

**Evaluation of novel optical imaging and therapeutic agents for the
detection and treatment of bladder cancer.**

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DEDICATION

This dissertation is dedicated to my parents, Gail and Lawton Bourn, who encouraged me to pursue my dreams and supported me no matter where those dreams took me.

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ABSTRACT

Several types of cancer, including bladder cancer, overexpress multiple receptor tyrosine kinases (RTKs), such as the c-Kit receptor and platelet derived growth factor receptor (PDGFR). Inhibitors of RTKs (RTKIs) are therefore used as targeted treatment options for patients with cancers that overexpress RTKs. A challenge associated with targeted therapies is the activation of alternative pro-survival signaling pathways, such as the cyclooxygenase-2 (COX-2) pathway, resulting in drug resistance. Although COX-2 is over-expressed and promotes tumorigenesis in bladder cancer, it can be used as a biomarker for bladder cancer detection and treatment strategies.

Conventional optical imaging technologies can detect advanced stages of bladder cancer; however, they have several limitations to detect early stages. Novel optical imaging agent, fluorocoxib A, is a rhodamine-conjugated analog of indomethacin, which selectively targets COX-2 expressing tissues. Fluorocoxib A can be used for the detection and monitoring of COX-2-expressing tumors during treatment.

We evaluated the effects of RTKIs and COX inhibitors, alone and in combination, on human and canine bladder cancer cell lines *in vitro*. Despite inhibiting cellular proliferation and increasing apoptosis, tested RTKIs increased COX-2 expression. Co-treatment of RTKIs and COX inhibitors abrogated the RTKI-induced COX-2 expression, indicating co-treatment may be more effective than either treatment alone.

To evaluate the ability of fluorocoxib A for the detection of bladder cancer in mice, we used the carcinogen (*N*-butyl-*N*-4-hydroxybutyl nitrosamine, BBN) -induced bladder cancer mouse model that resembles human bladder cancer. The specific

uptake of fluorocoxib A by the bladder tissues correlated with the progression of bladder carcinogenesis and increased COX-2 expression, in contrast to normal bladder urothelium where no fluorocoxib A uptake was detected.

We also investigated the RTKI-induced upregulation of COX-2 by evaluating the effects of RTKIs *in vivo*. Treatment of K9TCC#5Lilly xenograft tumors with AB1010 and imatinib increased COX-2 levels in the tumors of treated mice compared to the control group. The specific uptake of fluorocoxib A correlated with the treatment-induced increased COX-2 expression.

Our results suggest that fluorocoxib A is a valuable optical imaging agent that can be used for detecting and monitoring early responses to targeted therapies in patients diagnosed with COX-2-expressing bladder cancer.

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CHAPTER I INTRODUCTION: A REVIEW OF CURRENT TREATMENT STRATEGIES AND DETECTION METHODS FOR BLADDER CANCER

BLADDER CANCER

Bladder cancer in the United States is the 6th most common type of cancer, with an estimated 81,000 newly diagnosed cases and 17,000 deaths per year (1, 2). Bladder cancer incidence is three times higher in men than in women, which increases with age, as patients are generally diagnosed after 65 years of age. According to the World Health Organization (WHO), because of increased life expectancies, the rates of bladder cancer diagnoses are expected to increase over time (3, 4).

The most common type of bladder cancer is urothelial carcinoma, also known as transitional cell carcinoma (TCC). Urothelial carcinoma arises from the epithelial cells that line the inner part of the bladder, known as the urothelium, and accounts for 90% of all bladder cancer cases. There are several additional subtypes of bladder cancer, including squamous cell carcinoma, small-cell carcinoma, and adenocarcinoma. These rare forms of bladder cancer are more aggressive, associated with high rates of metastasis, and patients generally have a poor response to standard therapy regimens. The most common symptom of bladder cancer is the presence of blood in the urine (hematuria), which occurs in most patients (5). There is a correlation between macroscopic hematuria and the diagnosis of advanced stage bladder cancer. Currently, there are no active screening protocols for bladder cancer, so many patients who present with microscopic hematuria are not adequately evaluated and are diagnosed at a later disease state (6).

There are five primary stages of bladder cancer, with stage IV being the most advanced disease state (7). Stage 0 is classified as a non-invasive papillary carcinoma

or a flat, carcinoma-in-situ (CIS). Stage I, the cancer has spread to the layer of connective tissue in the bladder but not to the muscle layer of the bladder wall (non-muscle invasive bladder cancer, NMIBC). Stage II, the cancer has progressed to the muscle layer of the bladder wall (muscle-invasive bladder cancer, MIBC). Stage III, the cancer has spread through the fatty layer surrounding the bladder as well as surrounding pelvic organs (prostate, uterus, or vagina). Stage IV, the most advanced stage, is characterized by metastatic bladder cancer throughout the lymphatic system and other organs. Patients diagnosed with Stage IV bladder cancer have poor prognostic outcomes (8). Approximately 75% of newly diagnosed cases are classified as NMIBC, and 25% are MIBC, with or without metastatic disease (9-11).

The development of bladder cancer is associated with several risk factors including cigarette smoking, environmental factors, diet, occupational exposures, chronic bladder infections, and familial history of bladder cancer. Cigarette smoking accounts for almost half of the diagnosed cases, with a long lag time between exposure to tobacco and time of diagnosis (12). Environmental factors (contaminated drinking water, air pollution, etc.), diet, and alcohol consumption are indirect risk factors, and, when in combination with multiple risk factors, the risk of bladder cancer development increases (11, 13, 14). Occupational exposures include industrial workplaces, such as chemical processing plants, factories processing paint, rubber, and petroleum products, and mining locations, and prove to be the highest occupational risk areas (11, 15). Exposure to these hazards over time significantly increases the risk of developing bladder cancer. Another factor that accounts for approximately 10% of bladder cancer

cases is urbanization and the spread of manufacturing companies to under developed countries (16). There is an increasing indication that certain individuals due to their genetic makeup and mutations in specific genes have an increased risk for developing bladder cancer. More specifically, mutations in genes that are responsible for the breakdown of carcinogens, for example N-acetyl transferases, which play a role in the activating or inactivating of amine carcinogens, and *GSTM1*-null genotypes, where *GSTM1* plays a role in detoxifying environmental carcinogens (17, 18). A previously published study determined that women who smoke cigarettes and have the *GSTM1*-null genotype have a greater increased risk of developing bladder cancer compared to non-smoking women with the *GSTM1*-null genotype (19).

As there is no approved screening method for the early detection of bladder cancer, a current focus of research is to evaluate the genetic components of bladder cancer for biomarkers correlated with the tumorigenesis of bladder cancer. These biomarkers can be used as a screen, equivalent to testing for mutations in the *BRCA1/2* genes for breast and ovarian cancers, as well as potential drug targets for the treatment of bladder cancer. To date, several genes with known mutations have been found to aid in the development and progression of bladder cancer, including activating mutations in the oncogenes *Hras* and fibroblast growth factor receptor (*FGFR3*). Mutations in the tumor suppressor genes *Tp53*, *Rb1*, and *Pten* also lead to the development and progression of many types of cancer, including bladder cancer. (20-25). The development of a screen for patients with a high risk of developing bladder cancer would prove to be extremely beneficial for early detection but the relatively low

incidence and lack of bladder cancer specific biomarkers proves to be a challenge at this time. To overcome these challenges, preclinical and clinical research has shifted in the direction of enhancing the detection methods currently used, including improving the evaluation of hematuria, cytology practices, and novel optical imaging during cystoscopy procedures.

TREATMENT FOR BLADDER CANCER

Treatment for bladder cancer is dependent on whether the cancer is non-muscle invasive bladder cancer (NMIBC) or muscle-invasive bladder cancer (MIBC) at time of initial diagnosis. Standard treatment for NMIBC is a transurethral resection of the bladder tumor (TURBT) for the visualization and resection of the tumor. TURBT procedures are followed by intravesical adjuvant therapy due to high risk of recurrence and progression after TURBT procedures. TURBT procedures serve as a diagnostic and a therapeutic tool because the procedure can potentially provide a curative outcome for low-risk patients, depending on the location and pathology of the tumor. Intravesical adjuvant therapies following a TURBT procedure include standard chemotherapy (mitomycin C or gemcitabine) or immunotherapy (Bacille Calmette-Guérin), either alone or in combination (26-28). Adjuvant therapies significantly decrease the risk of recurrence rates after the initial diagnosis when compared to no adjuvant therapy after TURBT procedures. The tumors from patients diagnosed with NMIBC are further characterized to determine the appropriate treatment regimen after TURBT procedures (29). Despite the benefits of TURBT procedures, there is a high-risk of recurrence (>50%) associated with these procedures because of incomplete tumor

resection due to several factors, including the size and location of the tumor (30). This drives the need for accurate tumor staging after the initial TURBT procedures and repeat procedures for patients with known incompletely resected tumors to increase recurrence-free survival. Nonetheless, combined TURBT procedures and adjuvant therapy is highly beneficial to patients diagnosed with early stage bladder cancer as compared to those diagnosed with late stage bladder cancer.

MIBC cases are managed with more aggressive treatment regimens. Currently, the gold-standard treatment for MIBC is an open radical cystectomy (ORC) followed by adjuvant platinum-based chemotherapy (31, 32). Due to higher complications and invasiveness of the ORC procedure, less invasive methods have been explored including a laparoscopic radical cystectomy (33). The benefits of a less invasive procedure include reduced blood loss, equivalent outcomes to invasive procedures, decreased complications during the procedures, and improved quality of life after the procedure (34-36). Regardless of how the radical cystectomy procedure is performed, a urinary diversion procedure is required to restore normal urine flow (37). Bladder preservation surgery is also an option for qualified patients followed by adjuvant therapies to significantly improve quality of life after surgery (38). These patients are considered low risk and the surgery must prove to be curative to prevent a radical cystectomy procedure (i.e. complete tumor resection with negative margins) (39).

Survival outcomes for patients diagnosed with MIBC depends on initial pathological tumor staging. Patients who undergo a radical cystectomy and have negative lymph nodes (no metastasis) have a significantly higher progression free

survival rate when compared to patients diagnosed with metastatic disease state (40). The standard chemotherapy protocol for these patients is platinum-based therapeutics in combination with other chemotherapeutic agents. These drug combinations include cisplatin, methotrexate, vinblastine, doxorubicin, and gemcitabine. Despite high rates of recurrence and low patient median survival times, this protocol is still considered to be the standard first-line treatment option for patients diagnosed with advanced stage bladder cancer.

For patients who do not respond to the standard chemotherapy regimens or their disease state is too advanced, second-line options may be available (41). Current FDA-approved second-line therapies for late stage bladder cancer include systemic immunotherapy, for example the programmed cell death ligand (PD-L1) monoclonal antibody, atezolizumab (42). Additional immunotherapies are being evaluated in preclinical and clinical studies for the treatment of metastatic bladder cancer. The role of adjuvant therapy either prior to or after a radical cystectomy procedure has greatly improved survival for patients diagnosed with NMIBC and MIBC. Resistance to standard chemotherapy regimens has increasingly become a challenge for patients diagnosed with bladder cancer.

Bladder cancer is one of the most expensive malignancies to treat due to lack of improved therapies over the past few decades and high rates of recurrence and resistance. To combat resistance, improve the standard of the care for patients, and increase patient survival, novel targeted therapies are being explored as second-line treatment options, including systemic immunotherapies and receptor tyrosine kinase

inhibitors, alone and in combination with current therapeutic options. Recently, immunotherapy has played a pivotal role in the treatment of many types of cancer due to the development and success of monoclonal antibodies, cancer vaccines, and cytokine therapies. Based on the success of immunotherapy for treatment of both hematologic cancers and solid tumors, the notion of using immunotherapy for the treatment of late-stage bladder cancer has emerged.

The most commonly studied target for immunotherapy is the PD-1/PD-L1 interaction due to the pivotal role it plays in the immune response to tumor development and the tumor microenvironment (43). The PD-1 receptor is expressed on the surface of activated T cells, and PD-L1, which is overexpressed on cancer cells, binds to the receptor to suppress the innate immune response. Cancer cells, therefore evade the innate immune response and result in tumor progression. Targeting both the PD-1 receptor and its ligand, PD-L1, to block the binding of PD-L1 to the receptor can enhance the innate immune response and result in tumor suppression. There are several clinical trials in the process of evaluating monoclonal antibodies for the treatment of late-stage bladder cancer. As mentioned above, the FDA recently approved atezolizumab, which demonstrated a high success rate for patients diagnosed with PD-L1 positive metastatic bladder cancer (44). This treatment had minimal side effects compared to standard chemotherapy. Additionally, there are two early stage clinical trials being performed to evaluate anti-PD-1 monoclonal antibodies, pembrolizumab and nivolumab. Both have demonstrated to be well-tolerated, effective at inhibiting tumor growth, and have minimal side effects but these drugs are still in the

process of being evaluated for their effectiveness in increasing the overall progression-free survival. Preliminary data indicates the one-year survival time for patients diagnosed with metastatic bladder cancer and who receive pembrolizumab, is 53% (45). These agents, and others, are still under investigation but have delivered promising results as second-line therapeutic options for patients diagnosed with metastatic bladder cancer.

In addition to the PD-1/PD-L1 immune checkpoint, another pathway of interest for immunotherapy is targeting the cytotoxic T-lymphocyte-antigen 4 (CTLA4). By targeting CTLA4, the expected outcome is to increase the immune response directed towards the tumor cells by restoring activation of T lymphocytes and, therefore, activating the innate immune response to kill cancer cells. In 2010, the FDA approved the CTLA4 monoclonal antibody, ipilimumab, as a second-line therapeutic for metastatic melanoma (46). Again, based on the results demonstrated by ipilimumab to significantly increase patient survival with metastatic melanoma, the principle was applied to metastatic bladder cancer. Ipilimumab administration prior to radical cystectomy procedures demonstrated an increased anti-tumor immune response and significantly improved patient outcomes (47). Ipilimumab, in combination with gemcitabine and cisplatin, is currently in phase II clinical trials as a first-line agent for metastatic bladder cancer. An early phase clinical trial is also under investigation for the combination of nivolumab and ipilimumab as a second-line therapeutic strategy for advanced stage bladder cancer (with or without metastases).

More recently due to predicted challenges of targeted immunotherapies, cancer vaccines have been under investigation for the treatment of advanced stage bladder cancer as second-line therapeutic agents. There are several early phase clinical trials being conducted to evaluate the safety and efficacy of bladder cancer vaccines to activate the immune system of the patients, specifically targeting bladder cancer specific tumor antigens (48). Cancer vaccines are being evaluated alone and in combination with standard therapies in patients who demonstrate a high risk for recurrence (49, 50). More research still needs to be conducted to determine the efficacy of cancer vaccines for treatment bladder cancer.

In addition to immunotherapy, another area of focus for the treatment of advanced stage bladder cancer is receptor tyrosine kinase inhibitors (RTKIs) (51). Receptor tyrosine kinases (RTKs), such as the c-Kit receptor, epidermal growth factor receptors (EGFR), fibroblast growth factor receptors (FGFR), platelet derived growth factor receptors (PDGFR), and the vascular endothelial growth factor receptors (VEGFR), are prime therapeutic targets because they are overexpressed in a variety of cancers, including bladder cancer, due to mutations that affect RTK signaling (52-54). Once activated by their specific ligands, RTKs play a key role in tumorigenesis by activating downstream, pro-survival signaling pathways that promote cell proliferation, differentiation, migration, increased angiogenesis, and anti-apoptotic cellular responses (55-58). First discovered in the 1980s and extensively studied since, RTKIs are small-molecule inhibitors that bind to the active tyrosine kinase domain of RTKs to inhibit phosphorylation and activation of downstream signaling pathways to inhibit tumor

progression (59-62). RTKIs have proven to be highly selective, have minimal side effects compared to standard cytotoxic chemotherapy, and have significant treatment responses for patients diagnosed with cancer, including breast cancer, leukemia, lung cancer, renal cell carcinoma (RCC), and gastrointestinal stromal tumors (GIST) (63-71). To date, there are more than 20 FDA-approved RTKIs for cancer treatment and many more in the pre-clinical and clinical trial stages. Based on the success of RTKIs for treating solid tumors, the efficacy of RTKIs for the treatment of advanced stage bladder cancer is currently under pre-clinical and clinical investigation (72).

Of the many genomic alterations associated with bladder cancer, there are several highlighted for effective RTKIs therapy, and clinical trials are underway. The most common pathways currently being evaluated are the epidermal growth factor receptors (EGFR), fibroblast growth factor receptors (FGFR), and phosphoinositide-3-kinase/protein kinase b (PI3K/Akt) signaling pathways (73). There are several other RTKs overexpressed in bladder cancer, but the above-mentioned pathways are the pathways currently being evaluated, and inhibitors of those targets show the most promise for potentially becoming FDA-approved second-line therapeutic options for patients.

The FGFR family of receptors has been extensively evaluated as a potential therapeutic target for advanced stage bladder cancer because of its role in many physiological processes, including tumorigenesis, angiogenesis, and drug resistance (74, 75). FGFR is highly overexpressed in advanced stage bladder cancer compared to NMIBC and is associated with a more aggressive bladder cancer phenotype (52, 76-

78). The overexpression of FGFR and its roles in tumor progression indicate that FGFR is a strong therapeutic target. Several clinical trials are being conducted to evaluate the efficacy of novel FGFR-specific RTKIs, including the small-molecule inhibitors JNJ-42756493, BGJ398, and dovitinib as second-line therapeutic options for patients with advanced metastatic bladder cancer (72, 79, 80). Preliminary results from these studies indicate effective anti-tumor responses and increased progression-free survival times for patients who received the therapy. The RTKIs demonstrated high specificity for the tumors with minimal side effects. Additional studies need to be performed due to intrinsic drug resistance discovered in patients that harbor FGFR3 mutations (81, 82). Nonetheless, RTKIs specific to the FGFR seem to be a promising therapeutic option for patients who do not respond to standard chemotherapeutic options.

The EGFR family of receptors is highly overexpressed in human cancers arising from epithelial cells, including lung and bladder cancer. This family of receptors plays a key role in regulating cell proliferation, angiogenesis, apoptosis, and metastasis (83). EGFR is commonly expressed in MIBC, and expression correlates with poor patient prognosis (84, 85). The overexpression of EGFR in bladder cancer cells makes this receptor a suitable therapeutic target for the treatment of bladder cancer. Several pre-clinical and clinical studies are being conducted to evaluate the efficacy of RTKIs that specifically target EGFR. These studies are based on the results from a previously published pre-clinical study that evaluated the efficacy of the monoclonal antibody, cetuximab, alone and in combination with chemotherapy (86). Cetuximab is FDA-approved for treating EGFR-expressing non-small-cell lung, head and neck, and

colorectal cancers. Results from this study indicated that by inhibiting EGFR, angiogenesis was decreased, and anti-tumor effects were enhanced when combined with chemotherapy. This study confirmed that EGFR is a strong target for novel bladder cancer therapies.

There are two FDA-approved RTKIs, gefitinib and erlotinib, demonstrating strong anti-tumor effects in EGFR-expressing cancers, primarily lung cancer. Both gefitinib and erlotinib are EGFR-selective inhibitors. Pre-clinical studies show gefitinib, alone and in combination with chemotherapeutic agents, has strong anti-tumor effects by inhibiting EGFR phosphorylation and downstream pro-survival Akt signaling (87). A clinical study by the Southwest Oncology Group, determined that administration of gefitinib alone to patients, who previously had been treated with chemotherapy, did not see success with this therapy (88). Progression free survival was significantly decreased, and more than half of the patients experienced tumor progression. Further clinical investigation into the efficacy of gefitinib as a second-line therapy option determined that patients, who had not received prior chemotherapy and were treated simultaneously with gefitinib and standard chemotherapy simultaneously, had an increased progression-free survival time (89). The results of this study did not demonstrate that gefitinib significantly increased anti-tumor responses compared to chemotherapy alone. The studies evaluating gefitinib as a second-line therapy option for advanced stage bladder cancer are similar to the results obtained when gefitinib was evaluated as a therapeutic agent for the treatment of lung cancer (54, 88-91). Patients may initially respond to the

therapy, but overtime there is disease progression and eventually no response to the therapy. These results demonstrate one of the major challenges of targeted therapies.

Erlotinib is an EGFR-selective RTKI that has been evaluated in many types of EGFR-expressing cancers. Erlotinib was evaluated in clinical studies as a second-line therapeutic option for patients diagnosed with advanced stage bladder cancer, but similar to the results obtained during the gefitinib studies, erlotinib does not prove to be an effective treatment option for bladder cancer patients (91, 92). In these studies, there was a poor patient response, despite high EGFR expression in the primary tumor cells. An investigation as to why the patients did not respond is currently being conducted. Poor response to EGFR-targeted therapy has been a challenge for the treatment of bladder cancer. The mechanisms of resistance are being investigated, but it may be related to the number of EGFR mutations that are found in bladder cancer (93). These results would be like those of lung cancer, and many patients do not respond to certain EGFR-targeted therapies due to specific mutations found in the tumor cells. These results drive the need for better characterization of the mechanisms of drug resistance and further enhance the need for early detection of therapy responses for improved treatment of bladder cancer.

Despite being located downstream of RTKs, the PI3K/Akt signaling pathway plays a crucial role in promoting cell proliferation, angiogenesis, anti-apoptosis, and tumor progression. This pathway is commonly overexpressed in many types of cancer, including advanced stage bladder cancer. (52). To inhibit this pathway, the current protocol is to administer mammalian target of rapamycin (mTOR) inhibitors to patients

with high PI3K/Akt mutations. mTOR is downstream of PI3K/Akt, and by inhibiting mTOR, activation of downstream pro-survival signaling pathways is inhibited, resulting in cell death. There are several mTOR inhibitors being evaluated alone and in combination with cytotoxic chemotherapy, including rapamycin and its analogs - everolimus, temsirolimus, and sirolimus, as second-line therapy options for patients with advanced stage bladder cancer. Preliminary results from these studies indicate that mTOR inhibitors, when in combination with another therapeutic agent (chemotherapy or targeted therapy), increase in patient progression-free survival (94-97). Further studies need to be conducted to evaluate mTOR inhibitors alone as a second-line therapy option due to mixed patient results (95, 96, 98). Results of the above-mentioned studies indicate that mTOR inhibitors alone may not elicit an anti-tumor response and increase patient survival superior to chemotherapy alone. There are also several early stage clinical trials being conducted, evaluating the efficacy of PI3K inhibitors for metastatic bladder cancer, including the GSK458 and buparlisib inhibitors (99).

Briefly, VEGFR is also highly overexpressed in bladder cancer and is a prime target for monoclonal antibody therapies (i.e. bevacuzimab). The RTKI sunitinib has been evaluated in patients who do not respond to standard chemotherapy. In pre-clinical studies, sunitinib has enhanced the anti-tumor effects of the chemotherapy combination, cisplatin and gemcitabine (100). When administered to patients, sunitinib, like the FGFR inhibitor dovitinib, mixed results were observed (101). When used in combination with standard chemotherapy, the side effects were not tolerable, and the combination was too toxic for patients to continue with this regimen. At best, stable

disease was achieved, but progression-free survival was not superior to chemotherapy alone (102). Pre-clinical studies are still being conducted to evaluate the efficacy of sunitinib, but clinical trials are not being continued at this time.

The current and novel therapeutic strategies described in the section above provides insight into why there has been little change in the standard of care protocols for patients diagnosed with bladder cancer. Patients diagnosed with NMIBC have a significantly greater 5-year survival rate than patients diagnosed with MIBC, because, if caught early, surgery can be a curative procedure. These patients also respond better to standard intravesical chemotherapy and immunotherapy regimens than those who are diagnosed at a later disease state. Many of the novel therapeutic strategies are focused on improving patient survival outcomes for those diagnosed with advanced stage bladder cancer. The pre-clinical studies in this dissertation evaluate RTKIs for the treatment of bladder cancer *in vitro* and *in vivo*. These studies also address some of the challenges associated with targeted therapies, including resistance, and evaluate therapeutic strategies to overcome these challenges.

DETECTION OF BLADDER CANCER

The detection of cancer through imaging has been one of the most widely studied areas in cancer research. It is well known that early detection of cancer results in significantly improved patient prognostic outcomes and curative treatment options (4). There has been a recent shift in clinical diagnostic imaging to better visualize the tumors and sites of metastasis. Traditional FDA-approved imaging modalities for cancer detection include computed x-ray tomography (CT), magnetic resonance imaging (MRI),

ultrasound (US), and more recently positron emission tomography (PET) (103-106). These imaging modalities have changed the way cancer is detected and have significantly improved patient outcomes. They are still widely used throughout clinical settings and demonstrate continued success. Researchers and clinicians combined have demanded more information to be extracted from the obtained images and overcome limitations of traditional modalities which drives the need for improved molecular imaging modalities. There are several goals of molecular imaging research that are being investigated including the development of molecular biomarkers that can aid in the early detection of cancers, the ability to evaluate the early responses to therapy, and the development of imaging tools to evaluate tissues microscopically in real-time, not just macroscopically (106-108). The need for early detection of bladder cancer falls in line with these goals. Current imaging modalities have several limitations for the detection of the early stages of bladder cancer and therefore drives the need for improved optical imaging modalities. Those limitations and promising novel optical imaging modalities are further discussed in this section as well as the following *in vitro* and *in vivo* studies of this dissertation.

The initial process for evaluating patients presenting with bladder cancer symptoms is by performing an optical imaging procedure known as a cystoscopy (109). This procedure allows for the visualization of the inner surface layer of the bladder to determine if any abnormal lesions are present. White light cystoscopy (WLC) is the current standard of care for detecting papillary lesions or large growths. WLC has been used for several decades to detect and remove bladder tumors, but there are several

limitations associated with WLC, including the inability to detect early-stage bladder cancer (carcinoma in situ and low-grade tumors), as well as the inability to detect tumor margins during resection procedures, leading to incomplete resection of the tumor (110). If abnormal lesions are found during the procedure, histological evaluation is required to confirm the diagnosis of bladder cancer and determine the stage of the tumor. A urine cytology can also be performed along with the cystoscopy procedure to detect any missed cancer cells as the early stages of bladder cancer (flat lesions and carcinoma in situ) are often missed. As previously mentioned, there are currently no bladder cancer biomarkers present in urine that have demonstrated the necessary success rates for approval in clinical diagnostic use (111-113).

WLC is the gold standard for initial bladder cancer detection, but there are several other imaging modalities that are currently used, including CT, MRI, and PET imaging (114). These imaging modalities are most commonly used to aid in the detection of advanced stage bladder cancer as there are several limitations for these modalities to be used for early stage bladder cancer detection. CT or MRI scans are necessary for patients diagnosed with advanced stage bladder cancer to determine the infiltration of the tumor through the layers of the bladder and if there are any sites of metastasis. These scans, along with histopathology, will aid in determining the stage of the bladder tumors. CT scans are relatively inexpensive, easy to obtain, and can detect advanced stage tumors. There are several limitations associated with CT, including exposure of patients to ionizing radiation, lack of ability to determine lymph node involvement, large range of specificity, low resolution to differentiate between the

different layers of the bladder, and cannot detect flat or CIS bladder tumors (115-119). MRI scans can be used for the detecting and staging advanced bladder cancer. This method demonstrates high sensitivity and specificity, while not exposing patients to ionizing radiation. MRI can also be used to improve the accuracy of tumor staging after a cystoscopy or TURBT procedure and proves to be superior to CT scans in differentiating between the early stages of NMIBC (120, 121). Despite these improvements in detecting bladder cancer, there are several limitations of MRI as an imaging modality. These limitations include high cost for patients, reliance on normal functioning kidneys due to the contrast agent used (gadolinium), and limited ability to determine the extent of the primary tumor invasion through the layers of the bladder (122).

An emerging imaging modality commonly used for metastasis detection is PET scans. This imaging technique relies on the administration of a contrast agent, fluorodeoxyglucose (FDG), which takes advantage of one of the hallmarks of cancer, increased glycolysis activity within the cancer cells or the Warburg effect. Unfortunately, FDG is excreted through the urinary system and therefore is not a good contrast agent for the detection of tumors in the bladder. Therefore, FDG-PET scans can be used for the detection of metastatic lesions throughout the body and a review of previous clinical studies demonstrate the success of detecting metastatic bladder cancer in patients using FDG-PET scans (123). FDG-PET scans also prove to be superior at detecting lymph node involvement and metastatic lesions before and after therapy when compared to MRI or CT scans (124-126). The improved detection of advanced stage

bladder cancer has significantly improved patient prognostic outcomes by extending the duration of progression-free survival times. A significant limitation of FDG-PET scans is the inability to detect primary bladder tumors.

Conventional optical imaging technologies can detect advanced stages of bladder cancer; however, they are limited in detecting bladder cancer at the early stages. Newer imaging technologies have been developed to prevent disease recurrence and progression (127, 128). There are four primary novel optical imaging technologies that have recently been investigated for clinical diagnostic use: fluorescence cystoscopy/photodynamic diagnosis (PDD), narrow band imaging (NBI), confocal laser endomicroscopy (CLE), and optical coherence tomography (OCT) (129, 130). Fluorescence cystoscopy/PDD and NBI are used as diagnostic tools to better visualize the tumors and optimize detection of early stage bladder cancer lesions. On the contrary, CLE and OCT are used to characterize the detected lesions to improve accuracy in determining the grade and stage of the lesions.

Fluorescent cystoscopy requires the administration of a contrast agent, which selectively binds to the cancer cells. Photodynamic diagnosis/blue-light cystoscopy (BLC) is an FDA-approved procedure, which requires the administration of 5-aminolevulinic acid (5-ALA) dye directly into the bladder (131, 132). The dye is absorbed by the cancerous tissue and emits a red color under blue light to better visualize the tumor during cystoscopy. This method is currently being used during TURBT procedures and for detecting early-stage bladder cancer. Previous studies suggest BLC can detect more bladder tumors than WLC, both early and late stage

tumors (132-136). There are several advantages to fluorescent cystoscopy, including better visualization and differentiation of the cancer tissue from normal urothelium, detection of early stage bladder cancer, and tumor margins can be easily visualized during resection procedures (137). This technique improves diagnosis and decreases recurrence rates after TURBT procedures. There are some inconsistencies with high-false positive rates and high equipment costs. This drives the need for improved optical imaging modalities for detecting bladder cancer.

Narrow band imaging (NBI) is a novel endoscopy imaging modality that has been FDA-approved for clinical diagnostic use in the United States. This method of imaging does not require the administration of a contrast agent prior to imaging and relies on filters specialized for the blue and green light spectrum wavelengths (415 nm and 540 nm, respectively) that are absorbed by hemoglobin. Based on this principle, during cystoscopy procedures enough contrast is provided to visualize increased blood vessel formation indicative of bladder cancer development. This non-invasive method has significantly improved NMIBC detection compared to WLC alone (138). There have been a few clinical studies evaluating the effectiveness of NBI for detecting bladder cancer, but the results of those studies confirm NBI is more effective than WLC alone for detecting NMIBC (139-141). Consistent with fluorescent cystoscopy, it can be inferred that better detection of initial tumor sites can result in more effective treatment and decreased recurrence rates of bladder cancer. Consistent with all endoscopic techniques, NBI is not capable of determining tumor grade and stage during the

procedure. Histopathology analysis is required for further evaluation and treatment options.

To have the ability detect and properly stage bladder tumors during the diagnostic process, microscopic imaging modalities, such as CLE and OCT, are required. Both modalities are in the early stages of investigation and have not been approved by the FDA for clinical diagnostic use yet. These modalities allow for real-time histopathology information to be collected and evaluated during the diagnostic cystoscopy procedures. CLE has previously been evaluated and demonstrated a high success rate for the detection of gastrointestinal tumors. During cystoscopy procedures when CLE is performed, the administration of a fluorescent contrast agent is required. The administration of a contrast agent allows for microscopic visualization of the tissue being examined. Due to high resolution and depth of imaging penetration, CLE allows for morphology identification of individual cells in real-time, which can be used to determine the grade (high or low) of the bladder tumors during the actual diagnostic procedure (142). There are several limitations of CLE, including CLE does not visualize flat or CIS lesions superior to WLC, and due to limited field of view during the procedure, this modality is time consuming and does not offer deep tissue penetration. Further studies are being conducted to merge different fields of view for analysis at one time.

Another optical imaging modality under investigation for the detection of bladder cancer is OCT, which, like CLE, allows for real-time microscopic imaging of the bladder during cystoscopy procedures. This method is comparable to ultrasound and relies on

the signal produced by light waves in the near-infrared spectrum to form an image of the bladder tissue. Like CLE, OCT demonstrates high resolution and tissue penetration for the detection of bladder cancer. OCT can differentiate between normal urothelium and cancerous tissue, but unlike CLE, OCT cannot determine the grade of tumors detected. OCT can determine how far the tumor has infiltrated into the bladder, which is known as the stage of the tumor. It is important to distinguish between NMIBC and MIBC because the course of treatment is dependent on the diagnosis of NMIBC or MIBC. Recent studies evaluating OCT for the detection of bladder cancer have demonstrated high sensitivity and specificity, indicating success of this modality (143, 144). OCT also have several limitations, including the difficulty to image the whole bladder. A recent study demonstrated that OCT, in combination with PDD, resulted in higher sensitivity and specificity than OCT alone (145). These results indicate that currently fluorescent cystoscopy and NBI are more superior to WLC alone. Further evaluation needs to be conducted to determine the efficacy of CLE and OCT as clinical diagnostic tools for the detection of bladder cancer, either alone or in combination with current detection modalities.

As described above, the limitations of conventional optical imaging modalities have driven the need for the development of novel imaging modalities for the detection of early and late stage bladder cancer. An area of advancement in imaging is the evaluation of cancer-specific biomarkers for the detection of early stage cancer and determining complete tumor location for improved resection procedures. A biomarker of interest for the detection and treatment of bladder cancer is cyclooxygenase-2 (COX-2).

COX-2 converts arachidonic acid to prostaglandins and is highly inducible during periods of inflammation and cancer, including bladder cancer. COX-2 is not expressed in normal tissues, making it a suitable biomarker for COX-2-selective imaging and therapeutic agents. It is also one of the key proteins responsible for increased angiogenesis and tumorigenesis (146-149). Previous studies have demonstrated that increased prostaglandins production positively correlates with COX-2 expression levels and the progression of cancer (150-153). High COX-2 expression in bladder cancer is also indicative of poor patient prognostic outcomes (154). To inhibit COX-2 expression, non-steroidal anti-inflammatory drugs (NSAIDs) are commonly used for the prevention and treatment of cancer (155-163). A side effect of long-term NSAIDs use is gastrointestinal toxicity (164). To reduce toxicity, COX-2 selective inhibitors (COXIBs), such as celecoxib and rofecoxib, have been developed (165, 166). These selective inhibitors have demonstrated success in effectively reducing the side effects in patients, while still effectively inhibiting COX-2 (160, 167-169). Due to increased heart attacks and strokes in patients with prolonged COX-2 selective inhibitors use, celecoxib remains the only COX-2 selective inhibitor on the market (170). These side effects further drive the need for improved novel therapies for patients with chronic inflammation and cancer.

The overexpression of COX-2 in bladder cancer tissues can therefore be used as a biomarker for detecting bladder cancer. Fluorescently-labeled COX-2 inhibitors are suitable candidates for targeted optical imaging due to their highly selective uptake by cancer cells. Fluorocoxib A is a rhodamine-conjugated analog of indomethacin that

selectively targets COX-2 in solid tumors (171). This imaging agent selectively targets the COX-2 enzyme, and when bound to the active site of COX-2, fluorescence is emitted. Fluorocoxib A has been previously validated for the detection of LPS-induced inflammation in a rat model (171) and in COX-2-expressing cancers *in vitro* and *in vivo* (172-174). A previous study conducted by Cekanova et al., demonstrated that fluorocoxib A can specifically detect COX-2-expressing bladder cancer in dogs, and there is no uptake by the normal bladder urothelium (172). Fluorocoxib A has demonstrated high specificity and sensitivity for the detection of COX-2 expressing inflammation and cancer with little to no side effects *in vivo*. These pre-clinical results indicate that fluorocoxib A can be used for the detection and monitoring the responses to therapy in COX-2-expressing bladder cancer. The following studies in this dissertation evaluate fluorocoxib A as a novel optical imaging agent for the detection of early and late stage bladder cancer, as well as monitoring the responses to targeted therapies *in vivo* using two established bladder cancer mouse models.

REFERENCES

1. N. C. Institute. (2016).
2. N. C. Institute. (Bethesda, MD, 2018), vol. 2018.
3. S. Antoni *et al.*, Bladder Cancer Incidence and Mortality: A Global Overview and Recent Trends. *Eur Urol* **71**, 96-108 (2017).
4. J. Ferlay *et al.*, Cancer incidence and mortality worldwide: sources, methods and major patterns in GLOBOCAN 2012. *Int J Cancer* **136**, E359-386 (2015).
5. D. Ramirez *et al.*, Microscopic haematuria at time of diagnosis is associated with lower disease stage in patients with newly diagnosed bladder cancer. *BJU Int* **117**, 783-786 (2016).
6. K. Elias, R. S. Svatek, S. Gupta, R. Ho, Y. Lotan, High-risk patients with hematuria are not evaluated according to guideline recommendations. *Cancer* **116**, 2954-2959 (2010).
7. A. C. Society, K. Eidsmoe, Ed. (2016), vol. 2018.
8. M. E. Charlton, M. P. Adamo, L. Sun, S. Deorah, Bladder cancer collaborative stage variables and their data quality, usage, and clinical implications: a review of SEER data, 2004-2010. *Cancer* **120 Suppl 23**, 3815-3825 (2014).
9. M. Burger *et al.*, Epidemiology and risk factors of urothelial bladder cancer. *Eur Urol* **63**, 234-241 (2013).
10. A. B. Smith *et al.*, Muscle-invasive bladder cancer: evaluating treatment and survival in the National Cancer Data Base. *BJU Int* **114**, 719-726 (2014).

11. N. D. Smith *et al.*, Bladder Cancer Mortality in the United States: A Geographic and Temporal Analysis of Socioeconomic and Environmental Factors. *J Urol* **195**, 290-296 (2016).
12. S. H. Moolgavkar, R. G. Stevens, Smoking and cancers of bladder and pancreas: risks and temporal trends. *J Natl Cancer Inst* **67**, 15-23 (1981).
13. F. Cantiello *et al.*, Association between metabolic syndrome, obesity, diabetes mellitus and oncological outcomes of bladder cancer: a systematic review. *Int J Urol* **22**, 22-32 (2015).
14. C. Pelucchi, C. La Vecchia, Alcohol, coffee, and bladder cancer risk: a review of epidemiological studies. *Eur J Cancer Prev* **18**, 62-68 (2009).
15. M. G. Cumberbatch, B. Windsor-Shellard, J. W. Catto, The contemporary landscape of occupational bladder cancer within the United Kingdom: a meta-analysis of risks over the last 80 years. *BJU Int* **119**, 100-109 (2017).
16. M. P. Purdue, S. J. Hutchings, L. Rushton, D. T. Silverman, The proportion of cancer attributable to occupational exposures. *Ann Epidemiol* **25**, 188-192 (2015).
17. Y. An *et al.*, Meta-analysis of the relationship between slow acetylation of N-acetyl transferase 2 and the risk of bladder cancer. *Genet Mol Res* **14**, 16896-16904 (2015).
18. J. Lin *et al.*, Dietary intake of vegetables and fruits and the modification effects of GSTM1 and NAT2 genotypes on bladder cancer risk. *Cancer Epidemiol Biomarkers Prev* **18**, 2090-2097 (2009).

19. D. S. Yee, N. M. Ishill, W. T. Lowrance, H. W. Herr, E. B. Elkin, Ethnic differences in bladder cancer survival. *Urology* **78**, 544-549 (2011).
20. N. H. Chow *et al.*, Papillary urothelial hyperplasia is a clonal precursor to papillary transitional cell bladder cancer. *Int J Cancer* **89**, 514-518 (2000).
21. J. Gao *et al.*, p53 deficiency provokes urothelial proliferation and synergizes with activated Ha-ras in promoting urothelial tumorigenesis. *Oncogene* **23**, 687-696 (2004).
22. L. Mo *et al.*, Hyperactivation of Ha-ras oncogene, but not Ink4a/Arf deficiency, triggers bladder tumorigenesis. *J Clin Invest* **117**, 314-325 (2007).
23. E. C. Obermann *et al.*, Frequent genetic alterations in flat urothelial hyperplasias and concomitant papillary bladder cancer as detected by CGH, LOH, and FISH analyses. *J Pathol* **199**, 50-57 (2003).
24. A. M. Puzio-Kuter *et al.*, Inactivation of p53 and Pten promotes invasive bladder cancer. *Genes Dev* **23**, 675-680 (2009).
25. Z. T. Zhang, J. Pak, E. Shapiro, T. T. Sun, X. R. Wu, Urothelium-specific expression of an oncogene in transgenic mice induced the formation of carcinoma in situ and invasive transitional cell carcinoma. *Cancer Res* **59**, 3512-3517 (1999).
26. R. J. Sylvester, M. A. van der, D. L. Lamm, Intravesical bacillus Calmette-Guerin reduces the risk of progression in patients with superficial bladder cancer: a meta-analysis of the published results of randomized clinical trials. *J Urol* **168**, 1964-1970 (2002).

27. R. Colombo *et al.*, A new approach using local combined microwave hyperthermia and chemotherapy in superficial transitional bladder carcinoma treatment. *J Urol* **153**, 959-963 (1995).
28. M. D. Shelley, A. Cleves, T. J. Wilt, M. D. Mason, Gemcitabine chemotherapy for the treatment of metastatic bladder carcinoma. *BJU Int* **108**, 168-179 (2011).
29. S. S. Chang *et al.*, Diagnosis and Treatment of Non-Muscle Invasive Bladder Cancer: AUA/SUO Guideline. *J Urol* **196**, 1021-1029 (2016).
30. H. W. Herr, Role of Repeat Resection in Non-Muscle-Invasive Bladder Cancer. *J Natl Compr Canc Netw* **13**, 1041-1046 (2015).
31. C. Advanced Bladder Cancer Meta-analysis, Neoadjuvant chemotherapy in invasive bladder cancer: a systematic review and meta-analysis. *Lancet* **361**, 1927-1934 (2003).
32. H. B. Grossman *et al.*, Neoadjuvant chemotherapy plus cystectomy compared with cystectomy alone for locally advanced bladder cancer. *N Engl J Med* **349**, 859-866 (2003).
33. I. S. Gill *et al.*, Laparoscopic radical cystectomy and continent orthotopic ileal neobladder performed completely intracorporeally: the initial experience. *J Urol* **168**, 13-18 (2002).
34. G. Novara *et al.*, Systematic review and cumulative analysis of perioperative outcomes and complications after robot-assisted radical cystectomy. *Eur Urol* **67**, 376-401 (2015).

35. B. Yuh *et al.*, Systematic review and cumulative analysis of oncologic and functional outcomes after robot-assisted radical cystectomy. *Eur Urol* **67**, 402-422 (2015).
36. B. E. Yuh, N. Ruel, T. G. Wilson, N. Vogelzang, S. K. Pal, Pooled analysis of clinical outcomes with neoadjuvant cisplatin and gemcitabine chemotherapy for muscle invasive bladder cancer. *J Urol* **189**, 1682-1686 (2013).
37. R. K. Lee *et al.*, Urinary diversion after radical cystectomy for bladder cancer: options, patient selection, and outcomes. *BJU Int* **113**, 11-23 (2014).
38. N. D. James *et al.*, Radiotherapy with or without chemotherapy in muscle-invasive bladder cancer. *N Engl J Med* **366**, 1477-1488 (2012).
39. E. Solsona *et al.*, Feasibility of radical transurethral resection as monotherapy for selected patients with muscle invasive bladder cancer. *J Urol* **184**, 475-480 (2010).
40. J. N. Eble, R. H. Young, Carcinoma of the urinary bladder: a review of its diverse morphology. *Semin Diagn Pathol* **14**, 98-108 (1997).
41. N. S. Alimohamed, S. S. Sridhar, Options in metastatic urothelial cancer after first-line therapy. *Curr Opin Support Palliat Care* **9**, 255-260 (2015).
42. N. M. Donin *et al.*, Immunotherapy for the Treatment of Urothelial Carcinoma. *J Urol* **197**, 14-22 (2017).
43. C. Robert, J. C. Soria, A. M. Eggermont, Drug of the year: programmed death-1 receptor/programmed death-1 ligand-1 receptor monoclonal antibodies. *Eur J Cancer* **49**, 2968-2971 (2013).

44. T. Powles *et al.*, MPDL3280A (anti-PD-L1) treatment leads to clinical activity in metastatic bladder cancer. *Nature* **515**, 558-562 (2014).
45. J. Bellmunt *et al.*, Pembrolizumab as Second-Line Therapy for Advanced Urothelial Carcinoma. *N Engl J Med* **376**, 1015-1026 (2017).
46. F. S. Hodi *et al.*, Improved survival with ipilimumab in patients with metastatic melanoma. *N Engl J Med* **363**, 711-723 (2010).
47. C. I. Liakou *et al.*, CTLA-4 blockade increases IFN γ -producing CD4⁺ICOS^{hi} cells to shift the ratio of effector to regulatory T cells in cancer patients. *Proc Natl Acad Sci U S A* **105**, 14987-14992 (2008).
48. J. W. Kim, Y. Tomita, J. Trepel, A. B. Apolo, Emerging immunotherapies for bladder cancer. *Curr Opin Oncol* **27**, 191-200 (2015).
49. M. Bilusic, J. L. Gulley, Endpoints, patient selection, and biomarkers in the design of clinical trials for cancer vaccines. *Cancer Immunol Immunother* **61**, 109-117 (2012).
50. M. Bilusic, R. A. Madan, Therapeutic cancer vaccines: the latest advancement in targeted therapy. *Am J Ther* **19**, e172-181 (2012).
51. E. Seront, J. P. Machiels, Molecular biology and targeted therapies for urothelial carcinoma. *Cancer Treat Rev* **41**, 341-353 (2015).
52. N. Cancer Genome Atlas Research, Comprehensive molecular characterization of urothelial bladder carcinoma. *Nature* **507**, 315-322 (2014).
53. J. P. Crew, Vascular endothelial growth factor: an important angiogenic mediator in bladder cancer. *Eur Urol* **35**, 2-8 (1999).

54. Y. N. Wong *et al.*, Phase II trial of cetuximab with or without paclitaxel in patients with advanced urothelial tract carcinoma. *J Clin Oncol* **30**, 3545-3551 (2012).
55. S. R. Hubbard, W. T. Miller, Receptor tyrosine kinases: mechanisms of activation and signaling. *Curr Opin Cell Biol* **19**, 117-123 (2007).
56. M. A. Lemmon, J. Schlessinger, Cell signaling by receptor tyrosine kinases. *Cell* **141**, 1117-1134 (2010).
57. E. Li, K. Hristova, Role of receptor tyrosine kinase transmembrane domains in cell signaling and human pathologies. *Biochemistry* **45**, 6241-6251 (2006).
58. A. Ullrich, J. Schlessinger, Signal transduction by receptors with tyrosine kinase activity. *Cell* **61**, 203-212 (1990).
59. A. Arora, E. M. Scholar, Role of tyrosine kinase inhibitors in cancer therapy. *J Pharmacol Exp Ther* **315**, 971-979 (2005).
60. D. Fabbro, D. Parkinson, A. Matter, Protein tyrosine kinase inhibitors: new treatment modalities? *Curr Opin Pharmacol* **2**, 374-381 (2002).
61. Q. Jiao *et al.*, Advances in studies of tyrosine kinase inhibitors and their acquired resistance. *Mol Cancer* **17**, 36 (2018).
62. P. Yaish, A. Gazit, C. Gilon, A. Levitzki, Blocking of EGF-dependent cell proliferation by EGF receptor kinase inhibitors. *Science* **242**, 933-935 (1988).
63. M. Burotto, E. E. Manasanch, J. Wilkerson, T. Fojo, Gefitinib and erlotinib in metastatic non-small cell lung cancer: a meta-analysis of toxicity and efficacy of randomized clinical trials. *Oncologist* **20**, 400-410 (2015).

64. A. L. Cheng *et al.*, Sunitinib versus sorafenib in advanced hepatocellular cancer: results of a randomized phase III trial. *J Clin Oncol* **31**, 4067-4075 (2013).
65. N. De Silva *et al.*, Molecular effects of Lapatinib in the treatment of HER2 overexpressing oesophago-gastric adenocarcinoma. *Br J Cancer* **113**, 1305-1312 (2015).
66. I. C. Haznedaroglu, Monitoring the Response to Tyrosine Kinase Inhibitor (TKI) Treatment in Chronic Myeloid Leukemia (CML). *Mediterr J Hematol Infect Dis* **6**, e2014009 (2014).
67. P. J. Johnson *et al.*, Brivanib versus sorafenib as first-line therapy in patients with unresectable, advanced hepatocellular carcinoma: results from the randomized phase III BRISK-FL study. *J Clin Oncol* **31**, 3517-3524 (2013).
68. E. A. Kuczynski, C. R. Lee, S. Man, E. Chen, R. S. Kerbel, Effects of Sorafenib Dose on Acquired Reversible Resistance and Toxicity in Hepatocellular Carcinoma. *Cancer Res* **75**, 2510-2519 (2015).
69. L. Pusztai *et al.*, Gene signature-guided dasatinib therapy in metastatic breast cancer. *Clin Cancer Res* **20**, 5265-5271 (2014).
70. S. H. Tirumani *et al.*, Imatinib and beyond in gastrointestinal stromal tumors: A radiologist's perspective. *AJR Am J Roentgenol* **201**, 801-810 (2013).
71. C. Wertheimer *et al.*, EGFR inhibitor Gefitinib attenuates posterior capsule opacification in vitro and in the ex vivo human capsular bag model. *Graefes Arch Clin Exp Ophthalmol* **253**, 409-417 (2015).

72. P. J. Cheetham, D. P. Petrylak, New Agents for the Treatment of Advanced Bladder Cancer. *Oncology (Williston Park)* **30**, 571-579, 588 (2016).
73. V. Vashistha, D. I. Quinn, T. B. Dorff, S. Daneshmand, Current and recent clinical trials for perioperative systemic therapy for muscle invasive bladder cancer: a systematic review. *BMC Cancer* **14**, 966 (2014).
74. E. di Martino, D. C. Tomlinson, M. A. Knowles, A Decade of FGF Receptor Research in Bladder Cancer: Past, Present, and Future Challenges. *Adv Urol* **2012**, 429213 (2012).
75. M. V. Dieci, M. Arnedos, F. Andre, J. C. Soria, Fibroblast growth factor receptor inhibitors as a cancer treatment: from a biologic rationale to medical perspectives. *Cancer Discov* **3**, 264-279 (2013).
76. G. Iyer *et al.*, Prevalence and co-occurrence of actionable genomic alterations in high-grade bladder cancer. *J Clin Oncol* **31**, 3133-3140 (2013).
77. G. Iyer, M. I. Milowsky, Fibroblast growth factor receptor-3 in urothelial tumorigenesis. *Urol Oncol* **31**, 303-311 (2013).
78. J. Qing *et al.*, Antibody-based targeting of FGFR3 in bladder carcinoma and t(4;14)-positive multiple myeloma in mice. *J Clin Invest* **119**, 1216-1229 (2009).
79. M. I. Milowsky *et al.*, Phase 2 trial of dovitinib in patients with progressive FGFR3-mutated or FGFR3 wild-type advanced urothelial carcinoma. *Eur J Cancer* **50**, 3145-3152 (2014).
80. M. Rouanne, Y. Loriot, T. Lebreton, J. C. Soria, Novel therapeutic targets in advanced urothelial carcinoma. *Crit Rev Oncol Hematol* **98**, 106-115 (2016).

81. J. Bellmunt *et al.*, Identification of ALK gene alterations in urothelial carcinoma. *PLoS One* **9**, e103325 (2014).
82. E. A. Guancial *et al.*, FGFR3 expression in primary and metastatic urothelial carcinoma of the bladder. *Cancer Med* **3**, 835-844 (2014).
83. J. Bellmunt, M. Hussain, C. P. Dinney, Novel approaches with targeted therapies in bladder cancer. Therapy of bladder cancer by blockade of the epidermal growth factor receptor family. *Crit Rev Oncol Hematol* **46 Suppl**, S85-104 (2003).
84. P. Bue *et al.*, Expression of epidermal growth factor receptor in urinary bladder cancer metastases. *Int J Cancer* **76**, 189-193 (1998).
85. P. Lipponen, M. Eskelinen, Expression of epidermal growth factor receptor in bladder cancer as related to established prognostic factors, oncoprotein (c-erbB-2, p53) expression and long-term prognosis. *Br J Cancer* **69**, 1120-1125 (1994).
86. K. Inoue *et al.*, Paclitaxel enhances the effects of the anti-epidermal growth factor receptor monoclonal antibody ImClone C225 in mice with metastatic human bladder transitional cell carcinoma. *Clin Cancer Res* **6**, 4874-4884 (2000).
87. S. Rebouissou *et al.*, EGFR as a potential therapeutic target for a subset of muscle-invasive bladder cancers presenting a basal-like phenotype. *Sci Transl Med* **6**, 244ra291 (2014).
88. D. P. Petrylak *et al.*, Results of the Southwest Oncology Group phase II evaluation (study S0031) of ZD1839 for advanced transitional cell carcinoma of the urothelium. *BJU Int* **105**, 317-321 (2010).

89. G. K. Philips *et al.*, A phase II trial of cisplatin (C), gemcitabine (G) and gefitinib for advanced urothelial tract carcinoma: results of Cancer and Leukemia Group B (CALGB) 90102. *Ann Oncol* **20**, 1074-1079 (2009).
90. M. Hussain *et al.*, A randomized phase 2 trial of gemcitabine/cisplatin with or without cetuximab in patients with advanced urothelial carcinoma. *Cancer* **120**, 2684-2693 (2014).
91. R. S. Pruthi *et al.*, A phase II trial of neoadjuvant erlotinib in patients with muscle-invasive bladder cancer undergoing radical cystectomy: clinical and pathological results. *BJU Int* **106**, 349-354 (2010).
92. R. E. Strowd, 3rd, M. A. Knovich, G. J. Lesser, The therapeutic management of bleeding and thrombotic disorders complicating CNS malignancies. *Curr Treat Options Oncol* **13**, 451-464 (2012).
93. A. Chaux *et al.*, High epidermal growth factor receptor immunohistochemical expression in urothelial carcinoma of the bladder is not associated with EGFR mutations in exons 19 and 21: a study using formalin-fixed, paraffin-embedded archival tissues. *Hum Pathol* **43**, 1590-1595 (2012).
94. G. Iyer *et al.*, Genome sequencing identifies a basis for everolimus sensitivity. *Science* **338**, 221 (2012).
95. M. I. Milowsky *et al.*, Phase II study of everolimus in metastatic urothelial cancer. *BJU Int* **112**, 462-470 (2013).

96. E. Seront *et al.*, Phase II study of everolimus in patients with locally advanced or metastatic transitional cell carcinoma of the urothelial tract: clinical activity, molecular response, and biomarkers. *Ann Oncol* **23**, 2663-2670 (2012).
97. N. Wagle *et al.*, Activating mTOR mutations in a patient with an extraordinary response on a phase I trial of everolimus and pazopanib. *Cancer Discov* **4**, 546-553 (2014).
98. N. Houede, P. Pourquier, Targeting the genetic alterations of the PI3K-AKT-mTOR pathway: its potential use in the treatment of bladder cancers. *Pharmacol Ther* **145**, 1-18 (2015).
99. R. Aggarwal, J. Grabowsky, N. Strait, A. Cockerill, P. Munster, Impact of patient ethnicity on the metabolic and immunologic effects of PI3K-mTOR pathway inhibition in patients with solid tumor malignancies. *Cancer Chemother Pharmacol* **74**, 359-365 (2014).
100. G. Sonpavde *et al.*, Sunitinib malate is active against human urothelial carcinoma and enhances the activity of cisplatin in a preclinical model. *Urol Oncol* **27**, 391-399 (2009).
101. D. J. Gallagher *et al.*, Phase II study of sunitinib in patients with metastatic urothelial cancer. *J Clin Oncol* **28**, 1373-1379 (2010).
102. P. D. Grivas *et al.*, Double-blind, randomized, phase 2 trial of maintenance sunitinib versus placebo after response to chemotherapy in patients with advanced urothelial carcinoma. *Cancer* **120**, 692-701 (2014).
103. R. Etzioni *et al.*, The case for early detection. *Nat Rev Cancer* **3**, 243-252 (2003).

104. F. A. Jaffer, R. Weissleder, Molecular imaging in the clinical arena. *JAMA* **293**, 855-862 (2005).
105. M. E. Juweid, B. D. Cheson, Positron-emission tomography and assessment of cancer therapy. *N Engl J Med* **354**, 496-507 (2006).
106. R. Weissleder, Molecular imaging in cancer. *Science* **312**, 1168-1171 (2006).
107. R. Weissleder, Scaling down imaging: molecular mapping of cancer in mice. *Nat Rev Cancer* **2**, 11-18 (2002).
108. R. Weissleder, M. Nahrendorf, Advancing biomedical imaging. *Proc Natl Acad Sci U S A* **112**, 14424-14428 (2015).
109. D. S. Aaronson *et al.*, Meta-analysis: does lidocaine gel before flexible cystoscopy provide pain relief? *BJU Int* **104**, 506-509; discussion 509-510 (2009).
110. P. Jichlinski, H. J. Leisinger, Fluorescence cystoscopy in the management of bladder cancer: a help for the urologist! *Urol Int* **74**, 97-101 (2005).
111. M. Babjuk *et al.*, EAU Guidelines on Non-Muscle-invasive Urothelial Carcinoma of the Bladder: Update 2016. *Eur Urol* **71**, 447-461 (2017).
112. N. M. Passoni *et al.*, Concordance in Biomarker Status Between Bladder Tumors at Time of Transurethral Resection and Subsequent Radical Cystectomy: Results of a 5-year Prospective Study. *Bladder Cancer* **2**, 91-99 (2016).
113. M. N. van der Aa *et al.*, Cystoscopy revisited as the gold standard for detecting bladder cancer recurrence: diagnostic review bias in the randomized, prospective CEFUB trial. *J Urol* **183**, 76-80 (2010).

114. M. H. Bagheri *et al.*, Advances in medical imaging for the diagnosis and management of common genitourinary cancers. *Urol Oncol* **35**, 473-491 (2017).
115. S. Hilton, L. P. Jones, Recent advances in imaging cancer of the kidney and urinary tract. *Surg Oncol Clin N Am* **23**, 863-910 (2014).
116. C. M. Huguenot, V. Duddalwar, S. Daneshmand, Preoperative Imaging for Clinical Staging Prior to Radical Cystectomy. *Curr Urol Rep* **17**, 62 (2016).
117. S. Rais-Bahrami, J. A. Pietryga, J. W. Nix, Contemporary role of advanced imaging for bladder cancer staging. *Urol Oncol* **34**, 124-133 (2016).
118. I. A. Sternberg *et al.*, Upper tract imaging surveillance is not effective in diagnosing upper tract recurrence in patients followed for nonmuscle invasive bladder cancer. *J Urol* **190**, 1187-1191 (2013).
119. L. J. Wang *et al.*, Multidetector computerized tomography urography is more accurate than excretory urography for diagnosing transitional cell carcinoma of the upper urinary tract in adults with hematuria. *J Urol* **183**, 48-55 (2010).
120. S. F. Shariat *et al.*, Discrepancy between clinical and pathologic stage: impact on prognosis after radical cystectomy. *Eur Urol* **51**, 137-149; discussion 149-151 (2007).
121. S. Verma *et al.*, Urinary bladder cancer: role of MR imaging. *Radiographics* **32**, 371-387 (2012).
122. B. D. Niederhauser, A. Kawashima, B. F. King, N. Takahashi, Utility of gadolinium-enhanced MR urography in detection of bladder carcinoma. *Eur J Radiol* **82**, 472-477 (2013).

123. Y. Y. Lu *et al.*, Clinical value of FDG PET or PET/CT in urinary bladder cancer: a systemic review and meta-analysis. *Eur J Radiol* **81**, 2411-2416 (2012).
124. P. Giannatempo *et al.*, Interim fluorine-18 fluorodeoxyglucose positron emission tomography for early metabolic assessment of therapeutic response to chemotherapy for metastatic transitional cell carcinoma. *Clin Genitourin Cancer* **12**, 433-439 (2014).
125. A. S. Kibel *et al.*, Prospective study of [18F]fluorodeoxyglucose positron emission tomography/computed tomography for staging of muscle-invasive bladder carcinoma. *J Clin Oncol* **27**, 4314-4320 (2009).
126. H. Ozturk, Comparing RECIST with EORTC criteria in metastatic bladder cancer. *J Cancer Res Clin Oncol* **142**, 187-194 (2016).
127. M. A. D'Hallewin, L. Bezdetnaya, F. Guillemin, Fluorescence detection of bladder cancer: a review. *Eur Urol* **42**, 417-425 (2002).
128. D. Jocham, H. Stepp, R. Waidelich, Photodynamic diagnosis in urology: state-of-the-art. *Eur Urol* **53**, 1138-1148 (2008).
129. A. Kolodziej, W. Krajewski, M. Matuszewski, K. Tupikowski, Review of current optical diagnostic techniques for non-muscle-invasive bladder cancer. *Cent European J Urol* **69**, 150-156 (2016).
130. J. J. Liu, M. J. Droller, J. C. Liao, New optical imaging technologies for bladder cancer: considerations and perspectives. *J Urol* **188**, 361-368 (2012).

131. J. Leveckis, J. L. Burn, N. J. Brown, M. W. Reed, Kinetics of endogenous protoporphyrin IX induction by aminolevulinic acid: preliminary studies in the bladder. *J Urol* **152**, 550-553 (1994).
132. M. Kriegmair *et al.*, Fluorescence photodetection of neoplastic urothelial lesions following intravesical instillation of 5-aminolevulinic acid. *Urology* **44**, 836-841 (1994).
133. M. Kriegmair *et al.*, Detection of early bladder cancer by 5-aminolevulinic acid induced porphyrin fluorescence. *J Urol* **155**, 105-109; discussion 109-110 (1996).
134. M. Kriegmair *et al.*, Transurethral resection for bladder cancer using 5-aminolevulinic acid induced fluorescence endoscopy versus white light endoscopy. *J Urol* **168**, 475-478 (2002).
135. G. Mowatt *et al.*, Photodynamic diagnosis of bladder cancer compared with white light cystoscopy: Systematic review and meta-analysis. *Int J Technol Assess Health Care* **27**, 3-10 (2011).
136. D. Zaak *et al.*, Endoscopic detection of transitional cell carcinoma with 5-aminolevulinic acid: results of 1012 fluorescence endoscopies. *Urology* **57**, 690-694 (2001).
137. C. R. Riedl *et al.*, Fluorescence endoscopy with 5-aminolevulinic acid reduces early recurrence rate in superficial bladder cancer. *J Urol* **165**, 1121-1123 (2001).
138. C. Zheng, Y. Lv, Q. Zhong, R. Wang, Q. Jiang, Narrow band imaging diagnosis of bladder cancer: systematic review and meta-analysis. *BJU Int* **110**, E680-687 (2012).

139. R. T. Bryan, L. J. Billingham, D. M. Wallace, Narrow-band imaging flexible cystoscopy in the detection of recurrent urothelial cancer of the bladder. *BJU Int* **101**, 702-705; discussion 705-706 (2008).
140. B. Geavlete *et al.*, Narrow band imaging cystoscopy and bipolar plasma vaporization for large nonmuscle-invasive bladder tumors--results of a prospective, randomized comparison to the standard approach. *Urology* **79**, 846-851 (2012).
141. S. Naito, S. van Rees Vellinga, J. de la Rosette, Global randomized narrow band imaging versus white light study in nonmuscle invasive bladder cancer: accession to the first milestone-enrollment of 600 patients. *J Endourol* **27**, 1-3 (2013).
142. K. Wu *et al.*, Dynamic real-time microscopy of the urinary tract using confocal laser endomicroscopy. *Urology* **78**, 225-231 (2011).
143. A. C. Goh, N. J. Tresser, S. S. Shen, S. P. Lerner, Optical coherence tomography as an adjunct to white light cystoscopy for intravesical real-time imaging and staging of bladder cancer. *Urology* **72**, 133-137 (2008).
144. A. Karl *et al.*, Optical coherence tomography for bladder cancer -- ready as a surrogate for optical biopsy? Results of a prospective mono-centre study. *Eur J Med Res* **15**, 131-134 (2010).
145. J. Schmidbauer *et al.*, Fluorescence cystoscopy with high-resolution optical coherence tomography imaging as an adjunct reduces false-positive findings in the diagnosis of urothelial carcinoma of the bladder. *Eur Urol* **56**, 914-919 (2009).

146. G. Gakis, The role of inflammation in bladder cancer. *Adv Exp Med Biol* **816**, 183-196 (2014).
147. S. Gately, W. W. Li, Multiple roles of COX-2 in tumor angiogenesis: a target for antiangiogenic therapy. *Semin Oncol* **31**, 2-11 (2004).
148. K. M. Leahy *et al.*, Cyclooxygenase-2 inhibition by celecoxib reduces proliferation and induces apoptosis in angiogenic endothelial cells in vivo. *Cancer Res* **62**, 625-631 (2002).
149. T. Qayyum *et al.*, A prospective study of the role of inflammation in bladder cancer. *Curr Urol* **6**, 189-193 (2013).
150. P. J. Bostrom, V. Aaltonen, K. O. Soderstrom, P. Uotila, M. Laato, Expression of cyclooxygenase-1 and -2 in urinary bladder carcinomas in vivo and in vitro and prostaglandin E2 synthesis in cultured bladder cancer cells. *Pathology* **33**, 469-474 (2001).
151. D. Wang, D. Xia, R. N. Dubois, The Crosstalk of PTGS2 and EGF Signaling Pathways in Colorectal Cancer. *Cancers (Basel)* **3**, 3894-3908 (2011).
152. D. L. Wheeler, E. F. Dunn, P. M. Harari, Understanding resistance to EGFR inhibitors-impact on future treatment strategies. *Nat Rev Clin Oncol* **7**, 493-507 (2010).
153. Y. Yamada *et al.*, Cyclooxygenase-2 expression in invasive transitional cell carcinoma of the urinary bladder. *Mol Med Rep* **1**, 791-795 (2008).
154. M. Komhoff *et al.*, Enhanced expression of cyclooxygenase-2 in high grade human transitional cell bladder carcinomas. *Am J Pathol* **157**, 29-35 (2000).

155. W. C. Black *et al.*, 3,4-Diaryl-5-hydroxyfuranones: highly selective inhibitors of cyclooxygenase-2 with aqueous solubility. *Bioorg Med Chem Lett* **13**, 1195-1198 (2003).
156. S. M. Crusz, F. R. Balkwill, Inflammation and cancer: advances and new agents. *Nat Rev Clin Oncol* **12**, 584-596 (2015).
157. A. J. Dannenberg, K. Subbaramaiah, Targeting cyclooxygenase-2 in human neoplasia: rationale and promise. *Cancer Cell* **4**, 431-436 (2003).
158. D. L. DeWitt, Cox-2-selective inhibitors: the new super aspirins. *Mol Pharmacol* **55**, 625-631 (1999).
159. R. DuBois, M. C. Wallace, The emerging role and potential of COX-2 inhibitors. *Clin Adv Hematol Oncol* **1**, 97-98 (2003).
160. L. J. Marnett, The COXIB experience: a look in the rearview mirror. *Annu Rev Pharmacol Toxicol* **49**, 265-290 (2009).
161. L. J. Marnett, R. N. DuBois, COX-2: a target for colon cancer prevention. *Annu Rev Pharmacol Toxicol* **42**, 55-80 (2002).
162. L. J. Marnett, A. S. Kalgutkar, Cyclooxygenase 2 inhibitors: discovery, selectivity and the future. *Trends Pharmacol Sci* **20**, 465-469 (1999).
163. D. G. Menter, R. N. Dubois, Prostaglandins in cancer cell adhesion, migration, and invasion. *Int J Cell Biol* **2012**, 723419 (2012).
164. F. E. Silverstein *et al.*, Gastrointestinal toxicity with celecoxib vs nonsteroidal anti-inflammatory drugs for osteoarthritis and rheumatoid arthritis: the CLASS

- study: A randomized controlled trial. Celecoxib Long-term Arthritis Safety Study. *JAMA* **284**, 1247-1255 (2000).
165. L. H. Rome, W. E. Lands, Structural requirements for time-dependent inhibition of prostaglandin biosynthesis by anti-inflammatory drugs. *Proc Natl Acad Sci U S A* **72**, 4863-4865 (1975).
 166. M. Vosooghi, M. Amini, The discovery and development of cyclooxygenase-2 inhibitors as potential anticancer therapies. *Expert Opin Drug Discov* **9**, 255-267 (2014).
 167. F. G. Buchanan, V. Holla, S. Katkuri, P. Matta, R. N. DuBois, Targeting cyclooxygenase-2 and the epidermal growth factor receptor for the prevention and treatment of intestinal cancer. *Cancer Res* **67**, 9380-9388 (2007).
 168. J. J. Prusakiewicz, A. S. Felts, B. S. Mackenzie, L. J. Marnett, Molecular basis of the time-dependent inhibition of cyclooxygenases by indomethacin. *Biochemistry* **43**, 15439-15445 (2004).
 169. K. Rathore, M. Alexander, M. Cekanova, Piroxicam inhibits Masitinib-induced cyclooxygenase 2 expression in oral squamous cell carcinoma cells in vitro. *Transl Res* **164**, 158-168 (2014).
 170. S. X. Sun, K. Y. Lee, C. T. Bertram, J. L. Goldstein, Withdrawal of COX-2 selective inhibitors rofecoxib and valdecoxib: impact on NSAID and gastroprotective drug prescribing and utilization. *Curr Med Res Opin* **23**, 1859-1866 (2007).

171. M. J. Uddin *et al.*, Selective visualization of cyclooxygenase-2 in inflammation and cancer by targeted fluorescent imaging agents. *Cancer Res* **70**, 3618-3627 (2010).
172. M. Cekanova *et al.*, Molecular imaging of cyclooxygenase-2 in canine transitional cell carcinomas in vitro and in vivo. *Cancer Prev Res (Phila)* **6**, 466-476 (2013).
173. M. Cekanova *et al.*, Single-dose safety and pharmacokinetic evaluation of fluorocoxib A: pilot study of novel cyclooxygenase-2-targeted optical imaging agent in a canine model. *J Biomed Opt* **17**, 116002 (2012).
174. H. Ra *et al.*, Detection of non-melanoma skin cancer by in vivo fluorescence imaging with fluorocoxib A. *Neoplasia* **17**, 201-207 (2015).

**CHAPTER II CYCLOOXYGENASE INHIBITORS POTENTIATE
RECEPTOR TYROSINE KINASE THERAPIES IN BLADDER CANCER
CELLS *IN VITRO***

Research article described in this chapter is a slightly modified version of an article that was published in the *Dove Press* journal, *Drug Design, Development, and Therapy*, by Jennifer Bourn and Maria Cekanova in June 2018.

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In this paper, “our” or “we” refers to my co-author and me. My contribution to this manuscript includes: 1) Compiling and interpretation of literature, 2) Providing comprehensive structure to the paper, 3) Acquisition and analysis of data, and 4) Writing and editing of the manuscript.

ORIGINAL RESEARCH

Running title: COX inhibition potentiates RTKI therapies.

Bourn and Cekanova.

Cyclooxygenase inhibitors potentiate receptor tyrosine kinase therapies in bladder cancer cells *in vitro*.

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ABSTRACT

Purpose: Receptor tyrosine kinase inhibitors (RTKIs) are used as targeted therapies for patients diagnosed with cancer with highly expressed RTKs, including the platelet-derived growth factor receptor (PDGFR) and c-Kit receptor. Resistance to targeted therapies is partially due to the activation of alternative pro-survival signaling pathways, including cyclooxygenase-2 (COX-2). In this study, we validated the effects of two RTKIs, axitinib and AB1010, in combination with COX inhibitors on the Akt and COX-2 signaling pathways in bladder cancer cells.

Methods: The expression of several RTKs and their downstream signaling targets were analyzed by western blot analysis in three human and two canine bladder transitional cell carcinoma (TCC) cell lines. The effects of RTKIs and COX inhibitors in bladder TCC cells were assessed by cell viability, Caspase 3/7 and Annexin V assay for apoptosis, western blot analysis for detection of COX-2 and Akt signaling pathways, and by ELISA assay for detection of PGE₂ levels.

Results: All tested TCC cells expressed the c-Kit and PDGFR α receptors, except human 5637 cells that had low RTKs expression. In addition, all tested cells expressed COX-1, COX-2, Akt, ERK1/2, and NF- κ B proteins, except human UM-UC-3 cells, where no COX-2 expression was detected by WB analysis. Both RTKIs inhibited cell viability and increased apoptosis in a dose-dependent manner in tested bladder TCC cells, which positively correlated with their expression levels of the PDGFR α and c-Kit receptors. RTKIs increased the expression of COX-2 in h-5637 and K9TCC#1Lillie cells. Co-treatment with the non-selective COX inhibitor, indomethacin, abrogated the

AB1010-induced COX-2 expression leading to an additive effect in inhibition of cell viability and PGE₂ production in tested TCC cells.

Conclusions: Co-treatment of RTKIs with indomethacin inhibited cell viability and inhibited AB1010-induced COX-2 expression resulting in decreased PGE₂ production in tested TCC cells. Thus, COX inhibition may further potentiate RTKIs therapies in bladder cancer.

Key words: transitional cell carcinoma, axitinib, masitinib, cyclooxygenase-2, prostaglandin E2, indomethacin

INTRODUCTION

Bladder cancer is the 6th most common cancer in the United States and accounts for 4.6% of all new cancer cases (1). An estimated 81,000 new patients will be diagnosed with bladder cancer, and an estimated 17,000 deaths will occur as a result of the disease each year.¹ Bladder cancer incidence is three times higher in men than in women. The most commonly diagnosed type of bladder cancer is urothelial carcinoma, also known as transitional cell carcinoma (TCC), which accounts for over 90% of all bladder cancer cases in the United States (1).

The early detection and development of novel targeted therapies with higher efficacy and fewer adverse events as compared to commonly used chemotherapy treatments are currently a primary focus in research for bladder cancer treatment (2). Receptor tyrosine kinase inhibitors (RTKIs) are used for patients diagnosed with bladder cancer that have high expression of RTKs, including the platelet-derived growth factor receptor (PDGFR), c-Kit receptor, epidermal growth factor receptor (EGFR) (3,4), or vascular endothelial growth factor receptor (VEGFR) (5). Currently used RTKIs for the treatment of bladder cancer are monoclonal antibodies, including cetuximab (4,6) and bevacizumab (7,8), and small molecules, including gefitinib (9), sunitinib (10), and axitinib (11). Axitinib (also known as AG013736 or Inlyta[®] Pfizer, USA) is a potent RTKI (VEGFR IC₅₀ = 0.1-0.3 nM, c-Kit IC₅₀ = 1.7 nM, and PDGFR IC₅₀ = 1.6nM) therapy option for patients diagnosed with metastatic clear cell renal cell carcinoma (RCC) (12). Axitinib significantly increases progression-free survival rates in patients with RCC when compared to those treated with sorafenib (13). AB1010 (known also as Masitinib[®],

Masivet[®], Kinavet[®] AB Science, France) is a novel RTKI that targets the c-Kit (IC_{50} = 200 nM) and PDGFR α/β (IC_{50} = 540-800 nM) receptors (14). Previous studies demonstrate the effectiveness of AB1010 as a viable treatment option for canine mast cell tumors by reducing cell viability and degranulation of mast cells without cytotoxic effects (15,16). AB1010 can act as a chemo-sensitizer by increasing the sensitivity of canine bladder, breast, and osteosarcoma cancer cells to chemotherapy agents *in vitro* (17-19). Similar favorable results have also been demonstrated with AB1010 in combination with gemcitabine in human pancreatic cancer cells *in vitro* (20). Preliminary results from a pre-clinical trial with AB1010 for patients diagnosed with imatinib-resistant gastrointestinal stromal tumors (GIST) indicate that AB1010 is well-tolerated and increases overall patient survival (21).

Cyclooxygenase-2 (COX-2) is highly expressed in bladder cancer and is one of the key proteins responsible for angiogenesis (22,23) and tumorigenesis (24,25). Increased prostaglandins production positively correlates with COX-2 expression levels and progression of cancer (26-29). Non-steroidal anti-inflammatory drugs (NSAIDs), inhibitors for COX enzymes, are commonly used for the prevention and treatment of cancers (30-34). One of the common side effects of long-term NSAIDs usage is gastrointestinal toxicity (35,36). The development of COX-2 selective inhibitors (COXIBs), such as celecoxib (37,38), effectively reduces the side effects in patients (39-43). Several studies have shown that RTKIs increase COX-2 expression, which can possibly lead to therapy resistance (39,44,45). The cross-talk between the RTK and the COX-2 signaling pathways plays a key role in tumorigenesis (46,47).

In this study, we evaluated the efficacy of axitinib and AB1010 (RTKIs) in a co-treatment with indomethacin (NSAID) in bladder TCC cells *in vitro*. We have validated the anti-proliferative and pro-apoptotic effects of axitinib and AB1010 alone and in combination with indomethacin through regulation of COX-2 activity in bladder TCC cells *in vitro*.

MATERIALS AND METHODS

Reagents and antibodies

AB1010 (Masitinib®) was obtained from AB Science (Paris, France); axitinib was purchased from Sigma-Aldrich (St. Louis, MO, USA) and indomethacin was obtained from a laboratory of Dr. Lawrence Marnett (Vanderbilt University, Nashville, TN). The antibodies for COX-2 (C-20), COX-1 (H-62), PDGFR α (C-20), p-PDGFR α (Tyr720), p-ERK1/2 (E-4), p-Akt (Ser473), c-Kit (961-976), p-c-Kit (Tyr721), Akt1/2/3 (H136), ERK1/2 (K-23), caspase-3 (H-277), actin, and rabbit anti-goat secondary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA); antibody for p65 (NF- κ B) was purchased from BD Biosciences (San Jose, California); PARP (recognize total and cleaved PARP), cleaved caspase-3 (D175), secondary anti-rabbit and anti-mouse antibodies were obtained from Cell Signaling (Boston, MA). All other chemicals and reagents were purchased from Thermo Fisher Scientific (Pittsburgh, PA), unless otherwise specified.

Cell lines

Human bladder transitional cell carcinoma (TCC) cell lines h-UM-UC-3, h-T24, and h-5637 were purchased from American Type Culture Collection (ATCC, Manassas, VA). Cell lines were authenticated via short tandem repeat (STR) DNA profiling by Genetica DNA laboratories (Burlington, NC). Human T24 cells were grown in McCoy's media (GE Healthcare Bio-Sciences, Pittsburgh, PA, USA), human UM-UC-3 cells were grown in minimum essential media (GE Healthcare Bio-Sciences), and human 5637 cells were grown in RPMI-1640 media (GE Healthcare Bio-Sciences) supplemented with 10% fetal bovine serum, 100 I.U. penicillin, and 100 µg/mL streptomycin. Canine bladder transitional cell carcinoma (K9TCC) cell lines, K9TCC#1Lillie and K9TCC#5Lilly were established and characterized in the laboratory of Dr. Cekanova as described previously in detail (40,48,49). The K9TCC#1Lillie and K9TCC#5Lilly were grown in complete RPMI-1640 media supplemented with 10% fetal bovine serum, 100 I.U. penicillin, and 100 µg/mL streptomycin. Cells were grown at 37°C and 5% CO₂.

Proliferation (MTS) assay

Cells were plated in 96-well plates at concentration of 5×10^3 cells/well in complete media. After 24 h incubation, cells were treated with AB1010, axitinib, indomethacin, or celecoxib (data not shown) in dose-dependent manners alone or in combinations (as specifically shown in Results section) in complete media for an additional 48 h. Vehicle, DMSO, was used as a control. After treatment, cell viability was measured using MTS CellTiter 96® Aqueous One Solution Cell Proliferation Assay

(Promega, Madison, WI) according to manufacturer's protocol. Briefly, 20 μ l of the MTS reagent was added to each well and incubated with cells at 37°C for 1 h. Absorbance was measured at 490 nM using an FLx800 plate reader (Bio-Tek instruments, Winooski, VT). The data were shown as mean $\pm$ S.E. of four replicates of three independent experiments and normalized to the DMSO controls.

Annexin V-FITC detection of apoptosis by flow cytometry

Human 5637 and canine K9TCC#1Lillie bladder TCC cells were plated in 6-cm tissue culture dishes at density of 1×10^6 cells/dish in complete media. After 24 h of cell seeding, the cells were treated with DMSO (vehicle), axitinib (5 μ M), and AB1010 (5 μ M). After 24 h treatment, both the media and attached cells were collected for analysis. Cells were stained with Annexin V-FITC and propidium iodide (PI) according to the TACS[®] Annexin V kit protocol (Trevigen, Inc., Gaithersburg, MD). The apoptotic cells were identified using a MACSQuant10 Flow Analyzer (Miltenyi Biotec Inc., Auburn, CA).

Caspase 3/7 assay

Cells were plated in 10-cm tissue culture dishes at density of 2×10^6 cells/dish in complete media. Twenty-four hours after seeding, cells were treated with AB1010 or axitinib (1, 5, 10 μ M) for an additional 24 h in media without serum. After treatment, cell lysates were harvested using RIPA buffer supplemented with protease and phosphatase inhibitors cocktail (1 mM PMSF; 1 μ g/ml aprotinin; 1 μ g/ml leupeptin; 0.1 mM Na_3VO_4 ; 10 mM NaF). Protein concentrations were measured using Pierce[®]

BCA protein assay (Thermo Scientific, Rockford, IL). Twenty-five micrograms of proteins were used for detection of caspases 3/7 activities following the Caspase Glo[®] 3/7 Substrate manufacturer's protocol (Promega, Madison, WI). After 1 h incubation with reagents, the luminescence signals were measured using an FLx800 plate reader (Bio-Tek instruments, Winooski, VT). The data were shown as mean $\pm$ S.E. of two replicates of three independent experiments and normalized to the DMSO-treated controls.

Western blotting

Human and canine bladder TCC cells were plated in 10-cm tissue culture dishes at density of 2×10^6 cells/dish in complete media and allowed to attach for 24 h. The cells were then treated with or without serum, with drugs in a dose-dependent manner alone, or in combinations for 24 h as mentioned in figure legends. After treatments, the cells were lysed in ice-cold RIPA buffer supplemented with protease and phosphatase inhibitors cocktail and kept at -80°C until WB analyses were performed. Protein concentrations were measured using the BCA protein assay. Equal amount of proteins was loaded onto SDS-PAGE gels and transferred to a nitrocellulose membrane. After blocking, the membranes were incubated with primary antibodies overnight at 4°C . Next day, membranes were incubated with horseradish peroxidase-conjugated secondary antibodies (1: 3,000 dilution) for 45 min at room temperature, and immuno-reactive bands were visualized using the chemiluminescence system using ECL reagents. The

images were captured using the BioSpectrum® 815 imaging system (UVP, Upland, CA).

PGE₂ ELISA assay

Human 5637 and K9TCC#1Lillie bladder TCC cells were plated in 10-cm tissue culture dishes at density of 2×10^6 cells/dish in complete media for 24 h followed by the treatment with 5 μ M AB1010 and 50 μ M indomethacin alone or in combination for an additional 24 h in serum free media. After the treatment, the media were collected, and dead cells and debris were removed by spinning down the media in pre-chilled tubes. Collected media were stored at -80°C until the PGE₂ analysis was performed. One hundred microliters of media were used for PGE₂ analysis following the manufacturer's protocol for the PGE₂ EIA kit (Enzo Life Sciences, Plymouth Meeting, PA). PGE₂ concentration was measured at an optical density of 405 nm with correction between 570 and 590 nm using an FLx800 plate reader (Bio-Tek instruments, Winooski, VT). A standard curve was generated by using the Gen5™ 4 parameter logistic curve fitting software (Bio-Tek instruments) based on the PGE₂ standards and the treated sample concentrations were calculated accordingly. The data is shown as mean $\pm$ S.E. of six replicates of three independent experiment and normalized to the control groups (DMSO, vehicle treated groups).

Statistical analysis

Statistical analyses were conducted using the Student's *t*-test to establish significant differences among treatment groups. Results were considered statistically significant at $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$ to control, DMSO group. Student's *t*-tests were performed to compare co-treatment AB + Indo to AB treatment groups and results were considered statistically significant at $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, and $^{\#\#\#}p < 0.001$. Student's *t*-tests were performed to compare co-treatment AB + Indo to Indo treatment groups and results were considered statistically significant at $^{\dagger}p < 0.05$, $^{\dagger\dagger}p < 0.01$, and $^{\dagger\dagger\dagger}p < 0.001$.

RESULTS

The expression profile of RTK- and COX-signaling pathway proteins in tested bladder TCC cells

The expression profile of RTKs, COXs, and their downstream targeted proteins were tested in human (h-UM-UC-3, h-T24, and h-5637) and canine (K9TCC#1Lillie and K9TCC#5Lilly) bladder TCC cells by WB analysis as shown in Fig. 2.1. While h-UM-UC-3 cells expressed relatively high levels of c-Kit and PDGFR α receptors, h-T24 cells had only low and h-5637 had no detectable protein levels of PDGFR α and c-Kit receptors. K9TCC cell lines, K9TCC#5Lilly and K9TCC#1Lillie had relatively higher expression of PDGFR α and c-Kit receptors as compared to levels detected in h-TCC cell lines. All tested bladder TCC cells had high levels of phosphorylated ERK1/2 (p-ERK1/2) protein. In addition, h-5637 cells had the lowest levels of p-ERK1/2 expression, but the highest

expression of phosphorylated Akt (p-Akt) protein as compared to the other human bladder TCC cells. Both tested canine bladder TCC cell lines had higher p-ERK1/2 expression, but no detectable p-Akt expression, when compared to h-TCC cell lines. Human UM-UC-3 and T24 cells had very low detectable COX-2 expression, whereas h-5673, K9TCC#1Lillie, and K9TCC#5Lilly cells had high COX-2 protein expression. All five tested bladder TCC cell lines had high expression levels of COX-1 and NF- κ B proteins.

Axitinib and AB1010 inhibited cell proliferation and induced apoptosis in bladder TCC cells

Both tested RTKIs, axitinib and AB1010, significantly reduced cell viability in a dose-dependent manner in all tested human and canine bladder TCC cell lines as is shown in Fig. 2.2. To further analyze the possible mechanism of axitinib and AB1010 in tested cells, we have selected h-5637 and K9TCC#1Lillie cells with the lowest and with the highest basal expression levels of PDGFR α and c-Kit receptors, respectively. The inhibition of cell viability in bladder TCC cells by AB1010 treatment positively correlated with the expression levels of PDGFR α and c-Kit receptors in tested cells. AB1010 inhibited phosphorylation of c-Kit receptor in a dose-dependent manner causing the inhibition of cell viability in K9TCC#1Lillie cells (Fig. 2.5). No expression of phosphorylated PDGFR α (p-PDGFR α) was detected in either tested cell line after RTKIs treatment. Total PDGFR α protein expression was detected in K9TCC#1Lillie, but not in h-5637 cells (Fig. 2.5).

The effects of axitinib and AB1010 on cell apoptosis were evaluated using Annexin V-FITC staining by flow cytometry analysis and the caspase-3/7 activities assay. Doxorubicin (1 μ M) treatment was used as a positive control for the detection of apoptosis (data not shown). After 24 h treatment, both 5 μ M axitinib and 5 μ M AB1010 significantly increased apoptosis in h-5637 cells with a 3- and 1.5-fold increase, respectively, when compared to control (DMSO) as is shown in Fig. 2.3A. Axitinib treatment in h-5637 cells increased the number of apoptotic cells by 2-fold as compared to AB1010 treatment. In the K9TCC#1Lillie cells, both 5 μ M axitinib and 5 μ M AB1010 significantly increased apoptosis by 1.8-fold when compared to the control (DMSO) group as is shown in Fig. 2.3B. Axitinib, but not AB1010, significantly increased Caspase 3/7 activity in h-5637 cells after 24 h treatment as shown in Figure 4A. Neither axitinib nor AB1010 statistically significantly increased the caspase-3/7 activity in K9TCC#1Lillie cells, as shown in Fig. 2.4A. The apoptosis induced by axitinib was also confirmed by upregulation of cleaved caspase-3 (cCaspase-3) and cleaved PARP (cPARP) in tested human 5637 cells by WB analysis (Fig. 2.4B, 2.4C). There were no detectable levels of cCaspase-3 expression in the h-5637 cells by AB1010 treatment (Figure 2.4B and 2.4C). Axitinib (10 μ M) and AB1010 (10 μ M) treatments increased cCaspase-3 expression when compared to control in the K9TCC#1Lillie cells by 3- and 18-fold, respectively (Fig. 2.4B and 2.4C). Axitinib and AB1010 treatments increased cPARP expression by approximately 4-fold and 2-fold, respectively, when compared to control in h-5637 cells as shown by densitometry analysis of WB data in Fig. 2.4B and

2.4C. The cleavage of PARP was not detected after treatment with AB1010 or axitinib in K9TCC#1Lillie cells (Fig. 2.4B).

Axitinib and AB1010 increased COX-2 expression in bladder TCC cells

Both RTKIs, axitinib and AB1010, increased expression of COX-2 in h-5637 and K9TCC#1Lillie cells in dose-dependent manner (Fig. 2.6A). Based on the densitometry analysis of COX-2 protein expression in h-5637 cells, a 2-fold increase in COX-2 expression was detected by both axitinib and AB1010 treatments when compared to the control. AB1010 treatment increased COX-2 protein expression in the K9TCC#1Lillie cells by 4-fold when compared to the control as shown in Fig. 5B. RTKI treatments had no effects on NF- κ B expression in human 5637 cells (Figure 2.6A). AB1010 treatment increased NF- κ B expression in a dose-dependent manner in the K9TCC#1Lillie cells. Axitinib and AB1010 significantly reduced the phosphorylation of Akt in human 5637 cells in a dose-dependent manner as shown in Fig. 2.6A. In contrast, axitinib, but not AB1010, increased the phosphorylation of Akt in K9TCC#1Lillie cells (Fig. 2.6A and 2.6B). Neither axitinib nor AB1010 treatments had effects on the phosphorylation of ERK1/2 in human 5637 or K9TCC#1Lillie cells (data not shown).

NSAIDs inhibited AB1010-induced COX-2 expression in bladder TCC cells in vitro

To test our hypothesis that inhibition of AB1010-induced COX-2 expression might lead to more effective inhibition of cell viability of bladder cancer cells by RTKIs, bladder TCC cells were co-treated with RTKIs and indomethacin (non-selective NSAID). Co-

treatment of 5 μ M AB1010 with 5, 10, 50, or 100 μ M indomethacin was more effective inhibiting cell viability of K9TCC#1Lillie as compared to either treatment alone as is shown in Fig. 2.7A. Co-treatment of AB1010 and indomethacin was more effective in the inhibition of cell viability of K9TCC#1Lillie cells as compared to h-5637 cells (Fig. 2.7A) due to higher expression of the target receptors, c-Kit and PDGFR α in K9TCC#1Lillie cells (Fig. 1A). In addition, indomethacin inhibited AB1010-induced COX-2 expression in K9TCC#1Lillie cells as shown in Fig. 2.7B and 2.7C. Co-treatment of AB1010 with indomethacin did not affect the expression of NF- κ B in either tested TCC cells (Fig. 2.7B). Co-treatment of AB1010 with indomethacin reduced Akt expression levels in K9TCC#1Lillie cells as shown in Fig. 2.7B. Co-treatment of AB1010 with indomethacin increased the cleaved PARP (cPARP) expression in h-5637 cells; however, no cPARP levels were detected in K9TCC#1Lillie cells Fig. 2.7B and 2.7C.

NSAIDs inhibited AB1010-induced PGE₂ production in K9TCC#1Lillie cells in vitro

To evaluate the effects of AB1010 and indomethacin co-treatment on COX-2 activity in h-5637 and K9TCC#1Lillie cells, released levels of PGE₂ in media were measured by ELISA assay as shown in Fig. 2.8A and 2.8B. Indomethacin alone and in combination with AB1010 significantly inhibited PGE₂ production in human 5637 cells. No significant change in PGE₂ production was detected after AB1010 treatment in human 5637 cells (Fig. 2.8A). AB1010 treatment significantly increased PGE₂ levels by 1.75-fold in K9TCC#1Lillie cells. In addition, indomethacin inhibited AB1010-induced PGE₂ production in K9TCC#1Lillie cells by 5-fold (Fig. 2.8B).

DISCUSSION

Bladder TCC comprises 90% of all bladder cancer cases in the United State. Selection of treatment for patients diagnosed with bladder TCC depends on the grade (low or high) and invasiveness of the tumor. Surgery and conventional chemotherapy are used for low-grade TCC cases, but patients with advanced TCC rely on non-conventional treatment options, such as radical surgery, novel therapeutics, and combination therapies (1). Axitinib is a US Food and Drug Administration-approved second-line RTKI therapy for patients with metastatic RCC (13). AB1010 is used for patients diagnosed with GIST and significantly improves prognostic outcomes (21). In this study, we have confirmed that AB1010 as compared to axitinib more efficiently inhibited cell viability of K9TCC#1Lillie cells through inhibition of the c-Kit receptor and inhibition of Akt signaling pathways. In addition, RTKIs increased apoptosis in both tested bladder TCC cells. RTKIs treatment caused increased expression of COX-2 in tested bladder TCC cells. Co-treatment of RTKIs with indomethacin inhibited cell viability and inhibited AB1010-induced COX-2 expression resulting in decreased PGE₂ production in tested bladder TCC cells.

Overexpression of multiple RTKs (PDGFR α/β , c-Kit, and VEGFR) and COX-2 is a common characteristic of bladder cancer (3-5,24,25). Basal expression profile of these RTKs indicate that tested h-TCC cells had lower expression of the RTKs (c-Kit and PDGFR α) when compared to the tested K9TCC cells (Fig. 2.1). Our results are consistent with a previously published study where low or no levels of mRNA or proteins of c-Kit were detected in human 5637 and T24 cells (50). Only UM-UC-3 cells have high

levels of PDGFR α mRNA, but no expression of c-Kit mRNA as detected by RT-PCR analysis (50). The activation of the PI3K/Akt pathway and loss of PTEN correlates with tumor progression and poor patient prognosis (51,52). The expression of phosphorylated Akt was not detected in either tested canine bladder TCC cells. Human T24 and UM-UC-3 cells have little to no detectable levels of COX-2 expression, respectively when compared to K9TCC#1Lillie and K9TCC#5Lilly cells (Fig. 2.1). Our results concur with previously published studies where no protein levels of COX-2 are detected in UM-UC-3 cells (53,54).

While AB1010 decreased cell viability and inhibited phosphorylation of c-Kit, increased levels of COX-2 and NF- κ B expressions were detected in h-5637 and K9TCC#1Lillie cells (Fig. 2.6A). The increased COX-2 expression has been demonstrated *in vitro* and *in vivo* using several primary K9TCC cell lines, but the mechanisms, by which AB1010 increases COX-2 expression is still not well understood (39). We hypothesize that the increased COX-2 levels by RTKI could possibly lead to drug-resistance in COX-2 expressing tumors. To test our hypothesis, we evaluated the effects of co-treatment of AB1010 with indomethacin (NSAID) in h-5637 and K9TCC#1Lillie cells. Indeed, the co-treatment of AB1010 with indomethacin significantly reduced cell viability and COX-2 expression in h-5637 and K9TCC#1Lillie cells. Similar results have been demonstrated in patients with advanced pancreatic cancer, who received a combination therapy with AB1010 and gemcitabine compared to either treatment alone (18).

NSAIDs are commonly used for the treatment of inflammation as well as cancer (30,55). We evaluated the effects of celecoxib (a COX-2 selective inhibitor; data not shown) and indomethacin (non-selective NSAID) on cell viability of bladder TCC cells. Our results indicated that co-treatment of AB1010 with indomethacin significantly inhibited cell viability in h-5637 and K9TCC#1Lillie cells (Fig 2.7A). Co-treatment of AB1010 with indomethacin decreased the phosphorylation of Akt (Ser473) in both bladder TCC cell lines, and decreased COX-1 and COX-2 protein expression levels in K9TCC#1Lillie cells (Fig 2.7B and 2.7C). The decreased levels of the AB1010-induced COX-2 was detected after co-treatment with indomethacin in both bladder TCC cell lines. Increased prostaglandins production positively correlates with COX-2 expression levels and progression of cancer (26-29). To validate whether AB1010 and indomethacin affect the activity of COX-2 in tested cells, we have measured the levels of PGE₂ released into media 24 h after treatment of cells. AB1010 increased significantly the levels of COX-2 and production of PGE₂ in K9TCC#1Lillie cells as shown in Fig. 2.8. Indomethacin inhibited the production of PGE₂ in both tested cells and in addition, in co-treatment with AB1010 inhibited AB1010-induced COX-2 activity resulting in inhibition of PGE₂ production in canine bladder TCC cells. Currently, co-treatments of conventional chemotherapy agents with molecular targeted agents have been successfully applied for patients with drug-resistant cancers, including breast, lung, and colon cancer (56-58). COX-2/PGE₂ signaling pathway exerts pro-oncogenic effects through activation of receptor tyrosine kinases in colorectal cancer (59) and non-small cell lung cancer (60) that supports our finding that co-treatment of RTKIs and

NSAIDs might indicate better results for bladder cancer. Further experiments *in vivo* need to be conducted to confirm our findings of beneficial role of NSAIDs in co-treatment with RTKIs in bladder TCC cells *in vitro*. In conclusion, drug-resistance is one of the many challenges in the treatment of bladder cancer. Thus, co-treatment of RTKIs with COX inhibitors might indicate better clinical outcomes for patients diagnosed with bladder cancer.

Author's Contributions: **JB** has made substantial contributions to acquisition and analysis of data for in vitro assays; performed the statistical analysis; has been involved in writing the manuscript; has given a final approval of the version to be published; and agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. **MC** made substantial contributions to conception and design of experiments, analysis, and interpretation of data; has been involved in writing the manuscript and revising it critically for important intellectual content; has given a final approval of the version to be published; and agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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The authors declare that they have no competing interests.

ABBREVIATIONS

AB, AB1010 (Masitinib®); Akt, V-akt murine thymoma oncogene homolog 1; COX-1, Cyclooxygenase-1; COX-2, Cyclooxygenase-2; EGFR, Epidermal growth factor receptor; ERK1/2, Extracellular signal regulated kinases 1/2; Indo, Indomethacin; NF- κ B, Nuclear factor kappa-light-chain-enhance of activated B cells; NSAIDs, Non-steroidal anti-inflammatory drugs; cPARP, cleaved poly (ADP-ribose) polymerase; PDGFR α , Platelet-derived growth factor receptor alpha; PGE₂, prostaglandin E2; PI3K, Phosphatidylinositol-3-kinase; PTEN, Phosphatase and tensin homolog deleted on chromosome 10; RTK, Receptor tyrosine kinase; RTKI, Receptor tyrosine kinase inhibitor; TCC, Transitional cell carcinoma; VEGFR, vascular endothelial growth factor receptor

REFERENCES

1. N. C. Institute. (2016).
2. E. Seront, J. P. Machiels, Molecular biology and targeted therapies for urothelial carcinoma. *Cancer Treat Rev* **41**, 341-353 (2015).
3. N. The Cancer Genome Atlas Research, Comprehensive molecular characterization of urothelial bladder carcinoma. *Nature* **507**, 315-322 (2014).
4. Y. N. Wong *et al.*, Phase II trial of cetuximab with or without paclitaxel in patients with advanced urothelial tract carcinoma. *J Clin Oncol* **30**, 3545-3551 (2012).
5. J. P. Crew, Vascular endothelial growth factor: an important angiogenic mediator in bladder cancer. *Eur Urol* **35**, 2-8 (1999).
6. M. Hussain *et al.*, A randomized phase 2 trial of gemcitabine/cisplatin with or without cetuximab in patients with advanced urothelial carcinoma. *Cancer* **120**, 2684-2693 (2014).
7. A. V. Balar *et al.*, Phase II study of gemcitabine, carboplatin, and bevacizumab in patients with advanced unresectable or metastatic urothelial cancer. *J Clin Oncol* **31**, 724-730 (2013).
8. N. M. Hahn *et al.*, Phase II trial of cisplatin, gemcitabine, and bevacizumab as first-line therapy for metastatic urothelial carcinoma: Hoosier Oncology Group GU 04-75. *J Clin Oncol* **29**, 1525-1530 (2011).

9. D. P. Petrylak *et al.*, Results of the Southwest Oncology Group phase II evaluation (study S0031) of ZD1839 for advanced transitional cell carcinoma of the urothelium. *BJU Int* **105**, 317-321 (2010).
10. D. J. Gallagher *et al.*, Phase II study of sunitinib in patients with metastatic urothelial cancer. *J Clin Oncol* **28**, 1373-1379 (2010).
11. T. E. Hutson *et al.*, Axitinib versus sorafenib as first-line therapy in patients with metastatic renal-cell carcinoma: a randomised open-label phase 3 trial. *Lancet Oncol* **14**, 1287-1294 (2013).
12. H. S. Rugo *et al.*, Phase I trial of the oral antiangiogenesis agent AG-013736 in patients with advanced solid tumors: pharmacokinetic and clinical results. *J Clin Oncol* **23**, 5474-5483 (2005).
13. B. I. Rini *et al.*, Comparative effectiveness of axitinib versus sorafenib in advanced renal cell carcinoma (AXIS): a randomised phase 3 trial. *The Lancet* **378**, 1931-1939.
14. P. Dubreuil *et al.*, Masitinib (AB1010), a potent and selective tyrosine kinase inhibitor targeting KIT. *PLoS One* **4**, e7258 (2009).
15. K. A. Hahn *et al.*, Evaluation of 12- and 24-month survival rates after treatment with masitinib in dogs with nonresectable mast cell tumors. *Am J Vet Res* **71**, 1354-1361 (2010).

16. K. A. Hahn *et al.*, Masitinib is safe and effective for the treatment of canine mast cell tumors. *J Vet Intern Med* **22**, 1301-1309 (2008).
17. M. Humbert *et al.*, Masitinib combined with standard gemcitabine chemotherapy: in vitro and in vivo studies in human pancreatic tumour cell lines and ectopic mouse model. *PLoS One* **5**, e9430 (2010).
18. E. Mitry *et al.*, Safety and activity of masitinib in combination with gemcitabine in patients with advanced pancreatic cancer. *Cancer Chemother Pharmacol* **66**, 395-403 (2010).
19. D. H. Thamm *et al.*, Masitinib as a chemosensitizer of canine tumor cell lines: a proof of concept study. *Vet J* **191**, 131-134 (2012).
20. G. Deplanque *et al.*, A randomized, placebo-controlled phase III trial of masitinib plus gemcitabine in the treatment of advanced pancreatic cancer. *Ann Oncol* **26**, 1194-1200 (2015).
21. A. Adenis *et al.*, Masitinib in advanced gastrointestinal stromal tumor (GIST) after failure of imatinib: a randomized controlled open-label trial. *Ann Oncol* **25**, 1762-1769 (2014).
22. K. M. Leahy *et al.*, Cyclooxygenase-2 inhibition by celecoxib reduces proliferation and induces apoptosis in angiogenic endothelial cells in vivo. *Cancer Res* **62**, 625-631 (2002).

23. S. Gately, W. W. Li, Multiple roles of COX-2 in tumor angiogenesis: a target for antiangiogenic therapy. *Semin Oncol* **31**, 2-11 (2004).
24. G. Gakis, The role of inflammation in bladder cancer. *Adv Exp Med Biol* **816**, 183-196 (2014).
25. T. Qayyum *et al.*, A prospective study of the role of inflammation in bladder cancer. *Curr Urol* **6**, 189-193 (2013).
26. M. A. Wheeler, D. A. Hausladen, J. H. Yoon, R. M. Weiss, Prostaglandin E2 production and cyclooxygenase-2 induction in human urinary tract infections and bladder cancer. *J Urol* **168**, 1568-1573 (2002).
27. H. Yamada *et al.*, Prostaglandin E2 down-regulates viable Bacille Calmette-Guerin-induced macrophage cytotoxicity against murine bladder cancer cell MBT-2 in vitro. *Clin Exp Immunol* **128**, 52-58 (2002).
28. P. J. Bostrom, V. Aaltonen, K. O. Soderstrom, P. Uotila, M. Laato, Expression of cyclooxygenase-1 and -2 in urinary bladder carcinomas in vivo and in vitro and prostaglandin E2 synthesis in cultured bladder cancer cells. *Pathology* **33**, 469-474 (2001).
29. Z. Wang *et al.*, In vitro effects of prostaglandin E2 or indomethacin on the proliferation of lymphokine-activated killer cells and their cytotoxicity against bladder tumor cells in patients with bladder cancer. *Prostaglandins* **54**, 769-779 (1997).

30. S. M. Crusz, F. R. Balkwill, Inflammation and cancer: advances and new agents. *Nat Rev Clin Oncol* **12**, 584-596 (2015).
31. D. L. DeWitt, Cox-2-selective inhibitors: the new super aspirins. *Mol Pharmacol* **55**, 625-631 (1999).
32. L. J. Marnett, A. S. Kalgutkar, Cyclooxygenase 2 inhibitors: discovery, selectivity and the future. *Trends Pharmacol Sci* **20**, 465-469 (1999).
33. R. DuBois, M. C. Wallace, The emerging role and potential of COX-2 inhibitors. *Clin Adv Hematol Oncol* **1**, 97-98 (2003).
34. D. G. Menter, R. N. Dubois, Prostaglandins in cancer cell adhesion, migration, and invasion. *Int J Cell Biol* **2012**, 723419 (2012).
35. F. E. Silverstein *et al.*, Gastrointestinal toxicity with celecoxib vs nonsteroidal anti-inflammatory drugs for osteoarthritis and rheumatoid arthritis: the CLASS study: A randomized controlled trial. Celecoxib Long-term Arthritis Safety Study. *JAMA* **284**, 1247-1255 (2000).
36. L. J. Marnett, The COXIB experience: a look in the rearview mirror. *Annu Rev Pharmacol Toxicol* **49**, 265-290 (2009).
37. M. Vosooghi, M. Amini, The discovery and development of cyclooxygenase-2 inhibitors as potential anticancer therapies. *Expert Opin Drug Discov* **9**, 255-267 (2014).

38. L. H. Rome, W. E. Lands, Structural requirements for time-dependent inhibition of prostaglandin biosynthesis by anti-inflammatory drugs. *Proc Natl Acad Sci U S A* **72**, 4863-4865 (1975).
39. K. Rathore, M. Alexander, M. Cekanova, Piroxicam inhibits Masitinib-induced cyclooxygenase 2 expression in oral squamous cell carcinoma cells in vitro. *Transl Res* **164**, 158-168 (2014).
40. K. Rathore, M. Cekanova, Animal model of naturally occurring bladder cancer: characterization of four new canine transitional cell carcinoma cell lines. *BMC Cancer* **14**, 465 (2014).
41. F. G. Buchanan, V. Holla, S. Katkuri, P. Matta, R. N. DuBois, Targeting cyclooxygenase-2 and the epidermal growth factor receptor for the prevention and treatment of intestinal cancer. *Cancer Res* **67**, 9380-9388 (2007).
42. L. J. Marnett, R. N. DuBois, COX-2: a target for colon cancer prevention. *Annu Rev Pharmacol Toxicol* **42**, 55-80 (2002).
43. J. J. Prusakiewicz, A. S. Felts, B. S. Mackenzie, L. J. Marnett, Molecular basis of the time-dependent inhibition of cyclooxygenases by indomethacin. *Biochemistry* **43**, 15439-15445 (2004).
44. D. L. Wheeler, E. F. Dunn, P. M. Harari, Understanding resistance to EGFR inhibitors-impact on future treatment strategies. *Nat Rev Clin Oncol* **7**, 493-507 (2010).

45. S. A. Rosenzweig, Acquired resistance to drugs targeting receptor tyrosine kinases. *Biochem Pharmacol* **83**, 1041-1048 (2012).
46. A. J. Dannenberg, S. M. Lippman, J. R. Mann, K. Subbaramaiah, R. N. DuBois, Cyclooxygenase-2 and epidermal growth factor receptor: pharmacologic targets for chemoprevention. *J Clin Oncol* **23**, 254-266 (2005).
47. A. J. Dannenberg, K. Subbaramaiah, Targeting cyclooxygenase-2 in human neoplasia: rationale and promise. *Cancer Cell* **4**, 431-436 (2003).
48. M. Cekanova, K. Rathore, Animal models and therapeutic molecular targets of cancer: utility and limitations. *Drug Des Devel Ther* **8**, 1911-1921 (2014).
49. M. Cekanova *et al.*, Molecular imaging of cyclooxygenase-2 in canine transitional cell carcinomas in vitro and in vivo. *Cancer Prev Res (Phila)* **6**, 466-476 (2013).
50. J. Knievel *et al.*, Multiple mechanisms mediate resistance to sorafenib in urothelial cancer. *International journal of molecular sciences* **15**, 20500-20517 (2014).
51. C. H. Sun, Y. H. Chang, C. C. Pan, Activation of the PI3K/Akt/mTOR pathway correlates with tumour progression and reduced survival in patients with urothelial carcinoma of the urinary bladder. *Histopathology* **58**, 1054-1063 (2011).

52. H. Lee, S. K. Choi, J. Y. Ro, Overexpression of DJ-1 and HSP90alpha, and loss of PTEN associated with invasive urothelial carcinoma of urinary bladder: Possible prognostic markers. *Oncol Lett* **3**, 507-512 (2012).
53. D. Dhawan, A. B. Jeffreys, R. Zheng, J. C. Stewart, D. W. Knapp, Cyclooxygenase-2 dependent and independent antitumor effects induced by celecoxib in urinary bladder cancer cells. *Mol Cancer Ther* **7**, 897-904 (2008).
54. J. Gee *et al.*, Selective cyclooxygenase-2 inhibitors inhibit growth and induce apoptosis of bladder cancer. *Oncology reports* **15**, 471-477 (2006).
55. W. C. Black *et al.*, 3,4-Diaryl-5-hydroxyfuranones: highly selective inhibitors of cyclooxygenase-2 with aqueous solubility. *Bioorg Med Chem Lett* **13**, 1195-1198 (2003).
56. K. Miller *et al.*, Paclitaxel plus bevacizumab versus paclitaxel alone for metastatic breast cancer. *N Engl J Med* **357**, 2666-2676 (2007).
57. A. Sandler *et al.*, Paclitaxel-carboplatin alone or with bevacizumab for non-small-cell lung cancer. *N Engl J Med* **355**, 2542-2550 (2006).
58. H. Hurwitz *et al.*, Bevacizumab plus irinotecan, fluorouracil, and leucovorin for metastatic colorectal cancer. *N Engl J Med* **350**, 2335-2342 (2004).
59. J. Shao, B. M. Evers, H. Sheng, Prostaglandin E2 synergistically enhances receptor tyrosine kinase-dependent signaling system in colon cancer cells. *J Biol Chem* **279**, 14287-14293 (2004).

60. S. M. Gadgeel *et al.*, Phase II study of gefitinib, an epidermal growth factor receptor tyrosine kinase inhibitor (EGFR-TKI), and celecoxib, a cyclooxygenase-2 (COX-2) inhibitor, in patients with platinum refractory non-small cell lung cancer (NSCLC). *J Thorac Oncol* **2**, 299-305 (2007).

APPENDIX

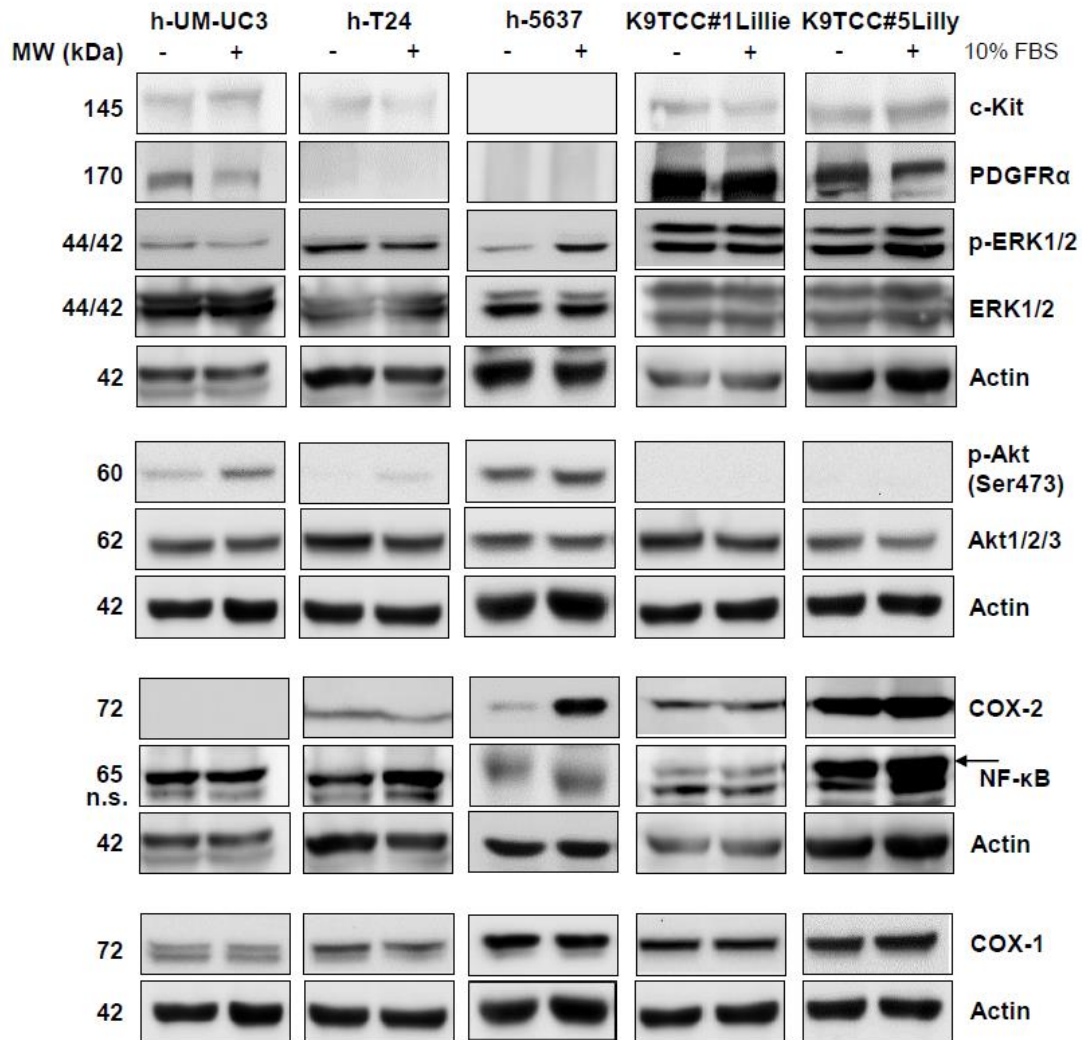


Figure 2.1 Expression profile of RTKs and COXs signaling pathway proteins in the human and primary canine bladder TCC by WB analysis.

Cells were grown in the absence (-) or presence (+) of fetal bovine serum (FBS) for 24 h. The expression of c-Kit, PDGFRα, p-ERK1/2, ERK1/2, p-Akt (Ser473), Akt1/2/3, COX-1, COX-2, and NF-κB proteins were evaluated by WB analysis. Actin was used as a loading control. Tested K9TCC cells had higher expression of RTK and COX proteins as compared to h-T24, h-5637, and h-UM-UC-3 cells.

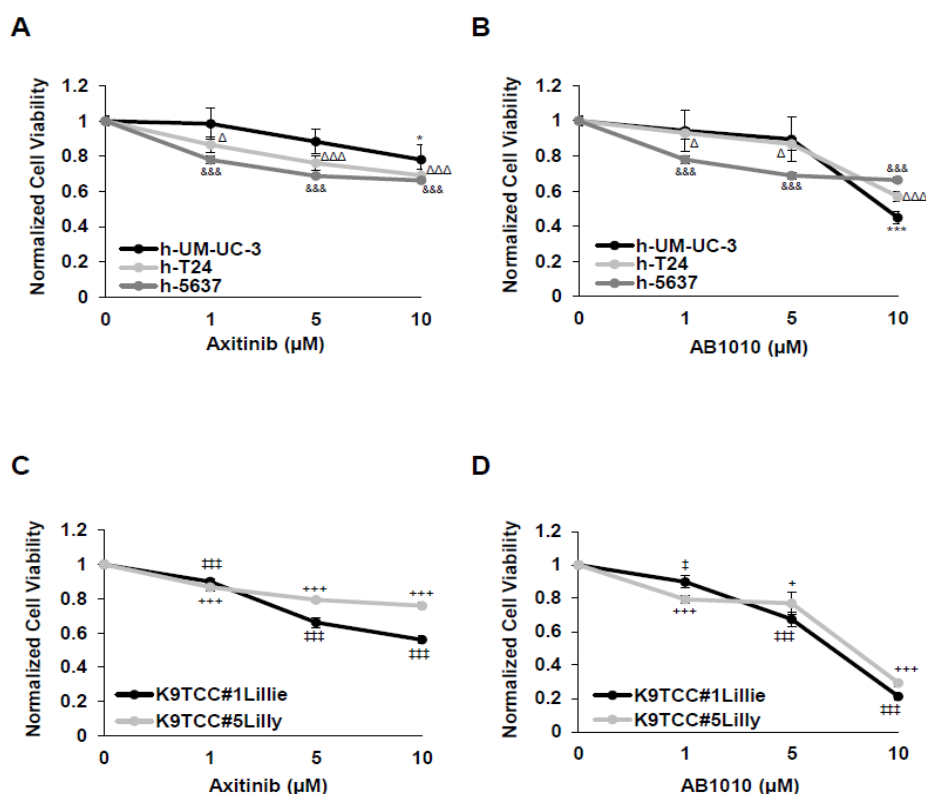


Figure 2.2 Axitinib and AB1010 inhibited cell proliferation in bladder TCC cells in a dose-dependent manner.

(A, B) Human UM-UC-3, T24, and 5637 and canine **(C, D)** K9TCC#1Lillie and K9TCC#5Lilly cells were treated with axitinib and AB1010 at dose of 0, 1, 5, and 10 μM for 48 h and cell viability was assessed by MTS assay. Both tested RTKIs inhibited cell viability of bladder TCC cells in a dose-dependent manner. Relative cell growth rates were normalized to the controls. Values represent mean ± S. E. of four replicates from three independent experiments; paired Student's *t*-test, **p* < 0.05, ***p* < 0.01, and ****p* < 0.001. Symbols denote *p*-value for each cell line - * (UM-UC-3), Δ (T24), & (5637), ‡ (Lillie), and + (Lilly).

Figure 2.3 Axitinib and AB1010 induced apoptosis in h-5637 cells.

(A) Human 5637 and **(B)** K9TCC#1Lillie cells were treated with 5 μ M axitinib and 5 μ M AB1010 for 24 h and cells were stained with Annexin V-FITC and propidium iodide followed by flow cytometry analysis. Percentage of apoptotic cells (positive for both Annexin V-FITC and PI staining) of treatment groups were normalized to the control (DMSO). Both axitinib and AB1010 significantly increased apoptosis in h-5637 and K9TCC#1Lillie cells. Values represent mean $\pm$ S.E. of two replicates from three independent experiments, paired Student's *t*-test, * p < 0.05, ** p < 0.01, and *** p < 0.001.

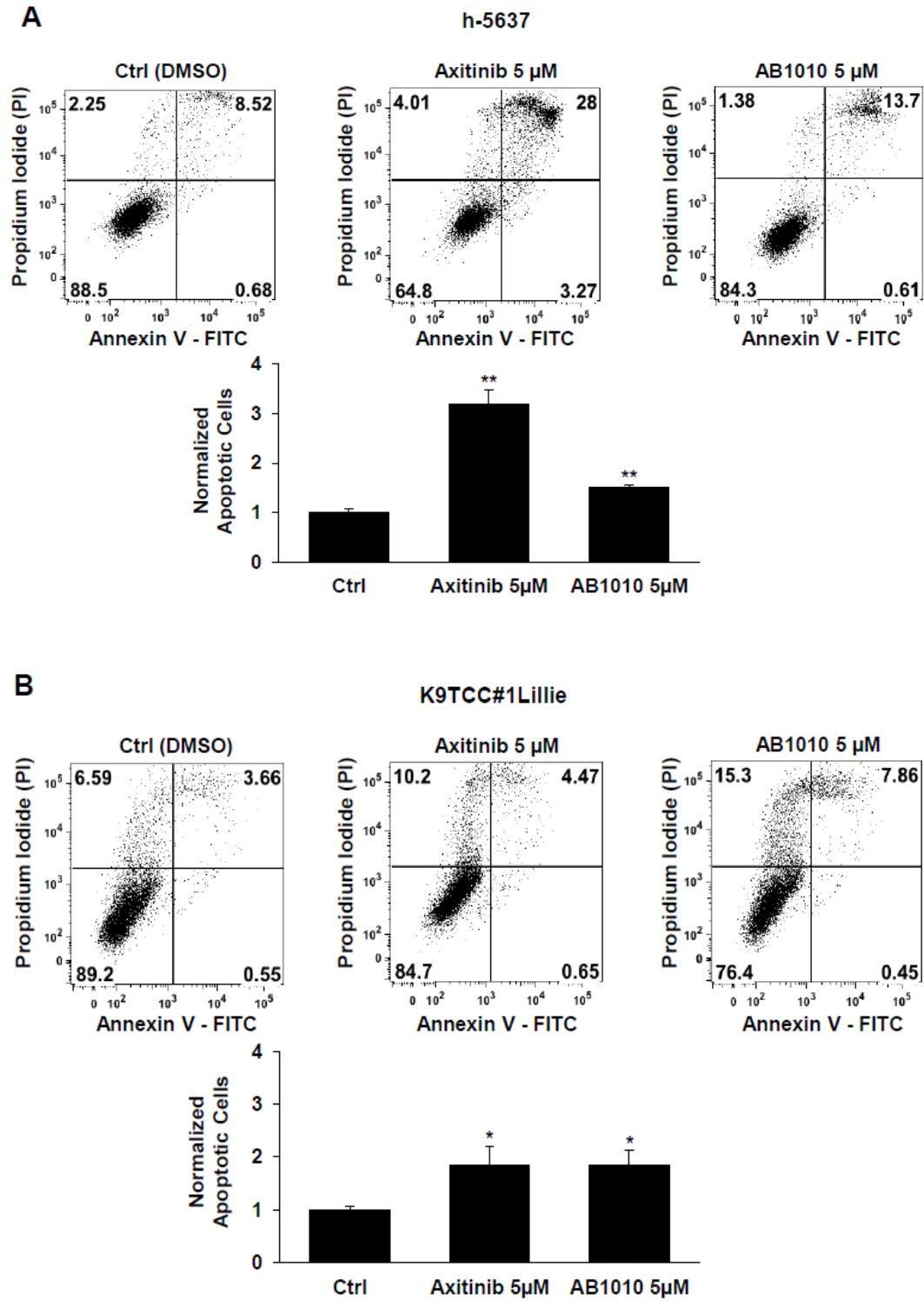
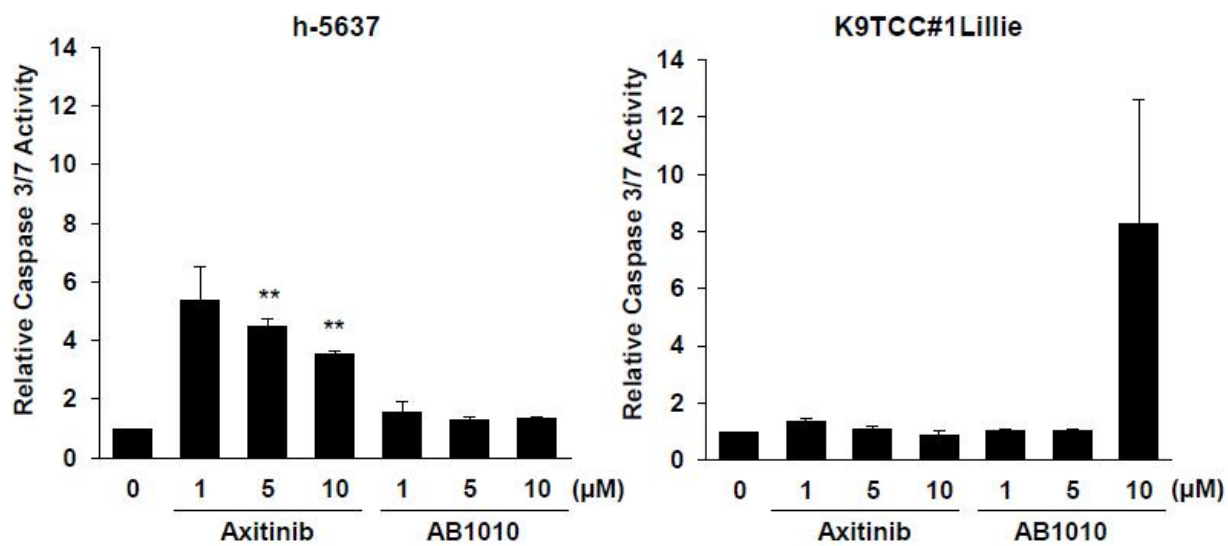


Figure 2.3

Figure 2.4 Axitinib activates caspase3/7 and cleavage of caspase-3 in bladder TCC cells.

Human 5637 and K9TCC#1Lillie cells were treated with 0, 1, 5, and 10 μ M axitinib or AB1010 for 24 h. **(A)** Relative activities of caspase 3/7 were detected from cell lysates using the Caspase-Glo 3/7 luminescence assay. Both RTKIs treatments increased the caspase-3/7 activities in h-5637 cells, but not in K9TCC#1Lillie. Relative caspase-3/7 activities were normalized to control, and values represent mean $\pm$ S.E. of two replicates of three independent experiments. Student's *t*-test, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$. **(B)** Axitinib treatment significantly increased the expression of cleaved PARP (cPARP) in h-5637 cells and cleaved caspase-3 (cCaspase-3) in both h-5637 and K9TCC#1Lillie cells as detected by WB analysis. The treatment of 10 μ M AB1010 significantly increased cCaspase-3 expression in K9TCC#1Lillie cells. Actin was used as a loading control. **(C)** Densitometry analyses of cCaspase-3 and cPARP protein levels from WB analysis were performed using VisionWorks analysis software. Values represent the mean $\pm$ S.E. of the measured densitometry of each protein band from three independent experiments. Paired Student's *t*-test was used to compare axitinib and AB1010 treatments to control groups ($*p < 0.05$, $**p < 0.01$, $***p < 0.001$).

A



B

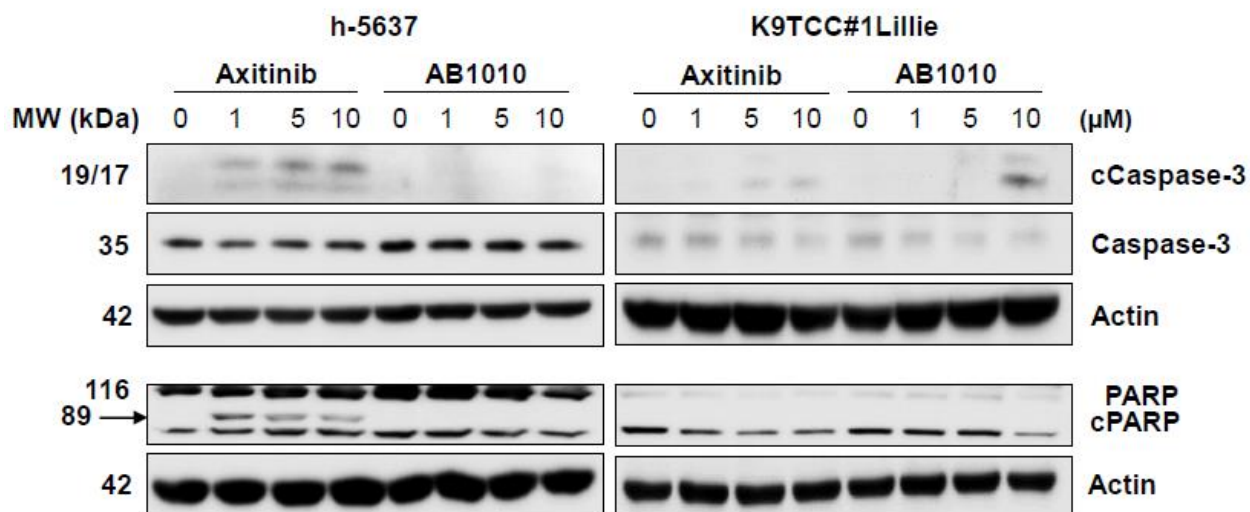


Figure 2.4

C

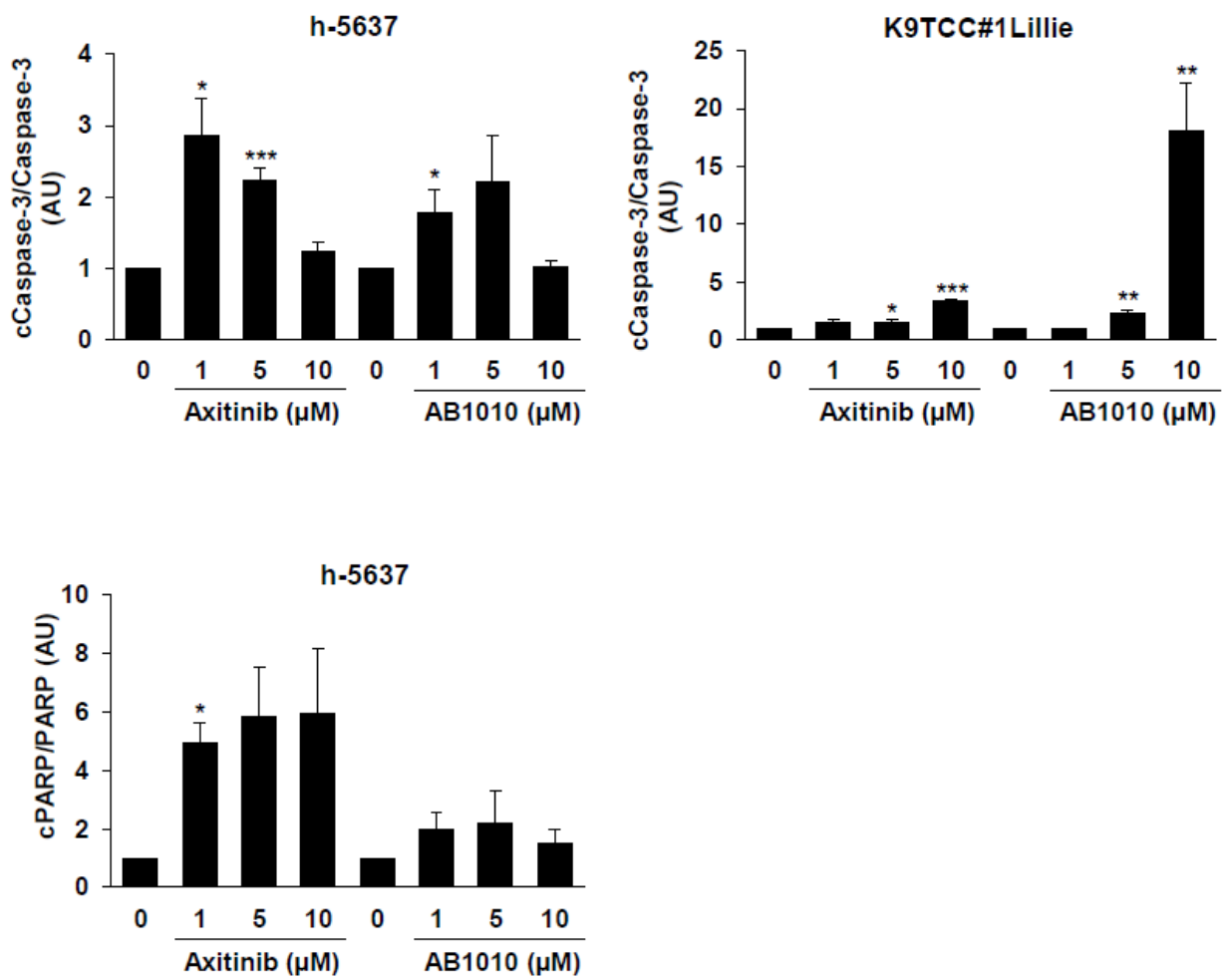


Figure 2.4 (continued)

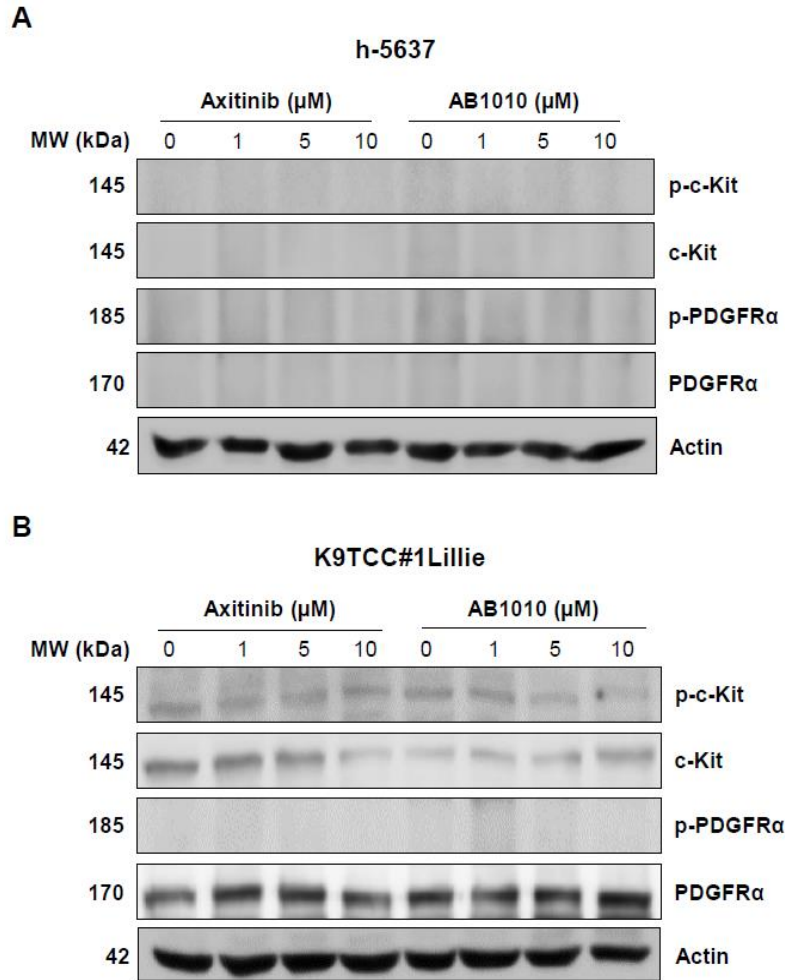


Figure 2.5 Axitinib and AB1010 inhibit phosphorylation of c-Kit in K9TCC#1Lillie cells.

Human 5637, and K9TCC#1Lillie cells were treated with 0, 1, 5, and 10 μ M axitinib and AB1010 for 24 h. The expression of p-c-Kit, c-Kit, p-PDGFR α , and PDGFR α proteins were determined by WB analysis. Actin was used as a loading control. No phosphorylated or total c-Kit expressions were detected in h-5637 cells. Both tested RTKIs inhibited phosphorylation of c-Kit in K9TCC#1Lille cells, but no p-PDGFR α levels were detected in either of the tested cell lines.

Figure 2.6 Axitinib and AB1010 increased COX-2 expression in a dose-dependent manner in tested bladder TCC cells by WB analysis.

Figure 2.6 (continued) (A) Human 5637 and K9TCC#1Lillie cells were treated with axitinib and AB1010 at doses of 0, 1, 5, and 10 μ M for 24 h. Actin was used as a loading control. Axitinib and AB1010 increased COX-2, but not NF- κ B expression in h-5637 cells. AB1010 increased COX-2 and NF- κ B protein expression in a dose-dependent manner in K9TCC#1Lillie cells. **(B)** Densitometry analysis of COX-2 and p-Akt protein bands from WB analysis was performed using VisionWorks analysis software. Values represent the mean $\pm$ S.E. of the measured densitometry of each protein band from three independent experiments. Paired Student's *t*-test was used to compare axitinib and AB1010 treatments to controls ($*p < 0.05$, $**p < 0.01$, $***p < 0.001$).

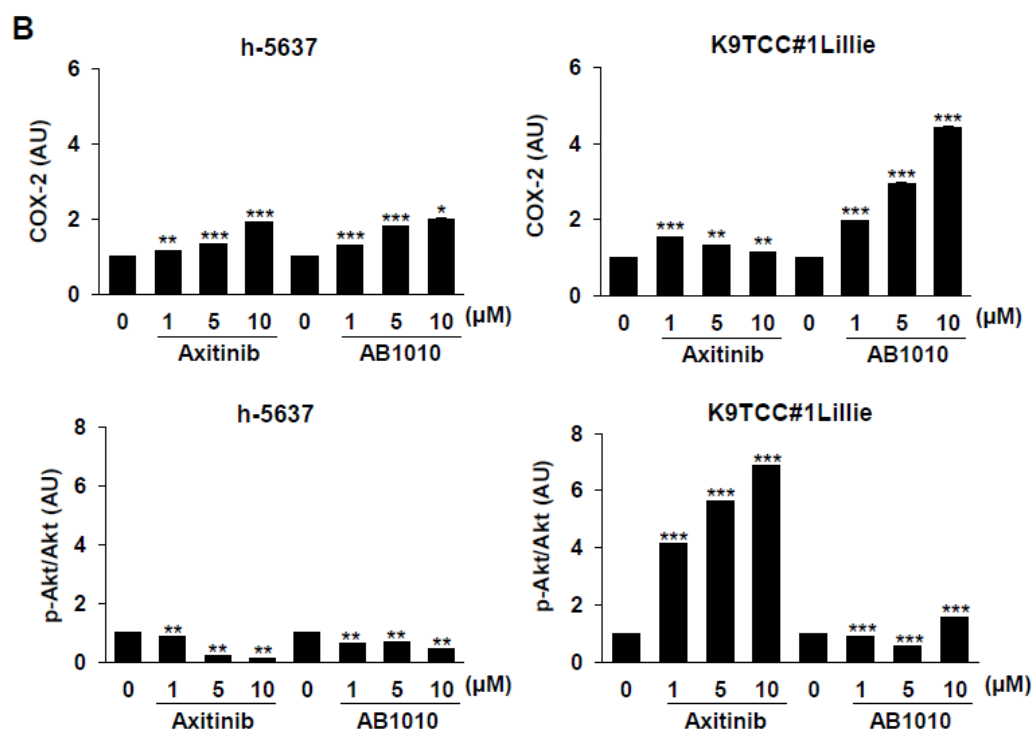
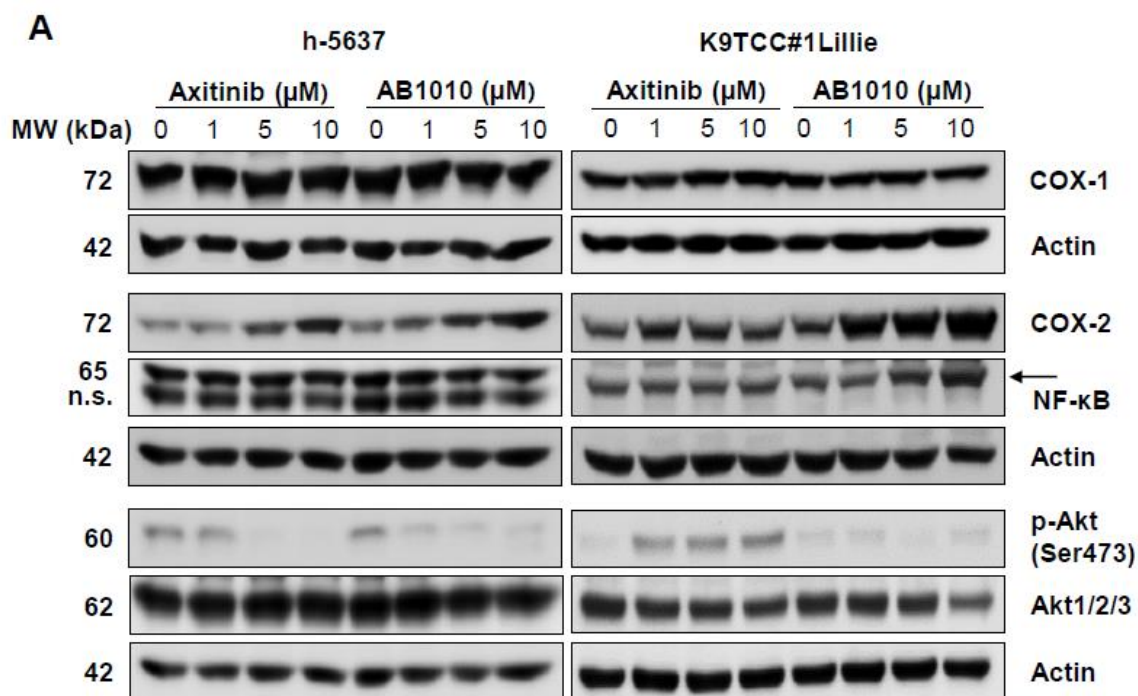


Figure 2.6

Figure 2.7 Co-treatment of AB1010 with indomethacin inhibited cell viability and AB1010-induced COX-2 expression in bladder TCC cells.

(A) Human 5637 and K9TCC#1Lillie cells were treated either with 5 μ M AB1010 (AB), or 5, 10, 50, and 100 μ M indomethacin (Indo), or combination treatments AB + Indo. Cell viability of bladder TCC cells was determined by MTS assay, and relative cell growth rates were normalized to the controls. Co-treatment of AB1010 with indomethacin inhibited cell viability more effectively than either treatment alone in bladder cancer cells. Values represent mean $\pm$ S.E. of four replicates of three independent experiments; paired Student's *t*-test was used to compare the treatment to control groups, **p* < 0.05, ***p* < 0.01, and ****p* < 0.001. Student's *t*-test was used to compare AB + Indo to AB treatment groups, #*p* < 0.05, ##*p* < 0.01, and ###*p* < 0.001. Student's *t*-test was used to compare AB + Indo to Indo treatment groups, †*p* < 0.05, ††*p* < 0.01, and †††*p* < 0.001. **(B)** Human 5637 and K9TCC#1Lillie cells were co-treated with 50 μ M indomethacin and 5 μ M AB1010, or each drug alone for 24 h. The expression of COX-1, COX-2, NF- κ B, p-Akt (Ser473), Akt1/2/3, and cleaved PARP (cPARP) proteins were determined by WB analysis. Actin was used as a loading control. AB1010-induced COX-2 expression was inhibited by co-treatment with indomethacin in h-5637 and K9TCC#1Lillie cells. **(C)** Densitometry analysis of COX-2, p-Akt (Ser473), and cPARP protein bands from WB analysis was performed using VisionWorks analysis software. Values represent the mean $\pm$ S.E. of the measured densitometry of each protein band of three independent experiments. Paired Student's

t-test was used to compare AB, Indo, and AB + Indo treatments to control groups,

p* < 0.05, *p* < 0.01, ****p* < 0.001.

A

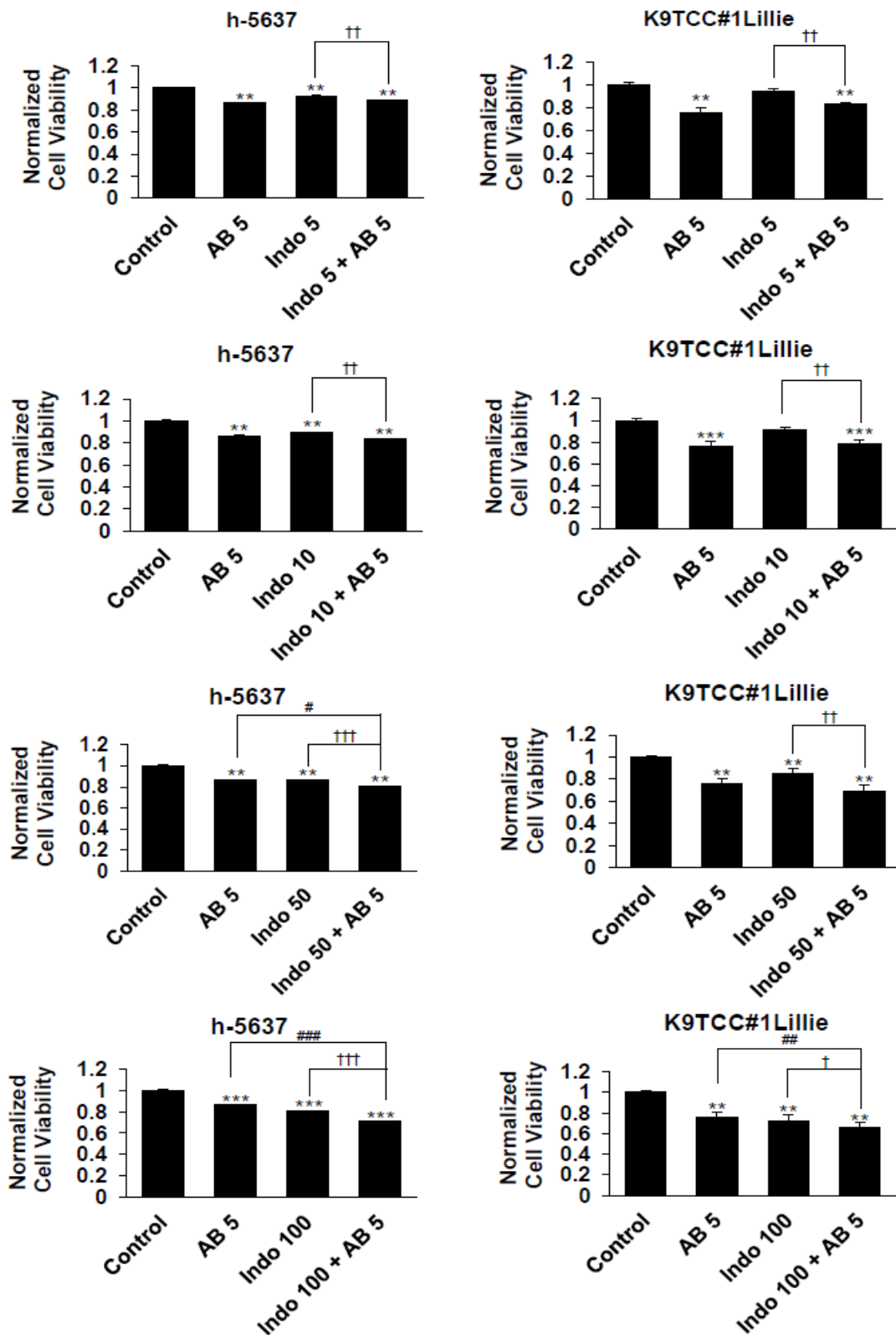


Figure 2.7

B

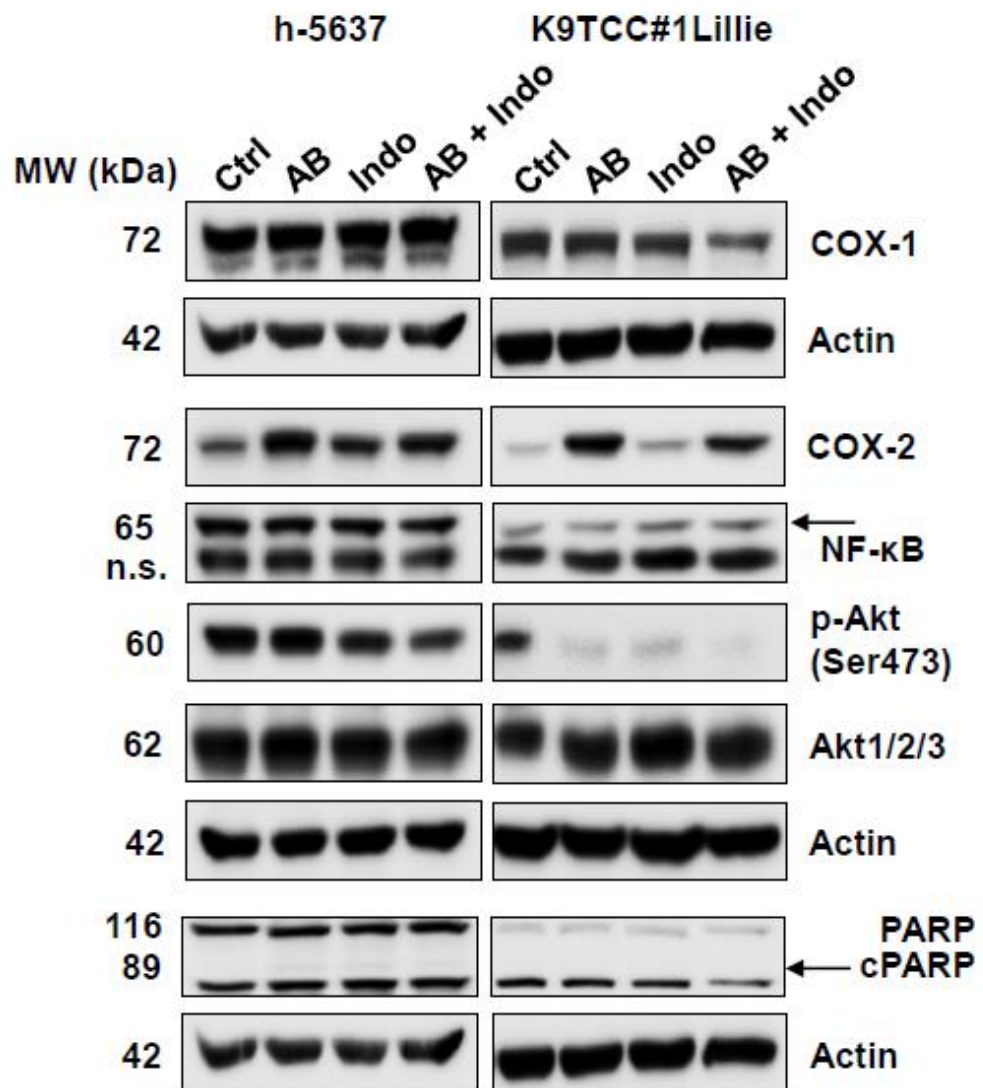


Figure 2.7 (continued)

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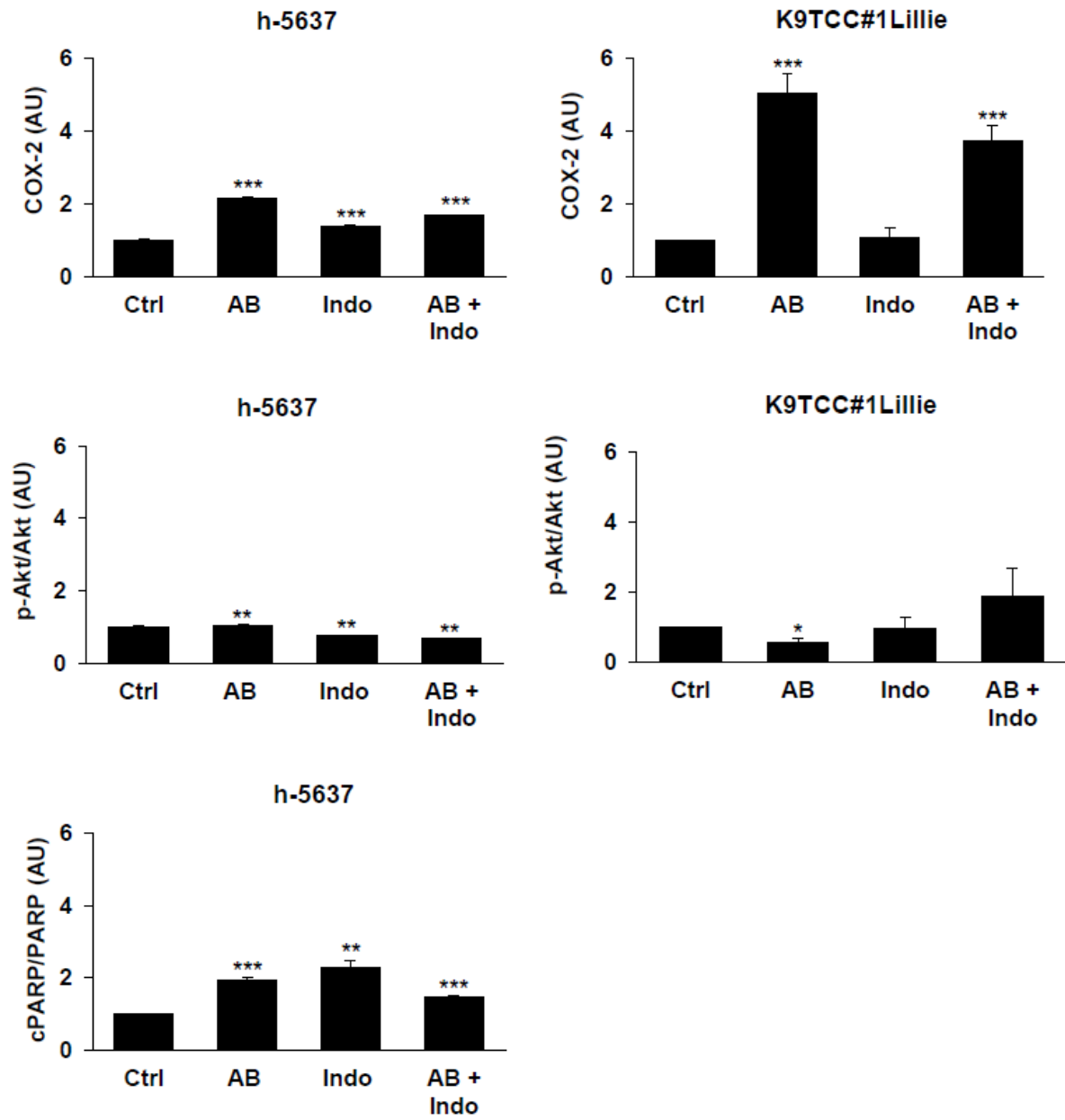


Figure 2.7 (continued)

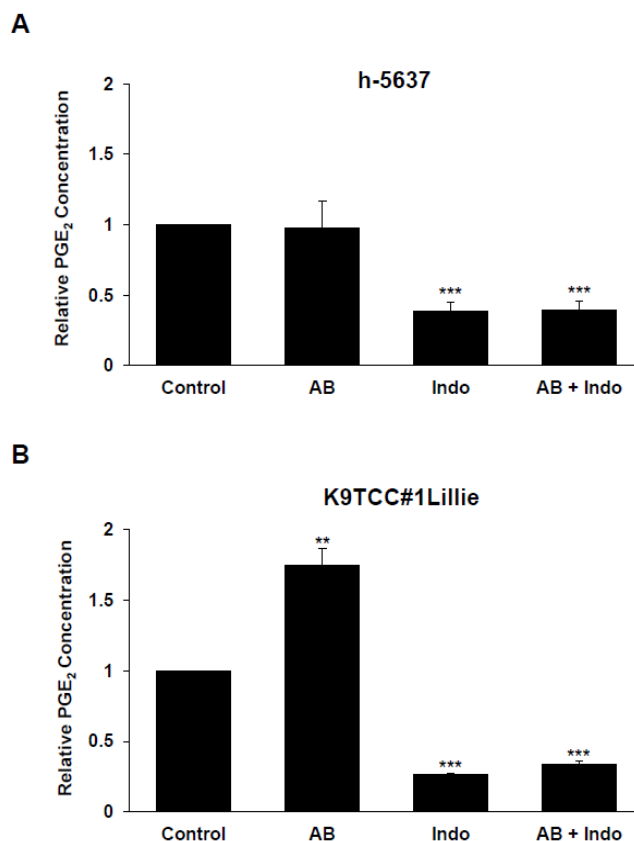


Figure 2.8 Co-treatment of AB1010 with Indo inhibited PGE₂ levels produced by bladder TCC cells.

(A) Human 5637 and **(B)** K9TCC#1Lillie cells were treated with 5 μ M AB1010 (AB) and 50 μ M indomethacin (Indo) alone or in combination (AB + Indo). PGE₂ levels in cell culture media collected 24 h after treatments were determined by ELISA. Indomethacin significantly reduced production of PGE₂ by bladder TCC cells. Co-treatment of AB1010 with indomethacin significantly decreased the AB1010-induced PGE₂ levels produced by K9TCC#1Lillie cells. Values represent mean $\pm$ S.E. of six replicates of three independent experiments; paired Student's *t*-test was used to compare treatments to the control groups, **p* < 0.05 and ****p* < 0.001.

CHAPTER III DETECTION OF CARCINOGEN-INDUCED COX-2- EXPRESSING BLADDER CANCER BY FLUOROCOXIB A

Research article described in this chapter is a slightly modified version of an article that was prepared for submission to the *American Association for Cancer Research journal, Cancer Research*, by Jennifer Bourn, Robert Donnell, Jashim Uddin, Lawrence Marnett, and Maria Cekanova in October 2018.

Detection of carcinogen-induced Cox-2-expressing bladder cancer by fluorocoxib A.
Jennifer Bourn, Robert Donnell, Jashim Uddin, Lawrence Marnett, and Maria Cekanova
Cancer Research (Manuscript in Progress)

In this paper, “our” or “we” refers to my co-authors and me. My contribution to this manuscript includes: 1) Compiling and interpretation of literature, 2) Providing comprehensive structure to the paper, 3) Acquisition and analysis of data, and 4) Writing and editing of the manuscript.

Detection of carcinogen-induced Cox-2-expressing bladder cancer by fluorocoxib

A.

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Running title: Monitoring bladder carcinogenesis.

Keywords: Bladder cancer, optical imaging, Cox-2, carcinogenesis, fluorocoxib A

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ABSTRACT

Conventional optical imaging technologies can detect advanced stages of bladder cancer; however, they have several limitations to detect bladder cancer at the early stages. Fluorocoxib A, a rhodamine-conjugated analog of indomethacin, is a novel fluorescent imaging agent that selectively targets cyclooxygenase-2 (Cox-2)-expressing cancers. In this study, we have used a well-established *N*-butyl-*N*-4-hydroxybutyl nitrosamine (BBN)-induced bladder cancer mouse model that strongly resembles human low-grade papillary and high-grade invasive urothelial carcinoma. We evaluated the ability of fluorocoxib A to detect the progression of carcinogen-induced bladder cancer in mice. Specific fluorocoxib A uptake by Cox-2-expressing bladder tumors was detected *ex vivo* using the IVIS Lumina imaging system. After *ex vivo* imaging, the progression of bladder carcinogenesis from normal urothelium to hyperplasia, carcinoma *in-situ*, and carcinoma with increased inflammation was assessed by H&E staining, increased Ki67, and decreased uroplakin-1A expression. The specific uptake of fluorocoxib A correlated with increased Cox-2 expression in bladder urothelial cells. In conclusion, fluorocoxib A detected the progression of bladder carcinogenesis in a mouse model with selective uptake in Cox-2-expressing bladder cancer in contrast to normal bladder urothelium where no fluorocoxib A uptake was detected. Fluorocoxib A is a novel, targeted optical imaging agent that could be applied for the detection of Cox-2 expressing human bladder cancer.

INTRODUCTION

Bladder cancer is the 6th most common type of cancer with an estimated 80,000 newly diagnosed cases and 17,000 deaths per year in the United States (1). Bladder cancer incidence is 4 times higher in men than in women. The most common type of bladder cancer is urothelial carcinoma, also known as transitional cell carcinoma, which accounts for over 90% of all bladder cancer cases in the United States. There are five stages of bladder cancer, with stage IV being the most advanced metastatic disease stage. Stages 0 and I are classified as non-muscle invasive bladder papillary carcinoma or flat carcinoma-in-situ (CIS) (NMIBC). Stages II – IV are more advanced stages, as the cancer has progressed through the muscle layer of the bladder wall, to surrounding local pelvic, and later to distal organs (muscle-invasive bladder cancer, MIBC) (2).

Treatment management depends on whether the bladder cancer is NMIBC or MIBC. Currently the gold-standard treatment for MIBC is open radical cystectomy (ORC) followed by adjuvant platinum-based chemotherapy (3,4). Due to high complications and invasiveness of ORC, less invasive procedures have been explored, including a laparoscopic radical cystectomy (5,6). Adjuvant therapies include standard chemotherapy (mitomycin C or gemcitabine) or immunotherapy (Bacille Calmette-Guérin) either alone or in combination (7-9). Standard treatment for NMIBC is a transurethral resection of bladder tumor (TURBT) followed by intravesical adjuvant therapy due to high risk of recurrence and progression of bladder cancer after procedure. The detection of bladder cancer at the early stages and more accurate detection of cancer during TURBT procedures is needed to improve patient outcomes.

Conventional optical imaging technologies can detect advanced stages of bladder cancer; however, they have several limitations to detect the early stages of bladder cancer (10). Newer technologies have been developed to significantly improve the quality of detection initial cancerous lesions and tumor margins TURBT procedures for prevention of disease recurrence and progression (11,12). There are four primary novel optical imaging technologies that have recently been investigated for clinical diagnostic use: fluorescence cystoscopy/photodynamic diagnosis (PDD), narrow band imaging (NBI), confocal laser endomicroscopy (CLE), and optical coherence tomography (OCT) (13,14). Fluorescence cystoscopy/PDD and NBI are used as diagnostic tools to better visualize the tumors and optimize detection of early stage bladder cancer lesions. On the contrary, CLE and OCT are used to further character the detected lesions to improve accuracy in determining the grade and stage of the lesions. For the purposes of this study, we will focus on the fluorescence cystoscopy technology.

White light cystoscopy (WLC) is the current standard of care for the detection of papillary or large growths lesions in the bladder. WLC has been used for several decades to detect bladder tumors but there are several limitations associated with WLC, including the inability to detect early-stage bladder cancer (CIS and low-grade tumors), as well as the inability to detect tumor margins during resection procedures leading to incomplete resection of the tumor (10). Fluorescent cystoscopy requires the administration of a contrast agent, which selectively binds to the cancer cells to improve visualization and differentiation of the cancer from normal tissue during resection procedures (15). Photodynamic diagnosis/blue-light cystoscopy (BLC) is an FDA-

approved procedure, which requires the administration of 5-aminolevulinic acid (5-ALA) dye directly into the bladder (16,17). The dye is absorbed by the cancerous tissue and emits a red color under blue light allowing the better visualization of the tumor during the cystoscopy procedure. Previous studies indicates that BLC can detect bladder tumors more effectively than WLC, at both early and late stages (18-20).

Cyclooxygenase-2 (Cox-2) is aberrantly expressed in bladder cancer and is one of the key proteins responsible for angiogenesis (21,22) and tumorigenesis (23,24). Increased Cox-2 expression has also been reported to be correlated with tumor grade in bladder cancer (25). The overexpression of Cox-2 in bladder cancer tissue be used as a biomarker for the detection bladder cancer. Fluorescently-labeled Cox-2 inhibitors are suitable candidates for targeted optical, non-radioactive imaging due to their selective uptake by cancer cells. Fluorocoxib A is a rhodamine-conjugated analog of indomethacin that selectively targets Cox-2 in solid tumors (26). Fluorocoxib A has been validated previously for the detection of LPS-induced inflammation in a rat model (26) and in Cox-2-expressing cancer *in vitro* (27) and *in vivo* (28,29).

In this study, we evaluated fluorocoxib A for detection of the early stages of Cox-2-expressing, carcinogen-induced bladder cancer in immunocompetent mice. We validated the specificity of fluorocoxib A to detect both the early and late stages of Cox-2-expressing bladder cancer *in vivo*. Our results indicate that fluorocoxib A can specifically detect Cox-2-expressing bladder cancer and has the potential to be used as an optical imaging agent during cystoscopy and TURBT procedures for human bladder cancer.

MATERIALS AND METHODS

Antibodies and reagents

The antibodies for UP1a (C-18, sc-15173) and actin (C-11, sc-1615) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA); antibody for Ki67 (SP6, ab16667) was purchased from Abcam Inc. (Cambridge, MA); antibody for Cox-2 (aa 570-598, 160106) was purchased from Cayman Chemical (Ann Arbor, MI); and secondary anti-rabbit antibody was obtained from Cell Signaling Technology (Danvers, MA). All other chemicals and reagents were purchased from Thermo Fisher Scientific (Pittsburgh, PA), unless otherwise specified.

Animals

All animal experiments were performed in accordance with approved UT IACUC protocols. Thirty 5-wk old, female B6D2F1 mice (Taconic, Hudson, NY) were randomly divided into three groups (n=10/group). Group 1 served as the control group of mice that received only tap drinking water. The other two groups of mice were treated with BBN for 12 weeks (Group 2) and 18 weeks (Group 3). Carcinogen N-butyl-N-(4-hydroxybutyl) nitrosamine (BBN) was obtained from Sigma-Aldrich (St. Louis, MO) and was supplied *ad libitum* at 0.05% in drinking water to mice for 12 or 18 weeks, respectively.

Fluorocoxib A uptake detection by IVIS Lumina optical imaging system

After the treatment with BBN at 12 and 18 weeks, respectively, fluorocoxib A (1mg/kg) was administered subcutaneously (SC). Specific fluorocoxib A uptake was

detected 4 h after administration by whole body imaging performed using the Xenogen IVIS Lumina optical imaging system with DsRed filters with excitation 500 to 550 nm, emission 575 to 650 nm, and background 460 to 490 nm. After imaging, the mice were sacrificed, tissues were dissected, and imaged *ex vivo*. The obtained total flux (p/s) and average radiant efficiency [p/s/cm²/sr]/ [μW/cm²] of labeled regions of interest of dissected bladder and other tissues (blood, kidney, liver, lung, heart, muscle, spleen, pancreas, and fat) were evaluated. Tumor-to-noise ratio (TNR) values were generated by dividing the average radiant efficiency of the bladder tissue to blood as the background fluorescence (noise). After imaging, each dissected bladder was divided into three pieces. One piece of bladder was fixed in 10% neutral buffered formalin for histology and immunohistochemistry (IHC) analysis. The second piece of bladder was placed into O.C.T. media for immunofluorescence (IF) detection of fluorocoxib A uptake by neoplastic tissue using fluorescence microscopy and the third piece of bladder was kept in RNA*later* solution and stored at -80°C until Western blotting (WB) analysis was performed.

Immunohistochemistry

Dissected tissues from mice were formalin-fixed, paraffin-embedded, and sectioned at 7 μm. Hematoxylin and eosin (H&E) was performed following standard histological staining protocol. The IHC staining was performed as described previously (29). After de-paraffinization, the antigen retrieval using sodium citrate pH 6.0 was performed for 20 min in antigen retriever (Electron Microscopy Sciences, Hatfield, PA).

The blocking of endogenous peroxidase activity was performed by 3% H₂O₂ in 50% methanol for 5 min, followed by the blocking of non-specific signal using protein block solution (BioGenex, Fremont, CA). Tissues were incubated with primary antibodies (Ki67, UP1a, and Cox-2), followed by the incubation with the specific biotinylated secondary antibodies, streptavidin/HRP detection system, and visualized by 3,3'-diaminobenzidine (DAB) staining. Nuclei were counter-stained with hematoxylin and slides were cover-slipped and evaluated using a Leitz DMRB microscope. The images were captured by a DP73 camera attached to microscope using CellSens Standard software (Olympus, Pittsburgh, PA).

Western blotting analysis

The tissue samples were lysed in ice-cold RIPA buffer supplemented with a protease and phosphatase inhibitors cocktail (1 mM PMSF; 1 µg/ml aprotinin; 1 µg/ml leupeptin; 5 mM Na₃VO₄; 5 mM NaF) and briefly sonicated on ice. Protein concentrations were measured using Pierce® BCA protein assay (Thermo Scientific, Rockford, IL). Equal amount of proteins (50 µg) were loaded onto SDS-PAGE gels and transferred to nitrocellulose membranes. After blocking, the membranes were incubated with primary antibodies overnight at 4°C, followed by incubation with horseradish peroxidase-conjugated secondary antibodies (1: 3,000 dilution) for 1 h at room temperature. The immuno-reactive bands were visualized using the ECL prime chemiluminescence system (GE Healthcare Life Sciences, Marlborough, MA) and the images were captured using the BioSpectrum® 815 imaging system (UVP, Upland,

CA). Densitometry analysis was performed using the VisionWorks acquisition and analysis software (UVP).

Statistical analysis

Statistical analysis was conducted using the paired Student's *t*-test to establish significant differences among treatment groups. Results were considered statistically significant at $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$.

RESULTS

BBN-induced bladder cancer mouse model

Thirty female 5-week old B6D2F1 mice were randomly divided into three groups (n=10/group). Group 1, served as the control and received only tap water for 18 weeks (Group 1 – 18wks H₂O). The other two groups were exposed to the carcinogen, BBN for 12 weeks (Group 2 – 12wks BBN) and 18 weeks (Group 3 – 18wks BBN). BBN was administered *ad libitum* at 0.05% in drinking water (Fig. 3.1A). Body weight of each mouse was recorded weekly and average weight per mouse (g) is presented in Fig. 3.1B. BBN had no adverse effect on the growth of the mice over time as no remarkable differences in body weight was observed between groups. Water consumption (ml) was recorded weekly and compared between groups (Fig. 3.1C). There was significant increase in water consumption for Group 2 and Group 3 when compared to the control (Group 1). Values represent mean $\pm$ standard error of each weekly recording from n=10 mice; paired Student's *t*-test, $***p < 0.001$.

Fluorocoxib A uptake by BBN-induced bladder cancer *in vivo*

After BBN exposure over time, fluorocoxib A was administered subcutaneously (1 mg/kg) followed by imaging of the mice. Imaging of the whole body and dissected tissues was performed using the Xenogen IVIS Lumina optical imaging system to detect fluorocoxib A uptake. The control group (Group 1 – 18wks H₂O) was imaged at the same time as Group 3 (18wks BBN). After euthanasia, whole body photographs were obtained of the individual mice bladders (Fig 3.2A). As shown by the representative images, the bladders from mice in Group 2 and Group 3 were larger when compared to the control group mice (Group 1). The dissected tissues were photographed and imaged *ex vivo* (Fig. 3.2B). Higher uptake and increased intensity of fluorocoxib A was observed in Group 3 when compared to the Group 1 (control) and Group 2 as shown in Fig. 3.2C. No fluorocoxib A uptake was detected in the bladders from control mice (Group 1). Moderate uptake of fluorocoxib A was observed in both the liver and muscle tissues of all three groups. Obtained average radiant efficiencies ($[p/s/cm^2/sr]/[\mu W/cm^2]$) of each organ were plotted (Fig. 3.2D). There is an observed increase in average radiant efficiency in the bladders of mice from Group 3 when compared to the control, indicating an increased uptake of fluorocoxib A. Average radiant efficiencies values of bladders were normalized to blood (TNR) and fold changes in fluorocoxib A uptake were plotted (Fig. 3.2E). The significant 1.8- and 3.2-fold increase in fluorocoxib A uptake was observed in bladders from mice in Group 2 and 3, respectively, when compared to bladders from untreated mice (Group 1).

Progression of bladder carcinogenesis by BBN

The histopathology of the dissected bladder tissues was assessed by H&E and IHC staining for detection of Ki67 and uroplakin-1A (UP1a) expression (Fig. 3.3). The BBN-induced bladder cancer progression from normal urothelium (Group 1) to hyperplasia, (Group 2) and to invasive carcinoma (Group 3) with presence of intensive inflammation in bladder of the mice as confirmed by H&E staining (left). The histological analysis of the H&E sections of the bladder tissue from each mouse was recorded to quantify the prevalence of BBN-induced inflammation, hyperplasia, CIS, and carcinoma among the experimental groups (Table 3.1). Mice from Group 2 (12wks BBN) and Group 3 (18wks BBN) had increased incidence of inflammation, hyperplasia, and bladder carcinoma when compared to mice from Group 1 (control). Tables 3.2 and 3.3 provide the description of the scoring summary used for histology evaluation of the bladder tissues and the individual evaluation of each mouse from all treatment groups. The progression of bladder carcinogenesis was also confirmed by the presence of increased expression of Ki67 expressing cells in bladder carcinomas from mice treated with BBN – Group 2 and Group 3 (middle). In addition, UP1a, a protein that is highly expressed in normal bladder urothelium (Group 1), was downregulated in BBN-induced bladder cancer (Group 2 and 3) as confirmed by IHC analysis (right). Fluorocoxib A uptake correlated with presence of neoplastic lesions in the bladders of mice exposed to BBN compared to mice in the control group.

Upregulation of Cox-2 by BBN in bladder carcinoma

The upregulation of Cox-2 in BBN-induced bladder cancer was detected by IHC (Fig. 3.4A) and WB analysis (Fig. 3.4B). Bladder carcinomas in mice from Group 3 (n=10; 18wks BBN) had significantly higher Cox-2 expression when compared to normal urothelium in mice from Group 1 (n=7; control) and bladder inflammation and hyperplasia in mice from Group 2 (n=9; 12wks BBN). This result was also confirmed by WB analysis of the dissected bladder tissues from each group. The tissues from Group 2 and Group 3 had higher Cox-2 expression when compared to the control (Group 1) where there was no detectable Cox-2 expression. Densitometry analysis of Cox-2 protein bands from WB analysis was performed using VisionWorks acquisition and analysis software (UVP) (Fig. 3.4C). There is a significant 2.7- and 8.6-fold increase in Cox-2 expression for Group 2 and Group 3, respectively, when compared to Group 1. Paired Student's *t*-test was used to compare the upregulation of Cox-2 expression in BBN-treated groups (Group 2 and 3) with control group (Group 1), $*p < 0.05$. The specific uptake and increased intensity of fluorocoxib A (Fig. 3.2) in carcinogen-induced bladder cancer of mice exposed to BBN correlated with the progression of bladder carcinogenesis (Fig. 3.3) and with the increased Cox-2 expression (Fig. 3.4).

DISCUSSION

Bladder cancer is the 6th most common type of cancer in the United States, with higher incidence in men than in women and is one of the most expensive malignancies to treat (1). Current treatment options for patients diagnosed with bladder cancer is

dependent on the stage of cancer. The standard treatment regime for bladder cancer is typically a TURBT procedure followed by adjuvant therapy (4). There is a high risk of recurrence and progression following TURBT procedures driving the need for improved diagnostic tools to aid in improved detection and prevention of bladder cancer recurrence. This study demonstrates the visualization of Cox-2-expressing, carcinogen-induced early and late stage bladder cancer in mice *in vivo* by use of the novel optical imaging agent, fluorocoxib A.

There are several models currently available for the study of bladder carcinogenesis, including genetically altered rodents that can develop spontaneously occurring tumors or the use of carcinogens to induce tumors in the rodents (30,31). Nitrosamines are a highly carcinogenic group of compounds that have been known to induce hepatic, gastric, and bladder cancer (32,33). The most common nitrosamine used for the induction of bladder cancer is N-butyl-N-(4-hydroxybutyl) (BBN), which is found in cigarette smoke (34). After ingestion, BBN is metabolized by the liver into several metabolites, which are further excreted from the body through urine. In the bladder, the metabolites come in contact with the urothelium and initiate the carcinogenic process resulting in DNA damage and development of both the early (hyperplasia and CIS) and late stages (MIBC) bladder cancer in immunocompetent mice (35,36). Bladder cancer in mice develops relatively early after BBN exposure with early-stage tumors developing after 12 weeks of exposure and late-stage tumors presenting after 18 weeks of exposure. To ensure the development of heterogeneous bladder tumor phenotypes, 30 B6D2F1 mice were randomly divided into 3 groups (n=10

mice/group). BBN is administered orally either in drinking water or by oral gavage at doses that range from 0.01-0.05% (37). As shown in Fig. 3.1A, 0.05% BBN was administered *ad libitum* in drinking water for 12 (Group 2) and 18 (Group 3) weeks. Despite significant increase in water consumption by the treatment groups compared to the control group, the prevalence of inflammation and tumor development within the treatment groups was not known until after euthanizing the mice from each group and histopathological analysis was performed.

Fluorescence cystoscopy is an attractive modification to WLC procedures because this technique allows for the visualization of the accumulation of the contrast agent in the cancerous cells. Previous studies indicate better diagnostic outcomes for patients with NMIBC using fluorescent cystoscopy than with WLC alone (38,39). There are several limitations to this method; however, including rapid photo-bleaching during the cystoscopy procedure, high false-positive rate (up to 30%), and this technique has not been approved for repeat procedures in patients as there is limited data to support how patients will react to the contrast agent after repeat exposures (40,41). The photosensitizing agent administered before the cystoscopy procedures, while it is selective for the increased blood flow to the tumor sites, the agent is not directly selective for the cancer cells. Increased inflammation following intravesical adjuvant therapies has been demonstrated to be correlated with false-positive rates of detection after fluorescent cystoscopy procedures (40,42). The non-selective properties of the contrast agent can contribute to an increase in false positive detection rates. These limitations drive the need for a selective contrast agent that will bind directly to the

cancer cells and decrease the false-positive rates while improving the detection of the neoplastic lesions in the bladder. The development of targeted optical imaging agents for preclinical and clinical studies has shifted the direction of current imaging research to further improve diagnostic detection methods for patients diagnosed with cancer to better patient prognosis. Fluorocoxib A, which is a Cox-2-selective imaging agent, is a prime candidate for the detection of bladder cancer during cystoscopy procedures due to its highly stable properties, ease of administration, and non-toxic effects. Previous preclinical studies indicate that fluorocoxib A has demonstrated to be highly specific and selective for detecting Cox-2-expressing inflammation and cancers, including bladder and non-melanoma skin cancer (26-29).

In this study following BBN exposure, fluorocoxib A was administered subcutaneously followed by *ex vivo* imaging of the BBN-induced bladder tumors in mice. Higher uptake and increased intensity of fluorocoxib A was observed in Group 3 (18wks BBN) when compared to Group 1 (control) and Group 2 (12wks BBN), which also correlated with the progression of bladder cancer (Fig. 3.2C & D). Moderate uptake of fluorocoxib A was observed in both the liver and muscle tissue of all three groups. Non-specific uptake in these tissues is indicative of the breakdown of fluorocoxib A in the mice (26). The significant 1.8- and 3.2-fold increase in fluorocoxib A uptake was observed in bladders from mice in Group 2 and 3, respectively, when compared to the bladders from untreated mice (Group 1) (Fig. 3.2E). These results demonstrate the specificity of fluorocoxib A uptake by the cancerous bladder tissue when compared to normal urothelium (Group 1) where no fluorocoxib A uptake was recorded. Furthermore,

the range of fluorocoxib A uptake between the groups indicates the ability of fluorocoxib A to detect different stages of bladder tumors, including early and late stage tumors.

The BBN-induced bladder cancer progression from normal urothelium (Group 1), to inflammation and hyperplasia (Group 2), and to invasive carcinoma (Group 3) in mice was confirmed by H&E staining (Fig. 3.3 - left). As shown in table 1, all bladder tissue from mice in Group 2 (12wks BBN) displayed signs of inflammation and the majority had signs of early stage bladder cancer (hyperplasia and CIS). All bladder tissue from mice in Group 3 (18wks BBN), displayed signs of inflammation and hyperplasia as well as developed bladder carcinomas. These results demonstrate a heterogeneous tumor population induced by BBN exposure and are consistent with previous BBN studies (32,34,36). Ki67 is a cellular marker associated with proliferation and is present in all cell cycle phases. The fraction of Ki67 positive tumor cells is often correlated with patient prognosis and aids in determining clinical course of treatment. The progression of bladder carcinogenesis was confirmed by the presence of increased expression of Ki67 positive cells in bladder tissues from mice treated with BBN – Group 2 and Group 3 (Fig. 3.3 - middle). Uroplakin-1A (UP1a) is a member of a group of cell-surface proteins and is highly expressed in normal bladder urothelium (Group 1). UP1a plays a role in maintaining normal urothelium tissue and aids in tumor suppression. In this study, UP1a expression is downregulated in BBN-induced bladder cancers (Group 2 and 3) as confirmed by IHC analysis (Fig. 3.3 - right). The downregulation of UP1a indicates a loss of normal urothelium and change in morphology of the bladder tissues as the cancer progresses. The fluorocoxib A uptake correlates with the progression of

neoplastic lesions in the bladder, higher uptake in the treatment Groups 2 and 3, when compared to the control, Group 1.

The upregulation of Cox-2 expression by BBN in bladder tumors was confirmed by IHC and WB analysis (Fig. 3.4A & B). Developed bladder carcinomas in mice from Group 3 had significantly higher Cox-2 expression when compared to normal urothelium in mice from Group 1 and bladder inflammation and hyperplasia in mice from Group 2. There is a significant 2- and 8-fold increase in Cox-2 expression for Group 2 and Group 3, respectively, when compared to Group 1 (Fig. 3.4C). These results are consistent with the protein expression profile of Cox-2, where Cox-2 is not expressed in normal tissue but is easily inducible during periods of inflammation and cancer. Cox-2 plays a key role in modulating cellular proliferation, apoptosis, and tumor invasion. The upregulation of Cox-2 may also be indicative of poor patient prognosis. These results also correlate with the increased uptake of fluorocoxib A in the bladders of mice that were positive for inflammation, hyperplasia, and developed carcinomas. Taken together, the results of this study demonstrate the selective and specific properties of fluorocoxib A uptake in Cox-2-expressing tissues.

In conclusion, the specific uptake and increased intensity of fluorocoxib A (Fig. 3.2) in the carcinogen-induced bladder cancers of mice treated with BBN *in vivo* correlated with the progression of bladder carcinogenesis (Fig. 3.3) and with the increased Cox-2 expression (Fig. 3.4). Currently, conventional imaging technologies for the detection of bladder cancer have several limitations, primarily lacking the ability to detect early stage bladder cancer and poor visualization of tumor margins during

resection procedures. The development of novel techniques and imaging agents, such as fluorocoxib A, can greatly improve patient prognosis by aiding in both the diagnostic and treatment procedures. Further molecular imaging studies need to be conducted to evaluate fluorocoxib A during cystoscopy procedures in different animal models but here we have demonstrated the ability of fluorocoxib A to detect both early stage and late stage Cox-2-expressing bladder cancer using a carcinogen-induced bladder cancer mouse model.

Authors' Contributions

Conception and design: M. Cekanova

Development of methodology: M. Cekanova

Acquisition of data: J. Bourn, M. Cekanova

Analysis and interpretation of data: J. Bourn, M. Cekanova, R. Donnell

Writing, review, and/or revision of the manuscript: J. Bourn, M. Cekanova, R. Donnell

Administrative, technical, or material support: M. Cekanova, J. Uddin, L. Marnett

Study supervision: M. Cekanova

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REFERENCES

1. N. C. Institute. (2016).
2. A. C. Society, K. Eidsmoe, Ed. (2016), vol. 2018.
3. C. Advanced Bladder Cancer Meta-analysis, Neoadjuvant chemotherapy in invasive bladder cancer: a systematic review and meta-analysis. *Lancet* **361**, 1927-1934 (2003).
4. H. B. Grossman *et al.*, Neoadjuvant chemotherapy plus cystectomy compared with cystectomy alone for locally advanced bladder cancer. *N Engl J Med* **349**, 859-866 (2003).
5. I. S. Gill *et al.*, Laparoscopic radical cystectomy and continent orthotopic ileal neobladder performed completely intracorporeally: the initial experience. *J Urol* **168**, 13-18 (2002).
6. N. D. James *et al.*, Radiotherapy with or without chemotherapy in muscle-invasive bladder cancer. *N Engl J Med* **366**, 1477-1488 (2012).
7. R. J. Sylvester, M. A. van der, D. L. Lamm, Intravesical bacillus Calmette-Guerin reduces the risk of progression in patients with superficial bladder cancer: a meta-analysis of the published results of randomized clinical trials. *J Urol* **168**, 1964-1970 (2002).
8. R. Colombo *et al.*, A new approach using local combined microwave hyperthermia and chemotherapy in superficial transitional bladder carcinoma treatment. *J Urol* **153**, 959-963 (1995).

9. M. D. Shelley, A. Cleves, T. J. Wilt, M. D. Mason, Gemcitabine chemotherapy for the treatment of metastatic bladder carcinoma. *BJU Int* **108**, 168-179 (2011).
10. P. Jichlinski, H. J. Leisinger, Fluorescence cystoscopy in the management of bladder cancer: a help for the urologist! *Urol Int* **74**, 97-101 (2005).
11. M. A. D'Hallewin, L. Bezdetnaya, F. Guillemin, Fluorescence detection of bladder cancer: a review. *Eur Urol* **42**, 417-425 (2002).
12. D. Jocham, H. Stepp, R. Waidelich, Photodynamic diagnosis in urology: state-of-the-art. *Eur Urol* **53**, 1138-1148 (2008).
13. J. J. Liu, M. J. Droller, J. C. Liao, New optical imaging technologies for bladder cancer: considerations and perspectives. *J Urol* **188**, 361-368 (2012).
14. A. Kolodziej, W. Krajewski, M. Matuszewski, K. Tupikowski, Review of current optical diagnostic techniques for non-muscle-invasive bladder cancer. *Cent European J Urol* **69**, 150-156 (2016).
15. C. R. Riedl *et al.*, Fluorescence endoscopy with 5-aminolevulinic acid reduces early recurrence rate in superficial bladder cancer. *J Urol* **165**, 1121-1123 (2001).
16. J. Leveckis, J. L. Burn, N. J. Brown, M. W. Reed, Kinetics of endogenous protoporphyrin IX induction by aminolevulinic acid: preliminary studies in the bladder. *J Urol* **152**, 550-553 (1994).
17. M. Kriegmair *et al.*, Fluorescence photodetection of neoplastic urothelial lesions following intravesical instillation of 5-aminolevulinic acid. *Urology* **44**, 836-841 (1994).

18. G. Mowatt *et al.*, Photodynamic diagnosis of bladder cancer compared with white light cystoscopy: Systematic review and meta-analysis. *Int J Technol Assess Health Care* **27**, 3-10 (2011).
19. M. Kriegmair *et al.*, Detection of early bladder cancer by 5-aminolevulinic acid induced porphyrin fluorescence. *J Urol* **155**, 105-109; discussion 109-110 (1996).
20. D. Zaak *et al.*, Endoscopic detection of transitional cell carcinoma with 5-aminolevulinic acid: results of 1012 fluorescence endoscopies. *Urology* **57**, 690-694 (2001).
21. K. M. Leahy *et al.*, Cyclooxygenase-2 inhibition by celecoxib reduces proliferation and induces apoptosis in angiogenic endothelial cells in vivo. *Cancer Res* **62**, 625-631 (2002).
22. S. Gately, W. W. Li, Multiple roles of COX-2 in tumor angiogenesis: a target for antiangiogenic therapy. *Semin Oncol* **31**, 2-11 (2004).
23. G. Gakis, The role of inflammation in bladder cancer. *Adv Exp Med Biol* **816**, 183-196 (2014).
24. T. Qayyum *et al.*, A prospective study of the role of inflammation in bladder cancer. *Curr Urol* **6**, 189-193 (2013).
25. M. Komhoff *et al.*, Enhanced expression of cyclooxygenase-2 in high grade human transitional cell bladder carcinomas. *Am J Pathol* **157**, 29-35 (2000).
26. M. J. Uddin *et al.*, Selective visualization of cyclooxygenase-2 in inflammation and cancer by targeted fluorescent imaging agents. *Cancer Res* **70**, 3618-3627 (2010).

27. M. Cekanova *et al.*, Molecular imaging of cyclooxygenase-2 in canine transitional cell carcinomas in vitro and in vivo. *Cancer Prev Res (Phila)* **6**, 466-476 (2013).
28. H. Ra *et al.*, Detection of non-melanoma skin cancer by in vivo fluorescence imaging with fluorocoxib A. *Neoplasia* **17**, 201-207 (2015).
29. M. Cekanova *et al.*, Single-dose safety and pharmacokinetic evaluation of fluorocoxib A: pilot study of novel cyclooxygenase-2-targeted optical imaging agent in a canine model. *J Biomed Opt* **17**, 116002 (2012).
30. R. J. van Moorselaar *et al.*, The rat bladder tumor model system RBT resembles phenotypically and cytogenetically human superficial transitional cell carcinoma. *Urol Res* **21**, 413-421 (1993).
31. H. C. Arentsen, K. Hendricksen, E. Oosterwijk, J. A. Witjes, Experimental rat bladder urothelial cell carcinoma models. *World J Urol* **27**, 313-317 (2009).
32. H. Druckrey *et al.*, [Selective Induction of Bladder Cancer in Rats by Dibutyl- and N-Butyl-N-Butanol(4)-Nitrosamine]. *Z Krebsforsch* **66**, 280-290 (1964).
33. S. S. Mirvish, Role of N-nitroso compounds (NOC) and N-nitrosation in etiology of gastric, esophageal, nasopharyngeal and bladder cancer and contribution to cancer of known exposures to NOC. *Cancer Lett* **93**, 17-48 (1995).
34. G. Akagi, A. Akagi, M. Kimura, H. Otsuka, Comparison of bladder tumors induced in rats and mice with N-butyl-N-(4-hydroxybutyl)-nitrosoamine. *Gan* **64**, 331-336 (1973).
35. C. Vasconcelos-Nobrega, A. Colaco, C. Lopes, P. A. Oliveira, Review: BBN as an urothelial carcinogen. *In Vivo* **26**, 727-739 (2012).

36. N. Ito, S. Fukushima, T. Shirai, K. Nakanishi, Effects of promoters on N-butyl-N-(4-hydroxybutyl)nitrosamine-induced urinary bladder carcinogenesis in the rat. *Environ Health Perspect* **50**, 61-69 (1983).
37. N. Ito, T. Shirai, S. Fukushima, M. Hirose, Dose-response study of urinary bladder carcinogenesis in rats by N-butyl-N-(4-hydroxybutyl)nitrosamine. *J Cancer Res Clin Oncol* **108**, 169-173 (1984).
38. F. Koenig *et al.*, Diagnosis of bladder carcinoma using protoporphyrin IX fluorescence induced by 5-aminolaevulinic acid. *BJU Int* **83**, 129-135 (1999).
39. P. Jichlinski *et al.*, Hexyl aminolevulinate fluorescence cystoscopy: new diagnostic tool for photodiagnosis of superficial bladder cancer--a multicenter study. *J Urol* **170**, 226-229 (2003).
40. M. Babjuk *et al.*, EAU Guidelines on Non-Muscle-invasive Urothelial Carcinoma of the Bladder: Update 2016. *Eur Urol* **71**, 447-461 (2017).
41. D. V. Zlatev, E. Altobelli, J. C. Liao, Advances in imaging technologies in the evaluation of high-grade bladder cancer. *Urol Clin North Am* **42**, 147-157, vii (2015).
42. E. R. Ray *et al.*, Hexylaminolaevulinate fluorescence cystoscopy in patients previously treated with intravesical bacille Calmette-Guerin. *BJU Int* **105**, 789-794 (2010).

APPENDIX

Table 3.1 Prevalence of BBN-induced inflammation, hyperplasia, carcinoma in situ (CIS), and carcinoma among treatment groups.

	Inflammation	Hyperplasia	CIS	Carcinoma
Group 1 – 18wks H₂O	2/8	2/8	0/8	0/8
Group 2 – 12wks BBN	9/9	5/7	2/7	2/7
Group 3 – 18wks BBN	10/10	10/10	3/10	6/10

Table 3.2 Description of scoring summary used for the histology evaluation of bladder tissue from mice.

	Score	Description
Inflammation	0	no presence of inflammation
	1	low presence of inflammatory cells
	2	moderate presence of inflammatory cells
	3	high presence of inflammatory cells
	N, L, P, M	neutrophils, lymphocytes, plasma cells, macrophages
Hyperplasia	0	no presence of hyperplasia (less than 2 cells in layer)
	1	low hyperplasia (between 3-5 cells in urothelial tissue)
	2	severe hyperplasia (more than 5 cells in layer)
	D, F, M	Diffuse, focal, multi-focal
Carcinoma in situ (CIS)	No	no CIS present
	Yes	CIS present
Carcinoma	No	no carcinoma present
	Yes	carcinoma present
	AC, SCC, TCC	Adenocarcinoma, squamous cell carcinoma, transitional cell carcinoma

Table 3.3 Histology evaluation of bladder tissue from individual mice among treatment groups.

	Mouse	Inflammation	Hyperplasia	Carcinoma in situ	Carcinoma
Group 1 – 18wks H₂O	1	1 (L)	0	0	0
	2	0	1 (F)	0	0
	3	3 (LF)	1(F)	0	0
	4	0	0	0	0
	5	0	0	0	0
	6	0	0	0	0
	7	0	0	0	0
	8†	-	-	-	-
	9†	-	-	-	-
	10	0	0	0	0
Group 2 – 12wks BBN	1*	-	-	-	-
	2	2 (NPL)	0	0	0
	3	1 (NL)	1 (M)	0	0
	4	3 (LP)	no epithelium	no epithelium	no epithelium
	5	3 (LP)	no epithelium	no epithelium	no epithelium
	6	2 (NPL)	2 (L)	yes	yes
	7	2 (LP)	1 (M)	0	0
	8	1 (L)	minimal epithelium	0	0
	9	1 (NPL)	1 (M)	0	0
	10	1 (NLP)	1 (M)	yes	yes
Group 3 – 18wks BBN	1	1 (LPN)	1 (D)	yes	0
	2	3 (PNL)	2 (M)	yes	yes
	3	2 (LP)	1 (+2)	0	0
	4	3 (LN)	1 (+2)	yes/ hyperplasia	0
	5	2 (L)	1	0	yes
	6	2 (L)	1	0	yes
	7	2 (LN)	1	0	yes
	8	2 (LN)	1 (+2)	0	yes
	9	3 (LNP)	1	0	yes
	10	2 (LN)	1 (D)	0	0

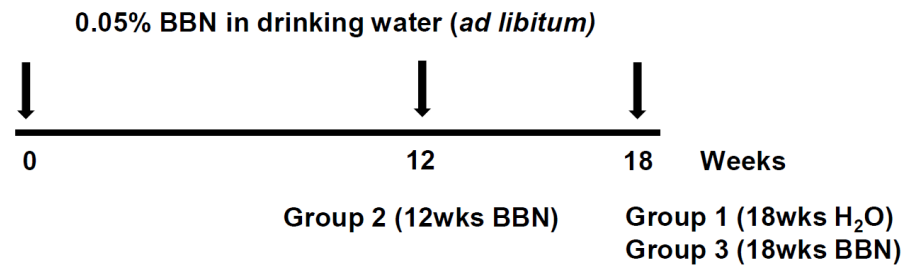
†No bladder tissue for histology evaluation

*Mouse died before the end of experiment

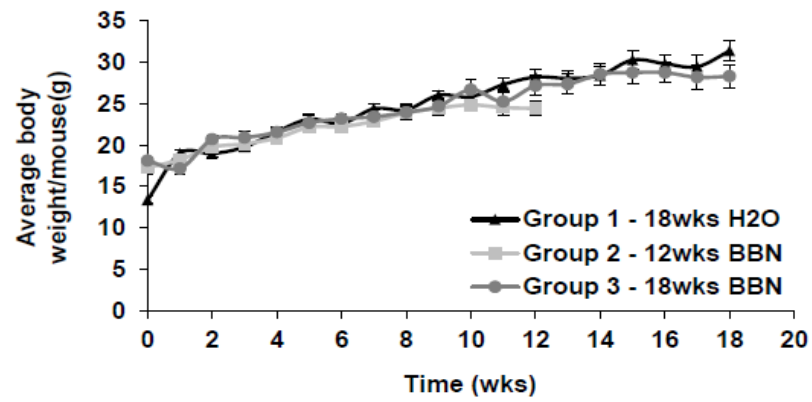
Figure 3.1 BBN-induced bladder cancer mouse model.

(A) B6D2F1 female mice were exposed to N-butyl-N-(4-hydroxybutyl) nitrosamine (BBN) in drinking water for 12 weeks (n=10; Group 2) and 18 weeks (n=10; Group 3). Mice without BBN for 18 weeks (n=10; Group 1) served as a control. After 12 and 18 weeks, mice were injected subcutaneously with Fluorocoxib A (1mg/kg) and imaged using the IVIS Lumina optical imaging system. **(B)** Body weight (g) measurements were recorded weekly to monitor the effect BBN consumption on growth. No remarkable differences in body weight among the different groups was seen. **(C)** Daily drinking water consumption (ml) per mouse was measured. There is a significant increase in water consumption for Group 2 and Group 3 when compared to the control (Group 1). Values represent mean $\pm$ standard error of each weekly body weight (g) and water consumption (ml) recording from n=10 mice; paired Student's *t*-test, ****p* < 0.001.

A



B



C

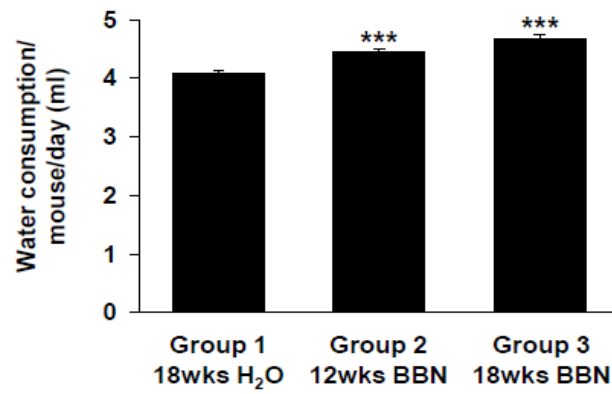


Figure 3.1

Figure 3.2 Fluorocoxib A uptake by BBN-induced bladder cancer *in vivo*.

After 4 h fluorocoxib A uptake, whole body and organ imaging was performed using the IVIS Lumina optical imaging system. **(A)** After sacrificing mice, representative images of mice bladders were obtained. **(B)** The dissected organs were photographed and imaged *ex vivo*. Organs from left to right: Row 1 - bladder, heart, lung; Row 2 - kidney, muscle, blood; Row 3 - liver, pancreas & spleen, fat. **(C)** Higher uptake and increased intensity of fluorocoxib A was observed in Group 3 when compared to the Group 1 (control). **(D)** Obtained average radiant efficiencies ($[p/s/cm^2/sr] / [\mu W/cm^2]$) of each organ were plotted. **(E)** Average radiant efficiency values of bladders were normalized to blood (TNR) and fold changes in fluorocoxib A uptake were plotted. The 1.8- and 3.2-fold increase in fluorocoxib A uptake was observed in bladders from mice in Group 2 and 3, respectively, when compared to the bladder from untreated mice. Paired Student's *t* test was used to compare the increase in fluorocoxib A uptake in BBN-exposed groups (Group 2 and 3) with control group (Group 1), ****p* < 0.001.

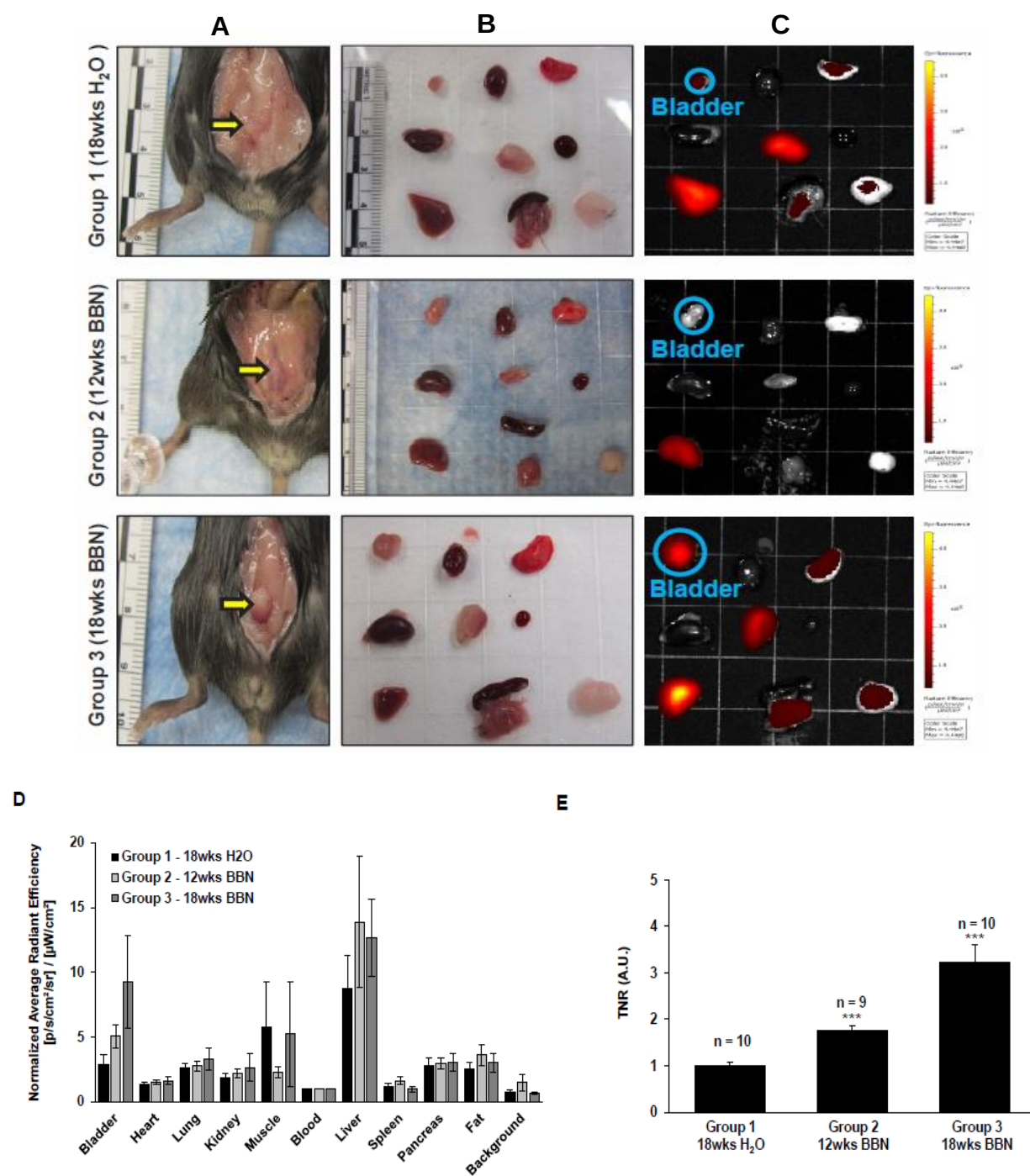


Figure 3.2

Figure 3.3 Progression of bladder cancer by BBN.

The BBN-induced bladder cancer progression from normal urothelium (Group 1), to hyperplasia, and carcinoma in situ (Group 2 and 3), and to invasive carcinoma (Group 2 and 3) with increased inflammation of bladder in mice was confirmed by H&E staining (left). The progression of bladder carcinogenesis was confirmed by increased expression of Ki67 in bladder from mice exposed to BBN (middle). In addition, uroplakin-1A, a protein that is highly expressed in normal bladder urothelium (Group 1), was downregulated in BBN-induced bladder cancers (Group 2 and 3) as confirmed by IHC analysis (right). Brown color is positive staining for tested proteins. Images taken by Leitz DMRB microscope, objective 10x, scale bar 100µm. C – carcinoma; CIS – carcinoma in situ; H – hyperplasia; I - Inflammation; U - Normal urothelium.

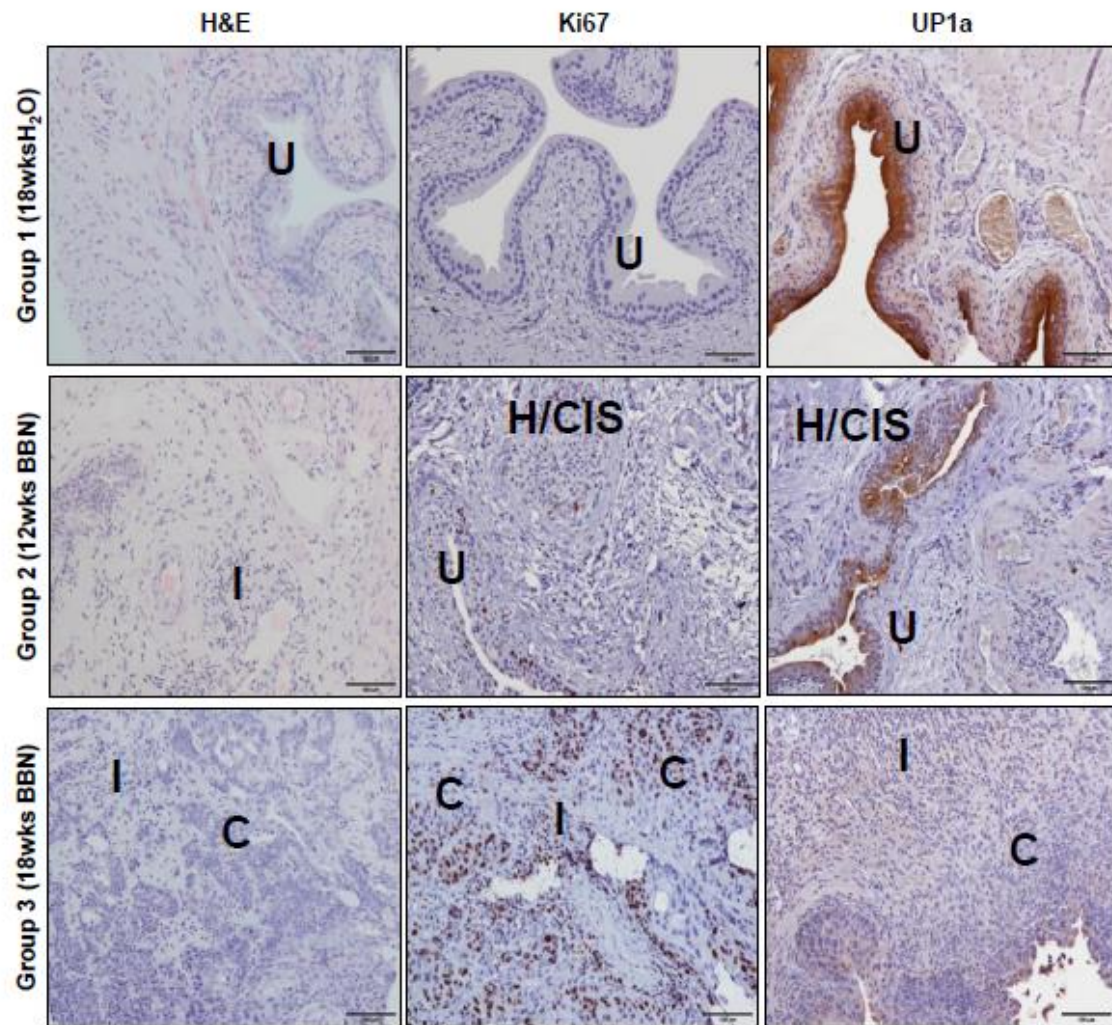


Figure 3.3

Figure 3.4 Upregulation of Cox-2 by BBN in bladder carcinoma.

The upregulation of Cox-2 in BBN-induced bladder carcinoma was detected by **(A)** IHC and **(B)** WB analysis. Bladder carcinoma in mice from Group 3 (n=10; 18wks BBN) had higher Cox-2 expression when compared to normal urothelium in mice from Group 1 (n=7; control) and bladder hyperplasia in mice from Group 2 (n=9; 12wks BBN). The higher Cox-2 expression in Group 3 correlates to higher fluorocoxib A uptake. Brown color is positive staining for tested protein expression. Images taken by Leitz DMRB microscope, objective 10x, scale bar 100µm. Actin was used as a WB loading control. **(C)** Densitometry evaluation of Cox-2/actin protein bands from WB analysis was performed using VisionWorks acquisition and analysis software (UVP). Paired Student's *t*-test was used to compare the upregulation of Cox-2 expression in BBN-exposed (Group 2 and 3) with control group (Group 1), **p* < 0.05.

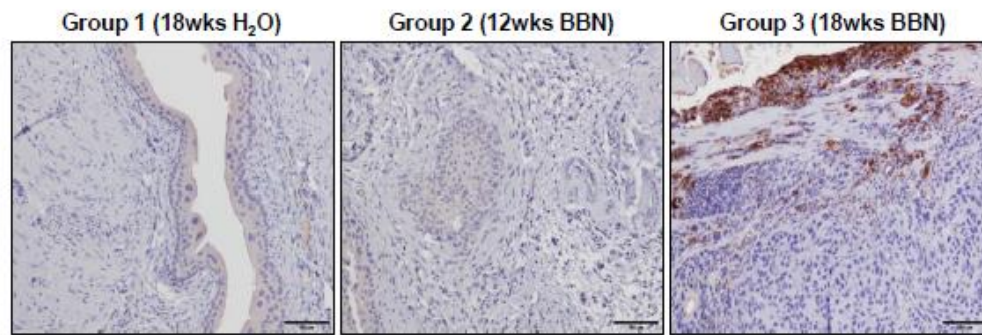
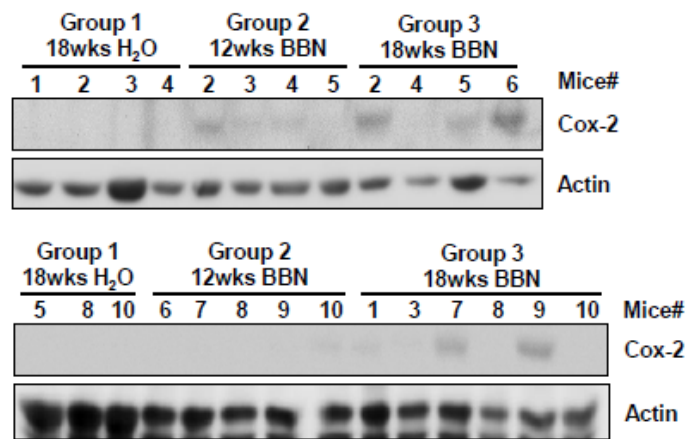
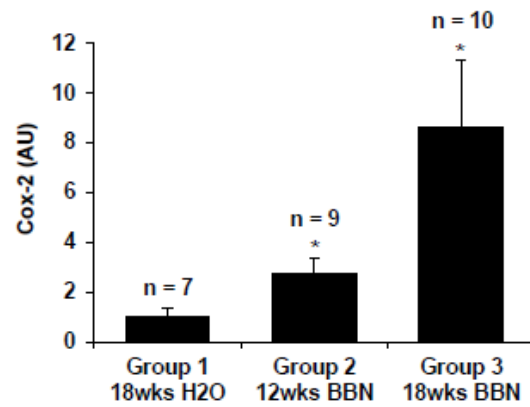
A**B****C**

Figure 3.4

**CHAPTER IV DETECTION OF RECEPTOR TYROSINE KINASE
INHIBITORS-INDUCED COX-2 UPREGULATION IN BLADDER CANCER
BY FLUOROCOXIB A**

Research article described in this chapter is a slightly modified version of an article that was prepared for submission to the *World Molecular Imaging Society journal, Molecular Imaging and Biology*, by Jennifer Bourn, Sony Pandey, Jashim Uddin, Lawrence Marnett, and Maria Cekanova in November 2018.

Detection of receptor tyrosine kinase inhibitor-induced COX-2 upregulation in bladder cancer by fluorocoxib A.

Jennifer Bourn, Sony Pandey, Jashim Uddin, Lawrence Marnett, and Maria Cekanova
Molecular Imaging and Biology (Manuscript in Progress)

In this paper, “our” or “we” refers to my co-authors and me. My contribution to this manuscript includes: 1) Compiling and interpretation of literature, 2) Providing comprehensive structure to the paper, 3) Acquisition and analysis of data, and 4) Writing and editing of the manuscript.

Detection of receptor tyrosine kinase inhibitors-induced COX-2 upregulation in bladder cancer by fluorocoxib A.

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Running title: Detection of RTKI-induced COX-2 upregulation.

Manuscript category: Article

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ABSTRACT

Purpose: A challenge with targeted therapies is the activation of alternative pro-survival signaling pathways, such as COX-2 upregulation, resulting in acquired drug resistance. COX-2 is overexpressed in bladder cancer cells, making it an attractive target for the detection and treatment of cancer. Fluorocoxib A is an optical imaging agent that selectively targets COX-2 which can be used for the detection and monitoring of bladder cancer. In this study, we evaluated the effects of RTKIs on bladder cancer cell lines *in vitro* and *in vivo*. We also evaluated the ability of fluorocoxib A to detect the response to targeted therapies *in vivo*.

Procedures: The effects of RTKIs on bladder cancer cells *in vitro* were analyzed by MTS and WB analysis. The effects of RTKIs *in vivo* were evaluated using the K9TCC#5Lilly xenograft tumor model and fluorocoxib A uptake was detected using the IVIS Lumina imaging system. COX-2 expression in the K9TCC#5Lilly xenografts was confirmed by IHC and WB analysis.

Results: Despite inhibiting cell viability, AB1010 and imatinib, increased COX-2 expression in bladder TCC cells *in vitro*. Specific uptake of fluorocoxib A correlated with the RTKI-induced COX-2 upregulation in the K9TCC#5Lilly xenografts *in vivo*.

Conclusions: These results indicate that fluorocoxib A can be used for monitoring the responses to targeted therapies in patients diagnosed with COX-2-expressing bladder cancer.

Keywords: Bladder cancer, RTKIs, COX-2, targeted therapies response, optical imaging, fluorocoxib A

INTRODUCTION

Bladder cancer is the 6th most common type of cancer in the United States and one of the most expensive malignancies to treat due to high recurrence rates and lack of improved treatment options over the past several decades (1-3). In 90% of all cases, bladder cancer originates from the epithelial lining of the bladder known as the urothelium. This type of bladder cancer is known as transitional cell carcinoma (TCC) or urothelial carcinoma (4). Early detection of bladder cancer proves to provide better prognostic outcomes for patients diagnosed with bladder cancer (5). Despite demonstrating the need for improved diagnostic screening, prevention, and treatment options, bladder cancer still remains one of the most commonly diagnosed malignancies in the United States (6). This drives the need for improved detection and novel therapeutic options for patients diagnosed with both non-muscle invasive bladder cancer (NMIBC) and muscle invasive bladder cancer (MIBC).

Receptor tyrosine kinases (RTKs) mediate key signaling pathways involved in cell proliferation, differentiation, survival, and cell migration (7-8). Mutations of RTKs can affect the expression of downstream signaling pathways such as the MAP kinase, PI3K/Akt, and cyclooxygenase (COX)-2 pathways, resulting in aberrant cell function which plays an important role in a number of biological processes, including the progression of cancer (9). RTKs, such as the c-Kit receptor, platelet derived growth factor receptor (PDGFR), and vascular endothelial growth factor receptor (VEGFR), are overexpressed in many types of cancer, including bladder cancer (10-12). Inhibitors of RTKs (RTKIs) are therefore used as targeted therapy options for patients diagnosed

with cancer that overexpress RTKs to inhibit auto-phosphorylation and downstream signal transduction, halting tumor progression (13-14). For many decades, kinases have been extensively studied as potential drug targets and to date, 38 RTKIs have been FDA-approved for the treatment of cancer, including imatinib (Gleevec) (15-16). There are even more RTKIs under development in pre-clinical research and clinical trial phases, including AB1010 (Masitinib or Masivet) (17).

Imatinib is a potent and selective inhibitor of the c-Kit ($IC_{50} = 100 \text{ nm}$) and PDGFR α/β ($IC_{50} = 100 \text{ nm}$) receptors (18-19). It currently is used as a first-line therapy option for treatment of chronic myeloid leukemia (CML) and for advanced stage gastrointestinal stromal tumors (GIST) (20-22). AB1010 is a novel RTKI that also selectively targets the c-Kit ($IC_{50} = 200 \text{ nm}$) and PDGFR α/β ($IC_{50} = 540 \text{ nm}$ and 800 nm , respectively) receptors (17). AB1010 is approved in Europe to be used as a treatment option for canine mast cell tumors and has been investigated for the treatment of patients diagnosed with GIST and pancreatic cancer, alone and in combination with chemotherapy agents (23-28). Both imatinib and AB1010 are well-tolerated with few side effects, unlike chemotherapeutic agents. A common challenge associated with targeted therapies is the activation of alternative pro-survival signaling pathways, such as the COX-2 pathway, resulting in acquired drug resistance. Previous studies indicate treatment with chemotherapeutic agents and even targeted therapies, such as RTKIs, increased COX-2 expression in bladder cancer cells, glioma cancer stem cells (CSCs), and oral squamous cell carcinoma cells *in vitro* (29-32). This drives the need for the

early detection and monitoring of responses to targeted therapies for patients diagnosed with bladder cancer to improve prognostic outcomes.

During periods of inflammation and cancer, COX-2 is one of the key proteins responsible for promoting angiogenesis, cell proliferation, and inhibiting apoptosis (33-36). COX-2 is overexpressed in many types of cancer, including bladder cancer, and is often an indicator of poor patient prognosis (37). Overexpression of COX-2 in bladder cancer tissue can therefore be used as a biomarker for the treatment and detection of bladder cancer. Conventional optical imaging modalities, primarily cystoscopy, for bladder cancer have several limitations, including poor ability to detect bladder cancer at the early stages and poor differentiation of tumor margins during resection procedures (38). To improve diagnostic imaging tools during cystoscopy procedures, a key focus in pre-clinical research is to evaluate the ability of fluorescently labeled contrast agents for the detection and monitoring of bladder cancer. Currently, inhibitors of COX-2 (non-steroidal anti-inflammatory drugs, NSAIDs) are used both for the prevention and treatment of cancer but recently have been investigated for the detection of inflammation and cancer. Previously published studies indicate that fluorescently-labeled COX-2 inhibitors, which bind to the active site of COX-2, are suitable candidates for targeted optical imaging because of their stable properties, high specificity for the target protein (i.e. COX-2), and ease of administration (39-42). Fluorocoxib A, a novel optical imaging agent, is a rhodamine-conjugated analog of indomethacin that selectively targets COX-2 expressing tissues, including solid tumors (43). This imaging agent has been extensively studied both *in vitro* and *in vivo* for the detection of

inflammation and cancer, demonstrating highly selective and specific uptake by COX-2-expressing tissues when compared to surrounding normal tissues (44-46).

In this study, we evaluated the effects of RTKIs in human and canine bladder TCC cell lines *in vitro*. We have validated the effects of tested RTKIs to inhibit cell proliferation and activity of target RTKs *in vitro*. Treatment with several RTKIs increased COX-2 expression in both human and canine COX-2 positive bladder TCC cell lines. We also evaluated the ability of fluorocoxib A to detect and monitor the response to RTKIs-induced COX-2 expression *in vivo* using the K9TCC#Lilly xenograft mouse model. Specific and selective fluorocoxib A uptake correlated with the RTKIs-induced COX-2 expression in K9TCC#5Lilly xenograft tumors. These results indicate that fluorocoxib A can be used for monitoring the responses to targeted therapies in patients diagnosed with COX-2-expressing bladder cancer.

MATERIALS AND METHODS

Antibodies and reagents

The antibodies for p-c-Kit (Tyr721, sc-18077), p-PDGFR β (F-10, sc-365464), COX-2 (C-20, sc-1745), actin (C-4, sc-47778), and secondary donkey anti-goat (sc-2020) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA); antibody for COX-2 (aa 570-598, 160106) was purchased from Cayman Chemical (Ann Arbor, MI); antibody for c-Kit (961-976, PC34) was purchased from Millipore Sigma (Burlington, MA); and secondary anti-rabbit (cs-7074) were purchased from Cell Signaling

Technology (Danvers, MA). The receptor tyrosine kinase inhibitors (RTKIs) AZD 5438 (AZD) and toceranib (Toc) were purchased from Tocris Bioscience (Minneapolis, MN); RTKIs erlotinib (Erl), gefitinib (Gef), imatinib (Ima), sorafenib (Sor), SP600125 (SP), vandetanib (Van), and UO126 (UO) were purchased from Cell Signaling Technology; RTKI axitinib (Ax) was purchased from Millipore Sigma (Burlington, MA); and RTKI AB1010 (AB) was purchased from ApexBio (Boston, MA) (Table 4.1). All other chemicals and reagents were purchased from Thermo Fisher Scientific (Pittsburgh, PA), unless otherwise specified.

Cell Lines

Human bladder TCC cell lines J82, RT4, T24, UM-UC-3, 5637, SW780, and TCCSUP were purchased from American Type Culture Collection (ATCC, Manassas, VA). Cell lines were authenticated via short tandem repeat (STR) DNA profiling by Genetica DNA laboratories (Burlington, NC). J82 and TCCSUP cells were grown in MEM media supplemented with MEM non-essential amino acids and sodium pyruvate; RT4 and T24 cells were grown in McCoy's media; UM-UC-3 cells were grown in MEM media; and 5637 and SW780 cells were grown in RPMI-1640 media. All culture media was supplemented with 10% fetal bovine serum, 100 I.U. penicillin, and 100 µg/mL streptomycin. Canine bladder TCC (K9TCC) cell lines, K9TCC#1Lillie, K9TCC#2Dakota, and K9TCC#5Lilly were established and characterized in the laboratory of Dr. Cekanova as described previously in detail (47). The K9TCC cell lines were grown in complete RPMI-1640 media supplemented with 10% fetal bovine serum,

100 I.U. penicillin, and 100 µg/mL streptomycin. All TCC cells were grown at 37°C and 5% CO₂.

Proliferation (MTS) assay

The tested bladder TCC cells were seeded in 96-well plates at a concentration of 5×10^3 cells/well in complete media. After 24 h incubation, cells were treated with 5 µM concentration of the tested RTKIs (as shown in Figure 1) in complete media for an additional 48 h. DMSO was used as the control. After 48 h, cell viability was measured using the MTS CellTiter 96® Aqueous One Solution Cell Proliferation Assay (Promega, Madison, WI) according to the manufacturer's protocol. Briefly, 20 µl of the MTS reagent was added to each well and incubated with cells at 37°C for 1 h. Absorbance was measured at 490 nM using an FLx800 plate reader (Bio-Tek instruments, Winooski, VT). The data were shown as mean $\pm$ S.E. of four replicates of two independent experiments and normalized to the DMSO controls.

Animals

All animal experiments were performed in accordance with approved UTK IACUC protocols. Thirty 5-wks old, female athymic nude mice (Charles River, Boston, MA) were randomly divided into three groups (n=10/group). The COX-2 positive primary bladder TCC cell line, K9TCC#5Lilly, was injected subcutaneously (SC) at a density of 1.7×10^5 cells mixed with a 1:1 ratio of PBS and Matrigel. After 2 weeks of tumor development, treatment with the RTKIs, AB1010 and imatinib, was initiated. Group 1,

served as the control group of mice that received the vehicle (DMSO+0.9% NaCl). The other two groups received the RTKIs treatments where Group 2 received AB1010 (3.75 mg/kg) and Group 3 received imatinib (7.5 mg/kg). Injections were made intraperitoneal (i.p.) twice per week for three weeks. Tumor volume was calculated as: length $\times$ width² $\times$ 0.52 (mm³).

Fluorocoxib A uptake by K9TCC#5Lilly xenograft tumors detected by IVIS Lumina optical imaging system *in vivo*

Following the RTKIs treatment, fluorocoxib A (1 mg/kg) was administered SC. After fluorocoxib A administration, the mice were sacrificed, tissues were dissected, and imaged *ex vivo*. Specific fluorocoxib A uptake was detected 4 h after administration using the Xenogen IVIS Lumina optical imaging system with DsRed filters with excitation 500 to 550 nm, emission 575 to 650 nm, and background 460 to 490 nm. The obtained total flux (p/s) and average radiant efficiency ([p/s/cm²/sr] / [μ W/cm²]) of labeled regions of interest (ROI) of dissected tumor and other tissues (heart, lung, kidney, liver, blood, spleen, pancreas, small intestine, muscle, and fat) were evaluated. Tumor-to-noise ratio (TNR) was generated using the average radiant efficiency of the tumor as the tumor fluorescence and blood as the background fluorescence (noise). The TNR for each treatment group (AB1010 and imatinib) was normalized to Group 1 (control) and normalized TNR was plotted. TNR was calculated using the following equation:

$$\text{TNR} = (\text{tumor fluorescence}) / (\text{background fluorescence})$$

Each dissected tumor was divided into three pieces, one piece of tumor was fixed in 10% neutral buffered formalin for histology and immunohistochemistry (IHC) analysis. The second piece of tumor was placed into O.C.T. media for immunofluorescence (IF) detection of fluorocoxib A uptake by the tumors using fluorescence microscopy and the third piece of tumor was kept in RNA/later solution and stored at -80°C until Western blotting (WB) analysis was performed.

Immunohistochemistry

Dissected tissues from mice were formalin-fixed, paraffin-embedded, and sectioned at 7 µm. Hematoxylin and eosin (H&E) was performed following standard histological staining protocol. The IHC staining was performed as described previously (45). After de-paraffinization, the antigen retrieval using sodium citrate pH 6.0 was performed for 20 min in antigen retriever (Electron Microscopy Sciences, Hatfield, PA). The blocking of endogenous peroxidase activity was performed by 3% H₂O₂ in 50% methanol for 5 min, followed by the blocking of non-specific signal using protein block solution (BioGenex, Fremont, CA). Tissues were incubated with the COX-2 primary antibody, followed by the incubation with the specific biotinylated secondary antibodies, streptavidin/HRP detection system, and visualized by 3,3'-Diaminobenzidine (DAB) staining. Nuclei were counter-stained with hematoxylin and slides were cover-slipped and evaluated using a Leitz DMRB microscope. The images were captured by a DP73 camera attached to microscope using CellSens Standard software (Olympus, Pittsburgh, PA).

Western blotting analysis

The tested bladder TCC cells were seeded in 10 cm tissue culture dishes at a concentration of 2×10^6 cells/dish in complete media. After 24 h incubation, cells were treated with 5 μ M concentration of the tested RTKIs (as shown in Figure 2) and with 0, 1, 5, and 10 μ M of AB1010 and imatinib (as shown in Figure 3) in complete media for an additional 24 h. DMSO was used as the control. The cell lysates and tissue samples were lysed in ice-cold RIPA buffer supplemented with protease and phosphatase inhibitors cocktail (1 mM PMSF; 1 μ g/ml aprotinin; 1 μ g/ml leupeptin; 5 mM Na_3VO_4 ; 5 mM NaF) and briefly sonicated on ice. Protein concentrations were measured using Pierce® BCA protein assay (Thermo Scientific, Rockford, IL). Equal amount of proteins was loaded onto SDS-PAGE gels and transferred to nitrocellulose membranes. After blocking, the membranes were incubated with primary antibodies overnight at 4°C, followed by incubation with horseradish peroxidase-conjugated secondary antibodies (1:3,000 dilution) for 1 h at room temperature. The immuno-reactive bands were visualized using the ECL prime chemiluminescence system (GE Healthcare Life Sciences, Marlborough, MA) and the images were captured using the BioSpectrum® 815 imaging system (UVP, Upland, CA). Densitometry analysis was performed using the VisionWorks acquisition and analysis software (UVP).

Statistical analysis

Statistical analysis was conducted using the paired Student's *t*-test to establish significant differences among treatment groups. Results were considered statistically significant at $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$.

RESULTS

RTKIs treatment inhibited cell viability but increased COX-2 expression in tested bladder TCC cells

Tested RTKIs treatment significantly inhibited cell viability in all human and canine bladder TCC cells as shown in Fig. 4.1. Response to RTKIs treatment correlated with the RTKs expression profile of the tested bladder TCC cells and the specific target of the RTKIs. For instance, AZD5438 is a potent cyclin dependent kinase inhibitor and demonstrates significant anti-proliferative activity in tumor cells (48). Cell viability was significantly inhibited in tested bladder TCC cells after AZD treatment as compared to the control. Bladder TCC cells that do not have high RTKs expression, such as h-J82 and h-UM-UC-3 cells, did not respond to RTKIs that target PDGFR and c-Kit activity as compared to bladder TCC cells that have high RTKs expression, such as h-T24, h-TCCSUP, and K9TCC cells.

Previously published studies demonstrate that treatment with RTKIs increases COX-2 expression in oral squamous cell carcinoma (32) and bladder TCC (29) cells *in vitro*. To investigate the effects of RTKIs treatment in the tested bladder TCC cells, the

cells were treated with 5 μ M doses of each inhibitor for 24 h. Cell lysates were collected, and WB analysis was performed. As shown in Fig. 4.2, there was no increase in COX-2 expression in the COX-2 negative bladder TCC cell lines (h-J82, h-RT4, h-T24, and h-UM-UC-3). In the COX-2 positive bladder TCC cell lines (h-5637, h-SW780, h-TCCSUP, K9TCC#1Lillie, K9TCC#2Dakota, and K9TCC#5Lilly), several of the tested RTKIs treatment significantly increased COX-2 expression including AB1010 and imatinib.

AB1010 and imatinib inhibited phosphorylation of RTKs but increased COX-2 expression in tested bladder TCC cells in a dose-dependent manner

We further investigated the effects of RTKIs, AB1010 and imatinib, using the COX-2 positive bladder TCC cell lines, h-5637, h-TCCSUP, K9TCC#1Lillie, and K9TCC#5Lilly. The cells were treated with 0, 1, 5, 10 μ M concentrations of AB1010 and imatinib for 24 h. Cell lysates were collected, and WB analysis was performed. AB1010 and imatinib selectively targets the c-Kit and PDGFR receptors which are overexpressed in bladder TCC cells. As shown in Fig. 4.3A, p-c-Kit expression was detected in either human bladder TCC cell line and total c-Kit expression was not detected in h-5637 cells. Both AB1010 and imatinib decreased c-Kit expression in h-TCCSUP cells in a dose-dependent manner. AB1010 and imatinib inhibited the phosphorylation of PDGFR β in h-5637 and h-TCCSUP cells. Both AB1010 and imatinib increased COX-2 expression in the h-5637 cells in a dose-dependent manner. Imatinib, but not AB1010, increased COX-2 expression in the h-TCCSUP cells in a dose-dependent manner.

As shown in Fig. 4.3B, p-c-Kit expression was detected in either K9TCC cell line but both AB1010 and imatinib decreased c-Kit expression in K9TCC#1Lillie cells in a dose-dependent manner. AB1010 increased c-Kit expression whereas imatinib decreased c-Kit expression in K9TCC#5Lilly cells. AB1010 increased p-PDGFR β expression in both tested K9TCC cell lines in a dose-dependent manner while imatinib decreased the phosphorylation of PDGFR β . Despite inhibiting cell viability and RTKs protein expression, both AB1010 and imatinib increased COX-2 expression in the tested K9TCC cell lines in a dose-dependent manner. These results are consistent with the response to RTKIs treatment in the h-TCC cell lines (Fig. 4.3A) and suggest the activation of alternative pro-survival signaling pathways.

Detection of RTKI-induced COX-2 expression in K9TCC#5Lilly xenograft tumors by fluorocoxib A

Thirty 5-wks old, female athymic nude mice were randomly divided into three groups (n=10/group). The COX-2 positive primary TCC cell line, K9TCC#5Lilly, was injected subcutaneously (SC) at a density of 1.7×10^5 cells mixed with a 1:1 ratio of PBS and Matrigel. After 2 weeks of tumor development, treatment with the RTKIs, AB1010 and imatinib, was initiated. Group 1, served as the control group of mice that received the vehicle (DMSO+0.9% NaCl). The other two groups received the RTKIs treatments where Group 2 received AB1010 (3.75 mg/kg) and Group 3 received imatinib (7.5 mg/kg). Injections were made intraperitoneal (i.p.) twice per week for three weeks. Body weight (g) and tumor volume (mm³) for each mouse were recorded three

times per week. Average body weight per mouse (g) and relative tumor volume was plotted as shown in Fig. 4.4A and 4.4B. Neither the injection of K9TCC#5Lilly cells nor RTKIs treatment had any adverse effects on the growth of the mice over time as no significant differences in body weight was observed between groups. After two weeks of RTKIs treatment, there was an increase in relative tumor volume for Group 2 – AB1010 and Group 3 – imatinib when compared to the control group. Values represent mean $\pm$ standard error of each recording.

After RTKIs treatment, fluorocoxib A was administered subcutaneously (1 mg/kg) followed by imaging of the mice. After 4 h uptake, the mice were sacrificed, and the dissected tissues were photographed *ex vivo* (Fig. 4.4C). Fluorescent imaging of the dissected tissues was performed using the Xenogen IVIS Lumina optical imaging system to detect fluorocoxib A uptake. Higher uptake and increased intensity of fluorocoxib A was observed in Group 2 and Group 3 when compared to Group 1 (control) as shown in Fig. 4.4D. Obtained average radiant efficiencies ($[p/s/cm^2/sr]/[\mu W/cm^2]$) of each organ were plotted (data not shown). Average radiant efficiency values of the tumors were normalized to blood (TNR) and fold changes in fluorocoxib A uptake were plotted (Fig. 4.4E). There is an increase in average radiant efficiency in the tumors of mice from Group 2 and Group 3 when compared to the control, indicating an increased uptake of fluorocoxib A. Values represent mean $\pm$ standard error of each TNR.

RTKI-induced COX-2 expression in K9TCC#5Lilly xenograft tumors detected by IHC and WB analysis

The RTKI-induced COX-2 expression in K9TCC#5Lilly xenograft tumors was detected by IHC (Fig. 4.5A) and WB analysis (Fig. 4.5B). Tumors from mice in Group 2 (AB1010) had higher COX-2 expression when compared to tumors from mice in Group 1 (control) and Group 3 (imatinib). This result was also confirmed by WB analysis of the dissected tumors from each group. The tumors from Group 2 and Group 3 had higher COX-2 expression when compared to the tumors from Group 1. Densitometry analysis of COX-2 protein bands from WB analysis was performed using VisionWorks acquisition and analysis software (UVP) (Fig. 4.5C). The specific uptake and increased intensity of fluorocoxib A (Fig. 4.4) in the K9TCC#5Lilly xenograft mouse model correlated with the RTKI-induced COX-2 expression both *in vitro* (Fig. 4.2 and 4.3) and *in vivo* (Fig. 4.5).

DISCUSSION

Bladder cancer remains one of the most expensive malignancies to treat primarily due to high rates of recurrence (1). In recent years, targeted therapies are commonly used as a treatment option for patients diagnosed with cancer because they are well-tolerated with few side effects, unlike standard chemotherapeutic agents. A common challenge associated with targeted therapies is the activation of alternative pro-survival signaling pathways, such as the COX-2 pathway, resulting in acquired drug resistance. Many of the molecular mechanisms of drug resistance remains unknown. Another challenge for the detection of bladder cancer, is that conventional optical

imaging modalities have several limitations, including poor ability to detect bladder cancer at the early stages and poor differentiation of tumor margins during resection procedures (38). These challenges drive the need for novel therapeutic options and better detection of bladder cancer to improve prognostic outcomes for patients.

In this study, we evaluated the effects of RTKIs on human and canine bladder TCC cell lines *in vitro*. We have validated the effects of tested RTKIs to inhibit cell proliferation and the activity of RTKs, c-Kit and PDGFR β , *in vitro*. Treatment with several RTKIs increased COX-2 expression in both human and canine COX-2 positive bladder TCC cell lines. Response to RTKIs treatment correlated with the RTKs expression profile of the tested bladder TCC cells and the specific target of the RTKIs as shown in Fig. 4.1. Bladder TCC cells that do not have high RTKs expression, such as h-J82, T24, and h-UM-UC-3 cells, did not respond to RTKIs as compared to bladder TCC cells that have high RTKs expression, such as h-5637, h-TCCSUP, and K9TCC cells. As shown in Fig. 4.2, in the COX-2 positive bladder TCC cell lines, several of the tested RTKIs treatment increased COX-2 expression, including AB1010 and imatinib. AB1010 and imatinib selectively target the c-Kit and PDGFR α/β receptors which are overexpressed in bladder TCC cells (17-19). Despite inhibiting the phosphorylation of the c-Kit and PDGFR β receptors, AB1010 and imatinib increased COX-2 expression in a dose-dependent manner in the tested COX-2 positive bladder TCC cell lines (Fig. 4.3).

When induced, COX-2 plays a key role in promoting angiogenesis, cell proliferation, and inhibiting apoptosis (33-36). COX-2 is overexpressed in many types of

cancer, including bladder cancer, and is often an indicator of poor patient prognosis (37). Previously published studies indicate treatment with chemotherapeutic agents and targeted therapies increased COX-2 expression in bladder cancer cells, glioma cancer stem cells (CSCs), non-small cell lung cancer cells, and oral squamous cell carcinoma cells *in vitro* (29-32, 49). These results suggest that COX-2 expression can be affected by the intrinsic tumor microenvironment and the response to therapies. The results from our study are consistent with previous findings that following treatment with RTKIs, COX-2 expression is upregulated by several RTKIs, including AB1010 and imatinib. Based on these results, we proposed that despite inhibiting cell viability and the activity of RTKs, the COX-2 signaling pathway is activated which leads to acquired drug resistance. Therefore, the molecular mechanism of action for the RTKIs-induced COX-2 expression needs to be elucidated.

To further investigate the RTKIs-induced COX-2 expression, we evaluated the ability of fluorocoxib A to detect and monitor the effects of RTKIs treatment *in vivo* using the K9TCC#5Lilly xenograft mouse model. Neither the injection of K9TCC#5Lilly cells nor RTKIs treatment had any adverse effects on the growth of the mice over time as no significant differences in body weight was observed between groups (Fig. 4.4A). After two weeks of RTKIs treatment, there was an increase in relative tumor volume for Group 2 – AB1010 and Group 3 – imatinib when compared to the control group (Fig. 4.4B). The increase in relative tumor volume in Groups 2 and 3 indicates a negative response to RTKIs treatment and suggests acquired drug resistance. Following RTKIs treatment, fluorocoxib A was administered and *ex vivo* imaging was performed. Higher

uptake and increased intensity of fluorocoxib A was observed in the K9TCC#5Lilly xenograft tumors from Group 2 (AB1010) and Group 3 (imatinib) when compared to Group 1 (control) as shown in Fig. 4.4D. The RTKI-induced COX-2 expression in K9TCC#5Lilly xenograft tumors was confirmed by IHC (Fig. 5.5A) and WB analysis (Fig. 5.5B). The xenograft tumors from mice in Group 2 (AB1010) had higher COX-2 expression when compared to tumors from mice in Group 1 (control) and Group 3 (imatinib). The specific uptake and increased intensity of fluorocoxib A (Fig. 4.4) by the K9TCC#5Lilly xenograft tumors correlated with the RTKI-induced COX-2 expression both *in vitro* (Fig. 4.2 and 4.3) and *in vivo* (Fig. 5.5).

The challenges associated with the detection of bladder cancer also contribute to the high bladder cancer recurrence rates, driving the need for improved diagnostic imaging techniques. To improve diagnostic imaging tools during cystoscopy procedures, a key focus in pre-clinical research is to evaluate the ability of fluorescently labeled contrast agents for the detection and monitoring of bladder cancer (38, 50-52). Previously published studies indicate that fluorescently-labeled COX-2 inhibitors (i.e. fluorocoxib A) are suitable candidates for targeted optical imaging because high specificity for the target protein (i.e. COX-2) (39-42). Fluorocoxib A has been extensively studied both *in vitro* and *in vivo* for the detection of inflammation and cancer, demonstrating highly selective and specific uptake by COX-2-expressing tissues when compared to surrounding normal tissues (44-46, 53). Our results are consistent with previously published studies that fluorocoxib A specifically detects COX-2 expression bladder cancer cells *in vivo*. In this study, we also validated the ability of fluorocoxib A to

detect molecular changes in COX-2 expression after treatment with the targeted therapies, AB1010 and imatinib. These results indicate that fluorocoxib A is sensitive and specific enough to detect the early responses to therapy in COX-2 expressing bladder cancer. A limitation of fluorescent probes is that they typically target a specific biomarker expressed within the tumor (i.e. site of inflammation or tumor). Fluorocoxib A specifically targets COX-2 expressing cells and tissues which suggests that if there is no COX-2 expression present, there will be no uptake of the fluorocoxib A.

We previously published a study which concludes that co-treatment of AB1010 with indomethacin, an NSAID, abrogates the AB1010-induced COX-2 expression in TCC cells *in vitro* (29). Future *in vivo* studies need to be conducted to evaluate the ability of fluorocoxib A to monitor the effects of RTKIs and NSAIDs co-treatment using the COX-2 positive TCC xenograft mouse model. In conclusion, the results of this study indicate that optical imaging agent, fluorocoxib A, can be used for monitoring the early responses to targeted therapies in patients diagnosed with COX-2-expressing bladder cancer. Early detection and monitoring of treatment responses can provide better prognostic outcomes for patients diagnosed with bladder cancer.

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Conflict of Interest: Authors from the University of Tennessee have no potential conflicts of interest to disclose. Vanderbilt University holds a patent for the fluorocoxib compounds and their use for COX-2-targeted imaging.

Ethical approval: All applicable institutional and/or national guidelines for the care and use of animals were followed.

REFERENCES

1. N. C. Institute. (Bethesda, MD, 2018), vol. 2018.
2. R. S. Svatek *et al.*, The economics of bladder cancer: costs and considerations of caring for this disease. *Eur Urol* **66**, 253-262 (2014).
3. M. F. Botteman, C. L. Pashos, A. Redaelli, B. Laskin, R. Hauser, The health economics of bladder cancer: a comprehensive review of the published literature. *Pharmacoeconomics* **21**, 1315-1330 (2003).
4. A. C. Society, K. Eidsmoe, Ed. (2016), vol. 2018.
5. B. L. Jacobs, C. T. Lee, J. E. Montie, Bladder cancer in 2010: how far have we come? *CA Cancer J Clin* **60**, 244-272 (2010).
6. D. S. Morris *et al.*, Understanding bladder cancer death: tumor biology versus physician practice. *Cancer* **115**, 1011-1020 (2009).
7. A. Ullrich, J. Schlessinger, Signal transduction by receptors with tyrosine kinase activity. *Cell* **61**, 203-212 (1990).
8. M. A. Lemmon, J. Schlessinger, Cell signaling by receptor tyrosine kinases. *Cell* **141**, 1117-1134 (2010).
9. P. Blume-Jensen, T. Hunter, Oncogenic kinase signalling. *Nature* **411**, 355-365 (2001).
10. N. Cancer Genome Atlas Research, Comprehensive molecular characterization of urothelial bladder carcinoma. *Nature* **507**, 315-322 (2014).
11. J. P. Crew, Vascular endothelial growth factor: an important angiogenic mediator in bladder cancer. *Eur Urol* **35**, 2-8 (1999).

12. C. X. Pan *et al.*, c-kit Expression in small cell carcinoma of the urinary bladder: prognostic and therapeutic implications. *Mod Pathol* **18**, 320-323 (2005).
13. A. Arora, E. M. Scholar, Role of tyrosine kinase inhibitors in cancer therapy. *J Pharmacol Exp Ther* **315**, 971-979 (2005).
14. J. T. Hartmann, M. Haap, H. G. Kopp, H. P. Lipp, Tyrosine kinase inhibitors - a review on pharmacology, metabolism and side effects. *Curr Drug Metab* **10**, 470-481 (2009).
15. B. Nagar *et al.*, Crystal structures of the kinase domain of c-Abl in complex with the small molecule inhibitors PD173955 and imatinib (STI-571). *Cancer Res* **62**, 4236-4243 (2002).
16. K. S. Bhullar *et al.*, Kinase-targeted cancer therapies: progress, challenges and future directions. *Mol Cancer* **17**, 48 (2018).
17. P. Dubreuil *et al.*, Masitinib (AB1010), a potent and selective tyrosine kinase inhibitor targeting KIT. *PLoS One* **4**, e7258 (2009).
18. M. C. Heinrich *et al.*, Inhibition of c-kit receptor tyrosine kinase activity by STI 571, a selective tyrosine kinase inhibitor. *Blood* **96**, 925-932 (2000).
19. E. Buchdunger *et al.*, Abl protein-tyrosine kinase inhibitor STI571 inhibits in vitro signal transduction mediated by c-kit and platelet-derived growth factor receptors. *J Pharmacol Exp Ther* **295**, 139-145 (2000).
20. S. Bauer *et al.*, Imatinib mesylate therapy in patients with gastrointestinal stromal tumors and impaired liver function. *Anticancer Drugs* **13**, 847-849 (2002).

21. M. W. Deininger, B. J. Druker, Specific targeted therapy of chronic myelogenous leukemia with imatinib. *Pharmacol Rev* **55**, 401-423 (2003).
22. P. Vigneri, J. Y. Wang, Induction of apoptosis in chronic myelogenous leukemia cells through nuclear entrapment of BCR-ABL tyrosine kinase. *Nat Med* **7**, 228-234 (2001).
23. K. A. Hahn *et al.*, Masitinib is safe and effective for the treatment of canine mast cell tumors. *J Vet Intern Med* **22**, 1301-1309 (2008).
24. K. A. Hahn *et al.*, Evaluation of 12- and 24-month survival rates after treatment with masitinib in dogs with nonresectable mast cell tumors. *Am J Vet Res* **71**, 1354-1361 (2010).
25. M. Humbert *et al.*, Masitinib combined with standard gemcitabine chemotherapy: in vitro and in vivo studies in human pancreatic tumour cell lines and ectopic mouse model. *PLoS One* **5**, e9430 (2010).
26. E. Mitry *et al.*, Safety and activity of masitinib in combination with gemcitabine in patients with advanced pancreatic cancer. *Cancer Chemother Pharmacol* **66**, 395-403 (2010).
27. G. Deplanque *et al.*, A randomized, placebo-controlled phase III trial of masitinib plus gemcitabine in the treatment of advanced pancreatic cancer. *Ann Oncol* **26**, 1194-1200 (2015).
28. A. Adenis *et al.*, Masitinib in advanced gastrointestinal stromal tumor (GIST) after failure of imatinib: a randomized controlled open-label trial. *Ann Oncol* **25**, 1762-1769 (2014).

29. J. Bourn, M. Cekanova, Cyclooxygenase inhibitors potentiate receptor tyrosine kinase therapies in bladder cancer cells in vitro. *Drug Des Devel Ther* **12**, 1727-1742 (2018).
30. A. V. Kurtova *et al.*, Blocking PGE2-induced tumour repopulation abrogates bladder cancer chemoresistance. *Nature* **517**, 209-213 (2015).
31. H. I. Ma *et al.*, Celecoxib and radioresistant glioblastoma-derived CD133+ cells: improvement in radiotherapeutic effects. Laboratory investigation. *J Neurosurg* **114**, 651-662 (2011).
32. K. Rathore, M. Alexander, M. Cekanova, Piroxicam inhibits Masitinib-induced cyclooxygenase 2 expression in oral squamous cell carcinoma cells in vitro. *Transl Res* **164**, 158-168 (2014).
33. K. M. Leahy *et al.*, Cyclooxygenase-2 inhibition by celecoxib reduces proliferation and induces apoptosis in angiogenic endothelial cells in vivo. *Cancer Res* **62**, 625-631 (2002).
34. S. Gately, W. W. Li, Multiple roles of COX-2 in tumor angiogenesis: a target for antiangiogenic therapy. *Semin Oncol* **31**, 2-11 (2004).
35. G. Gakis, The role of inflammation in bladder cancer. *Adv Exp Med Biol* **816**, 183-196 (2014).
36. T. Qayyum *et al.*, A prospective study of the role of inflammation in bladder cancer. *Curr Urol* **6**, 189-193 (2013).
37. M. Komhoff *et al.*, Enhanced expression of cyclooxygenase-2 in high grade human transitional cell bladder carcinomas. *Am J Pathol* **157**, 29-35 (2000).

38. P. Jichlinski, H. J. Leisinger, Fluorescence cystoscopy in the management of bladder cancer: a help for the urologist! *Urol Int* **74**, 97-101 (2005).
39. H. M. Schuller *et al.*, Detection of overexpressed COX-2 in precancerous lesions of hamster pancreas and lungs by molecular imaging: implications for early diagnosis and prevention. *ChemMedChem* **1**, 603-610 (2006).
40. M. J. Uddin, B. C. Crews, K. Ghebreselasie, M. N. Tantawy, L. J. Marnett, [I]-Celecoxib Analogues as SPECT Tracers of Cyclooxygenase-2 in Inflammation. *ACS Med Chem Lett* **2**, 160-164 (2011).
41. A. Bhardwaj *et al.*, Hybrid fluorescent conjugates of COX-2 inhibitors: search for a COX-2 isozyme imaging cancer biomarker. *Bioorg Med Chem Lett* **23**, 163-168 (2013).
42. A. Bhardwaj, J. Kaur, F. Wuest, E. E. Knaus, Fluorophore-labeled cyclooxygenase-2 inhibitors for the imaging of cyclooxygenase-2 overexpression in cancer: synthesis and biological studies. *ChemMedChem* **9**, 109-116, 240 (2014).
43. M. J. Uddin *et al.*, Selective visualization of cyclooxygenase-2 in inflammation and cancer by targeted fluorescent imaging agents. *Cancer Res* **70**, 3618-3627 (2010).
44. M. Cekanova *et al.*, Molecular imaging of cyclooxygenase-2 in canine transitional cell carcinomas in vitro and in vivo. *Cancer Prev Res (Phila)* **6**, 466-476 (2013).

45. M. Cekanova *et al.*, Single-dose safety and pharmacokinetic evaluation of fluorocoxib A: pilot study of novel cyclooxygenase-2-targeted optical imaging agent in a canine model. *J Biomed Opt* **17**, 116002 (2012).
46. H. Ra *et al.*, Detection of non-melanoma skin cancer by in vivo fluorescence imaging with fluorocoxib A. *Neoplasia* **17**, 201-207 (2015).
47. K. Rathore, M. Cekanova, Animal model of naturally occurring bladder cancer: characterization of four new canine transitional cell carcinoma cell lines. *BMC Cancer* **14**, 465 (2014).
48. K. F. Byth *et al.*, AZD5438, a potent oral inhibitor of cyclin-dependent kinases 1, 2, and 9, leads to pharmacodynamic changes and potent antitumor effects in human tumor xenografts. *Mol Cancer Ther* **8**, 1856-1866 (2009).
49. N. K. Altorki *et al.*, Chemotherapy induces the expression of cyclooxygenase-2 in non-small cell lung cancer. *Clin Cancer Res* **11**, 4191-4197 (2005).
50. Y. Fradet *et al.*, A comparison of hexaminolevulinate fluorescence cystoscopy and white light cystoscopy for the detection of carcinoma in situ in patients with bladder cancer: a phase III, multicenter study. *J Urol* **178**, 68-73; discussion 73 (2007).
51. H. B. Grossman *et al.*, A phase III, multicenter comparison of hexaminolevulinate fluorescence cystoscopy and white light cystoscopy for the detection of superficial papillary lesions in patients with bladder cancer. *J Urol* **178**, 62-67 (2007).

52. M. Kriegmair *et al.*, Transurethral resection for bladder cancer using 5-aminolevulinic acid induced fluorescence endoscopy versus white light endoscopy. *J Urol* **168**, 475-478 (2002).
53. M. J. Uddin *et al.*, Fluorocoxib A loaded nanoparticles enable targeted visualization of cyclooxygenase-2 in inflammation and cancer. *Biomaterials* **92**, 71-80 (2016).

APPENDIX

Table 4.1 Receptor tyrosine kinase inhibitors (RTKIs) treatments.

Receptor Tyrosine Kinase Inhibitors (RTKIs) Treatments			
Drug	Abbreviation	Dose	Target
1. Control	DMSO	-	-
2. Axitinib	Ax	5 μ M	c-kit, PDGFR
3. AB1010	AB	5 μ M	c-Kit, PDGFR
4. Toceranib	Toc	5 μ M	c-Kit, PDGFR
5. Imatinib	Ima	5 μ M	c-Kit, PDGFR
6. Erlotinib	Erl	5 μ M	EGFR
7. Gefitinib	Gef	5 μ M	EGFR
8. Sorafenib	Sor	5 μ M	B-Raf, c-Kit, VEGFR
9. Vandetanib	Van	5 μ M	B-Raf, c-Kit, VEGFR
10. AZD5438	AZD	5 μ M	CDKI
11. SP600125	SP	5 μ M	cJUN
12. UO126	UO	5 μ M	MEK

Figure 4.1 RTKIs treatment inhibited cell viability in tested bladder TCC cells.

(A) Human bladder transitional cell carcinoma (TCC) cell lines, J82, RT4, T24, UM-UC-3, 5637, SW780, TCCSUP; and **(B)** canine bladder TCC cell lines, K9TCC#1Lillie, K9TCC#5Lilly, and K9TCC#2Dakota were treated with 5 μ M concentration of tested receptor tyrosine kinase inhibitors (RTKIs) for 48 h. Cell viability was measured by MTS assay and compared to the control (DMSO) groups. Values represent mean $\pm$ standard error of four replicates from two independent experiments; paired Student's *t* test, $*p < 0.05$ and $**p < 0.01$.

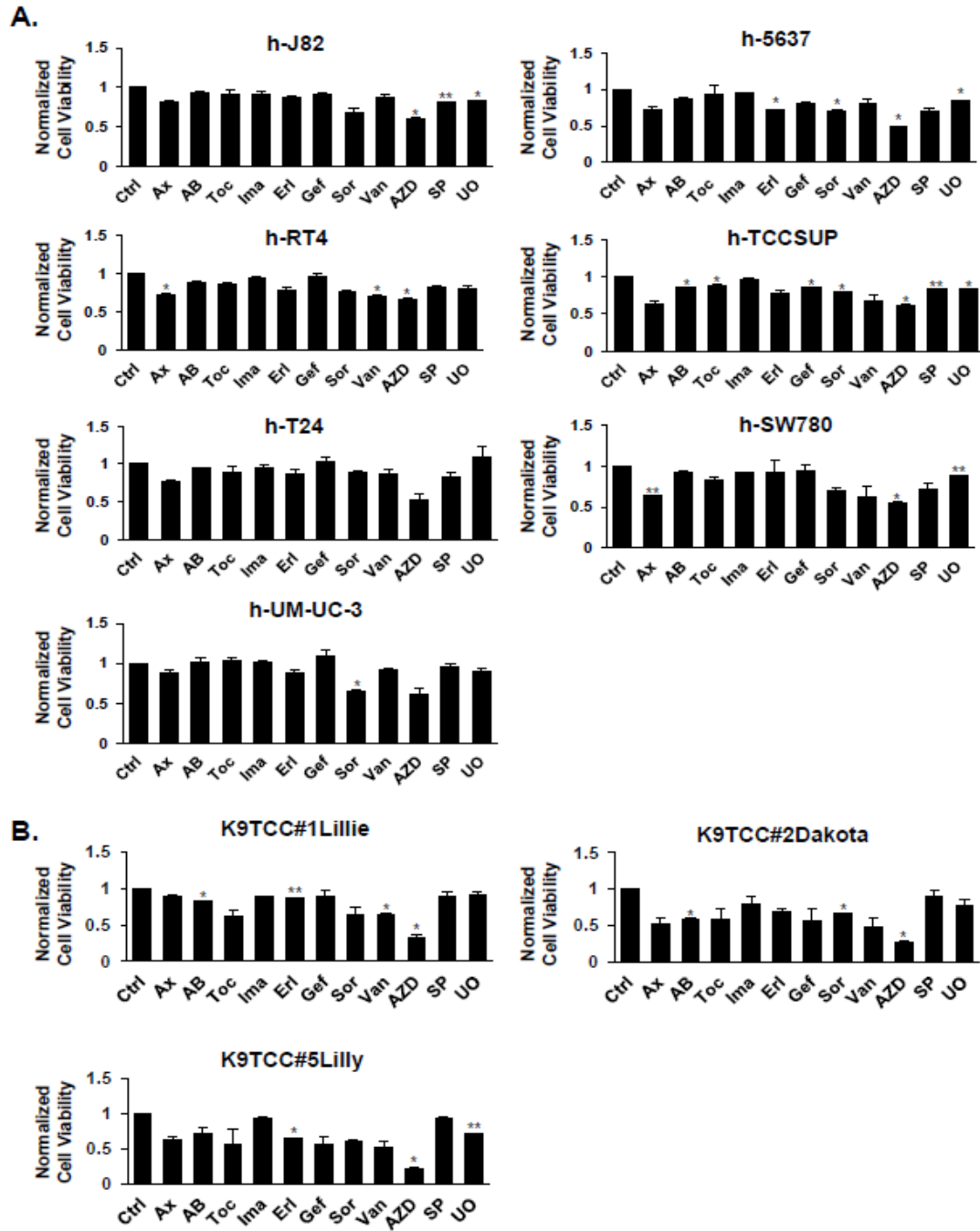


Figure 4.1

Figure 4.2 RTKIs treatment increased COX-2 expression in tested bladder TCC cells as detected by WB analysis.

(A) Human bladder TCC cell lines, J82, RT4, T24, UM-UC-3, 5637, SW780, TCCSUP; and **(B)** canine bladder TCC cell lines K9TCC#1Lillie, K9TCC#5Lilly, and K9TCC#2Dakota were treated with 5 μ M concentration of tested RTKIs for 24 h. The expression of COX-2 was determined by WB analysis and actin was used as a loading control. Several of the tested RTKIs increased COX-2 expression in the COX-2 positive bladder TCC cell lines, including AB1010 and imatinib. No increase in COX-2 expression was detected in the COX-2 negative bladder TCC cell lines. Densitometry evaluation of COX-2/actin protein bands from WB analysis was performed using VisionWorks acquisition and analysis software (UVP). Values represent mean $\pm$ standard error from two independent experiments; paired Student's *t* test, $*p < 0.05$.

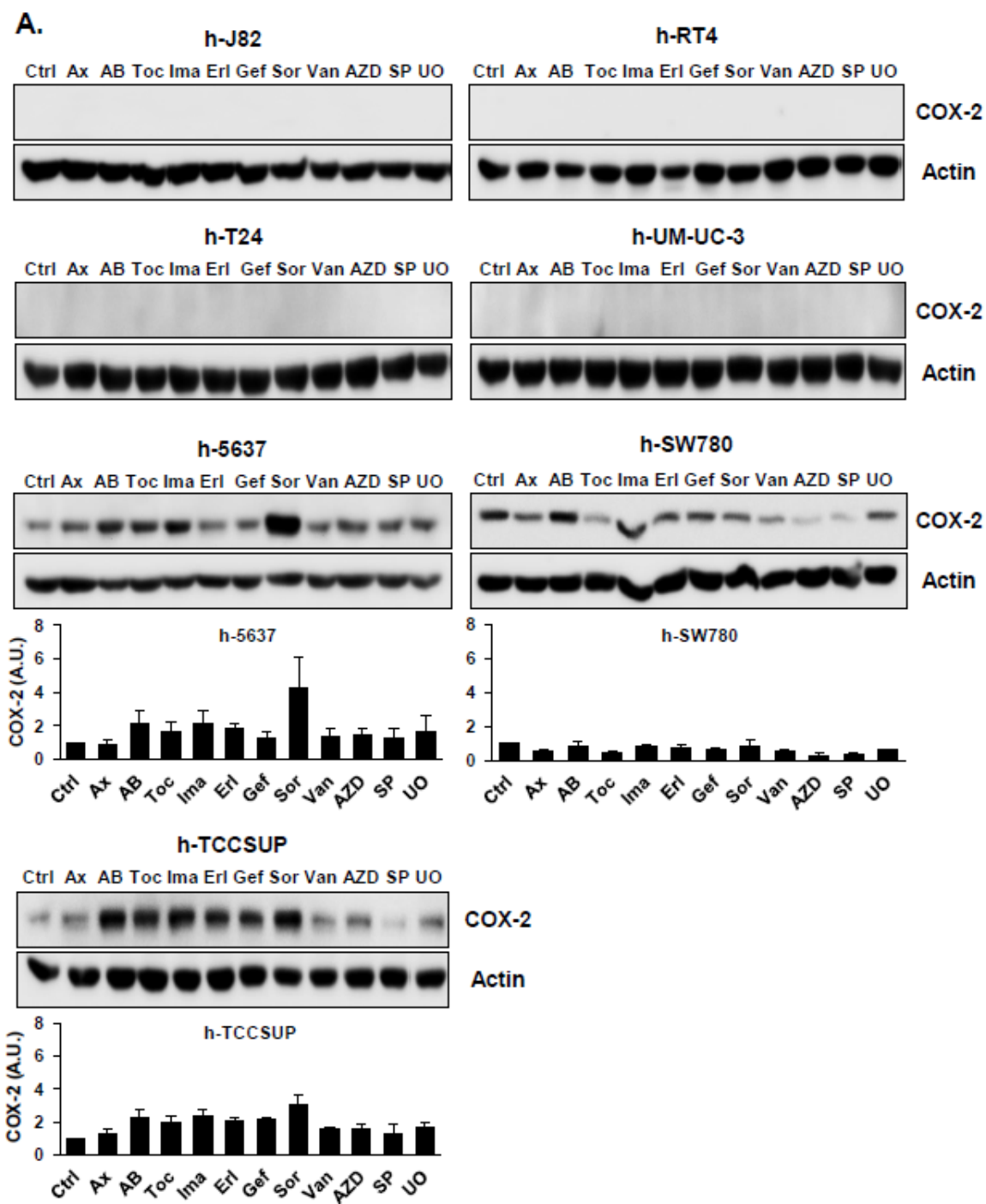


Figure 4.2

B.

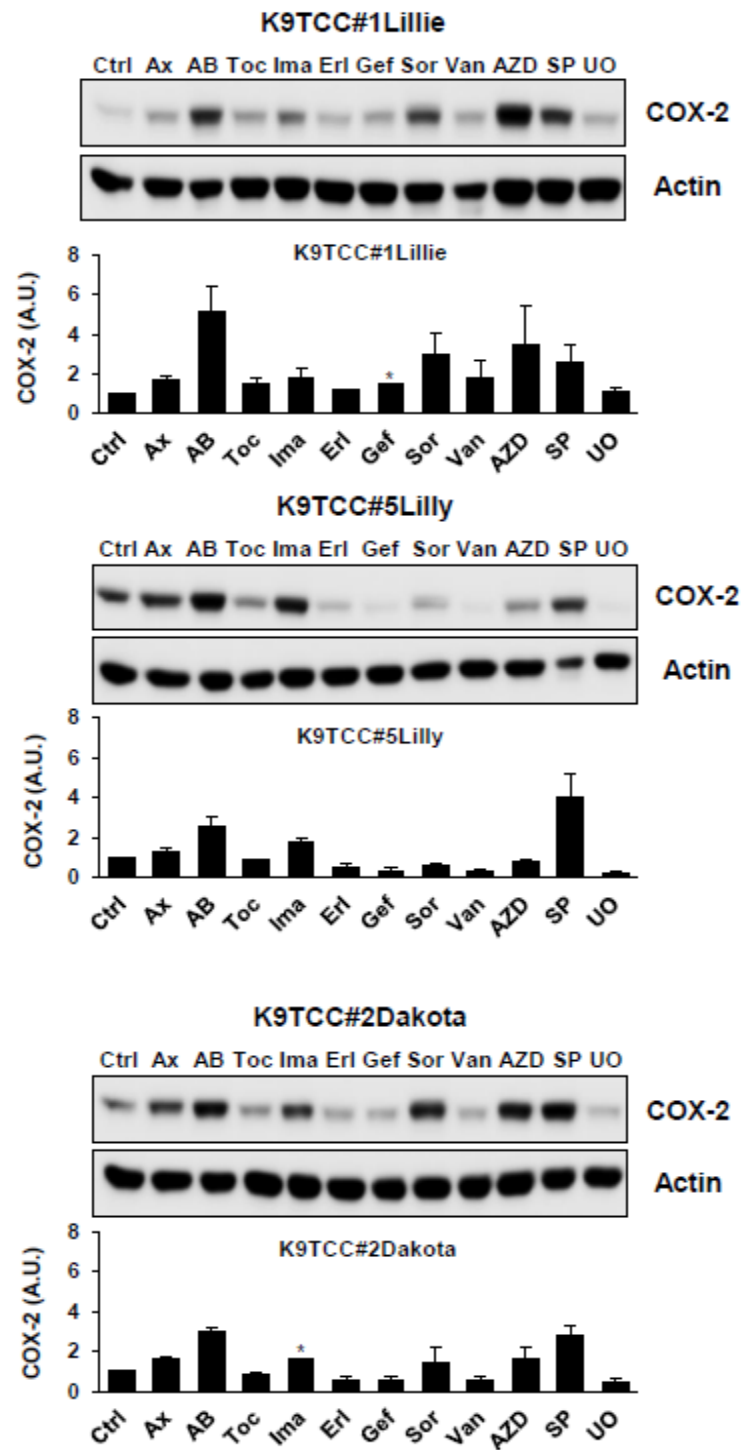


Figure 4.2 (continued)

Figure 4.3 AB1010 and imatinib inhibited the phosphorylation of RTKs but increased COX-2 expression in tested bladder TCC cells in a dose-dependent manner.

(A) Human bladder TCC cell lines, 5637, and TCCSUP; and **(B)** canine bladder TCC cell lines, K9TCC#1Lillie and K9TCC#5Lilly were treated with 0, 1, 5, and 10 μ M of AB1010 and imatinib for 24 h in serum free media. The expression of p-c-Kit, c-Kit, p-PDGFR β , and COX-2 proteins were determined by WB analysis. Actin was used as a loading control. No p-c-Kit expression was detected in the tested bladder TCC cells. Both AB1010 and imatinib inhibited the phosphorylation of PDGFR β in the h-TCC cell lines in a dose-dependent manner. AB1010 increased the phosphorylation of PDGFR β in both K9TCC#1Lillie and K9TCC#5Lilly while AB1010 inhibited the phosphorylation of PDGFR β in the tested K9TCC cell lines. Despite inhibited phosphorylation of RTKs, both AB1010 and imatinib increased COX-2 expression in a dose-dependent manner in the tested human and canine bladder TCC cell lines.

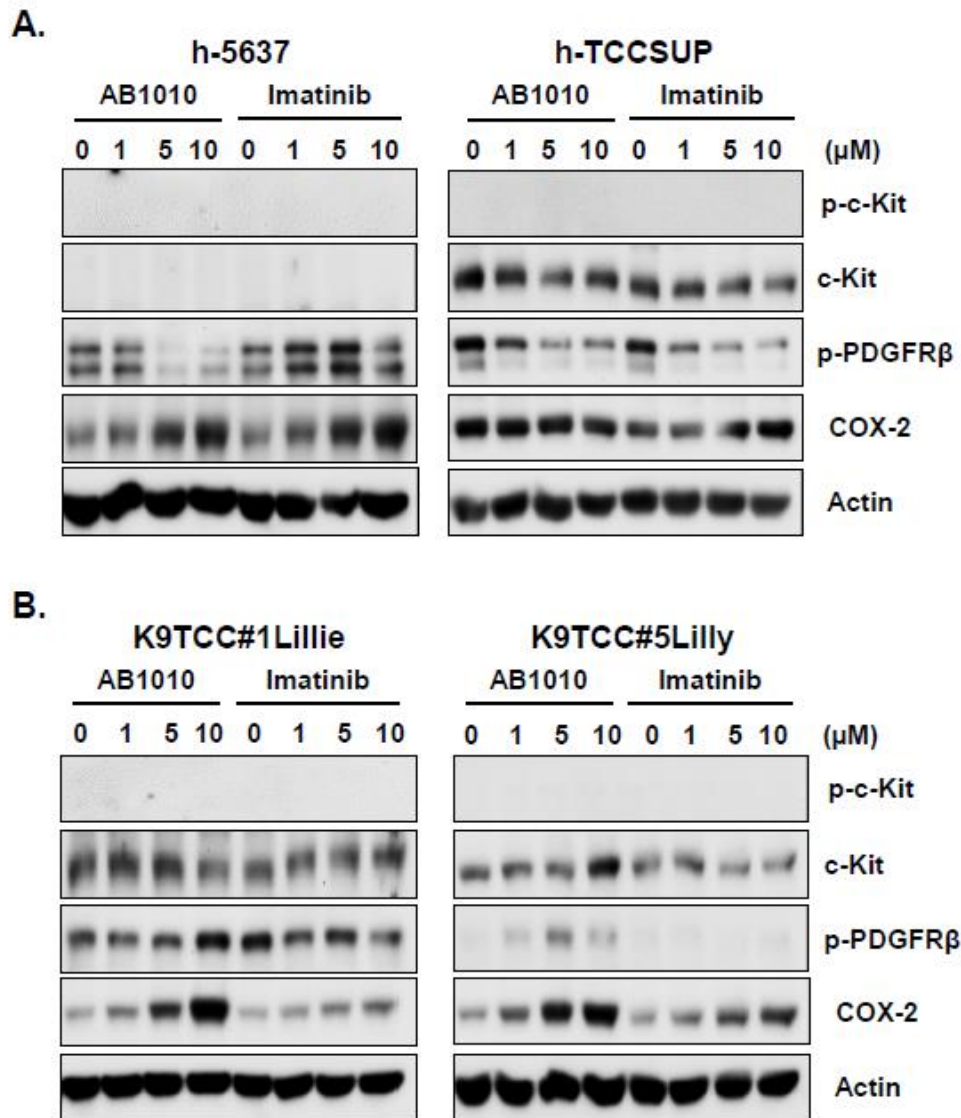


Figure 4.3

Figure 4.4 Detection of RTKI-induced COX-2 expression in K9TCC#5Lilly xenograft tumors by fluorocoxib A.

(A, B) Average body weight and relative tumor volume for each group were plotted as shown. After 2wks of treatment, mice were injected with fluorocoxib A (1mg/kg, s.c.) and imaged using the IVIS Lumina system. **(C, D)** After sacrificing the mice, the dissected organs were photographed and imaged *ex vivo*. Organs from left to right: Row 1 - tumor, heart, lung; Row 2 - kidney, liver, blood; Row 3 - pancreas & spleen, small intestine, muscle, and fat. Blue circle depicts the region of interest (ROI) area used for IVIS image data analysis. **(E)** Relative average radiant efficiency values of fluorocoxib A uptake by the K9TCC#5Lilly xenograft tumors were plotted as tumor-to-noise ratio (TNR). TNR values were calculated as described in the methods section. Higher uptake and increased intensity of fluorocoxib A was detected in Group 2 (AB1010) and Group 3 (imatinib) when compared to Group 1 (control).

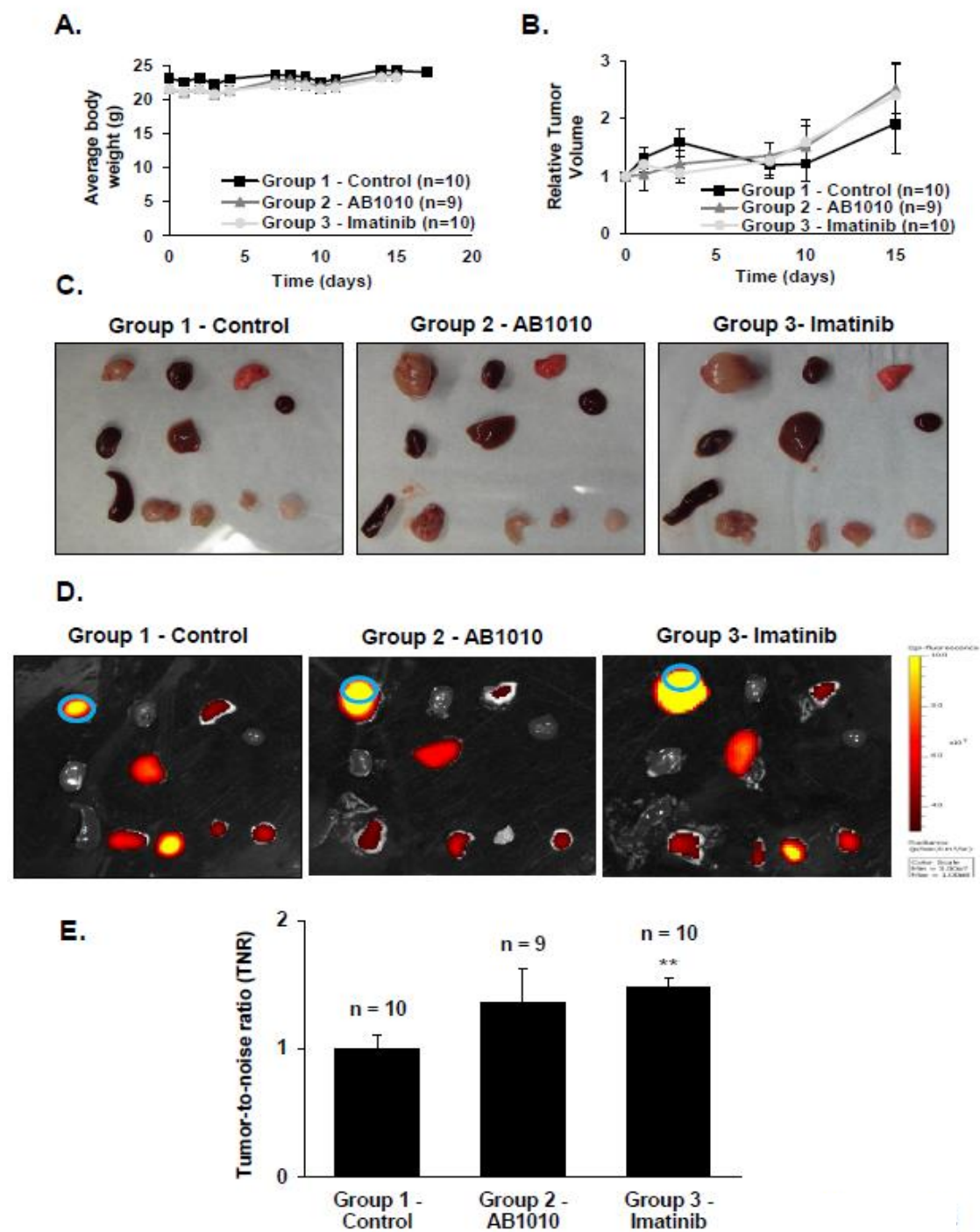


Figure 4.4

Figure 4.5 RTKI-induced COX-2 expression in K9TCC#5Lilly xenograft tumors detected by IHC and WB analysis.

The upregulation of COX-2 by AB1010 and imatinib in the K9TCC#5Lilly xenograft tumors was detected by **(A)** IHC and **(B)** WB analysis. K9TCC#5Lilly xenografts in mice from Group 2 (n=8, AB1010) and Group 3 (n=10, imatinib) treatment had higher COX-2 expression when compared to mice from Group 1 (n=9, control). The higher COX-2 expression in Group 2 and 3 correlates to increased fluorocoxib A uptake. Brown color is positive staining for tested COX-2 protein expression. Representative images were taken by Olympus Microscope, objective 10x, scale bar 100µm. Actin was used as a WB loading control. **(C)** Densitometry evaluation of COX-2/actin protein bands from WB analysis was performed using the VisionWorks acquisition and analysis software (UVP).

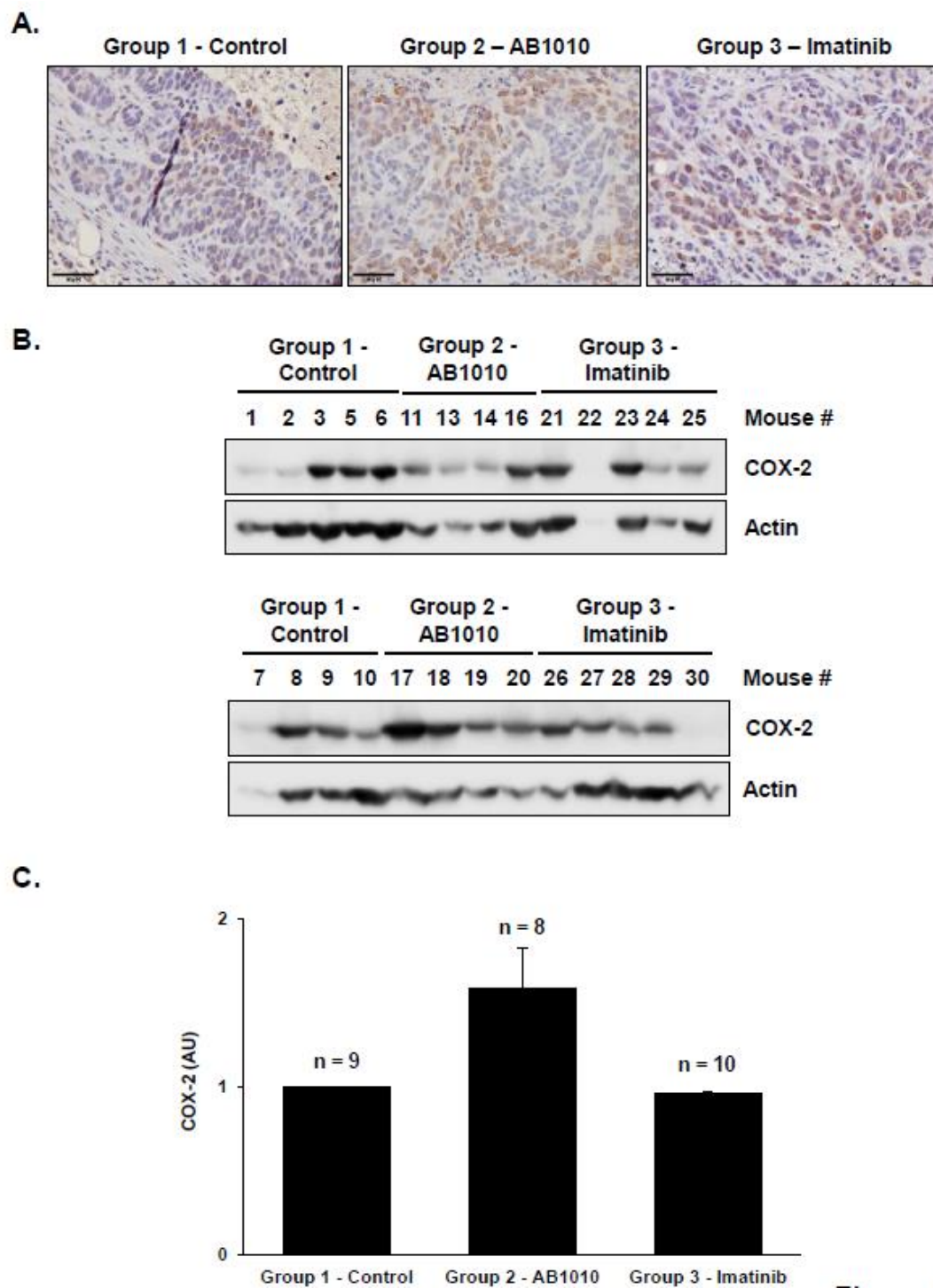


Figure 4.5

CHAPTER V DISCUSSION

GENERAL DISCUSSION

The studies presented in this dissertation were designed to: 1) evaluate the effects of several receptor tyrosine kinase inhibitors (RTKIs) and/or COX inhibitors (non-steroidal anti-inflammatory drugs, NSAIDs) both *in vitro* and *in vivo* using human and canine bladder transitional cell carcinoma (TCC) cell lines, 2) evaluate the novel optical imaging agent, fluorocoxib A, for the detection of bladder cancer *in vivo* using the established carcinogen-induced bladder cancer mouse model, and 3) evaluate the ability of fluorocoxib A to monitor the responses to targeted therapies for the treatment of bladder cancer *in vivo* using the xenograft mouse model.

SUMMARY OF RESULTS

Inhibition of RTKIs-induced COX-2 expression in bladder TCC cells *in vitro*

The basal expression profile of RTKs, COXs, and their downstream targeted proteins were tested in human and canine bladder TCC cells by WB analysis as shown in Fig. 2.1. The K9TCC cell lines had relatively higher expression of the RTKs, PDGFR α and c-Kit, as compared to levels detected in the h-TCC cell lines. Human UM-UC-3 and T24 cells had very low detectable COX-2 expression, whereas h-5673, K9TCC#1Lillie, and K9TCC#5Lilly cells had high COX-2 expression. All tested bladder TCC cell lines had high expression levels of Akt1/2/3, COX-1, ERK1/2, and the COX-2 transcription factor, NF- κ B.

This study demonstrates the effectiveness of the RTKIs, axitinib and AB1010, to significantly inhibit cell viability and induce apoptosis of human and canine bladder TCC

cell lines *in vitro* in a dose-dependent manner. To evaluate the effects of the tested RTKIs on cell viability, an MTS assay was performed. Both axitinib and AB1010 significantly inhibited cell viability in a dose-dependent manner in both human and canine bladder TCC cells, despite the varying expression of the RTKs (c-Kit and PDGFR) in the different bladder TCC cell lines. Axitinib may also be effective in inhibiting additional signaling pathways, other than the ones tested in this study and further evaluation is required to determine the direct mechanism of action for axitinib in those cell lines with low RTKs expression. AB1010 compared to axitinib was more effective in inhibiting cell viability at a 10 μ M dose in both the human and canine cell lines (Fig. 2.2). The bladder TCC cell lines with high c-Kit and PDGFR expression (h-UM-UC-3, K9TCC#1Lillie, and K9TCC#5Lilly) were more sensitive to axitinib and AB1010 treatment after 48 h when compared to the cell lines that do not have high RTKs expression.

We evaluated the effects of the RTKIs on apoptosis by performing Annexin V staining to determine the percentage of apoptotic cells. Axitinib and AB1010 significantly induced apoptosis in both human and canine bladder TCC cells after 24 h treatment (Fig. 2.3). This result was confirmed by evaluating the caspase 3/7 activity and downstream cleavage of PARP (cPARP) in the h-5637 and K9TCC#1Lillie bladder TCC cells (Fig. 2.4). A significant increase in caspase 3/7 activity and cPARP was observed in the h-5637 and K9TCC#1Lillie bladder TCC cells after axitinib treatment in a dose-dependent manner. AB1010 significantly increased caspase 3/7 activity at a 10 μ M dose in the K9TCC#1Lillie cells.

The inhibition of cell viability and induced apoptosis in bladder TCC cells by AB1010 treatment positively correlated with the expression levels of PDGFR α and c-Kit receptors in tested cells. AB1010 inhibited phosphorylation of the c-Kit receptor in a dose-dependent manner causing the inhibition of cell viability and activation of caspase 3/7 in K9TCC#1Lillie cells (Fig. 2.5). Despite AB1010 significantly inhibiting cell viability and phosphorylation of c-Kit, increased levels of COX-2 and NF- κ B expression were detected in both h-5637 and K9TCC#1Lillie bladder TCC cells (Fig. 2.6A). The increase in COX-2 and NF- κ B expression suggests drug resistance to the AB1010 therapy. This is also supported by the reduced effectiveness of axitinib and AB1010 to induce apoptosis and increased p-Akt expression after treatment in the K9TCC#1Lillie cells (Fig. 2.3 and 2.6). These results are consistent with previous studies that indicate after treatment with chemotherapy and targeted therapies (i.e. AB1010) there is an upregulation of COX-2 expression in glioma cancer stem cells, non-small cell lung cancer, and oral squamous cell carcinoma (1-4).

To further investigate the AB1010-induced COX-2 expression in the h-5637 and K9TCC#1Lillie cells, co-treatment of AB1010 (AB) and indomethacin (indo) was performed. MTS results demonstrate that co-treatment of AB + Indo is more effective at inhibiting cell viability than either treatment alone (Fig. 2.7A). We evaluated the effects of the AB + Indo co-treatment on the AB1010-induced COX-2 expression by performing WB analysis. These results show that the AB1010-induced COX-2 expression in both h-5637 and K9TCC#1Lillie bladder TCC cells can be inhibited by co-treatment of AB + Indo (Fig. 2.7 B & C). To confirm the inhibition of COX-2 activity, we measured the

levels of prostaglandins E2 synthase (PGE₂) released into the cell culture media after AB + Indo co-treatment. PGE₂ levels were significantly decreased by the AB + Indo co-treatment in both h-5637 and K9TCC#1Lillie cells (Fig. 2.8). Our results are consistent with previous studies that indicate the COX-2/PGE₂ signaling pathway exerts pro-oncogenic effects through activation of RTKs in colorectal cancer and non-small cell lung cancer (5, 6). By inhibiting the activity of the COX-2 signaling pathway, the cancer cells are more sensitive to the RTKIs therapy, resulting in a more effective treatment option for bladder cancer.

Our results indicate that co-treatment of RTKIs and NSAIDs can abrogate the RTKIs-induced COX-2 expression in bladder TCC cells and result in a more effective treatment than either therapy alone (Fig. 5.1). These results are consistent with previous studies that demonstrate the effectiveness of NSAIDs in combination with other therapies (i.e. chemotherapy and RTKIs) for the treatment of cancer and support our findings that co-treatment of RTKIs and NSAIDs might indicate better clinical success for the treatment of bladder cancer (5-11).

Bladder cancer has an extremely high recurrence rate due to many contributing factors, including the lack of novel therapies for the treatment of bladder cancer over the past several decades. However, as previously mentioned, drug resistance is a common challenge associated with targeted therapies (i.e. RTKIs) due to several reasons, including the activation of alternative signaling pathways (i.e. COX-2 signaling pathway), activation of a bypass oncoprotein, and the presence of secondary mutations in the RTKs which ultimately results in tumor progression. This drives the need for the

characterization of the molecular mechanisms of resistance and early detection of the responses to targeted therapies to improve patient prognostic outcomes. Further studies need to be conducted to evaluate the molecular mechanisms of resistance for the RTKI-induced COX-2 expression in bladder TCC cells following treatment with AB1010.

Detection of early and late stage bladder cancer by fluorocoxib A *in vivo*

Fluorocoxib A is a novel optical imaging agent that selectively targets Cox-2, which commonly is overexpressed in several types of cancers, including bladder cancer. In this study, we confirmed that fluorocoxib A could detect both the early and late stages of Cox-2-expressing bladder cancer using the carcinogen-induced bladder cancer mouse model.

Over the course of 12- and 18- weeks, immunocompetent mice were exposed to the carcinogen, *N*-butyl-*N*-4-hydroxybutyl nitrosamine (BBN), in drinking water (Fig. 3.1A). Following BBN exposure, fluorocoxib A was administered subcutaneously followed by *ex vivo* imaging of the BBN-induced bladder tumors in mice using the IVIS Lumina imaging system. As shown by the representative white light images, the bladders from BBN-exposed mice were larger when compared to the control mice (Fig. 3.2A). Significantly higher uptake and increased intensity of fluorocoxib A was observed in the bladders from mice exposed to BBN when compared to the control mice (Fig. 3.2C, D, & E). These results demonstrate the specificity of fluorocoxib A uptake by the

cancerous bladder tissue when compared to normal urothelium, where no fluorocoxib A uptake was recorded.

The BBN-induced bladder cancer progression from normal urothelium, to inflammation and hyperplasia, and to invasive carcinoma in mice was confirmed by H&E staining (Fig. 3.3 - left). The progression of bladder carcinogenesis was also confirmed by the presence of increased expression of Ki67 expressing cells in bladder carcinomas from mice exposed to BBN (Fig. 3.3 - middle). In addition, uroplakin 1a (UP1a), a protein that is highly expressed in normal bladder urothelium, was downregulated in BBN-induced bladder cancer (Fig. 3.3 - right). These results are consistent with previous studies using the BBN carcinogen for the development of bladder cancer in rodents (12-14). In addition to the development of bladder cancer, fluorocoxib A uptake correlated with presence of neoplastic lesions in the bladders of mice exposed to BBN when compared to mice from the control group.

To confirm the ability of fluorocoxib A to detect Cox-2-expressing bladder cancer, the upregulation of Cox-2 in BBN-induced bladder cancer was confirmed by immunohistochemistry (IHC) (Fig. 3.4A) and western blot (WB) analysis (Fig. 3.4B). Bladder carcinomas in mice from Group 3 had significantly higher Cox-2 expression when compared to normal urothelium in mice from Group 1 and bladder inflammation and hyperplasia in mice from Group 2. The specific uptake and increased intensity of fluorocoxib A (Fig. 3.2) in carcinogen-induced bladder cancer of mice exposed to BBN correlated with the progression of bladder carcinogenesis (Fig. 3.3) and with the increased Cox-2 expression (Fig. 3.4). The detection of fluorocoxib A uptake by the

tumors and upregulation of Cox-2 in BBN-induced bladder cancer can also be detected by immunofluorescence (IF) staining in fresh tissue samples that have been preserved in O.C.T. media. The results from the IF evaluation would further confirm the correlation between increased fluorocoxib A uptake and Cox-2 expression in the tumors.

The results from this study demonstrate the range of fluorocoxib A uptake between the groups and indicates the ability of fluorocoxib A to detect different stages of Cox-2-expressing bladder inflammation and tumors, including the early and late stages of bladder cancer. These results are consistent with pre-clinical studies that demonstrated the effectiveness of fluorocoxib A to be highly specific and selective for detecting Cox-2-expressing inflammation and cancers, including bladder and non-melanoma skin cancer (15-18).

Collectively, these results demonstrate the strong potential for fluorocoxib A to be used as a diagnostic tool for the detection of early and late stage bladder cancer *in vivo*. As previously mentioned, a limitation to targeted optical imaging agents is the necessity for the molecular target (i.e. Cox-2) to be expressed in the cancerous cells. If there is no Cox-2 expression in the bladder tumors, no fluorescence uptake will be detected and further histopathology will be required to confirm the presence of neoplastic lesions. This is a common challenge with precision medicine as there is no one method or technique that can encompass all cases, but the development of novel optical imaging agents such as fluorocoxib A can significantly improve diagnostic and resection procedures for bladder cancer patients to reduce the risk of recurrence. Further studies can be conducted to evaluate the improved efficacy of fluorocoxib A for the detection of

bladder cancer compared to current detection imaging techniques including white light and fluorescent cystoscopy procedures.

Detection of RTKIs-induced COX-2 expression in bladder TCC cells by fluorocoxib A *in vivo*

Previously published studies from our lab have demonstrated that treatment with the RTKI, AB1010, increases COX-2 expression in oral squamous cell carcinoma (4) and TCC (19) cells *in vitro*. To further investigate these results, we evaluated the effects of several RTKIs on COX-2 expression in human and canine bladder TCC cells by WB analysis. Consistent with previous results from our studies, despite inhibiting the phosphorylation of the c-Kit and PDGFR β receptors, AB1010 and imatinib increased COX-2 expression in the tested COX-2 positive human and canine bladder TCC cell lines *in vitro* (Fig. 4.2 & 4.3).

Prior to evaluating fluorocoxib A *in vivo*, we evaluated the specificity of fluorocoxib A to detect COX-2-expressing bladder TCC cells *in vitro*. We confirmed the specific uptake over time of fluorocoxib A is correlated to COX-2 expression in bladder TCC cells *in vitro* (Fig. 5.2A&B). The detection of fluorocoxib A also correlated with COX-2 expression in bladder TCC cells *in vitro* (Fig. 5.3). Based on the results from this study and our previous studies, we proposed that fluorocoxib A can detect and monitor the RTKIs-induced COX-2 expression in bladder cancer cells using the K9TCC#5Lilly xenograft mouse model *in vivo*.

Immunosuppressed mice were randomly divided into three groups and the COX-2 positive bladder TCC cell line, K9TCC#5Lilly, was injected subcutaneously into the shoulder of the mice. After tumor development, treatment with the RTKIs AB1010 and imatinib was initiated. There was an observed increase in relative tumor volume for both treatment groups when compared to the control group (Fig. 4.4B). Following RTKIs treatment, fluorocoxib A was administered subcutaneously, followed by *ex vivo* imaging of the xenograft tumors using the IVIS Lumina imaging system. Significantly higher uptake and increased intensity of fluorocoxib A was observed in the K9TCC#5Lilly xenograft tumors from mice with AB1010 and imatinib treatments when compared to the control mice (Fig. 4D & E).

The RTKI-induced COX-2 expression in K9TCC#5Lilly xenograft tumors was confirmed by IHC (Fig. 4.5A) and WB analysis (Fig. 4.5B). The xenograft tumors from mice in Group 2 (AB1010) had higher COX-2 expression when compared to tumors from mice in Group 1 (control) and Group 3 (imatinib). The specific uptake and increased intensity of fluorocoxib A (Fig. 4.4) by the K9TCC#5Lilly xenograft tumors correlated with the RTKI-induced COX-2 expression both *in vitro* (Fig. 4.2 and 4.3) and *in vivo* (Fig. 4.5)

These results indicate that fluorocoxib A is sensitive and specific enough to detect early responses to targeted therapies in COX-2 expressing bladder cancer. The results from this study are valuable in the fact that the responses to targeted therapies can be monitored over time to determine if a patient is responding to therapy or if drug resistance is occurring. The ability to monitor the responses to therapy using a targeted

imaging agent, such as fluorocoxib A, can significantly improve patient outcomes over time.

FUTURE DIRECTIONS

The results of our studies have demonstrated that: 1) Co-treatment of RTKIs and NSAIDs proves to be a more effective treatment strategy than either therapy alone in COX-2 positive human and canine bladder TCC cells *in vitro*, 2) Fluorocoxib A has the ability to detect both the early and late stages of COX-2-expressing bladder cancer *in vivo*, and 3) Fluorocoxib A is sensitive and specific enough to detect the early responses to targeted therapies in COX-2-expressing bladder cancer *in vivo*.

Previously published studies have indicated that there is cross-talk between the RTKs and COX-2 signaling pathways which promotes the progression of cancer and can result in intrinsic or acquired drug resistance (2, 20-25). These studies also demonstrated that simultaneous inhibition of both signaling pathways results in a more effective treatment response than targeting the RTKs or COX-2 signaling pathway alone. The mechanisms of action for the RTKI-induced COX-2 upregulation is currently unknown and needs to be further elucidated. To determine the mechanisms for the RTKIs-induced COX-2 expression, the role of the RTKs (c-Kit, EGFR, PDGFR, VEGFR, etc.) signaling pathway can be evaluated *in vitro*. The cross-talk between the RTKs and COX-2 signaling pathways in bladder cancer can be evaluated via knockdown of the RTKs by siRNA in human and canine TCC cells, followed by RTKIs treatment *in vitro*. The results of these experiments would further test the idea that there is a direct

connection between the signaling pathways if the RTKI-induced COX-2 expression is not observed when the RTKs expression is knocked down. Evaluation of the RTKs downstream signaling targets, such as the PI3K/Akt and MAPK pathways, would be necessary to determine which downstream signaling proteins have a connection to the COX-2 signaling pathway. These results would suggest that when the RTKs signaling pathway is inhibited by RTKIs treatment, the COX-2 signaling pathway is activated as a pro-survival pathway to promote tumor progression and drug resistance to the RTKIs. In addition, these results would further support the findings that co-treatment of RTKIs and NSAIDs is necessary for better therapeutic responses for bladder cancer than either treatment alone due to cross-talk between the signaling pathways.

To further investigate the efficacy of the co-treatment of RTKIs (ex. AB1010 and imatinib) with COX inhibitors (ex. indomethacin) for the treatment of bladder cancer needs to be validated *in vivo*. A xenograft mouse model of bladder cancer would be necessary to test the efficacy of the individual treatments alone and in combination. At the end of the treatment regimen, fluorocoxib A can be administered prior to euthanasia to allow for *in vivo* imaging of the responses to therapy. The administration of fluorocoxib A would allow for the visualization of COX-2 expression in the xenograft tumors after treatment to confirm the conclusion that co-treatment of RTKIs with NSAIDs abrogates the RTKI-induced COX-2 expression in TCC cells. The results of these studies would also confirm the ability of fluorocoxib A to detect and monitor molecular changes within the tumor in response to targeted therapies. Following success of the xenograft mouse model, these results can be applied to other COX-2-

expressing cancers, such as colorectal cancer and oral squamous cell carcinoma, to determine if this treatment strategy can be applied to different types of cancer *in vitro* and *in vivo*.

To date, many RTKIs are FDA-approved for treatment of human cancers including breast, colorectal, lung, and renal cell carcinoma (26-32). To overcome both intrinsic and acquired resistance to standard chemotherapy and targeted therapies, combination treatment strategies have proven to be more effective than a single therapeutic agent. The results of our studies are consistent with this notion and demonstrate the effectiveness of co-treatment with RTKIs and NSAIDs. This therapeutic strategy can be applied to other COX-2 positive cancers that overexpress RTKs, such as lung and colorectal cancer, where FDA-approval has already been granted to treat patients with RTKIs. Both RTKIs and NSAIDs have been extensively evaluated and are well-tolerated compared to standard chemotherapy and radiation. Pending results from *in vitro* and *in vivo* pre-clinical studies, the effectiveness of co-treatment with RTKIs and NSAIDs in humans, can be evaluated by performing clinical trials with patients who qualify for RTKIs therapy and are diagnosed with COX-2 expressing cancers. These studies have the potential to provide a more effective treatment strategy for patients diagnosed with cancers that have high RTKs and COX-2 expression for a better prognostic outcome and reduce the likelihood of developing drug resistance.

CONCLUSION

These studies indicate that in human and canine COX-2-positive bladder cancer, several receptor tyrosine inhibitors (RTKIs) upregulate COX-2 expression after

treatment despite inhibiting cell viability, inducing apoptosis, and inhibiting the phosphorylation and downstream signaling of the RTKs, indicating acquired drug resistance. Co-treatment of RTKIs and COX inhibitors increases the efficacy of RTKIs targeted therapy *in vitro*. Furthermore, the optical imaging agent, fluorocoxib A, is specific and sensitive enough to detect molecular changes in COX-2 expression to have the ability to monitor the responses to targeted therapies *in vivo*. In conclusion, these results can be used to study novel targeted therapeutic strategies for the treatment of bladder cancer and as a model for monitoring the responses to targeted therapies in patients diagnosed with COX-2 positive bladder cancer to improve prognostic outcomes.

REFERENCES

1. N. K. Altorki *et al.*, Chemotherapy induces the expression of cyclooxygenase-2 in non-small cell lung cancer. *Clin Cancer Res* **11**, 4191-4197 (2005).
2. A. V. Kurtova *et al.*, Blocking PGE2-induced tumour repopulation abrogates bladder cancer chemoresistance. *Nature* **517**, 209-213 (2015).
3. H. I. Ma *et al.*, Celecoxib and radioresistant glioblastoma-derived CD133+ cells: improvement in radiotherapeutic effects. Laboratory investigation. *J Neurosurg* **114**, 651-662 (2011).
4. K. Rathore, M. Alexander, M. Cekanova, Piroxicam inhibits Masitinib-induced cyclooxygenase 2 expression in oral squamous cell carcinoma cells in vitro. *Transl Res* **164**, 158-168 (2014).
5. S. M. Gadgeel *et al.*, Phase II study of gefitinib, an epidermal growth factor receptor tyrosine kinase inhibitor (EGFR-TKI), and celecoxib, a cyclooxygenase-2 (COX-2) inhibitor, in patients with platinum refractory non-small cell lung cancer (NSCLC). *J Thorac Oncol* **2**, 299-305 (2007).
6. J. Shao, B. M. Evers, H. Sheng, Prostaglandin E2 synergistically enhances receptor tyrosine kinase-dependent signaling system in colon cancer cells. *J Biol Chem* **279**, 14287-14293 (2004).
7. W. C. Black *et al.*, 3,4-Diaryl-5-hydroxyfuranones: highly selective inhibitors of cyclooxygenase-2 with aqueous solubility. *Bioorg Med Chem Lett* **13**, 1195-1198 (2003).

8. S. M. Crusz, F. R. Balkwill, Inflammation and cancer: advances and new agents. *Nat Rev Clin Oncol* **12**, 584-596 (2015).
9. H. Hurwitz *et al.*, Bevacizumab plus irinotecan, fluorouracil, and leucovorin for metastatic colorectal cancer. *N Engl J Med* **350**, 2335-2342 (2004).
10. K. Miller *et al.*, Paclitaxel plus bevacizumab versus paclitaxel alone for metastatic breast cancer. *N Engl J Med* **357**, 2666-2676 (2007).
11. A. Sandler *et al.*, Paclitaxel-carboplatin alone or with bevacizumab for non-small-cell lung cancer. *N Engl J Med* **355**, 2542-2550 (2006).
12. G. Akagi, A. Akagi, M. Kimura, H. Otsuka, Comparison of bladder tumors induced in rats and mice with N-butyl-N-(4-hydroxybutyl)-nitrosoamine. *Gan* **64**, 331-336 (1973).
13. H. C. Arentsen, K. Hendricksen, E. Oosterwijk, J. A. Witjes, Experimental rat bladder urothelial cell carcinoma models. *World J Urol* **27**, 313-317 (2009).
14. H. Druckrey *et al.*, [Selective Induction of Bladder Cancer in Rats by Dibutyl- and N-Butyl-N-Butanol(4)-Nitrosamine]. *Z Krebsforsch* **66**, 280-290 (1964).
15. M. Cekanova *et al.*, Molecular imaging of cyclooxygenase-2 in canine transitional cell carcinomas in vitro and in vivo. *Cancer Prev Res (Phila)* **6**, 466-476 (2013).
16. M. Cekanova *et al.*, Single-dose safety and pharmacokinetic evaluation of fluorocoxib A: pilot study of novel cyclooxygenase-2-targeted optical imaging agent in a canine model. *J Biomed Opt* **17**, 116002 (2012).
17. H. Ra *et al.*, Detection of non-melanoma skin cancer by in vivo fluorescence imaging with fluorocoxib A. *Neoplasia* **17**, 201-207 (2015).

18. M. J. Uddin *et al.*, Selective visualization of cyclooxygenase-2 in inflammation and cancer by targeted fluorescent imaging agents. *Cancer Res* **70**, 3618-3627 (2010).
19. J. Bourn, M. Cekanova, Cyclooxygenase inhibitors potentiate receptor tyrosine kinase therapies in bladder cancer cells in vitro. *Drug Des Devel Ther* **12**, 1727-1742 (2018).
20. T. R. Lyons *et al.*, Cyclooxygenase-2-dependent lymphangiogenesis promotes nodal metastasis of postpartum breast cancer. *J Clin Invest* **124**, 3901-3912 (2014).
21. S. M. Thomas *et al.*, Cross-talk between G protein-coupled receptor and epidermal growth factor receptor signaling pathways contributes to growth and invasion of head and neck squamous cell carcinoma. *Cancer Res* **66**, 11831-11839 (2006).
22. A. V. Timoshenko, C. Chakraborty, G. F. Wagner, P. K. Lala, COX-2-mediated stimulation of the lymphangiogenic factor VEGF-C in human breast cancer. *Br J Cancer* **94**, 1154-1163 (2006).
23. X. Wang *et al.*, EGFR signaling promotes inflammation and cancer stem-like activity in inflammatory breast cancer. *Oncotarget* **8**, 67904-67917 (2017).
24. D. Wang, D. Xia, R. N. Dubois, The Crosstalk of PTGS2 and EGF Signaling Pathways in Colorectal Cancer. *Cancers (Basel)* **3**, 3894-3908 (2011).

25. R. Pai, T. Nakamura, W. S. Moon, A. S. Tarnawski, Prostaglandins promote colon cancer cell invasion; signaling by cross-talk between two distinct growth factor receptors. *FASEB J* **17**, 1640-1647 (2003).
26. K. S. Bhullar *et al.*, Kinase-targeted cancer therapies: progress, challenges and future directions. *Mol Cancer* **17**, 48 (2018).
27. I. Walker, H. Newell, Do molecularly targeted agents in oncology have reduced attrition rates? *Nat Rev Drug Discov* **8**, 15-16 (2009).
28. C. Toniatti, P. Jones, H. Graham, B. Pagliara, G. Draetta, Oncology drug discovery: planning a turnaround. *Cancer Discov* **4**, 397-404 (2014).
29. F. S. Collins, H. Varmus, A new initiative on precision medicine. *N Engl J Med* **372**, 793-795 (2015).
30. P. Wu, T. E. Nielsen, M. H. Clausen, FDA-approved small-molecule kinase inhibitors. *Trends Pharmacol Sci* **36**, 422-439 (2015).
31. P. Wu, T. E. Nielsen, M. H. Clausen, Small-molecule kinase inhibitors: an analysis of FDA-approved drugs. *Drug Discov Today* **21**, 5-10 (2016).
32. W. Jeong, J. H. Doroshow, S. Kummar, United States Food and Drug Administration approved oral kinase inhibitors for the treatment of malignancies. *Curr Probl Cancer* **37**, 110-144 (2013).

APPENDIX

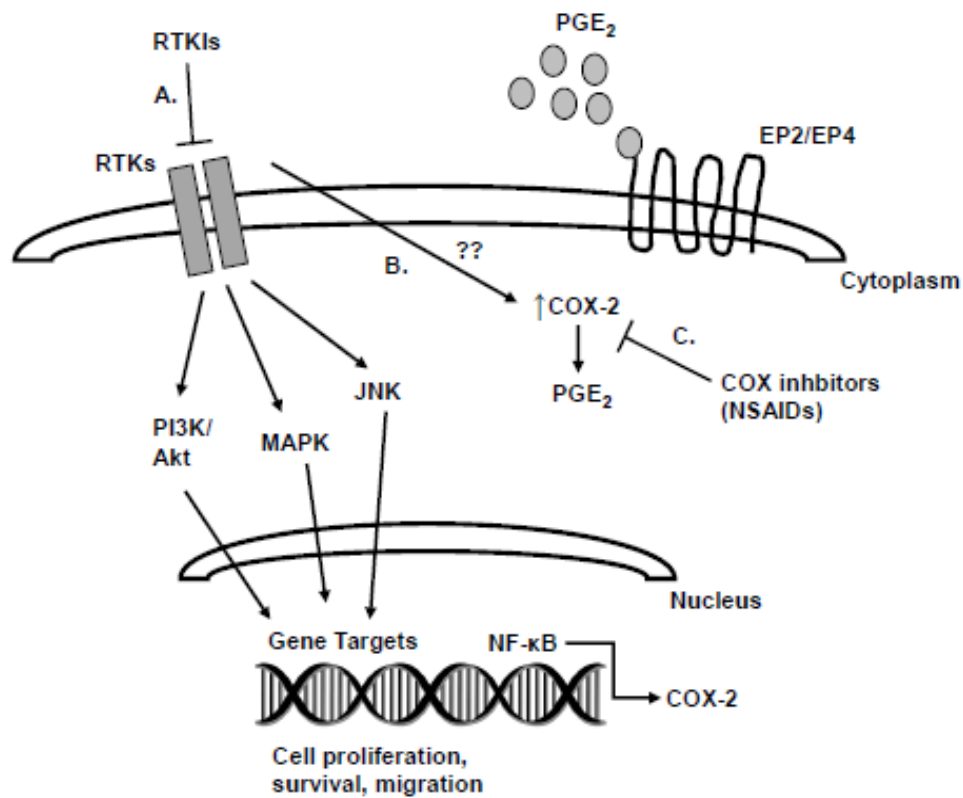


Figure 5.1 Representation of RTKIs-induced COX-2 expression in bladder cancer cells.

(A) Receptor tyrosine kinase inhibitors (RTKIs) inhibit the activity of their target receptors and downstream signaling targets (PI3K/Akt, JNK, MAPK) to inhibit cell proliferation and induce apoptosis. **(B)** RTKIs treatment also shows increased COX-2 expression and subsequent PGE₂ levels in the cells, indicating drug resistance. The mechanism of action for this is still unknown. **(C)** Co-treatment of RTKIs with COX-inhibitors abrogates the RTKI-induced COX-2 expression resulting in a more effective treatment option than either treatment alone.

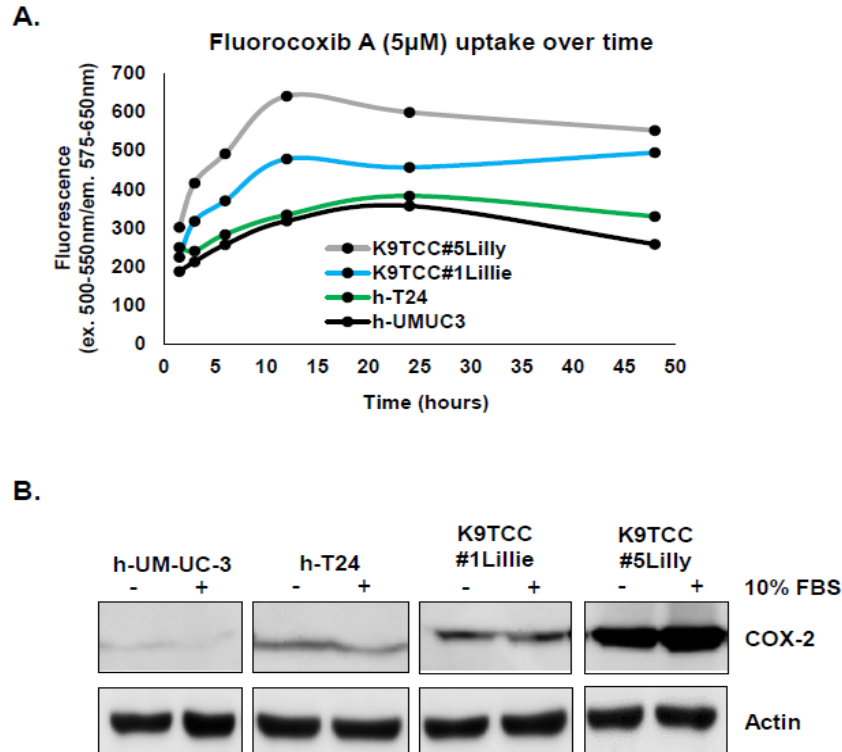


Figure 5.2 Fluorocoxib A uptake correlates with COX-2 expression in human and canine bladder TCC cells *in vitro*.

(A) Human and canine bladder TCC cells were treated with 5 μ M fluorocoxib A at various time points (0.5, 1, 6, 12, 24, and 48 h). Fluorescence was measured to determine fluorocoxib A uptake by bladder TCC cells. Fluorescence readings were normalized to the corresponding cell viability values which were obtained by performing an MTS assay after fluorocoxib A treatment. **(B)** Cells were grown in the absence (-) or presence (+) of fetal bovine serum (FBS) for 24 h. The expression of COX-2 was evaluated by WB analysis. Actin was used as a loading control. The specific uptake of fluorocoxib A by bladder TCC cells correlated with the basal expression of COX-2 in the bladder TCC cells *in vitro*.

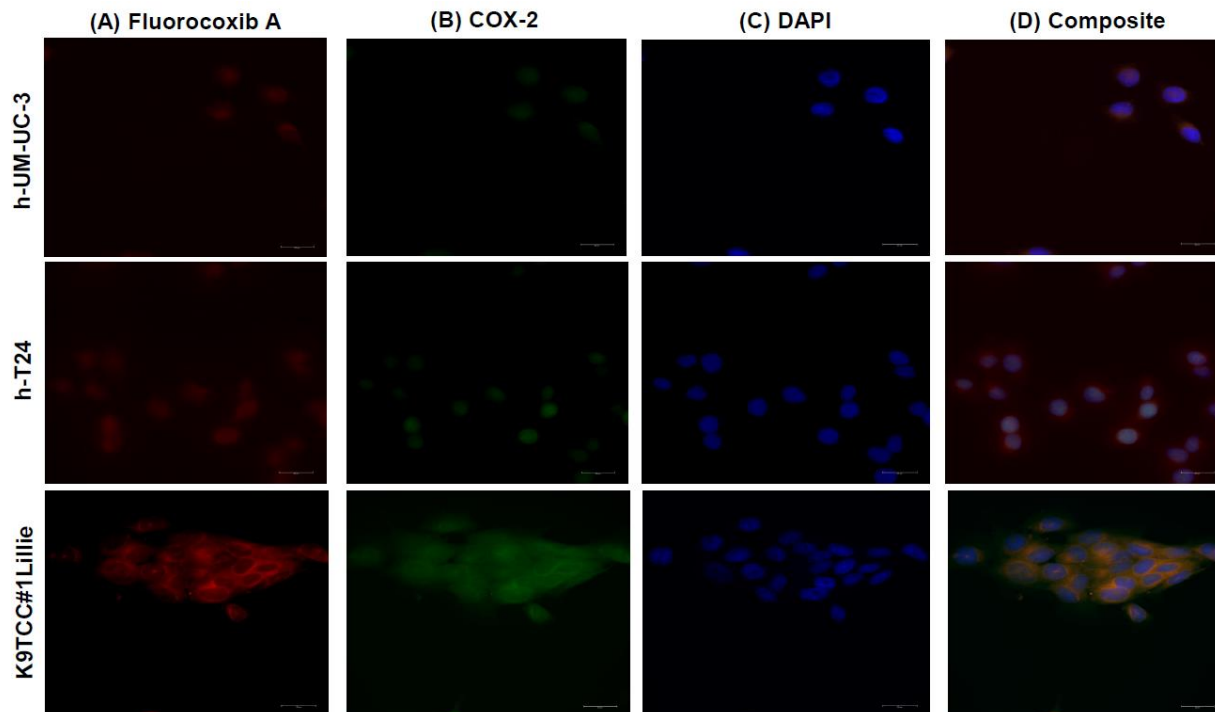


Figure 5.3 Fluorocoxib A detection correlates with COX-2 expression in human and canine bladder TCC cells *in vitro*.

(A) Specific uptake of fluorocoxib A (1 μ M; 1 h R.T.) by human and canine bladder TCC cells *in vitro* (red color). **(B)** Basal COX-2 expression of bladder TCC cells was detected by Alexa Fluor488nm dye (green color) **(C)** Cells were counterstained with DAPI for nuclei detection (blue color). **(D)** Co-localization of fluorocoxib A and COX-2 was confirmed by merging the individual images (yellow color).

VITA

Jennifer Bourn was born in Laconia, New Hampshire to the parents of Lawton and Gail Bourn. She attended elementary school at Holy Trinity Catholic School and continued to Laconia High School in Laconia, NH. After graduating high school in 2007, she pursued and received her bachelor's degree in Genetics from Purdue University (Indiana) in 2011. After received her B.S. degree, she worked as a clinical laboratory assistant at Lakes Region General Healthcare in Laconia, NH. In August 2014, she joined the University of Tennessee – Oak Ridge National Lab (UT-ORNL) Genome Science and Technology graduate program where under the mentorship of Dr. Maria Cekanova, she successfully defended her dissertation and graduated with a Doctor of Philosophy degree in Life Sciences in December 2018. Jennifer plans on pursuing her post-doctoral training at an academic institution.