Cytotoxic effect of thieno[2,3-b]pyridine derivatives on human bladder cancer cells

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UNIVERSITY OF SPLIT
SCHOOL OF MEDICINE

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CYTOTOXIC EFFECT OF THIENO[2,3-B]PYRIDINE DERIVATIVES ON HUMAN BLADDER CANCER CELLS

Diploma thesis

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Mentor:
Assoc. Prof. Vedrana Čikeš Čulić, PhD

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I want to thank my family and friends that suffered with me through this time. There were moments when you feel lost and lonely on this journey. You were all just one call away and always helped me out the darkness.

In particular I have to thank my mom. THANK YOU for all the countless hours being there for me. Not only this journey but my entire life. I could not have done it without you.

Time to conclude this acknowledgement with the same words the journey started with…

…why not?!

1. INTRODUCTION

1.1 Cancer

The generic term cancer is used for a large group of diseases. Any part of the body can be affected by them. Malignant tumors and neoplasms are other commonly used terms for cancer. Rapid creation of abnormal cells that grow beyond their usual boundaries, and which can then invade adjoining parts of the body and spread to other organs are a defining feature of cancer. The latter process is known as metastasis (1). The primary reason why cancer patients die is due to widespread metastases. They account to 90% of deaths (2). "The Hallmarks of Cancer" by Douglas Hanahan *et al*. shows that all known cancers have general hallmarks that can be found in every known type. Those allow a normal healthy cell to change itself to a cancerous cell. These are the 10 aforementioned hallmarks: growth signals self-sufficiency, insensitivity to inhibitory signals, resistance to apoptosis, immense replicative possibility, ability of invasion and metastases to surrounding tissues, the stimulation of angiogenesis, disrupted metabolic pathways, immune system evasion, genomic mutability and inflammation (3).

1.2 Bladder cancer

1.2.1 Etiology and epidemiology

Bladder cancer is the 10th most common type of cancer worldwide. It is the 6th most common in men and 17th in women. There were greater than 573,000 newly made diagnosis of bladder cancer in 2020. As shown in Table 1A, Greece has the highest rate followed by the Netherlands and Italy (4).

Older persons are more likely to develop bladder cancer (5,6) Nine out of ten patients with this malignancy are older than 55. People are typically 73 years old when they receive a diagnosis. Overall, males have a 1 in 27 chance of developing this cancer during their lifetime. The likelihood for women is roughly 1 in 89 (5). Men certainly have a larger risk of developing bladder cancer than women do (6).

Men are more prone to smoke and work in industries where they are exposed to carcinogens, as can be seen. The hormonal and general physiological variations between the sexes are the other aspect that distinguishes men and women. In recent years, both the percentage of women who smoke and their presence in the industrial workforce have increased.

Although those rates are changing, it was shown in the early 1990s that men still experience cancer at a rate of 2.7 times that of women (7).

An age standardized death rate, also known as an age-adjusted rate, is a summary indicator of the mortality rate a population would experience if it directly or indirectly possessed a standard age. Age-standardized rates make it possible to compare populations with various age demographics (8).

The age standardized rate (ASR) is a calculation of the rate at which a population would grow if its age distribution were uniform. Age has a significant impact on the probability of developing cancer; hence standardization is required when comparing groups that vary in age. It shows a rate worldwide of 5.6. This is much lower than Greece with an ASR of 21.2. The Netherlands has an ASR of 17.2 and Italy of 16.4.

If we look at gender-specific findings from Table 1B and C, we can see that women have a significantly lower rate of bladder cancer. There are 132,414 cases in women worldwide newly diagnosed in 2020, while there were 440,864 in men. The ASR is also widely different. 2.4 in female and 9.5 in the male population. When comparing the most prevalent countries by gender, we see that Hungary, the Netherlands and Germany have the highest rates of newly diagnosed bladder cancers. Their ASR are 9.1, 8.1 and 7.5 respectively. The leading countries for the male population are Greece, Italy, Spain and the Netherlands. In those countries the age-standardized rate is visibly higher. 39.7, 28.4 while Spain and the Netherlands share a rate of 27.3. Figure 1 also visualizes this trend between male and female population nicely. It also shows that southern Europe is leading the regional prevalence over the rest of the world.

Looking at the mortality, we see a worldwide total number of 212,536 people dying in 2020 and an ASR of 1.9. Table 1 shows also the country the highest mortality is Egypt with an ASR of 7.8.

The region with the highest incidence to mortality rate is Northern Africa. By utilizing the ASR, we can see that 8.9 people out of 100,000 are getting bladder cancer and 5.2 die from it each year (Figure 1) (9).

Several causes of bladder cancer have been identified. Smoking, *Schistosoma haematobium* infection, work-related contact with metalworking fluids, and contact with aromatic amines and polyaromatic hydrocarbons. These are substances utilized in the chemical

and plastic industries. Arsenic-tainted water is also known to raise the risk of bladder cancer (4).

Patients who were observed with schistosome eggs in the specimens had bladder cancer at a younger age (46.7 years) than patients with egg-negative instances (53.2 years). This finding confirms the importance of *Schistosoma haematobium* infection and/or associated issues in the development of bladder cancer (10).

Genetic factors have a crucial role in the probability of getting bladder cancer. There is a two-fold increased chance of developing urothelial carcinoma in patients with first-degree relatives with the disease. The inheritance of certain genetic traits has been identified as probable risk factors for urinary bladder cancer, including glutathione S-transferase mu 1 (GSTM1)-null genotypes and genetic slow acetylator N-acetyltransferase 2 (NAT2) variations. Slow acetylation is one factor that may not directly cause bladder cancer but may increase the risk of exposure to carcinogens like tobacco products (11).

Table 1 A. Age-standardized incidence rates of bladder cancer worldwide sorted by country

Source: (4)

Rank	Country	Number	ASR/100,000
	World	*573,278*	*5.6*
1	Greece	5,645	21.2
2	The Netherlands	7,417	17.2
3	Italy	28,336	16.4
4	Denmark	2,323	16.2
5	Belgium	4,527	16.0
6	Spain	18,512	15.8
7	Germany	35,147	15.3
8	Hungary	3,239	15.2
9	Serbia	2,549	14.1
10	Croatia	1,398	14.1

B. Age-standardized incidence rates of bladder cancer of the female population worldwide sorted by country

Rank (women)	Country	Number	ASR/100,000
	World	*132,414*	*2.4*
1	Hungary	1,097	9.1
2	The Netherlands	1,752	8.1
3	Germany	9,205	7.5
4	Denmark	542	7.1
5	Norway	453	7.0
6	Switzerland	722	6.7
7	Mali	345	6.6
8	Serbia	638	6.5
9	Croatia	352	6.5
10	Belgium	976	6.4

C. Age-standardized incidence rates of bladder cancer of the male population worldwide sorted by country

Rank (men)	Country	Number	ASR/100,000
	World	*440,864*	*9.5*
1	Greece	4,842	39.7
2	Italy	22,524	28.4
3	Spain	14,838	27.3
4	The Netherlands	5,665	27.3
5	Belgium	3,551	27.1
6	Denmark	1,781	26.3
7	Germany	25,942	24.4
8	Croatia	1,046	24.0
9	Hungary	2,142	23.9
10	Serbia	1,911	23.1

D. Age-standardized mortality rates of bladder cancer worldwide sorted by country

Rank	Country	Number	ASR/100,000
	World	*212,536*	*1.9*
1	Egypt	6,170	7.8
2	Tunisia	822	5.2
3	Libya	242	5.0
4	Poland	5,026	4.9
5	Mali	426	4.8
6	Slovakia	629	4.7
7	Latvia	271	4.5
8	São Tomé and Príncipe	5	4.4
9	Algeria	1,861	4.3
10	Serbia	931	4.3

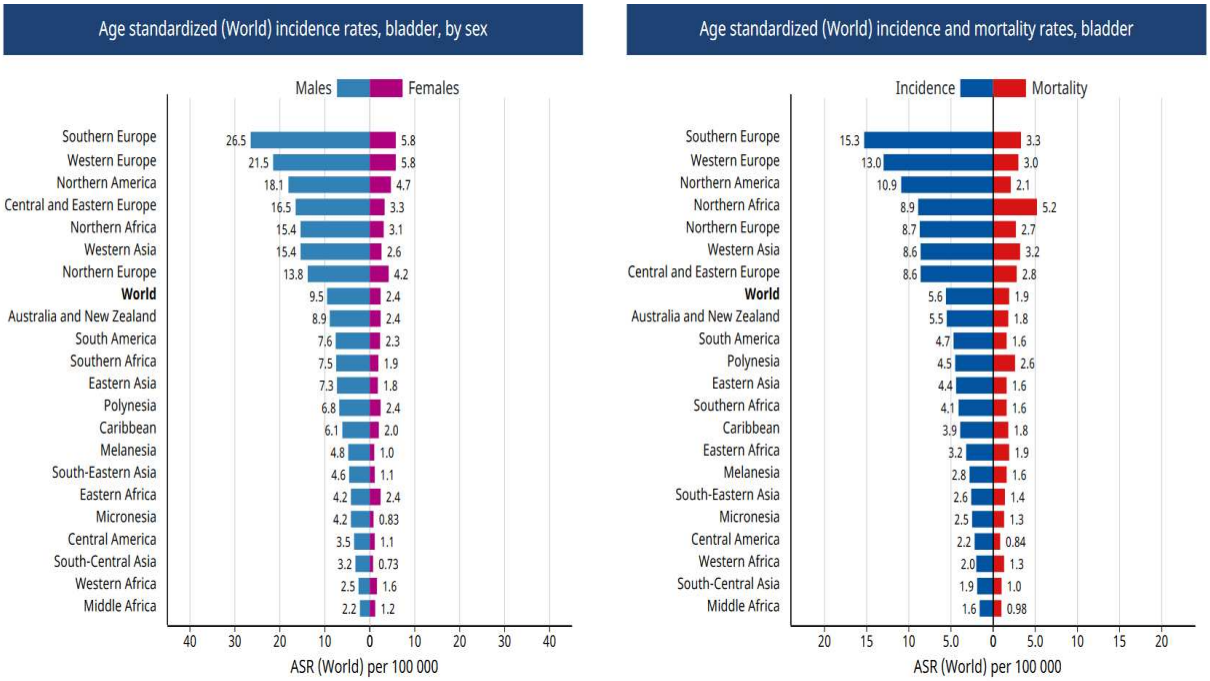

Figure 1. Age-standardized incidence and mortality rates by sex and region for bladder cancer

Source: globocan2020 (9)

1.2.2 Types of cancer

By far the most widespread variant of bladder cancer is the so-called transitional cell carcinoma. It is also known as urothelial carcinoma (12). As the name suggest, this type of cancer starts in the urothelial layer of the bladder that lines its inside. The inside of the bladder isn't the only part of the urogenital system that is covered by those cells. Hence, tumors in those patients can be found in the renal pelvis, the ureters and the urethra. Other types are a lot less common (13).

Squamous cell carcinoma has a prevalence of 1-2% in the US. Nearly all squamous cell carcinomas of the bladder are invasive (13). It is more common in parts of the world where schistosomiasis is a common cause of bladder infections (14).

Adenocarcinomas are only around 1% of all bladder cancers. They arise from cells that make up mucus-secreting glands in the bladder. Just like squamous cell carcinomas, they are very invasive (13,14).

Small cell carcinomas make up less than 1%. They start in neuroendocrine cells and has a very fast growth rate (13).

The last group of bladder cancers is sarcoma. Those cancers are arising from the muscle layer. With an extremely low probability of this type to occur, it marks the lowest of all the groups mentioned above (13).

1.2.3 Pathology

The grading of bladder cancer subdivides it into 4 stages.

1. Papilloma
2. Papillary urothelial neoplasm of low malignancy potential (PUNLMP)
3. Low grade papillary urothelial carcinoma
4. High grade papillary urothelial carcinoma (15)

Histologically, bladder cancer can be divided into urothelial and non-urothelial types. Divergent differentiation is a characteristic of urothelial cancer that has been widely noticed in recent years as a result of increased awareness and enhanced immunohistochemical methods. With its enumeration of 13 histologic types of urothelial carcinoma, the most current

classification of urothelial malignancies by the World Health Organization (WHO) further clarified this matter. Squamous, glandular, micropapillary, nested, lymphepithelioma-like, plasmacytoid, and sarcomatoid types of urothelial carcinoma are only a few examples of the many differentiation patterns (16).

Given its rarity, little is known about micropapillary bladder cancer (MPBC), a variant histology of urothelial carcinoma (UC) that is linked with poor prognosis (17).

Looking at the TNM staging, we see the following division (18):

Stage 0a: This is a very early form of cancer only seen on the bladder's inner lining's surface. Cancer cells tend to cluster in conjunction and are frequently simple to eliminate. Connective tissue or muscle in the bladder wall have not yet been affected by the malignancy. Non-invasive papillary urothelial carcinoma is another name for this particular form of bladder cancer (Ta, N0, M0).

Cancer that is at stage 0 is sometimes referred to as a flat tumor or carcinoma in situ (CIS), because it solely affects the bladder's interior lining. It has not become bigger or extended to the bladder's dense layer of muscle or connective tissue, nor has it gotten closer to the hollow region of the bladder (Tis, N0, M0).

This is always a high-grade malignancy (see "Grades," below), and because it can progress to muscle-invasive illness, it is regarded as aggressive.

Stage I: The malignancy has penetrated the lamina propria and the bladder's inner lining. Lymph nodes, other organs, or the dense layer of muscle in the wall of the bladder have not been affected by its spread (T1, N0, M0).

Stage II: The bladder's strong muscular wall has been invaded by the malignancy. Additionally, it is known as muscle-invasive cancer or invasive cancer. The tumor has not migrated to lymph nodes or other organs, and it has penetrated to the fatty tissue covering the bladder (T2, N0, M0).

Stage III: The cancer has migrated from the muscular wall to the prostate, the uterus, the vagina, or to the fatty layer of tissue surrounding the bladder (perivesical tissue), or the lymph nodes in the area have been affected by the malignancy.

Stage IIIA: The tumor has penetrated the peritoneum or reached the prostate, uterus or vagina, but not the lymph nodes or other organs (T3a, T3b, or T4a; N0; M0).

Alternatively, one local lymph node has been affected by the malignancy (T1 to T4a, N1, M0).

Stage IIIB: Two or more regional lymph nodes or the common iliac lymph nodes have shown evidence of malignant dissemination (T1 to T4a, N2 or N3, M0).

Stage IV: The cancer has spread to lymph nodes outside of the pelvis or to other regions of the body, or the tumor has penetrated the abdominal wall or pelvic wall.

Stage IVA: The tumor has extended to the abdominal wall or pelvic wall but has not yet reached additional bodily sections (T4b, any N, M0). Or the lymph nodes outside of the pelvis have been affected by the malignancy (any T, any N, M1a).

Stage IVB: The cancer has spread to other body areas (any T, any N, M1b) (18).

1.2.4 Signs, symptoms and diagnostics

Symptoms of bladder cancer that can be observed include blood in the urine which can vary from slightly rusty to bright red in color. The urinary frequency is increased and lower back or abdominal pain is present. Furthermore, dysuria is evident, which is pain during urination (18, 19).

Physical examination and medical history: An examination of the body to check for general health indicators, including tumors or anything else that looks out of the ordinary. Additionally, patient history, health practices, diseases, and past treatments will be recorded.

Internal examination: A review of the rectum and/or vagina. To check for lumps, the doctor places his or her gloved, lubricated fingers within the vagina and/or the rectum.

An examination of the urine's color and its composition, including the presence of sugar, protein, red and white blood cells, is known as a urinalysis.

Urine cytology is a laboratory test that involves looking for abnormal cells in a urine sample under a microscope.

A diagnostic and treatment method called cystoscopy involves visualizing the bladder and urethra to search for any abnormalities. A cystoscope is a small, tube-shaped device with a light and viewing lens. The urethra is used to put it into the bladder. It is giving direct access to a tumor so that a biopsy, fulguration, and/or excision with a tool for removing tissue samples

can be performed. Afterwards the sample can be examined under a microscope for cancerous cells (17, 19, 21).

A series of X-rays of the kidneys, ureters, and bladder are taken during an intravenous pyelogram (IVP) to determine whether malignancy is present in these organs. A vein receives an injection of contrast dye. X-rays are obtained as contrast dye passes through the kidneys, ureters, and bladder to check for any obstructions (19).

A biopsy is the excision of tissue or cells, enabling a pathologist to examine them under a microscope looking for indications of malignancy. Typically, a bladder cancer biopsy is performed during a cystoscopy. During the biopsy, the entire tumor may be able to be removed (19,22).

To check whether the cancer has spread from the bladder elsewhere it is crucial to utilize CT scans, MRI, PET scans, chest X-rays and bone scans in the treatment plan of cancer patients. (19).

1.2.5 Therapeutic options and advances

There are 5 standard types of therapy for cancer:

1.2.5.1 Surgery

Depending on the decision of the multidisciplinary team, any of the following surgical procedures might be performed:

Surgery in which a cystoscope is put into the bladder through the urethra is known as a transurethral resection (TUR) with optional fulguration. The malignancy is then removed, or the tumor is burned away using an instrument that has a little wire loop on the end. This process is called fulguration (19).

Radical cystectomy: No matter the variant or typical urothelial histology of the bladder cancer, radical cystectomy is a cornerstone of treatment (23). It is a surgical procedure that involves the removal of the bladder, any cancerous lymph nodes and any surrounding organs. This surgery may be necessary if the cancer has spread to the muscular wall or if a significant portion of the bladder is affected by superficial cancer. The prostate and seminal vesicles are neighboring organs that are taken out in male patients. The uterus, ovaries and a portion of the

vagina are removed in women. Cystectomy can be performed to decrease cancer-related urinary symptoms if the cancer has spread outside the bladder and cannot be entirely removed (18). A transitional cell cancer that invades muscle (T2 or higher) with no signs of distant metastasis is the most frequent indication for radical cystectomy (23). The surgeon develops a different pathway for urine to exit the body when the bladder must be removed.

Part of the bladder is surgically removed during a partial cystectomy. Patients whose tumors were low-grade, meaning that the cancer has only spread to a small portion of the bladder's wall were eligible to undergo this operation. Patients can urinate regularly following this procedure since only a portion of the bladder is removed. This technique is also known as segmental cystectomy.

Urinary diversion is a surgery that facilitates the creation of a new path for the body to store and release urine. Adjuvant chemotherapy is given in patients following a surgery to lower the risk of reoccurrence (19).

1.2.5.2 Radiotherapy

High-energy X-rays or other forms of radiation are used in radiation therapy, to either kill or cease the growth of tumor cells. A device outside the body is used in external radiation treatment to direct radiation toward the cancerous part of the body (19).

Radical radiotherapy has shown good therapeutic results in T2 and T3 tumors but paradoxically limited success in non-invasive Ta and superficial T1 (25).

1.2.5.3 Chemotherapy

Chemotherapy is a form of cancer treatment that utilizes medications to eliminate tumor cells or prevent them from proliferating in order to limit the development of cancer cells. Chemotherapy enters the circulation whether administered orally or by an injection into a vein or muscle, where it can target cancer cells that are found throughout the body. This intervention is known as systemic chemotherapy. Chemotherapy mostly targets cancer cells in various regions when administered directly into the cerebrospinal fluid (CSF), an organ, or a bodily cavity like the abdomen (regional chemotherapy). Regional chemotherapy in the bladder has the property to be intravesicular meaning being put into the bladder through a tube inserted into the urethra. The variant of cancer along with the stage determine the kind of chemotherapy

administration. Multiple anticancer medications are used during combination chemotherapy (19).

Neoadjuvant chemotherapy, administered prior to local therapy, may decrease the size of the main tumor and may be useful in controlling metastatic malignancy when the volume of the micrometastases is predicted to be little (26).

1.2.5.4 Immunotherapy

Immunotherapy is a form of cancer treatment that activates the patient's immune system. The body's natural defenses against cancer are boosted, directed, or restored using substances produced by the body or in a lab. This is part of biologic therapy.

Therapy with Programmed cell death protein 1 (PD-1) and Programmed death-ligand 1 (PD-L1) inhibitors: T cells have a protein called PD-1 on their surface that aids in controlling the body's immunological reactions. Some cancer cell types include a protein called PD-L1. The T cell cannot attack the cancer cell when PD-1 binds to PD-L1. Inhibitors of PD-1 and PD-L1 prevent the proteins from binding to one another. As a result, cancer cells can be killed by T lymphocytes. PD-1 inhibitors include drugs like pembrolizumab and nivolumab. PD-L1 inhibitors include atezolizumab, avelumab, and durvalumab (19). The PD-1 inhibitor pembrolizumab was the first systemic drug to be approved by the FDA for the treatment of high-risk non-muscle-invasive bladder cancer (NMIBC) (27).

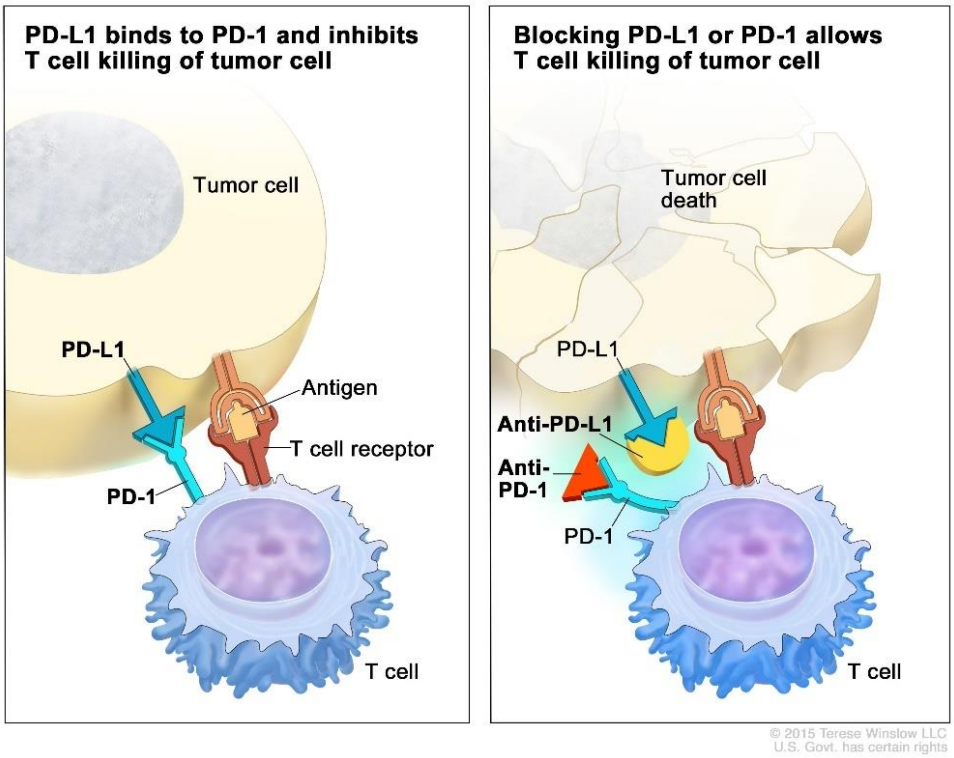

Figure 2. PD-1 and PD-L1 inhibitors allow the immune system´s T cells to recognize the tumor as a hostile/foreign cell and not as part of our own body. This allows the cytotoxic effect of the T cells to eliminate those cells.

source: (19) https://www.cancer.gov/types/bladder/patient/bladder-treatment-pdq

1.2.5.5 Targeted therapy

Targeted therapy identifies and kills specific cancer cells using medicines or other chemicals. Compared to chemotherapy or radiation therapy, targeted therapies often have a lower impact on healthy cells.

Monoclonal antibodies, tyrosine kinase inhibitors or blockers of vascular endothelial growth factor (VEGF) fall under this category and can be used for the treatment in bladder cancer patients (19).

Since urothelial carcinoma targeted therapy development is still in its early stages, significant therapeutic advancements have not yet been made. The growth of clinical trials, however, has led to a better knowledge of the biology of urothelial carcinoma and solid tumors, which may result in major improvements in therapeutic approaches (28).

Agents that target the VEGF/R, FGF/R, or EGF/R pathways are currently being researched but do not yet appear to have any clinically significant advantages (29).

1.3 Phospholipase C (PLC)

Activated phospholipase C (PLC) isozymes are interconnected with several pathways, such as the PI3K/protein kinase B (PKB/Akt)/mammalian target of rapamycin (mTOR) (PI3K/Akt/mTOR) pathway, RAS/rapidly accelerated fibrosarcoma (RAF)/mitogen activated protein kinase (MAPK)/extracellular signal-related kinase (ERK) pathway and the Janus kinase (JAK)/signal transducer and activator of transcription (STAT) pathway. They have a significant role in regulating cancer cell growth and proliferation as seen in figure 3 (30).

In this thesis, newly-synthesized compounds from the thieno-pyridine family, synthesized as potential inhibitors of PLC by virtual high throughput screening (vHTS), are tested on a bladder cancer cell line. Similar compounds from the same family of PLC inhibitors (thieno-pyridine) were previously tested and showed significant cytotoxic effect in prostate cancer and triple negative breast cancer cell lines (31,32).

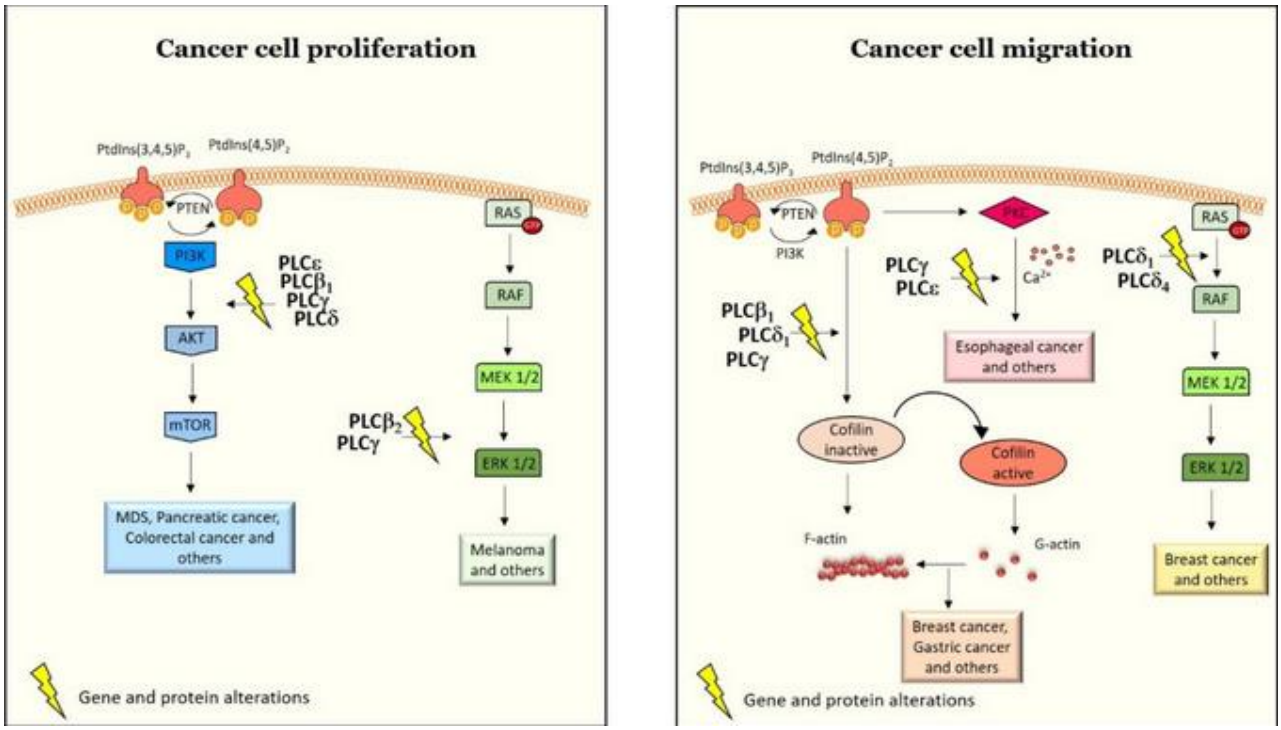

Figure 3. PLC inhibitors act on multiple points of cancer cell proliferation and migration process and can be used to stop them. It has been demonstrated that critical pathways including the PI3K/Akt/mTOR and the RAS/RAF/MAPK/ERK are affected by the expression of PLC isoforms at both the genetic and protein levels in various cancer types (30).

Source: (30)

2. OBJECTIVES AND HYPOTHESES

2.1 Objectives

The aim of this study is to determine the cytotoxic effects of treating T24 bladder cancer cells with the newly synthesized thieno[2,3-b]pyridine anticancer compounds.

2.2 Hypotheses

1. Thieno[2,3-b]pyridine anticancer compounds will show cytotoxic effect on T24 bladder cancer cells.

2. Cytotoxicity will be proportional to the increase in concentration and time of exposure.

3. MATERIALS AND METHODS

3.1 Cell culture

Cells were purchased from ATCC (LGC Standards). Human bladder cancer cell line T24 was cultured in a humidified atmosphere with 5% CO_2 at 37ºC, in a Dulbecco's modified Eagle's medium (DMEM Euroclone, Milano, Italy) containing 4.5 g/L glucose, 10% fetal bovine serum (FBS) and 1% antibiotics (Penicillin Streptomycin, EuroClone, Milano, Italy).

We can see the cell culture in figure 4 where a cell micrograph of the T24 cell line was taken.

The table below depicts the known characteristics of the cell line (Table 2).

Table 2. Characteristics of T24 cell line

Characteristics:	
Organism:	Homo sapiens, human
Tissue:	Urinary bladder; transitional cells
Disease:	Transitional Cell Carcinoma
Age:	81 years
Ethnicity:	Caucasian
Gender:	Female
Clinical data:	Leukocytes and sera from patients were cytotoxic to T24 and related lines
Product format:	Frozen
Applications:	3D cell culture
	Cancer research
Oncogenes:	Contains the ras (H-ras) oncogene

Source: https://www.atcc.org/products/htb-4 (33)

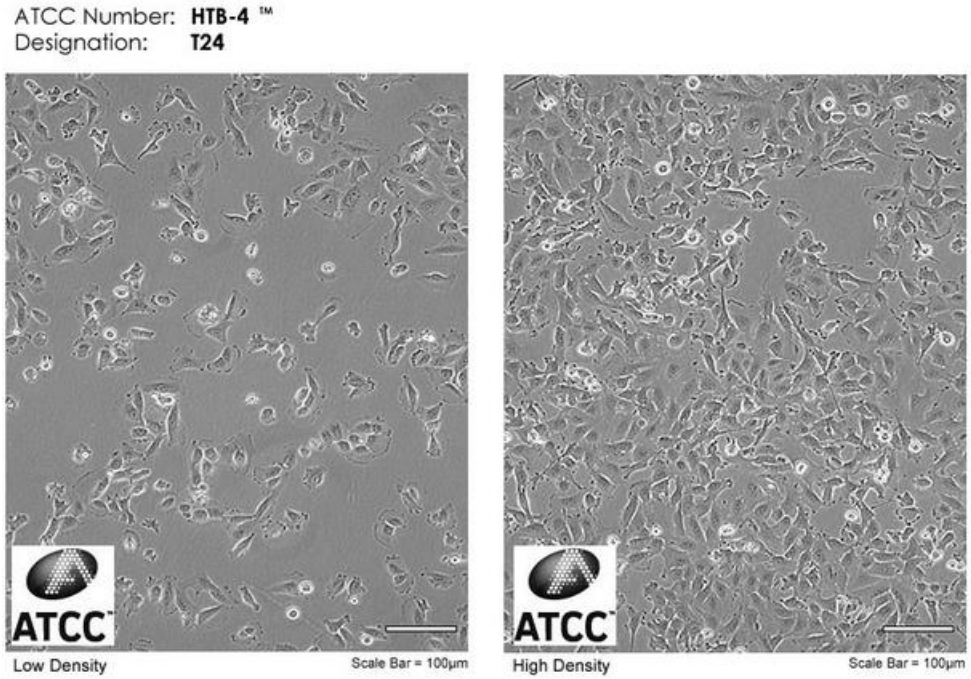

Figure 4. Cell micrograph of T24 cell line

Source: https://www.atcc.org/products/htb-4 (33)

3.2 Cell proliferation assay (MTT)

Cells were resuspended in a diluted solution of Trypan blue and counted through a binocular inverted microscope, MOTIC AE30, using Neubauer chambers. The cell number was calculated according to the formula: number of counted cells x 10 × 10^4/mL. The cells were then plated in 96-well plates at a density of 11,000 cells/well and incubated overnight. The cells were treated with thieno[2,3-b]pyridine compounds (Inhibitors 5,6,8&9) at concentration of 0.05, 0.2, 0.5, 1, 2.5, 5, 10 µg/mL in a complete medium (in triplicate) for 4, 24, 48 and 72 h. Then, the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] assay was performed in such a manner that after the treatment with the agents, the cells were incubated with 0.5 g MTT/L at 37°C for 2 hours. Afterwards, the medium was removed and dimethylsulphoxide (DMSO) was added and incubated for another 10 min at 37°C with shaking. The degree of formazan formation, an indicator of living and metabolically active cells, was measured photometrically at 570 nm with microplate photometar (HiPo MPP-96, Biosan, Riga, Latvia). The data was calculated in relation to the untreated control (100%) from three independent measurements.

The thieno[2,3-b]pyridine compounds used can be seen in the following Figures 4, 5, 6 and 7.

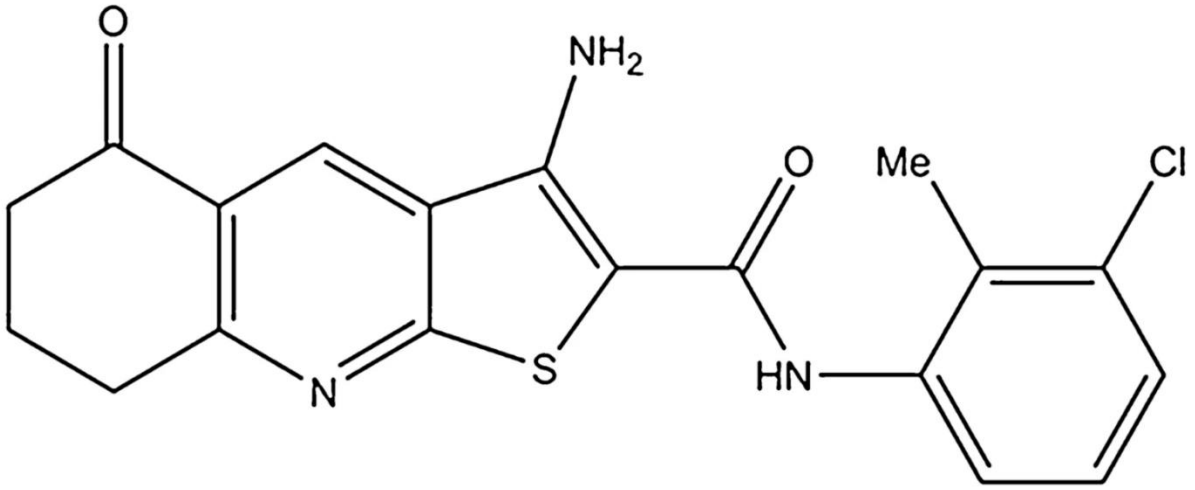

Figure 5. Inhibitor 5

3-amino-*N*-(3-chloro-2-methylphenyl)-5-oxo-5,6,7,8-tetrahydrothieno[2,3-*b*]quinoline-2-carboxamide

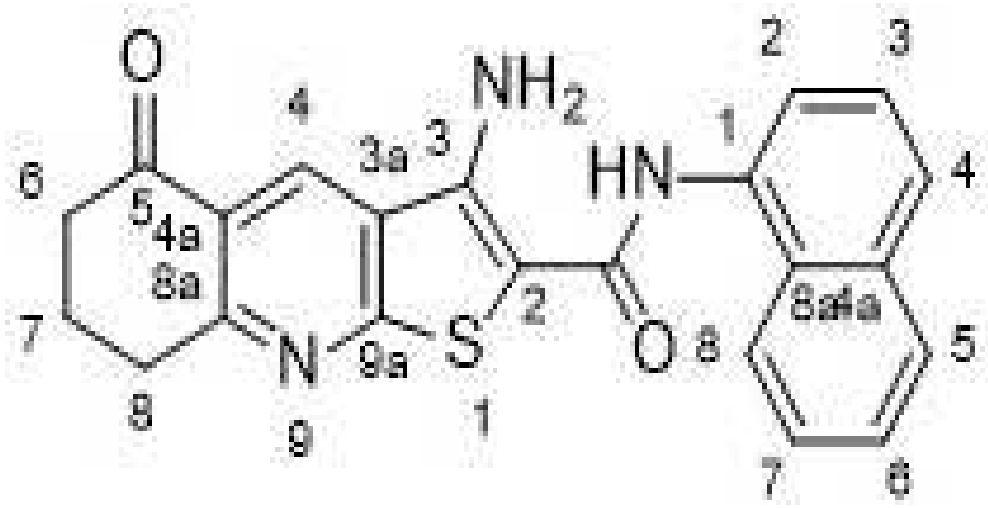

Figure 6. Inhibitor 6

3-amino-*N*-(naphthalen-1-yl)-5-oxo-5,6,7,8-tetrahydrothieno[2,3-*b*]quinoline-2-carboxamide

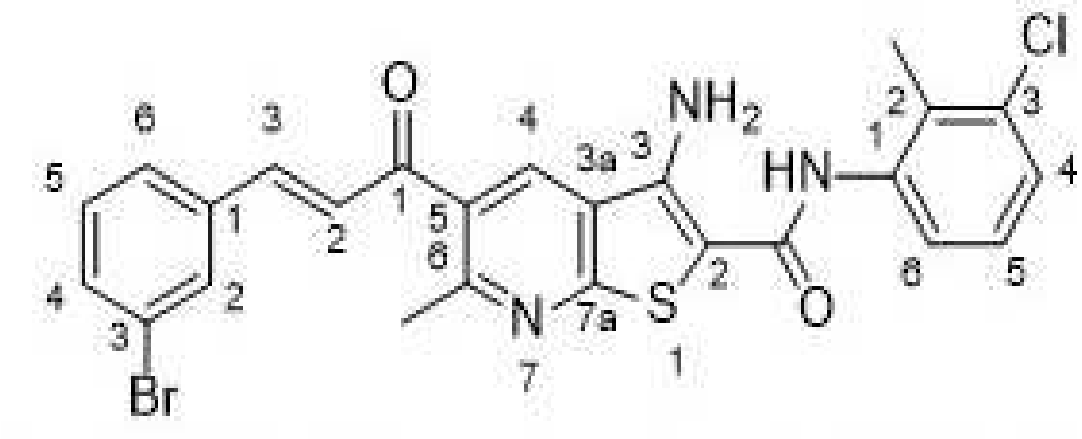

Figure 7. Inhibitor 8

(E)-3-amino-5-(3-(3-bromophenyl)acryloyl)-*N*-(3-chloro-2-methylphenyl)-6-methylthieno[2,3-*b*]pyridine-2-carboxamide

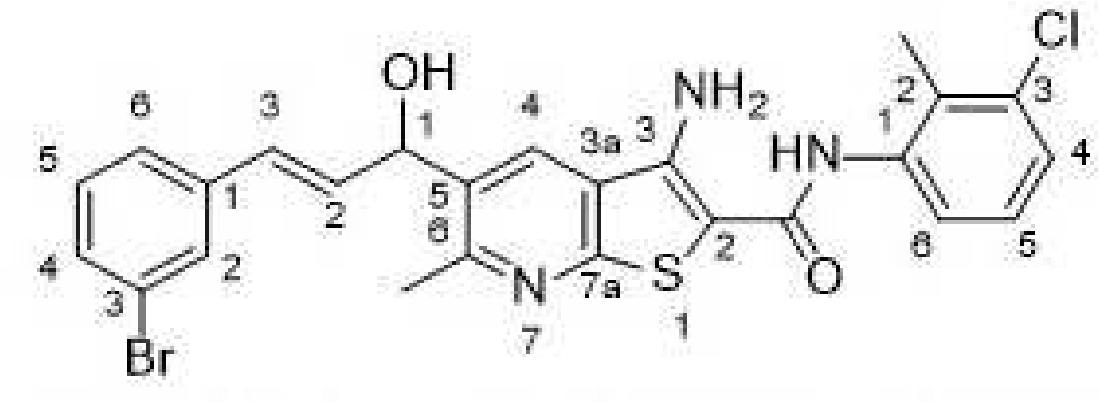

Figure 8. Inhibitor 9

(E)-3-amino-5-(3-(3-bromophenyl)-1-hydroxyallyl)-*N*-(3-chloro-2-methylphenyl)-6-methylthieno[2,3-*b*]pyridine-2-carboxamide

4. RESULTS

By using the MTT assay we determined the activity of the T24 cell lines after administration of the inhibitors 5, 6, 8 and 9. We depicted the activity of the cells after 4, 24, 48 and 72 hours. This was done with a dosage of 0.05, 0.20, 0.50, 1, 2.5, 5 and 10 µg/mL, respectively.

For inhibitor 5, we observed that the half maximal inhibitory concentration (IC50) after 48 hours was reached with 2.672 µg/mL. Consequently, after 72 hours the IC50 was 0.5987 µg/mL. For 4 and 24 hours, IC50 could not be determined (Figure 9).

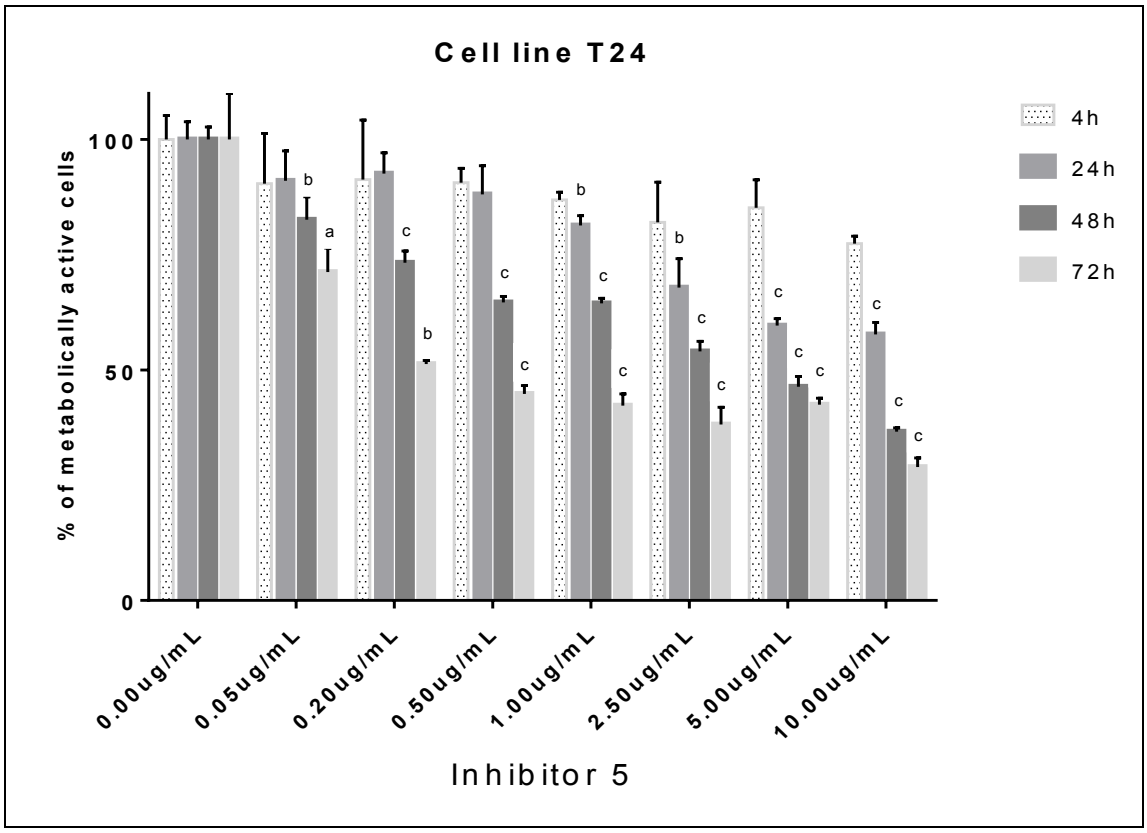

Figure 9. Inhibitor 5 produces a dose- and time-dependent metabolic defect in T-24 cell line. Cells were treated with a dose-dependent curve of Inhibitor 5 as shown in the figure for 4, 24, 48 and 72 h and cell metabolism evaluated by the MTT assay. NOTES: h, hours; a, $P<0.05$; b, $P<0.01$; c, $P<0.001$.

Table 3. Inhibitor 5 concentrations in µg/mL with exact P values. NOTES: h, hours; a, $P<0.05$; b, $P<0.01$; c, $P<0.001$.

conc / µg/mL	P value		conc / µg/mL	P value	
0.05	0.241639		0.05	0.138826	
0.2	0.341205		0.2	0.098248	
0.5	0.055353		0.5	0.048383	
1	0.014175		1	0.001867	b
2.5	0.058107		2.5	0.001657	b
5	0.032543		5	7.28E-05	c
10	0.001988		10	9.75E-05	c

conc /µg/mL	P value		conc /µg/mL	P value	
0.05	0.006157	b	0.05	0.011483	a
0.2	0.00024	c	0.2	0.001086	b
0.5	3.53E-05	c	0.5	0.000707	c
1	3.09E-05	c	1	0.000631	c
2.5	2.18E-05	c	2.5	0.00055	c
5	1.21E-05	c	5	0.000588	c
10	2.69E-06	c	10	0.000268	c

Inhibitor 6 showed a IC50 at a value of 1.528 µg/mL after 72 hours. For 4, 24 and 48 hours, IC50 could not be determined (Figure 10).

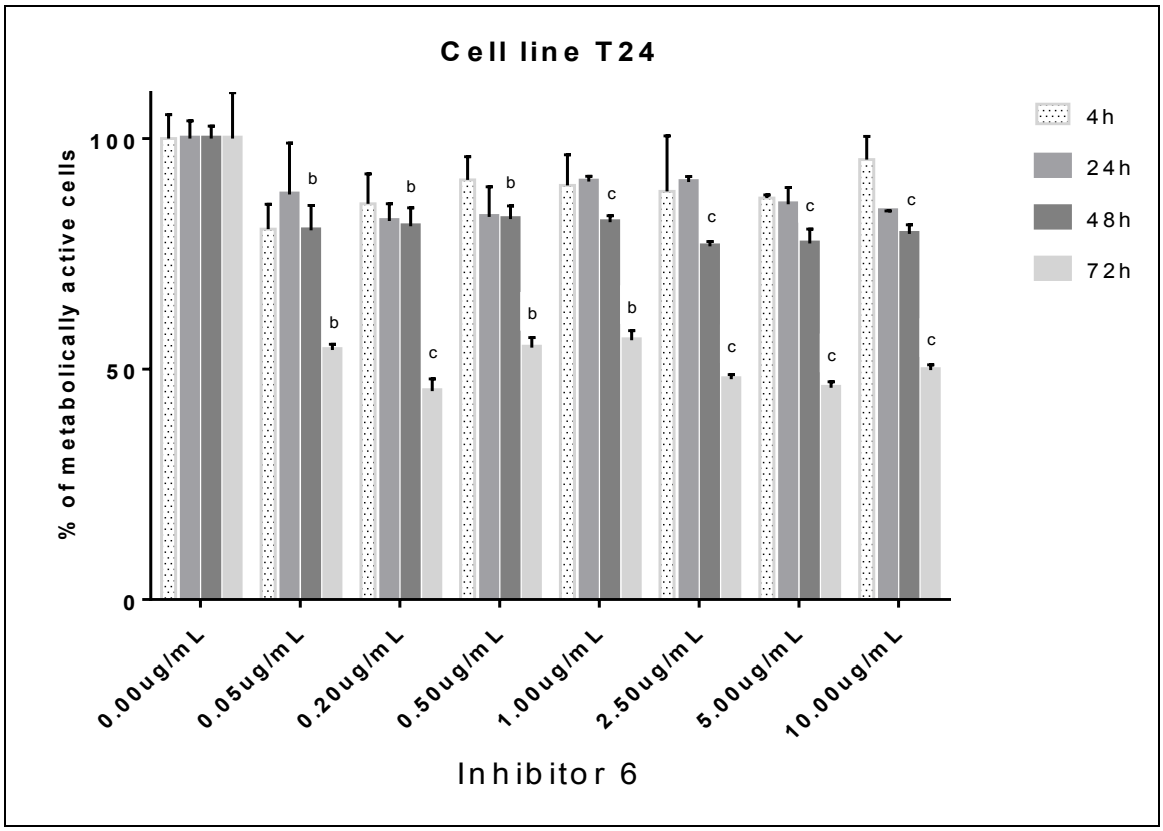

Figure 10. Inhibitor 6 produces a dose- and time-dependent metabolic defect in T-24 cell line. Cells were treated with a dose-dependent curve of Inhibitor 6 as shown in the figure for 4, 24, 48 and 72 h and cell metabolism evaluated by the MTT assay. NOTES: h, hours; a, $P<0.05$; b, $P<0.01$; c, $P<0.001$.

Table 4. Inhibitor 6 concentrations in µg/mL with exact P values. NOTES: h, hours; a, $P<0.05$; b, $P<0.01$; c, $P<0.001$.

conc / µg/mL	P value		conc / µg/mL	P value	
0.05	0.010528		0.05	0.150298	
0.2	0.042296		0.2	0.004555	
0.5	0.09866		0.5	0.017928	
1	0.105785		1	0.051386	
2.5	0.205811		2.5	0.015992	
5	0.01304		5	0.009692	
10	0.340014		10	0.01216	
conc / µg/mL	P value		conc / µg/mL	P value	
0.05	0.004617	b	0.05	0.001393	b
0.2	0.002409	b	0.2	0.000781	c
0.5	0.001557	b	0.5	0.001539	b
1	0.000514	c	1	0.001755	b
2.5	0.000155	c	2.5	0.00084	c
5	0.000675	c	5	0.000743	c
10	0.000436	c	10	0.000981	c

Inhibitor 8 was the only one that had a IC50 value at 24 hours reached with a value of 3.384 µg/mL. After 48 hours it was measurable at 1.582 µg/mL and after 72 hours an IC50 of 0.2114 µg/mL was observed. For 4 hours, IC50 could not be determined (Figure 11).

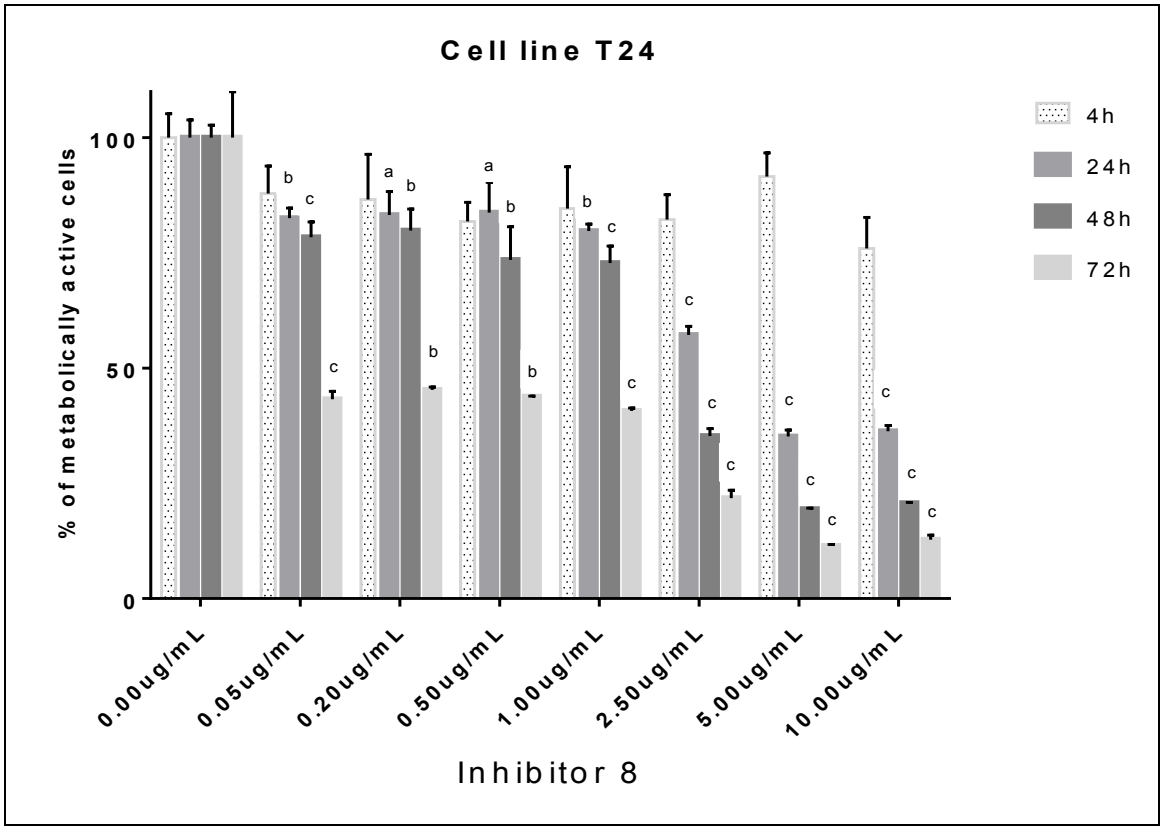

Figure 11. Inhibitor 8 produces a dose- and time-dependent metabolic defect in T-24 cell line. Cells were treated with a dose-dependent curve of Inhibitor 8 as shown in the figure for 4, 24, 48 and 72 h and cell metabolism evaluated by the MTT assay. NOTES: h, hours; a, $P<0.05$; b, $P<0.01$; c, $P<0.001$.

Table 5. Inhibitor 8 concentrations in µg/mL with exact P values. NOTES: h, hours; a, $P<0.05$; b, $P<0.01$; c, $P<0.001$.

conc / µg/mL	P value		conc / µg/mL	P value	
0.05	0.057097		0.05	0.002441	b
0.2	0.104003		0.2	0.010374	a
0.5	0.009064		0.5	0.021072	a
1	0.063554		1	0.00108	b
2.5	0.014853		2.5	6.8E-05	c
5	0.116395		5	1.1E-05	c
10	0.008143		10	1.11E-05	c
conc / µg/mL	P value		conc / µg/mL	P value	
0.05	0.000976	c	0.05	0.000632	c
0.2	0.002954	b	0.2	0.005213	b
0.5	0.003981	b	0.5	0.004821	b
1	0.000503	c	1	0.000508	c
2.5	3.7E-06	c	2.5	0.000181	c
5	8.81E-07	c	5	0.000105	c
10	9.35E-07	c	10	0.000113	c

Inhibitor 9 had a value of 4.197 µg/mL after 48 hours and 0.4041 µg/mL after 72 hours. For 4 and 24 hours, IC50 could not be determined (Figure 12).

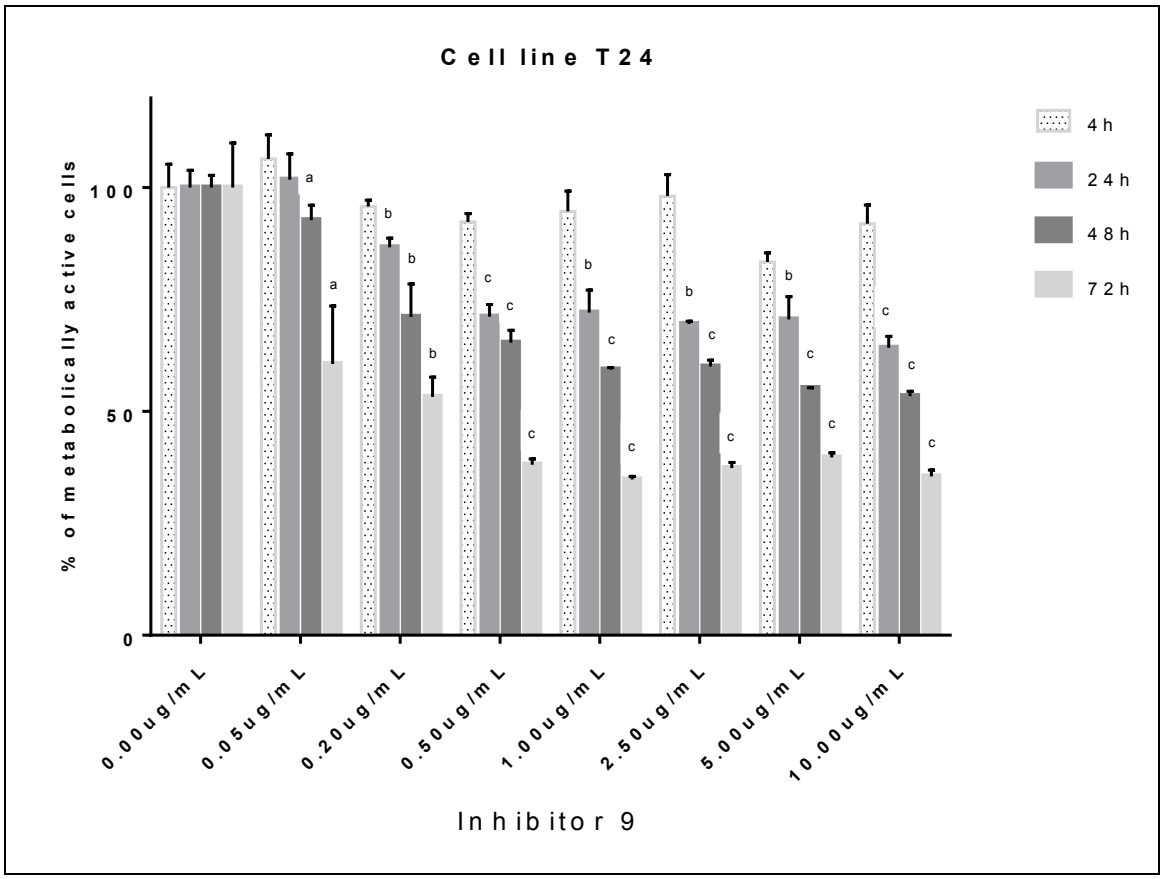

Figure 12. Inhibitor 9 produces a dose- and time-dependent metabolic defect in T-24 cell line. Cells were treated with a dose-dependent curve of Inhibitor 9 as shown in the figure for 4, 24, 48 and 72 h and cell metabolism evaluated by the MTT assay. NOTES: h, hours; a, $P<0.05$; b, $P<0.01$; c, $P<0.001$.

Table 6. Inhibitor 9 concentrations in µg/mL with exact P values. NOTES: h, hours; a, $P<0.05$; b, $P<0.01$; c, $P<0.001$.

conc / µg/mL	P value		conc / µg/mL	P value	
0.05	0.213752		0.05	0.679471	
0.2	0.363284		0.2	0.006226	b
0.5	0.073729		0.5	0.000451	c
1	0.252333		1	0.001628	b
2.5	0.665637		2.5	0.001866	b
5	0.006736		5	0.004984	b
10	0.103535		10	0.000182	c
conc / µg/mL	P value		conc / µg/mL	P value	
0.05	0.042723	a	0.05	0.013989	a
0.2	0.003078	b	0.2	0.001768	b
0.5	0.000102	c	0.5	0.00044	c
1	1.42E-05	c	1	0.000351	c
2.5	2.26E-05	c	2.5	0.000419	c
5	9.1E-06	c	5	0.000482	c
10	1.03E-05	c	10	0.000376	c

5. DISCUSSION

Previous studies have shown that thieno[2,3-b]pyridine derivatives show a dose- and time-dependent cytotoxicity (32). Inhibitor 5 3-amino-*N*-(3-chloro-2-methylphenyl)-5-oxo-5,6,7,8-tetrahydrothieno[2,3-*b*]quinoline-2-carboxamide, Inhibitor 6 3-amino-*N*-(naphthalen-1-yl)-5-oxo-5,6,7,8-tetrahydrothieno[2,3-*b*]quinoline-2-carboxamide, inhibitor 8 (E)-3-amino-5-(3-(3-bromophenyl)acryloyl)-*N*-(3-chloro-2-methylphenyl)-6-methylthieno[2,3-*b*]pyridine-2-carboxamide and Inhibitor 9 (E)-3-amino-5-(3-(3-bromophenyl)-1-hydroxyallyl)-*N*-(3-chloro-2-methylphenyl)-6-methylthieno[2,3-*b*]pyridine-2-carboxamide all show this property. By performing a colorimetric MTT assay to analyze the cell viability of bladder cancer cells treated with various concentrations for different time intervals. We discovered that the newly synthesized anticancer compounds (Inhibitors 5,6,8&9) show a dose- and time-dependent cytotoxicity for bladder cancer T24 cancer cells.

Any palliative treatment for bladder cancer should aim to enhance quality of life, decrease morbidity, prevent cystectomy, give long-lasting control, and increase overall survival. It is assumed that a combination of chemotherapy and radiotherapy represents an alternative to radical cystectomy due to the effectiveness of cisplatin-based chemotherapy in patients with invasive bladder carcinoma, the frequently favorable response of the primary tumor to combination chemotherapy, and the synergistic effect of chemotherapy and radiotherapy (34).

Thieno[2,3-b]pyridine derivatives have the potential to be great additives to the cisplatin-based chemotherapy. Further studies should be done to evaluate the correspondence of those therapies in combination.

All inhibitors have shown a great effectiveness on the T24 line´s cell activity. All of them reached a IC50 value within 72 hours. The most effective was Inhibitor 8. It had an IC50 of 0.4041 µg/mL after 72 hours.

A longer period of incubation time leads to lower IC50 values because less dosage of inhibitor is needed to achieve an inhibition of 50% of the T24 cells. The lower IC50 values means the better the drug acts on the cancer cells without harming the patient's own organism and occurrence of side effects.

Other agents have also proven effective on the T24 cell line. Crude brazilin extract from Sappan wood works on the c-Fos proto-oncogene (35). It is the product of the signaling cascade that includes ERK, which is inhibited by thieno[2,3-b]pyridine derivatives. Both compounds

combined might show an increased potential of fighting bladder cancer. Further studies should be conducted to show if there is a correlation.

Other studies have also used thieno[2,3-b]pyridine anticancer compounds to determine their effectiveness in apoptosis or cytotoxicity. Glycophenotype of breast and prostate cancer stem cells all showed significant results in these studies. The use of these anticancer agents has comparable outcomes in regards to the cytotoxicity in our study conducted on T24 bladder cancer cells. Consequently, further elaborate studies should be conducted regarding apoptosis in the T24 bladder cancer cell line using thieno[2,3-b]pyridine anticancer compounds (31,32).

The study was performed in triplicate decreasing its limiting factors. Nonetheless some limiting factors have to be taken into consideration in our study. Being an in vitro study, no conclusions regarding patients' safety can be made. Additionally, the utilization of a different bladder cancer cell lines other than T24 could provide even more clarity over the effectiveness of the inhibitors.

6. CONCLUSION

- The treatment of human T24 bladder cancer cells with compounds from thieno[2,3-b]pyridine family (Inhibitor 5, 6, 8 and 9) has a cytotoxic effect on the investigated cell line.
- All inhibitors have a dose- and time-dependent activity - the cytotoxicity was proportional with the increase in concentration and time of incubation.
- The most effective was Inhibitor 8 with an IC50 value of 0.4041 μg/mL after 72 hours.

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8. SUMMARY

Objectives: The purpose of this study was to determine the effects of treating bladder cancer cells with the newly synthesized thieno[2,3-b]pyridine anticancer agents, by focusing on its cytotoxic effects on the investigated human cell line T24.

Methods: The T24 bladder cancer cell line was treated with a newly developed thieno[2,3-b]pyridine anticancer compounds: Inhibitor 5 3-amino-*N*-(3-chloro-2-methylphenyl)-5-oxo-5,6,7,8-tetrahydrothieno[2,3-*b*]quinoline-2-carboxamide, Inhibitor 6 3-amino-*N*-(naphthalen-1-yl)-5-oxo-5,6,7,8-tetrahydrothieno[2,3-*b*]quinoline-2-carboxamide, Inhibitor 8 (E)-3-amino-5-(3-(3-bromophenyl)acryloyl)-*N*-(3-chloro-2-methylphenyl)-6-methylthieno[2,3-*b*]pyridine-2-carboxamide and Inhibitor 9 (E)-3-amino-5-(3-(3-bromophenyl)-1-hydroxyallyl)-*N*-(3-chloro-2-methylphenyl)-6-methylthieno[2,3-*b*]pyridine-2-carboxamide to determine its cytotoxic effect on T24 cells. The 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT) assay was performed to analyze the cellular metabolic activity and determine the cytotoxic effect.

Results: Inhibitors 5, 6, 8 and 9 all showed significant cytotoxic effect on the bladder cancer cells in a dose- and time-dependent manner. The most effective was Inhibitor 8 with an IC50 value of 0.4041 μg/mL after 72 hours.

Conclusion: Due to their cytotoxic effect on bladder cancer cells, Inhibitors 5, 6, 8 and 9 deserve further attention as a potential treatment for transitional cell cancer.

9. CROATIAN SUMMARY

Naslov: Citotoksično djelovanje tieno[2,3-b]piridinskih spojeva na stanice humanog karcinoma mokraćnog mjehura

Ciljevi: Svrha ove studije bila je utvrditi učinke tretiranja stanica raka mokraćnog mjehura novosintetiziranim tieno[2,3-b]piridin antitumorskim spojevima, fokusirajući se na njihove citotoksične učinke na ispitivanu humanu staničnu liniju T24.

Materijali i metode: T24 stanična linija raka mokraćnog mjehura tretirana je novosintetiziranim tieno[2,3-b]piridin antitumorskim spojevima: Inhibitor 5 - 3-amino-N-(3-kloro-2-metilfenil)-5-okso-5,6,7,8-tetrahidrotieno[2,3-b]kinolin-2-karboksamid, Inhibitor 6 - 3-amino-N-(naftalen-1-il)-5-okso-5,6,7,8-tetrahidrotieno[2,3 -b]kinolin-2-karboksamid, Inhibitor 8 - (E)-3-amino-5-(3-(3-bromofenil)akriloil)-N-(3-klor-2-metilfenil)-6-metiltieno[2,3-b]piridin-2-karboksamid i Inhibitor 9 - (E)-3-amino-5-(3-(3-bromofenil)-1-hidroksialil)-N-(3-klor-2-metilfenil)-6- metiltieno[2,3-b]piridin-2-karboksamid, kako bi se odredio njihov citotoksični učinak na T24 stanice. Proveden je test 3-(4,5-dimetiltiazolil-2)-2,5-difeniltetrazolij bromida (MTT) kako bi se analizirala stanična metabolička aktivnost i odredio citotoksični učinak.

Rezultati: Svi inhibitori 5, 6, 8 i 9 pokazali su značajan citotoksični učinak na stanice raka mokraćnog mjehura i taj učinak je ovisan o dozi i vremenu inkubacije. Najučinkovitiji je bio Inhibitor 8 s IC50 vrijednošću od 0,4041 μg/mL nakon 72 sata.

Zaključci: Zbog svog citotoksičnog učinka na stanice raka mokraćnog mjehura, Inhibitori 5, 6, 8 i 9 zaslužuju daljnju pozornost kao potencijalni tretman za rak prijelaznih stanica.