.[30]

One of the most important processes in growth and metastases of cancer is cell migration. A simple, inexpensive, and repeatable technique for examining the migration of cancer cells in-vitro is the wound healing assay. It is based on the finding that, following the formation of an artificial wound, cells growing in a monolayer migrate to re-establish cell connections. Cancer stem cells are also implicated in cell migration, leading to metastasis. For the test wound is created in the monolayer of cells, and images are taken during wound closure, and the area of migration is compared with a negative control.[31]

Differentiation therapy with ATRA has shown that its effect on differentiation of CSCs can be augmented with standard anti-cancer agents for better results, similarly treating the cancer with BMP followed by an anti-cancer drug has also been proven to be effective in glioblastoma but the effectiveness of the BMP alternative such as Sb4 along with an anti-cancer drug hasn't been tested in prostate cancer cells as of yet. Therefore, the hypothesis of this study is that

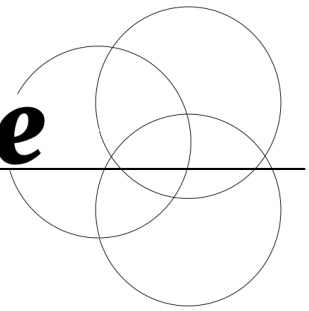
treatment of the prostate cancer cells with Sb4, will increase the effectiveness of the standard chemotherapeutic compound. Hence the current study will try to explore the effect of Sb4 and compare it with the effects of ATRA as well as their effects in conjunction with standard chemotherapeutic drug.

# Objectives



1. To assess the effect of Sb4 on prostate cancer cells (DU145)
2. To assess the cytotoxic effect of Sb4 - Docetaxel combination on prostate cancer cells (DU145)
3. To compare the cytotoxic effect of Sb4 - Docetaxel combination with All-trans retinoic acid - Docetaxel combination on prostate cancer cells (DU145)

# Review of Literature



## Prostate Cancer

Neoplasm which impacts the prostate is known as prostate cancer. A number of categories have been established to assess the prognosis and possibility for malignancy. In the Gleason scoring system, two histologic patterns are given numbers 1 through 5 (best to least differentiated) separately. The sum of these figures yields a total score ranging from 2 to 10. Highly differentiated cancers (Gleason scores 2 to 6) have the better prognosis as compared to the majority of poorly differentiated tumors (Gleason scores 7 to 10).[32]

The most prevalent non-skin cancer in males is now prostate cancer, surpassing lung cancer. Prostate cancer incidence rises with age. Due to widespread PSA testing, the lifetime risk of prostate cancer has risen to around 16%. The rate of new diagnoses of prostate cancer has also doubled. Additionally, the incidence of clinically "silent" tumors has grown from 17% in 1989 to 48% in 2001 with the introduction of PSA screening, and prostate cancer is detected sooner. Nowadays, around 80% of cases are classified as localized. The average age upon diagnosis is 72 years old. Prostate cancers can be passed down in families in around 9% of cases. One risk factor for prostate cancer is obesity. A diet heavy in fat and lacking in fiber raises the risk. Prostate cancer risk may also be raised by elevated insulin levels. Vitamin E dietary supplements have been shown to dramatically raise the probability of prostate cancer in otherwise healthy men. According to linkage studies, chromosome 17p21-22 may include a gene that predisposes individuals for prostate cancer. A markedly elevated risk of familial prostate cancer is linked to germline mutations in HOXB13 gene. Prostate cancer mortality rates have significantly decreased over the last 15 years, from 34% in 1990, to less than 20% at now.[32]

Earlier stages of the disease are typically silent. Prostate cancer may first manifest as bone discomfort and pathologic fractures. Micturition blockage symptoms may be brought on by local development. 10% of patients will have a negative digital rectal examination (DRE), which may show an area of increased hardness. The prostate may be hard, fixed, or in advanced stages, the tumor may have spread to the seminal vesicles.[32]

The diagnosis includes physical examination, in digital rectal examination (DRE) Prostate size, consistency, and anomalies inside or outside the gland are the main concerns. Numerous malignancies may be felt on DRE and are found in the peripheral zone. Carcinomas are characterized by their hardness, nodules, and irregularities; calculi or benign prostatic hyperplasia (BPH) can also cause induration. Prostate cancer affects 20–25% of males with an irregular DRE overall.[33]

The kallikrein-related serine protease known as prostate specific antigen (PSA) (kallikrein-related peptidase 3; KLK3) liquefies the seminal coagulum. It is specific to the prostate rather than prostate cancer since it is generated by both malignant and nonmalignant epithelial cells. Prostate cancer, BPH, or prostatitis can all cause a rise in PSA serum values. PSA in circulation is inactive and mostly exists as free PSA alongside the protease inhibitor α1-antichymotrypsin. Glomerular filtration rapidly eliminates free PSA from the blood, having a half-life of 12–18 hours.[33]

The PSA test's widespread application has contributed significantly to the percentage of men who receive an early-stage cancer diagnosis: between 70 and 80 percent of newly detected malignancies are clinically organ-confined. The risk and prognosis of prostate cancer are closely linked to the blood level of PSA. The lifetime risk of surviving from prostate cancer is linked to

a single PSA taken at age 60. Only a small percentage of men with PSA levels within the top quartile (>2 ng/ml) may develop deadly prostate cancer, yet the majority of prostate cancer deaths (90%) happen to patients having PSA levels >2ng/ml. Despite this, and the mortality rate declines reported in major randomized prostate cancer screening trials, everyday use of the test is still contentious.[33]

Over time, the PSA standards for recommending an exploratory prostate biopsy have changed. But according to the standard cut off for prostate biopsies (a total PSA ≥4 ng/ml), the majority of men with elevated PSA do not exhibit histologic indications of prostate cancer upon biopsy. Prostate cancer cells are also present in many men whose PSA levels fall below this threshold. Even at normal PSA value, one can not completely rule out the risk of prostate cancer, according to data provided by the Prostate Cancer Prevention Trial.[33]

The diagnosis can be confirmed with an ultrasound-guided transrectal biopsy and prostate fine-needle aspiration. An elevated PSA level, an abnormal DRE, or a prior biopsy specimen demonstrating prostatic intraepithelial carcinoma or prostatic atypia are among the indications for a biopsy.[32] MRI can be used to help decide whether to do biopsies in men with increased PSA levels during prostate cancer screening. Additionally, MRI makes targeted biopsies of uncertain locations easier.[34] However, the risk of overdiagnosis is cut in half when MRI-directed targeted biopsy is used instead of systematic biopsy in screening and early detection in individuals with increased PSA levels. This comes at the cost of a small proportion of patients' intermediate-risk malignancies being identified later.[35]

## Treatment Strategies for Prostate Cancer

Patients having localized prostate cancer are typically considered for radical prostatectomy. Radical prostatectomy lowers the risk of metastasis and local progression, as well as disease-specific and total mortality.[32] With the emergence of techniques such as nerve-sparing radical prostatectomy, the open radical prostatectomy has established itself as the gold standard for the management of confined prostate cancer by delivering robust long-term disease control and ever-improving side-effect profiles. All of the new information on robotic radical prostatectomy and minimally invasive laparoscopic prostatectomy aims to build on this advancement in the treatment of prostate cancer by reproducing the results achieved with the open approach.[36]

At the University of Chicago in the 1940s, Drs Huggins and Hodges established the role of androgens in prostate cancer. They discovered that hormone signaling regulates prostate cancer cell proliferation and dissemination. Huggins and Hodges received the Nobel Prize in 1966 for the discovery that prostate cancer is androgen-dependent. This weakness in prostate cancer was utilized throughout half a century. Androgen deprivation therapy (ADT) is still the cornerstone of advanced prostate cancer treatment. More than 70 years following the discovery of prostate cancer's androgen dependence, ADT is still an essential component of many prostate cancer therapies.[37]

Historically, chemotherapy was thought to be ineffective for metastatic castration-resistant prostate cancer (mCRPC), with a rate of response of less than 20% and no substantial influence on overall survival (OS). Surprisingly, in the recent decade, a few chemotherapy drugs have exhibited improved OS, positioning chemotherapy as a common

treatment choice in advanced cancer.[38] Radiation therapy is still the best option for both curative and palliative treatment of confined malignancies. Treatment procedures have developed, and this field stands to benefit from rapid technological advancements.[39] More details regarding radiotherapy and chemotherapy in prostate cancer will be reviewed in later sections.

## Tumor Heterogeneity and Cancer Stem Cells

When in regards to morphology, cell surface markers, genetic mutations, cell proliferation kinetics, and responsiveness to treatment, tumors show a great deal of variation. All of these characteristics can also be heterogeneous within a single clonal tumor (i.e., originating from a single cell). Single-cell investigations have demonstrated the existence of variability in genetic and epigenetic anomalies among various cells despite the fact that individual cells in a tumor all share similar genetic anomalies suggestive of their clonal source. For many years, cancer researchers have been interested in the fundamental issue of the cellular and molecular foundation of this heterogeneity. According to one theory all tumor cells should be physiologically identical, but heterogeneity arises from internal or extrinsic factors that produce random reactions.[40] The tumor could also be an aberration of healthy tissue growth, maintaining a hierarchical structure with cancer stem cells at the top.[41]

Cancer stem cells (CSCs) diverge from the majority of the tumor cells and are responsible for maintaining tumor growth in a number of cancers.[42] Although the evidence is stronger for acute leukaemias research has found that CSCs are present in an increasing variety of solid tumors, such as the brain, breast, and colon.[8] The development and assessment of successful anticancer treatments, as well as researchers attempting to comprehend the processes

behind tumor beginning and progression, are possibly going to be altered by this conceptual change. Although there is currently not much therapeutic significance for CSCs outside of experimental models, the high rate of relapse following traditional cytotoxic chemotherapies indicates that CSCs are likely to survive standard treatments.

Since the late 1800s, human trials have been conducted in which tumors were removed, single-cell suspensions were prepared, and various cell dosages were auto transplanted into the thigh.[43] These investigations demonstrated that tumor reinitiation was uncommon, varying, and frequently required more than $10^6$ cells. The ability of malignant teratocarcinoma cells to spontaneously develop into mature benign cells was finally demonstrated in 1960.[44] Therefore, similar to how normal tissue development takes place, a tumor can be thought of as a hierarchy characterized by a maturation process. The theory that neoplastic cells represent distortions of normal development and that this maturation process may be used as a therapeutic tool was put forward by Pierce and Speers in 1961.[45]

The cytokinetic investigations were initially conducted in cell lines and mouse models of acute leukemias. It was evident from the in vivo results that most leukemic blasts were postmitotic and required constant replenishing from a very small proliferative proportion.[46] In vivo, only around 5% of leukemic blasts were cycling quickly. Nevertheless, a thorough examination revealed that patients had two proliferative fractions: a smaller, slow-cycling fraction, and a bigger, fast-cycling subgroup with a 24-hour cell cycle time. Because of their similar cytokinetic characteristics to those of normal hematopoietic stem cells (HSCs), it was concluded that the slow cycling fraction was actually giving rise to the fast cycling fraction and proposed that these represented a leukemic stem cell (LSC) population.[47] However, the investigators were unable to establish if the cycling fractions were representative of a single population with cells that were either proliferative or nonproliferative, or if these findings indicate a hierarchical relationship between two functionally different cell types. However, based

on these findings, it was suggested that the failure of antiproliferative chemotherapies, which were the primary chemotherapeutic strategy at the time, was due to the inability to eliminate these LSCs.[48]

These rapidly proliferating leukemic cells (AML-colony-forming unit [CFU]) from various patients can be divided into three categories: primitive (similar to multipotent normal CFU), early lineage committed (similar to day 7 CFU-GM), late blasts (similar to normal day 14 CFU-GM). Leukemic cells within a single colony may be more differentiated than the particular AML-CFU that gave rise to the colony. AML-CFU's quantity did not predict clinical disease characteristics or response to treatment but the secondary recurrence post treatment had a higher predictive potential.[49]

Histopathologically, a prostate can be graded according to Gleason score as a high grade tumor, which is known to contain poorly or undifferentiated cells which are more aggressive and pose a higher risk, while lower grade tumors tend to be well differentiated and have relatively better prognosis. This grading could be related to the process of dedifferentiation, where tumor cells lose their specialized characteristics and adopt less differentiated phenotypes suggestive of early embryonic development or regenerative processes. Resistance to treatment and enhanced tumor cell invasiveness are known to be linked to loss of differentiation.[50] Studies have found that many high grade tumors have higher prevalence of poorly differentiated, highly proliferative cancer stem cells.[51-52]

## Tumorigenesis and Metastasis of Prostate Cancer

Human prostate stem cells express the cell surface marker CD133. In 2005, primary human Prostate cancer (PC) was used to identify prostate cancer stem cells (PCSCs). Using the sphere formation assay and cell surface markers, PCSCs have also been detected in PC cell lines.

CD44+ cells from continuous human prostate cancer cell lines were more carcinogenic and showed increased expression in stemness genes.[53,54] PCSCs can also be isolated from DU145 cells using sphere formation assays. In NOD/SCID mice, these PCSCs demonstrated a 100-fold increase in tumor initiation.[55]

The prostate is composed of two layers of epithelial cells: the luminal and basal. While luminal cells are typically thought to be differentiated cells with minimal stem/progenitor cell capability, basal cells—similar to the basal cells in the mammary gland—are the primary source of prostate stem cells (PSCs). The profile of human PCs is similar to that of luminal cells, indicating that the target cells for neoplastic transformation are the luminal epithelial cells.[56] The fact that the great majority of PC cells have a profile similar to luminal epithelial cells does not rule out basal epithelial cells as a potential PC origin, given that PCSCs make up just a small percentage of PC cells and are AR-negative. PSCs with CD133+ surface antigens are found in the basal layer of the human prostate. The idea that aberrations in PSCs eventually result in PCSCs that start PC is supported by accumulating data. Glioblastoma also needs tissue stem cells, and the elimination of Nestin+ CSCs leads to the suppression of glioblastoma.[57] The findings also confirm the claim that Lgr5+ intestinal stem cells are the source of colorectal cancer.[58]

The process by which tumor cells migrate from their original location and develop into secondary tumors at a different location is known as tumor metastasis. It has been demonstrated that in epithelium-derived cancer, a process known as the epithelial-mesenchymal transition (EMT) is essential for promoting metastasis. A group of essential transcription factors that carry out EMT, such as SNAIL, TWIST, and ZEB, are at the core of EMT.[59] The irregular gene expression of either TWIST1 or SNAIL in human mammary epithelial cells (HMECs) triggers

EMT; the resulting HMECs exhibited characteristics of breast epithelial stem cells, including the formation of mammospheres, the expression of the $CD44^{high}CD24^{low}$ antigen profile.[60] ZEB1 deletion prevented stemness and metastasis in the murine model of pancreatic cancer.[61] There is evidence that TWIST1 has a role in bone metastases in PC.[62]

## Resistance to Treatment in CSCs

Drug resistance develops as a result of resistance of CSCs to DNA damage and their high expression of transport and anti-apoptotic proteins.[63] Drug resistance is also believed to be linked to angiogenesis and the epithelial–mesenchymal transition (EMT).[64] Drug resistance in CSCs enables them to withstand prolonged therapy and encourages distant metastasis and subsequent tumor recurrence. Several studies on solid tumors have verified this process.[65]

CSCs have a number of traits that give them an innate ability to withstand and adjust to environmental disturbances. ATP-Binding Cassette (ABC) transporters are expressed in greater quantities in CSCs.[66] By exporting a wide range of harmful medicines from cells, these proteins have been shown to contribute to the multidrug resistant phenotype.[67] Given that the majority of ABC transporters directly contribute to the occurrence of resistance and that resistance is reversed when their efflux activity is reduced, they may be regarded as oncogenic proteins.[68]

Hypoxia is a critical element in modulating the microenvironment and medication resistance. Hypoxia activates multiple signaling pathways, including hypoxia-inducible factor-1α and 2α (HIF1α, HIF2α) and the PI3K/AKT pathway, which binds to promoters that contain the hypoxia-response element (HRE). The PI3K/AKT pathway further activates HIF1α and HIF2α,

promoting CSCs through a positive feedback loop. The activation cascade induces stemness and resistance to chemotherapy.[65]

CSCs may circumvent apoptotic signals via numerous mechanisms, like the following: i) an altered state in the regulation of the cell cycle; ii) an imbalance in the proteins that induce apoptosis (Bax, Bak, Bid, Bik, Noxa, and Puma) and anti-apoptotic (Bcl-2, BCLXL, and Mcl-1); iii) the downregulation of death receptors and the upregulation of c-FLIT; iv) an increase in the production of inhibitors of apoptosis family proteins (IAPs); and v) an absence of mitochondria-mediated apoptosis. Furthermore, the fact that CSCs have an extended G2/M phase throughout the cell cycle, which leads to increased expression of checkpoint proteins such as CHK1 and CHK2 allows them to repair DNA damage which might otherwise result in apoptosis.[65]

Increasing evidence suggests that CSCs are the primary cause of cancer radioresistance in a large number of tumor types. Furthermore, it has been found that a tumor's radioresistance is determined by the quantity of CSCs present in the tumor.[69] As a result, it can be hypothesized that CSCs were responsible for radiation failure. CSC radioresistance is thought to be caused by a variety of reasons. The radioresistance of human pulmonary CSCs was shown to be caused by altered expression of DNA repair genes, helping to improve double-strand break repair.[70] A study has also found that breast CSCs have superior ability to eliminate the free radicals compared to non-tumorigenic cells due to enhanced production of free radical removal systems, which may lower reactive oxygen species-mediated damage to DNA and cell death following radiation.[71]

## Chemotherapy and Radiotherapy for Prostate Cancer

Docetaxel is the standard choice for therapy for metastatic castration-resistant prostate cancer. Docetaxel has exhibited objective response rates (ORR) in up to 38% of patients, a PSA reduction of more than 50% in up to 69% of patients, and a median overall survival of 20 months to 23 months. These findings independently verified that Docetaxel treatment enhanced overall survival in mCRPC.[38]

Docetaxel is a semisynthetic taxane that is thought to act via two different mechanisms of antitumor action. First, it inhibits microtubule depolymerization. Second, Docetaxel has been shown to reverse the effects of bcl-2 and bcl-xL. Docetaxel binds to β-tubulin to promote polymerization, which is the most commonly acknowledged mechanism of action, known as microtubule stabilization. Under normal circumstances, guanosine triphosphate (GTP), which binds with β-tubulin, and microtubule-associated proteins cause microtubules to polymerize (Fig 3.1). However, it is known that taxanes preferentially bind to β-tubulin, causing microtubule

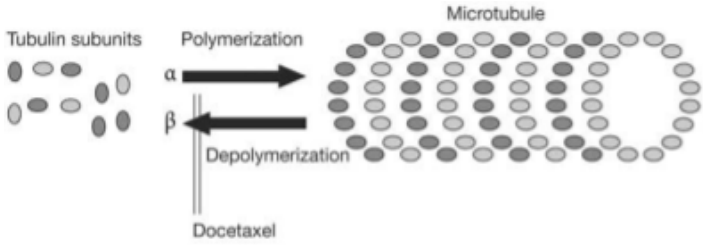



Fig 3.1: Mechanism of action of Docetaxel on β-tubulin[72]

formation even without the presence of GTP or other cofactors. When taxanes bind microtubules, they cannot be broken down, not even at 4°C or with $Ca^{+2}$ ions present (two common in vitro techniques for microtubule depolymerization). Apoptosis results from this static polymerization, which interferes with the regular mitotic process and usually stops cells in the G2/M phase of the cell cycle.[72]

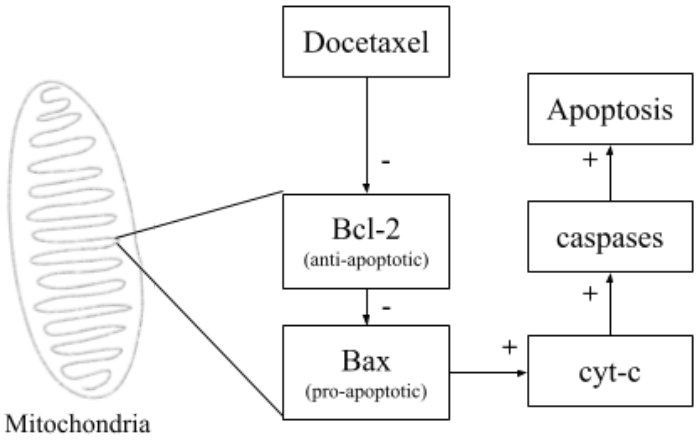



Fig 3.2: Mechanism of action of Docetaxel on Bcl-2

Docetaxel is known to cause cell arrest during the G2/M phase of the cell cycle. Another apoptotic indicator, bcl-2 phosphorylation, is also seen in cells that experience this cell cycle termination. The bcl-2 gene belongs to a group of oncogenes that increase tumor cell survival by preventing apoptotic cell death, hence promoting the development of cancer. As part of the G2/M interaction, bcl-2 is routinely phosphorylated. Increased apoptosis results from the taxanes' deactivation of bcl-2 by phosphorylation, which forces the caspase cascade to continue activating. Physiologically, Bcl-2 dimerizes with the proapoptotic protein bax and then stops it from activating the caspase cascade. Overexpression of bcl-2 can also protect prostate cancer cells from apoptosis following androgen depletion (Fig 3.2).[72]

Radiation treatment was utilized to treat cancers within a short time after X-rays were discovered in 1895. By 1902, Emil Grubbe published an article summarizing his own and hundreds of other writers' findings on the use of radiation therapy to treat cancer. Over a hundred years later, these principles still hold true in the treatment of prostate cancer. Radiation therapy utilizes ionizing radiation to cause damage to the molecules of living tissues. Ionizing radiation

is described as a radiation with enough energy to remove the electrons from the orbit of an atom, leading the atom to be ionized. Ionizing radiation includes alpha and beta particles, gamma-rays, X-rays, and shortwave UV light.[39]

Individual charged elements, like neutrons, electrons, protons, and heavy-charged ions with sufficient kinetic energy, can directly disturb the atomic structure of the substance being targeted, causing significant damage. Radiation's biological impacts include DNA damage, which is a major target in cells. X-rays and gamma-rays can directly interact with DNA. Thus, these beams may ionize or excite atoms in the target, resulting in a biological effect. They may cause DNA lesions such as single-strand breaks in phosphodiester bonds, base damage, double-stranded breaks, protein-DNA crosslinks, and protein-protein crosslinks. Radiation therapy can also be utilized for palliation.[39]

## Chemoresistance and Radioresistance in Prostate Cancer

There are many mechanisms by which metastatic castration-resistant prostate cancer becomes resistant to chemotherapy as well as radiotherapy. Docetaxel binds to β-tubulin, which is integrated into cytoskeletal microtubules in the G2/M phase of the cell cycle. This inhibits microtubule disintegration and causes cell death in dividing cells.[73] Docetaxel's efficiency can be hindered because of significant affinity for P-glycoprotein. P-glycoprotein is an ATP-dependent drug efflux pump that reduces Docetaxel's intracellular concentration, hence inhibiting its activity.[74] P-glycoprotein is a member of the ATP-binding cassette transporter family of proteins.

It has been demonstrated that cancer cells that produce P-glycoprotein develop resistance to both Docetaxel and paclitaxel.[75]

Another cause of Docetaxel resistance is alterations in the structure or function of microtubules. β-tubulin mutations that impact Docetaxel binding, elevated total cellular β-tubulin content, changed expression of β-tubulin isotypes (e.g., overexpression of βIII-tubulin), and post-translational β-tubulin modifications are examples of structural alterations that may result in taxane resistance.[76] Microtubule alterations brought on by interactions with other cytoskeletal elements (such γ-actin) or the overexpression of microtubule-associated proteins can also result in taxane resistance.[77]

Docetaxel can cause apoptosis by phosphorylation induced inhibition of the antiapoptotic protein Bcl-2 along with stabilizing microtubules.[78] However, Bcl-2 and secretory clusterin, another antiapoptotic cytoprotective protein, are overexpressed when prostate tumors are treated with taxanes. This reduces the efficiency of Docetaxel by preventing apoptosis.[79] Additionally, following taxane therapy, elevated expression of serine-threonine kinases (like Pim-1 kinase) and lipid kinases (like sphingosine kinase-1) has been observed, which further promotes the growth of prostate cancer cells.[80]

Prostate cancer cells have an exceptional capacity to repair the damage of the DNA from ionizing radiation and a low susceptibility to ROS-induced cellular damage. ROS are molecules that include oxygen and are chemically reactive. They include the hydroxyl free radical (HO•), hydrogen peroxide ($H_2O_2$), and superoxide ($O^{2-}$). They are produced as a byproduct of oxygen metabolism normally. Despite their brief lifespan, ROS interact quickly with a variety of intracellular biomolecules, including DNA.[81] More DNA damage is caused by ROS produced

within 2 mm of DNA than by direct ionization.[82] Stem cell activity also depends on keeping ROS at a low level.[83]

Through the adhesion molecule CD44 (also a widely used stem cell marker) the ROS defense mechanism is activated. By encouraging the production of intracellular glutathione, an antioxidant that lowers intracellular ROS levels[84]. The DNA repair proteins γ-H2AX, Ku70, and Ku80 are also highly expressed in the prostate cancer cells after administration of ionizing radiation, suggesting that these cells have a high potential to repair the damage to the DNA caused by ionizing radiation. According to earlier research, these resistant cells exist in a variety of tumor types and have effective DNA repair mechanisms.[85]

The features of CSCs which were described earlier, such as presence of abundant ATP-binding cassette transporter proteins, robust DNA repair mechanisms, increased expression of anti-apoptotic genes such as bcl-2, and expression of cell surface marker CD44 are described here as tools by which prostate cancer can achieve resistance to standard therapeutic measures.

## Inducing Differentiation

The stimulation of terminal differentiation of CSCs is included in the idea of CSC differentiation therapy. Additionally, it can cause them to transform into non-stem cells that are vulnerable to traditional anticancer therapies. A tumor's aggressive behavior and resistance to treatment may be suppressed or its malignant potential reduced by cancer stem cell

differentiation treatment. Additionally, it provides a therapeutic approach to eliminate cancer by reducing the number of CSCs.[11]

CD44, CD47, and CD133 are cell surface markers that are considered to be the primary targets for CSC differentiation. In mice with AML, treatment by using antibodies targeting CD44 dramatically reduced CSCs. Since CD47 is also prominently produced by acute lymphoblastic leukemia (ALL), it may be possible to target CSCs specifically in ALL for therapy. One cell surface marker for a variety of malignancies, such as glioblastomas, colon, and prostate cancers, is CD133. The prognosis for patients with elevated CD133 expression is poor. Targeting CSCs that express high amounts of CD133 has been the aim of many studies. Several cancers have been found to have aberrant activation of stemness signaling pathways, including Hedgehog, Notch, Wnt, JAK/STAT, RA, NF-κB, and BMP as well. Targeting CSCs may therefore be possible by inhibiting or resolving these aberrant stemness signaling pathways.[86]

Numerous studies have documented the use of CSCs to eliminate cancers through the regulation of stem cell signaling mechanisms and novel approaches to induce differentiation. Nonetheless, the majority of the research concentrated on employing chemical reagents to control the CSC signaling pathway. According to one study, inducing hepatocyte differentiation in mice inhibits the formation of hepatocellular carcinoma (HCC) tumors.[87] In preclinical studies of colorectal cancer, it was reported that anti-R-Spondin 3 antibody therapy reduced tumor development and caused loss of stemness by causing CSC differentiation, which eventually resulted in significant therapeutic response.[88]

## Physiological Role of Retinoic Acid

Numerous biological processes are regulated by retinoic acid (RA) in both developing and mature organisms. The ability of cells to convert Retinol in diet to Retinoic acid is necessary for retinoic acid signaling. Retinol is converted to retinal by retinol dehydrogenases, whereas retinal is converted to RA by aldehyde dehydrogenases. Certain cells secrete retinoic acid, which creates gradients that regulate nearby cells. The availability of vitamin A (retinol), the activity of the enzymes, and the catabolism of RA by CYP26 enzymes all affect the concentration of RA.[89]

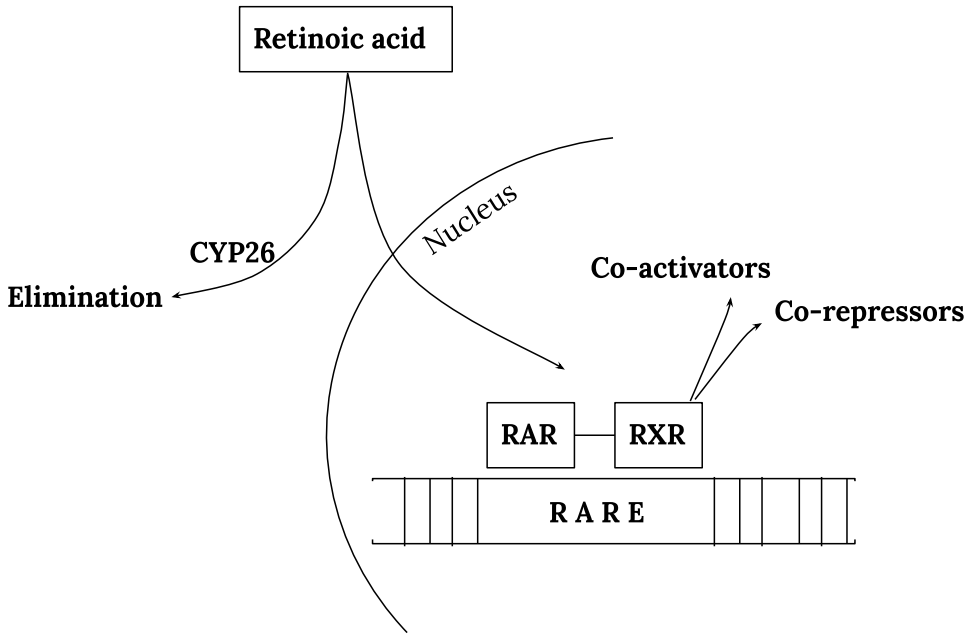



Fig 3.3: Mechanism of action of Retinoic acid

Retinoic acid influences transcription through its interactions with retinoid X receptors (RXR) and nuclear RA receptor (RAR) heterodimers attached to RA response elements (RAREs) in target gene promoters. RA can upregulate or downregulate the expression of more than 500 genes. When in the resting unbound state, the RAR–RXR heterodimer attracts co-repressors such as nuclear receptor co-repressor 1 and 2. These co-repressors then attract PRC2 and histone

deacetylase (HDAC) protein complexes. These lead to histone H3 lysine 27 trimethylation, which gives rise to chromatin condensation, and silences of the gene. When retinoic acid binds to RAR–RXR, the heterodimer undergoes a conformational change which allows co-activators such as nuclear receptor co-activator 1, 2, or 3 to replace repressive factors. Histone acetylase (HAT) complexes are recruited by these co-activators, and they mediate chromatin relaxation, and gene activation (Fig 3.3).[90]

Secretion of precise quantities of RA is known to be critical in many aspects of embryonic development such as proximal-distal patterning of limbs, cranio-caudal patterning of the body axis, and various organ development such as eye, brain, heart and liver.

## History of All-trans Retinoic Acid

In 1925, there were many conflicting reports regarding effects of Vitamin A deficiency in the various organ systems of the body, to rectify this an exhaustive study of effects of vitamin A deficiency was conducted on albino rats. The study had many conclusive findings, peculiar among them being the effects on epithelium. It was previously hypothesized that vitamin A deficiency could lead to diminution of proliferation of epithelium, but on the contrary the study found that the growth of the epithelium is greatly augmented. They also reported development of neoplastic changes such as increased mitotic activity and proliferation of vasculature.[91] Thus, at the time it was believed that vitamin A had a preventive role against these neoplasms.

In 1980, after the establishment of various cell lines, the supposed “anti-neoplastic” effects of vitamin A and its derivatives were assessed on HL-60 cell lines (derived from a patient

with acute promyelocytic leukemia). This study found that retinoic acid was effective in converting the proliferating HL-60 leukemic promyelocytes into terminally differentiated, functionally mature granulocytes.[92]

In 1988, a clinical trial was carried out to estimate the efficacy of the pharmaceutical form of retinoic acid known as all-trans retinoic acid (ATRA) in patients with acute promyelocytic leukemia (APL). The study included newly diagnosed patients as well as patients with resistance to chemotherapy. The study concluded that ATRA could be used in treatment to achieve complete remission.[13] In the following years it was discovered that treatment of APL with ATRA in conjunction with standard chemotherapy is more efficacious compared to ATRA monotherapy.[93] The trend of ATRA along with chemotherapy in APL treatment is followed to this day.

## ATRA as an Anti-cancer Drug

Over the past decades, a growing number of pre-clinical investigations on various solid tumors have been carried out. In glioma,[14] non-small cell lung adenocarcinoma,[15] breast cancer,[16] cervical cancer,[17] ovarian cancer,[94] hepatocellular carcinoma,[95] and prostate cancer[18] these studies show that ATRA can reduce the aggressiveness of the cancer cells and enhance the benefits of chemotherapy. This has made ATRA a standard for differentiation therapy research in a number of cancer types.

ATRA has been linked to the increased expression of Tight Junctions (TJs) proteins, including occludins, claudins, & Junctional Adhesion Molecules (JAMs), according to some

studies.[96] The loss of TJ proteins, which results in a deficiency of cell-cell adhesion, is thought to be the origin of the early phases of the aggressive and metastatic progression of tumor cells, which permits their diffusion to a remote site within the body.[97] In this case, it is clear that ATRA restores TJs in order to carry out its anti-tumor action. A study also showed that ATRA may significantly boost JAML (JAM-Like) protein expression in NB4 APL cells, ultimately encouraging inhibition of the growth and promoting differentiation in such cells.[96]

Notably, a number of pre-clinical studies revealed that ATRA activated a number of kinases, including PKA and MAPK, which are essential for intracellular signaling in various cell types and contexts.[98] This activation is likely responsible for the transcription-independent effects of ATRA on multiple cancer-driving pathways.[99]

Despite the encouraging outcomes of pre-clinical studies for solid tumor therapy, ATRA-based treatments proved unsuccessful in clinical trials. According to reports, a number of mechanisms, including enhanced removal through CYP26 or active efflux encouraged by ABC transmembrane transporters, contribute to the decrease of ATRA intracellular content in cancer cells.[100] Some of ATRA's characteristics, like its lipophilic nature, that hinders parenteral delivery and may restrict its clinical efficacy especially in solid tumors. Although drug carriers such as liposomes[101] and nanotechnology based drug delivery systems[102] are in development to overcome these limitations.

## Physiological Role of Bone Morphogenetic Proteins

The bone morphogenetic proteins (BMP) are a group of ligands that regulate cellular lineage determination, differentiation, proliferation, as well as apoptosis of different cell types

throughout the body, and thus plays a significant role in many processes throughout embryonic development and adult homeostasis.

Initially identified as proteins that promote ectopic bone growth, BMPs are today recognized as multifunctional cytokines found in both vertebrates and invertebrates. It was discovered in 1889 that decalcified bone may be used to restore aseptic bone cavities.[103] In 1965 it was revealed that ectopic formation of bone tissues and cartilage with bone marrow is induced by demineralized bone matrix transplanted in muscle tissues.[104] These results suggested that demineralized bone matrix included one or more bioactive factors that were responsible for promoting bone formation. The factor or factors that produce ectopic bone growth have been termed "bone morphogenetic proteins" because trypsin, a common protease, eliminated this activity.[105]

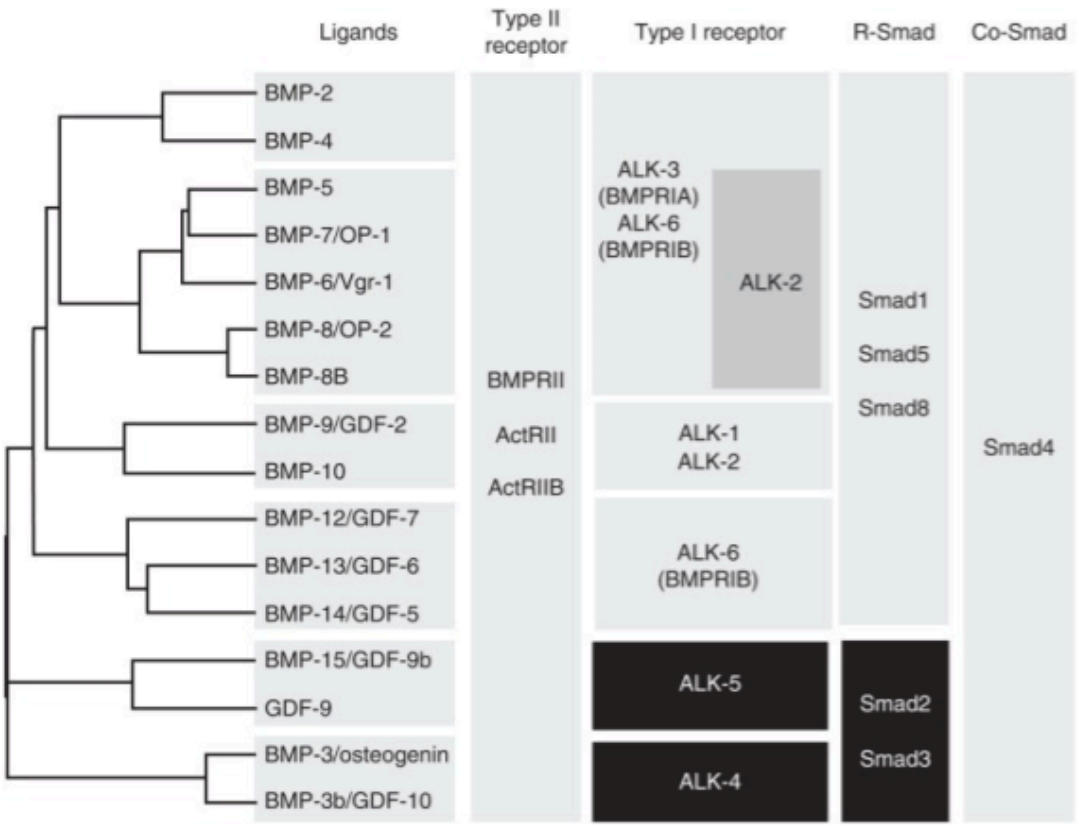



Fig 3.4: BMP ligands, receptors and their downstream signaling[107]

With over 20 members discovered, the BMP signaling pathway has a variety of ligands. Notable ligands in the pathway include anti-Müllerian hormone,[106] growth differentiation factors (GDFs) 5 and 9, and BMPs 2, 4, 6, 7, 9, and 15. There are two types of serine-threonine kinase transmembrane receptors that BMPs bind to, type I and type II TGF-β family receptors. Both receptors are necessary for signal transmission. The presence of type II receptors facilitates the binding affinity of BMPs to type I receptors. Mammals have five distinct type II receptors, and BMPs bind to the BMP type II receptor (BMPR-II), the activin type II receptor (ActR-II), and the activin type IIB receptor (ActR-IIB). From the seven Type I receptors, they can bind with Activin receptor-like kinase (ALK) 1, 2, 3, and 6 (Fig 3.4). [107]

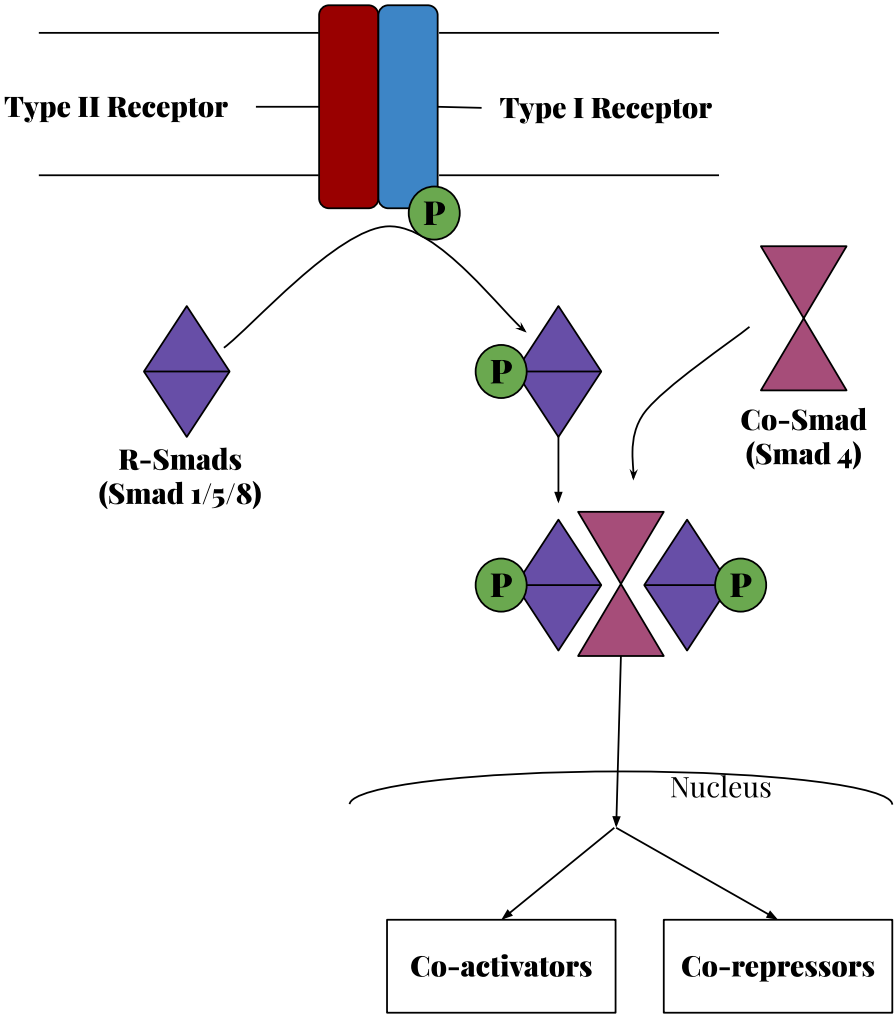



Fig 3.5: Mechanism of action of BMPs

Receptor-regulated Smads (R-Smads), namely Smad 1, 5, and 8/9 (R-Smads), are phosphorylated by activated BMP type I receptors. The phosphorylated R-Smad proteins enter the nucleus and bind with Smad 4 (co-Smad). Smad complexes comprising two R-Smads and one Smad4 bind to their regulatory elements and interact with different transcriptional co-activators or co-repressors to regulate gene expression (Fig 3.5).[107]

Apart from these Smad-dependent signaling pathways, there are non-canonical pathways that BMPs also activate, including IP3, Erk, MAPK, and small GTPase pathways. Through its effects on cell development, survival, and regeneration in a variety of organs and tissues, including skin, bone, and the CNS, BMP signaling also contributes to the maintenance of tissue homeostasis in adult organisms.[108]

Beyond tissue maintenance and development, BMP signaling has become an important factor in disease settings. Numerous clinical conditions, such as malignancies, cardiovascular diseases, and disorders of development have been linked to the dysregulation of BMP signaling.[109]

## Role of BMPs in Cancers

According to several lines of evidence, BMP signals modulate tumor microenvironments, including tumor vasculature, as well as cancer cells, suggesting that they contribute to the development and spread of cancer.[110] Notably, BMPs have context-dependent roles as both cancer promoters and suppressors. Most sporadic colorectal cancers (CRCs) have been shown to contain mutations in BMP signaling components, including BMPRII and Smad4, in conjunction

with a lack of phosphorylation of Smad1/5/9.[111] BMPs have been shown to suppress the growth of colorectal cancers (CRCs) either in vitro or in vivo.[112] The effects of BMPs on the cancer cells are thought to be brought about by their ability to suppress tumor promoting genes as well as inducing differentiation of CSCs and making the tumor more responsive to therapy.

For early-stage carcinomas, EMT is a crucial mechanism that accelerates the development of invasive malignancies.[113] Recent research has shown that EMT is closely related to the development of CSCs and plays a crucial role in both tumor metastasis and tumor recurrence.[114] However, BMPs have some shortcomings in terms of clinical application in therapy. Thus, a compound was developed based on the 3D structure of BMP7, a small molecule BMP mimetic, known as P123. It has shown favorable results by inhibiting the proliferation of breast cancer cells and shows potential to revert the EMT changes.[115]

However, most of the studies discussed here have assessed the effects of BMP signaling in cancer, in other words, these studies aimed to understand the role of BMP signaling in various malignancies and found that it was the source of pathology in certain cancers, but it was a biomarker of good prognosis in other cancers. Very few studies have aimed to use BMPs as therapeutic compounds against cancers, a study in 2012 aimed to assess the effect of BMP treatment on HCC cells in-vitro. This study reported that, at very low concentrations, the BMP mimics the endogenous paracrine and autocrine secretion and promotes the proliferation of the cells. But at high concentrations, it acts to suppress the growth of cells by inducing differentiation of CSCs and reducing CD133+ count.[116]

In colorectal cancer, endogenous BMP signaling promotes tumor growth and aggressiveness,[117] but exogenous treatment suppresses the proliferation and metastasis of the

malignant cells.[118] In breast cancer the endogenous BMP pathways are linked to increasing proliferation of the tumor and invasiveness of breast cancer and poor prognosis,[119] but exogenous administration of BMPs suppresses cancer growth and aggressiveness.[120] This is also seen in hepatocellular carcinoma, where endogenous BMP signaling is essential for tumor initiation and proliferation[121] but exogenous BMP administration suppresses the tumor growth.[116] Whereas, in glioblastoma, the tumorigenicity is suppressed by both endogenous as well as exogenous BMPs.[122-123] Studies on effects of BMP signaling in prostate cancer state that it is responsible for increased invasiveness, metastasis and proliferation of the tumor.[124] But the studies where prostate cancer was treated with exogenous BMPs showed reduction in tumor size and aggressiveness.[125,26]

## Sb4

In 2020, Bradford STJ et al published a study which aimed to identify a small molecule compound via high throughput screening, which is able to activate the BMP signaling pathway in human renal cell line (HEK293s). This study was concerned with utilization of BMP agonists for their use in chronic kidney disease.[28] In CKD, the BMPs have shown to have a protective and therapeutic role.[27]

The high throughput screening study found that a benzoxazole compound, 2-[[(4-Bromophenyl)methyl]thio]benzoxazole or Sb4, which is able to significantly increase the levels of intracellular phosphorylated Smad-1/5/9 within 1 hour and increased expression of BMP target genes. This study also showed that Sb4 is also able to overcome the endogenous

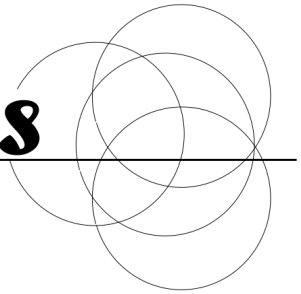
antagonists of BMP such as noggin. Although it also showed that Sb4 is not as effective as BMP4 at phosphorylation of Smad-1/5/9 complex.[28]

In 2022, multiple studies were done to further explore the effects of Sb4 on inducing differentiation. Hayaei Tehrani RS et al suggested that Sb4 is capable of germline fate determination in in-vitro embryonic stem cells.[126] Another study by Yan H. et al in the same year demonstrated that Sb4 could be used as a reliable activator of Smad 1/5/9 pathway in in-vitro mesenchymal stem cell culture.[127]

In 2024, Xu et al studied the effectiveness of Sb4 as a therapeutic agent against pituitary neuroendocrine tumors. They found that Sb4 is capable of bringing about inhibition of growth of cancer cells in-vitro via Smad-dependent pathway. They also studied this effect in vivo with a pituitary neuroendocrine mouse model having cushing's disease phenotype, where Sb4 administration showed inhibition of tumor growth as well as reduction in circulating ACTH levels.[128]

Hence, the role of Sb4 as a cost effective alternative for utilization of BMP pathway signaling is established. Even though the role of BMP signaling agonists as therapeutic agents in prostate cancer remains inconclusive, active pursuit of learning about the effects of such compounds has the potential to improve future prospects of cancer treatment.

# Material & Methods



The study was conducted at the cell culture lab of KLE's Dr. Prabhakar Kore Basic Science Research Center from April 2023 to February 2025.

Prostate cancer cell line (DU-145) was procured from National Centre for Cell Sciences, Pune.

Standard anti-cancer drug Docetaxel was procured from KLE's Dr. Prabhakar Kore Hospital & Medical Research Centre, Belagavi.

Test Compounds:

- All-trans retinoic acid (ATRA) (CAT No. R2625) was procured from Sigma-Aldrich
- 2-[[(4-Bromophenyl)methyl]thio]benzoxazole (Sb4) (CAT No. SML2636) was procured from Sigma-Aldrich

Ethical clearance for the study was provided by the JNMC institutional ethics committee (Ref No.MDC/JNMCIEC/129).

Stock solution of ATRA was prepared by diluting the ATRA powder in dimethyl Sulfoxide (DMSO) and phosphate buffer saline (PBS) (solubility: 40 mg/ml in DMSO). A stock solution was prepared with concentration of 1 mg/ml to make further dilutions. Care was taken to ensure the concentration of DMSO in the final treatment solution does not reach >0.1%v/v. Because of the photosensitive nature of ATRA, the container of ATRA stock solution was wrapped in aluminium foil and protective measures were taken to maintain a dark environment while carrying out the assays. The ATRA stock solution was stored in -20℃.

Stock solution of Sb4 was prepared by diluting the Sb4 powder in DMSO and PBS (solubility: 2 mg/ml in DMSO). The stock solution was prepared with the concentration of 0.1 mg/ml to prepare further concentrations. Care was taken to ensure the concentration of DMSO in the final treatment solution does not reach >0.1%v/v. The Sb4 stock solution was stored at 2-8℃.

The assessment of the effect of test compounds on DU-145 cells was done using two assays; Cytotoxicity assay and Migration assay (Scratch assay). While cytotoxicity assay provides results regarding the cytotoxicity of the individual compound and whether differentiation of the cells increases their sensitivity to the standard anticancer cancer drug, Migration assay provides results regarding whether the induction of differentiation affects the cancer cell migration and motility.

Cytotoxicity assay was performed in two phases. In the first phase, the direct cytotoxic effects of the test compounds on the DU-145 prostate cancer were assessed and compared. For the second phase, to assess the effect of combinations of compounds, the protocol was modified where the cells were treated with the differentiation inducing compound first, following that, the cytotoxic effect of the standard chemotherapeutic drug was assessed.

The effective concentrations of the test compounds were prepared by diluting the stock solution to the highest required concentration, from here two fold serial dilution was done to make eight decreasing concentrations of compounds (Fig 4.1). The known effective

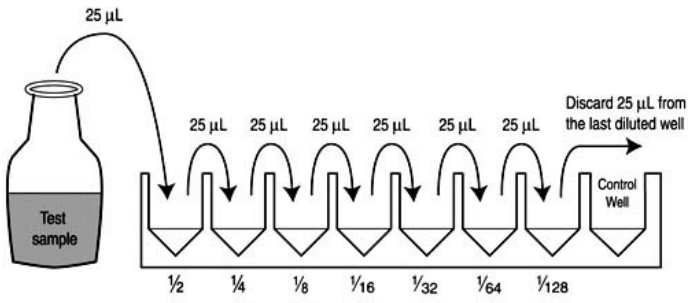



Fig 4.1: Serial dilution

concentrations of the test compounds are based on previous studies involving these compounds (Table 4.1).[28,129-130]

**Table 4.1: Concentrations for MTT assay phase I**

| No. | Compounds | Concentrations | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| 1. | Negative Control | - | - | - | - | - | - | - | - |
| 2. | Docetaxel (Positive control) | 0.975 nM | 1.95 nM | 3.9 nM | 7.8 nM | 15.6 nM | 31.25 nM | 62.5 nM | 125 nM |
| | | 0.775 ng/ml | 1.55 ng/ml | 3.1 ng/ml | 6.25 ng/ml | 12.5 ng/ml | 25 ng/ml | 50 ng/ml | 0.1 µg/ml |
| 3. | All-trans retinoic acid (ATRA) | 4.68 µM | 9.37 µM | 18.75 µM | 37.5 µM | 75 µM | 150 µM | 300 µM | 600 µM |
| | | 1.4 µg/ml | 2.8 µg/ml | 5.6 µg/ml | 11.25 µg/ml | 22.5 µg/ml | 45 µg/ml | 90 µg/ml | 180 µg/ml |
| 4. | Sb4 | 15.6 nM | 31.25 nM | 62.5 nM | 125 nM | 250 nM | 500 nM | 1 µM | 2 µM |
| | | 5 ng/ml | 10 ng/ml | 20 ng/ml | 40 ng/ml | 80 ng/ml | 0.16 µg/ml | 0.32 µg/ml | 0.64 µg/ml |

The first phase consisted of the MTT based cytotoxicity assay to assess the direct cytotoxic effect of the compounds. The MTT assay is usually done to estimate the proportion of viable cells in presence of a compound compared to control, which is calculated as viability percentage. At first, the cells are seeded in a 96 well plate (Fig 4.2). Once the cells are in the exponential phase of growth they are exposed to the test compound for a period of time. 8 concentrations of three compounds were prepared (ATRA, Sb4 and Docetaxel). Cells in three wells were treated with the same concentration, thus for 8 concentrations, 24 wells were treated

with the same compound. Three wells were taken as negative control which were not treated with any compound and the media in these wells was changed every 48 hrs.

At the end of the compound treatment phase, the compounds were removed and growth medium was added. After removal of the drug, the cells were allowed to proliferate during a period of recovery to distinguish between cells that remain viable and are capable of proliferation and those that remain viable but cannot proliferate. The number of surviving cells was then determined indirectly by MTT dye reduction. The mitochondria in the living cells are able to convert the MTT dye (yellow in color) into formazan product (purple in color). The amount of formazan produced wes determined spectrophotometrically using ELISA reader after the formazan was dissolved in DMSO. Proportions of viable cells in each well were calculated by measuring the optical density of each well at 570 nm and comparing it to the optical density of negative control, which has not been treated with any compound.[30] Viability is calculated as,

$$\text{Viability \%} = \frac{Mean\ OD\ of\ test\ compound}{Mean\ OD\ of\ control(untreated\ cells)} \times 100$$

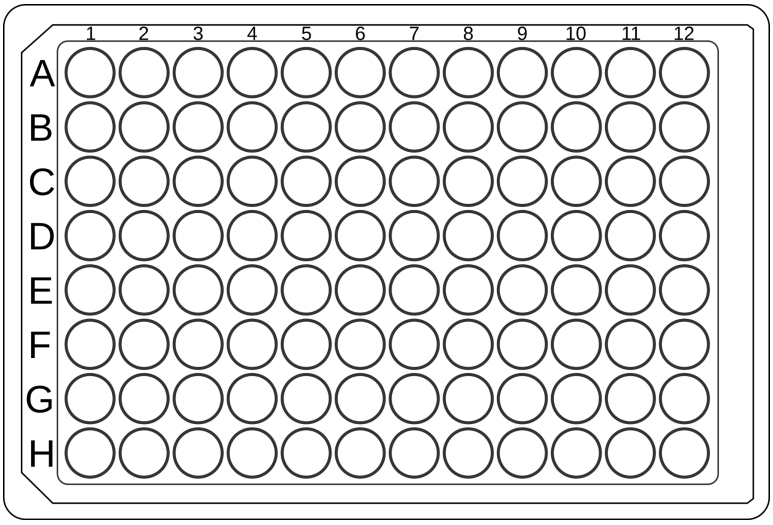



Fig 4.2: 96 well plate[30]

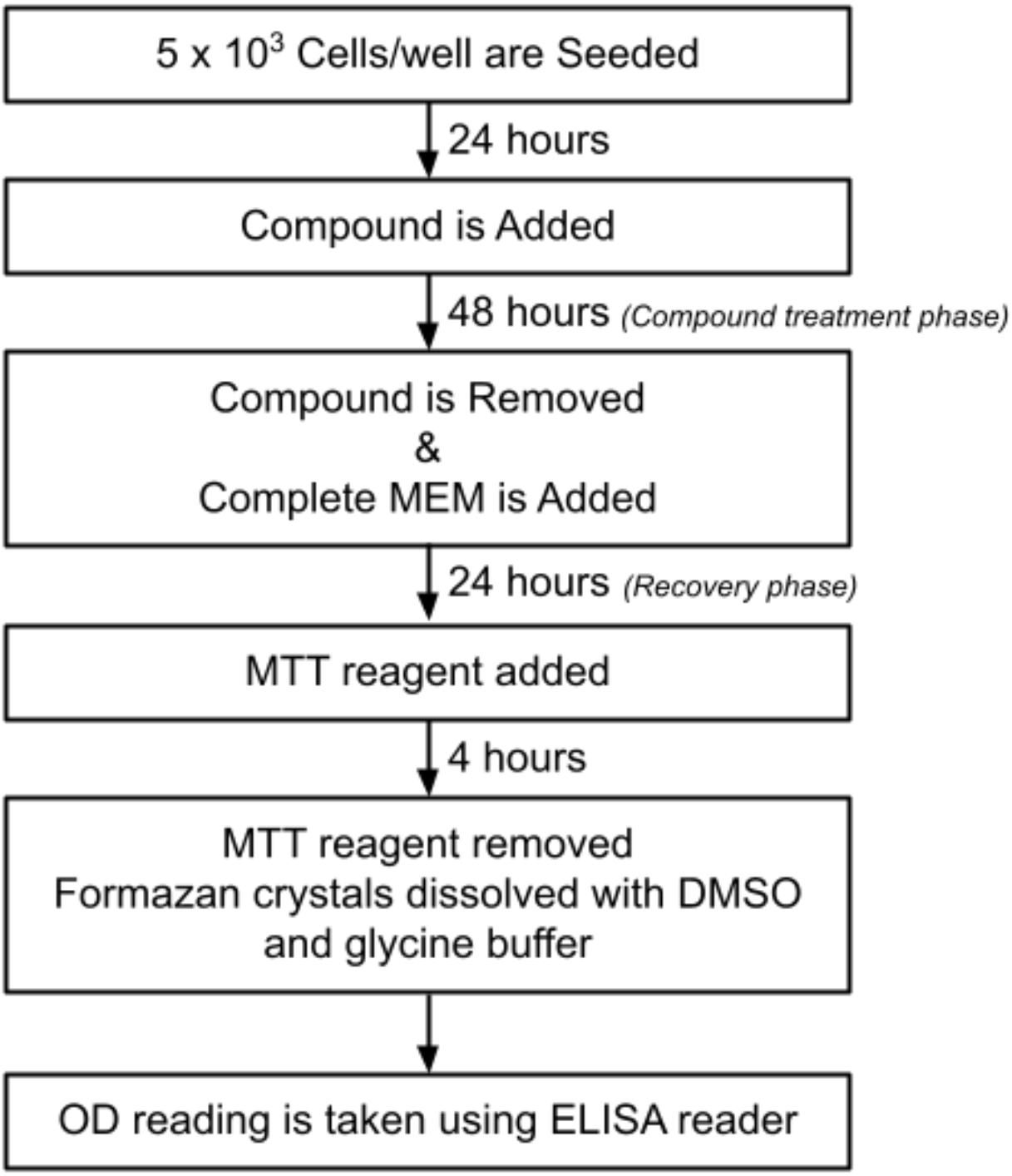



Fig. 4.3: MTT assay phase I protocol

MTT assay phase I protocol:

- Day 1:
    - 5 x $10^3$ cells - cell suspension was seeded in each well in a 96 well microtiter plate and final volume was made up to 150 µl by adding complete MEM media, cells were incubated for 24 hours in 5% $CO_2$ at 37℃.
- Day 2:
    - Dilutions of test compounds were prepared in MEM media.
    - 100 µl of the test compounds of different concentrations were added to the wells and incubated for 48 hours, in presence of 5% $CO_2$ at 37℃.
- Day 4:
    - Test compounds were removed and 150 µl fresh MEM media added and incubated for 24 hours in 5% $CO_2$ at 37℃.
- Day 5:
    - 200 µl of complete MEM and 20 µl of 5 mg/ml MTT reagent was added to the wells. The plate was incubated for 4 hours in a dark place at room temperature (The plate was covered with aluminium foil since MTT reagent is photosensitive).
    - The supernatant was carefully removed without disturbing the precipitated formazan crystals and 100 µl of DMSO was added to dissolve the crystals formed. Glycine buffer was added to neutralize the changes in pH.
    - The optical density of the dissolved formazan crystals was measured at a wavelength of 570 nm.

- The study was performed in triplicates. The result represents the mean of three readings.

From the results of cell viabilities at eight different concentrations of the tested compounds, a graph can be plotted. By plotting a graph of cell viability against concentrations for the compounds, the slope of the graph was calculated. Based on the slope, the concentration of the compound required to kill 50% of the living cells in a population (IC 50) was determined.

The second phase was done to assess whether the differentiation inducing compounds (ATRA & Sb4) were able to affect the effectiveness of standard care cytotoxic agent (Docetaxel). The cells were seeded in 24 well plate instead of 96 well plate, more surface area can allow for a longer duration of the protocol by preventing the cells from reaching confluency during the protocol. The concentrations of the test compounds used here were determined based on the results of the phase I MTT assay. For the differentiation inducing compounds, two concentrations were selected for each compound, both below the IC 50 value of the compound (Fig 4.4). In addition, the same molarity of the concentrations (100 nM and 10 nM) was selected for Sb4 and ATRA to more accurately compare their effects. The concentration of the standard care cytotoxic drug (Docetaxel) was kept constant at IC 50 so that it does not become a variable affecting the cell viability. The principle of the MTT assay remains the same but standard protocol was modified to account for a period of induction of differentiation by the test compounds (ATRA & Sb4)

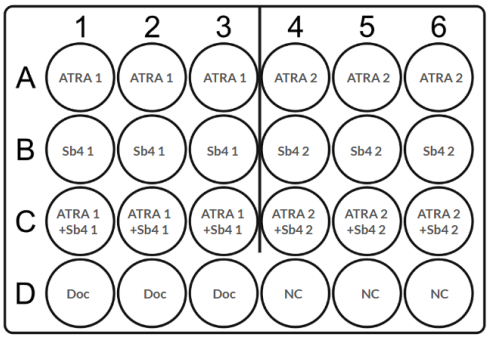



Fig 4.4: 24 well plate layout

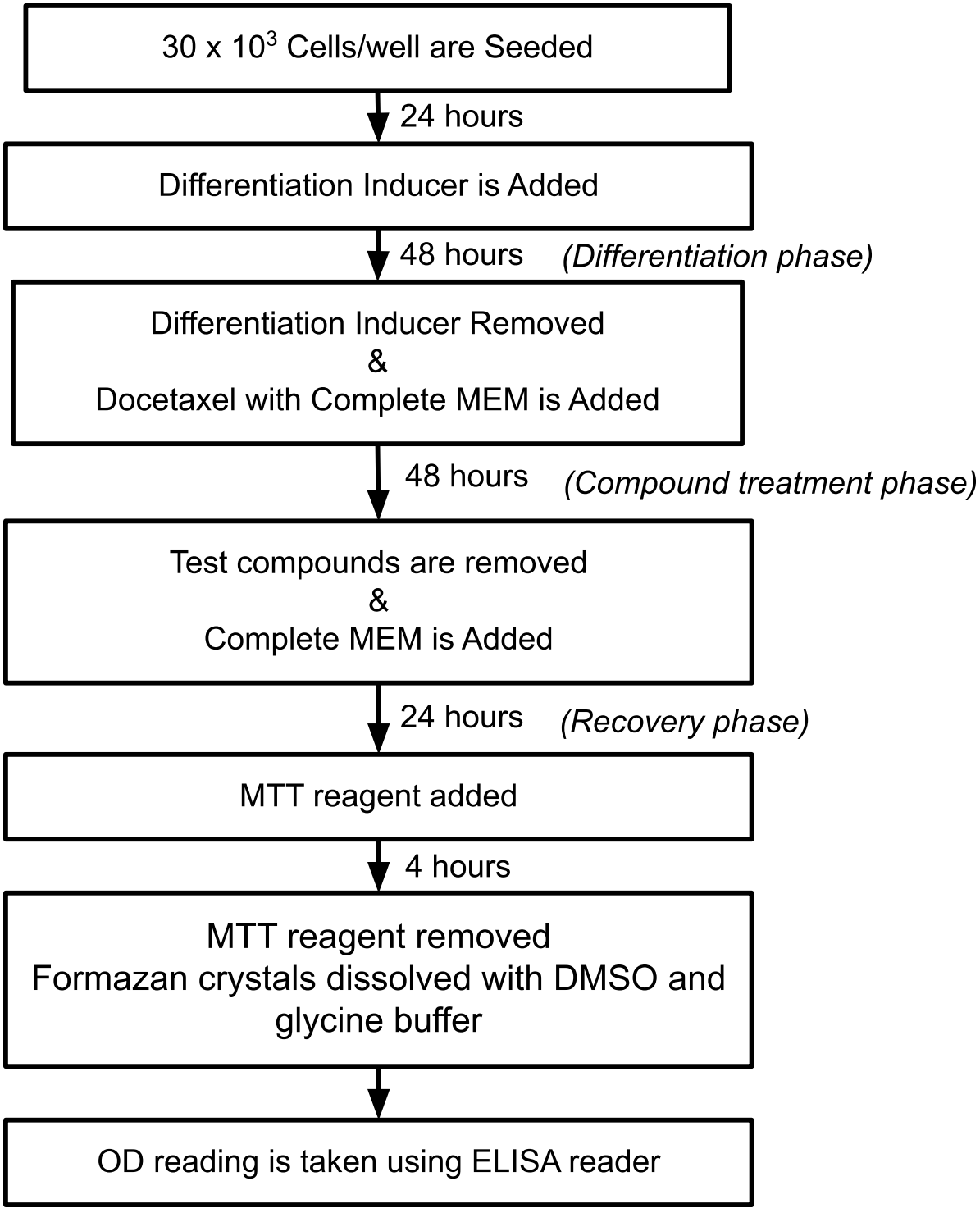



Fig. 4.5: MTT assay phase II protocol

MTT assay phase II protocol:

- Day 1:
    - 30 x $10^3$ cells - cell suspension was seeded in each well in a 24 well plate and final volume was made up to 500 µl by adding complete MEM media, cells were incubated for 24 hours in 5% $CO_2$ at 37℃.
- Day 2:
    - Dilutions of Differentiation inducing agents were prepared in MEM media.
    - 500 µl of the test compounds were added to the wells and incubated for 48 hours, in presence of 5% $CO_2$ at 37℃.
- Day 4:
    - Differentiation inducing agents were removed and standard care drug (Docetaxel at IC 50) and 500 µl fresh MEM media were added and incubated for 48 hours in 5% $CO_2$ at 37℃.
- Day 6:
    - Test compounds removed and 500 µl fresh MEM media added and incubated for 24 hours in 5% $CO_2$ at 37℃.
    - In vitro growth inhibition effects of test compounds were assessed by colorimetric or spectrophotometric determination of conversion of MTT to Formazan by living cells.

- Day 7:
    - 400 µl of complete MEM and 40 µl of 5 mg/ml MTT reagent was added to the wells. The plate was incubated for 4 hours in a dark place at room temperature (The plate was covered with aluminium foil since MTT reagent is photosensitive).
    - The supernatant was carefully removed without disturbing the precipitated formazan crystals and 200 µl of DMSO was added to dissolve the crystals formed. Glycine buffer was added to neutralize the changes in pH.
    - The optical density of the dissolved formazan crystals was measured at a wavelength of 570 nm.

The population doubling time was determined alongside the MTT assay. PDT is based on the doubling time of the cell population. The number of cells seeded per well were estimated. By considering different concentrations of test compounds and negative control, doubling time was determined at the end of the assay using this formula,

$$\text{Doubling time} = \frac{\text{Duration} \cdot ln(2)}{ln(\frac{\text{Final concentration}}{\text{Initial concentration}})}$$

Migration assay, also known as scratch assay or wound healing assay was carried out to assess the effects of the test compounds on cancer cell motility and migration. Cells are seeded in a 6 well plate and allowed to attach and grow until a monolayer of cells is formed. Once the monolayer is observed, the cells in all wells are treated with Mitomycin-C (5 µg/ml). This prevents the synthesis of DNA and proteins, thus the cell cycle is halted. Then the tip of a 10 µl

pipette is used to create a scratch or wound on the monolayer in all wells. Now the test compounds (ATRA, Sb4 and Docetaxel) are added to the wells. No compound is added in one well, which is treated as negative control. Approximation of the wound is captured with an inverted microscope. This approximation is captured every 2 hours until 24 hours or until the wound is closed.

The surface area of the wound is calculated by ImageJ analysis software. Based on the surface area of the wound in the monolayer at 0 hours($A_0$) and the surface area of the wound in the monolayer at the end of the assay ($A_t$), using this formula the wound closure can be calculated as a percentage,

$$\textbf{Wound Closure\%} = \frac{A_0 - A_t}{A_0} \times 100$$

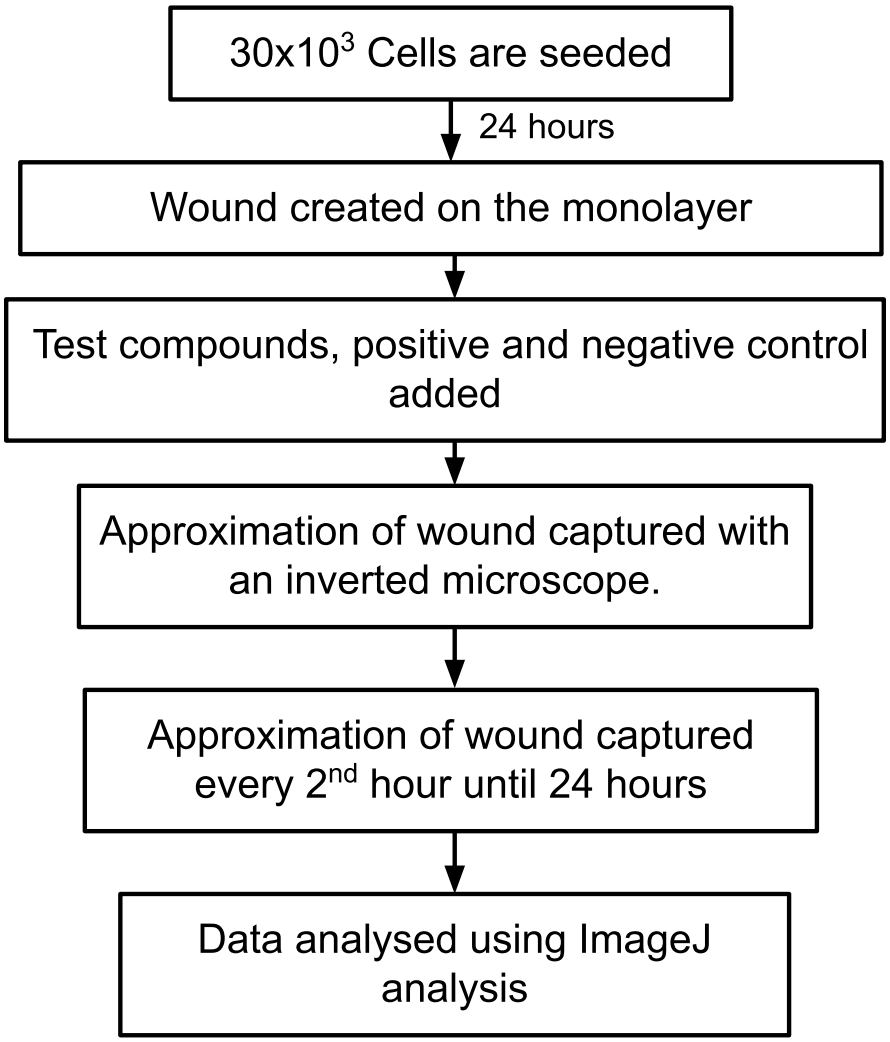



Fig 4.6: Migration assay protocol

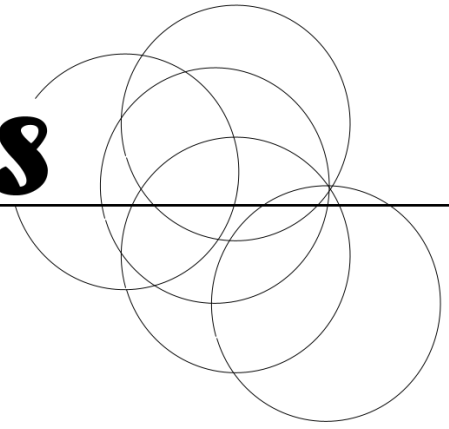
Migration assay protocol:

- Day 1:
    - Thirty thousand cells per 6 well plate were seeded and 1 ml of complete media was added to develop monolayer of cells

- Day 2:
    - After 24 hrs, development of Monolayer of cells to be observed. If required media was added.
- Day 3:
    - After 48 hrs, the monolayer was observed. Once monolayer was developed the cells are treated with mitomycin-c
    - Mitomycin-C (5 µg/ml) was prepared in complete media
    - The monolayer was treated with mitomycin-C for two hours by keeping in $CO_2$ chamber
    - Then slowly without disturbing the monolayer, mitomycin-C was removed by the edge of well
    - To neutralize the action of mitomycin-C, the monolayer was washed twice with complete media
    - A wound was created with the help of a 10 µl pipette tip and again fitted into a small tip at the 45-degree angle (one stroke from top and other from bottom joining the top stroke).
    - Then the monolayer was treated with the test compound by considering positive control and negative control
    - Approximation of the wound captured with the help of inverted microscope
    - Approximation of the wound repeated every $2^{nd}$ hour until 24 hours (Until the wound is closed in the negative control)
    - Analysis of the data done using ImageJ software.

Statistical analysis of data was performed by analysis of variance (ANOVA) and level of statistical significance between groups using GraphPad Prism version 7.00. P value $<0.05$ was considered statistically significant.

# Results



The aim of this study was to assess the effect of the compound Sb4 on DU 145 prostate cancer cells. To assess whether the compound increases the sensitivity of the cells to standard chemotherapy, MTT cytotoxicity assay was done along with ATRA, which is known to increase the effectiveness of the treatment. To assess the effect of the compound on cell motility, migration assay (or scratch assay) was done. The results from the first phase, MTT assay reveal the cytotoxicity of each compound individually. In the second phase, MTT assay reveals the effects of the test compounds on the cytotoxicity of the standard chemotherapeutic drug Docetaxel.

**Table 5.1: Cytotoxic effects of Docetaxel on DU145 cells**

| Compound | Concentration (nM) | Viability % | Population doubling time (PDT) (in hrs) |
|---|---|---|---|
| Docetaxel (IC50 = 10.78 nM) | 125 | 44.55 | 61.56 |
| | 62.5 | 46.31 | 59.85 |
| | 31.25 | 46.47 | 59.7 |
| | 15.62 | 47.27 | 58.97 |
| | 7.812 | 53.68 | 54.1 |
| | 3.90 | 54.16 | 53.78 |
| | 1.95 | 59.93 | 50.48 |
| | 0.97 | 63.46 | 48.79 |

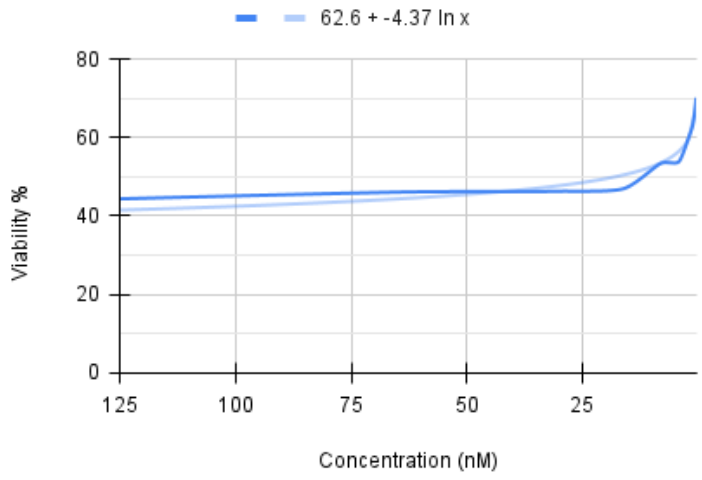
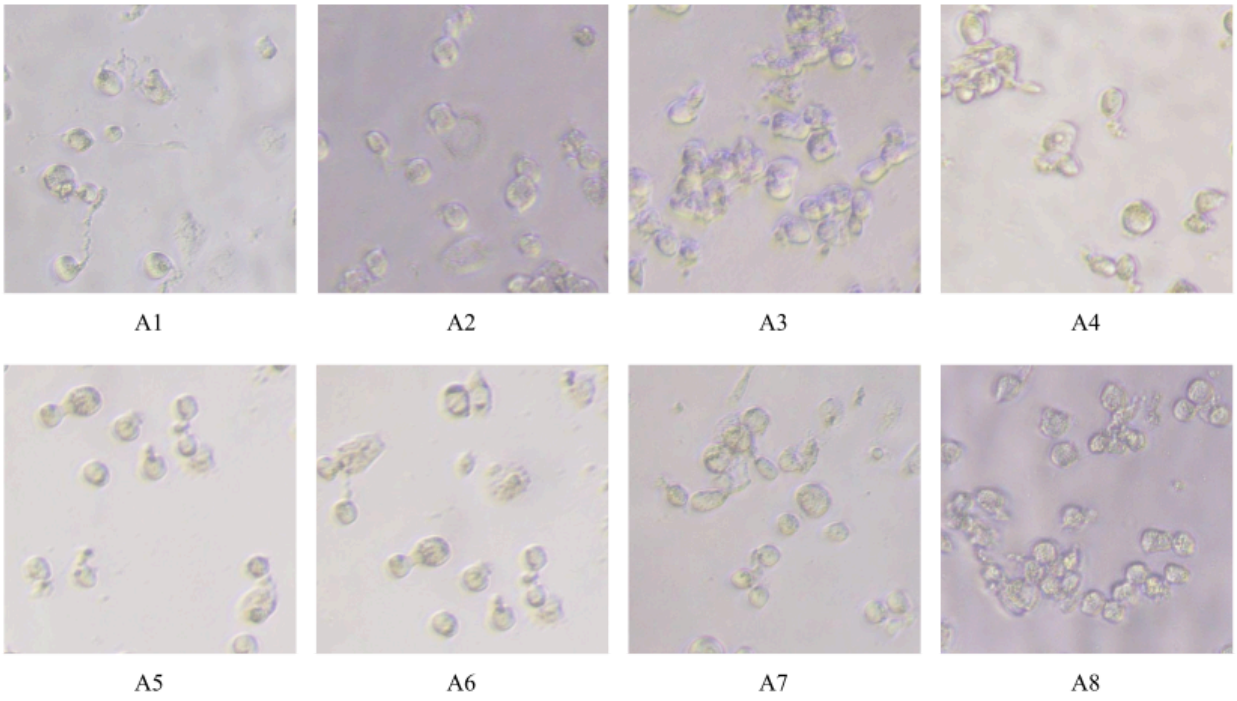



Fig. 5.1: Cytotoxic effects of Docetaxel on DU145 cells



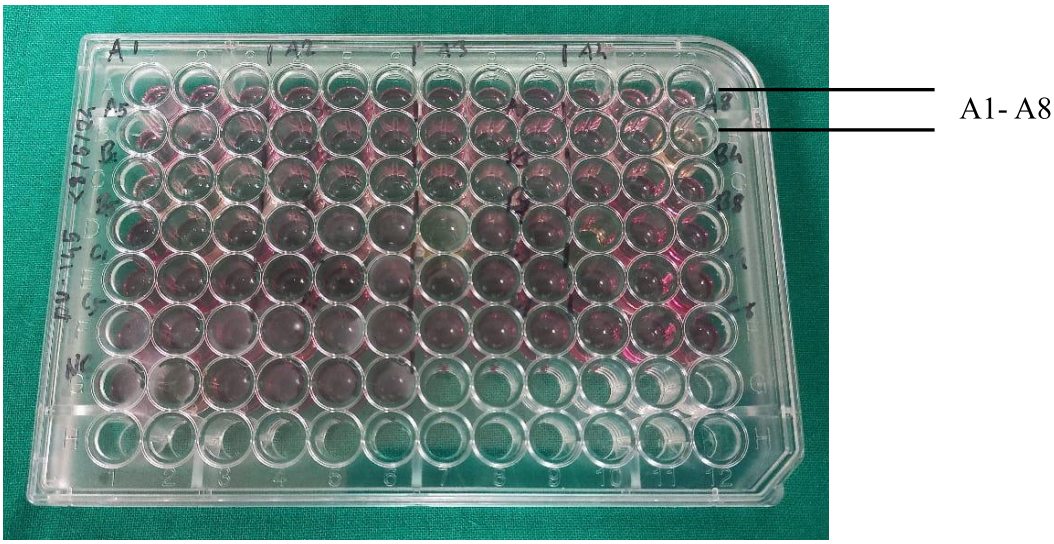




Fig. 5.2: DU145 cells under effects of different concentrations of Docetaxel

The table 5.1 shows the results of cytotoxicity of Docetaxel in prostate cancer cells. The IC 50 of Docetaxel was calculated based on the slope from the graph of cell viability against concentration of the compound. As one would expect from a chemotherapeutic drug, Docetaxel has the IC 50 value of 10.78 nM which is lowest among the compounds assessed. Population doubling time (PDT) was also calculated for different concentrations of Docetaxel. PDT showed a steady increase proportional to increasing concentration, even at lowest concentration, it was increased. One way ANOVA was done to compare the effect of concentration of Docetaxel on cell viability. It showed that there was a statistically significant difference between different concentration groups ($F=5.95$, p-value=<0.001) (Table 5.1) (Fig. 5.1). Upon treatment with Docetaxel, cells start to go through apoptosis. This manifests as cells losing their normal epithelial morphology and becoming spherical and detached. Upon gentle shaking of the plate under the microscope, the cells can be identified as floating dead cells. (Fig. 5.2).

**Table 5.2: Cytotoxic effects of ATRA on DU145 cells**

| Compound | Concentration (µM) | Viability % | PDT (in hrs) |
|---|---|---|---|
| ATRA (IC50 = 99.5 µM) | 600 | 20.56 | 131.59 |
| | 300 | 32.09 | 77.2 |
| | 150 | 35.89 | 69.93 |
| | 75 | 52.08 | 53.26 |
| | 37.5 | 52.73 | 52.84 |
| | 18.75 | 53.01 | 52.66 |
| | 9.37 | 62.32 | 47.77 |
| | 4.68 | 64.06 | 47.02 |

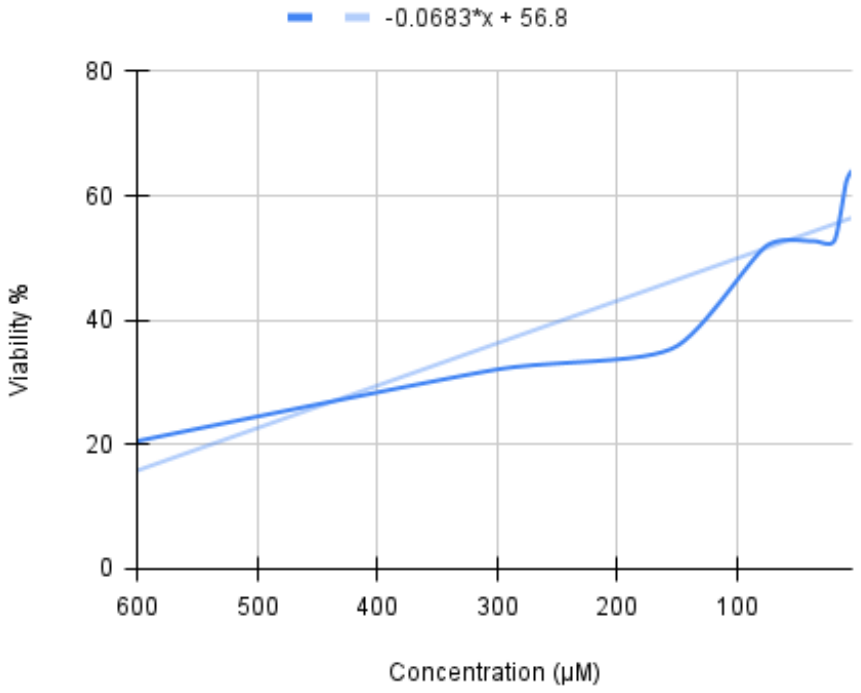
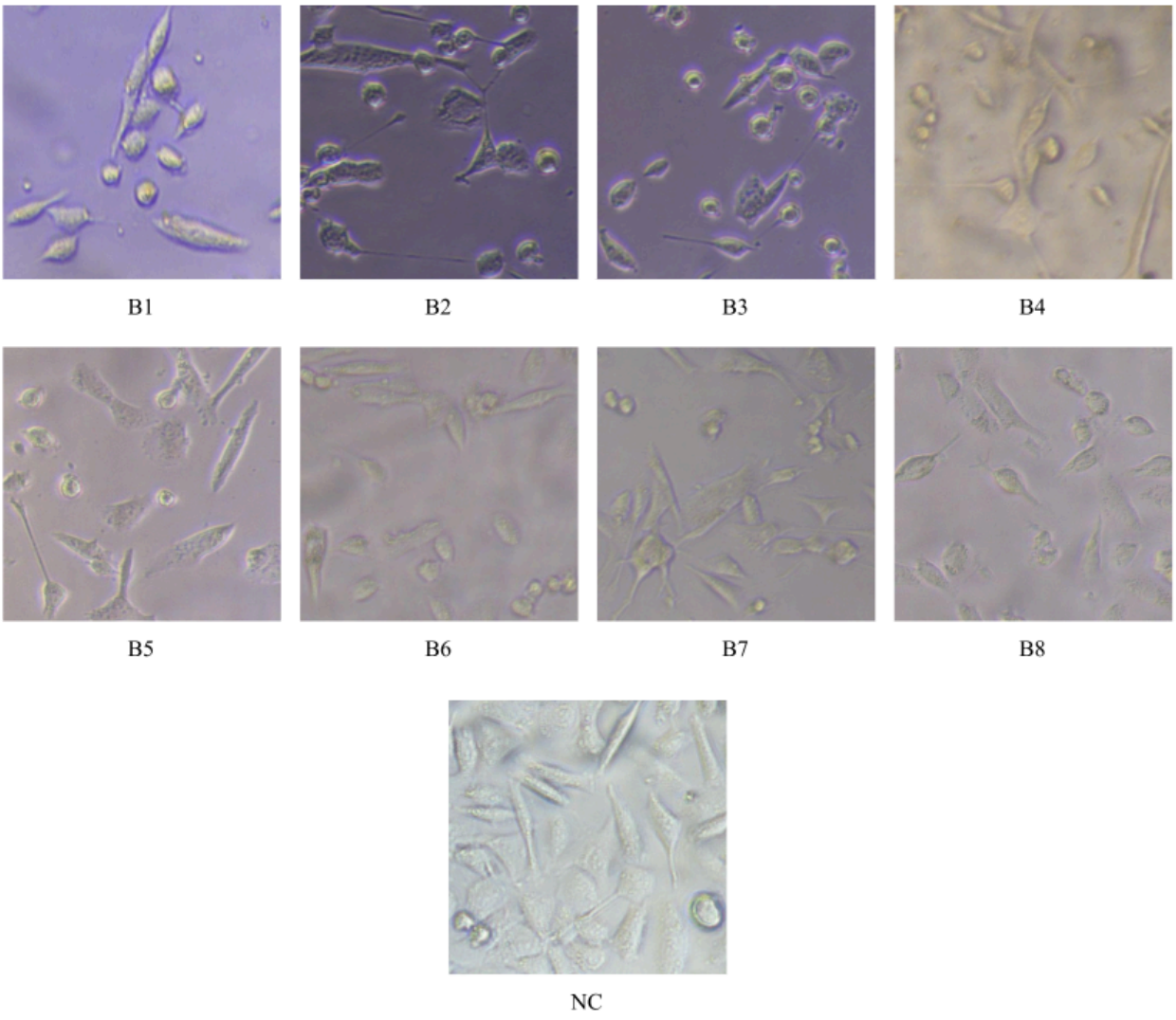



Fig. 5.3: Cytotoxic effects of ATRA on DU145 cells



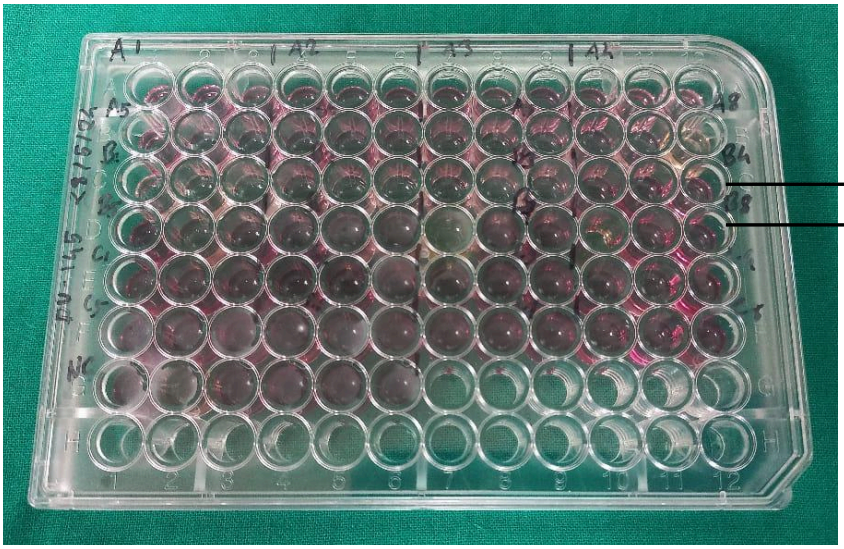




Fig. 5.4: DU145 cells under effects of different concentrations of ATRA

Table 5.2 shows the cytotoxic effect of ATRA. The effect of ATRA on prostate cancer cells showed drastic effects on cell viability between high concentration and low concentrations. Based on the graph of cell viability against concentration, IC 50 value was calculated. ATRA was found to have an IC 50 value of 99.5 µM. Population doubling time was also calculated for different concentrations of ATRA. PDT showed a drastic increase at highest concentration. One way ANOVA was done to compare the effect of concentration of ATRA on cell viability. It showed that there was a statistically significant difference between different concentration groups ($F=8.45$, p-value=<0.001) (Table 5.2) (Fig. 5.3). When treated with ATRA, at high concentration, even visually the low cell density can be appreciated. The cells do not have significant morphological differences (Fig. 5.4).

**Table 5.3: Cytotoxic effects of Sb4 on DU145 cells**

| Compound | Concentration (nM) | Viability % | PDT (in hrs) |
|---|---|---|---|
| Sb4 (IC 50 = 4.4 µM) | 2000 | 72.33 | 49.42 |
| | 1000 | 74.13 | 48.77 |
| | 500 | 74.83 | 47.98 |
| | 250 | 76.57 | 49.42 |
| | 125 | 83.81 | 43.54 |
| | 62.5 | 85.71 | 42.96 |
| | 31.25 | 88.38 | 42.21 |
| | 15.625 | 89.52 | 41.49 |

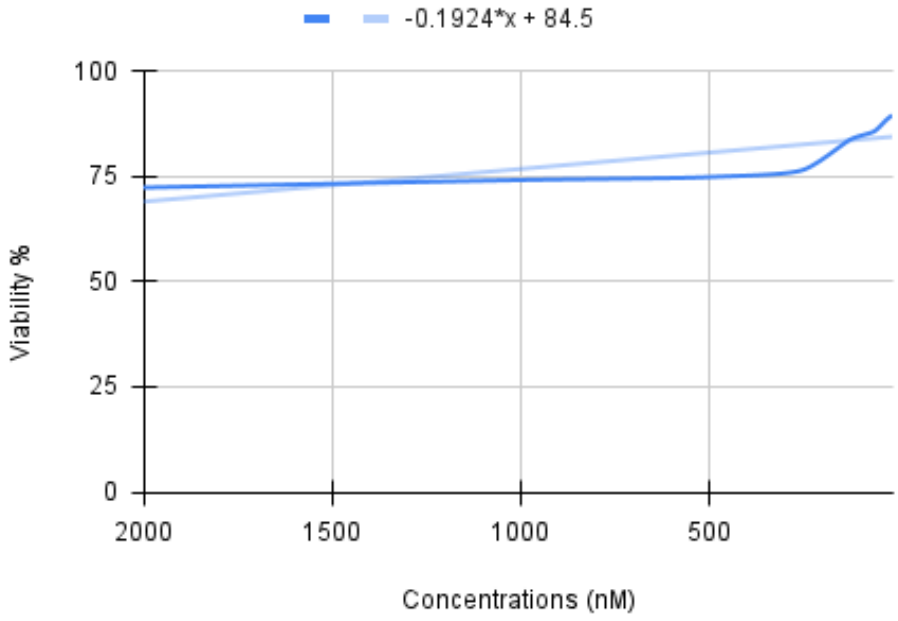
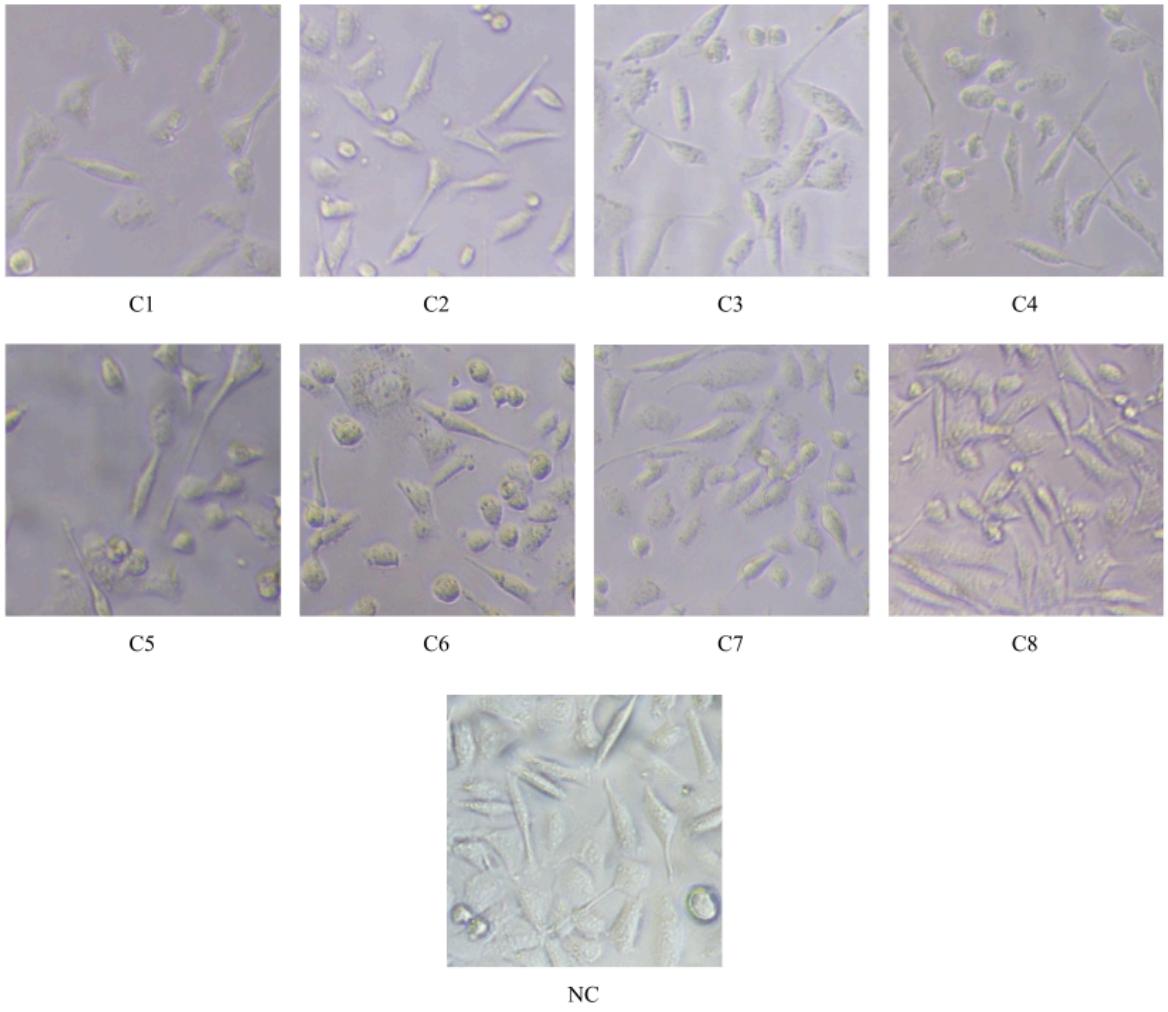



Fig. 5.5: Cytotoxic effects of Sb4 on DU145 cells



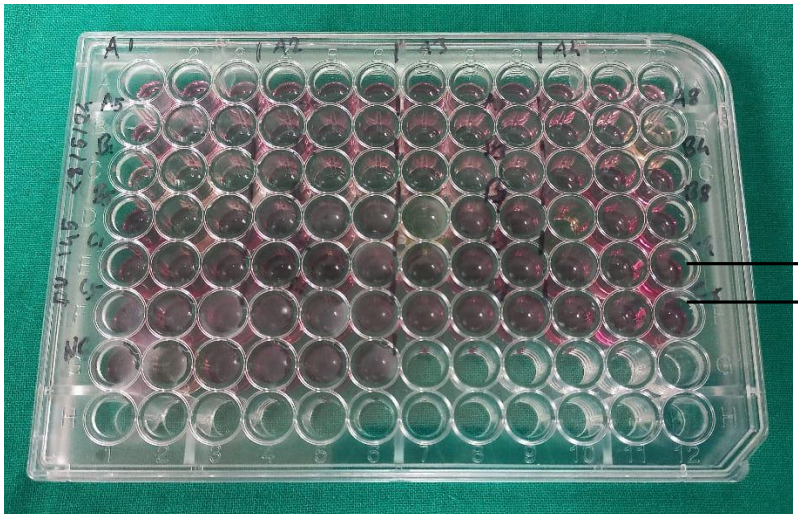




Fig. 5.6: DU145 cells under effects of different concentrations of Sb4

The table 5.3 shows the cytotoxic effect of Sb4. The effect of Sb4 on prostate cancer cells showed minimal effects on cell viability. By plotting the graph of cell viability against concentration, an IC 50 value of 4.4 µM was calculated. Population doubling time was also calculated for different concentrations of Sb4. PDT showed an increase with increasing concentration, but at lower concentrations, it was marginally increased. One way ANOVA was done to compare the effect of concentration of ATRA on cell viability. It showed that there was a statistically significant difference between different concentration groups (F=3.02, p-value=0.02) (Table 5.3) (Fig. 5.5). After Sb4 treatment, the cells do not show significant changes in either cellular density or morphology (Fig. 5.6).

**Table 5.4: Cytotoxic effects of combinations of compounds on DU145 cells**

| Compound | Concentration | Viability% |
|---|---|---|
| Docetaxel | Docetaxel 10.78 nM | 45.97 |
| ATRA → Docetaxel | ATRA 1 µM \| Docetaxel 10.78 nM | 41.59 |
| | ATRA 100 nM \| Docetaxel 10.78 nM | 43.81 |
| Sb4 → Docetaxel | Sb4 1 µM \| Docetaxel 10.78 nM | 35.44 |
| | Sb4 100 nM \| Docetaxel 10.78 nM | 54.17 |
| ATRA+Sb4 → Docetaxel | ATRA 1 µM + Sb4 1 µM \| Docetaxel 10.78 nM | 35.7 |
| | ATRA 100 nM + Sb4 100 nM \| Docetaxel 10.78 nM | 45.61 |

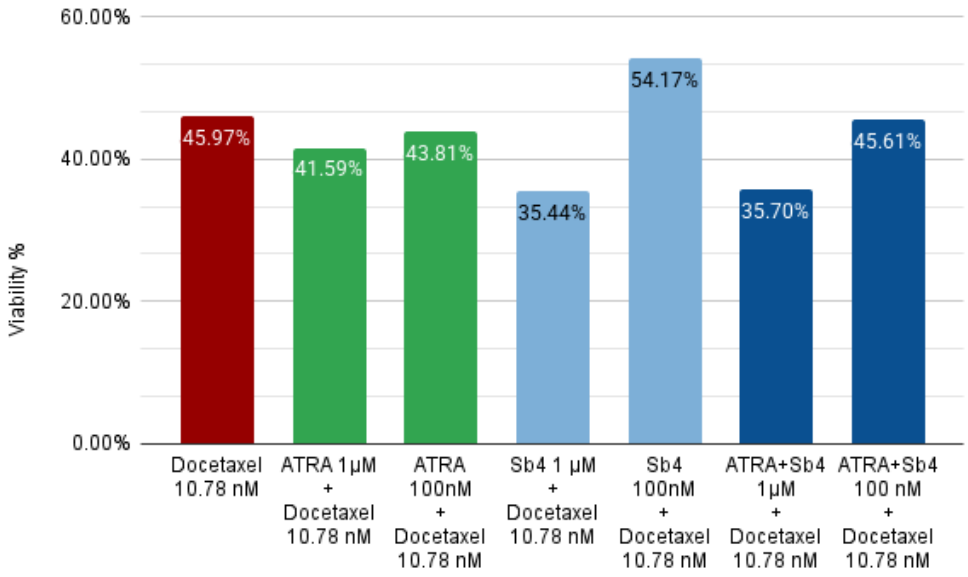



Fig. 5.7: Cytotoxic effects of combinations of compounds on DU145 cells

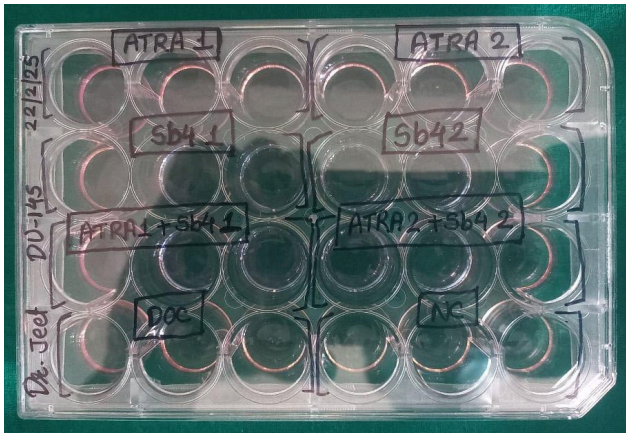



Fig 5.8: 24 well plate layout

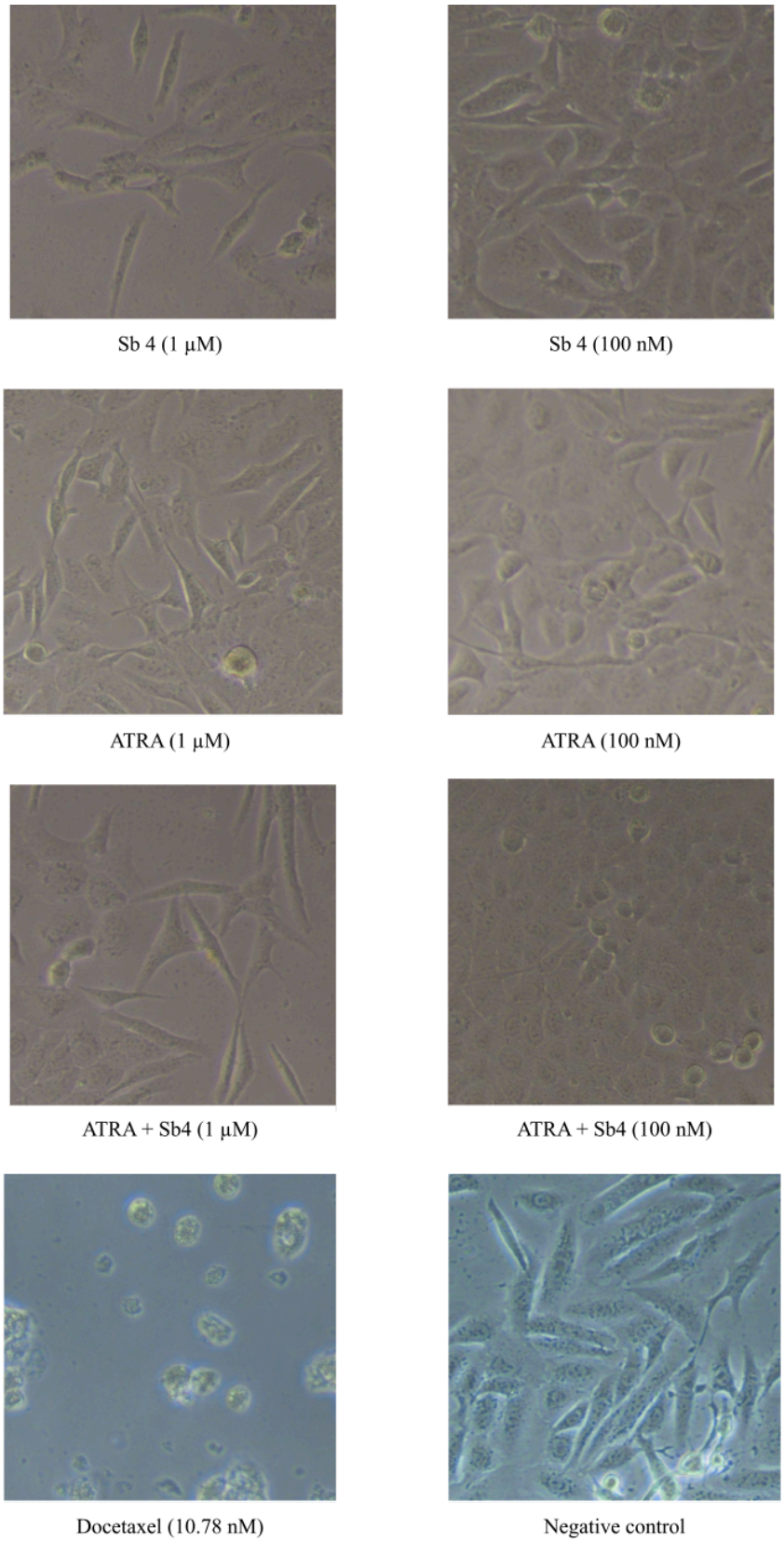



Fig. 5.9: DU145 cells under effects of different concentrations of combinations of compounds

Table 5.4 shows the results of the MTT assay of phase 2. MTT assay was done to assess the effect of the differentiation inducing compound on the cytotoxicity of Docetaxel. The concentration of Docetaxel was kept at 10.78 nM (IC 50) for all wells (Fig 5.9). Three wells were treated with only Docetaxel that showed cell viability of 45.97%. When exposed to ATRA before Docetaxel treatment, it showed a reduction in cell viability. At high concentration (1 µM) the effect of Docetaxel was augmented and cell viability was 41.59%. At low concentration (100 nM) the effect of Docetaxel was also reduced, the cell viability was 43.81%. When exposed to Sb4 before Docetaxel treatment, it showed both increase or decrease in cell viability based on the concentration. At high concentration (1 µM) the effect of Docetaxel was increased, and the cell viability was 35.44%, which was the lowest cell viability in phase 2. At low concentration (100 nM) the effect of Docetaxel was reduced, cell viability showed an increase instead of 54.17%. When exposed to the combination of ATRA+Sb4, before Docetaxel treatment, it showed reduction in cell viability. At high concentrations (1 µM) the effect of Docetaxel was increased, cell viability was 35.7%. At lower concentration (100 nM) the effect of Docetaxel was not significantly altered, cell viability was 45.61%. Two way ANOVA was done to determine the effects of different compounds and different concentrations on cell viability. It was found that there was a significant interaction between different compounds and different concentrations on cell viability ($F=12.63$, p-value= $<0.01$) (Table 5.4) (Figure 5.7). The effects of these compounds of cellular morphology is seen in Fig. 5.9.

**Table 5.5: Wound closure % of DU145 cells treated with different compounds**

| Compound | Wound closure % |
|---|---|
| NC | 96.58 |
| ATRA | 89.8 |
| Sb4 | 73.62 |
| ATRA+Sb4 | 72.81 |
| Docetaxel | 31.81 |

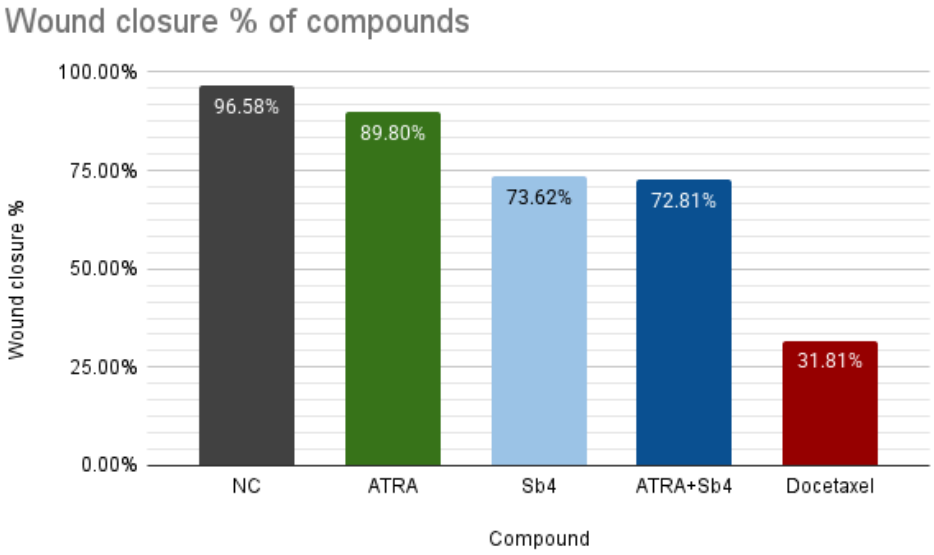



Fig. 5.10: Wound closure % of DU145 cells treated with different compounds

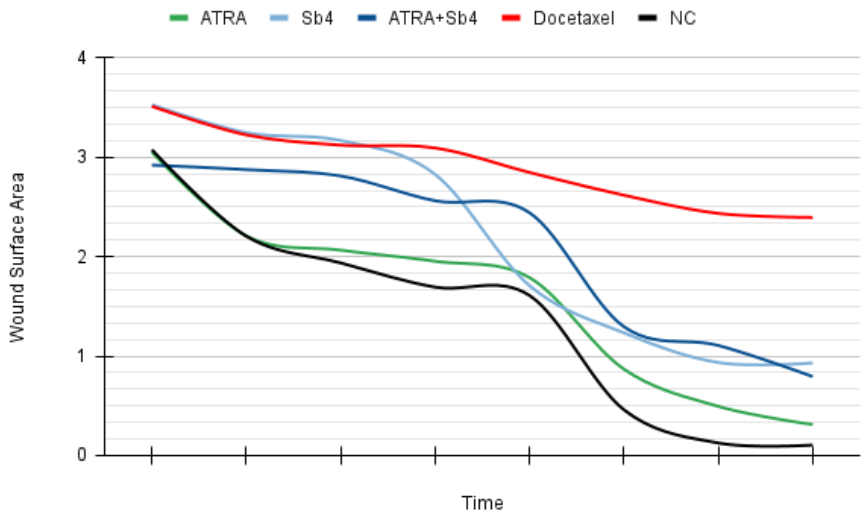



Fig. 5.11: Wound closure % of DU145 cells treated with different compounds over time

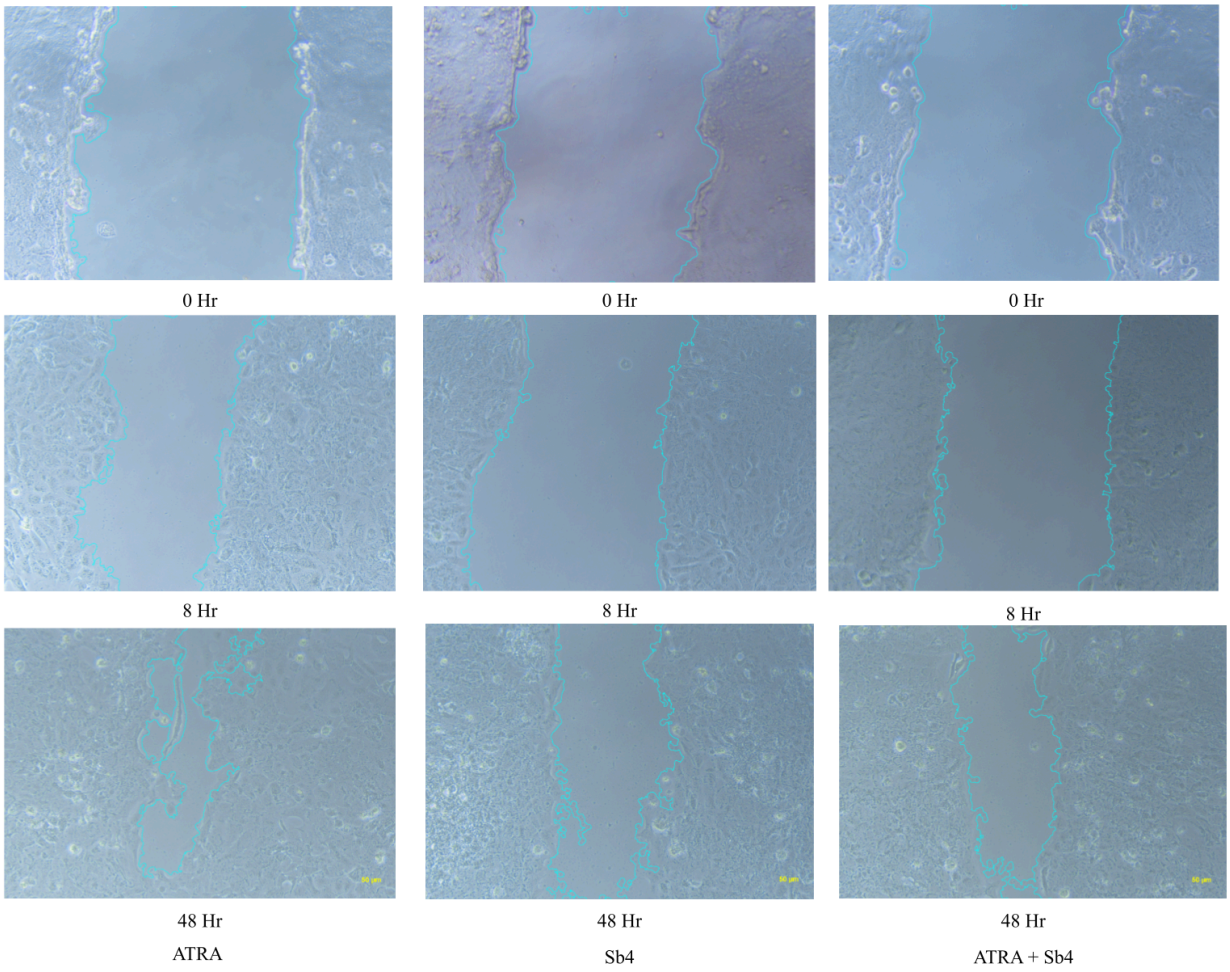



Fig 5.12: Wound closure of DU145 cells treated with different compounds

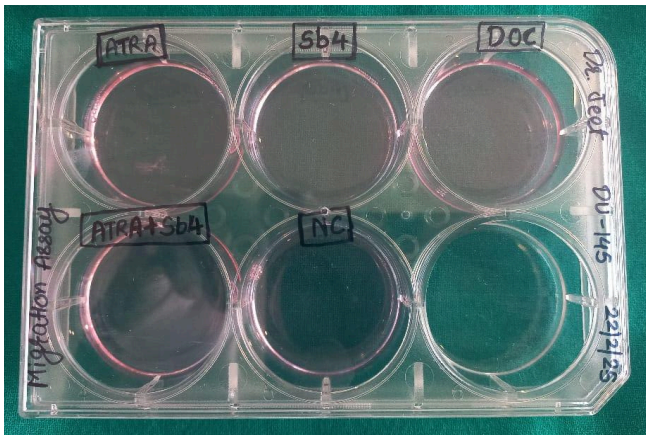



Fig 5.13: Migration assay 6 well plate

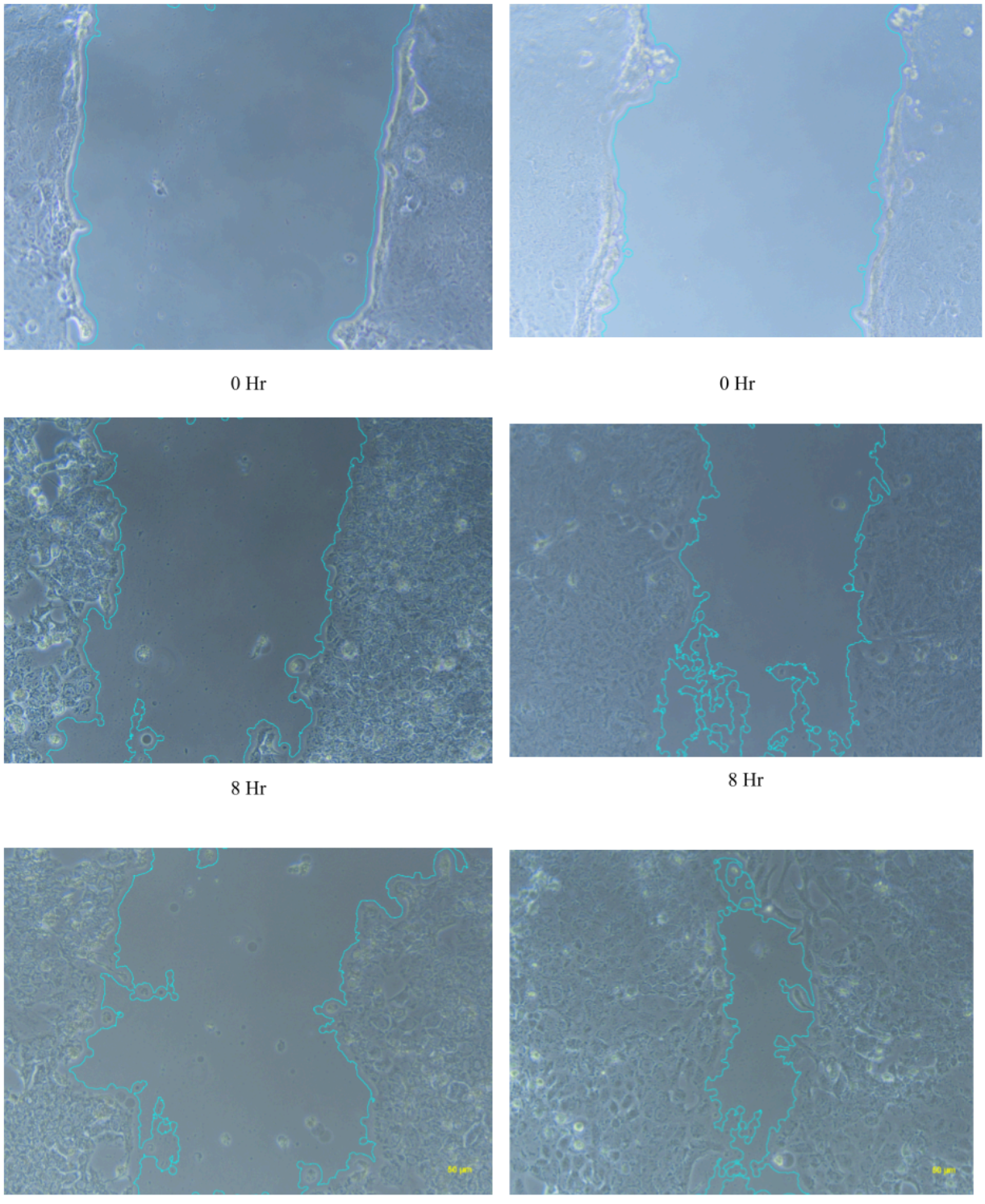
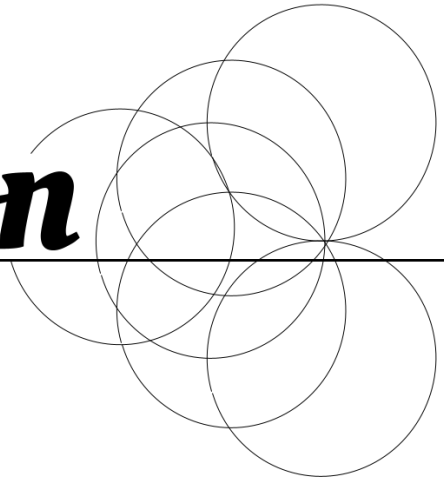



Fig. 5.14: Wound closure of DU145 cells treated with different Docetaxel and negative control

The results of migration assay are shown in table table 5.5. The migration assay was done to assess the effects on the test compounds on cellular motility and migration. The negative control showed 96.58% wound closure at the end of 48 hrs. The cells treated with ATRA (1 µM) showed 89.8% wound closure at the end of the assay. The treatment with Sb4 (1 µM) was found to have 73.62% wound closure and the combination of ATRA+Sb4 (1 µM) had 72.81% wound closure. Wound closure of Docetaxel was lowest at 31.81%. One way ANOVA was done to compare the effect of different compounds on percentage of wound closure. It was found that there was a statistically significant difference between different compounds in percentage of wound closure (F=3.28, p-value=0.021) (Table 5.5) (Fig. 5.10 & 5.11). The monolayer with the wound and its closure over time in presence of the compounds can be appreciated. (Fig. 5.12 & 5.14)

# Discussion



Prostate cancer contributes significantly to the burden of cancer among men worldwide. The condition doesn't become apparent until later stages. Primary examinations such as digital rectal examination may also be negative. Prostate specific antigen (PSA) and biopsies are more reliable for diagnosis. Although there are many interventions available, they have their limitations.[33] Surgical intervention via radical prostatectomy is effective for localized prostate cancer. Androgen deprivation therapy (ADT) is more helpful when there is metastatic spread, but even then, resistance to this approach develops, like castration resistant prostate cancer.[37,9] Radiotherapy and Chemotherapy with docetaxel is effective but high grade aggressive tumors are known to develop resistance to these approaches as well.[80-83]

Cancer stem cells or cancer initiating cells are cells with certain characteristics of adult stem cells, they are also known to contribute to resistance from chemotherapy and radiotherapy,[65] along with their resistance to therapy, they are also known to contribute to higher grade tumors with poor prognosis.[49,54] One approach to treatment focusing on cancer stem cells is by inducing differentiation of these cells to a type of cell that is more responsive to standard therapies, it has shown promising results,[11] although there is much to be understood regarding the mechanisms governing this approach.

All-trans retinoic acid (ATRA) is a compound that is known as an inducer of differentiation in many physiologic conditions[90] as well as in cancers. It is also used clinically in cases with acute promyelocytic leukemia and it shows better responsiveness to chemotherapy and lower rates of recurrence.[93] ATRA acts by binding to retinoic acid receptor and retinoid x receptor forming a heterodimer and regulating the expression of many genes.[90] There are many such signaling pathways which regulate cellular differentiation which can be targeted to control

the proliferation of cancer stem cells. Bone morphogenetic proteins (BMPs) have one such signaling pathway with a potent effect on regulation of gene transcription.[107] Many studies have been carried out in order to learn more about the contributions of BMP signaling pathway in cancer. BMP signaling is indicated as tumorigenic in many cancers, but the studies assessing the effectiveness of administration of recombinant human BMPs (rhBMPs) have found that they have a biphasic effect, with lower concentrations having endogenous BMP-like tumor promoting effects and higher concentrations (exogenous) having tumor suppressive effects.[116] This effect is also seen in prostate cancer cells with BMP administration.[125,26] However, there are many hurdles to usage of rhBMP for therapeutic purposes, principal among them being its high cost, which also hinders the progress of research.

Sb4 is a compound that was identified by Bradford STJ et al as a potent stimulator of the BMP signaling pathway. It has shown its reliability in activating the downstream messengers of BMP pathway such as Smad dependent gene regulation in many studies.[128-129] Xu L et al have also studied its effectiveness against neuroendocrine tumors.[128] Sb4 being a more cost effective alternative provides avenues for more research and even therapeutic approaches in cancers. Therefore, this study was taken up to determine if Sb4 has any effect on the prostate cancer cell line.

This study consisted of cytotoxicity assays and a migration assay. ATRA was chosen as a positive control for its history of being recognized as a differentiation inducer. Docetaxel was chosen as the standard chemotherapeutic drug as per the clinical regimen for prostate cancer. Cytotoxicity assays were carried out to determine whether the compound has any direct cytotoxic effects on the prostate cancer cells or not, following that, the compound was assessed

for its ability to improve the efficacy of standard chemotherapeutic drug, docetaxel on DU 145 cells. Migration assay was done to determine the effectiveness of the Sb4 on cell motility. Due to budget constraints, confirmation of the shrinkage of cancer stem cell population by flow cytometry or assessment of downstream messengers like Smads and effect on gene expression could not be done, so the mechanism of its action could not be determined by observation.

First the MTT based cytotoxicity assay was done to assess the cytotoxic effects of the individual compound and compare it with cytotoxicity of ATRA and Docetaxel. This assay showed that the half-maximal inhibitory concentration (IC 50) for docetaxel was the lowest at 10.78 nM followed by Sb4 at 4.4 μM and ATRA at 99.5 μM. This suggests that the concentration of docetaxel required to eliminate 50% of the cell population is the lowest which is typical of many cytotoxic drugs. The utility of this assay was two fold, one was to identify the concentrations of Sb4 which are cytotoxic to the cells so that those concentrations can be avoided in the next assay, since the hypothesis states that Sb4 should make the cells more sensitive to the chemotherapy not exhibit cytotoxic activity by itself. Second was to determine the IC 50 value of docetaxel, since the IC 50 value of a compound may not have reliable external validity, as it relies significantly on experimental conditions such as cell specifications, assay format, and duration of the assay. Thus, IC 50 values obtained in one study may not be directly comparable to those from different experiments or in vivo conditions. Once the IC 50 concentration of docetaxel is identified it can be used as a static baseline to determine whether the test compound has any effect on its cytotoxic efficacy.

In the second phase MTT assay was done to determine the effects of test compound Sb4 on the cytotoxicity of docetaxel. Since ATRA and Sb4 are known to have different mechanisms

of action, combinations of both compounds were attempted to determine whether they are more beneficial.

The results show that the treatment with docetaxel alone yields 45.97% cell viability which is not surprising considering the concentration used was IC 50. The cells treated with ATRA at both high and low concentrations show marginal reduction in cell viability, meaning the cytotoxic effect of docetaxel was augmented to some extent. Similar results were reported by Petrie K et al in 2020 which showed that higher concentrations of ATRA are cytotoxic to prostate cancer cell lines, but the lower concentrations ($10^{-10}$ M) may help in proliferation of cells.[131] Given the lowest concentration used in the present study was $10^{-7}$ M one can not expect to find such proliferative effects, although the cytotoxic effects of ATRA on DU 145 cells reported by Petrie K et al was lowest, here it did show lower cell viability at 41.59% when in combination with docetaxel.

The wells treated with Sb4 at high concentration (1 μM) and docetaxel (IC 50) showed viability at 35.44%. While the wells treated with Sb4 at low concentration (100 nM) and docetaxel (IC 50) showed viability at 54.17%. At high concentration, the cell viability reduces drastically, this degree of cytotoxicity is not seen in the treatment of the cells with Sb4 alone. This implies that treatment with Sb4 enhanced the cytotoxic effect of docetaxel. This could be due to the activation of the BMP signaling pathway by Sb4, which increases the phosphorylation of the Smad-dependent pathway. This could lead to differentiation of the chemoresistant CSCs, similar to what Lombardo Y et al reported with administration of BMP4 in colorectal cancer.[118] However, there is no evidence suggesting that the increased sensitivity of the cells is due to differentiation. There may be other mechanisms other than differentiation which contribute to

this cytotoxicity like autophagy induced cell death which was reported by Xu L et al.[128] At low concentration however, the cell viability was increased which suggests that Sb4 can promote cell survival and proliferation. This biphasic response is similar to findings by Zhang et al who reported the lower concentrations of BMP4 being proliferative but higher concentrations contributing to cytotoxicity when in combination with a cytotoxic drug.[116] This could also explain the majority of findings from literature that suggest the tumor promoting role of BMPs, the low concentrations, which mimic the endogenous BMP secretion are known to promote cell proliferation and resistance to treatment, the exact mechanism behind this phenomenon remains to be understood.

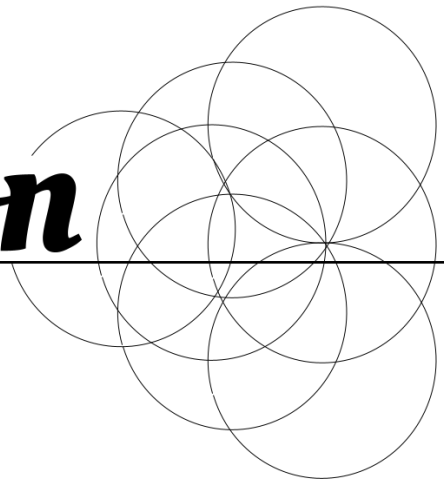
Migration assay or scratch assay was done to assess the effect of Sb4 on cell motility which is an indicator of EMT and subsequent metastatic potential. Assays like invasion assay and transwell assay are more specific for determining invasiveness of the cells, which can be pursued in future studies. Migration assay showed that percentage of wound closure for cells treated with docetaxel was 31.81% which suggests that docetaxel is a potent inhibitor of cell migration, but in a broader context, it is understood that this anti migratory effect is just a manifestation of the cytotoxic effect of docetaxel. Wound closure for ATRA was 89.80% which is not very significant. The cells treated with Sb4 showed a percentage of wound closure of 73.62 % which is not drastically lower but the difference is significant. It implies that treatment with Sb4 was able to slow the rate of migration of cancer cells compared to negative control. The combination of Sb4 and ATRA showed similar wound closure percentage; it can be suggested that in the combination, the major contributor of inhibition of migration was Sb4, since Sb4 also has a more prominent effect when treated in isolation. Buijs JT et al have also reported similar

findings with BMP administration in breast cancer, where BMP was capable of antagonizing the TGF-β induced invasion, but even in their study BMP administration did not completely halt the invasion of cancer cells.[120]

## Limitations & Future studies

This study also had some limitations, only providing basic data on the apparent effectiveness of Sb4 and its combination with standard care drugs against prostate cancer cell lines. This study did not provide evidence regarding the exact mechanism of its effect on prostate cancer cell lines. Gene expression studies with luciferase assay and estimation of proteins expressed by western blotting may help shed some light on its mechanisms. Confirmation of the differentiation effects requires isolation of cancer stem cells and assessment of CD133+ cells by flow cytometry in future studies. Other prostate cancer cell lines such as PC3 and LNCaP can be used in future studies to make a clearer conclusion. Being an in-vitro study, it also carries the limitation of requiring many further studies before it could be implemented in a clinical setting.

# Conclusion

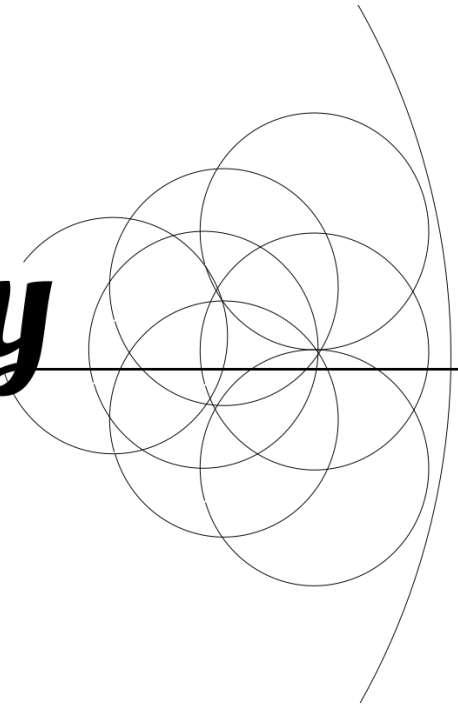


Treatment for prostate cancer includes many modalities such as surgical resection and radiotherapy for localized disease as well as androgen deprivation therapy and chemotherapy for metastatic tumors. However, many high grade tumors show resistance to most therapies and many even result in recurrence and relapse. To overcome such obstacles one approach is to target the cells responsible for the resistance to therapy and convert them into cells which are more sensitive to standard therapy. Many cellular signaling pathways could be targeted to achieve elimination of such resistant cells. BMP signaling pathway is a potent candidate for this approach. However, use of BMPs is considered only in research settings so far due to its high cost and the scarce research conducted shows inconclusive results on its effectiveness. The complex effects of BMP need to be studied more in order to better understand its role in pathophysiology and treatment of many cancers. Recently, a small molecule mimetic of BMP signaling, Sb4, was identified and has shown promising effects on a small number of studies.

This study aimed to assess the effect of Sb4 on prostate cancer cells. The prestated hypothesis was that Sb4 would increase the efficacy of the standard care chemotherapeutic drug Docetaxel. Based on the results, this study concludes that treatment of prostate cancer cells (DU 145) with high concentration Sb4 leads to an increase in efficacy of Docetaxel treatment on these cells, while low concentration Sb4 treatment increases resistance of the cells to the chemotherapy, along with its effect on cytotoxicity, it also inhibits the migratory capacity of DU 145 cells to some extent. This study did not provide evidence regarding the mechanism of action of Sb4 nor did it give evidence of induction of differentiation among the cancer cells. Future studies should seek to uncover the intricacies of Sb4 to further understand its effectiveness as

well as its unintended effects so that through it, the capability of the BMP signaling pathway can be utilized in many cancers.

# Summary



Prostate cancer is the second most common cancer among men throughout the world. Metastasis of the disease poses a difficult challenge, this arises from the resistance to chemotherapy and radiotherapy developed by a subpopulation of cancer cells, to overcome this resistance, one approach dictates that these resistant cells could be converted into more sensitive cells by inducing differentiation. One such candidate for this approach is the BMP signaling pathway. Although, not many studies have been carried out considering BMPs as therapeutic agents due to its high cost. Recently, one small molecule compound known as Sb4 has shown its effectiveness as a BMP agonist. This study aims to assess the effects of Sb4 on DU 145 prostate cancer cell line.

This study conducted MTT based cytotoxicity assays to assess the cytotoxic concentrations of Sb4 on DU 145 cells as well as assessing the response of DU 145 cells to the standard chemotherapeutic drug Docetaxel in presence of Sb4 treatment. This study also conducted a migration assay to determine whether Sb4 treatment has any effect on the cellular motility and migratory capability of DU 145 cells.

The results showed that the cells treated with Sb4 (1μM) followed by Docetaxel (10.78 nM) showed 35.44% cell viability compared to 45.97% viability when treated with Docetaxel (10.78 nM) alone, but the cells treated with Sb4 (100nM) showed increased cell viability (54.17%) ($F=12.63$, p-value= $<0.01$). The migration assay showed that when treated with Sb4, the wound closure was 73.62% compared to 96.58% in negative control ($F=3.28$, p-value=0.021).

In conclusion, this study shows the effects of Sb4 on DU 145 prostate cancer cell line. The effect of high concentration Sb4 is tumor suppressive and low concentration is tumor promoting. Further studies with Sb4 need to be carried out to understand the intricacies of the mechanisms as well as gather evidence on its mechanism of action in prostate cancer.

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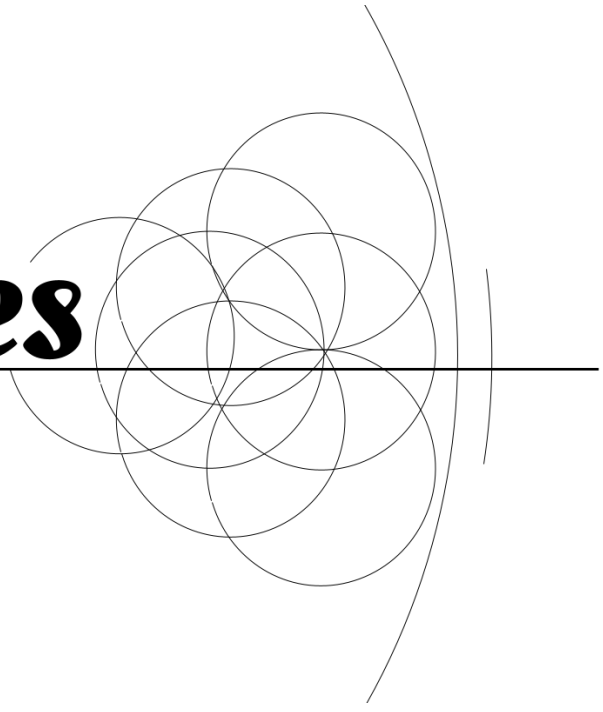

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