Impact of Urinary Tract Analgesics on Bladder Cancer Growth *In Vitro*

Thesis Submitted to Flinders University

By

Bindu Kshetri Bhattarai

Student ID: 2289223

Master of Biotechnology

College of Medicine and Public Health

19th November 2024

Supervisors

Dr. Luke Grundy, Ms. Georgia Bourlotos

TABLE OF CONTENT

ABSTRACT

Bladder cancer is the 10th most common cancer in the world. Approximately 70-75% of bladder cancers are classified as non-muscle invasive (NMIBC). The gold standard treatment for NMIBC is surgical resection of the bladder tumor. Additionally, patients with intermediate and high risk NMIBC will undergo further BCG-immunotherapy. BCG (Bacillus Calmette-Guérin), a live attenuated strain of *Mycobacterium bovis*, is the most successful immunotherapy for bladder cancer. While BCG therapy is effective in preventing cancer progression and recurrence, up to 70% of patients experience local bladder side effects including bladder pain, urinary urgency, and urinary frequency that significantly reduce quality of life during treatment. In 7-20% of patients these side effects are so severe that they require cessation of therapy. If BCG therapy ceases, limited alternative treatment options exist. Despite this, there is no standard-of-care therapy to ease NMIBC patient suffering and improve treatment adherence. Urinary tract analgesics represent a possible therapeutic option, but the impact of analgesics on bladder cancer growth is unclear. This study aimed to investigate the impact of urinary tract analgesics lidocaine and phenazopyridine in bladder cancer growth *in vitro* using mouse bladder cancer cell line (MB49). Cell proliferation was measured using an Incucyte S3, cytotoxicity measured using a nuclear stain for cell death, and metabolism measured using an XTT assay. Lidocaine and phenazopyridine (30 μM, 10 μM, 3 μM, 1 μM, 300nM) had no impact on cell proliferation ($p > 0.05$), metabolism ($p > 0.05$) or cytotoxicity ($p > 0.05$). These findings suggest that phenazopyridine and lidocaine may be a potential therapeutic option for managing the side effects of BCG-immunotherapy for NMIBC. However, further studies are required to determine the potential impacts of urinary tract analgesics on the BCG response.

Keywords: NMIBC, BCG therapy, XTT , lidocaine, phenazopyridine, MB49 cells

DECLARATION

I, Bindu Kshetri Bhattarai hereby declared that this thesis entitled "**Impact of Urinary Tract Analgesics on Bladder Cancer Growth *In Vitro*"** is entirely original and has been conducted under the guidance of my supervisor Dr Luke Grundy. It has not been submitted for any other degree without the permission of Flinders University and does not contain any material previously written or published by another person except where due reference is made in the text.

Signed: Bindu Kshetri Bhattarai

ACKNOWLEDGEMENT

I would like to express my sincere gratitude to my supervisor Dr. Luke Grundy for his support and guidance throughout my research journey. His expertise has helped me broaden my knowledge in this field. I would also like to thank to my co-supervisor Georgia Bourlotos who helped me in completing this project.

I am really grateful for my husband who emotionally supported me and motivated me throughout my research journey. Also, I am very thankful to our Topic coordinator Associate professor Alistair Standish for conducting the workshop which helped me lot while writing thesis.

LIST OF FIGURES

LIST OF ABBREVIATION

BCG: Bacillus Calmette Guerin

CNS: Central nervous system

DMEM: Dulbecco's Modified Eagle Medium

FN: Fibronectin

GM-CSF: Granulocyte-Macrophage Colony-Stimulating Factor (GM-CSF)

LUTS: lower urinary tract symptoms

MIBC: Muscle invasive bladder cancer

MHC: major histocompatibility complex class II

NMIBC: Non-muscle invasive bladder cancer

NSAID: Nonsteroidal Anti-Inflammatory Drugs

PBS: Phosphate Buffered Saline

PTEN: Phosphatase and tensin homolog

TURBT: Trans urethral resection of bladder tumor.

TNF: Tumor necrosis factor

XTT : 2,3-Bis-(2-Methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide

CHAPTER 1: INTRODUCTION

1.1 Overview:

Bladder cancer is a significant health problem worldwide. Bladder cancer is the tenth most prevalent cancer and the thirteenth leading cause of cancer deaths globally (Zhang et al., 2023). According to GLOBOCAN report of 2020, there were 573000 new cases, and 213000 deaths attributed to bladder cancer. The incidence and mortality rates of bladder cancer are higher in men than women with rates of 9.5 and 3.3 per 100,000 respectively (Sung et al., 2021). Among bladder cancer, the most prevalent cancer is transitional cell carcinoma which is also known as urothelial carcinoma which consist about 90%-95% (Yaxley, 2016). It is categorized either as muscle invasive (MIBC) or non-muscle invasive bladder cancer (NMIBC) based on the depth of invasion into the bladder wall (Lenis et al., 2020). Approximately 70-75% of bladder cancers are classified as NMIBC. The gold standard treatment for NMIBC is surgical resection of the bladder tumor. Intermediate and high risk NMIBC patients will also undergo Bacillus Calmette–Guérin (BCG) immunotherapy to reduce the risk of disease progression and recurrence (Chang et al., 2016). BCG, a live attenuated strain of *Mycobacterium bovis* is considered as the best immunotherapy for bladder cancer and perhaps the most successful microbial cancer treatment to date (Jiang & Redelman-Sidi, 2022).

NMIBC patients receiving BCG have progression-free survival rates typically falling between 60% and 80% (Durant et al., 2024). Although BCG therapy is the mainstay for the treatment of NMIBC patients its use comes with certain challenges. Most patients experience BCG induced lower urinary tract symptoms (LUTS) including increased urgency and frequency, dysuria (painful urination) and pelvic pain (Alexandroff et al., 1999). Due to these side effects, up to 20% of patients undergoing BCG-immunotherapy discontinue the treatment (Liu et al., 2019; Sharma et al., 2020). Despite this, there is no standard care therapy to ease patient suffering and improve therapy persistence in NMIBC patients undergoing BCG therapy.

Bladder sensation plays an important role in maintaining healthy functioning of bladder. During bladder filling, the bladder wall stretches to accommodate an increase in urine volume (Eastham & Gillespie, 2013). At this time sensory afferent nerves innervating throughout the bladder wall are activated, and sensory signals are sent to the CNS (central nervous system) to alert us of the degree of bladder fullness (de Groat & Yoshimura, 2009). As the urine volume increases so does

the intensity of bladder sensation. These sensory afferent nerves are spread throughout the detrusor, lamina propria and urothelium of the bladder wall (Grundy et al., 2024). Exaggerated sensory signaling from the bladder leads to changes in normal function and sensation which lead to the development of LUTS including bladder pain and overactive bladder (Grundy et al., 2024).

To manage LUTS associated with BCG therapy, directly targeting bladder sensory nerves with urinary tract analgesics may be useful therapeutic strategy. There are different kinds of analgesics recommended by The European Association of Urology (EAU) such as acetaminophen, NSAIDS, or phenazopyridine. However, there is limited information available on the potential impact of these analgesics on the bladder cancer growth and progression or impacts on BCG efficacy. So, our aim is to address these knowledge gaps by investigating the effects of analgesics on bladder cancer cell growth in-vitro.

1.2 Hypothesis and Aims:

We hypothesize that urinary tract analgesics will be effective in relieving BCG-induced LUTS in NMIBC patients. We hypothesize that urinary tract analgesics will not have significant impacts on bladder cancer cell proliferation.

General aim

To confirm the safety of urinary tract analgesics on the proliferation of bladder cancer cells and their compatibility with BCG therapy in an *in vitro* setting.

Specific aims

1. To evaluate the effects of urinary tract analgesics on bladder cancer cell proliferation invitro.
2. To determine the effects of urinary tract analgesics on bladder cancer cell viability and metabolism.

1.3. Literature review

Bladder cancer: An overview

Bladder cancer, also known as urinary bladder cancer is the tenth common cancer globally and its incidence is rapidly increasing worldwide (Sung et al., 2021). Bladder cancer can range in severity from very low-grade tumors with low mortality to very high-grade tumors with high mortality. It is clearly correlated with exposure to the environment, including smoking. Selecting the best course of treatment requires accurate staging and grading (Sharma et al., 2009) .

1.3.1 Epidemiology:

According to GLOBOCAN data, 573,000 new cases of bladder cancer have been reported in 2020. The highest incidence of bladder cancer is found in Western and Southern Europe where Greece has the highest rates of bladder cancer in men and Hungary has highest rate in women in the world (Sung et al., 2021) . It is less common in central Asia, Western and Middle Africa and this correlated with the reduced exposure of industrial chemicals and tobacco (Bray et al., 2018; Sung et al., 2021). Globally, the incidence rate of bladder cancer is 9.5 per 10000 in men while women have rates of 3.3 per 10000 which means it is four times more common in men than women. Although bladder cancer ranks tenth globally in terms of frequency of occurrence, it ranks thirteenth in terms of fatality (Sung et al., 2021) with a 212,536 expected deaths in 2020 alone, representing 2.1% of all cancer-related deaths (Ng, 2022). This indicates that bladder cancer remains one of the main causes of cancer related death (Bray et al., 2018; Ng, 2022). Bladder cancer is predominantly found in older populations, with the highest prevalence among individuals aged above 60 years (Sharma et al., 2009). While the incidence is higher in white individuals, delayed diagnosis contributes to higher mortality rates among black individuals (Sharma et al., 2009).

1.3.2 Risk factors:

The principle risk factor of bladder cancer by far is cigarette smoking, accounting for about 50-60% of new cases annually (Freedman et al., 2011). Freedman et al., (2011) reported that smoking increases the risk of bladder cancer by three to four times. After smoking, the second most significant risk factor is occupational exposure to carcinogenic chemicals such as aromatic amines, chlorinated hydrocarbons and polycyclic aromatic hydrocarbons and is believed that 18% of all bladder cancers are associated with this (Saginala et al., 2020). These classes of compound are

used for manufacturing dyes, rubber, paint or petroleum products in industries (Chen et al., 2005; Saginala et al., 2020). Another important risk factor is age, with bladder cancer significantly more prevalent over the age of 55. Other factors including chronic inflammation of the bladder and certain genetic factors are linked to an increase in bladder cancer (Saginala et al., 2020).

1.3.3 Bladder cancer stages:

The bladder is responsible for storing urine that is received from the kidneys through uterus until it is expelled during urination. There are different layers which together form the bladder wall. The inner most layer is known as urothelium and the layer beneath the urothelium which consists of nerves and blood vessels is the lamina propria and the third major layer of tissue is known as smooth muscle (Pashos et al., 2002).

Approximately 75% of bladder cancers are characterized as non-muscle invasive bladder cancer which are confined with the first two layers of bladder i.e., lamina propria and urothelium (Ta or T1 stage) as shown in **Figure 1** and the remaining tumors are either present as muscle invasive or metastatic (**Figure 1**) (Compérat et al., 2019; Kamat et al., 2016).

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1.3.4 Non muscle invasive bladder cancer (NMIBC) and its treatment

It has been reported that 75% of bladder cancer is diagnosed as NMIBC which is also known as superficial cancer. Tumors that are restricted to the urothelium (stage Ta) or lamina propria (stage T1) are considered as NMIBC. Remaining patients have either muscle invasive bladder cancer (MIBC) or metastatic bladder cancer (Kamat et al., 2016). There are 3 different types of NMIBC which are divided as low risk, intermediate and high risk based on likelihood of progression and recurrence according to European Association of Urology (EAU) (Babjuk et al., 2019). The standard treatment for low risk NMIBC is only through transurethral resection of bladder tumor (TURBT) and for intermediate and high risk, the recommended treatment is TURBT followed by Bacillus Calmette-Guérin (BCG) immunotherapy (Babjuk et al., 2019). In contrast, MIBC are treated with radical cystectomy, chemotherapy and/or radiotherapy (Babjuk et al., 2019; McConkey & Lerner, 2019). Intravesical BCG-immunotherapy once weekly for 6 weeks is considered as a standard treatment for patients with intermediate- and high-grade NMIBC (Nargund et al., 2012). Depending upon the stage of cancer, weekly BCG via intravesical instillation is proven to have 55-75% of success rate in prevention of tumor recurrence (Nargund et al., 2012; Pettenati & Ingersoll, 2018; Tang & Chang, 2015; Thyavihally et al., 2022). Further maintenance BCG therapy is advised for patients sensitive to BCG, in order to maintain the effect of tumor suppression. There are specific maintenance protocols depending upon the countries, however the most common protocol followed is Southwest Oncology Group (SWOG) that recommends 3 times installations of BCG at 3 and 6 months, then every 6 months up to 3yrs post induction (Kamat et al., 2015).

1.3.5. Mechanisms of BCG

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1.3.5.1 BCG attachment to urothelial cells

After the instillation of BCG into bladder, it attaches to the bladder urothelium, and this is facilitated by fibronectin (FN) which can be found in urine in soluble form. Through the fibronectin attachment protein, BCG attaches to urothelial cells via the α5β1 receptor. However, this attachment was observed in the areas where urothelium is damaged and not healthy urothelial cells (Pettenati & Ingersoll, 2018; Redelman-Sidi et al., 2014).

1.3.5.2 BCG internalization by bladder cancer cells

The internalization of BCG typically occurs through phagocytosis with the help of macrophages (Pettenati & Ingersoll, 2018; Redelman-Sidi et al., 2014). The macrophages and neutrophils are the only ones which are capable of phagocytosis. Therefore, it is surprising that bladder cancer which originated from the non-phagocytic epithelial cells can internalize BCG. The other mechanism is macropinocytosis which can encompass the larger particles, for example in dendritic cells and can be induced in other cell types such as epithelial cells. The internalization of BCG through macropinocytosis relies on particular oncogenic alterations. The uptake of BCG increase in different cell lines such as the cell line carrying the activating mutation of RAS family or the cell lines that harbor the deletion of PTEN (Pettenati & Ingersoll, 2018; Redelman-Sidi et al., 2014). Additionally, it is possible to make cell lines resistant to BCG uptake responsive by inducing oncogenic RAS or knocking down PTEN, suggesting that the activation of these oncogenic genes makes the bladder cancer cells sensitive to BCG (Pettenati & Ingersoll, 2018; Redelman-Sidi et al., 2014).

1.3.5.3 Immune response

After internalizing BCG, bladder cancer cells can directly secrete immune-activating effectors such as IL-6, IL-8, Granulocyte-Macrophage Colony-Stimulating Factor (GM-CSF), and tumor necrosis factor (TNF) (Pettenati & Ingersoll, 2018; Redelman-Sidi et al., 2014). Cytokines are released when bladder cancer cells are exposed to BCG. IL-6 has been shown to be released (Esuvaranathan et al., 1995) which aids in recruiting neutrophils to cancer sites and further promotes BCG binding via the upregulation of integrin α5β1 (Pettenati & Ingersoll, 2018; Redelman-Sidi et al., 2014).

After BCG exposure, bladder cancer cells can act as antigen-presenting cells. Bladder cancer cells activated by IFN-γ have the ability to expose CD4+ T cells to BCG antigens (Pettenati & Ingersoll, 2018; Redelman-Sidi et al., 2014). Early findings showed that BCG therapy causes bladder cancer cells from patients to express higher levels of major histocompatibility complex class II (MHC-II) and ICAM-1 (intercellular adhesion molecule) (El-Demiry et al., 1987; Jackson et al., 1994; Prescott et al., 1989). In a recent study, it has been reported that bladder cancer cells internalizing BCG causes this upregulation (Ikeda et al., 2002). The immune response to BCG leads to direct immune mediated cytotoxicity of bladder cancer cells.

1.3.6 BCG induced lower urinary tract symptoms

BCG therapy is the cornerstone for the management of NMIBC, but it is associated with several adverse side effects. In the first 48 hours after each infusion, about 70-80% of patients report experiencing lower urinary tract symptoms including urinary frequency, urgency, dysuria and pelvic pain (Alexandroff et al., 1999; Green et al., 2019; Guallar-Garrido & Julián, 2020; Unsworth-White et al., 2021). In most cases, LUTS can be locally managed and is considered an indication of the induction of an effective immune response. However, LUTS can dramatically impact patient quality of life during treatment and up to 20% of patients cease BCG treatments due to these adverse side effects (Liu et al., 2019; Sharma et al., 2020). It BCG-immunotherapy ceases, patients will be recommended for radical cystectomy to prevent chances of tumor progression and will experience permanent changes in their quality of life (Aldousari & Kassouf, 2010; Waked et al., 2020).

1.3.7 Mechanism behind BCG induced lower urinary tract symptoms

The mechanisms underlying the development of LUTS following BCG-immunotherapy have yet to be comprehensively explored, however, several possibilities exist that are introduced below:

1.3.7.1 Bladder afferent sensitization:

When sensory afferents come into contact with the molecular factors such as ATP (adenosine triphosphate, nerve growth factor (NGF), inflammatory cytokines and chemokines then it leads to development of afferent sensitization (Chu et al., 2020). Afferent sensitization plays an important role in providing useful sensory information regarding tissue damage and inflammation to change behavior and favors tissue healing (Baral et al., 2019). Different kinds of inflammatory mediators are responsible for the sensitization of bladder afferents, leading to enhanced peripheral signaling from the bladder to central nervous system (Brierley et al., 2020; de Groat & Yoshimura, 2009). During the pathophysiological condition, the bladder afferents provide high amount of signals to central nervous system for lower body capacities thus leading to exaggerated bladder sensations that are a key component of LUTS (Yoshimura et al., 2014).

1.3.7.2 Bladder inflammation:

Bladder inflammation plays an important role in the development of various bladder disorders such as bladder pain, urinary tract infections and is one of the major modulators of bladder hypersensitivity (Grundy et al., 2018; Mills et al., 2020). After the instillation of intravesical BCG,

it generates an immune response characterized by the infiltration of dendritic cells, macrophages and neutrophils (Simons et al., 2008). Additionally, patients receiving the BCG treatment have higher concentration of cytokines (IL-2, IL-6, IL-8, IL-18, IL-1ra, IFN-γ, IL-12[p70], TNF-a, GM-CSF), chemokines, CD4+ and CD8+ T cells present in the urine (Redelman-Sidi et al., 2014; Taniguchi et al., 1999). As there is continuation of BCG infusion then various cytokines and inflammatory markers are found in higher concentration in urine creating a sustained environment which is necessary for BCG efficacy and may further sensitize bladder-innervating afferents (Ingersoll & Albert, 2013; Simons et al., 2008).

1.3.7.3 Urothelial permeability

The urothelial barrier prevents the passage of urine and molecules into the underling tissue (Jafari & Rohn, 2022). Whenever the urothelial barrier is experimentally comprised it can lead to bladder afferent sensitization (Grundy et al., 2020). Clinically, it has been found that increased permeability of the urothelium is linked to LUTS, which is considered to be consequence of allowing urine to reach and active/sensitize afferent terminals in the suburothelium (Grundy et al., 2020; Hurst et al., 2015). However, there are no studies where BCG treatment is directly linked to breakdown of urothelial barrier, but it is thought that it may act indirectly as a result of inflammation for urothelial permeability (Grover et al., 2011; Redelman-Sidi et al., 2014). Additionally, when BCG is instilled intravesically then it releases higher concentration of cytokine in urine which may indirectly increase the permeability of urothelium.

1.3.8 Urinary tract analgesics for BCG induced LUTS

1.3.8.1 Non-steroidal inflammatory drugs (NSAID):

It has been suggested that NSAID helps in the onset of lower urinary tract symptoms. There are some studies which reported that there is a correlation between C-reactive protein levels and LUTS in both men and women (Kupelian et al., 2009; Rohrmann et al., 2005; St. Sauver et al., 2009). However, in men the prostatic inflammatory infiltrates have also been linked to acute urine retention and LUTS (St. Sauver & Jacobsen, 2008). Some additional studies also reported that NSAID inhibits cyclooxygenase 2 enzymes reducing the prostaglandins synthesis which helps to reduce the inflammatory response induced by BCG (Gruenenfelder et al., 2002). NSAID such as aspirin has been reported as an effective in preventing bladder and skin cancer (Fischer et al., 2011).

1.3.8.2 Phenazopyridine

Phenazopyridine is a urinary tract analgesic which is used to treat the symptoms of urinary tract infections (Eastham & Patel, 2022). According to US Food and Drug Administration (FDA) this drug was approved to treat patients with dysuria, urinary frequency, urgency and related pain associated with lower UTIs (Eastham & Patel, 2022). Phenazopyridine also helps in relieving the pain that arises from bladder surgery or cystoscopy procedures any of which may irritate the urinary tract's epithelial lining (Eastham & Patel, 2022). It is available both over the counter or as prescription (Pergialiotis et al., 2012). The exact mechanism underlying the clinical efficacy of phenazopyridine are not widely understood, but it is believed that it helps to alleviate discomfort in the urinary tract lining via interactions with the transient receptor potential channel (TRPM8) (Luyts et al., 2023). Studies suggested that this medication suppresses the bladder's nerve fibers that react to mechanical stimulation (Preynat-Seauve et al., 2021). Furthermore, this medication also inhibits the kinases involved in metabolism and cell proliferation (Aizawa & Wyndaele, 2010). It is generally considered safe with no severe adverse side effects when taken for short durations. Not following dosing and duration guidelines or taking an overdose, can be life threatening (Patel, 2024).

1.3.8.3 Lidocaine

Lidocaine is a local anesthetic which is used to inhibit the sensation of pain via direct interactions with sensory nerves. It consists of two types of subunits hydrophilic and lipophilic which are linked by hydrocarbon chain. The lipophilic portion is involved in the anesthetic activity (Karnina et al., 2021). This anesthetic reduces the membrane potential of sensory nerves by acting on sodium ion channels which blocks the depolarization and prevents the transmission of pain signal (Karnina et al., 2021).

Due to its potent anesthetic properties, rapid onset and broad safety profile, it has been used in various medical procedures. Furthermore, lidocaine can be administered through multiple routes such as perineural, epidural, intravenous, subcutaneous and intrathecal (Karnina et al., 2021). Besides its anesthetic procedure, it also acts as an analgesic which is used for treating acute and chronic pain, anti-inflammatory agent to protect against lung injury and to reduce the postoperative cognitive dysfunction (POCD). Moreover, the lidocaine has a promising anticancer property which

acts as a sensitizer on cancer drugs (Yang et al., 2018). Butrick et al., (2009) reported that lidocaine was most effective in alleviating the bladder pain in patients than heparin and triamcinolone.

Hypothesis:

We hypothesize that urinary tract analgesics will be effective in relieving BCG-induced LUTS in NMIBC patients. We hypothesize that urinary tract analgesics will not have significant impacts on bladder cancer cell proliferation.

Specific aims

1. To evaluate the effects of urinary tract analgesics on bladder cancer cell proliferation invitro.
2. To determine the effects of urinary tract analgesics on bladder cancer cell viability and metabolism.

CHAPTER 2: MATERIALS AND METHODS

2.1 Cell Lines and maintenance:

The murine bladder cancer cell line (MB49) was obtained from Sigma-Aldrich in a vial which contained $\geq 1\times10^6$ viable cells. The cells were stored in a liquid nitrogen until thawing. The stable cell lines were thawed and grown as per the manufacturer's instructions in Dulbecco's modified Eagle's medium high Glucose (Sigma Cat. No. D5796) with 10% FBS (Cat. No. ES-009-B) and 1X Penicillin/Streptomycin (optional; Cat. No. TMS-AB2- C).

2.1.1 Thawing Cells:

MB49 cells were thawed in incubator at 37° C and transferred into 15ml conical tube using a 1ml pipette in a laminar hood. Then, 9ml of DMEM with 10% FBS was added dropwise to avoid any bubbles in the tube followed by gently mixing. The cells were then centrifuged for 3 minutes at 300 RCF and the supernatant was decanted carefully. The cells were resuspended in 10ml of DMEM media and transferred into T75 tissue culture flask and incubated at 37° C with 5% CO_2. The following day, the media was exchanged with 10 ml of fresh DMEM medium, and cells were allowed to grow to confluence before further passage.

2.1.2 Subculturing of MB49 cells:

At 80-90% confluence, MB49 cells were subculture to allow continuous growth of cells and maintain cell health and growth characteristics. The floating cells were carefully collected in a 50 ml conical tube by aspirating all media in the flask. Cells were centrifuged at 300 RCF for 3 minutes. The supernatant was discarded, and the cell pellet was resuspended in 2ml of media and set aside. The T75 flask was rinsed twice with 10 ml 1X PBS (Cat. No. D8537) followed by 4ml of 0.25% trypsin (Cat. No. SM-2003-C) and incubated at 37° C with 5% CO_2 for 4 minutes. After incubation, cells were detached from the flask base and 8ml of media was added into the flask before transfer into a 15 ml conical tube and centrifuged for 4 minutes at 300 RCF. The supernatant was discarded, and the cell pellet was resuspended in the 2 ml of floating cells that was kept aside. Then 20 µl of trypan blue and 20 µl of cell suspension was mixed well in an Eppendorf tube and 10 µl of mixed cells suspension was placed on a hemocytometer and counted. Cells were diluted to a concentration of 1×10^6 cells/ml with fresh media and resuspended in 9ml of media and incubated at 37° C with 5% CO_2. Cells were passaged every 2-3 days based on confluence until required for experiments.

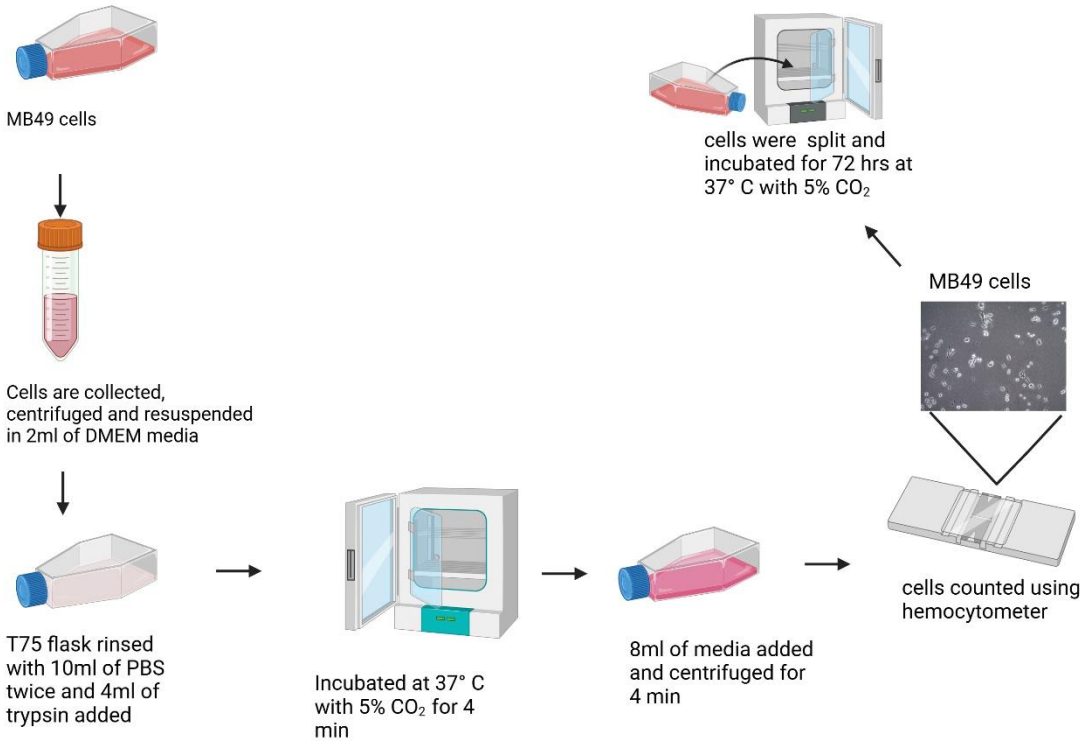

Figure 3 : Procedure of subculturing of MB49 cells. Created from BioRender.com

2.1.3 Preparation of different cell concentration and cell seeding:

To understand the growth characteristics of MB49 cells, we tested different seeding densities of cells. From the 1×10^6 cells/ml suspension, different concentrations of MB49 cells were prepared for seeding into 96 well plates at 50,000, 25000, 12500, 6250, 3125 and 1562 cells/ml.

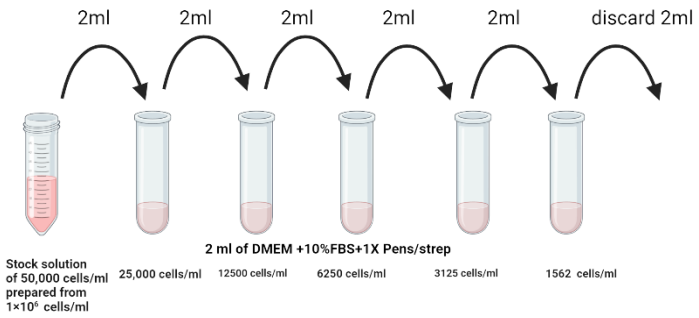

Figure 4: Procedure for preparation of different MB49 cell concentrations. Created from BioRender.com

2.2 Incucyte

2.2.1 Growth Curve:

MB49 cells were seeded into 96 well plates at concentrations of 50,000, 25000, 12500, 6250, 3125 and 1562 cells/ml to assess differences in growth rate for different cell concentrations. Our aim was to identify the cell concentration that generated a robust exponential growth curve that reached 80-90% confluence over 3 days to match cell proliferation rates during subculture. Each cell concentration was plated in 10 wells of a 96 well plate and incubated at 37° C with 5 % CO_2 for 18 hours. 18 hours after plating, the cells were placed into an Incucyte®S3 system for subsequent analysis of cell growth over time. The Incucyte®S3 generated phase contrast images every 6 hours over a 3-day period and these images were analyzed by Incucyte software to determine the cell confluence in percentage at different time points. The cell concentration that showed consistent generation of an exponential growth curve was selected for further experiments.

2.2.2 Drug dilutions

Phenazopyridine (**Cas No**. 136-40-3) and lidocaine (**Cas No**. 6108-05-0) stock solutions of 1mM and 10mM were prepared in distilled water. From the stock solution, different stock concentrations of 30 µM, 10 µM, 3 µM, 1 µM, 300nM were prepared in expansion media by serial dilution. Drugs were added 18hrs after cell seeding directly into the wells containing cells.

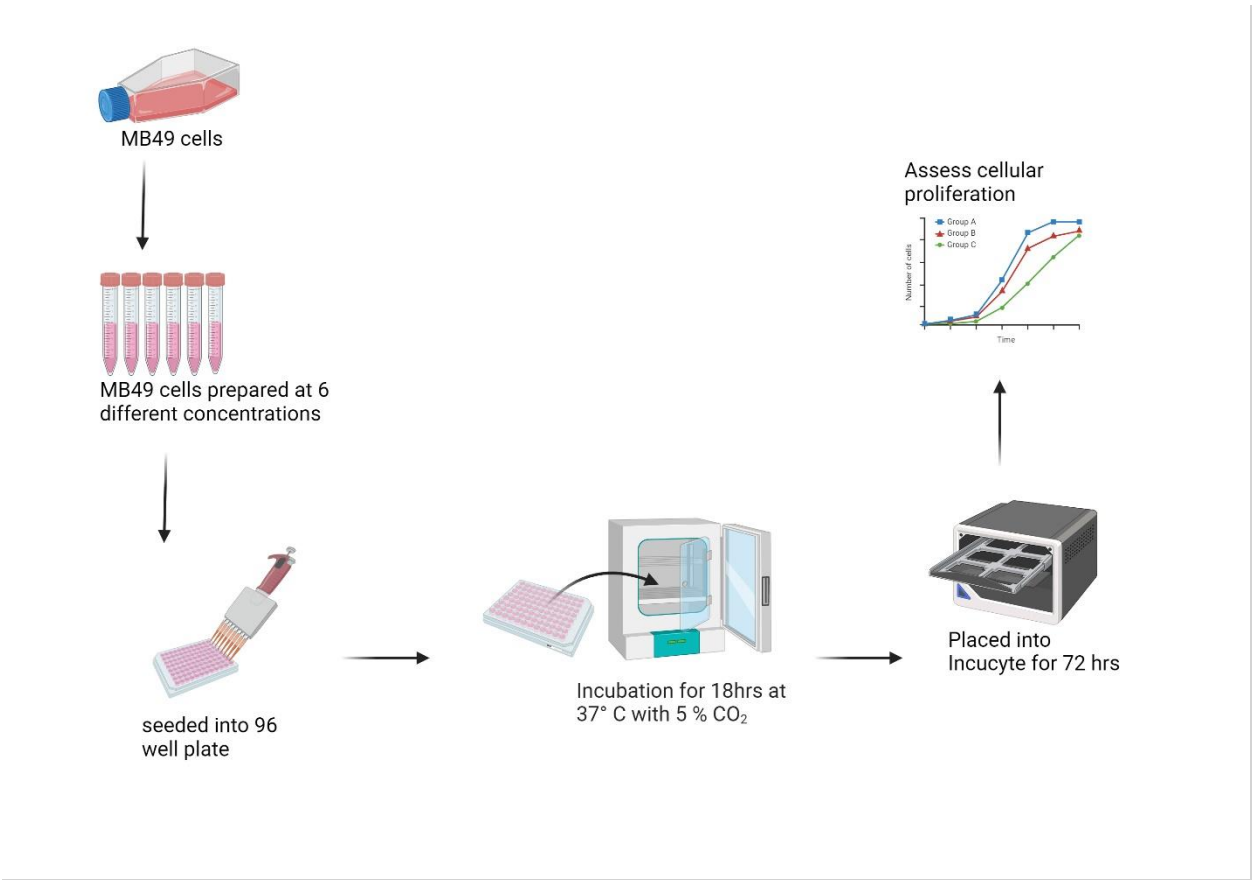

Figure 5. Procedure for determining the optimal cell concentration via growth curve analysis. Created from BioRender.com

From the above procedure, we selected 25000 cells/ml as the seeding density for all subsequent experiments testing analgesics. To setup plates for the Incucyte®S3, 10 ml of 25,000 cells/ml solution was prepared at 1×10^6 cells/ml and 10 µl of Sytox fluorescent nucleic acid stain (SYTOX™ Green Nucleic Acid Stain – Invitrogen S7020). Sytox green exhibits fluorescent upon binding nucleic acids and is an indicator of cell death. For cell seeding, 150 µl of 25,000 cells/ml solution was added to each well of a 96 well plate and placed in an incubator for 18hrs. At this time point the plate was removed from the incubator and 15 µl of either lidocaine (30µM, 10µM, 3 µM, 1µM, 300nM) or phenazopyridine (30µM, 10 µM, 3 µM, 1 µM, 300nM) were added in each row except a control row which received no drug. The plate was placed into the Incucyte®S3 where phase and green contrast images were taken every 6 hours to quantify the cell confluence in percentage at different time intervals for 72 hours. The Incucyte software was used to analyze the images and generate the growth curve for drugs treated cells and control.

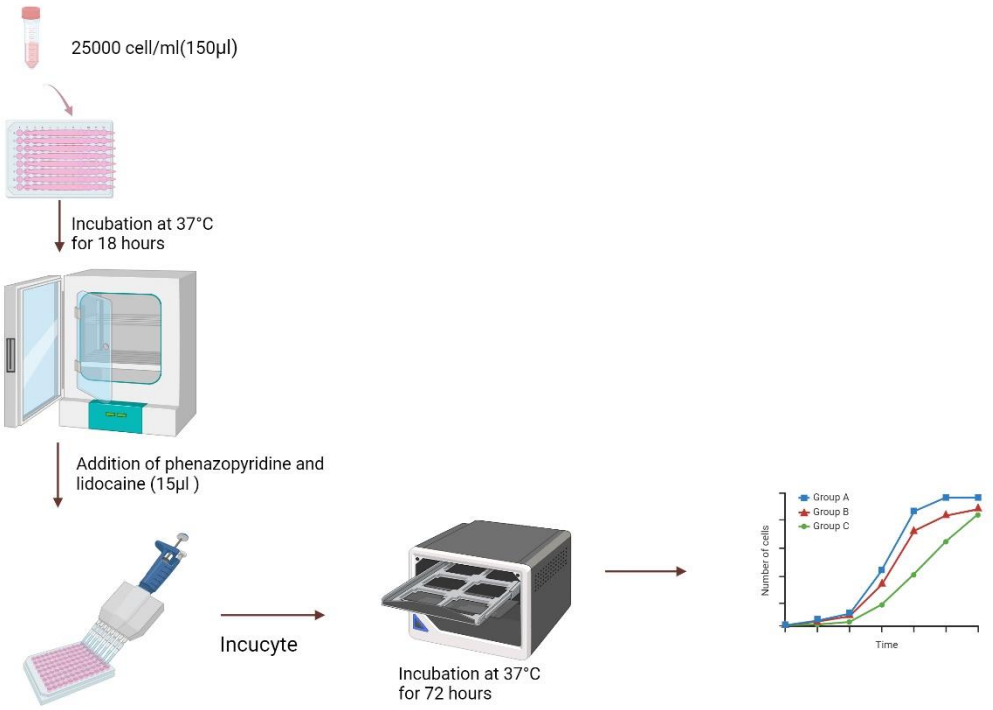

Figure 6:: Steps involved in measuring the cellular proliferation of MB49 cells with different concentrations of lidocaine and phenazopyridine. Created from BioRender.com

2.3 XTT assay:

In XTT assay, viable cells change the yellow color of XTT into orange color formazan product due to the presence of mitochondrial dehydrogenase enzyme in the live cells. Spectrophotometer is used to estimate the cell viability by measuring the amount of formazan product (Wang et al., 2011)

2.3.1 Preparation of XTT working solution:

CyQUANT XTT cell viability assay kit **(Cat. No. X12223**) consists of one vial of electron coupling reagent and one bottle of XTT reagent. The electron coupling reagent was thawed by keeping at room temperature while XTT reagent was thawed by keeping at water bath at 37°C and vortexed to ensure they are fully in solution. Then, 1 ml of electron coupling reagent and 6 ml of XTT reagent are mixed in a 15 ml tube and vortexed to ensure proper mixing. The solution was used immediately after preparation.

2.3.2 Growth curve:

MB49 cells were prepared at 75000, 37500, 18750, 9375 and 4678.5 and 2339.25 cells/ml and seeded into 96 well plate with 100 µl of 1X PBS in surrounding well. Each 100 µl of cell concentrations were plated in each of the 10 wells and incubated at 37° C with 5 % CO_2 for 18 hours.

The next day, cell metabolism was determined by XTT (2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide) assay. 70 µl of XTT reagent was added to each well containing cells and incubated at 37°C for 4 hours. Then the absorbance was measured at 450 nm using a SpectraMax iD5 Plate Reader.

From the above procedure, we selected 37500 cells/ml as our seeding density for future experiments. For the XTT assay, 37500 cells/ml cell suspension was prepared from 1×10^6 cells/ml. For cell seeding, 100 µl of 37500 cells/ml was added into 96 well plates except blank (media only) and incubated at 37°C for 18 hours.

The next day different lidocaine and phenazopyridine were added at 30 µM, 10 µM, 3 µM, 1 µM, 300nM into sequential rows except the control and blank wells. After drug addition, 70 µl of XTT reagent was added into all of wells and the plate was then incubated at 37°C for 4 hours. Absorbance was measured at 450 nm using a SpectraMax iD5 Plate Reader.

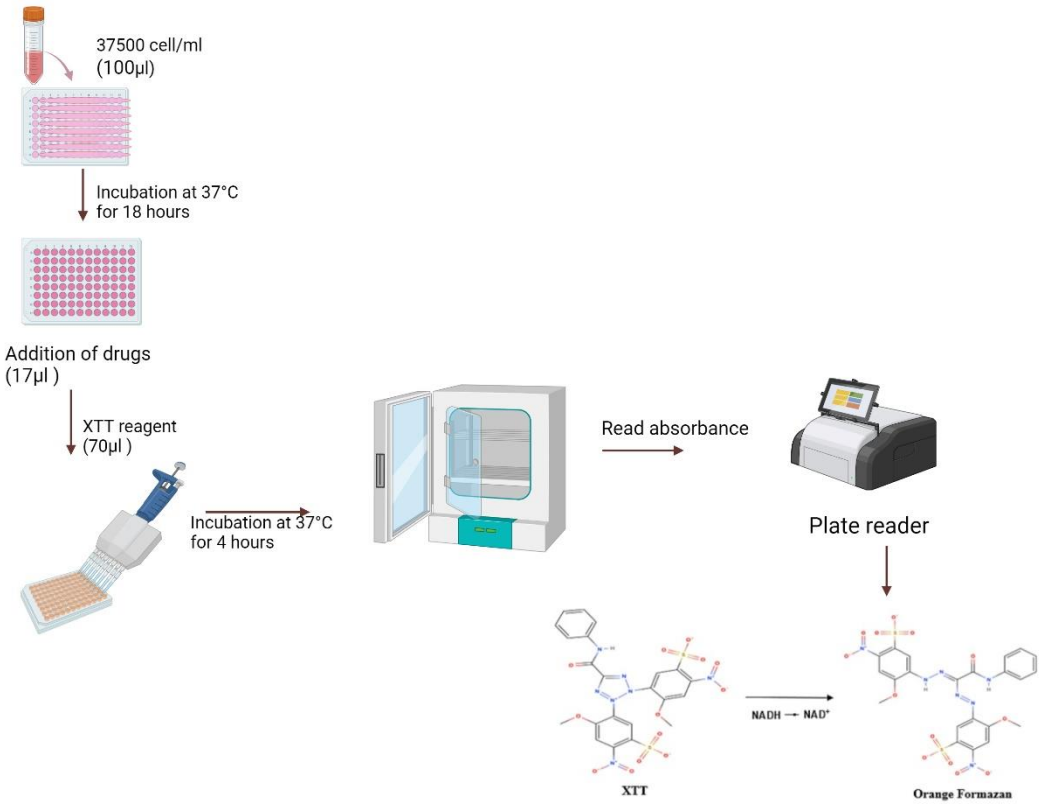

Figure 7: Procedure of XTT assay for measuring the cell viability of MB49 cells with different concentration of drugs (Lidocaine and phenazopyridine). Created from BioRender.com

2.4 Statistical analysis:

Data was exported from the Incucyte and plate reader into data sheets. Initial processing of data was performed in Microsoft excel prior to export into Graph Pad Prism for statistical analysis and to create graphical representation of data. Either One-way or Two-way ANOVA was used to determine statistical difference between the control and various drug treated cells either at single or multiple time points. $p<0.05$ was considered statistically significant.

CHAPTER 3: RESULTS

3.1 Optimization of cell concentration for Incucyte®S3

Before investigating the impacts of phenazopyridine and lidocaine on bladder cancer growth *in vitro*, it was crucial to find out the optimal seeding density of MB49 cells for our experiments. This was achieved by preparing different concentrations of MB49 cells seeded into 96 well plates and analyzing cell proliferation for 3 days.

The greater the number of MB49 cells starting in a well, the greater the final confluence after 3 days (**Figure 8A, B, C**). At an initial starting density of 50000 cells/ml, cells grew rapidly to reach a confluence of 77% over 3 days. Cell growth started to plateau around 48 hours. At a seeding density of 25000 cells/ml, we observed consistent cell growth throughout the experiment starting from 10% confluence to 72% by the end of period just as growth was starting to plateau. (**Fig 8A, B**). All lower cell seeding densities failed to reach confluence during the 3-day trial period (**Fig 1A, B**).

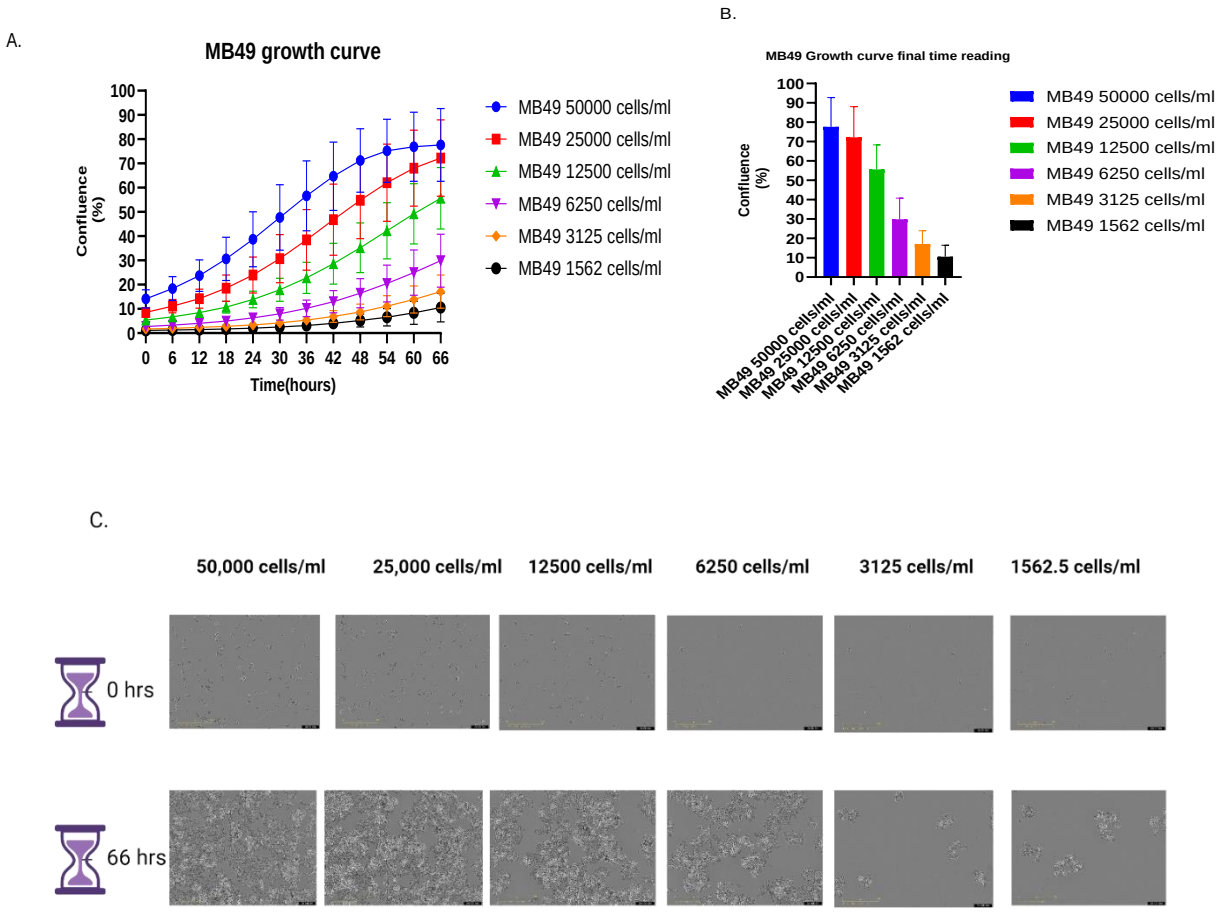

Figure 8: The growth curve of MB49 cells at different seeding densities. **Figure 8A** shows the growth curve of MB49 cells at 6 different concentrations (50,000, 25000, 12500, 6250, 1562 and 3125 cells/ml) over the period of 3 days (N=2, n=20). The y-axis represents the confluence in percentage while the x-axis represents the time in hours. At 66hr, 50000 cells/ml reached confluence of 77%, 25000 cells/ml reached around 72%. Similarly, 12500 cells/ml showed moderate growth with confluence of 55%. In contrast, the remaining cell concentrations showed minimal growth which are below 40%. The data were analyzed through GraphPad prism. **Figure 8B** represents the final time point reading of MB49 growth curve at 66 hours. The data were analyzed by using GraphPad Prism and are presented as mean ± SEM. **Figure 8C** shows the representative images captured from Incucyte®S3 which shows the morphological changes of MB49 cells at different concentrations.

3.2 Effects of Lidocaine on MB49 cell proliferation

After finding out appropriate cell seeding density for future experiments, lidocaine was evaluated for its impact on the proliferation of bladder cancer cells in an *in vitro* setting. Results showed that lidocaine had no significant effect on MB49 cell proliferation, with no change in MB49 proliferation over 3 days (Fig 9A). Lidocaine at 30 µM showed higher confluence compared to control cells after 72hrs of growth, this was not significant (**Fig 9B**). There were no obvious differences in well coverage in Incucyte images (**Fig 9C**)**.**

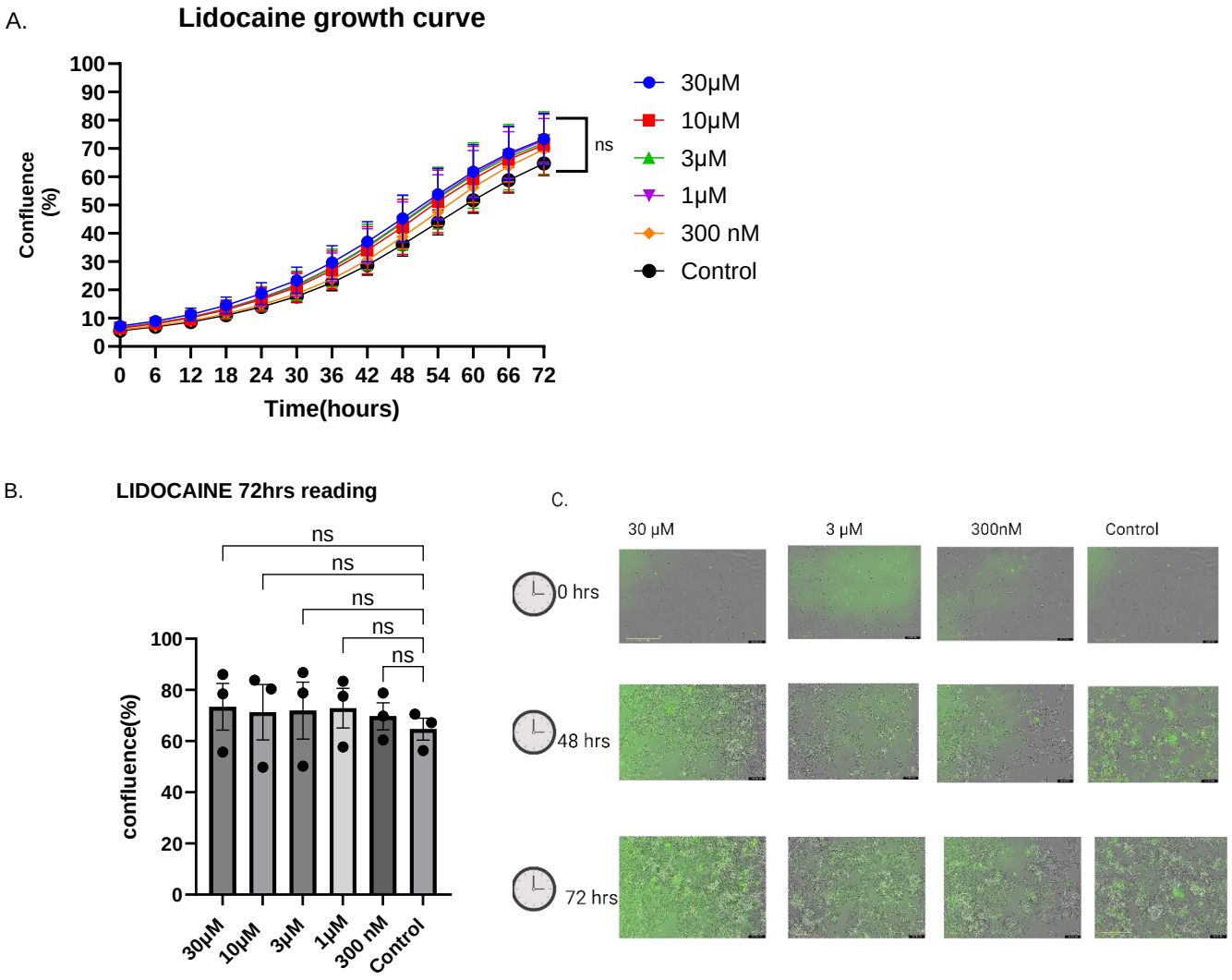

Figure 9. The impact of lidocaine on MB49 cell proliferation. In **Fig 9A**, various concentrations of lidocaine (30µM, 10µM, 3µM, 1µM and 300nM) (n=3) showed the increase in proliferation of bladder cancer cells where 30µM had the highest confluence of about 72% which can be further visually seen in the **Fig 9B, 9C**. **Fig 9B** showed the individual data of lidocaine of 72 hours where it can be observed that control had slightly less confluence than other lidocaine concentrations however, these concentrations are statistically non-significant when compared to control (Fig **9B**: 30µM P = 0.8438, 10µM P = 0.9555, 3µM P = 0.9438, 1µm P =0.8179 and 300nM P =0.9054) .The data were analyzed by using two-way ANOVA with Dunnett's multiple comparisons test , with single pooled variance and are presented as mean ± SEM. **Fig 9C:** The images of various concentration of lidocaine along with control obtained from Sartorius IncuCyte imaging system where green fluorescence indicates cell death.

3.3 Effect of lidocaine on MB49 cell via Sytox green analysis

Sytox green is a fluorescent dye that stains the nucleic acid of dead cells. Therefore, an increase in Sytox green represents an increase in cytotoxicity. To determine the cytotoxic effect of lidocaine, we performed Sytox green analysis. Our results showed that the fluorescent intensity of sytox green did not differ between control cells and cell treated with lidocaine concentrations was higher in the first few hours and then decreased by half at the end of 72 hours period(**Fig 10A, B**). This trend was similar to control and wasn't significant which suggested that lidocaine doesn't induce significant cell death.

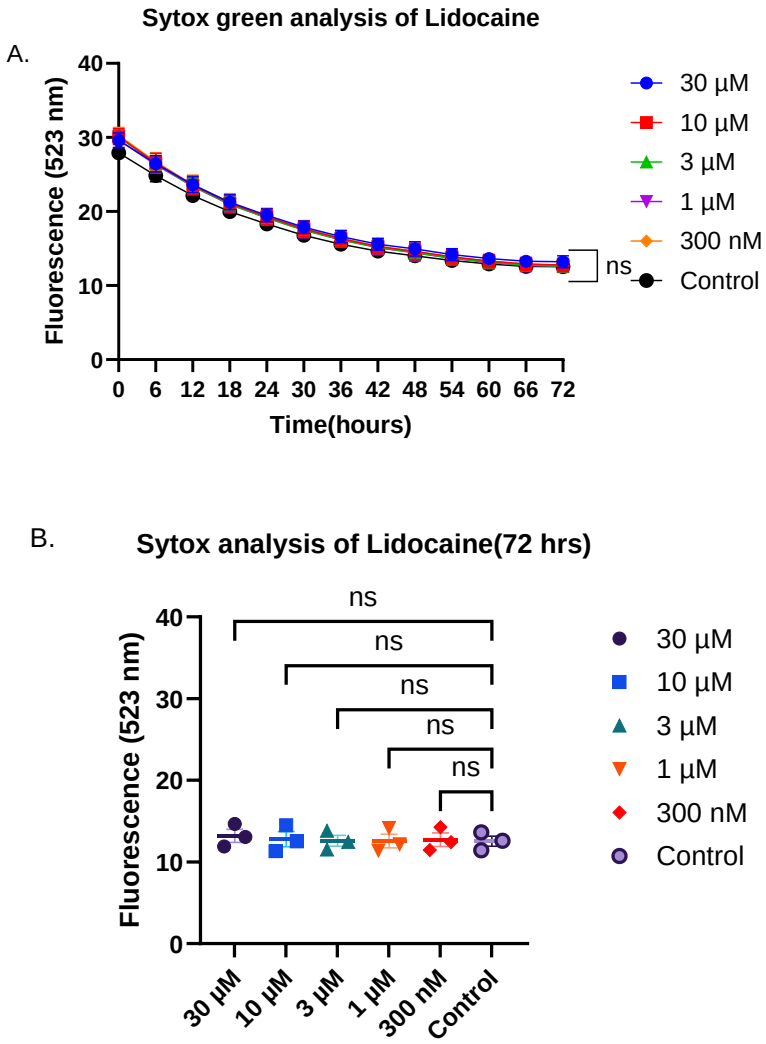

Figure 10: MB49 and lidocaine growth curve: The figure illustrates the effect of different concentration of lidocaine on MB49 cells over the period of 72 hours. **Fig 10A:** The line graph depicts the fluorescence intensity of lidocaine concentrations and control over 72 hours period where at the initial hours the fluorescence intensity was higher i.e., 30 but gradually decreases over the 3 days period and reached approximately about 12. **Fig 10B**. This bar graph shows the final time point readings of lidocaine at 72hrs where it can be seen that there was no significant difference in fluorescence intensity as compared to control indicating that lidocaine is not cytotoxic to the MB49 cancer cells. The data were analyzed by using two-way ANOVA with Dunnett's multiple comparisons test ,with single pooled variance and are presented as mean ± SEM (**Fig 10B**: 30µM P = 0.9764, 10µM P = 0.9995, 3µM P > 0.9999, 1µM P > 0.9999 and 300nM P = 0.9999).

3.4 Effect of Phenazopyridine on MB49 cell proliferation

After finding out optimal seeding density, the next step was to determine the oncological impact of phenazopyridine on MB49 cell proliferation in vitro. Results showed that various concentrations of phenazopyridine showed an upward trend of growth indicating cell proliferation (**Fig 11A**). The concentrations of phenazopyridine such as 10 µM, 3 µM, and 1 µM had higher confluence of 75%, 74% and 72% in compared to control (65%). However, all this concentration didn't have significant impact on cell proliferation when compared to control and this can be further supported by the data reading at 72hrs in **Fig 11B**.

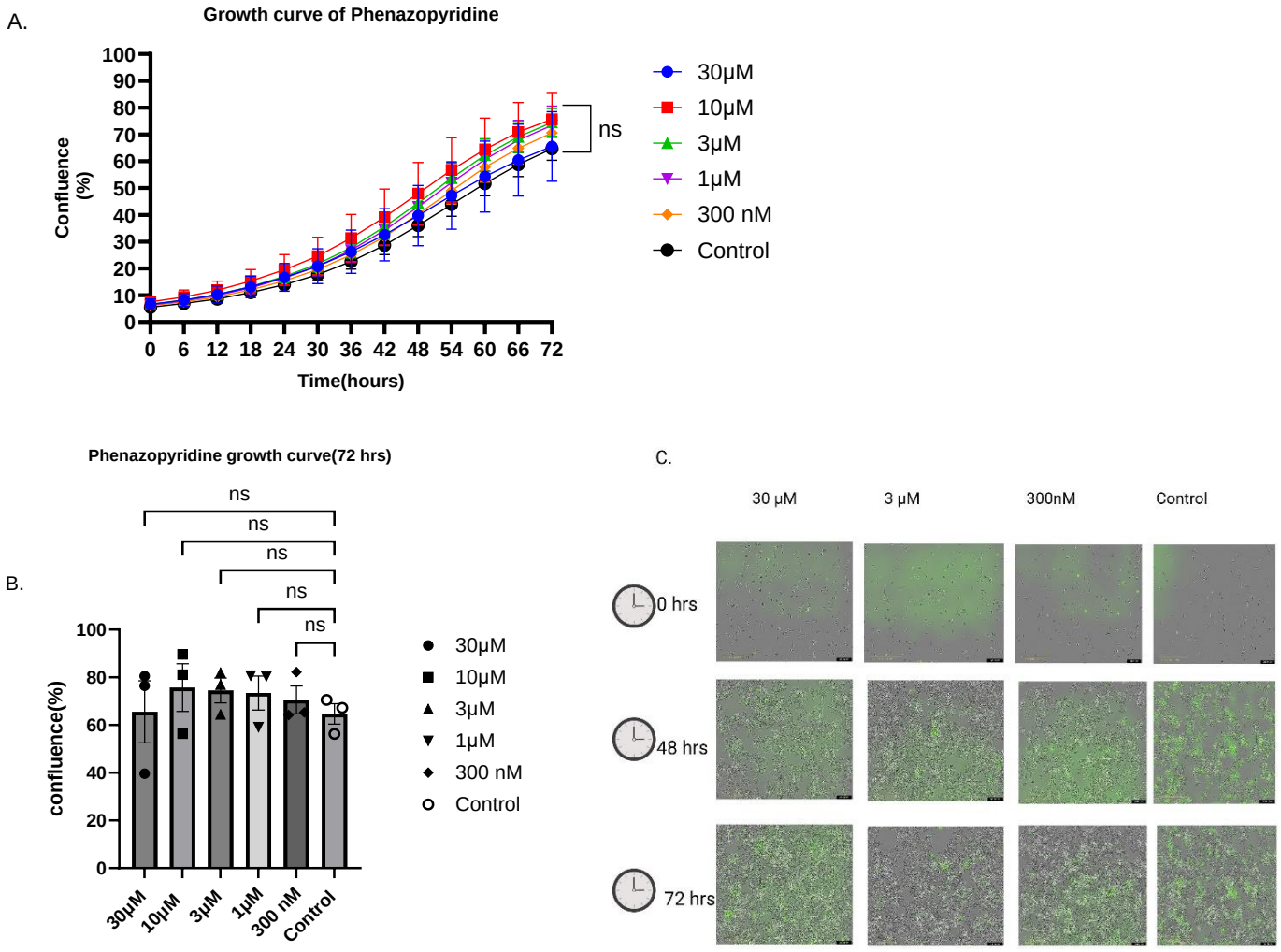

Figure 11: Impact of phenazopyridine at various concentration. Among the different concentrations tested, 30 µM, 10µM, 3µM, 1µM and 300nM showed the increase in proliferation of bladder cancer cells where 10µM had the highest confluence of about 75% **(Fig 11A).** In **Fig 11B** the bar graph showed the individual data of phenazopyridine of 72 hours where it can be observed that control and 30µM had similar percentage of confluence but was slightly less when compared to rest of phenazopyridine concentrations. These concentrations are statistically non-significant when compared to control (Fig **11B**: 30µM P > 0.9999, 10µM P = 0.7728, 3µM P = 0.5404, 1µm P =0.7525 and 300nM P =0.8709) .The data were analyzed by using two-way ANOVA with Dunnett's multiple comparisons test ,with single pooled variance and are presented as mean ± SEM. **Fig 11C:** The images of various concentration of phenazopyridine along with control obtained from Sartorius IncuCyte imaging system where green fluorescence indicates cell death.

3.5 Effects of phenazopyridine on MB49 cell via Sytox green analysis

After finding out its effect on the cell growth proliferation, we assessed its cytotoxic effect as well by performing sytox green analysis. Result showed that the fluorescent intensity was higher at first few hours which reached about 30 but there were no significant differences with compared to control for various concentrations (30 μM, 10μM, 3μM, 1μM and 300nM) throughout the period (**Fig 12A**). In 72 hours, it can be seen that the fluorescence intensity was similar for all concentrations tested as compared to control(**Fig 12B)**. These results suggested that phenazopyridine had no cytotoxic effect on MB49 cell indicating its safety profile for patients undergoing BCG therapy.

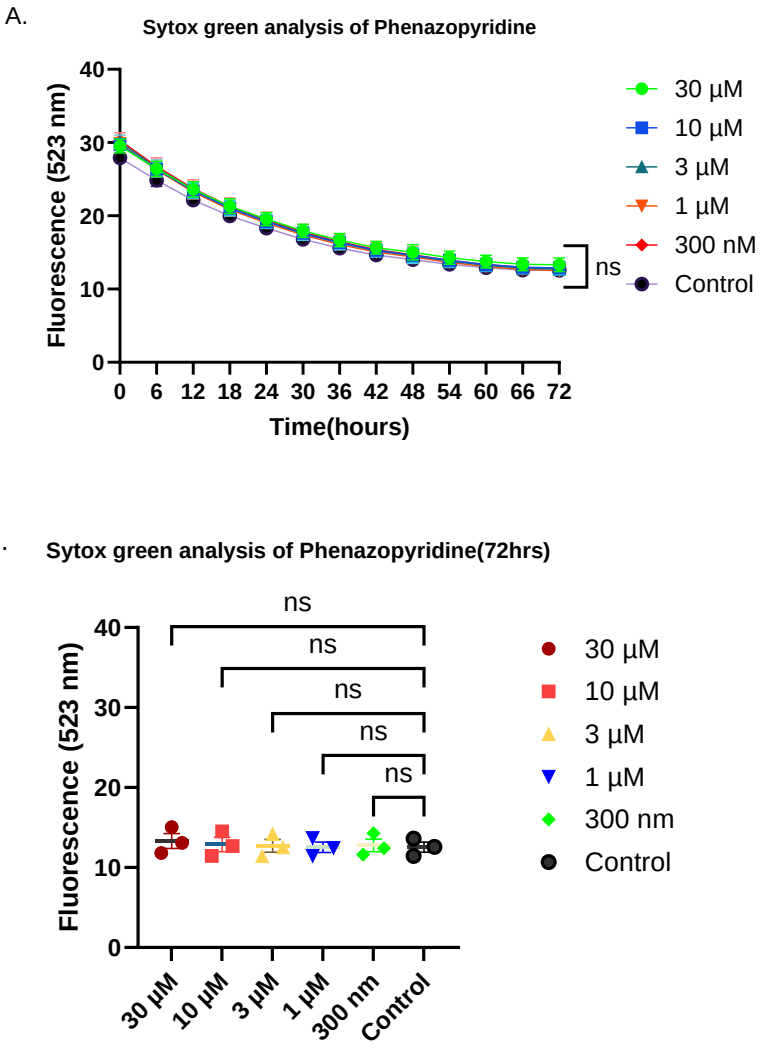

Figure 12: MB49 and phenazopyridine growth curve: The figure depicts the impact of various concentrations of phenazopyridine on MB49 cells over the period of 72 hours. Figure legend for D and E. **Fig 12A:** The line graph depicts the fluorescence intensity of phenazopyridine concentrations and control over 72hrs period where at the initial hours the fluorescence intensity was higher i.e., 30 but gradually decreases over the 3 days period and reached approximately about 12 which was similar as lidocaine. **Fig 12B**. This bar graph shows the final time point readings of phenazopyridine at 72hrs where it can be seen that there was no significant difference in fluorescence intensity as compared to control. The data were analyzed by using two-way ANOVA with Dunnett's multiple comparisons test ,with single pooled variance and are presented as mean ± SEM (**Fig 12B** 30µM $P > 0.9999$, 10µM $P = 0.9971$, 3µM $P > 0.9866$, 1µM $P > 0.9996$ and 300nM $P = 0.9999$).

3.6 XTT assay

After finding out the impact of phenazopyridine and lidocaine in MB49 cell proliferation, we performed XTT assay to determine if these drugs affect the metabolic activity of bladder cancer cell. An increase in fluorescent intensity reflects an increase in metabolic activity associated with an increase in the number of cells in the well. To determine the XTT assay, we need to find out the optimal seeding density which has consistent growth to conduct the further experiment. Result showed that 75000 cells/ml has the highest absorbance over 3 days. There was a sharp increase in metabolic activity in day 2 then it starts to plateau at day 3. However, 37500 cells/ml has consistent growth throughout the 3-day period. So, this concentration was chosen for further experiments.

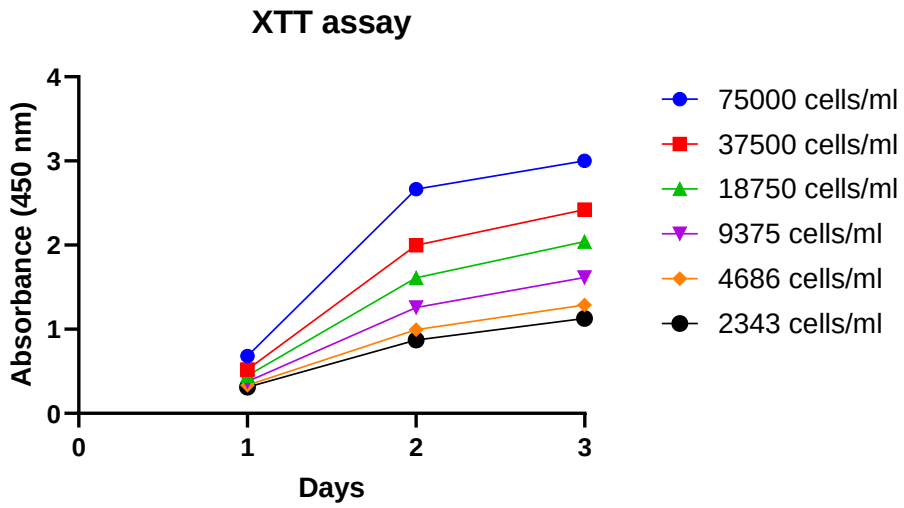

Figure 13: MB49 XTT assay: The graph depicts the growth curve of MB49 cells at different concentration of seeding densities over the 3 days period (N = 1, n =10). The y- axis represents the absorbance value at 450 nm whereas the x-axis represents the number of days. Higher seeding densities such as 75000 cells/ml and 25000 cells/ml showed higher absorbance values of 3.0 and 2.4 respectively by day 3 whereas the rest of concentration such as 18750, 9375, 4686 and 2343 cells/ml has lower absorbance value with around 1.5, 1.0, 0.8 and 0.6 suggesting slower growth rates. The data were analyzed by using GraphPad Prism and are presented as mean ± SEM.

3.6.1 Effects of Lidocaine on MB49 cell via XTT assay

After finding out the optimal seeding density i.e., 37500 cells/ml then we plated this concentration into 96 well plates and spiked it with various concentrations of lidocaine along with XTT reagent to assess its metabolic activity. Although the absorbance values seem similar to control until Day 2 there was slight increase in metabolic activity in some of the concentrations at day 3 with respect to control **(Fig 14A)**. However, they were all non-significant compared to control which can be further clearly seen in **Fig 14B**.

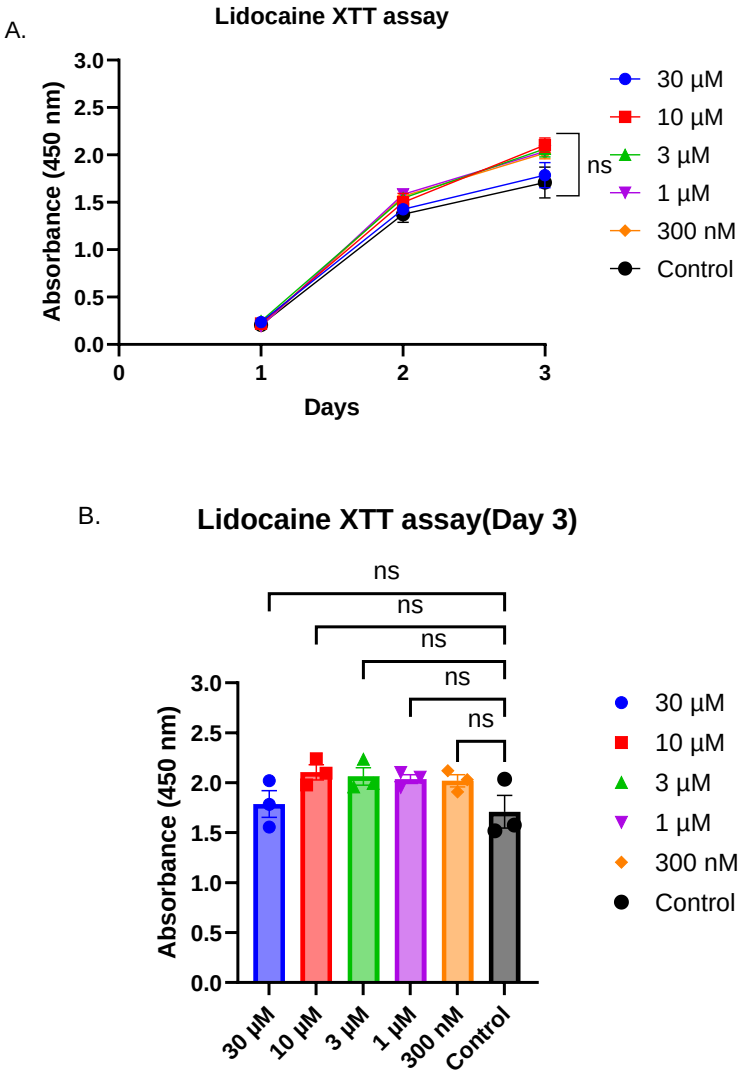

Figure 14. Lidocaine XTT assay: The graph represents the impact of different concentrations (30µM, 10µM, 3µM, 1µM and 300 nM) of lidocaine on MB49 cells (N=3, n=15) over three different days. All lidocaine concentration including control has similar growth pattern until day 2. Afterward, 10 µM, 3µM, 1µM and 300 nM and control showed slightly higher metabolic growth rate with an absorbance value of about 2.0 on day 3 however these increases were non-significant as compared to control ($p > 0.05$) (**Fig 14A**,**B)** which can be further supported in **Fig 14B**. This is the final time point reading where all the lidocaine concentrations are statistically non-significant (**Fig 14B**: 30µM P = 0.4399, 10µM P = 0.5053, 3µM P = 0.8444, 1µM P =0.9893 and 300 nM P =0.9993) .The data were analyzed by using two-way ANOVA with Dunnett's multiple comparisons test ,with single pooled variance and are presented as mean ± SEM.

3.6.2 Effect of phenazopyridine on MB49 cell via XTT assay

XTT assay was employed to assess whether phenazopyridine has any detrimental effect on MB49 cell viability. **Fig 15A** shows that all of the six concentrations of phenazopyridine had upward trend in absorbance values however all these concentrations are statistically non-significant as compared to control. This can be further supported by **Fig 15B** where all of the concentration is non-significant as compared to the control. This suggests that phenazopyridine doesn't have any effect on metabolic activity of MB49 cells.

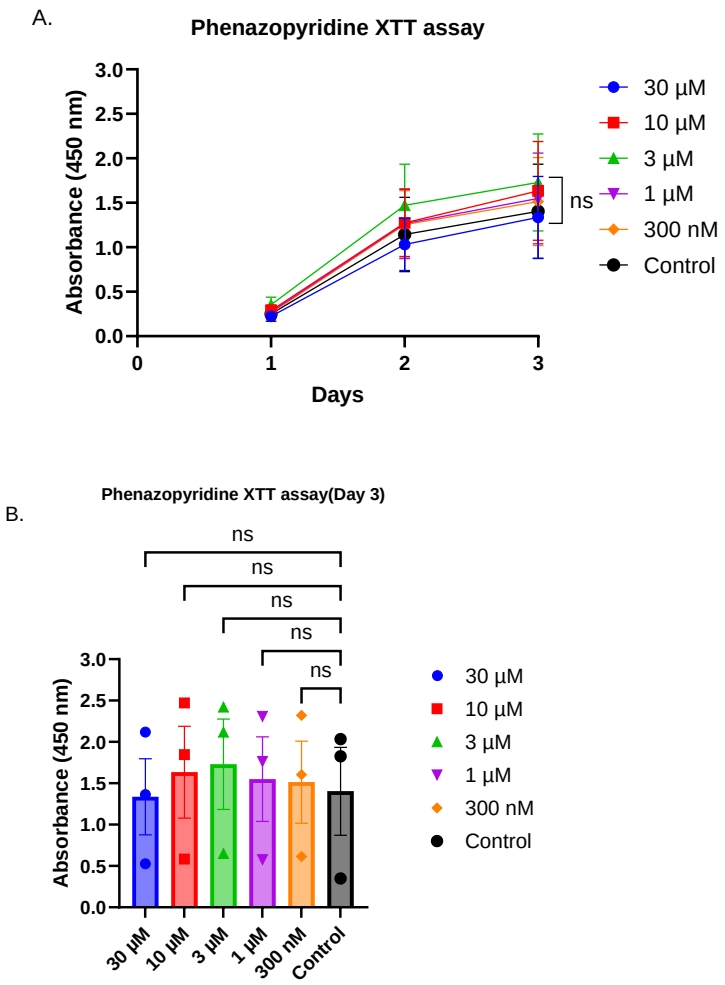

Figure 15. Phenazopyridine XTT assay. The graph illustrates the impact of various concentrations of phenazopyridine on MB49 cells (N=4, n=20) on 3 different days . In **Fig 15A,** the x-axis represents the number of days while y-axis represents the absorbance obtained from 450nm. The control and various concentration of phenazopyridine showed similar growth patterns throughout the period. 3µM had the highest absorbance value of about 1.7 followed by 10µM at 1.5. Furthermore, the remaining concentration including the control had absorbance value less than 1.5. **Fig 15B**. The bar graph represents the data of 3 days period where 3µM had highest cell viability than other concentrations however all these concentrations were non-significant when compared to control in terms of cell viability ((**Fig 15B**: 30µM P = 0.9999, 10µM P = 0.9971, 3µM P = 0.9866, 1µM P =0.9996 and 300nM P =0.9999). The data were analyzed by using two-way ANOVA with Dunnett's multiple comparisons test ,with single pooled variance and are presented as mean ± SEM.

CHAPTER 4: DISCUSSION

In our study we found that lidocaine and phenazopyridine have no significant impact on bladder cancer proliferation, cell death, or metabolism, indicating that these analgesics may be safe to use to relieve LUTS in patients undergoing BCG therapy.

Bladder cancer is the 10th most common cancer worldwide. NMIBC remains a significant clinical problem due to its recurrence even after treatment (Chang et al., 2016). BCG therapy is the most effective treatment for reducing the risk of progression and recurrence (Jiang & Redelman-Sidi, 2022). However, its administration causes patient to suffer from lower urinary tract symptoms including dysuria, urinary frequency, urgency and pelvic pain (Alexandroff et al., 1999). Despite this, there is no standard of care therapy to alleviate the pain and discomfort caused by BCG therapy.

When BCG is instilled into the bladder, it disrupts the GAG layer causing increased permeability of urothelial layer in bladder wall. This in turn allows the entry of urinary solutes and inflammatory mediators. These mediators are can sensitize the sensory nerves causing neuronal hypersensitivity which causes the development of LUTS along with other conditions such as interstitial cystitis/bladder pain symptoms (Bourlotos et al., 2024). So, urinary tract analgesics are used to alleviate the pain caused by LUTS along with other conditions to inhibit these sensory nerves. Phenazopyridine for example is commonly prescribed in treating urinary tract symptoms and intravesical lidocaine has been used to treat interstitial cystitis/bladder pain syndrome (Nickel et al., 2012). There are few clinical trials where phenazopyridine has been tested in patients undergoing BCG therapy (Kamali et al., 2020), however, its oncological outcomes along with that of lidocaine have not been explored.

So, our study aimed to provide insight on the impact of lidocaine and phenazopyridine on MB49 cell proliferation and BCG therapy in vitro and found that these drugs didn't have an impact on the cancer cell proliferation and can be used as an adjuvant therapy to alleviate the pain associated with BCG therapy.

4.1 Effects of Lidocaine and phenazopyridine on MB49 cell proliferation

Phenazopyridine, the commonly used urinary tract analgesic showed no statistical difference in cell proliferation as compared to control. These results suggest that phenazopyridine has no impact on bladder cancer growth and could have potential to be used for pain management caused by BCG therapy. This findings align with a clinical trial where efficacy of phenazopyridine was evaluated in reducing BCG-induced side effects and found that phenazopyridine alleviate the BCG induced symptoms (Kamali et al., 2020). Additionally, Purohit et al. (2023) reported that phenazopyridine metabolites showed significant reduction bladder pain in an animal model suffering from bladder pain syndrome. This suggests that phenazopyridine could be a potential analgesic for managing bladder pain following BCG-immunotherapy.

The lidocaine tested at all concentrations did not show any significant difference as compared to control. This showed that lidocaine to be potential to be used as analgesics for management of the LUTS related to BCG therapy. However, Yang et al. (2018) reported that lidocaine showed the antiproliferative effects on the BIU-87 bladder cancer cells at the inhibition rate of 23.64% for 1.25 mg/mL lidocaine, 45.88% for 2.5 mg/mL lidocaine and 75.49% for 5 mg/mL lidocaine which seems to be dose dependent. This variation in result may be due to the use of higher concentration of lidocaine or different cell line used. Irrespective, the study by Yang et al showed decrease proliferation which would not be a negative result.

4.2 Effects of Lidocaine and phenazopyridine on MB49 cell via Sytox green analysis

Sytox green is an fluorescent dye which is commonly used as dead cell markers that stains the nucleic acid of compromised plasma membranes without crossing the membranes of live cells (Lebaron et al., 1998). In our experiment, there were no significant differences compared to control for both various concentrations of drug throughout the period. These results suggested that both lidocaine and phenazopyridine had no cytotoxic effect on MB49 cell and aligned with the result of proliferation which did not show any detrimental effects on cell viability. However, in a study done by Teng et al. (2021) reported that lidocaine significantly reduced the growth of tumor in mice model of bladder cancer and increased the lactose dehydrogenase level in two bladder cancer cell line i.e., UMUC3 and T24 cells at the concentration of 4 and 8 mM. suggesting an anticancer effect as well as cytotoxic effect. This variation in result may be due to the use of different cell lines, cytotoxicity test and higher concentration of lidocaine. Additionally, the concentration of

lidocaine used in this study was much higher than our tested concentrations i.e., 4 and 8 mM of lidocaine. Additionally, in study by Sakaguchi et al. (2006) reported that lidocaine at the concentration of 400 μM suppress the human tongue cancer cells CAL27 without causing any cytotoxicity however in the concentration of 4000 μM it showed cytotoxic effect as well as antiproliferative effect on tongue cancer cells which suggest that lidocaine at lower concentrations doesn't exert cytotoxic effect which is consistent with our findings.

4.3 Effects of Lidocaine and phenazopyridine on MB49 cell via XTT assay

XTT (2,3-bis[2-methoxy4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxanilide) is a tetrazolium salt that is widely used to measure cell viability and proliferation (Wang et al., 2011). In this experiment, the XTT assay result showed that both the lidocaine and phenazopyridine does not affect significantly on metabolic activity of MB49 cancer cell as compared to control suggesting that these drugs do not affect the metabolic function of MB49 cells. This aligns with our data showing neither lidocaine nor phenazopyridine had any impact on cell proliferation but provides additional insight that there are no impacts beyond proliferation.

4.4 Therapeutic translation

In our experiment it was found that phenazopyridine and lidocaine have no impact on bladder cancer cell proliferation in vitro and therefore could be considered safe for patients with LUTS associated with BCG therapy. Phenazopyridine is given orally which makes it more convenient for bladder cancer patients. It has been successful in some clinical trials where it was found effective in pain management of BCG side effects (Kamali et al., 2020). Phenazopyridine would generally be given after BCG instillation as this drug alleviates the pain associated with BCG. However, it may be more useful to administer prophylactically to prevent the development of side effects in the first place.

Clinically, lidocaine has been used via an intravesical infusion route. This provides some advantages, as it may provide more direct analgesia at a faster rate. Yang et al (2018) reported that lidocaine is administered alongside BCG therapy and is effective for localized pain. While it is given intravesically, it can relieve the pain in the bladder more quickly. From a clinical perspective, it would be feasible to infuse lidocaine into the bladder prior to BCG, as the bladder is catheterized for the procedure anyway.

4.5 Challenges and limitations:

Due to time constraints, it was not possible to perform the second aim of our study which was to investigate the effect of lidocaine and phenazopyridine on the cytotoxic effects of BCG on MB49 cells. A further challenge encountered was with handling the XTT reagent as the reagent should be used immediately, which could degrade over time and end up affecting the variability in our results. Additionally, this study was conducted in invitro so further investigation should be done in an in vivo model to confirm the impact of the analgesics on MB49 cell proliferation. Moreover, these methods were tested on MB49 cells so further studies should be done on other bladder cancer cell lines to better understand the impact of these analgesics on BCG treatments.

4.6 Future directions:

The result obtained from this study provides a positive foundation for further study on the combined effect of urinary tract analgesics on the efficacy of BCG-immunotherapy. While our results suggested that the phenazopyridine and lidocaine does not cause a significant impact on the MB49 cell proliferation in vitro further experiment should be done to explore whether these drugs have an impact on BCG cytotoxicity and on the internalization process of BCG for its therapeutic efficacy. Additionally, the various drug concentrations used in this study showed no significant effect on the proliferation and cytotoxicity. Additionally, extending the incubation time more than 3 days of lidocaine and phenazopyridine could provide insights on the long-term effect on the proliferation, cytotoxicity and metabolic activity of MB49 cells.

Furthermore, after successful in vitro experiment, the study should be done in mouse model of bladder cancer to understand the impact of analgesics on cancer growth. Additionally, by combining analgesics and BCG in this model. we can determine if these drugs are safe for use in bladder cancer patients.

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