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Investigative Translational Study of NSAIDs and Chemotherapeutic Drugs for Canine Transitional Cell Carcinoma

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Abstract: Large animal models are being used more often for studying diseases including gastrointestinal (GI) disorders. The dog model shares similar environmental, genomic, anatomical, and intestinal physiologic features with humans. To expand the translational potential of the dog model, we developed a three-dimensional (3D) canine GI organoid system, which provides translational advantages over commonly used models, such as the rodent (1). Increases in our understanding of key signaling pathways within the intestinal stem cell niche growth and maintenance, has allowed for the development of fully differentiated epithelial cells in 3D organoids. Organoids have recently gained interest in translational research as this model system better recapitulates the physiological and molecular features of the tissue environment in comparison with two-dimensional cultures (1). These organoids are relevant in studying the absorption of oral medications for subjects who suffer from GI disorders. The purpose of this study was to investigate the biologic activity of the NSAID piroxicam, and chemotherapeutic agents Doxorubicin and Mitomycin C, using our organoid system, for the treatment of transitional cell carcinoma (TCC). Effective therapies for TCC are limited, with objective response rates to most chemotherapeutic regimens below 20% (2). Ongoing translational research on organoids derived from dogs with naturally occurring digestive disorders has the potential to improve the predictability of preclinical models used for optimizing the therapeutic management of severe chronic enteropathies in human patients.

Background: Transitional cell carcinoma (TCC) of the urinary bladder is the most common cancer of the canine bladder, and accounts for 1–2% of all cancers diagnosed in dogs. According to literature available, certain breeds are at higher risk for developing this disease including the Scottish Terrier, West Highland White Terrier, Beagles, and Shetland Sheep Dogs (2). Evidence suggest that other risk factors for the development of TCC in dogs include sex, obesity, and exposure to herbicides. There are no known effective therapies for TCC, and these tumors are often not amenable to surgical excision as they typically involve the trigone of the bladder and/or occur multifocally throughout the bladder secondary to intravesical seeding (2). When surgical removal can be performed it is usually palliative, as local recurrence and distant tumor relapse rates are high with median survival times of only 3–10 months. Aggressive surgical techniques, such as bladder replacement, have been attempted with very limited success. According to the literature, cystectomy with ureterocolonic anastomosis is associated with severe complications including metabolic acidosis, uremia, pyelonephritis and resulting in survival times of less than 5 months (2).

Available medical therapies for canine TCC are fairly limited, with insufficient outcomes. The COX1/COX2 inhibitor piroxicam has been used for over 20 years to treat canine TCC. According to evidence, the objective response rate and survival time prior to the administration of piroxicam are both low (18% and 6 months, respectively). Prior studies have probed a variety of chemotherapeutic agents to treat canine TCC including carboplatin, cisplatin, doxorubicin, cyclophosphamide and intravesical thiotepa. None of these drugs result in objective response rates greater than 10–15% (2). Piroxicam has been co-administered with carboplatin resulting in no significant improvements in survival. Previous studies showing the combination of cisplatin and piroxicam did improve the objective response rate in affected

dogs to 71%, however, fatal nephrotoxicity occurred in several patients making this therapeutic combination unreliable (2).

Radiation therapy has been previously used to successfully control TCC growth in the bladder in dogs. Although, radiation given in traditional doses when applied to the bladder can lead to harmful complications including a scarring and hypotrophy of the bladder (3). The bladder can relocate within the abdomen and take on a different shape depending on how much urine is in the bladder, which make applying radiation challenging. To use radiation therapy successfully in canine TCC, different treatment schemes need to be developed. Studies are underway, and results may help determine if radiation will have a role in treating TCC in dogs (3).

According to previous studies, in a limited number of cases radiation has been used as the sole treatment modality, with reported median survival times of 4–16 months (2). However, outcomes in canine TCC when adjuvant radiation therapy is incorporated into the treatment regimen are inconsistent (2).

Combination of piroxicam with an intravenous chemotherapy drug called mitoxantrone has been previously used as a treatment protocol. However, in a study performed by the Veterinary Cooperative Oncology Group, this combination treatment resulted in a remission rate of approximately 35% (3). In addition to dogs that had remission, 46% of the dogs also had "stable disease" where the cancer did not grow for a period of time. "Average" survival times with mitoxantrone/piroxicam have been in the 250-300-day range. Some dogs live much longer than this, while others do not live this long (3).

Vinblastine to treat TCC has been previously used as a treatment protocol. Vinblastine is a chemotherapy drug that is given intravenously at 2-week intervals in dogs with TCC. Vinblastine has been popularly used for decades to treat a multitude of cancers in dogs, but it has only recently been used to treat dogs with TCC. According to a study at Purdue University, vinblastine resulted in remission in 35% of dogs, and stable disease (cancer control, but not shrink) in 50% of dogs (3).

According to the literature, results of another treatment study defined another treatment option for dogs with TCC, that is "metronomic" chemotherapy. Metronomic chemotherapy is used to describe the frequent, low dose, oral administration of chemotherapy (3). The drug dose is low in order to be given daily. The research showed that at the dose concentrations used, chemotherapy is most likely not having direct cytotoxic activity to the cancer cells. This type of is thought to block the formation of new blood vessels in the cancer, producing an anti-angiogenic effect. This in turn will kill the cancer cells (3).

Evidence suggest that metronomic chemotherapy will stop the cancer from growing for a period of time. The cancer, however, is not expected to shrink, but to stabilize in growth. According to a Purdue study, a series of 31 dogs with TCC were treated with low dose oral chlorambucil (also called leukeran). In the study, 1 dog had remission, and 20 dogs had stable disease, for a cancer control rate of 70% (3). The median length of life from the start of chlorambucil to death was 7 months, and this extended life was after other therapies had stopped working. The therapy was well tolerated with toxicity being very uncommon (3).

There are other treatments that can also help dogs with TCC. One of the more active types of TCC treatment in humans is treatment with the drugs cisplatin and carboplatin. These drugs have had considerable antitumor activity against canine TCC as well (3). Cisplatin, however, is not used in dogs

very commonly anymore because of risk of damage to the kidneys. Carboplatin is used but needs to be dosed carefully to limit the risk of side effects (3).

Intravesical therapy, which refers to placing anticancer drugs directly into the bladder though a urinary catheter, is a form of treatment in humans with superficial TCC. Through this method of delivery, the drug is expected to stay in the bladder where high concentrations can come in direct contact with the cancer. According to literature, it was not known if intravesical therapy would be of benefit in dogs because TCC in dogs would be deeper in the bladder wall, and tumor masses would often be larger than those treated in humans. This in turn would limit the access of the drug to the tumor. A clinical trial of intravesical therapy (specifically intravesical mitomycin C) in 12 dogs with TCC at Purdue University revealed that the antitumor effects were encouraging. The study consisted of partial remission in 5 dogs and stable disease in 7 dogs, but in 2 dogs the drug appeared to pass from the bladder into the blood stream and then throughout the body (3). These dogs then developed toxicity similar to what would occur with high dose intravenous chemotherapy. This large amount of drug could have been absorbed into the blood stream, it could cause more serious and life-threatening side effects. For this reason, intravesical mitomycin C therapy is not typically given to dogs if there are other treatment options available (3).

Many pet owners have observed humans undergoing chemotherapy and are concerned that some of the serious side effects of chemotherapy in humans will also be observed in pet dogs. Fortunately, most dogs treated with chemotherapy, experience much less toxicity than humans receiving chemotherapy. The side effects of chemotherapy are considered acceptable in most dogs (3).

In this study, we have developed 3D canine bladder organoids from a large a healthy dog and dogs with TCC. The physiological relevance of the canine organoid system was further demonstrated using a functional assay, MTT assay, which measures cell metabolic activity. The 3D organoid model better reproduces the in vivo biology, structure, and function, as well as genetic and epigenetic signatures of original tissues, unlike widely used two-dimensional (2D) cell monolayer models that utilize cancer and immortalized cell lines (1).

In summary, 3D canine organoids are a relevant in vitro animal model with wide applications in veterinary and translational biomedical research. They are used to perform mechanistic studies for basic GI research, for applied preclinical drug permeability, efficacy, and safety testing, for personalized medicine in animal health, and for preclinical research prior to in vivo clinical trials in human patients (1).

Methods: Using canine urine and biopsy samples, we developed 3D bladder and colon organoids. Bladder organoids were obtained and maintained for over 15 passages. Intestinal stem cell isolation and maintenance required defined media that included Wnt3a, R-spondin-1, and Noggin, as well as inhibitors of Rock and GSK3β for the first 2–3 days of culture (1).

Canine bladder cancer cells for TCC organoids were isolated from urine (TCC#1) or biopsies (TCC#2) of dogs with transitional cell carcinoma. Briefly, urine was centrifuged and washed, or small biopsies (Healthy and TCC) were washed with 1X Complete Chelation Solution (CCS) and vortexed up to 6 times, then incubated in 20-30 mM EDTA for 1 hour at 4 C on a rocker. FBS and CCS was added to stop EDTA chelation, mixed, and the supernatant containing cancer cells was put in a new tube. Samples were centrifuged at 150 g for 5 minutes at 4 C, resuspended in DMEM/F12, centrifuged again, and the pellet

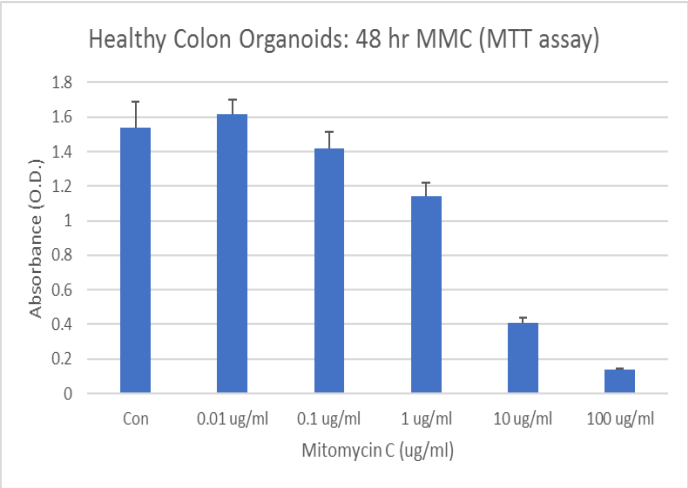
was resuspended in 120 µl of Matrigel. Matrigel droplets were then plated at 30 ul/well Matrigel/cells on 24-well plates. Complete Media with Growth Factors (CMGF+) containing Rock inhibitor and GSK inhibitor was added for 2-3 days at 37 C, and after 2-3 days, organoids were cultured in CMGF+ media without inhibitors. Organoids were passaged every 4-7 days with TrypLE Express to dissociate organoids to single cells.

Organoids from healthy colon or transitional cell carcinoma (TCC) were dissociated and plated at equal density in 30 µl/well Matrigel in 24-well plates in 500 µl/well CMGF+ growth medium. On Day 1-4 after passage, organoids were incubated with the indicated drug for 48 or 96 hours. Cytotoxicity was determined using 3-(4,5-dimethylthiazol-2-yl) 2,5-diphenyl tetrazolium bromide (MTT) at a final concentration of 0.5 mg/mL for 1.5 hr. After medium removal, 200 µl/well cold DMSO was used to dissolve the formazan dye crystals and absorbance was read at 570 nm using a plate reader (SpectraMax 190, Molecular Devices).

The general purpose of the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) assay is to measure viable cells within a 96-well plate without the need for cell counting. Our use of this method was to determine cytotoxicity of piroxicam, doxorubicin, and mitomycin C at different concentrations. The principle of the MTT assay is that for most viable cells mitochondrial activity is constant and thereby an increase or decrease in the number of viable cells is linearly related to mitochondrial activity (4). The mitochondrial activity of the cells is reflected by the conversion of the tetrazolium salt MTT into formazan crystals. These formazan crystals are then broken up mechanically via a pipette, becoming solubilized. Thus, any increase or decrease in viable cell number can be detected by measuring formazan concentration reflected in optical density (OD) using a plate reader at 540 and 720 nm (4). For drug sensitivity measurements the OD values of wells with cells incubated with drugs at various concentrations (ranging from 0.01 µg/ml to 100 µg/ml) are compared to the OD of wells with cells not exposed to drugs.

The MTT assay is suitable for the measurement of drug sensitivity in established cell organoids. For dividing cells, the decrease in cell number reflects cell growth inhibition and the drug sensitivity is then usually specified as the concentration of the drug that is required to achieve 50% growth inhibition as compared to the growth of the untreated control (50% inhibitory concentration, IC50) (4).

Results: The use of adjuvant intravesical chemotherapy agents after an initial transurethral resection of bladder tumor is well known throughout the veterinary community. The most commonly used of these types of agents is mitomycin C. Mitomycin C is an alkylating agent that inhibits DNA synthesis and causes single strand breakage of DNA and chromosomal breaks. It is considered a safe and effective therapy in decreasing tumor recurrence rates in non-invasive bladder cancers (5). The benefits of intravesical mitomycin C therapy in low-risk bladder tumors is well recognize, and our goal was to recapitulate mitomycin C therapy on 3D diseased TCC organoids and healthy colon organoids for translational purposes. Organoids were treated with mitomycin C at various doses (0.01 µg/ml, 0.1 µg/ml, 1.0 µg/ml, 10.0 µg/ml, 100.0 µg/ml) and incubated for 48 hrs. After the incubation period, MMC assay was conducted and analysis was done via Excel. As seen in Figure 1, the healthy colon organoids began to experience cytotoxicity around 1.0 µg/ml and at higher doses began to fully die off. This has been seen in previous studies when mitomycin C is administered directly at higher concentrations in individuals



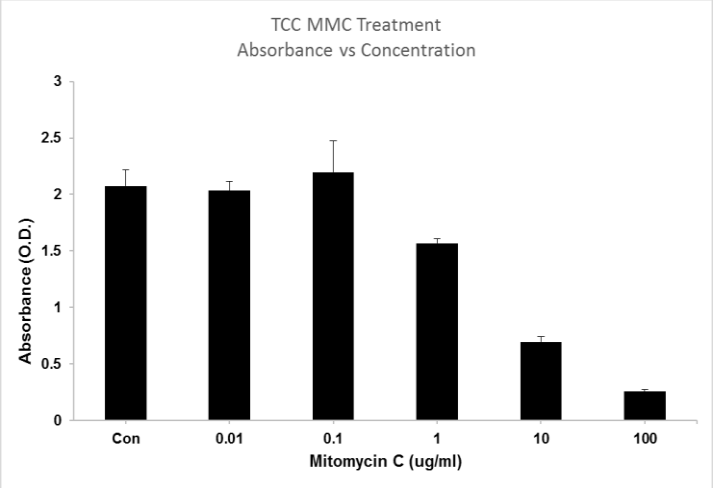


Figure 1 and 2: Healthy colon organoids incubated with mitomycin C for 48 hours at various concentrations and subjected to an MTT assay (Figure 1; Shown on the left). Diseased TCC bladder organoids incubated with mitomycin C for 48 hours at various concentrations and subjected to an MTT assay (Figure 2; shown on the right).

with TCC. When the diseased organoids were treated, as seen in Figure 2, the same results were observed. The organoids began to experience toxicity around 1 µg/ml, then began to die off at higher concentrations. This followed the same trend as the healthy colon organoids.

Doxorubicin is a type of chemotherapy drug called an anthracycline. It slows or stops the growth of cancer cells by blocking Topo Isomerase II. Cancer cells need this enzyme to divide and grow. Doxorubicin is an effective drug used in a variety of cancers and has shown the ability to kill TCC when combined with methotrexate, vinblastine, and cisplatin post-surgery. We paired the known knowledge of the lethality of Doxorubicin (observed in previous clinical trials) with our diseased TCC organoids and were able to kill them at 10.0 µM, As seen in Figure 3.

Piroxicam is a Nonsteroidal Anti-inflammatory drug that possesses both analgesic and antipyretic properties. Drugs, such as Piroxicam, that inhibit cyclooxygenase (cox) have recently been found to have chemopreventive and antitumor activity and may potentiate the effects of chemotherapy. According to a previous study done by Knapp, the antitumor activity of piroxicam is due to an unknown mechanism observed in dogs with TCC of the urinary bladder (6). According to Figure 3, Piroxicam showed inconclusive results on the TCC organoids at concentrations 0.1 µM and 1.0 µM. More data is required.

Conclusion:

In summary, we have developed and maintained long term canine 3D bladder and colon organoid models using urine and biopsy samples from 2 diseased dogs and 1 healthy dog. Our enteroid system complements existing preclinical animal models and provides a translational platform for drug permeability, efficacy, and safety screening. Finally, the tools developed and validated in our lab for the canine 3D organoid system includes a comprehensive set of reagents, probes, and functional assays, which will serve as a foundation for using the dog as a translational model for precision and regenerative medicine (1).

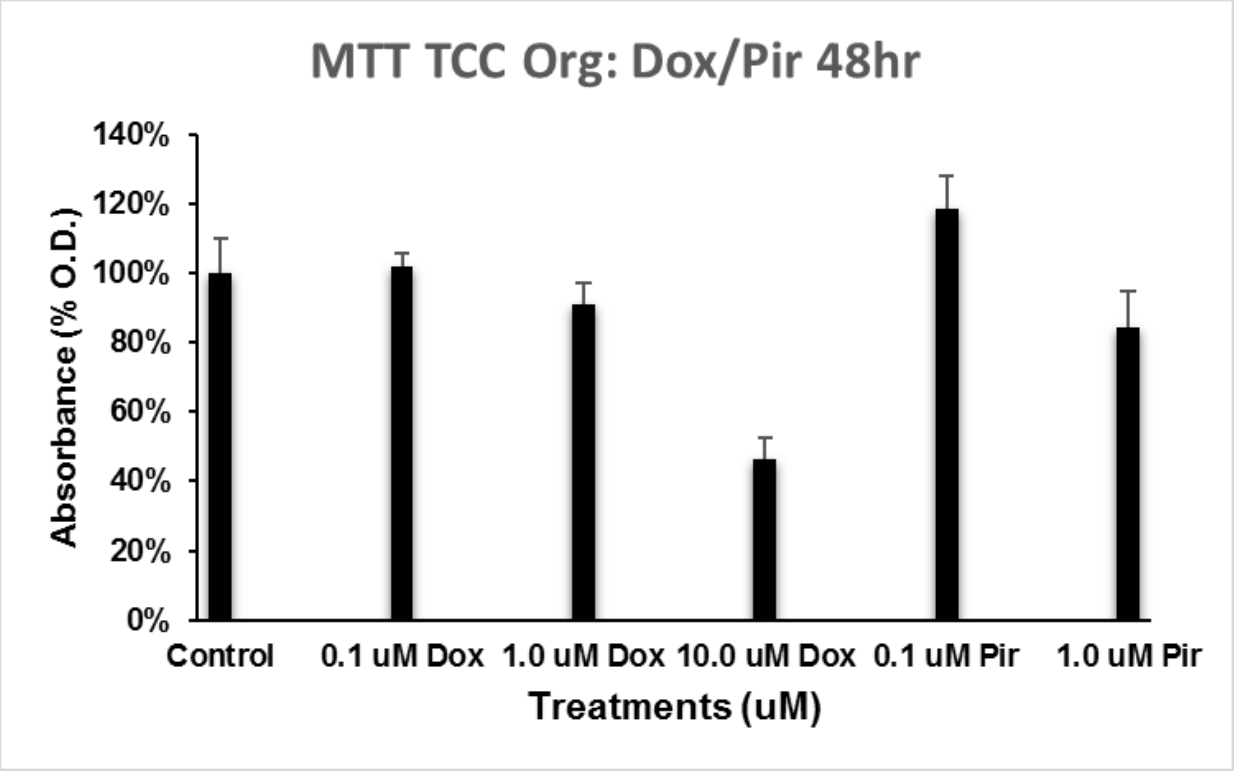


Figure 3: Diseased TCC bladder organoids incubated for 48 hours with doxorubicin or piroxicam at various concentrations then subjected to an MTT assay.

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