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MULTI-TARGET DIRECTED LIGANDS (MTDLS) AS POTENTIAL THERAPIES FOR BLADDER CANCER

by
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MULTI-TARGET DIRECTED LIGANDS (MTDLS) AS POTENTIAL THERAPIES FOR BLADDER CANCER

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ABSTRACT
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Introduction
Bladder cancer is the predominant type of cancer affecting males. It is also a malignancy that targets females as well. Recent studies implicated a sub-population of tumor cells with stem cell properties in imparting resistance to chemotherapy and radiation. Henceforth, it is critical to distinguish and explicitly target those cancer stem cells. Significantly, multiple inflammatory cascades are involved in the pathogenesis of proliferative disorders, namely the Arachidonic acid (AA) pathway involving cyclooxygenase-2 (COX-2) and 15-lipoxygenase (15-LOX). They assume a multitude of functions ranging from metabolic impairment leading to adipose tissue inflammation, to pro- and anti- tumorigenic effects making them viable targets for interference with tumor growth. However, such an intervention is often complicated by the uniqueness and often mutual regulatory effects of COX-2 and 15-LOX on different cellular targets mainly the Peroxisome Proliferator-Activated Receptor (PPARγ).

Hypothesis & Aim
Treating bladder cancer as a multifaceted disorder requires the regulation of more than one target. We hypothesize that the simultaneous targeting of COX-2, 15-LOX and PPARγ by MTDLs will be effective in suppressing human bladder cancer cell growth.

Methods
Two Human bladder cancer cell lines (RT4 & T24) will be treated with different concentrations of MTDL inhibitors of COX-2 & 15-LOX with or without PPARγ partial agonist activity. MTT and Trypan blue exclusion assays will be used to assess the anti-proliferative effect of these compounds *in vitro*. In addition, the ability of these drugs to inhibit cell migration will be tested using the wound-healing migration assay. Moreover, the 3D sphere-formation assay will be used to investigate their impact on the cancer stem/progenitor cells population in both cell lines. Molecular studies will also be performed to assess the potential mechanism involved.

Results
Treatment with MTDLs namely Pd3 was shown to decrease RT4 & T24 cell proliferation and migration as well as inhibit sphere-formation ability in a dose-dependent manner. Moreover, Pd3 was able to inhibit Monocyte differentiation into macrophages which, occurs during inflammation thus, is effective in targeting inflammation induced cancer. Molecular studies showed that the dual target drug was able to target inflammatory pathways involved

in the pathogenesis of cancer. This is the first study to assess the effect of double- and triple-target drugs on human bladder cancer cells *in vitro* and possibly demonstrate their potential therapeutic value.

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ABBREVIATIONS

12-LOX: Lipoxygenase 12

13(S)-HpODE:13-S-hydroxyoctadecadienoic acid

15 LOX-2: 5-Lipoxygenase-2

15(S)HpETE: 15-S-hydroxyeicosatetraenoic

15-LOX: 15- Lipoxygenase

5 HPETE: hydroperoxy-eicosatetraenoic acid

5-LOX: Lipoxygenase 5

AA: arachidonic acid

AC: apoptotic cells

AJCC: American Joint Committee on Cancer

APC: adenomatous polyposis coli

AR: androgen receptor

ATCC: American Type Culture Collection

Axin: axin inhibitor

CAC: colitis associated colon cancer

CIS: *in situ* carcinoma

CNS: Central nervous system

CO2: Carbone dioxide

COX: cyclooxygenase

COX-1: cyclooxygenase

COX-2: cyclooxygenase 2

CRC: colorectal cancer

CSC: cancer stem cells

CSCs: Cancer stem cells

CT: Computerized tomography

DHT: dihydrotestosterone

DMEM: Dulbecco's Modified Eagle Medium

DMSO: Dimethyl sulfoxide

EGF: epidermal growth factor

EGFR: endothelial growth factor receptor

EMT: Epithelial-mesenchymal transition

ER: endoplasmic reticulum

FAP: familial adenomatous polyposis

FBS: Fetal bovine serum

FDA: Food and drug administration

Fgfr: fibroblast growth factor receptor

GPCR: G-protein-coupled receptors

GSK-3β: Glycogen synthase kinase 3 beta

HBV: Hepatitis B virus

HIF-1: Hypoxia- inducible factor

Hrs: Hours

IL-10: Interleukin 10

IL-1β: Interleukin 1β

LA: linoleic acid

LEF: lymphoid enhancer-binding factor

LOX: lipoxygenase

MCP-1: macrophage chemoattractant protein

MIF: migration inhibitory factor

MKP-1: mitogen activated protein kinase phosphatase 1

MRI: Magnetic resonance imaging

mRNA: Messenger RNA

MTDD: multi targeted drug design

MTDL: multi target directed ligands

MTT: Mean transit time

NADH: Nicotinamide adenine dinucleotide

NF-κB,: nuclear factor kappa-light-chain-enhancer of activated B cells

NSAID: Non-steroidal anti-inflammatory drugs

NSCLC: non-small cell lung carcinoma

OD: Optical density

PBS: Phosphate buffered saline

PET scan: positron emission tomography

PG: prostaglandin

PGI2: Prostacyclin

PNET: Primitive neuroectodermal tumors

PPARs: Peroxisome Proliferator-Activated Receptors

PUFAs: polyunsaturated Fatty acids

RB: retinoblastoma protein

RNS: reactive nitrogen species

TB: Trypan blue

WH: wound healing

ROS: Reactive oxygen species

RXR: receptor; the retinoid X receptor

SCLC: small cell lung carcinoma

SFU: Sphere formation unit

STAT-3: Signal transducer and activator of transcription 3

TCC: transitional cell carcinoma

TCF: N-termini of DNA-binding proteins of the T-cell factor

TLR4: Toll-Like Receptor 4

TNF: tumor necrosis factor

TXA2: Thromboxanes A2

TZD: Thiazolidinediones

UCB: urothelial bladder cancer

UTI: Urinary Tract Infection

VEGF: vascular endothelial growth factor

VSMCs: vascular smooth muscle cells

YAP 1: Yes-associated protein

CHAPTER I

INTRODUCTION

A. The Urinary Bladder

1. Physiology of the Urinary Bladder

Being a hollow and stretchable organ, the urinary bladder is part of the pelvis, located in front of the rectum, above the reproductive organs. It mainly assumes a spherical shape, however it is important to mention that the size of the bladder is variable depending on the volume of urine it contains (1). Being the main organ of the urinary system, the urinary bladder plays two major roles. First, the bladder allows urine to be stored due to the distensible walls of the bladder. The kidneys produce urine which then travels down the two ureters to reach the bladder. The internal lining of the bladder, called rugae, allows it to store a volume of 400-600mL of urine in normal healthy adults. The urine then passes into the urethra to be expelled outside the body which is the second role of the bladder. During urination, the walls of the bladder contract and the sphincter relaxes thus, allowing urine to be expelled to the outside (2).

2. Epidemiology

Being the second most predominant malignancy in males after prostate cancer and the ninth most predominant malignancy in females, over 429, 793 new cases of bladder cancer (BC) are yearly identified in the worldwide. Concerning death rates, the estimate mortality rate is approximately 165,084. In Lebanon, almost 781 new cases of BC are reported yearly. Men are at a

higher incidence risk than women by a rate ratio of 3:1. Approximately 66% of the cases are of age 65 years or older (3).The majority of bladder cancer tumors, namely 70% are classified as superficial (Fig.1). Of which only 15% are likely to progress into a malignant type thus, increasing the risk of death. 30% are invasive initially, and 50% of the cases are have a high risk of metastasis along with a high incidence of death (4).

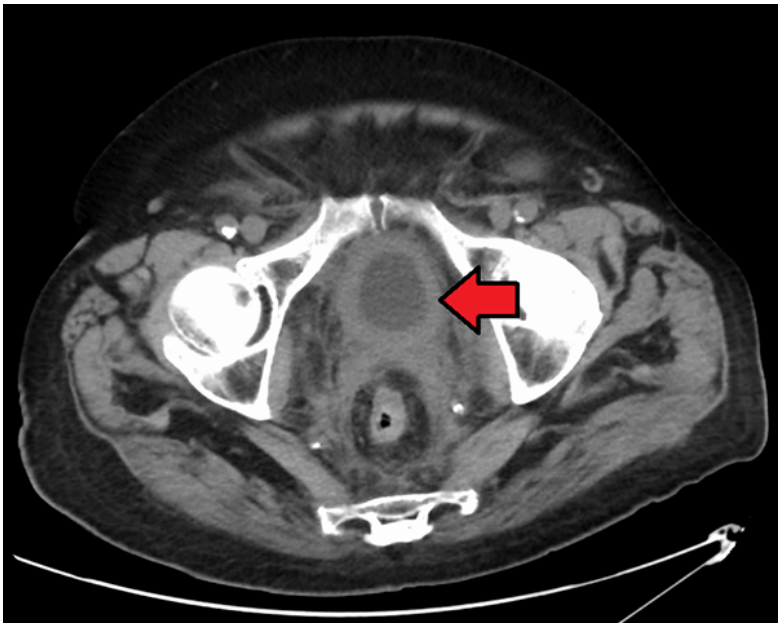

Fig.1: A CT scan of a bladder cancer patient with a thickened bladder wall (red arrow). (Adapted from NCI Staff, May 30, 2017)

3. History of Bladder Cancer

The history of bladder cancer incidence was strongly correlated with the patients being exposed to biological toxic amines mainly in dye, textile, rubber, leather, paint and printing industries. Moreover, exposure to tobacco and smoking is a potential

environmental risk factors for the development of bladder cancer, responsible for more than 50% of the total cases (5).

Furthermore, recurrent (UTI) may lead to the chronic irritation of the bladder thus, is also considered a major risk factor. Sex and the age of patients are also risk factors. People above the age of 70 are mostly prone. (6). Prior radiation to the pelvis and some chemotherapeutic drugs, namely cyclophosphamide, were shown to potentiate the long-term risk of developing bladder cancer. Finally, some diabetes medications mainly Pioglitazone (Actos®), which is recommended for patients diagnosed with type 2 diabetes, was labelled by FDA (food and drug administration) to be correlated with bladder cancer (7).

4. Prognosis

Several prognostic factors are correlated with the history of bladder cancer. Some of these include the tumor depth into the wall of the bladder, the detection of in situ carcinoma (CIS), histologic evaluation, among many others (8). However, they do not constitute the ultimate prognostic response of cancer patients because of the biologic origins the disease subset, which is not fully understood. In fact, some lesions showing a good prognostic histology end up developing into malignant and aggressive types of tumors despite the curative treatments implemented. On the other hand, some other tumors present with an unfavorable histological grade and yet respond well to therapy. Hence, histological assessment is not a major determinant in evaluating patients response to treatment (9).

5. Types and subtypes of Bladder cancer

There are several types and subtypes depending on the type of cell in which the tumor was initiated. Pathologists diagnose and classify the type of cancer by examining the tumor under a compound microscope. They are classified into three major classes (Fig 2):

First, there is the non-invasive bladder cancer; here the cancer is only confined to the bladder's inner layer (urothelium). Second, there is the Non-muscle-invasive bladder cancer in which the cancer has spread to reach the layer of connective tissue (lamina propria) (10).

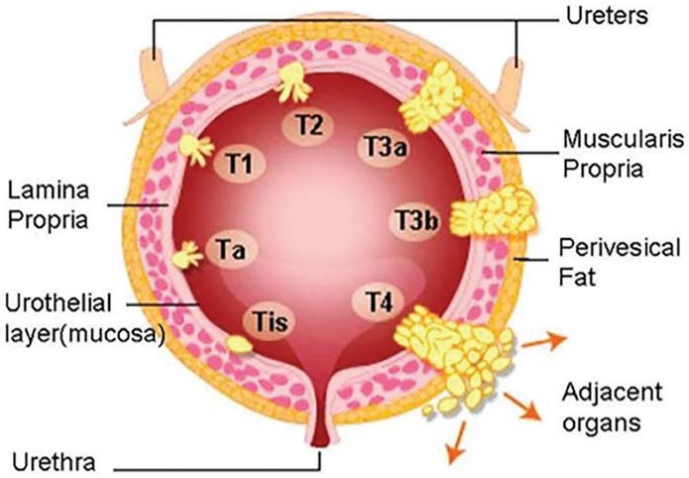

Fig.2: Schematic Representation of Bladder Cancer Stages. Non-invasive bladder cancer is only confined to the bladder's inner layer (urothelium). Second, the non-muscle-invasive bladder cancer has reached the connective tissue (lamina propria Finally, the aggressive one is the Muscle-invasive bladder cancer. Here, the cancer has targeted the bladder muscles (muscularis propria) along with the surrounding fat (Adapted from Nelson Durán and Wagner J. Fávaro, 2018)

Finally, the aggressive one is the Muscle-invasive bladder cancer. Here, the cancer has targeted the bladder muscles (muscularis propria) along with the surrounding fat (11). Transitional cell carcinoma (TCC) sometimes called urothelial cancer is the predominant

one. It accounts for almost 95% of bladder cancer cases. The cancerous cells of TCC are similar to those lining the inside of the urinary bladder. There are two different subtypes for TCC (12):

Papillary carcinoma starts in the inner surface and grows towards the center of the bladder. In most cases, they are non-invasive papillary cancer, in other words, they remain in the superficial layers of the bladder wall. This type of TCC is well responsive to therapy and is correlated with a good prognosis. On the other hand, Flat carcinomas remains on the surface (Fig.3). If non-invasive, this type is classified as *in situ* or non-invasive flat carcinoma (13). There are other rare types of bladder cancer including squamous cell cancer; which accounts for only 1- 2% of the cases diagnosed in the United States. Adenocarcinoma accounts for 1% of all bladder cancer cases in the United-States. These cancerous cells are similar to the gland-forming cells seen in patients with colon cancer. There are also extremely rare cases of bladder cancer, of which are small-cell carcinoma and sarcoma. The former starts in neuroendocrine cells and the latter in the muscular layer of the bladder.(14)

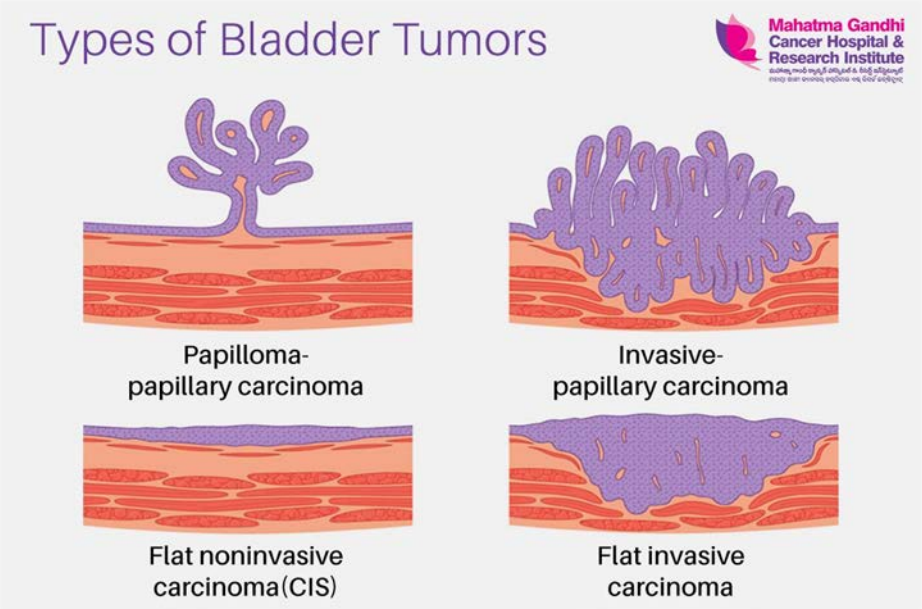

Fig.3: Schematic Representation of the Types of Bladder Tumors. Papillary carcinoma starts in the inner surface of the bladder and grows towards the center. Flat carcinoma remains on the surface of the bladder. If non-invasive, this type of cancer is classified as *in situ* or non-invasive flat carcinoma**.** (Adapted from Mahatma Gandhi Cancer Research Institute, 2018)

The (AJCC) proposed the TNM system evaluating three primary indices. First, the letter T (tumor) refers to the size of the cancer. N (node) specifies whether or not the cancer is found in the (LN) and lastly the M (metastasis) indicates whether or not the cancer has spread to other areas. Then a number ranging from 0-4 or the letter X is attributed. A higher number is correlated with a higher severity. The letter X indicates the lack of information. Now the cancer is staged (0-4) (15): Stage 0 has; Stage 0a which represents a noninvasive papillary carcinoma. At this stage, cancerous cells have not yet invaded the lymph nodes or any other site. On the other hand, Stage 0is which is a Flat, noninvasive carcinoma or carcinoma in situ. At this stage, cancerous cells have not yet invaded the lymph nodes or

any other site. Stage I: at this stage, the cancer has targeted the connective tissue layer underneath beneath the layer of the bladder wall. At this stage, cancerous cells have not yet invaded the lymph nodes or any other site. Stage II: at this stage, cancerous cells have metastasized to the muscle layer. Here, no lymph node spread is detected. Stage III: at this stage, cancerous cells have metastasized through the bladder to reach the fatty tissue layer. No metastasis has been detected. Stage IV: here the cancer has grown into the pelvis cavity with lymph nodes spread and in some cases several organs, such as bones, liver or lungs are also affected (16) (Fig.4)

Stage	Tumor (T)	Lymph node involvement (N)	Metastasis (M)
Stage 0a	Ta: noninvasive papillary carcinoma	N0	M0
Stage 0is	Tis: carcinoma in situ	N0	M0
Stage I	T1: has grown into connective tissue	N0	M0
Stage II	T2a: has grown into inner half of muscle layer	N0	M0
	T2b: has grown into outer half of muscle layer	N0	M0
Stage III	T3a: microscopic invasion of surrounding fatty tissue	N0	M0
	T3b: macroscopically detectable invasion of surrounding fatty tissue	N0	M0
Stage IV	T4a: spread into prostate (men) and uterus (women)	N0	M0
	T4b: has grown into pelvic or abdominal wall	N0	M0
	Any T	N1–3: lymph node involvement in proximal or distal lymph nodes	M0
	Any T	Any N: any lymph node involvement	M1: metastasis present

Notes: According to American Joint Committee on Cancer (AJCC). N0, no lymph node involvement; M0, No signs of metastasis.

Fig.4: Clinical Staging of urinary bladder cancer. The (AJCC) has established the TNM system evaluating three primary indices. First, the letter T (tumor) refers to the size, N (node) specifies whether or not the cancer is found in the (LN) and lastly the M (metastasis) indicates whether or not the cancer has spread to other areas. Then a number ranging from 0-4 or the letter X is assigned. A higher number corresponds to a higher severity. The letter X indicates the lack of. Now the cancer is staged (0-4) (Adapted from Smolensky D, Rathore K and Cekanova M, 2016)

6. Bladder cancer diagnosis

Several diagnostic methods are used for bladder cancer. These include mainly cystoscopy and urine cytology. Other diagnostic tools such as imaging techniques and genetic analysis are also of importance as they aim at evaluating the disease progression and developing adequate treatment regimens for each patient. First, cystoscopy and transurethral resection is a procedure during which a small tube with a camera is inserted into the urethra and slowly into the bladder. By doing so, doctors are able to examine the lining of the bladder wall and take a biopsy for further investigation (17). Second, urine cytology is also of importance whereby a urine sample is collected and checked for the presence of pre or cancerous cells. In order to make a definitive diagnosis of the patient, various imaging tests are used to examine the integrity of the Urinary tract. These imaging techniques are performed using a dye injected into the patient's arm and, that dye flows into the bladder and gives a fluorescent signal whenever a cancerous area is detected. These include a CT of the bladder, ureters and kidneys (to determine whether or not cancerous cells are only confined to the bladder area and have not spread to other regions in the body especially to the lymph nodes), a MRI; magnetic resonance imaging (to assess the depth of cancerous cells infiltration into the bladder musculature) a PET scan (to differentiate between active and dormant bladder tissues). A retrograde Pyelogram is also used in some patients allergic to the X-ray dye. This technique uses a dye to improve the view of the bladder area (18). For people with advanced bladder cancer stages in which cancerous cells have metastasized to other areas in the body, genetic analysis of bladder tumors is performed using the MSK-IMPACT, which has been used starting January 2014 to assess advanced stages. It operates on the basis of next-generation sequencing to detect the presence of genetic mutations that would help the oncologist in choosing the best treatment

regimens (19). On the other hand, some laboratory tests may also direct towards the diagnosis of bladder cancer, and these include a urine culture test to determine whether the urinary symptoms are due to a UTI (urinary tract infection) or bladder cancer. In addition, urine tumor marker tests are also performed to look for tumor markers indicative of bladder cancer. These include the NMP22 and BTA tests, the Immunocyt test and the UroVysion test (20).

B. Bladder Cancer Treatment

Regarding bladder cancer treatments, several techniques have been implemented and are in current use. A number of factors dictate the treatment regimens adopted by physicians such as type and subtype of bladder cancer, the cancer grade and stage. In addition to assessing the overall health, age and expected lifespan of the patient.

1. Surgical approach (Transurethral resection)

Surgery is performed early on because tumors are still found locally in the site of origin. In fact, the surgical intervention be total, meaning the whole bladder organ is removed sometimes for some patients, doctors proceed by adopting the bladder preserving approach. Transurethral resection is also adopted early on during the course of the disease whereby the cancer is confined to the superficial area of the bladder (Fig 5). By using a resectoscope, cancerous cells are removed from the bladder without damaging the abdomen (21).

2. Cystectomy

Cystectomy is a surgical procedure whereby the whole bladder is removed via an incision in the abdomen. It can be performed during a laparoscopic surgery, a novel surgical approach aiming at reducing pain and shortening the recovery time post-op. Cystectomy can be partial or Radical. Partial cystectomy is performed when the cancer has infiltrated the muscular layer of the bladder yet is not large enough to destroy the entire bladder. So, only the invaded part is removed during surgery. On the other hand, radical cystectomy is performed during metastasis. In this case, the entire bladder has to be removed (22)

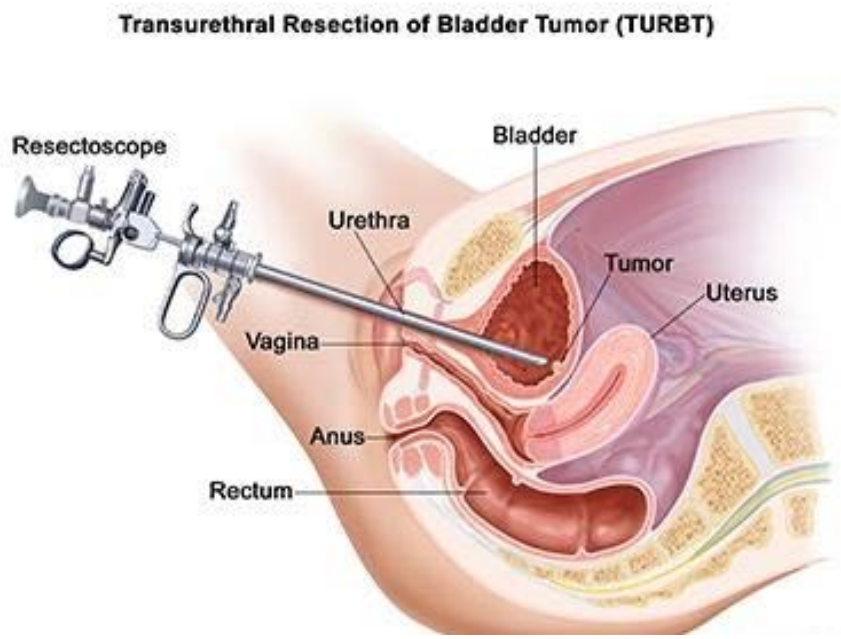

Fig.5: Bladder Cancer Transurethral Resection using a Resectoscope. It can be total, meaning the whole bladder organ is removed sometimes for some patients, doctors proceed by adopting the bladder preserving approach. Using a resectoscope, doctors are able to reach the inner lining of the bladder wall and remove the tumors without causing any damage to nearby organs**.** (Adapted from cancer support community https://www.cancersupportcommunity.org/bladder-cancer)

3. Immunotherapy

Immunotherapy is a technique that stimulate the body's defense mechanisms to kill cancerous cells. Four immunotherapeutic drugs have been approved by the (FDA) for the treatment of bladder cancer. They are known to be checkpoint inhibitors meaning, they work by "releasing the breaks" on the immune system by targeting signaling proteins that confer cancer cells the ability to escape the body's defense mechanisms. Pembrolizumab is used for patients who are intolerable to cisplatin-containing chemotherapy. Moreover, avelumab (Bavencio®) and Nivolumab (Opdivo®) respectively, are also prescribed by physicians. Finally, atezolizumab (Tecentriq®) was approved in patients who are intolerable of cisplatin-based chemotherapy due to kidney damage or heart failure. In most cases, immunotherapy is supplemented with radiation therapy for better outcomes. Also, radiation in combination with chemotherapy and/or surgery to reduce the size of the tumors and make surgery easier especially for difficult to reach bladder tumors (23).

C. The role of Inflammation in the Pathogenesis of Cancer

1. Arachidonic Acid Pathway

Evidence from recent studies have shown the role of several inflammatory mechanisms in a joint pathology in a number of diseases ranging from adipose tissue inflammation to pro and anti-tumorigenic effects. Importantly, is the arachidonic acid (AA) metabolism (24). Post injury or irritation, membrane phospholipids release arachidonic acid whereby it gets oxygenated by enzymes systems called lipoxygenases generating inflammatory mediators, the eicosanoids. Previous studies investigated the role of inflammation as a critical

component of tumor development. In fact, chronic inflammation promotes cancer development and tumor progression thus, favors metastatic spread (25).

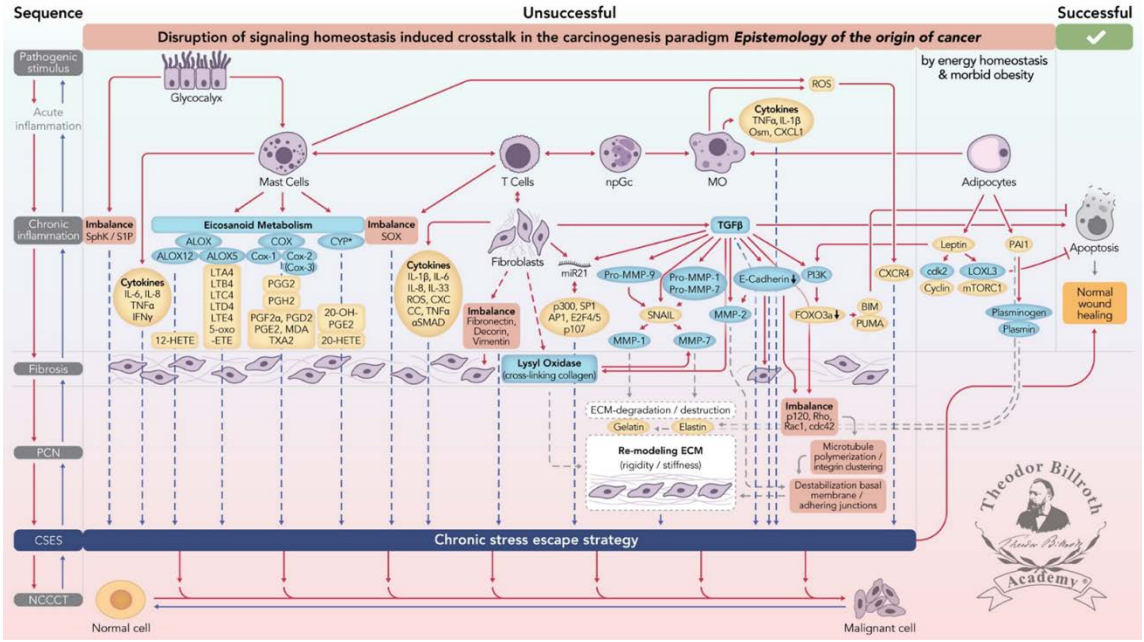

Fig.6: Schematic representation of the crosstalk between inflammation and carcinogenesis. A stimulus triggers inflammation which is accompanied by fibrosis and (EMT), thus a change in the surrounding microenvironment. Chronic inflammation leads to the development of stress which if it fails to resolve leads to the formation of cancer cells. (Adapted from Björn L.D.M. Brücher, 2019)

2. Body's defense against inflammation

The main players are inflammatory cells which provide the tumor microenvironment which is a crucial element in cancer development. Moreover, selectins and chemokines are used by cancer cells to aid in their invasion, migration and metastasis (26). In fact, Francis Peyton Rous, an American noble Prize-winning virologist was the first establish the that cancer arises from “subthreshold neoplastic states” due to somatic changes caused by the release of cytokines, (Fig.6) .These are the product of cancer cells attacking immune cells causing the silencing of tumor suppressor genes and thus, irreversible DNA alteration that

persist in normal tissue until the process of promotion takes place secreting a number of cytokines and chemokines by immune cells which promotes the survival and growth of these malignant neoplasms (27).Subsequently, the angiogenic switch is a critical step allowing tumor cells to get access to oxygen, nutrients and growth factors. Tumor progression is initiated by the release of cytokines and chemokines allowing cancerous cells to metastasize and invade other areas of the body. This process requires the (EMT)(28, 29).

3. Inflammation and cancer

Previous studies conducted by Mantovani *et al.* in 2008, emphasized the correlation and association between cancer and inflammation. This is facilitated via an intrinsic and extrinsic mechanism. Alterations in the genetic material activate the intrinsic pathway leading to inflammation and neoplasia. These alterations include the activation of mutation driven proto-oncogenes, silencing of tumor suppressor genes and chromosomal rearrangement and amplifications. Cells that acquired these mutations secrete a number of inflammatory mediators generating the inflammatory microenvironment (29). The extrinsic pathway is activated by inflammation which if chronic triggers the formation of tumors in different body organs including the bladder, prostate, breasts and colon among many others. Both, pathways activate a number of transcription factors thus, the development of pro-inflammatory mediators such COX-2, encoded by the PTGS2 gene and catalyzing the formation of prostaglandin H2 from AA, which is expressed in inflammation (30). These pro inflammatory mediators trigger the activation of a variety of leukocyte populations and their recruitment into the tumor microenvironment. Hence, the inflammatory process

becomes more pronounced thus, favoring tumor development and increasing invasiveness (31).

Thus, chronically inflamed tissues that have lost normal growth control start to divide and proliferate uncontrollably due alterations in the production of anti-inflammatory cytokines required to resolve an inflammatory response. In fact, a balance of cytokines and chemokines is required to regulate neoplastic outcome. Tumor cells that generate little pro-inflammatory cytokines and an abundance of anti-inflammatory cytokines leads to the induction of an inflammatory response, which slows down tumor progression, whereas high levels of pro inflammatory cytokines leads to the development of inflammation which favors angiogenesis thus, promoting neoplastic transformation (32). Moreover, a high number of inflammatory cells mainly monocytes and neutrophils via the imbalance of these cytokines favors the production of pro-inflammatory cytokines, leading to angiogenesis and thus, tumor proregression (Fig.7). In fact, Interleukin 10 (IL-10) is the major end product formed by tumor cells (25).

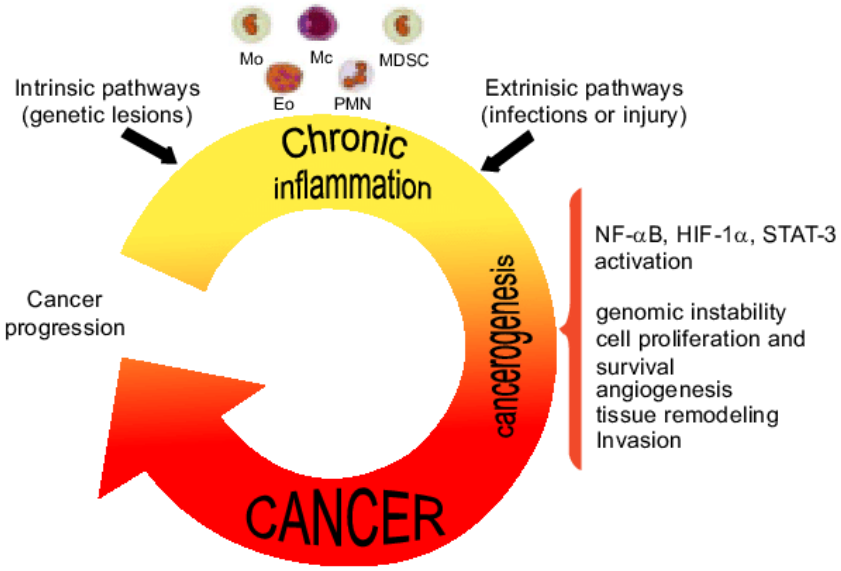

Fig.7: Cellular and molecular models linking inflammation to cancer development. Due to A stimulus either genetic alterations or inflammatory mediators trigger the development of inflammation and malignancy. Malignant cells along with leukocytes favors the inflammatory driven neoplasm transformation. Some transcription factors also contribute to the modulation of the inflammatory response favoring the development of cancer via genomic instability thus, altering gene expression, in addition to escaping apoptosis, favoring angiogenesis hence, tumor invasion and metastasis. (Adapted from Chiara Porta *et. al*, 2009).

Supporting evidence from previous studies confirm the interplay between inflammation and malignant neoplasms. Individuals suffering from inflammatory bowel diseases such as Crohn's disease are predisposed to colon cancer. In the same manner, infections with Hepatitis C in the liver favor the development of liver cancer. Schistosomiasis, also known as snail fever and bilharzia is also correlated with a high risk of colon cancer. Moreover, bacterial infection mainly Helicobacter pylori is the major causative agent of gastric cancer worldwide. They are all associated with DNA damage due to the action of inflammatory cells by expressing the (MIF). MIF is a protein encoded y the MIF gene and is the main regulator of the innate immunity in humans. This potent cytokine suppresses the transcription of the tumor suppressing protein p53. This

favors the proliferation and growth of defective cells by creating a microenvironment unable to respond to any DNA damage thus, leading to the formation of potential mutations that might lead to the development of cancer (31). In addition to bacterial infections, viral agents may also contribute to the development of malignant neoplasms via the addition of oncogenes to the cell's genome. Studies have showed that a minority of people infected with (HBV) or (EBV) develop malignancies due to the presence of the virus. This may be due to the suppression of the host 'immune system or the need of a number of cofactors necessary for the progression of a viral infection into a malignancy. In some instances, the viral infection has to target a stem cell or pluripotent progenitor. A typical example is the Rous sarcoma viral infection where a past inflammatory state is required for the development of cancer. This is mediated by a number of cytokines mainly TGF-β produced by inflammatory cells. (33)

One of the most important inflammatory pathways is the (AA). (AA) is a 20 carboon FA derived primarily from dietary linoleic acid and found in the phospholipid layer of cell membranes particularily in the brain, muscle and liver. (AA) is released from cell membranes phospholipids via the action of phospholipases activated by chemical mechanical or physical stimuli or in some cases by inflammatory mediators such as the complement component C5a (34). Because inflammation is implicated in the pathogenesis of a multitude of cancers, Arachidonic acid metabolites namely the prostanoids and eicosanoids, might serve as potential drivers of malignant neoplasms (35). Arachidonic acid (AA) metabolism proceeds along one or two major enzymatic pathways; the (COX) and the (LOX) pathway.

4. Cyclooxygenases

There are 2 cyclooxygenase (COX) isoforms; COX-1 is constitutive and the induced form is COX-2 (post-injury). COX-1 is located in platelets, GI mucosal cells etc. COX-2 has been identified in fibroblasts, endothelial cells, macrophages, and hence various cytokines such as TNF, mitogens such as EGF trigger its expression. It is upregulated at inflammatory sites. COX 1 catalyzes the conversion of Arachidonc acid (AA) Thromboxanes A2 (TXA2) which assumes a number of housekeeping fuctions such as platelet aggregation, mucosal protection renal blood flow and endothelial control in addition to Prostacyclin (PGI2) which causes vasodilation and inhibits platelt agregation . COX-2 is expressed in response to many physiological stimuli such as inflammatory conditions and tumorigenic disorders. It catalyzes the converion of (AA) to (PGD2 and PGE2) which are potent inflammatory mediators causing vasodilation and thus, redness and increased vascular permeability, inflammation induced hyperaglsesia among many others (36) (Fig.8)

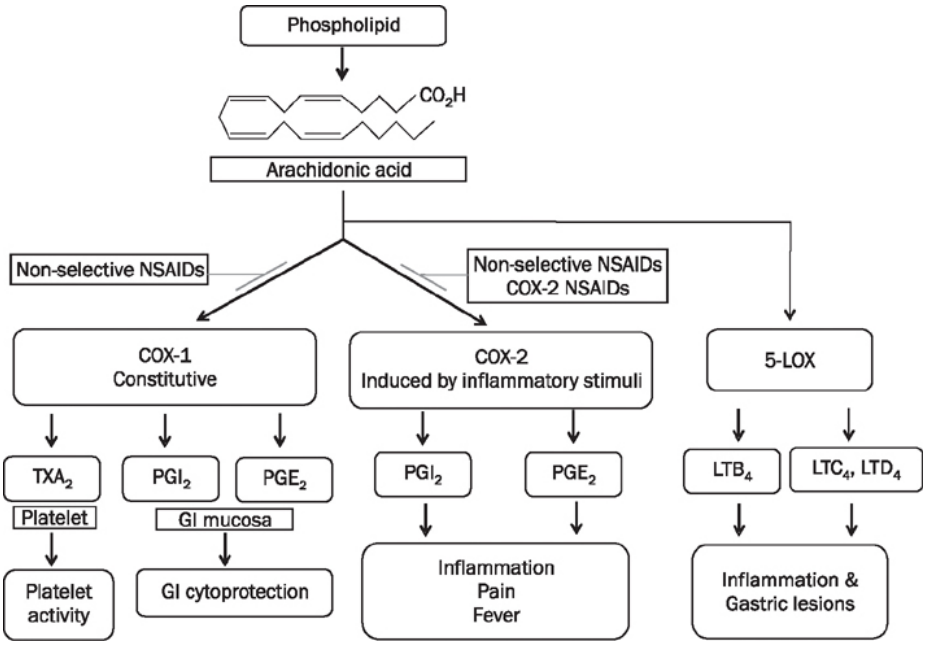

Fig.8: Cyclooxygenase (COX) Pathway. The 2 isoforms of COX namely (COX)-1 and COX-2 convert AA to (PGH2) which is in turn converted to a number of prostanoids such as prostacyclin (PGI2), thromboxane A2 (TXA2) etc. which exert a multitude of cellular responses using cell membrane receptors. (Adapted from Fitzgerald & Patrono 2001)

The (AA) pathway is of importance due to the pleiotropic effects of modulating this pathway which is pivotal in a number of disorders, especially in cancer progression. (NSAIDS) have been known for being inhibitors of cyclooxygenases. These include Aspirin which irreversibly acetylate COX 1 and COX 2. Others reversibly and competitively inhibit both COXs. Celecoxib for instance, reversibly and competitively inhibit COX 2, important contributor to cancer tumorigenesis (37)

a. Prostate Cancer

COX-2 was studied as being an important indicator of prostatic adenocarcinoma, whereby COX-2 expression was shown to be increased in comparison with normal prostatic tissues. Two important players act synergistically in accordance to induce the proliferation of prostatic cells by activating NF-κB. And these are COX-2 and (TLR4) (38). Moreover, metastasis of cancer cells is triggered by prostaglandins E2 (PGE2), a COX-2 metabolite via matriptase activation. In addition, tumor progression and survival were also shown to be induced by COX-2 via an association between fat cells and cancer. Remarkably, lipolysis of the adipocytes found in the bone marrow increased IL-1β in metastatic tumor cells thus, triggering the upregulation of COX-2 as well as MCP-1 in the adipocytes of the bone marrow(24).

b. Lung cancer

COX-2 is a major contributor to neoplastic transformation starting with cancer cell proliferation to angiogenesis and metastasis. Previous studies have showed that COX-2 gene polymorphisms were strongly associated with immune system suppression and invasion. Moreover, it was also found that this polymorphism in the COX-2 gene is as a marker for lung cancer diagnosis. Wang *et al.* demonstrated that COX-2 polymorphism showed a reduced risk of lung cancer. This is why COX-2 inhibitors were extensively studied throughout the past decade (39). Celecoxib was shown to potentiate and favor the lysis of lung cancer cells through the activation of ICAM-1, along with LFA-1 thus, favoring lymphokine-activated killer cells mechanism of cancer cell lysis. It was also found to be a potential ER stress inducer in lung tumors hence, causing cell death. Previous *in*

vivo studies in a mouse lung cancer model showed that celecoxib was able to accentuate the number of M1 macrophages and decrease that of M2 macrophages in the cancer cells. Also it attenuated MMPs and VEGF activation of PPARγ along with COX-2 downregulation thus, suggesting a positive outcome in treating patients with NSCLC (40).

Also, the inhibition of COX-2 increased the sensitivity to chemotherapy and radiation. Both EGFR- and COX-2-related signaling pathway blockade was shown to increase radiation effects via triggering apoptosis in lung cancer cells possibly through PI3K/AKT signaling pathway. Celecoxib was also shown to increase the effectiveness of radiation-induced apoptosis without causing any DNA damage. In fact, cancer cells treated with Celecoxib presented a low Akt, mTOR and COX-2 expression demonstrating the involvement of the Akt/mTOR pathway. Furthermore, celecoxib treatment combined with radiation therapy significantly decreased the size in a mouse model acting thus, synergistically reduce lung cancer cell survival by disrupting the chromosomal architecture of the COX-2 locus (41).

Additionally, COX-2 was shown to induce chemoresistance in lung cancer. In fact, studies have highlighted the importance of COX-2 in conferring patients with NSCLC cisplatin resistance. This is triggered by enhancing EMT via AKT signaling pathway activation. Moreover, the PI3K/Akt signaling pathway along with the interaction of COX-2 and EGFR was shown to confer patients with NSCLC Gefitinib resistance. This is why, chemotherapy along with the use of a COX-2 inhibitor could serve as a potential treatment regimen for patients with lung cancer. Indeed, studies have reported that a combination of celecoxib along with chemotherapy demonstrated an improved response to treatment in NSCLC patients (42).

c. Bladder cancer

COX-2 expression is more extensively studied in oncogenesis than COX-1. In fact, early on during the course of the disease, Cox-2 gene expression is induced and shown be involved in initiating carcinogenesis.

In fact, COX-2expression was upregulated in urothelial bladder. Previous studies reported an elevated inflammatory cell expression of COX-2 suggesting that higher levels of COX-2 gene increased invasion and recurrence. In fact, an elevated COX-2 expression causes an increase in prostaglandin synthesis thus, promoting cancerous cell proliferation, angiogenesis via tyrosine kinase receptor activation. COX-2 also stimulates the production pro-angiogenic factors for example VEGF (43).

Accordingly, COX-2 promote cancer progression and invasion. Not only it is associated with a poor survival rate but also predicts whether COX-2 inhibitors will be beneficial. Furthermore, it was shown that COX-2 expression in bladder cancer patients led to a shorter survival rate (44).

Supporting evidence showed a prominent COX-2 immunostaining in patients with advanced tumor stages. Thus, the degree of COX-2 immunostaining and urothelial bladder cancer muscle invasiveness are highly correlated. Moreover, tumors that present with a positive lymph nodes and vascular invasion show a high COX-2 immunostaining. Ongoing research experiments are currently in view examining if COX-2 inhibitors could be of potential benefit to bladder cancer patients along chemotherapy and radiotherapy (45)

5. Lipoxygenases

On the other hand, lipoxygenases are non-heme iron containing dioxygenases. (PUFAs) such as (AA) and linoleic acid are oxygenated via lipoxygenases, yielding oxygenated lipid mediators (24). The nomenclature of LOXs varies according to the oxygenation site in AA. There are different LOX isoforms have been identified and these are 5-, 12- and 15-LOX. Thus, LOXs include 5-, 8-, platelet type 12(*S*)-, epidermal-type 12(*R*)-, and 15-LOXs. LOXs are expressed in immune and tumor cells displaying a multitude of functions (Fig.9). 5-LOX is a crucial player in the development of asthma through (5- HPETE) which yields leukotrienes. 5-LOX is also involved in the synthesis of lipoxins (46).

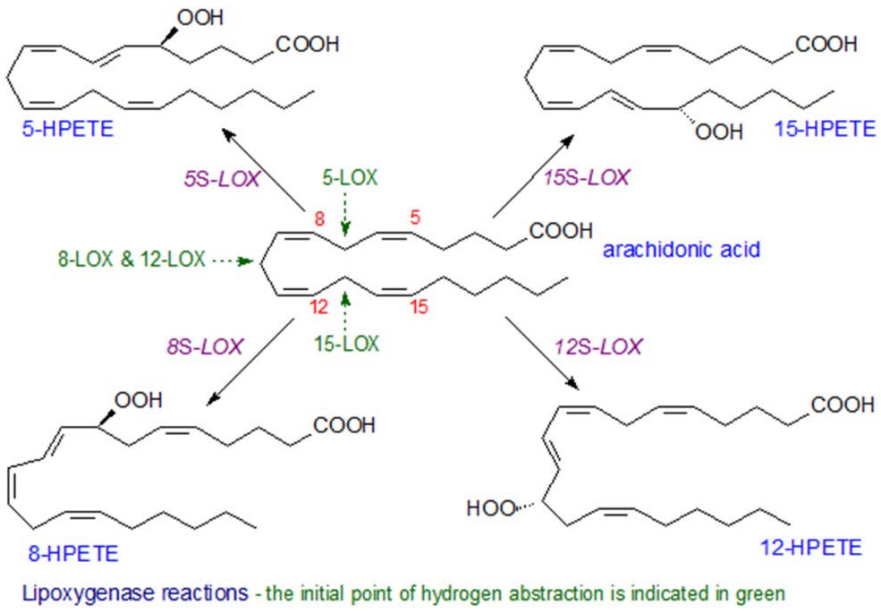

Fig.9: Family of Lipoxygenases (LOXs). AA is converted by the isoforms of LOX enzymes namely 12-LOX and 15-LOX into (HpETEs) hydroperoxides which in turn lead to the formation of (HETEs) at carbon-12 or carbon-15 respectively. This depends on the LOX isoform involved in this conversion. (Adapted from Shozo Yamamoto, 1999)

On the other hand, PUFAs are converted via 15-LOX to 15-S-HPETE which in turn is converted to either lipoxins or eoxins. Lipoxins are endogenous anti-inflammatory pro-resolving molecules involved in tissue injury and chronic inflammation by controlling components of the immune response, in addition to inflammatory cells. Eoxins, however, are pro inflammatory eicosanoids produced by eosinophils and mast cells. They are implicated in a number of disorders ranging from asthma to malignancies such as Hodgkin Lymphoma, with an inflammatory component. 15-LOX is subdivided based on tissue specificity, location and enzymatic properties into 15-LOX 1 and 15- LOX 2. LA and arachidonic acid are oxygenated by 15-LOX-1 yielding (13(S)-HpODE) and (15(S)HpETE) as main metabolites, respectively. 15-LOX-2 however, oxidizes (AA) to 15(S)HpETE (47) (Fig.10)

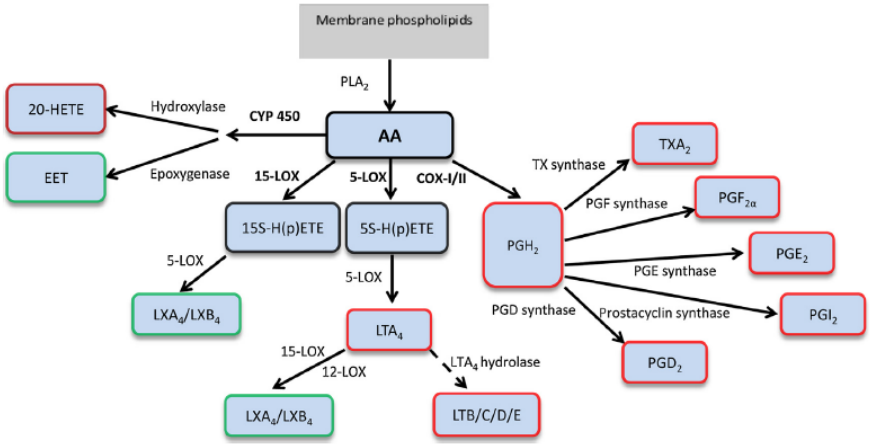

Fig.10: Lipoxygenases (LOXs) Pathway. The isoforms of LOX include 5-, 12- and 15-LOX. Thus, the family of LOXs includes 5-, 8-, platelet type 12(*S*)-, epidermal-type 12. Each of these isoforms undergoes further reactions yielding important metabolites exerting a multitude of cellular functions. (Adapted from Vernon E. Steele *et.al*, 1999).

In fact, 12/15-LOX enzyme metabolites exhibit differences between mammalian and murine thus, an interspecies variability. The major oxygenation product of the human 15-LOX-1 is 15(S)- HETE but also, a little amount of 12(S)-HETE is produced (ratio of 9:1) (Fig.11). On the contrary, the murine orthologue 12-LOX yields both 12(S)-HETE and 15(S)-HETE via the 8S-lipoxygenating activity (ratio 3:1). This still ill understood in respect to the role of 15-LOX in different tissue types. Therefore, these differences imposes limits to the extent of which our data could be replicated on humans (48).

Table 1
15-Lipoxygenase isoforms in humans. From Gene – National Center for Biotechnology Information.

Enzyme	Gene/location	Abbreviation	Tissue/cellular distribution	Substrates	Metabolites
15-LOX-1	Arachidonate 15-lipoxygenase/17p13.3	ALOX15	Reticulocytes, eosinophils, macrophages	Arachidonic acid Linoleic acid DHA	15S-H(p)ETE 13S-H(p)ODE 17S-H(p)DHA
15-LOX-2	Arachidonate 15-lipoxygenase, type B/17p13.1	ALOX15B	Skin, cornea, prostate, lung, esophagus	Arachidonic acid	15S-H(p)ETE

Humans express two 15-lipoxygenase isoforms. 15-Lipoxygenase-1 (15-LOX-1) can oxygenate various fatty acid substrates, and is expressed in multiple tissues. 15-Lipoxygenase-2 (15-LOX-2) is more tissue-localized and substrate-specific. See text for further details. 15S-H(p)ETE, 15S-hydroperoxy-5Z,8Z,11Z,13E-eicosatetraenoic acid; 13S-H(p)ODE, (13S,9Z,11E)-13-hydroperoxy-9,11-octadecadienoic acid; 17S-H(p)DHA, 17S-hydroperoxy-4Z,7Z,10Z,13Z,15E,19Z-docosahexaenoic acid.

Fig.11: Lipoxygenases (15-LOX) isoforms in Humans. 15-LOX is subdivided based on tissue specificity, location and enzymatic properties into 15-LOX 1 and 15- LOX 2. 15-LOX-1 yields 13(S)-HpODE) and (15(S)HpETE) as it's the main product. 15-Lipoxygenase-2 yields 15(S)HpETE. (Adapted from Ryan G. Snodgrass* and Bernhard Brüne, 2019**)**

The mechanisms by which 12 and 15-LOX controls pathophysiological immune cell function is still poorly understood.

Till now, we know that 15-LOX expression in monocytes and macrophages is induced via the stimulation of IL-4 or IL-13. Macrophage 15-LOX contributes to the generation of pro-resolving mediators (SPMs) in order to alleviate and reduce the inflammation and produce oxidized phospholipids (oxPLs) thus, regulating the removal of ACs non- immunogenically (49). Contradictory data has been gathered supporting the dual

pro- and antitumorigenic effects of 15 LOX. The metabolites generated by 15-LOX have been shown to have opposing actions on the development of inflammation and oncogenesis, which supports the evidence of the involvement of 15-LOX in promoting tumor development, growth and metastasis. On one hand, 15-LOX-1 that is found in allergic activated immune cells such as eosinophils and mast cells yield eoxins which are active proinflammatory mediators involved in increasing vascular permeability and causes vasodilation mediated by Histamine, the most important chemical mediator of acute inflammation. On the other hand, 15-LOX also produces anti-inflammatory metabolites called lipoxins and resolvins. 15-LOX-1 metabolizes DHA yielding resolvins of the series D and protectin D1 with a strong anti-inflammatory, anti-apoptotic and neuroprotective activity (24).

In fact, 15-LOX is involved in malignancies

a. Prostate Cancer

In 1997, Spindler *et al.* were the first to describe the existing role of 15-LOX in prostate cancer. The evaluation of prostate adenocarcinoma specimens using immunoassay techniques showed an increased level of 13-HODE. In addition, high levels of 15 LOX were reported in prostate cancer. Previous studies on prostate cancer have reported that 15-LOX-2 conferred protection against the development of prostate cancer. On one hand, it was shown that in prostatic hyperplasia, there is overexpression of 15-LOX-2 and its product15-(S) HETE (50). On the other hand, in prostate adenocarcinoma, 15-LOX-2 expression was decreased. It is negatively associated with the Gleason score. The inhibitory role exerted by 15-LOX -2 was shown successful in maintaining the tumor in an inactive

state. Thus, the loss of 15-LOX-2 expression is associated with the malignant phenotype in prostate cancer. Alternatively, in prostatic hyperplasia, higher 15-LOX-2 level was associated with cell senescence thus, blocking tumor development (51). On another hand, prostatic tumor growth and the degree of invasiveness was strongly correlated with 15-LOX-1 expression. In vivo studies have showed that introducing prostate cancer cells exhibiting higher than normal levels of 15-LOX-1 into athymic mice formed larger tumors, along with a higher level of the angiogenic factor VIII. Moreover, the Gleason grading system postulated that a higher 15-LOX-1 expression was strongly associated with an increased invasiveness and malignancy. 15-LOX-1 being a tumor promotor and 15-LOX-2 a tumor suppressor explains the opposing actions of their corresponding metabolites on (MAPK) and downstream PPARγ activity (52). 15-LOX-2 metabolite namely 15- (S)HETE was able to decrease the activity of MAPK in PC3 cells. this decreased the phosphorylation of PPARγ thus, increased in PPARγ transcriptional activity. In contrast, the metabolite generated by 15-LOX-1,13-HODE, was shown to enhance MAPK activity. This decreased PPARγ activity by the activation of (IGF-1). In fact, it was shown that inhibiting the IGF-1 receptor was strongly correlated with a decreased tumor growth thus, highlighting the role of 15-LOX-1 as being oncogenic (24).

b. Colorectal Cancer

Two contradicting approaches regarding the role of 15-LOX-1 in favoring the progression of colorectal cancer have been thoroughly investigated. The upregulation of 15-LOX-1 in CRC tissue, indicates its tumor- promoter effect. We showed that 15-LOX-1 was able to upregulate PPARγ activity by activating the MAPK signaling pathway in prostate

adenocarcinoma. On the contrary, other studies demonstrated a low level of both15-LOX-1 and its metabolite, thus, suggesting its effect as being beneficial in CRC when found in low levels so it exerts a tumor-protective effect in colorectal carcinoma. This could be due to the inhibition of 15-LOX-1 via GATA-6. In caco-2 CRC cell lines, the binding site of GATA-6 was identified, along a high RNA expression of GATA-6. in-line with the enhancement of GATA-6 RNA expression. In fact, inhibiting the the IL-6/STAT3 signaling pathway was shown to be responsible for conferring 15-LOX-1 its protective effect in (CAC) (53). Cancer progression may also be due a consequence of 15-LOX-1 activity, by regulating p53, whereby high levels of 15- LOX-1 was shown to activate p53 in HCT116 cells. Moreover, several studies investigated the biochemical pathway through which the enzyme is able to inhibit metastasis and invasion. The expression of (HIF)-1α, which is known to induce metastasis and angiogenesis, was shown to be suppressed 15-LOX-1. Consistently, another study proved that 15-LOX-1 was able to inhibit neo-angiogenesis (54). In fact, (TSP-1), and ICAM-1 levels were shown to be decreased following endothelial cells treatment with cancer media having high levels of 15- LOX-1, thus decreasing vessel formation. (MTA-1) expression, a component of the (NuRD) complex was shown to be decreased Thus, any 15-LOX-1 loss is severely associated with cancer metastasis and angiogenesis (24).Moreover, 15-LOX-1 and 15-LOX-2 were shown to have antagonistic effects in a number of cancers. In fact, supporting evidence have demonstrated the protective effect of 15-LOX 2 in epithelial derived carcinoma such as head and neck cancer (HNC), being a tumor suppressor molecule. It does so via its main metabolite 15S-HETE by acting as an endogenous ligand of PPARγ, Thus, activating the PPARγ receptor

was shown to reverse the malignant phenotype by inhibiting cancerous cell proliferation (55).

Moreover, (DHA) was able to induce apoptosis by activating several pathways involving (SDC-1). In fact,15-LOX-1 isoform was shown to mediate SDC-1 signaling and thus, prostate cancer apoptosis suggesting the preventive activity of DHA in prostate cancer (56).

Previous studies demonstrated that high DHA erythrocyte levels were a strong indicator of a high prostate cancer risk whereas low erythrocyte DHA levels were indicative of cancer in prostatic tissues compared advanced stages. However, low levels of 15-LOX-2 and its metabolite was strongly correlated with prostate cancer development and progression. This antagonistic effect can be explained by examining both 15-LOX-1 and 15-LOX-2 metabolites. 13-(*S*)-HODE, a 15-LOX-1 metabolite, was shown to upregulate the MAP kinase pathway thus, phosphorylating PPARγ whereas, on the other hand, 15-(*S*)-HETE, was able to decrease MAP kinase thus, dephosphorylating PPARγ. Moreover, IGF-1 activation was shown to activate 13-(*S*)-HODE which in turn upregulated both the MAPK and Akt pathways whereas and 15-(*S*)-HETE down-regulate both these pathways. These could possibly explain the antagonistic roles of 15-LOX-1 and 15-LOX-2 play in prostate cancer (57).

c. Bladder Cancer

While normal bladder tissues express 5 and 12-HETEs metabolites of 5 and 12 LOX respectively, bladder cancer tissues were shown to exhibit higher levels of 5 and 12-HETEs. 5 and 12-HETE were shown to promote tumorigenesis. Lipoxygenase inhibitors

triggered the condensation of the chromatin in addition to cellular shrinkage. Apoptotic bodies were also detected suggesting that lipoxygenase inhibitors may be used as a treatment regimen (58).

However, 15-LOX role in initiating bladder cancer is still ill understood. Minimal studies were conducted assessing the role of 15-LOX in cancer. Some literature reviews demonstrated that 15-LOX expression fluctuates normal and invasive bladder cancer stages. As bladder cancer progress, the expression of 15-LOX-1 was shown to be subsequently decreased. In normal tissues, there is higher levels of AA possibly due to high levels of D-5 and D-6 desaturase enzymes. Furthermore, D-6 desaturase and 15-LOX-1 enzymes competes for Linoleic acid. In addition 15-LOX-1 expression was shown to be upregulated, indicating that the D-6 desaturase/AA pathway is downregulated in bladder cancer tissues (59). While, elevated 15-LOX-1 expression may be suggestive of a normal phenotype, overexpression of this enzyme due to chronic bladder irritation and inflammation that occurs prior to the development of cancer could be a potential driver for epithelial transformation. In fact, the 15-LOX-1 gene is regulated by pro-inflammatory cytokines. Thus, because highest15-LOX-1 levels were demonstrated in bladder cancer, 15-LOX-1 could have exert a protective effect and suppress tumor progression(60)

6. PPAR γ

a. PPARγ as potential targets

(PPARs) belong to the nuclear hormone receptor superfamily of ligand-activated TF controlling cellular metabolism, proliferation and function. After binding PPAR ligands, PPARs receptors heterodimerize with (RXR) then, this complex translocates to the nucleus

whereby it undergoes a conformational change thus, activating transcription. There are three different members of the PPAR family and these are the PPARα, PPARγ, and PPARβ/δ (24).

To start with, PPARγ, is mainly found in the adipose tissues whereby it controls the differentiation of preadipocytes to adipocytes. It is also found in endothelial cells, and (VSMCs). Several disorders such as insulin resistance are the result of genetic polymorphisms (61). Furthermore, the use of PPARγ, agonists along with thiazolidinediones (TZDs) have been extensively studied in treating (T2D). Due to the effects of TZDs, the role PPARγ has been thoroughly investigated in a multitude of disorders such as metabolic diseases and cancer.
(62).

b. PPARγ and Cancer

Previous studies demonstrated the involvement of PPARγ in tumorigenesis. Upon binding to specific ligands, PPAR*γ* get activated causing apoptosis or triggering cellular growth. The use of PPAR*γ* agonists demonstrated antiproliferative effects in a number of cancers. Furthermore, several clinical trials have shown that Thiazolidinediones (TDZ) demonstrated an antitumor effect. There exist two different isoforms of PPAR*γ,* PPAR*γ* 1 and PPAR*γ* 2. Most tissues exhibit PPAR*γ* 1 whereas PPAR*γ*2 is selectively expressed in adipocytes (63).

PPAR*γ* 2 demonstrated antagonistic effects in prostate cancer whereby, PPAR*γ* 1 as an oncogene induces neoplastic transformation of benign prostate cancer epithelial cells. on the other hand, PPAR*γ* 2 is a tumor suppressor. Early on during the course of the disease

AR transition from AR directing cytodifferentiation of luminal epithelial cells to AR driving the uncontrolled proliferation of these cells thus, triggering cancer cell growth and development(64). This "malignancy switch" is essential in tumorigenesis thus, AR is considered to be the main driver of transformation. Remarkably, the most successful prostate cancer preventive treatment targets the AR via blockade of dihydrotestosterone (DHT) production via specific 5α-reductase inhibitors. Recent studies have demonstrated the correlation between the androgen receptor (AR) and PPAR*γ* whereby the reciprocal protein interaction mediates the oncogenic phenotype. In vivo studies in prostate adenocarcinoma rat models have shown that pioglitazone was able to attenuate the number of adenocarcinoma lesions thus, demonstrating a chemo-preventive potential (65). Moreover, it was found that CXCR4/CXCL12 signaling was inhibited upon treatment with Rosiglitazone, indicating the ability of Rosiglitazone to be used in prostate cancer. In fact, the combination of a PPARγ agonist along with a fatty acid synthase inhibitor demonstrated significant antitumorigenic effects.

Furthermore, polymorphisms in PPARγ and a loss of function mutation are strongly correlated with the development of colon cancer. Suppressing PPARγ expression epigenetically triggered the development of colon cancer in patients regardless of their BMI. On the other hand, PPARγ upregulation alleviated colitis-associated neoplasia *in vivo*. The overexpression of PPARγ in colon cancer cell lines was shown to inhibit the growth of cancerous. Troglitazone member of the thiazolidinediones family suppressed colon cancer cell development. This occurs by inhibiting NF-κB signaling via impeding GSK-3β activity. Thus, TZDs demonstrate potent antitumorigenic effect by sensitizing colon cancers that are resistant to therapy (24).

Furthermore, the development CRC is triggered by the Wnt/β-catenin signaling pathway via the Warburg effect. Genetic mutations occurring in the Wnt/β-catenin signaling pathway are at the basis of the development of CRC (66).The (APC) gene encodes the APC protein which acts as a tumor suppressor. A mutation in the APC gene is the main driving force for the development of (FAP) syndrome, which was found to be 80% mutated in sporadic CRC. Additional mutations may occur in the catenin or axin inhibitor (Axin) genes. Absence or dysregulation of APC leads to β-catenin overexpression, then moves to the nucleus to combine with TCF/LEF thus, it binds DNA and causes the subsequent transcription of CRC development (67).

c. Bladder cancer

Considering the invasiveness of the procedures used to treat the aggressive type of bladder cancer, new treatment regimens have been thoroughly investigated, the most important of which is the PPARγ. In fact, s PPARγ was shown to be a potential therapeutic agent in a number of cancers. The role of PPARγ in bladder cancer progression and development is under investigation. Recent studies suggested that PPARγ control bladder cancer development by targeting important cellular processes such as cellular proliferation, invasiveness, aggressiveness etc. possibly via two important pathways known as the PI3K-Akt signaling pathway, and the WNT/β-catenin signaling pathway (68).
Interestingly, it was found that PPARγ caused a blockage in the cell cycle by triggering apoptosis of bladder cancer cells. this impedes cancer cell growth thus, prevents further tumor expansion. This occurs via PI3K-Akt pathway blockage. Aberrant activation of

the PI3K/AKT pathway promotes cancer cell development and invasion, namely in bladder cancer (69).

7. Multi targeted directed ligand drugs

a. Concept & mode of action (MOA)

Several therapeutic approaches have been thoroughly investigated to treat complex diseases. These medical interventions belong to the so called traditional Chinese medicine class (TCM) which are manufactured based on the principle of “syndrome differentiation” meaning the pharmacological properties are based on patients' syndromes. In fact, recent pharmacological studies have established the multiple target network of these compounds to construct complex predictive models to evaluate the possible ligands along with their targets. Several therapeutic agents possess multi-targeting activities such as Aspirin in addition, it has been extensively used in treating patients with prophylaxis of ischemia, strokes and cancer.

b. Uses

In 2010, Metz and Hajduk presented a narrowed description of MTDD as therapeutic compounds capable of regulating several targets. Remarkably, MTDD are also intended to treat complex disorders that are the consequence of genetic abnormalities with a drug capable of targeting more than one component of the disease pathway (24). Interestingly, these MTDD are designed in a way that allow them to regulate multiple cellular targets that are involved in a specific pathological condition or in diseases that share similar or closely

related biochemical pathways and mechanisms. Moreover, designing MTDLs is a challenging and competitive task for it requires scientists to purposefully design these compounds. This allows scientists to discover and design new compounds having a higher safety profile but at the same time efficient. It also allows to try new drug combination for specific disorders, the combination of which might exhibit synergistic effects, additive effects or in some cases by simple escaping drug resistance. In this context, it is important to mention that in some cases partially inhibiting a cellular target can be of a higher benefit than inhibiting the whole target. (24).

Thus, designing a potent MTDL is a challenging task for it requires effective target combination keeping the druglike characters of the designed compounds. Therefore, due to our interest in the intertwining roles of COX-2 and 15-LOX in inflammatory changes encompassing malignant disorders, insights have encouraged the development of new anti-inflammatory therapeutic approaches to target cancer progression. Previous biological and pharmacological evaluation of different chemical compounds targeting COX-2 and/or 15-LOX- and PPARγ have been done which was narrowed down to choosing the compounds with the most potent effects.

c. Pd3: Dual COX-2/15-LOX Inhibitors

Several dual cycloxygenase and lipoxygenase inhibitors were synthesized as thiazolo-celecoxib hybrids. Out of seven Pyrazolyl Thiazolones hybrids, Pd3 was shown to be the compound with the most potent dual COX-LOX inhibitory activity.

Structure 1 and 2 are Phenolic arylidene thiazolidinones darbufelone with low ulcerogenicity and are strong inhibitors of both COX and LOX, Structure 3 was shown to

reduce ear edema in mice and possesses a COX inhibitory activity. Moreover, Structure 4 was shown to exhibit a higher COX-2 selectivity than celecoxib along with a higher potency for 15-LOX than meclofenamate sodium. Finally, Structure 5 relieved pain associated with musculoskeletal disorders. Thus, we proposed that a hybridization including both pyrazolyl and thiazolyl structure could serve as a potential dual COX-2/LOX inhibitor. In addition, we proposed that the addition of a cyclized secondary amine might increase and potentiate the anti-inflammatory activity (42) (Fig. 12-13)

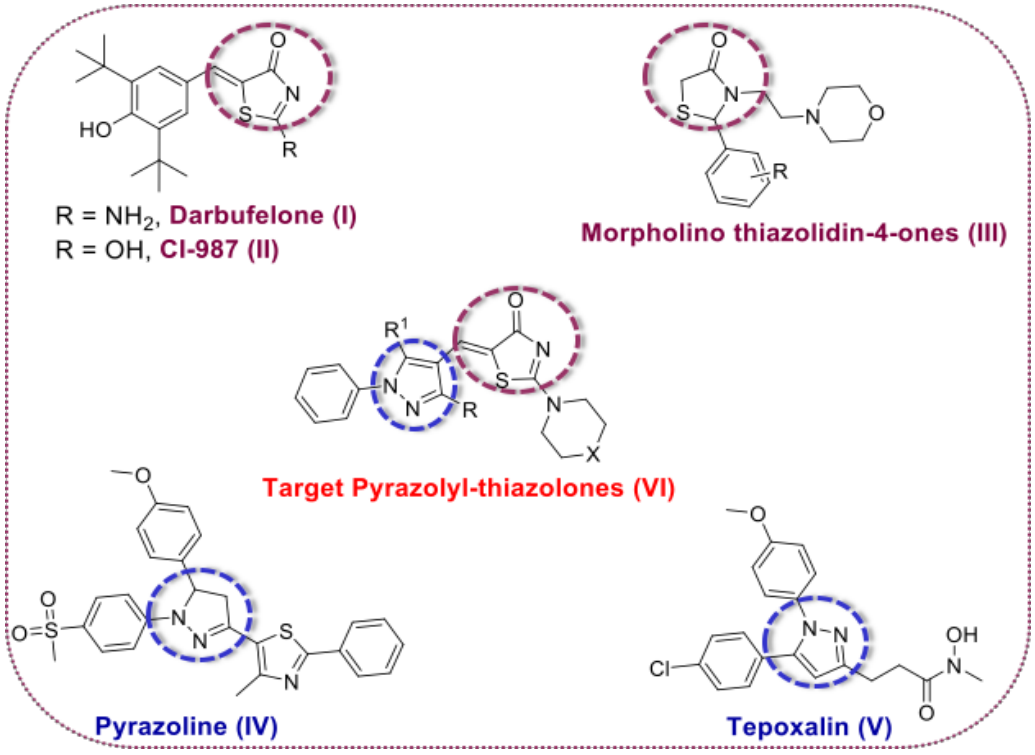

Fig.12: Rationale for the design of the dual target compound. Structure 1 and 2 are Phenolic arylidene thiazolidinones darbufelone with low ulcerogenicity and are strong inhibitors of both COX and LOX, Structure 3 was shown to reduce ear edema in mice and possesses a COX inhibitory activity. Moreover, Structure 4 was shown to exhibit a higher COX-2 selectivity than celecoxib along with a higher potency for 15-LOX than meclofenamate sodium. Finally, Structure 5 relieved pain associated with musculoskeletal disorders. (Adapted from Perihan Elzahhar *et. al*, 2021)

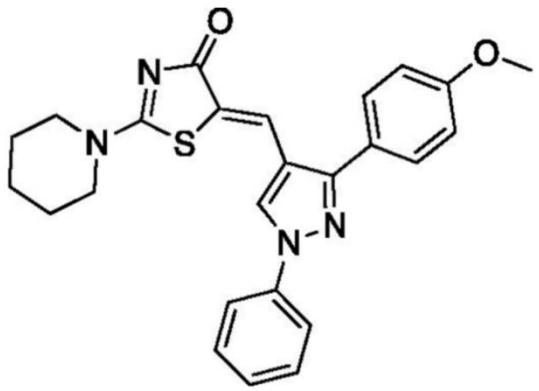

Fig.13: Structure of the dual target compound (Pd3) compound (Adapted from Perihan Elzahhar *et. al*, 2021)

d. 4b: Triple COX-2/15-LOX/PPARγ Targeting Compounds

By adopting a pharmacophoric molecular hybridization approach, a series of 1,2,3-triazolyl-thiazolidinedione/rhodamine hybrids were designed. These glitazone-like compounds were tested as being potential PPARγ partial agonists/COX-2 and 15-LOX inhibitors. Lyso 7 was shown to exhibit a potent COX 2 inhibitory activity. Pioglitazone is a known PPAR gamma agonist. TDZ was shown to be both a Dual PPARγ agonist and Lox inhibitor. Structure D demonstrated a dual Cox-2/15-Lox inhibitory activity. The polar acidic group indicated by the presence of the double bond is a partial PPAR gamma agonist. Finally, the hydrophobic tail is a 1,4-disubstituted- 1,2,3-triazole which is a pharmacophore exhibiting a dual COX-2 and LOX inhibitory activity (42) (Fig.14-15).

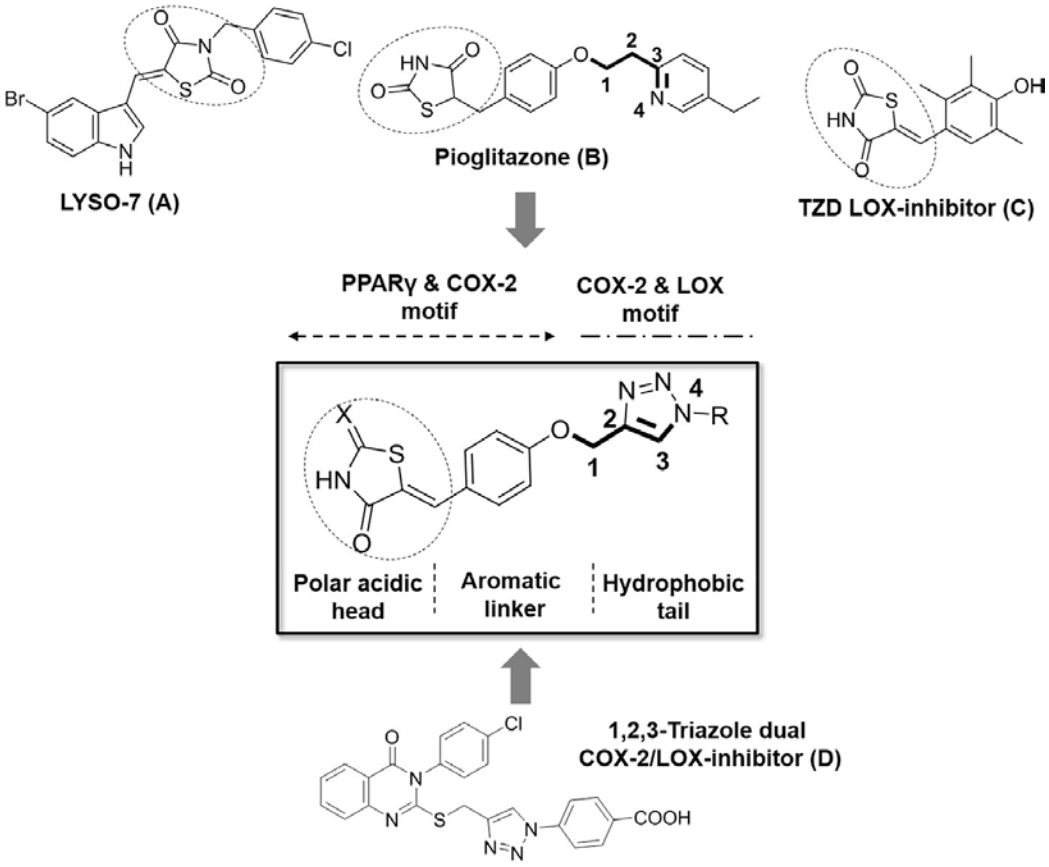

Fig.14: Rationale for the design of the triple target compound. Lyso 7 was shown to exhibit a potent COX 2 inhibitory activity. Pioglitazone is a known PPAR gamma agonist. TDZ: was shown to be both a Dual PPARγ agonist and Lox inhibitor. Structure D demonstrated a dual Cox-2/15-Lox inhibitory activity. The polar acidic group indicated by the presence of the double bond is a partial PPAR gamma agonist. Finally, the hydrophobic tail is a 1,4-disubstituted- 1,2,3-triazole which is a pharmacophore exhibiting a dual COX-2 and LOX inhibitory activity. (Adapted from Perihan A. Elzahhar *et.al*, 2019)

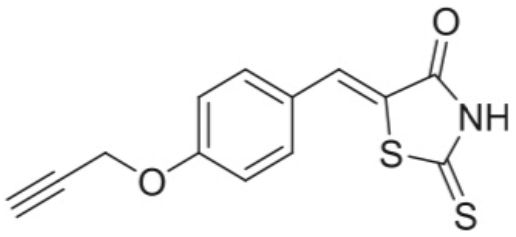

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Fig.15: Structure of the triple target compound (4b). (Adapted from Perihan A. Elzahhar *et.al*, 2019).

D. Aims of The Study

In our study, we hypothesized that multi-target directed ligands could be an adjuvant to radiation and chemotherapy for bladder cancer patients namely, dual COX-2/ 15-LOX inhibitors and triple COX-2/ 15-LOX inhibitors & partial PPARγ agonist. We aim to investigate the mechanisms behind the role of COX-2/ 15-LOX pathways in cancer progression. Hence, it highlights the evidence of repurposing previously established anti-inflammatory drugs for the use as anti-neoplastic agents. It also proposes novel therapeutic approaches for the effective targeting of inflammation induced cancers.

Accordingly, we evaluated cancer cell proliferation using the MTT cell growth assay whereby the MTT is converted to into formazan crystals, an indicator of mitochondrial activity. Then performing the TB assay is used to determine the viability of cells upon treatment with the dual and triple target drugs. Next the migratory ability of cancerous cells was assessed using the WH assay representing cell migration in vivo post wound creation. The 3D sphere forming assay was performed to target cancer stem cells (CSCs) can form (3D) spheres *in vitro* when grown in non-adherent serum-free conditions. Moreover, the monocyte recruitment assay was performed to assess the ability of these compounds to inhibit monocyte differentiation into macrophages, a process that occurs

during an inflammatory response which may help in stopping inflammation induced cancers.

Finally, some molecular studies were performed that aimed at evaluating the mechanisms and cellular pathways by which bladder cancer cells are able to grow, metastasize and invade other areas of the body thus to escape apoptosis. Accordingly, p53; a marker of apoptosis, full length-caspase-3; an executer of apoptosis by cleavage of cancer cells were analyzed by western blotting.

CHAPTER II

MATERIALS AND METHODS

A. Cell Lines

Two human urinary bladder cancer cell lines were used: RT4 is derived from a non-invasive superficial cancer (Derived from explants of a recurring papillary tumor of the bladder). T24 on the other hand is derived from an invasive bladder tumor with a metastatic profile (70). All cells were obtained from the American Tissue Culture Collection (ATCC). RT4 cells were cultured and maintained in DMEM F12-HAM (Sigma-Aldrich) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Sigma-Aldrich), 1% Penicillin/Streptomycin (Sigma-Aldrich), and 0.2% plasmocin prophylactic (InVivogen). T24 Cells were cultured and maintained in DMEM High glucose (Sigma-Aldrich) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Sigma-Aldrich), 1% Penicillin/Streptomycin (Sigma-Aldrich), and 0.2% plasmocin prophylactic (InVivogen). Cells were incubated at 37°C in a humidified incubator containing 5% CO2.

B. Preparation of Pd3

Both Pd3 and 4b compounds were reconstituted in DMSO, separated into aliquots of concentrations 10 and 20mM and, stored at -20 °C.

C. In vitro evaluation of COX-1 and COX-2 inhibitory activity

The enzymatic activity of COX enzyme is measured using the Cayman colorimetric COX (ovine) inhibitor screening assay kit (Catalog No. 560131) supplied by Cayman chemicals according to the manufacturer's instructions.

COX enzyme is a bifunctional enzyme exhibiting both COX and peroxidase enzymatic activities.

The COX activity of this enzyme converts (AA) to a hydroxy endoperoxide (PGG2). On the other hand, the peroxidase activity of this enzyme is involved in the reduction of endoperoxides to the corresponding alcohol (PGH2). The assay directly measures PGF2 α by $SnCl_2$ reaction of COX derived PGH2 that is formed by the COX reaction. Then the following prostanoid product is quantified using the competitive ELISA (enzyme immunoabsorbant assay) using a range of antibodies that bind to the prostaglandins. The basis of this assay is a competition between the prostaglandins and the Prostaglandins-Acetylcholinesterse (AChE) called the PG tracer using a limited number of PG antibodies. PG concentration remains constant while on the other hand the concentration of prostaglandins is variable hence, the amount of PG binding to the PG antibody will be inversely proportional to the concentration of prostaglandin found in each well. Then this rabbit antibody-prostaglandin complex whether free or the tracer binds to a mouse monoclonal anti rabbit antibody that has been previously attached to the well. Then 2x washes are performed to eliminate any unbound material followed by the Ellman's reagent that contains the substrate for acetylcholine. The product has a yellow color that absorbs light at 412nm. The intensity of this color is measured spectrophotometrically. It is

proportional to the amount of prostaglandin tracer found in the well which is in turn inversely proportional to the amount of free prostaglandin. (Fig.16)

Absorbance α to concentration of bound prostaglandins tracer 1/α prostaglandins

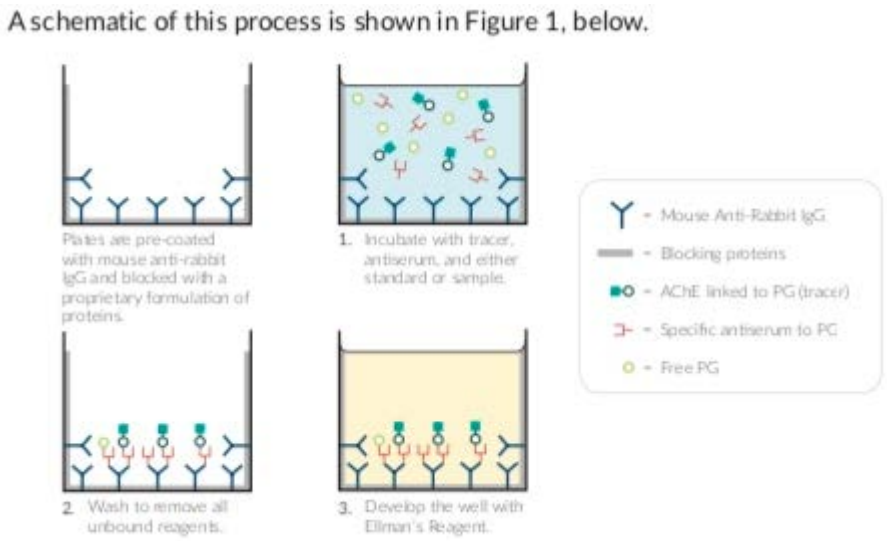

Fig.16: A schematic representation of COX-1 and COX-2 enzymatic activity *in vitro*. It measures PGF2 α by $SnCl_2$ reaction of COX derived PGH2 that is formed by the COX reaction. Then the following prostanoid product is quantified using the competitive ELISA (enzyme immunoabsorbant assay) using a range of antibodies that bind to the prostaglandins. The product has a yellow color whose intensity is measured spectrophotometrically. (Adapted from Cayman Chemicals, COX (ovine/human) inhibitor screening assay).

D. In vitro evaluation of 15-LOX inhibitory activity

The enzymatic activity of 15-LOX enzyme was measured using the using Cayman lipoxygenase inhibitor screening assay kit (Catalog No. 760700). Reagent preparation and the determination of the IC50 values of the tested compounds determined using the instructions given with the assay kit (Catalog No. 760700). Lipoxygenases are involved in

the molecular addition of oxygen to (FA) containing a cis—trans-1,3 conjugated penta-dienyl moiety within the unsaturated fatty acid. Cayman lipoxygenase inhibitory assay measures the hydroperoxides that are produced by lipoxygenation. It is important to mention that COX enzymes cannot be measured using this assay. The reaction is initiated by adding the substrate; either arachidonic acid or linoleic followed by the addition of the chromogen to stop the enzymatic catalysis and develop the reaction. The color produced will be proportional to the amount of hydroperoxides generated via the lipoxygenation reaction.

E. Monocyte to macrophage differentiation assay

THP-1 cells were seeded at a density of 20×10^5 cells/mL. Some of these cells were subjected to phorbol myristate-acetate (PMA, Calbiochem, Darmstadt, Germany) at a concentration of 25 nM for 24 h in order to induce cellular differentiation. The other cells were also treated with phorbol myristate-acetate (PMA, Calbiochem, Darmstadt, Germany) at a concentration of 25 nM for 24 h followed by 100 ng/ml of lipopolysaccharide (LPS, invivogen, San Diego, CA, USA) for 72 h. After incubation, the supernatant was aspired then using MTS colorimetric cell viability kit (Abcam, Cambridge, UK), the number of adherent monocytes was estimated allowing us to evaluate the ability of different compounds to inhibit monocyte cellular differentiation.THP-1 cells were preincubated with different concentrations of each compound for 6 h. after treatment, cellular viability was normalized to the reading after PMA exposure following a 6-h incubation with DMSO. It is important to mention that all experiments were conducted in triplicates. Treating the cells with diclofenac allowed us to establish a positive control for the effect of COX1/COX2

inhibition on monocyte-to-macrophage differentiation. The IC_{50} values determined by non-linear regression as the best fit values of the log [inhibitor] vs. response curve using GraphPad Prism software (71) (Fig.17). When treated with 10 μM of Pd3 this decrease was observed in a time and concentration dependent manner in both cell lines indicating that the dual target compound was able to decrease monocyte adherence to cancer cells.

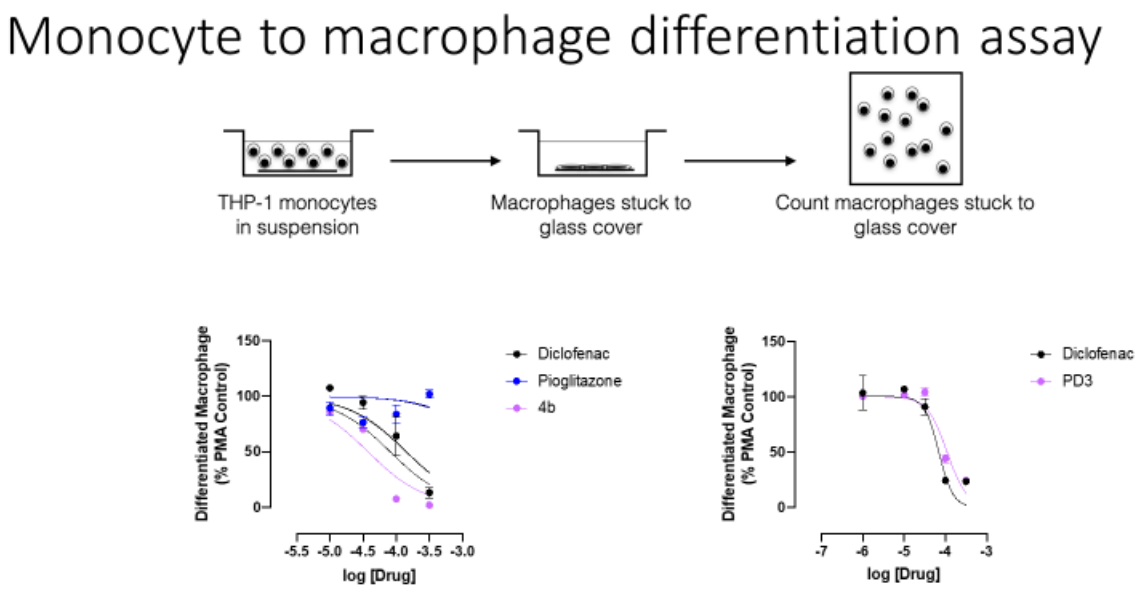

Fig.17: A schematic representation of the monocyte to macrophage differentiation assay *in vitro* THP-1 cells were seeded at a density of 20×10^5 cells/mL. Some of these cells were subjected to PMA at a concentration of 25 nM for 24 h in order to induce cellular differentiation. The other cells were also treated with PMA at a concentration of 25 nM for 24 h followed by 100 ng/ml of LPS for 72 h. After incubation, the supernatant was aspired then using MTS colorimetric cell viability kit the number of adherent monocytes was estimated allowing us to evaluate the ability of different compounds inhibit monocyte cellular differentiation.THP-1 cells were preincubated with different concentrations of each compound for 6 h. after treatment, cellular viability was normalized to the reading after PMA exposure following a 6-h incubation with DMSO. (Adapted from Elzahhar PA *et.al*,2021).

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F. MTT cell growth assay

The basis of the MTT cell growth assay is to evaluate mitochondrial activity of cancerous cells by testing their ability to convert the MTT to formazan crystals. (72). So, in this regard. The MTT assay was used to measure the anti-proliferative effect of Pd3 in vitro. MTT reacts with NADH through specific mitochondrial enzymes and thus, receives electrons. T is then converted from a yellow color agent into formazan, a product with a purple color. Human urinary Bladder cell lines RT4 and T24 were seeded at a density respectively of 1x 10^3 cells/well, in 100µl complete growth medium in three different 96-well culture plates, one plate per time point (24hrs, 48hrs and 72hrs). Cells were incubated overnight at 37°C in a humidified incubator containing 5% CO2. Cells were then treated in triplicates with 0.2% DMSO (vehicle condition) or different Pd3 concentrations for 24, 48, and 72hrs. Four concentrations of Pd3 were used: 3, 10, 30, and 100µM. These concentrations were chosen based on previous studies that were performed on THP-1 cells that showed potent anti -inflammatory activity for these compounds at around 30 µM. At each time point, media was removed and replaced with fresh media along with 10µl/well of 5mg/ml (in 1x PBS) MTT yellow dye and incubated at 37°C, 5% CO2 for 4hrs, after which 100µl/well of the solubilizing agent was added. After overnight incubation, the reduced MTT optical density (OD) was measured by the microplate ELISA reader (Multiscan EX) at a wavelength of 595nm. The percentage of cell proliferation was measured according to the following formula:

$$\%\ cell\ proliferation = (OD\ of\ treated\ cells - OD\ of\ blank)\ x100$$

Data are reported s means ± Standard Error of the Mean (SEM).

G. Trypan Blue Exclusion Assay

The TB assay is used to determine the viability of cells when treated with therapeutic agents. Cells are suspended in a mixture of PBS and trypan blue stain and then examined to identify the percentage of viable cells having a clear cytoplasm (viable cells) versus dead cells with a blue cytoplasm (73). Cell viability was assessed 24, 48, and 72 hrs after treatment. Briefly, human urinary Bladder cell lines RT4 and T24 were seeded in 24-well plates and incubated at 37°C in a humidified incubator containing 5% CO2. After reaching 40-60% confluency, both cell lines were treated with four concentrations of Pd3 (3, 10, 30, and 100μM of Pd3). After 24hrs, cells were trypsinized and counted by Trypan Blue method using a hemocytometer. Cell viability was calculated by the following formula:

$Cell\ Survival$ (%) =Number of living cells count$ed/\ Total\ number\ of\ cells$ ×100

The same procedure was repeated after 48 and 72 hrs. Data are reported as mean ±SEM.

H. Wound Healing Assay

The WH assay is the earliest simple and cheap method used to assess cellular migration in vitro. In fact, a "wound" in a cell monolayer is created and then images are captured at the beginning and at regular intervals. (74). *In vitro* directional cell migration *in vitro* was assessed using WH assay. Briefly, RT4 and T24 bladder cancer cell lines were seeded in 12-well plates at a density of 40x10^4cells/well and 30x10^4 cells/well, respectively. Collagen was added as a supplementation for the growth of RT4. Cells were incubated at 37°C in the humidified incubator containing 5% CO2 until reaching 90-100% confluence. Cells were then treated with 10mg/mL of Mitomycin C (Sigma, USA) for 1hr at 37°C to

inhibit cellular proliferation. Later on, Mitomycin C was removed and two uniform scratches of almost the same width were made using a 200µL micropipette tip. Cells were washed twice with PBS to eliminate the detached cells. Remaining cells were cultured in complete growth media with or without treatment (Pd3vs. control). Finally, bright field images were taken at different time points (0, 4, 18, 24 and 48 hrs.) until the wound closes completely or reduces in size in the untreated group (control) as compared to the treated conditions.

The distance of the wound was measured using (Zeiss) and data represent an average of three independent experiments and reported as mean ±SEM.

I. Three-Dimensional (3D) Culture and spheres-Formation Assay

CSCs are capable of forming (3D) spheres *in vitro* when suspended in non-adherent serum-free conditions which allows for their growth and propagation thus, evaluating the potential use of various drugs to target these tumor-initiating cells (75). Using 96-well plates, $1x10^3$ cells/well were suspended in cold growth factor-reduced Matrigel™/serum-free medium (1:1) in a total volume of 10µl (10µl mix: 5µl cold cell suspension + 5µl cold Matrigel™). The mix of cells and Matrigel™ was kept on ice and pipetted up and down to keep the cells uniformly suspended before plating. The solution was then plated gently around the bottom rim of individual wells uniformly and bubble-free in a circular manner and allowed to solidify for 45-60 minutes at 37°C in a humidified incubator containing 5% CO2. After solidification, 500µL of DMEM High glucose medium with 5% FBS, with or without Pd3, was added gently to the center of each well and the media was replenished

every 2-3 days. Spheres were counted at day 7 and the sphere forming unit was calculated using the following formula:

SFU (%) $= Number\ of\ spheres\ counted / Number\ of$ cells s$eeded$ i$nitially\ x$ 100

However, it is important to mention that the majority of the most of the currently used protocols for 3D culturing of tumor spheroids in suspension are subject to a mechanism of force floating which constitutes a major obstacle and imposes challenges regarding the efficiency and accuracy of assessing the number and size of cultured spheres, as they are mobile and can merge with one another (75).

J. Monocyte (THP-1 cells) Recruitment Assay

In this assay, RT4 and T24 cell lines were seeded into a 96-well plate at a density of 1500 cells/ well. Experiments were performed in triplicates. Cells were allowed 24 hours to attach to the plate. Next, the treatment is applied (Pd3) using two different concentrations; 3 and 10 μM. 2 x 10^4 THP-1 cells were added in each well on top of the seeded cells and allowed for incubation at 37°C for 30 mins. 2 x PBS washes were performed to eliminate debris and non-adherent monocytes. Fresh RPMI media aliquots were added to each well (100μl /well). The results are evaluated under a microscope and the average number of attached monocytes per cell was determined for each treatment.

K. Western Blotting

Western blotting was carried out as described in our previous studies (76). Briefly, Proteins were extracted from cell culture frozen at -80°C according to a protocol previously

designed and optimized at our laboratory. The extraction buffer was composed of 100 mM dithiothreitol, 1% SDS, 0.9% NaCl, and 80 mM Tris hydrochloride (pH 6.8). Protein samples that were extracted were put for heating at 95°C for 10 minutes and then kept overnight on a shaker at 4°C. Aliquots of equal protein content were loaded on a 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis, and the separated proteins were transferred and fixed on a nitrocellulose membrane. Membranes were then blocked with 5% skimmed milk and TBST (Tris-buffered saline with 0.1% Tween 20) for 2 hrs at room temperature (RT). The membranes were then incubated with a dilution of the primary antibodies against p53 (1:1000 primary antibody in TBS-T 0.1%, Cell Signaling) caspase 3 (1:1000 primary antibody in 1% skimmed milk and TBST 0.1%, Santa Cruz)overnight at 4°C. Afterward, the membranes were washed with 0.02% TBST (5 minutes/wash) and incubated with 1:40,000 biotinylated goat antirabbit Ig secondary antibody (Abcam) in 0.1% TBST for 1 hour at RT. Membranes are then washed with 0.02% TBST (5 minutes/wash) and incubated with 1:200,000 horseradish peroxidase-conjugated streptavidin (Abcam) in 0.1% TBST for 30 minutes at RT. After washing with 0.02% TBST (2 washes for 5 minutes each) and TBS (2 washes for 5 minutes each), membranes were developed using Clarity Western ECL substrate for 5 minutes prior to image detection by Chemidoc imaging system (BioRad). ImageJ software was used to measure optical density of protein bands. A ratio of arbitrary density units was obtained for the protein band of interest and the density of the band representing p53 and caspase 3. The bands were normalized to GAPDH

L. Statistical Analysis

Statistical analysis was done using GraphPad Prism 7 software. The significance of the data was determined using proper statistical tests, including the student t-test and two-way ANOVA. P-values of $p<0.05$ (*) were considered significant.

CHAPTER III

RESULTS

A. Cell lines morphology

Two human-bladder cancer cell lines were used for this study. RT4 is derived from a bladder transitional cell carcinoma (Fig.18) whereas, T24 is derived from a transitional cell carcinoma showing a mixed morphology of epithelial and fibroblasts (Fig.19).

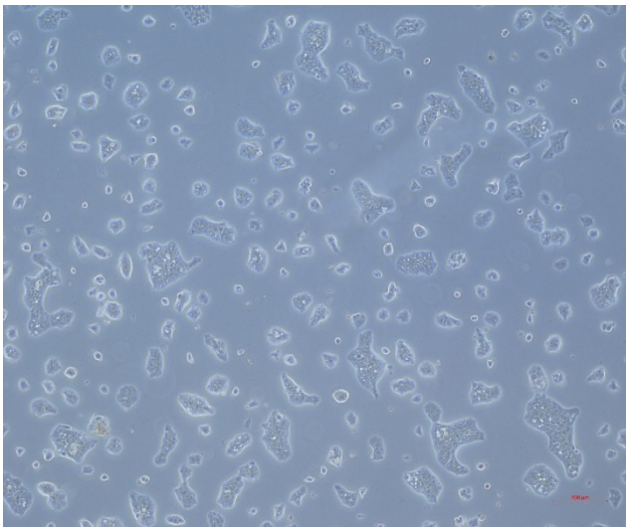


Fig.18: Bright field image displaying the morphological features of RT4 bladder cancer cell line. Images were acquired using Zeiss Axiovert light microscope, showing the morphology of RT4 cell line, on low magnification (5x)

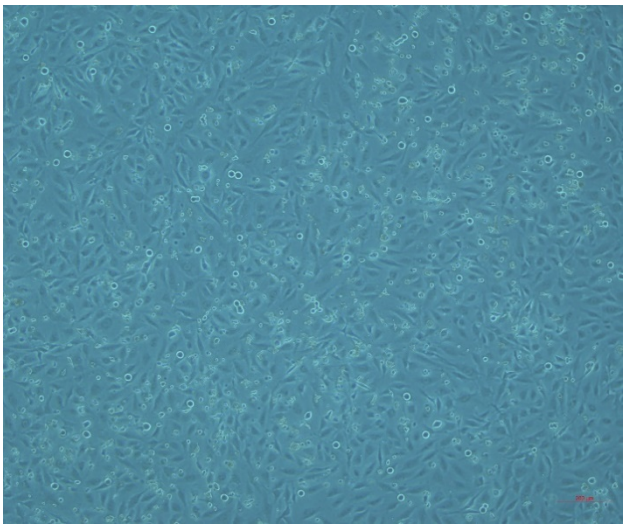


Fig.19: Bright field image displaying the morphological features of T24 bladder cancer cell line. Images were acquired using Zeiss Axiovert light microscope, showing the morphology of T24 cell line, on low magnification (5x)

B. Pd3 inhibits urinary bladder cancer cell proliferation in vitro in a dose-and time dependent manner

First, we assessed the *in vitro* anti-proliferative effect of increasing concentrations of 4b and Pd3 on RT4 and T24 cells using the MTT assay. Starting with the triple target compoud 4b, it showed a slight decrease in cellular proliferation in both bladder cancer cell lines reaching around 5% and 10% after 48 hours and 72 hours of treatment respectively at 10 μM for RT4. Regarding T24, it showed a slight decrease in cellular proliferation in both cell lines reaching around 25% and 15% 48- and 72-hours post-treatment at 10 μM respectively. On the other hand, the dual target drug Pd3 inhibited both cell lines, reaching around 60% and 65% after 48 and 72 hours of treatment at 10 μM respectively for RT4. Regarding T24, Pd3 showed a substantial decrease in cellular proliferation reaching around 60% and almost 58% after 48 and 72 hours of treatment respectively. Increasing the concentrations of Pd3 was able to significantly inhibit cell proliferation by approximately

60% at 30µM of Pd3 for RT4 and by 65% after 48 and72 hours respectively, and by almost 60% for T24 after and 65% after 48 and 72 hours. Further increase in Pd3 concentration to 100µM showed the maximum percentage of reduction in proliferation by almost 70% and 80% after 48 and 72 hours of treatment respectively for RT4 and, by approximately 80% at 48hrs and 85% at 72 hours respectively. Upon using a very low concentration of Pd3 (3µM), the proliferation was not significantly decreased in either cell line as at the three time points indicated. Since Pd3 appeared to be more potent on both cell lines, the remaining experiments were conducted on Pd3 only (Fig.20)

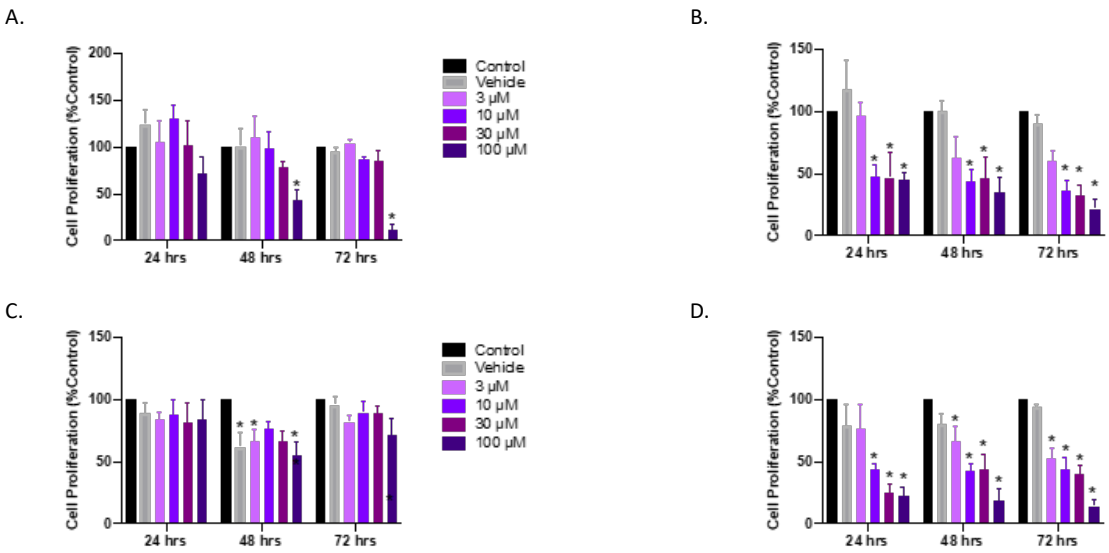


Fig.20: Pd3 reduces RT4 human bladder cancer cell line proliferation in dose- and time-dependent manner more effectively than 4b. Results are expressed as a percentage of the treated group compared to its control. **(A)** Upon treatment with 4b, a slight decrease in cellular proliferation was observed reaching around 5% and 10% after 48 hours and 72 hours of treatment respectively at 10 µM for RT4. **(B)** Upon treatment with 4b, a slight decrease in cellular proliferation was observed reaching around 25% and 15% after 48 and 72 hours of treatment at 10 µM respectively. **(C)** Upon treatment with Pd3, a significant decrease in cellular proliferation was observed reaching around 60% and 65% after 48 and 72 hours of treatment at 10 µM respectively for RT4. **(D)** Upon treatment with Pd3, a significant decrease in cellular proliferation was observed around 60% and almost 58%

after 48 and 72 hours of treatment respectively. Increasing the concentrations of Pd3 was able to significantly inhibit RT4 cell proliferation by approximately 60% at 30μM of Pd3 and by 65% after 48 and72 hours respectively, and by almost 60% for T24 after and 65% after 48 and 72 hours. Further increase in Pd3 concentration to 100μM showed the maximum percentage of reduction in proliferation by almost 70% and 80% after 48 and 72 hours of treatment respectively for RT4 and, by approximately 80% at 48hrs and 85% at 72 hours respectively. Data represent an average of three independent experiments and are expressed as mean ± SEM (error bars).

C. Pd3 reduces bladder cancer cell viability in vitro in a dose and time-dependent manner

The Trypan blue test evaluate the percentage of viable cells found in a cell suspension. Because viable cells exhibit intact cell membranes, they are able to exclude certain dyes, such as trypan blue dye, while on the other hand dead cells do not. So in this test, in order to visually examine whether cells are able to take up the dye or not, the cell suspension was mixed with the trypan blue dye, so theoretically a viable cell will have a clear cytoplasm whereas a nonviable cell will take up the stain and hence exhibit a blue cytoplasm (77).

Trypan blue results showed a time- and dose-dependent reduction in cells viability in response to Pd3 in both RT4 and T24, the inhibitory effect of Pd3 commenced at a concentration of 10μM at 24hrs, decreasing cell viability by almost 58% and by 30% in RT4 and T24 respectively. The maximum percentage of reduction in viability was shown when both cell lines were treated with 100 μM of Pd3 at 72hrs after treatment in RT4 was 90% compared to 94% in T24 (Fig.21). These results were consistent with MTT assay.

A. B.

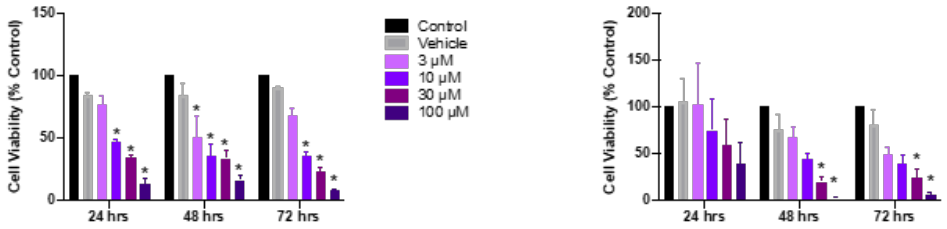


Fig.21: Pd3 decreases T24 human bladder cancer cell line viability in dose- and time-dependent manner. **(A)** Trypan blue results showed a time- and dose-dependent reduction in cells viability in response to Pd3 in RT4.The inhibitory effect of Pd3 commenced at a concentration of 10µM at 24hrs, decreasing cell viability by almost 58% in RT4. The maximum percentage of reduction in viability was shown when both cell lines were treated with 100 µM of Pd3 at 72hrs after treatment in RT4 was 90%. **(B)** Trypan blue results showed a time- and dose-dependent reduction in cells viability in response to Pd3 in T24, the inhibitory effect of Pd3 commenced at a concentration of 10µM at 24hrs, decreasing cell viability by 30% in T24. The maximum percentage of reduction in viability was shown when both cell lines were treated with 100 µM of Pd3 at 72hrs after treatment in T24 was 90%. Data represent an average of three independent experiments and are expressed as mean ± SEM

D. Pd3 inhibits bladder cancer cell migration in vitro

In this assay we are interested in assessing RT4 and T24 bladder cancer cell migratory ability and thus, determine how the migratory potential may be altered by Pd3 treatment.

We assessed cell migration under different experimental conditions, and we investigated the effect of Pd3 on cell migration of human Bladder cancer cells RT4 and T24. Hence, we used a wound-healing/ scratch assay where the cells were divided into 5 groups: untreated (control), vehicle, and treated with 3µM, and 10µM of Pd3. The main advantage of this assay is that it mimics migration of tumor cells *in vivo* and allows studying the cell–cell interactions and communication between cancer cells and their

extracellular matrix (ECM) in order to regulate the cell migration process. Our results showed that the wound was completely healed after 24hrs in the control and vehicle for T24, and the migration was significantly decreased after the treatment with 3μM and 10μM of Pd3. Nevertheless, Pd3 showed a reduction in the rate of wound closure after 48 hrs post treatment with 3μM and 10μM of Pd3 as compared to the untreated groups (control & vehicle). For RT4, our results show that upon treatment with 3μM and 10μM of Pd3, there is a slight decrease in the wound distance when compared to the control condition. This may be due to the collagen plating that could have possibly altered the migration of these cancerous cells, especially that Pd3 showed a potent inhibitory effect on the migratory ability of the invasive type of bladder cancer (T24 cell line). Our results showed that the migration of RT4 bladder cancer cell line was decreased by almost 20% 48 hrs after the treatment with 3μM and 10μM of Pd3 (Fig.22-23)

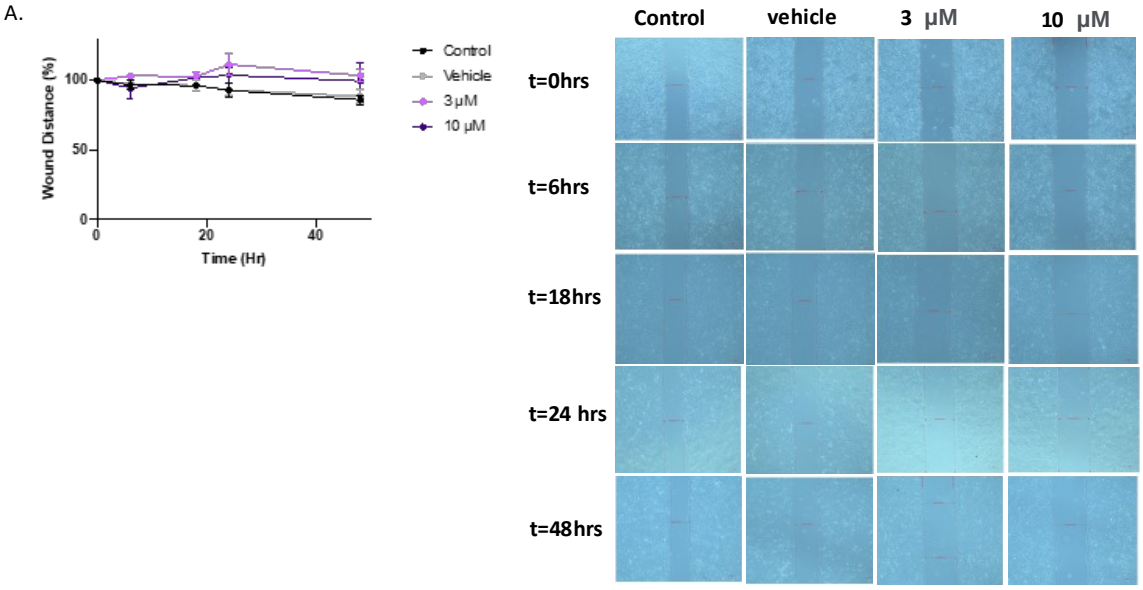


Fig.22: Pd3 reduces RT4 human bladder cancer cell line cell migration in dose- and time-dependent manners. After incubation of RT4 cell line with or without treatment (Pd3), cell migration was tested by scratch assay, and showing no wound healing after 48 hrs in the control and vehicle similarly to both the 3µM and 10µM treated conditions. **(A)** upon treatment with 3µM and 10 µM of Pd3, the wound distance decreased slightly by 5% and 7% respectively after 48 hrs. **(B)** bright field images of RT4 wound distance across 48 hrs Results indicate the inability of Pd3 to inhibit the cellular migration of RT4 at different time points (0,6,18, 24 and 48hrs),

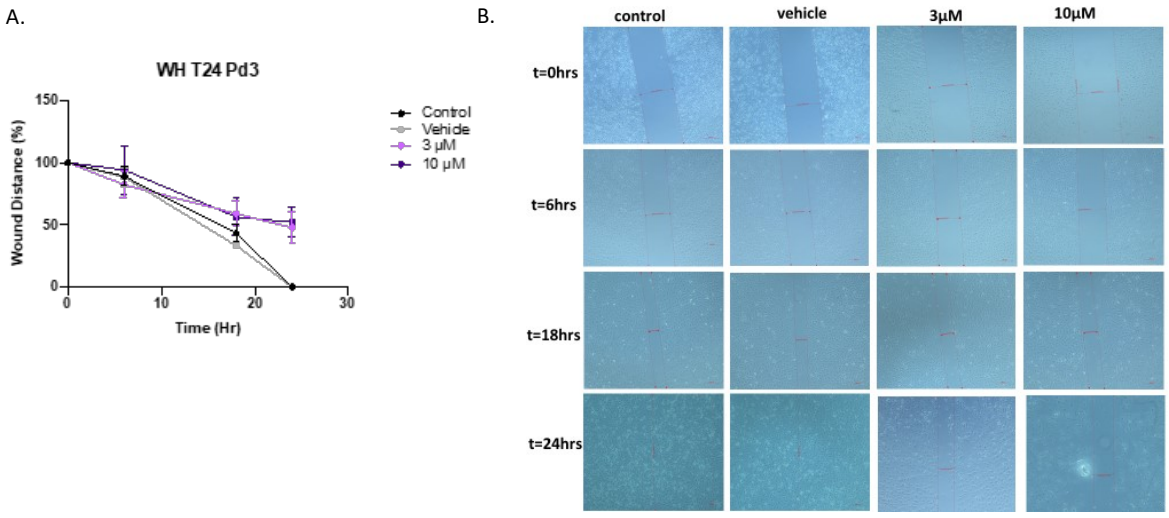


Fig.23: Pd3 reduces t24 human bladder cancer cell line cell migration in dose- and time-dependent manners. (A) . After incubation of T24 cell line with or without treatment (Pd3), cell migration was tested by scratch assay, and showing a total wound healing after 48 hrs in the control and vehicle and suppression of the wound closure after 48 hrs with 3µM and 10µM of Pd3. **(A)** upon treatment with 3µM and 10 µM of Pd3, **(B)** bright field images of T24 wound distance across 48 hrs Results indicate the ability of Pd3 to inhibit the cellular migration of T24 at different time points (0,6,18 and 24hrs).

E. Pd3 diminishes the sizes of human bladder cancer cell line cultured spheres in a dose-dependent manner

Having established Pd3's inhibitory effect on both cell lines in 2D, we next aimed at the ability of Pd3 to target the self-renewal capacity of Bladder Cancer stem cells (SC) enriched from RT4 cell line in 3D cultures using sphere formation and propagation assays. 50 µL of DMEM F12-Ham with 5% FBS, with or without Pd3 was added gently to the center of each well after solidification and the media was replenished every 2-3 days. Spheres were counted at day 7 for RT4 bladder cancer cell line after plating, and the

spheres formation efficiency or sphere formation unit (SFU) was calculated based on the following formula: SFU = (number of spheres counted ÷ number of input cells) × 100. Pd3 had significantly decreased the area of RT4 spheres, in addition to a remarkable decrease in the sphere formation unit, when treating with increasing concentration of Pd3 (1, 5 and 10μM) (Fig.24)

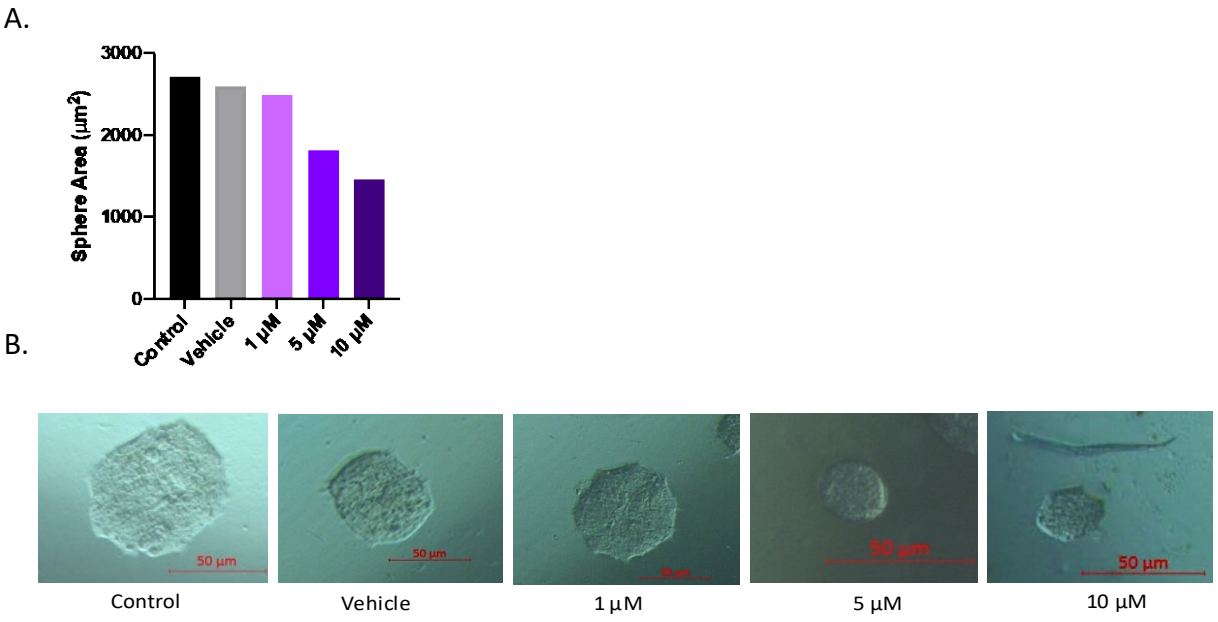


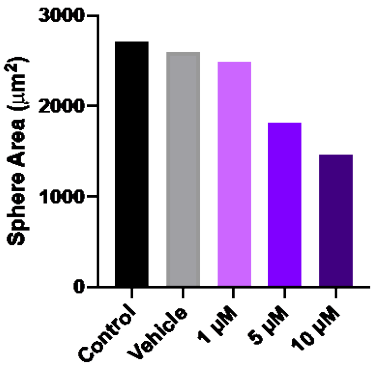
Fig.24: Pd3 diminishes the sizes of human bladder cancer cell line RT4 cultured spheres in a dose-dependent manner. We measured the diameter and calculated the area of 30 fully grown spheres. The average size of RT4 spheres (μm^2) decreased significantly with higher Pd3 concentrations. **(A)** upon treating with increasing concentrations of Pd3, the sphere area decreased significantly when compared to the untreated control conditions. Upon treating with 5μM of Pd3, RT4 spheroid size decreased by almost 68% when compared to the control group **(B)** Morphological representation of the spheroid diameter upon treatment with Pd3. Images of cultured spheres were visualized and taken by Zeiss Axiovert inverted microscope and analyzed by Carl Zeiss Zen 2012 images software Data are represented by mean ± SEM (error bars).

Fig.25: Pd3 reduces the sphere forming units of RT4 and T24 bladder cancer cell cultured spheres in a dose-dependent manner. Spheres formed in each well, referred to as generation 1 spheres (G1), were counted at day7 post- plating. **(A)** A 5µM of Pd3 was able to decrease the SFU by 45% for RT4 while a dose of 10µM of Pd3 significantly reduced the SFU by 50%. **(B)** A 5µM of Pd3 was able to decrease the SFU by 40% for T24, while a dose of 10µM of Pd3 significantly reduced the SFU by 60% respectively. Results are represented as mean ±SEM (**p<0.01, ***p<0.001).

F. Pd3 inhibited monocyte recruitment and their adhesion to bladder cancer cell line.

In this assay, RT4 and T24 cell lines were seeded into a 96-well plate at a density of 1500 cells/ well then, treated using two different concentrations; 3 and 10 µM. 2 x 10^4.THP-1 cells were added in each well on top of the seeded cells and allowed for incubation. Upon treating bladder cancer cells with increasing concentrations of the dual target compound Pd3, the average number of monocytes that were able to adhere to the cancer cells was evaluated, counted and compared to the control where no drug was added. Our results indicate that the number of adherent monocytes has decreased significantly in both bladder cancer cell lines RT4 and T24 when compared to the untreated control condition. For RT4, after 48 hours the average number of recruited monocytes was approximately 12 whereas approximately 9 monocytes were counted when treated with 10 µM of Pd3. Similarly, for T24, after 48 hours the average number of recruited monocytes was approximately 16 whereas approximately 10 monocytes were counted. (Fig.26-27)

A.



B.

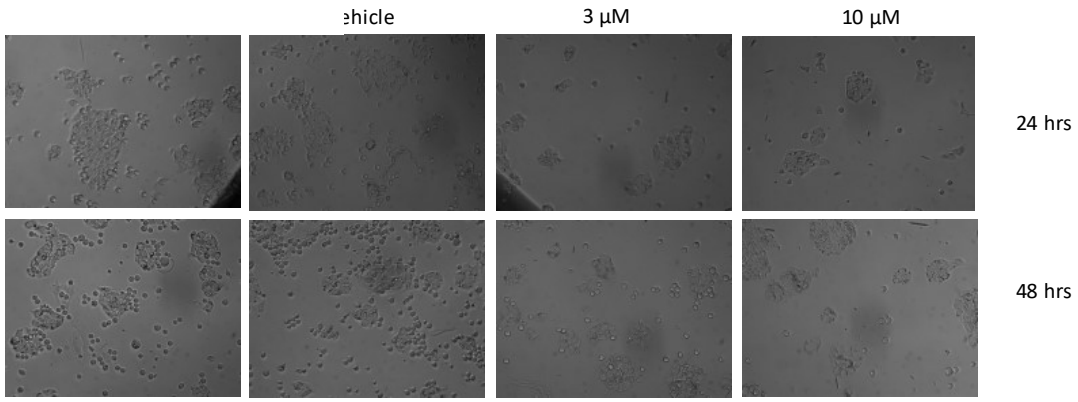
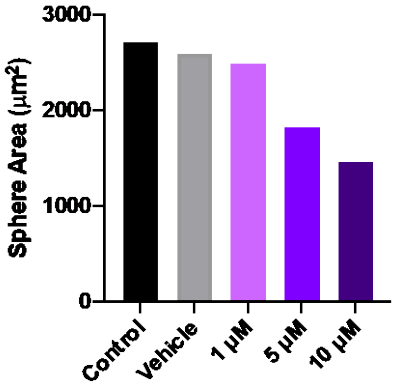


Fig.26: Pd3 inhibited monocyte recruitment and their adhesion to RT4 bladder cancer cell line. Monocytes were incubated with RT4 bladder cancer cell line over a period of 48 hrs, then two concentrations (3 and 10 μM) of the treatment Pd3 were applied. The number of monocytes that adhered to RT4 cancer cells were evaluated and counted under the microscope. **(A)**After applying 10 μM of Pd3, the average number of adherent monocytes decreased by 58% after 48 hrs when compared to the control condition where no treatment was applied. **(B)** Representative images evaluating the number of monocytes adherent to RT4 bladder cancer cell line. Results are represented as mean ±SEM (**p<0.01, ***p<0.001).

A.



B.

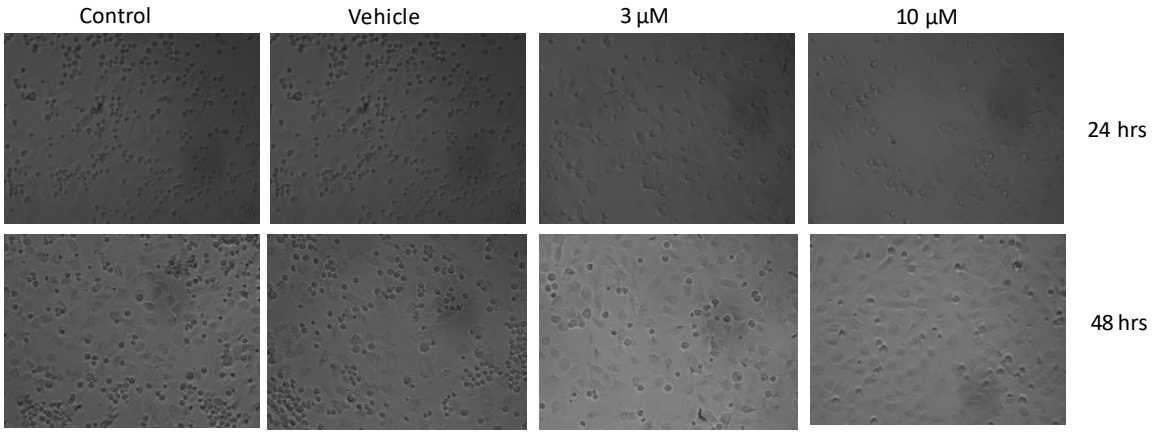


Fig.27: Monocyte recruitment assay performed on T24 bladder cancer cell line. Monocytes were incubated with T24 bladder cancer cell line over a period of 48 hrs, then two concentrations (3 and 10 µM) of the treatment Pd3 were applied. The number of monocytes that adhered to T24 cancer cells were evaluated and counted under the microscope. **(A)**After applying 10 µM of Pd3, the average number of adherent monocytes decreased by 53% after 48 hrs when compared to the control condition where no treatment was applied. **(B)** Representative images evaluating the number of monocytes adherent to T24 bladder cancer cell line Results are represented as mean ±SEM (**p<0.01, ***p<0.001).

G. Western Blot

Protein samples were extracted from both RT4 and T24 cell lines screened for the presence of p53, controlling cell death (78). Preliminary results showed that exposure to 3 μM of the dual target compound increased p53 expression in T24 cell line across 72 hrs when compared to the untreated control. On the other hand, p53 expression upon treating with 3μM of Pd3 across 72hrs was less significant for RT4.The bands were normalized to GAPDH a housekeeping gene indicating that the variabilities in the bands are neither due to loading inconsistencies nor a lack of protein in a lane.

Additionally, caspase 3 is also a marker for apoptosis(79). Our results show that upon treating the cells with 3 μM of the dual target compound, caspase 3 was significantly expressed in T24 cell line across 72 hrs when compared to the untreated control. Along the same lines, full-length caspase 3 expression was reduced following the same treatment possibly indicative of increased cleavage and apoptosis. Importantly and consistent with the reduced effect on RT4 cells, neither change was seen upon treatment with Pd3. (Fig.28)

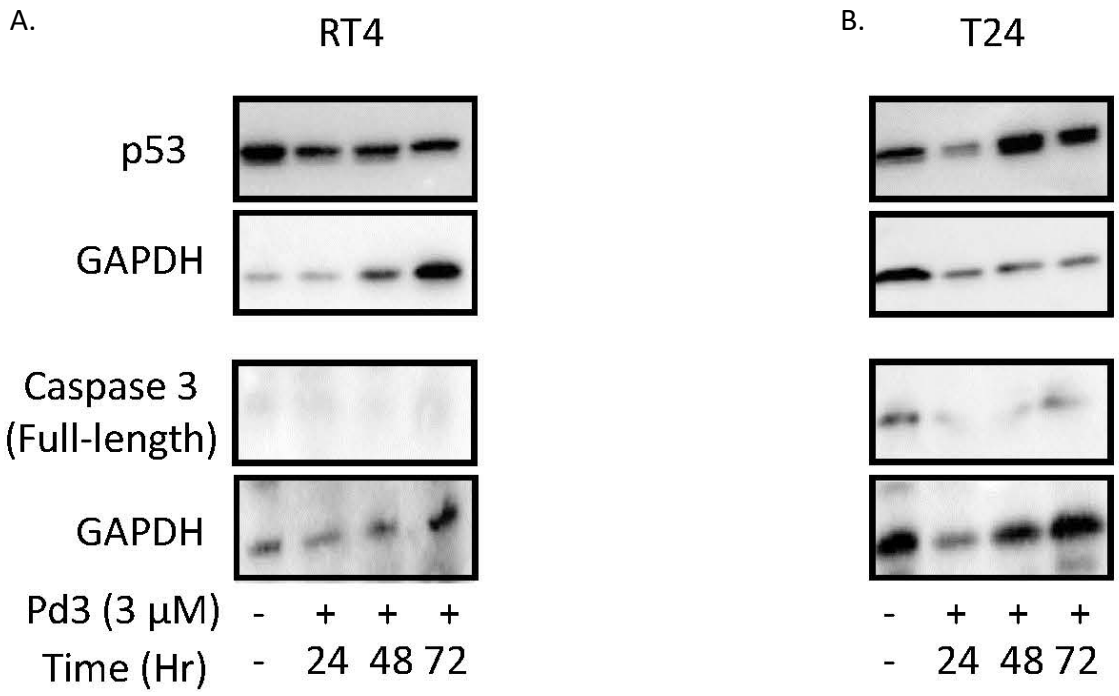


Fig.28: Western Blot assay for p53 and Caspase 3 proteins extracted from both RT4 and T24. Protein samples were extracted from both bladder cancer cell lines and screened for the presence of P53; a marker of apoptosis and full-length caspase 3; an executer of apoptosis. **(A)** upon treating the cells with 3 μM of Pd3, no change was seen regarding the expression of p53 and full-length caspase 3. **(B)** upon treating the cells with 3 μM of Pd3, caspase 3 was significantly expressed in T24 cell line across 72 hrs when compared to the untreated control. Along the same lines, full-length caspase 3 expression was reduced following the same treatment.

CHAPTER IV

DISCUSSION

Bladder cancer is classified as being the leading cause cancer associated death in males. In fact, it constitutes the sixth most predominant malignancy in addition to being the ninth most fatal. (CSCs) are classified as being the causative for cancer metastasis and invasiveness thus, are associated with aggressiveness. Henceforth, knowing their vital roles, targeting CSCs in bladder cancer therapy can increase treatment efficiency. In fact, bladder cancer is considered to be a stem cell disease. Identifying reliable diagnostic and prognostic markers remain an important hallmark in the diagnosis of the disease. Analyzing bladder cancer stem cells at the molecular levels allows physician to predict the patient's response to treatment, whether or not the patient will have a good prognosis and most importantly whether the patient will develop chemoresistance to these cytotoxic drugs. Therefore, a deep understanding of the molecular and biological basis of CSC is a prerequisite in ameliorating bladder cancer treatment regimens.

To start with, CD44 marker has been showed to be highly expressed in bladder cancer. CD44 marker is mainly found on cell surface adhesion molecule that is overexpressed in several neoplasms. It plays a major role in cancer cell proliferation, differentiation, migration, angiogenesis, and disease progression. In normal healthy individuals, $CD44^{+}$ cells are located in the basal layer of the normal urothelium. Remarkably, Chan et al. demonstrated that the ability of $CD44^{+}$ tumor cells to form a tumor in immunodeficient mice was 10–200 times higher than $CD44^{-}$ tumor cells. In addition, the expression analysis of CD44 in over 300 Bladder Cancer tissues indicates that the

subpopulation of $CD44^+$ cells is formed of around 40% of all tumor cells (80). Moreover, studies have reported an elevated ALDH1A1-positive cell population in bladder cancer patients and its upregulation is strongly associated with progression, aggressiveness, recurrence, shorter survival time, and a poor prognosis in patients with bladder cancer. Moreover, recent evidence demonstrated that the knockdown of the ALDH1A1 gene via its siRNA was associated with a decreased clonogenicity and tumorigenic potential in bladder Cancer Stem Cells. Su et al. found that $ALDH1A1^+$ BC cells have higher clonogenicity and tumorigenicity than ALDH1A1 ones. Additionally, the overexpression of SOX4 has been reported in a number of cancers including Bladder Cancer and is strongly correlated with tumorigenesis and cancer progression through the EMT process. Shen et al. showed that SOX4 plays an important role in the regulation of bladder Cancer Stem Cells properties. Moreover, SOX4 overexpression was strongly associated with advanced bladder cancer stages and a poor survival rate. They also found that SOX4 downregulation resulted in the inhibition of cell migration, colony formation, and EMT (80). Additionally, knockdown of the SOX4 gene was able to decrease the sphere formation however, increased cell populations carrying high levels of ALDH and thus, tumorigenic potentials.

Chemotherapy, radiation and transurethral resection represent the main bladder cancer therapeutic strategies; however, such treatments are accompanied with several side effects and hence long-term morbidities. In the process of discovery and development of novel compounds with anti-tumor effects, recent discoveries has led to the introduction of new harmless but also lucrative molecules(81).

Thus, designing a potent MTDL is a challenging task for it requires not also an effective target combination without introducing changes in the drug characteristics. Hence, because we are interested in the effects of COX-2,15-LOX and PPARγ in inflammatory changes encompassing malignant disorders, insights have encouraged the development of new compounds with an anti-inflammatory activity that can be potentially used therapeutically to treat malignant transformation. Previous biological and pharmacological evaluation of different chemical compounds targeting COX-2 and/or 15-LOX- and PPARγ have been done, which was narrowed down to choosing the compounds with the most potent effects.

Therefore, we were interested in studying the effects of a dual COX-2/15-LOX inhibitor (Pd3) and triple COX-2/15-LOX inhibitors/PPARγ agonist (4b), which have been used to treat different conditions such as metabolic disorders, hypertension, diabetes, and others. In addition, these molecules have been shown to be effective agents in treating a number of neoplastic diseases such as prostate, CRC, lung cancer among many others.

We examined the anti-cancer effect of double (Pd3) and triple (4b) target drugs on RT4 and T24, where Pd3 was able to decrease cell proliferation and viability more efficiently than the triple target drug. The triple COX-2/15-LOX inhibitor and PPARγ agonist showed a weak efficacy in inhibiting bladder cancer cell proliferation, migration, metastasis etc. compared to the dual COX-2/15-LOX inhibitor. Moreover, these anti-inflammatory compounds proved success in inhibiting monocytes recruitment and their adherence to both RT4 and T24 thus, targeting the body's first reactive mechanism which occurs during inflammatory responses. By doing so, we were able to target early mechanisms of inflammation driven neoplasm and therefore, these compounds may serve as potential adjuvant in treating malignancies among which bladder cancer. In fact, early

detection of inflammation is crucial in preventing the development of malignant neoplasm since it is associated with a good response and a better prognosis (82).

Moreover, several protein markers were screened for and evaluated in order to understand how COX-2 and 15-LOX inhibitors exert their effects on bladder cancer cells. P53, a tumor suppressor gene was evaluated as a marker of apoptosis. Interestingly, it was found that p53 expression was increased in both bladder cancer cell lines upon treating with the dual target compound indicating that dualCOX-2 and 15-LOX inhibitor work by possibly targeting the cellular apoptotic machinery and inducing apoptosis of cancer cells. Our results were supported by previous studies that demonstrated the importance of p53 as being an important regulator of EMT which occurs by activating miR-200c. This occurs through the binding to the miR-200c promoter. It was demonstrated that a low level of miR-200c was strongly associated with a loss of p53 thereby promoting EMT program. Therefore, p53 controls EMT via the involvement of p53-miR-200c pathway (83). Moreover, full length caspase-3 was also screened for and evaluated in our study as being a marker of apoptosis by cleaving a number of cellular targets (84). Interestingly, it was found that full length caspase-3 expression was decreased in both bladder cancer cell lines upon treating with the dual target compound indicating that dualCOX-2 and 15-LOX inhibitor work by possibly inducing apoptotic chromatin condensation and DNA fragmentation in bladder cancer cells (83). our preliminary results were supported by previous studies that demonstrated that caspase-3 sensitizes cancer cells to be more responsive to chemotherapy and radiotherapy by altering their ability to spread(84). However, the effect of caspase 3 on cancer growth and development is still poorly understood requiring further investigation. Some studies have showed that *CASP-3* gene is

able to generate an alternative type of caspase 3 called caspase-3s, which has antagonistic effects to the normal caspase 3 thus, inhibit apoptosis. It was found that the expression of the normal caspase 3 along with the new variant was seen in a number of malignancies, the ratio of which is considered to be a strong indicator of patients response to therapy.(85).

In addition, we used two different types of bladder cancer cell lines ranging from the superficial type (RT4) to the invasive and aggressive type associated with lower survival, poor prognosis, and higher rates of metastasis (T24). This is in order to assess the effects of these novel therapeutic approaches not only on the superficial noninvasive type but also on the metastatic type. Owing to the integral role of COX-2 and 15-LOX in the pathogenesis a number of neoplasms, the dual COX-2/15-LOX inhibitors are expected to be potent and effective therapeutic agents. Indeed, our data has showed encouraging results targeting bladder cancer cell lines and further studies, if proven successful, Pd3 could in the management of human bladder cancer by acting as an adjuvant to chemotherapy and radiation therapy.

Managing cancer patients remains a challenging task whereby metastasis is the greatest obstacle. Cell migration is a fundamental and ancient cellular behavior that contributes to metastasis. Interestingly, our data showed that low doses of Pd3 ranging from (3μM-10μM) were able to reduce cell migration in both bladder cancer cell lines in dose and time dependent manners. This comes along with previous conducted studies demonstrating that either COX-2 or 15-LOX inhibitors were able to attenuate cancer cell ability to metastasize and propagate in breast cancer cells, renal carcinoma among many others(86). One possible mechanism of Pd3 inhibiting cell migration can be via targeting the epithelial-to-mesenchymal transition program (EMT program) (87). Henceforth, it

remains important to further investigate the mechanisms behind Pd3-inhibiting cell migration on our bladder cancer cell models.

Previous studies demonstrated the pivotal role of COX-2 inhibitors in treating malignant disorders. In fact, COX-2 inhibitors owe to the enzyme inhibition not only in the tumor itself but also in stromal cells thus, decreasing the production of agents that favor cancer development. Thus, combining a potent COX-2 inhibitor along with chemotherapy may provide additional benefits in treating a number of malignancies (88). Others have suggested the possible involvement of VEGF in promoting neoplastic transformation. The VEGF pathway is a crucial pathway responsible for inducing neovascularization. In fact, a very high level of COX-2 was shown to increase VEGF, responsible for assuring angiogenesis for cancer cells(89). Interestingly, VEGF receptor inhibitors such as bevacizumab, aflibercept, and sorafenib were officially accepted to be used to treat cancer patients either alone or along with another treatment regimen such as chemotherapy or radiation therapy thus, increasing their effectiveness in targeting cancer cells (90). Moreover, (MMP)-9 is involved in controlling pathological remodeling processes involving inflammation and fibrosis in cardiovascular disease. In fact, COX-2 inhibitors, were shown successful in decreasing PGE_2 levels which were shown to be associated with cancer growth and development. Decreasing PGE2 was associated with low levels of MMP-9 production thus, altering the ability of cancer cells to spread (91). These are facilitated by (GPCRs) specific to PGE_2. It was shown that EP4 receptor is the one mediating the ability of cancer cells to metastasize hence be more aggressive. Thus, PGE_2 demonstrated its ability to control the invasion of NSCLC through the regulation of COX-2 via the EP4 receptor (91). Additionally, some inflammatory cytokines are able to significantly increase

COX-2 expression. In fact, these cytokines to induce high levels of COX-2 along with the production of PGE2 via NF-κB pathway activation in colon cancer. NF-κB pathway is a highly conserved TF complex that controls transcription of DNA, cytokine production particularly cell inflammatory responses, cellular growth and apoptosis (92). Interestingly, TNF-α allowed the overexpression of COX-2 in pancreatic cancer cells, postulating that inflammation creates the suitable milieu that allows high levels of COX-2 to be expressed in pancreatic cancers (92).Moreover, pro-inflammatory cytokines like IL-1β and IL-6 are able to trigger the expression of COX-2 in CRC through the NF-κB subunit p65 in CRC. Because COX-2 expression was shown to inhibit apoptosis of cancer cells, altering of NF-κB signaling pathway may be a good alternative in targeting CRC progression and metastasis(93).

Previous studies have suggested that (TME) favors the EMT in cancerous cells. In fact, cancer-associated fibroblasts (CAFs) activated by cancer cell make up the majority of (TME) cells. In fact, emerging evidence suggest that CAFs are crucial players in establishing the metastatic niche for cancerous cells thus, promoting tumor cell proliferation, invasion through the secretion of important mediators such as the cytokines into the surrounding milieu. Interestingly, elevated COX-2 correlated with a bad response to treatment in patients with nasopharyngeal carcinoma (NPC) by altering the death of NPC cells. It was found that COX-2 increases the levels of tumor necrosis factor-α expression in CAF whereby it increases the ability of NPC cells to metastasize and invade other areas of the body suggesting that COX-2 overexpression in CAF could be used as a strong metastatic indicator for NPC (94).

Little is known about the possible mechanisms behind the use of 15-LOX inhibitors in treating malignant neoplasms. Some studies have reported that NSAIDS are crucial inducive agents of apoptosis in CRC by acting on PUFAs via both LOXs and COXs (95). Colonic linoleic acid is primarily metabolized by 15-LOX-1 to (13-S-HODE), an inducer of apoptosis. In human CRC, 15-LOX-1 was shown to be decreased. Moreover, it was found that the use of NSAIDs triggered CRC cells apoptosis via high 15-LOX-1 expression.

Additionally, studies have demonstrated the inhibition of the IL-6/STAT3 signaling pathway by the action of 15-LOX-1 in (CAC) by down-regulating PPAR-δ. IL-6/STAT3 signaling is a pro-tumorigenic pathway for (CAC). It was shown that colonic epithelial cells transgenically expressed 15-LOX-1 and were thus able to inhibit the upregulation and expression of IL-6. This leads to the phosphorylation of STAT3 in mice models during CAC induction(96). These findings give rise to novel approaches that can be used to regulate IL-6/STAT3 signaling in CAC formation.

On the other hand, some tumor markers were extensively studied as potential targets for COX-2 inhibitors. To start with, CD44 is highly expressed in bladder cancer. In fact, CD44 is found at high levels in several neoplasms for it controls cancer cell growth and development. Moreover, CD44 is also used to trigger the association with MMP-9 thus, favoring MMP-9 activity. Interestingly, it was found that high levels of COX-2 lead to CD44-dependent invasion in (NSCLC) thus, increasing the metastatic potential. This suggest that targeting COX-makes it harder for cancer cells to metastasize in (NSCLC). (97).

Furthermore, recent evidence demonstrated that (YAP1), contributes to cancer cells growth and proliferation thus correlates with a bad response to treatment. It was shown that

YAP1 plays a major role in immunosuppression, and chemoresistance. Although evidence are still lacking, it is evident that activating YAP1 favors an immunosuppressive tumor microenvironment (98).

Finally, OCT4 (also known as POU5f1) is a key modulator of stem cells renewal. Its overexpression is strongly correlated with invasiveness, metastasis and hence, bad prognosis. In fact, Atlasi et al. demonstrated that almost all bladder cancer tumors showed a high OCT4 expression. In fact, in bladder high OCT4 was strongly associated with an increased aggressiveness of the cancer. Moreover, a recent study suggested that OCT4 expression is variable depending on a number of factors such as age, sex, tumor size and treatment regimens adopted thus, suggesting that OCT4 can be used to predict the outcomes and may become an adjuvant therapy to radiation and chemotherapy (99).

Given that multi-targeted directed ligands (MTDLs) are compounds that are designed in a way to target many disorders, and given the potential effect of both COX-2 and 15-LOX enzymes in physiological and pathophysiological conditions such as inflammation induced cancer, dual and triple target drugs involving dual COX-2, 15-LOX inhibitors and triple COX-2, 15-LOX inhibitors and PPARγ agonists were synthesized and evaluated to target human Bladder cancer tumors. This is accomplished while preserving the integrity and role of other cellular targets and enzymes that are beneficial thus, reducing possible risks. Moreover, 4b was designed to act as partial PPARγ agonists allowing scientists to escape the bad unwanted side effects a full PPARγ agonist would give.

However, the possibility of potential adverse events with the use MTDLs is still poorly understood and must be subjected to *in vivo* evaluation (24).

Our study investigated the effect of dual COX-2/15-LOX inhibitors and triple COX-2/15-LOX inhibitors and PPARγ agonists on bladder cancer cell lines where we showed that Pd3 inhibits cell viability, cell proliferation and migration. Moreover, this study is the first to show the inhibitory effect of low doses of dual COX-2/15-LOX inhibitors not only on the superficial type but also on the muscle invasive and metastatic bladder cancer cells. Furthermore, we provide initial evidence that Pd3 might targets Bladder cancer CSCs subpopulation.

In fact, a growing body of evidence have suggested additional targets such as choline esterases and carbonic anhydrases whereby, cellular and molecular assays in addition to in vivo models showed the potential role of these molecules in inhibiting inflammation-induced tumorigeneses which might be used to treat a number of cancers namely bladder cancer. However, further investigation is still needed to fully understand the potential effects of using newly and rationally designed compounds as effective therapeutic agents (42).

Limitations in our study arise from the bladder cancer spheroid diameter limit, where spheres <40 μm diameter (corresponding to a volume of 3.3×10^4 μm^3) were not counted and hence were not part of the analysis. Moreover, the discrepancy seen in 15-LOX activity is most probably due to differences in cancer cell lines (prostate, colon, breast, lung etc.), to different stages within the same cancer type (superficial, invasive, metastatic, benign etc.) and finally to inter-species differences, that's why we lack of a proper *in-vivo* model

Future directions would be to try the combination of both double and triple target drugs on Bladder cancer cell lines hoping that this combination would possibly boost the

therapeutic effects and increase their efficacy. Moreover, we could further investigate the effect of the MTDLs on normal Human bladder cells thus, compare the effect of these novel therapeutic approaches on both normal and cancerous cells. Moreover, we could further elucidate the mechanistic action behind the role of COX-2 and 15-LOX inhibitors in treating malignant neoplasms. This could be accomplished by screening a number of protein markers that may be involved in the pathogenesis of bladder cancer through the COX-2 and 15-LOX pathways. These include the, FGFR, RB, (MKP-1) among many others (100). Finally, the possibility of potential adverse events with the use MTDLs is still poorly understood and must be subjected to further *in vivo* evaluation.

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