THESIS

INVESTIGATION OF CLINICAL GASTROINTESTINAL TOXICITY AND UNDERLYING NORMAL TISSUE DAMAGE ASSOCIATED WITH CONCURRENT ABDOMINAL RADIATION THERAPY AND TYROSINE KINASE INHIBITION

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ABSTRACT

INVESTIGATION OF CLINICAL GASTROINTESTINAL TOXICITY AND UNDERLYING NORMAL TISSUE DAMAGE ASSOCIATED WITH CONCURRENT ABDOMINAL RADIATION THERAPY AND TYROSINE KINASE INHIBITION

Tyrosine kinase inhibitors (TKIs) may be combined with radiation therapy (RT) to enhance tumor control due to their anticancer and antiangiogenic effects; however, clinical evidence has emerged which suggests the treatment combination of RT and TKI may result in higher incidence of normal tissue side effects, dependent on the organs at risk in the radiation treatment field, than would be expected for either modality alone. We evaluated the incidence of gastrointestinal (GI) toxicity in canine cancer patients receiving concurrent hypofractionated abdominal RT and the TKI toceranib and compared to those receiving abdominal RT alone, toceranib alone, or concurrent non-abdominal RT and toceranib. Medical records of canine cancer patients were retrospectively reviewed and identified dogs were included in the following treatment categories: dogs which received RT to a portion of the abdomen and concurrent TOC (n = 19), abdominal RT alone (n = 29), TOC alone (n = 20), or non-abdominal RT plus TOC (n = 9). Toxicities were graded using the Veterinary Cooperative Oncology Group - Common Terminology Criteria for Adverse Events criteria and compared to published data on TOC-associated GI toxicity. Patients receiving TOC while undergoing abdominal RT had significantly increased rates of any grade of diarrhea ($p = 0.002$), hyporexia ($p = 0.0045$), and vomiting ($p = 0.003$), as well as severe hyporexia ($p = 0.003$) when compared across the treatment groups. This retrospective study revealed significantly increased incidences of GI toxicity when abdominal RT was combined with TOC in canine patients.

Following these findings, we investigated the morbidity and underlying histological changes associated with combined abdominal RT and the TKI sunitinib in a mouse model. Prior to the

experimental study, we identified a dose of abdominal RT in CD1 outbred mice which would induce mild GI toxicity according to weight loss and histologic changes in GI tissues harvested 7 days after irradiation; 12 gray (Gy) was selected as the optimal dose for the subsequent experiment. Twenty-five mice were then assigned to control (n = 5), sunitinib alone (n = 7), RT alone (n = 6), or RT + sunitinib (n = 7) groups and were weighed daily. All mice received daily oral gavage of vehicle or sunitinib (40 mg/kg) in vehicle for the entire study. On day 7 mice received 12 Gy abdominal RT or sham irradiation. On day 14 mice were euthanized and their entire GI tract was harvested for histopathologic evaluation, semiquantitative scoring of inflammation, and immunohistochemical quantification of cells positive for CD31 (vascularity) and Ki67 (proliferation). Major findings of this study included that mice in the combined therapy group, RT + sunitinib, lost significantly more weight than sunitinib alone ($p < 0.0001$) or RT alone ($p = 0.0258$). Mice in the RT alone group had a significant increase in GI vascular density, as determined by CD31, when compared to the SUN group ($p = 0.0252$). The mice in the RT + sunitinib group did not mount the same GI vascular response as the RT treated mice. The RT + sunitinib group had more crypt abscessation when compared to groups not receiving RT (vs. Control, $p = 0.0076$; vs. sunitinib alone, $p = 0.0023$). And, while it did not reach statistical significance when compared to the RT alone group, the RT + sunitinib group had more abscessation than RT alone ($p = 0.0862$) which could indicate a trend of higher levels of crypt abscessation with this combined treatment modality.

The results from our canine retrospective clinical study and the preclinical mouse model experiment suggest that abdominal RT + TKI increases morbidity and GI toxicity at the RT and TKI doses investigated. Continued investigation of the underlying normal tissue effects associated with concurrent TKI and abdominal RT are recommended in order to determine whether combining these therapies could be optimized for safety and efficacy, such that GI toxicity is minimalized while achieving optimal tumor control.

DEDICATION

To Phillip

You have taught me more than I could ever teach you. Your future is bright, the possibilities are endless, and you are my greatest achievement.

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INTRODUCTION

Radiation therapy (RT) and tyrosine kinase inhibition are two methods of cancer treatment in both veterinary and human cancer patients. In 2016 we began noticing what seemed to be a trend of increased gastrointestinal (GI) side effects in canine cancer patients being treated at our hospital with concurrent abdominal RT and toceranib, a tyrosine kinase inhibitor (TKI). These patients subjectively appeared to be exhibiting an increase in nausea, vomiting, or inappetence over what would normally be expected for either treatment modality alone, which prompted us to investigate further. A review of published literature and case reports at the time revealed clinical concerns raised in human medicine associated with the combination of RT and TKI use. We sought to better understand the significance of what we were observing clinically in our veterinary cancer patients. We performed a retrospective study examining rates of gastrointestinal (GI) toxicity in dogs receiving toceranib alone, abdominal RT alone, combined toceranib and abdominal RT, or combined toceranib and RT to an area outside of the abdomen. This study revealed statistically significant increases in any grade of nausea, vomiting, and hyporexia in the combined abdominal RT and toceranib group when compared to the other treatment groups, as well as a significant increase in severe hyporexia. This study was published in *Veterinary and Comparative Oncology* in 2022. Subsequently, we further sought to understand why this combination was leading to toxicity and developed a preclinical study in a mouse model. Across the treatment groups, we analyzed changes in weight, performed histopathological evaluation and semiquantitative scoring of inflammation of the GI tissues, as well as quantification of the density of vasculature and proliferating cells throughout the GI tract via immunohistochemisty. With future research we may be able to identify a safe and effective way to deliver these two therapies concurrently or sequentially, via

adjustments in dosage or timing of administration the TKI, or fractionation, dose, or treatment modality of RT.

CHAPTER 1

LITERATURE REVIEW

Treatment with radiation is a mainstay of cancer therapy. Radiation kills cells primarily through mitotic cell death and apoptosis.[1,2] Mitotic cell death occurs when cells with chromosomal damage die during cell division at the first or subsequent mitosis after irradiation.[1,3] The length of time of occurrence of this type of death varies based on the kinetics of the specific cell type, occurring within hours or up to months following RT.[4] This type of cell death occurs predominantly in cells types where natural apoptosis does not play a significant role[5] and is the most common way that cells die after irradiation.[3] Other tissues die via apoptosis, which is a two-stage programmed cell death where cells produce membrane-enclosed bodies that are then digested via phagocytosis or shed from the epithelial surface.[1] Cell types that undergo this type of radiation-induced death are lymphoid cells, serous cells in the salivary and lacrimal glands, and, within the GI tract, epithelial crypt cells and endothelial cells.[3–9] Due to endothelial sensitivity, the vascular system is very susceptible to radiation damage.[10] Apoptosis of endothelial cells in the GI has been shown to be the initiating cause of radiation-induced intestinal damage in mice followed by endothelial crypt cell apoptosis,[6] though this finding has been a source of controversy.[11,12] It is accepted, however, that both endothelial and epithelial damage play a role in GI toxicity following radiation.[12,13]

Normal tissue toxicities can be seen during RT and vary based on the organs at risk in the field, radiation dose, fractionation, and treatment modality.[10,14] These toxicities often limit the dose that can be delivered to the target, reducing efficacy and overall tumor response.[6,15,16] GI toxicities may be seen

when the RT field includes a section of the abdomen and commonly include nausea, vomiting, hyporexia, diarrhea, and abdominal cramping.[17,18] Epithelial cells within the GI Intestinal crypt stem cells die via apoptosis after exposure to radiation beginning within 3-6 hours.[8] As these cells die, they are no longer able to differentiate, move upward out of the crypt, and repopulate the epithelium.[7,8,19] This leads to villi shortening in the small intestine (SI) and a breakdown of the mucosal barrier and mucosal inflammation in both the SI and large intestine (LI).[7,13,19] Irradiated crypts in the SI and LI initially shrink[18,20] followed by hyperproliferation of surviving cells, which leads to a temporary increase in crypt size.[21] Endothelial cell apoptosis within in the GI also contributes to GI toxicities. After radiation, endothelial cell damage causes extensive microvascular injury and microvascular apoptosis in GI tissues.[6] Radiation has been shown to cause an increase in vascular permeability and endothelial detachment from the basement membrane in the microvasculature of the kidney, myocardium, and lungs in mice, rats and humans,[12,22,23] which may also be applicable to the microvasculature of the GI tract. The epithelial and endothelial cell damage in the GI tract leads to damage of the mucosal lining, a toxicity referred to as mucositis.[24]

Mucositis is defined as ulceration and/or inflammation of the oral cavity or GI tract which occurs as a complication following RT or chemotherapy.[24–26] Clinical symptoms of mucositis include the GI toxicities described above and can lead to malnutrition, fatigue, dehydration, and infection that can potentially lead to death.[25] The focus of this review will be mucositis of the GI tract following RT. A five step model for the pathogenesis of mucositis was proposed by Sonis in 2004 and is comprised of the stages of initiation, primary damage response, signal amplification, ulceration, and finally healing.[24] Initiation occurs quickly following radiation and consists of direct cellular injury resulting in clonogenic and apoptotic cell death and indirect damage from the generation of reactive oxygen species (ROS) leading to the beginnings of mucosal barrier breakdown and inflammation.[24–26] This initiation phase is followed by a primary damage response. Sonis describes this as a period wherein transcription factors,

such as p53 and nuclear factor-κB (NF- κB) are activated following the previous cell death and ROS generation, resulting in activation of many genes which have an effect on mucosal toxicity.[24] The upregulation of these genes in turn leads to production of pro-inflammatory cytokines, stimulating further damage to the endothelium and reducing epithelial oxygenation as the inflammatory response mounts.[24] The third step of mucositis is signal amplification and occurs as pro-inflammatory cytokines enter into a positive-feedback loop, amplifying the damage caused by the initial radiation injury.[12,24,26,27] This damage is seen in the basal epithelium and submucosa and occurs alongside inflammation-exacerbated endothelial dysfunction.[26,27] At this stage the tissue may appear clinically normal apart from erythema.[24,26] This signal amplification is followed by the ulceration phase of mucositis, hallmarked by the formation of painful lesions that are susceptible to bacterial colonization and infiltrated by a macrophages and mast cells.[24,26] At this stage a breakdown of the mucosa is seen as the epithelial barrier is discontinuous.[25] The final step of mucositis is healing of the epithelial tissues, as epithelial proliferation and differentiation from the crypts recommences and the mucosal barrier is repaired by re-epithelialization and angiogenesis.[24,26,28]

During the release of cytokines in response to radiation, growth factors are also released.[12] One of these upregulated growth factors is vascular endothelial growth factor (VEGF).[29] Exposure to radiation has been shown to increase VEGF in normal GI tissues in human prostate cancer patients, normal brain tissue in rats, and human glioblastoma cell lines *in vitro*.[29–31] VEGF is secreted by various types of cells, notably macrophages and endothelial cells in the GI tract, which induces proliferation and vascular permeability.[29] VEGF is considered the main regulatory cytokine of angiogenesis and as such is an important component of the wound healing angiogenic cascade.[32–34] This cascade begins with vasodilation and degradation of the basement membrane followed by endothelial cell migration and proliferation.[33] VEGF restores the proliferative capacity of endothelial cells and delays their senescence, allowing for angiogenesis and new capillary growth within the injured tissues, leading to healing.[33]

The VEGF receptor is one type of receptor tyrosine kinase (RTK).[34] RTKs span the plasma membrane and, when phosphorylated, lead to the activation of angiogenic and proliferation pathways.[35,36] Tyrosine kinases are enzymes that catalyze phosphorylation of tyrosine residues after activation of the RTK via ligand binding in the extracellular domain.[35,37] In this manner, they play a role in cellular differentiation, metabolism, migration, and programmed cell death.[35] Examples of the ligands that can bind to RTKs are platelet derived growth factor (PDGF), epidermal growth factor (EGF), and VEGF.[35] In normal cells this activity is tightly regulated, but the overexpression and mutation of some tyrosine kinases in cancer can lead to unchecked growth and malignancy.[35,36,38,39]

Tyrosine kinases can become deregulated in cancerous tissues via mutations, chromosomal rearrangements, autocrine activation, or overexpression.[35,37] May cancers have been identified as having such activity, including ovarian cancer, gliomas[40], non-small cell lung carcinoma (NSCLC)[38,40,41], colorectal cancer, gastric carcinoma, multiple myeloma, acute myeloid leukemia, chronic myeloid leukemia, hepatocellular carcinoma, neuroblastoma, and thyroid carcinoma.[38,39,41] Due to this overexpression in human cancers, RTKs emerged as a target for cancer treatment in the 1980s. The first TKI used for cancer treatment in the United States, imatinib (Gleevec, Novartis Pharmaceuticals, East Hanover, NJ) was introduced in 1998[42] and was approved for clinical use in 2001.[43] Almost immediately, based on the plethora of literature published in the subsequent two years, investigation of TKI use with concurrent RT or conventional chemotherapy for cancer control began.

The earliest investigations of combined TKI and RT reported in literature focused on epidermal growth factor receptor (EGFR) inhibition. *In vitro* and *in vivo* animal xenograft experiments with human epidermoid carcinoma, squamous cell carcinoma, ovarian carcinoma, breast carcinoma, and colon carcinoma cells showed inhibited cellular proliferation or increased tumor response when the modalities were combined, compared to either modality alone.[44–49] Bevacizumab (Avastin, Genetech, Inc, South San Francisco, CA) became the first anti-VEGF monoclonal antibody, whose effects are similar to TKI

inhibition of VEGFR, approved for the treatment of cancer in the United States in 2004.[50–52] It was initially approved for the treatment of advanced colon cancer and later for advanced lung cancer, kidney cancer, glioblastoma, and metastatic breast cancer.[50,52] Approval for the TKIs sorafanib and sunitinib followed in 2005 and 2006, respectively.[53] VEGFR TKIs also showed promising results in preclinical models when combined with radiation for tumor types including non-small cell lung cancer (NSCLC), esophageal adenocarcinoma, colon adenocarcinoma, squamous cell carcinoma, glioblastoma, and melanoma .[51,54–57] After successful preclinical trials, the combination of TKI use with RT naturally moved to clinical trials in human patients, specifically with the hopes that anti-angiogenic TKIs could transiently normalize the vasculature of solid tumors, improving their structure and function, causing tumor radiosensitivity due to an increase in oxygenation .[58–61]

By 2004 there were more than 30 clinical trials for bevacizumab in the US for monotherapy or combined therapies, including RT. At that time, clinical trials combining bevacizumab and concurrent RT focused on pancreatic cancer and solid tumors of the head and neck.[51] Bevacizumab was investigated in combination with fractionated RT for glioblastoma and rectal carcinoma and yielded promising results.[57] In 2020 a retrospective analysis of published studies of patients with brain metastases from renal cell carcinoma treated with RT +/- concurrent TKI was published.[62] The retrospective included 7 studies and concluded that the combination of RT and TKI yielded longer survival times and improved localcontrol.[62] Further favorable outcomes were seen for combined RT and TKI in primary NSCLC,[63] intracranial metastatic NSCLC,[64] and head and neck squamous cell carcinoma. [65]

Clinical trials in veterinary medicine were also emerging, as the first TKI for veterinary use, toceranib (Palladia, Zoetis, Parsippany, NJ), was approved in 2009. Toceranib was initially licensed for the treatment of canine cutaneous mast cell tumors[66] but is now used off-label for the treatment of carcinomas and sarcomas.[67–70] Just as in human medicine, toceranib has been combined with RT for a variety of cancer types including inflammatory mammary carcinoma[67], cutaneous mast cell tumors[68],

osteosarcoma, transitional cell carcinoma, lymphoma, and other sarcomas and carcinomas.[70] The radiation protocols in these veterinary studies were all hypofractionated, weekly protocols with either photons or electrons.[67,68,70]

Despite successes in tumor control, as the combination of RT and TKI became more widely used, toxicities began to be reported in human medicine. A 2008 case study of a patient receiving sorafenib for metastatic renal cell carcinoma (RCC) with concurrent single fraction 8 Gy RT to a spinal metastasis reported death 8 days after RT due to multiple colon perforations attributed to the combined treatment based on the normal tolerance of the bowel to RT being well above the 8 Gy administered.[71] In 2013 Barney, et al. reported a significant increase in the rate of serious bowel injury with intra-abdominal SBRT followed by administration of VEGF inhibition within 3 months of completion of SBRT.[72] The rate of serious bowel injury with the combination was higher than expected for either modality alone.[72] A retrospective study carried out in 2014 to examine late toxicity risks of SBRT to the esophagus found that esophageal fistulas were only present in their patient population when they had also been treated with VEGF inhibitors.[73] Sorafenib was combined with SBRT for hepatocellular carcinoma in a Phase I trial, however at the conclusion of this trial significant toxicities were seen and the recommendation was made not to move this combination out of clinical trial.[74] In veterinary medicine, skin toxicity was reported in the study of dogs receiving toceranib with electron RT for inflammatory mammary carcinoma[67] and we reported increased gastrointestinal toxicities in canine patients receiving hypofractionated RT to the abdomen with concurrent toceranib administration.[70]

One tool for examining such toxicities at a histological level, but more powerful than standard hematoxylin and eosin (E&E) staining, is immunohistochemical (IHC) staining. IHC staining allows for a deeper, cell-specific, investigation of tissues using antibody labeling to detect antigens in tissue samples.[75] The density of labeling within a tissue can then be quantified and used for diagnosis of disease, tumors, microorganisms, or establishment of normal levels of the antibody within a tissue.[75,76]

During the course of our research we utilized the IHC markers Ki67 and CD31. Ki67 is present in proliferating cells and has been used to identify cell proliferation in normal tissues and in tumor diagnostics.[77] Our research focused on Ki67 as a marker of proliferation within the GI tract, as an indicator of crypt epithelial stem cell activity. CD31, also called PECAM-1, is a marker of blood vessels and an indicator of angiogenesis that was first identified in the 1980s.[78,79] CD31 is expressed on vascular cells, including platelets, bone marrow cells, leukocytes, T lymphocytes, and the endothelium of all vessel types.[78] It is known that CD31 is upregulated in human umbilical vein endothelial cells and human dermal microvascular endothelial cells *in vitro* after irradiation.[80,81] CD31+ cells are known to be detectible in mouse intestine.[82] We chose to work with this marker for our mouse irradiation study due to its reliability as a marker of angiogenesis[78] and later learned of its upregulation in human endothelial cells after irradiation.

RT and chemotherapy have been mainstays of cancer treatment for many decades. Significant advancements in the treatment of cancer were seen with the development of TKIs. The identification of overexpression and mutations of RTKs in cancerous cells yielded a new target for therapeutic action. However, in addition to targeting cancerous cells, TKIs can have a negative impact on normal tissues. When combined with RT, tyrosine kinase inhibition can lead to increased toxicities depending on the normal tissues in the radiation treatment field. In evaluating these studies, we identified a gap in knowledge when comparing clinical signs with associated histological changes in an effort to identify the causes of acute toxicities. If these changes could be identified, it is possible that a safe protocol could be developed with regards to the optimal timing and dosing of TKIs, RT fractionation and RT planning, or supportive medications to be administered such that the two modalities could be safely delivered concurrently, yielding a greater outcome for the patient. Review of previously published literature reporting toxicities with combined TKI and RT prompted us to retrospectively study our own population of canine patients that were receiving this combined therapy, which identified GI toxicities and led to a

change in how we recommend treating these patients. We utilized the findings in the literature to guide our selection of RT dose, TKI dosing and administration, and IHC markers to investigate during our mouse study. Our findings add to the body of knowledge with regards to concurrent administration of TKIs and abdominal RT.

CHAPTER 2

INCREASED INCIDENCE OF GASTROINTESTINAL TOXICITY IN CANINE CANCER PATIENTS TREATED WITH CONCURRENT ABDOMINAL RADIATION THERAPY AND TOCERANIB PHOSPHATE

Introduction

Tyrosine kinases (TKs) are enzymes that play an important role in growth factor signaling. TKs function through the transfer of a phosphate group from adenosine triphosphate (ATP) onto a tyrosine residue of a protein. In tumor cells, aberrant TK activation can lead to unrestricted tumor growth and proliferation.[35] Tyrosine kinase inhibitors (TKIs) compete with the ATP binding site on these proteins, preventing phosphorylation, which prevents growth factor signaling. TKIs are used to treat human and veterinary cancers. Treatment of cancer patients with TKIs has been associated with a range of toxicities, including gastrointestinal (GI), hematologic, neuromuscular, and renal injuries.[83] The mechanism of action for many of these TKI-associated toxicities is undefined, however it is suspected that GI toxicities may be due to mucosal damage or disruption in the function of interstitial cells of Cajal.[71,74,83,84].

Toceranib phosphate (TOC; Palladia™, Zoetis, Parsippany, NJ) is a TKI used in veterinary medicine. TOC specifically targets the receptor TKs platelet derived growth factor receptors (PDGFRs), KIT, vascular endothelial growth factor receptor 2 (VEGFR2), Flt-3, and colony stimulating factor-1 receptor (CSF-1R).[66,68,83] Vascular endothelial growth factor (VEGF) plays an important role in angiogenesis, in both tumors and normal tissues; blocking VEGF signaling with TKIs can lead to vascular side effects such as bleeding and increased healing time, as reported in human medical literature.[59]

TOC was originally labeled for use for the treatment of canine mast cell tumors. This oral drug is currently used off-label for both cats and dogs to treat various tumor types due to its anti-tumor as well as antiangiogenic and potential immunomodulatory effects.[69] Although well-tolerated by most animals, with or without the need for modification to dosing, TOC has been shown to cause diarrhea, hyporexia, and vomiting in patients.[83] When given at the label dosage of 3.25 mg/kg, every other day, 50% of canine patients will develop diarrhea, 40% experience hyporexia, and 5% exhibit vomiting.[83] Toceranib has been shown to be an effective means of tumor control when given at doses as low as 2.4-2.9 mg/kg every other day.[85]

Radiation therapy (RT) is used routinely as a means of local tumor control for cancer patients. Preclinical models have demonstrated potential synergistic effects of the combination of TKIs and RT.[86,87] Both human and veterinary patients have been treated with RT and concurrent TKIs to enhance tumor control.[67,68,88,89] As TKIs elicit antiangiogenic effects via VEGF inhibition, they are attractive therapeutics to promote vascular normalization within the tumor microenvironment.[90–92] Tumor vascular normalization has been shown to decrease vascular permeability,[61,93] which may lead to greater RT efficacy via transiently improved tumor perfusion and oxygenation, enhancing RT-induced tumor cytotoxicity.[86] However, there is early evidence which suggests that this combination may exacerbate normal tissue toxicity in human patients.[60,71] It is suspected that effects on the vasculature induced by both TKIs and RT may lead to increased normal tissue toxicities, the type and severity of which may depend on the organs at risk in the treatment field.[84] With respect to the abdomen, irradiation of gastrointestinal organs is known to cause adverse effects such as diarrhea, vomiting, and nausea.[94] Radiation-induced GI toxicities are the result of the death of intestinal crypt cells, as well as mucosal barrier breakdown, which is caused by damage due to inflammation.[26,74,94] Disruption of angiogenesis and healing of irradiated GI tissues via the anti-angiogenic properties of tyrosine kinase inhibitors may exacerbate these abdominal RT side effects.[60]

After observing a concerning frequency of GI toxicity in our canine patient population receiving TOC concurrently with hypofractionated abdominal RT, we were moved to investigate this observation further through retrospective review of our cases. The goal of this study was to evaluate the incidence of GI toxicity in dogs receiving concurrent TOC and abdominal RT compared to those receiving abdominal RT alone, TOC alone, or concurrent TOC and non-abdominal RT.

Materials and Methods

Case selection

This retrospective study was performed at Colorado State University (CSU). Medical records from January 1, 2010 to December 31, 2018 were searched to identify canine patients that received hypofractionated RT at CSU, delivered with 6MV photons utilizing manual dose calculation, and with a treatment field which resulted in abdominal irradiation. Patients were excluded from the study if electron treatments or computer-based planning (3D-conformal RT, intensity modulated RT) was utilized for radiotherapy. Portal imaging was reviewed for each patient to verify that the treatment field included a portion of the abdomen, identified as extending from the diaphragm to the caudal aspect of the patient. Cranial and caudal portions of this area were divided at lumbar vertebra number 3 (L3). For the TOC and abdominal RT group, patients were excluded from the study if they received any form of chemotherapy other than TOC during the time of RT or within three weeks prior to the start of RT. The patients included in the RT alone group were excluded if any chemotherapy was given during RT or within 3 weeks prior to RT. Patients were also excluded if they were exhibiting any signs of GI abnormalities at the time of the first dose of RT, had any known GI diseases or GI-associated abnormalities, or if their tumors incorporated any of the GI tract. Patients in this group included dogs whose TOC treatment was initiated prior to the start of RT as well as those starting TOC treatment on

the date of the first fraction of RT. Patients receiving corticosteroids, H2 antagonists, or proton-pump inhibitors were included in the study.

Additionally, medical records were reviewed of dogs that received TOC alone from December 1, 2014 to February 7, 2018; the same exclusion criteria were applied. Additionally, canine patients were identified as receiving concurrent TOC and hypofractionated RT, but whose radiation fields did not include any portion of the abdomen. They were subject to the same inclusion criteria as the other patients receiving RT and evaluated for the same GI adverse effects. These patients were identified to determine if a general combination of RT, TOC, and repeated anesthetic events were driving GI adverse effects.

The recommended recheck schedule for RT patients was weekly during their radiation treatments, two weeks following their final dose of RT, and then monthly for those patients who were also receiving TOC, while the recommended recheck schedule for patients receiving TOC was every two weeks for the first month, then monthly for two months, and then every 6 weeks for the remaining period that the patient remained on TOC. The only patients included in the TOC arm of the study were those that strictly followed the recheck schedule and were seen at CSU at the recommended interval. Those patients that followed the recheck schedule but discontinued TOC due to GI side effects were included in the study so as not to bias this group towards patients who tolerated TOC. Those that were not seen at the recommended times were excluded.

Medical records review

Medical records of the identified patients were reviewed from the initiation of treatment with RT or TOC until 3 months after completing RT and/or initiating treatment with TOC. Data collected included signalment, tumor type, tumor location (external abdomen (body wall, rectal or vaginal

cavities, axial skeleton, or lymph nodes external to the abdomen), internal abdomen (bladder, prostate, or abdominal lymph nodes), or both external and internal abdominal targets), RT protocol, portion of abdomen included in RT field (<25% of abdomen in RT field, ≥25% of abdomen in RT field), region of RT field (cranial abdomen, caudal abdomen, or extending across both cranial and caudal abdomen), RT field size (calculated equivalent square), and concurrent medications, as well as changes in patient health, with a particular interest in GI side effects, seen during the first three months of therapy. The average dose of TOC received over 3 months and the number of discontinuations due to GI adverse effects were recorded for each patient receiving this drug protocol. Reported GI side effects of hyporexia, vomiting, and diarrhea were recorded from 24 hours after the first dose of RT up to 3 months after completion of the RT protocol. The records of patients receiving TOC alone were reviewed for the first 3 months of TOC treatment and their incidence of GI side effects was recorded in the same manner. Patients who discontinued TOC due to adverse side effects were included in this group, even if they did not have a full 3 months of treatment with TOC, so as not to bias this group with patients who tolerated TOC for an extended period of time. These incidents were scored based on the Veterinary Cooperative Oncology Group – Common Terminology Criteria for Adverse Events v1.1.[95] Toxicities were assigned retrospectively based on review of the medical records. The highest grade of each side effect per patient during the investigation timeline was recorded (**Table 1**).

Radiation Therapy

RT was delivered using a linear accelerator (Varian Trilogy, Varian Medical Systems, Palo Alto, CA, USA) using 6MV photons collimated to the treatment region using secondary collimators. Multileaf collimation was not used to collimate the RT field further than to create a rectangular or square shaped field. The field size was determined to account for the entire planning target volume (PTV) to be within central region of beam, i.e. 80% of the nominal field size and within the penumbra with the edge of the light field positioned to standard minimum of 1cm from the defined PTV margin. Clinical target volume

(CTV) expansions were not defined or recorded for the dogs included in this study. In all cases, manual (factor based on empirical data) isocentric treatment calculations were performed so that the prescribed dose was administered to the midline of the patient utilizing parallel opposed beams. Portal images (MV radiographs) were used to verify positioning prior to each radiation treatment.

Table 1: Criteria used for grading diarrhea, hyporexia, and vomiting as mild (grade 1-2) and severe (grade 3-4) categorization.[95] *IV, intravenous; SC, subcutaneous.*

Side Effect	Grade 1 (Mild)	Grade 2 (Mild)	Grade 3 (Severe)	Grade 4 (Severe)
Diarrhea	Increase of up to 2 stools per day over baseline; decreased consistency.	Increase of 3-6 stools per day over baseline; medications indicated	Increase of greater than 6 stools per day over baseline; IV fluids indicated for greater than 48 hours.	Life-threatening (e.g. hemodynamic collapse).
Hyporexia	Coaxing or dietary change required to maintain appetite.	Oral intake altered less than or equal to 3 days without significant weight loss; appetite stimulants may be indicated.	Oral intake altered greater than 3 days with greater than or equal to 10% weight loss; IV fluids and/or tube feeding indicated.	Life-threatening consequences; total parenteral nutrition indicated; greater than 5 days in duration.
Vomiting	Fewer than 3 episodes in 24 hours, medical intervention not indicated.	3-10 episodes in 24 hours; fewer than 5 episodes per day for less than or equal to 48 hours; IV or subcutaneous SC fluids indicated; medications indicated.	Multiple episodes over greater than 48 hours and IV fluids or partial to total parenteral nutrition indicated for greater than 48 hours.	Life-threatening (e.g. hemodynamic collapse).

Statistical Analysis

The treatment effects of patients included in this study were evaluated according to four groups: abdominal RT alone, concurrent TOC and abdominal RT, concurrent TOC and non-abdominal RT, and TOC alone. Categorical data were analyzed for significance using Chi Square and Fisher's Exact tests. Fischer's Exact test was used for pairwise comparisons of categorical data. One-way ANOVA was used to evaluate differences in continuous data of patient body weight and age across the treatment groups. Tukey's adjustment was used for pairwise comparison of continuous data. A power calculation was not performed prior to the retrospective review of the cases included in this study. Prism 8 was used for all

statistical analyses (GraphPad Software, San Diego, CA).[96] A p value of < 0.05 was used to determine significance.

Results

Patients

Fifty-nine canine patients during the defined evaluation period were identified as receiving hypofractionated RT with fields involving the abdomen. Eleven patients were excluded: three were treated with electron RT, three were treated with three-dimensional conformal RT (3D-CRT) plans, two were receiving concurrent chemotherapy other than TOC at the time of RT, one did not have portal images available for review, one developed an ocular ulcer due to trauma following the first fraction of the RT protocol and TOC was discontinued to help wound healing, and one patient only received one fraction of the RT protocol at CSU before transferring care to another institution to complete the protocol. Forty-eight patients met inclusion criteria for this study, including one patient that was treated at two separate anatomical sites at different times and was counted twice for the study, bringing the total cases to forty-nine. Eighty percent (n = 39/49) of the cases completed their prescribed RT protocol. Of the 10 patients that did not complete their protocol, five developed progressive disease during RT and their owners elected to proceed with an alternative therapy, four were euthanized during their RT protocol (progressive disease (n = 2), severe hyporexia (n = 1), tear in the urinary bladder possibly associated with diagnosed transitional cell carcinoma (n = 1)), and one patient was responding to RT but the owners decided to discontinue treatment for unknown reasons. These 49 cases were broken into two groups: concurrent TOC and abdominal RT (n = 19), and abdominal RT alone (n = 30).

An additional 20 canine patients were identified as receiving TOC alone with 3 months of regular follow up appointments at CSU, and nine canine patients were identified as receiving concurrent TOC and RT to an area that did not include the abdomen.

Patient demographics for each identified group are described in **Table 2**. There was no significant difference in patient body weight across the treatment groups (p = 0.058). There was a significant difference in patient age across the treatment groups (p = 0.02); however, with pairwise

Table 2: Summary of demographics and concurrent medications of canine patients receiving hypofractionated abdominal radiation therapy with or without toceranib, toceranib alone, or non-abdominal hypofractionated radiation therapy with toceranib. TOC, toceranib; RT, radiation therapy; KG, kilograms. Footnotes: ***a)*** *adenocarcinoma (1), sarcoma (1);* ***b)*** *adenocarcinoma (2), lymphoma (2), sarcoma (2), squamous cell carcinoma (2), histiocytic sarcoma (1), melanoma (1), carcinoma (1), mesenchymal neoplasia (1), transmissible venereal tumor (1);* ***c)*** *hemangiosarcoma (2), adenocarcinoma (1), lymphangiosarcoma (1), soft tissue sarcoma (1), squamous cell carcinoma (1)*

	TOC Alone	RT Abdomen Alone	TOC + RT abdomen	TOC + RT Non-abdominal RT	
	(n = 20) n (%)	(n = 30) n (%)	(n = 19) n (%)	(n = 9) n (%)	
Breed					
Mixed	6 (30%)	9 (30%)	4 (21%)	1 (11%)	
Labrador Retriever	4 (20%)	1 (3%)	5 (26%)	6 (67%)	
Golden Retriever	3 (15%)	4 (13%)	1 (5%)	0 (0%)	
Boxer	1 (5%)	2 (7%)	3 (16%)	0 (0%)	
Great Dane	0 (0%)	2 (7%)	0 (0%)	0 (0%)	
Other	6 (30%)	12 (40%)	6 (32%)	2 (22%)	
Sex					
Female spayed	11 (55%)	18 (60%)	10 (53%)	6 (67%)	
Male castrated	9 (45%)	9 (30%)	9 (47%)	3 (33%)	
Female intact	0 (0%)	1 (3%)	0 (0%)	0 (0%)	
Male intact	0 (0%)	2 (7%)	0 (0%)	0 (0%)	
Age (years), median (range)	9.5 (4.9-13)	10.1 (0.6-14.2)	7.2 (4.2-13.3)	7.8 (2.3-12)	p=0.02
Weight (kg), median (range)	32.9 (19.7-53.5)	23.5 (4.7-60.5)	39.8 (9.2-46.5)	25.1 (4.2-39.3)	p=0.058
Tumor Type					
Mast Cell Tumor	6 (30)	4 (13)	12 (63)	9 (100)	
Osteosarcoma	12 (60)	4 (13)	1 (5)	0 (0)	
Mammary Carcinoma	0 (0)	5 (17)	0 (0)	0 (0)	
Transitional Cell Carcinoma	0 (0)	4 (13)	0 (0)	0 (0)	
Other	2 (10)[a]	13 (43)[b]	6 (32)[c]	0 (0)	
Additional Medications					
Corticosteroids	6 (30%)	6 (20%)	13 (68%)	9 (100%)	*p=0.00004*
Proton Pump Inhibitors	5 (25%)	5 (17%)	8 (42%)	4 (44%)	p=0.16
H2 agonists	1 (5%)	4 (13%)	4 (21%)	3 (33%)	p=0.18

comparisons, this significance between the treatment groups was lost. Of the total cases across all groups (n = 79), 35% (n = 31) were treated for mast cell tumor, 22% (n = 17) for osteosarcoma, 6% (n = 5) for mammary carcinoma, and 5% (n = 4) for transitional cell carcinoma. Additional tumor types treated included lymphoma, squamous cell carcinoma, adenocarcinoma, and others. The distribution of tumor types per group is included in **Table 2**.

Radiation Protocols

Radiation protocols were prescribed according to clinician recommendations, with consideration of patient health, client goals, and logistics of treatment for both the patient and client. Of the cases with abdominal RT, 83% (n = 25/30) received weekly RT protocols and 16% (n = 5/30) received daily RT. One of these patients received a course of treatment of 16 Gy over two days (2 treatments of 4 Gy per day, separated by 6 hours, for two consecutive days) and this was repeated monthly for a total of three cycles (48Gy). For this study, the patient was considered to have received a daily protocol.[97] Of the cases with TOC and abdominal RT, all dogs received weekly RT protocols (100%, n = 19). The patients treated with TOC and non-abdominal RT all received weekly RT protocols (100%, n = 9). The patients treated with daily RT protocols were all in the abdominal RT group. Specifics of the various radiation protocols used are described in **Table 3**.

Toceranib Protocols

TOC dosing was consistent across the treatment groups receiving TOC (**Table 4**). TOC dosing, the number of patients requiring a temporary pause or discontinuation of TOC due to GI adverse effects, and timeline for starting TOC with respect to the first fraction of RT are reported in **Table 4**. The average dose of TOC for patients receiving TOC alone was 2.6mg/kg, and for patients in both TOC + abdominal RT and TOC + non-abdominal RT treatment groups the average dose of TOC was 2.5mg/kg. The rate of dose holidays or discontinuation due to adverse GI effects was not statistically significant across the three groups ($p = 0.283$). There was no difference in the percentage of patients in the TOC + RT

treatment groups who received TOC treatment prior to RT compared to TOC treatment initiated at the start of RT (p = 0.37).

Table 3: Distribution of radiation therapy protocols of canine patients receiving hypofractionated abdominal radiation therapy with or without toceranib, or non-abdominal hypofractionated radiation therapy with toceranib. RT, radiation therapy; TOC, toceranib; Gy, Gray.

		Abdominal RT Alone	TOC + Abdominal RT	TOC + Non-abdominal RT
		(n = 30)	(n = 19)	(n = 9)
	Protocol	n (%)	n (%)	n (%)
Daily n=5	**3.2Gy x 5**	1 (3.3%)	0 (0%)	0 (%)
	7Gy x 2	1 (3.3%)	0 (0%)	0 (%)
	8Gy x 2	3 (10%)	0 (0%)	0 (%)
Weekly n=44	**5Gy x 4**	1 (3.3%)	0 (0%)	0 (%)
	6Gy x 4	14 (46.7%)	13 (68.4%)	0 (%)
	6Gy x 5	1 (3.3%)	0 (0%)	0 (%)
	6Gy x 6	6 (20%)	2 (10.5%)	0 (%)
	8Gy x 3	3 (10%)	4 (21.1%)	9 (100%)

Gastrointestinal Adverse Effects

The incidence of the development of any grade GI adverse effects was compared across the four treatment groups. When diarrhea of any grade was recorded, 55% (n = 11/20) of patients receiving TOC alone, 63.2% (n = 12/19) of patients receiving TOC and abdominal RT, 33% (n = 3/9) of patients receiving TOC and non-abdominal RT, and 10% (n = 3/30) of patients receiving abdominal RT alone developed diarrhea at some point up to 3 months after RT (or up to 3 months after starting TOC in patients receiving TOC alone) (p = 0.0002 when compared across all 4 treatment groups). We further analyzed the significance between groups for any grade of diarrhea and found significantly increased incidence when TOC and abdominal RT was compared to abdominal RT alone (p = 0.0002), as well as when TOC

alone was compared to abdominal RT alone (p = 0.0009). For any grade of hyporexia, 40% (n = 8/20) of patients

Table 4: Summary of TOC dosing of canine patients receiving hypofractionated abdominal radiation therapy with toceranib, toceranib alone, or non-abdominal hypofractionated radiation therapy with toceranib. TOC, toceranib; RT, radiation therapy; MG, milligrams; KG, kilograms, GI, gastrointestinal; fx, fraction.

	TOC Alone (n = 20) n (%)	**TOC + abdominal RT** (n = 19) n (%)	**TOC + non-abdominal RT** (n = 9) n (%)	
TOC dose (average)	2.6mg/kg	2.5mg/kg	2.5mg/kg	
TOC dose (range)	2.0-2.8mg/kg	2.4-2.8mg/kg	2.4-2.8mg/kg	
Patients with adverse GI effects requiring:				
1 week dose holiday	2 (10%)	4 (21%)	2 (22.2%)	p=0.06
Dose reduction	5 (25%)	1 (5%)	1 (11.1%)	p=0.24
Discontinuation	1 (5%)	3 (15.7%)	0 (0%)	p=0.62
Patients with any change/discontinuation due to GI adverse effects	7 (35%)	8 (42%)	2 (22%)	p=0.62
TOC Timeline				
Started before RT		12 (63%)	7 (77.8%)	
Started at first fx of RT		7 (37%)	2 (22.2%)	p=0.37

receiving TOC alone, 57.9% (n = 11/19) of patients receiving TOC and abdominal RT, no patients receiving TOC and non-abdominal RT (0%), and 20% (n = 6/30) of patients receiving abdominal RT alone developed hyporexia at some point up to 3 months after RT (or up to 3 months after starting TOC in patients receiving TOC alone) (p = 0.0045 when compared across all 4 treatment groups). Between groups, significant increases in the incidence of any grade of hyporexia was found when TOC and abdominal RT was compared to both abdominal RT alone (p = 0.013) and TOC with non-abdominal RT (p

= 0.004), as well as when TOC alone was compared to TOC with non-abdominal RT (p = 0.033). For any grade of vomiting, 10% (n = 2/20) of patients receiving TOC alone, 52.6% (n = 10/19) of patients receiving TOC and abdominal RT, 22% (n = 2/9) of patients receiving TOC and non-abdominal RT, and 13.33% (n = 4/30) patients receiving RT alone developed vomiting at some point up to 3 months after RT (or up to 3 months after starting TOC in patients receiving TOC alone) (p = 0.0068 when compared across all 4 treatment groups) Between groups, significant increases in the incidence of any grade of vomiting was found when TOC and abdominal RT was compared to both abdominal RT alone (p = 0.008) and TOC alone (p = 0.006). These results are presented in **Figure 1** and **Table 5**.

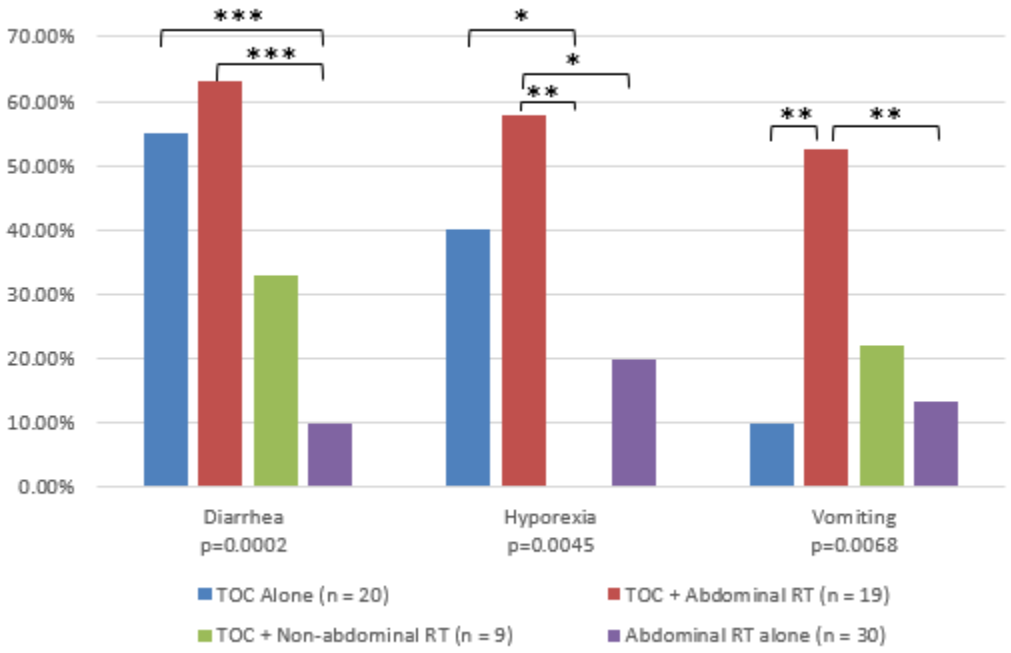

Figure 1: Any Grade (1-4) gastrointestinal toxicities of canine patients receiving hypofractionated abdominal radiation therapy with or without toceranib, toceranib alone, or non-abdominal hypofractionated radiation therapy with toceranib. (p<0.05; **p<0.01; ***p<0.001) RT, radiation therapy.*

The incidence of severe (grade 3 or 4) GI adverse effects was compared across the four treatment groups. Severe diarrhea was seen in 5% (n = 1/20) of patients receiving TOC alone, 15.8% (n = 3/19) of patients receiving TOC and abdominal RT, and not seen in patients receiving TOC and non-abdominal RT (0%) or abdominal RT alone (0%) during the same time periods as described above. There

Table 5: Incidence of adverse effects for canine patients receiving hypofractionated abdominal radiation therapy with or without toceranib, toceranib alone, or non-abdominal hypofractionated radiation therapy with toceranib. Bolded and italicized p values indicate significant findings. Abbreviations: TOC, toceranib; RT, radiation therapy; Abd, abdomen.

GI adverse event	n (%)	*p*-value	GI adverse event	n (%)	*p*-value
Any grade diarrhoea			Severe diarrhoea		
TOC alone (n = 20)	11 (55%)	.748	TOC alone (n = 20)	1 (5%)	.342
TOC + abd RT (n = 19)	12 (63.2%)		TOC + abd RT (n = 19)	3 (15.8%)	
TOC alone (n = 20)	11 (55%)	.47	TOC alone (n = 20)	1 (5%)	.999
TOC + non-abd RT (n = 9)	3 (33.3%)		TOC + non-abd RT (n = 9)	0 (0%)	
TOC alone (n = 20)	11 (55%)	**.0009**	TOC alone (n = 20)	1 (5%)	.4
Abd RT alone (n = 30)	3 (10%)		Abd RT Alone (n = 30)	0 (0%)	
TOC + abd RT (n = 19)	12 (63.2%)	.228	TOC + abd RT (n = 19)	3 (15.8%)	.53
TOC + non-abd RT (n = 9)	3 (33.3%)		TOC + non-abd RT (n = 9)	0 (0%)	
TOC + abd RT (n = 19)	12 (63.2%)	**.0002**	TOC + abd RT (n = 19)	3 (15.8%)	.053
Abd RT alone (n = 30)	3 (10%)		Abd RT alone (n = 30)	0 (0%)	
TOC + non-abd RT (n = 9)	3 (33.3%)	.123	TOC + non-abd RT (n = 9)	0 (0%)	1
Abd RT alone (n = 30)	3 (10%)		Abd RT alone (n = 30)	0 (0%)	
Any grade hyporexia			Severe hyporexia		
TOC alone (n = 20)	8 (40%)	.343	TOC alone (n = 20)	0 (0%)	**.008**
TOC + abd RT (n = 19)	11 (57.9%)		TOC + abd RT (n = 19)	6 (31.6%)	
TOC alone (n = 20)	8 (40%)	**.033**	TOC alone (n = 20)	0 (0%)	1
TOC + non-abd RT (n = 9)	0 (0%)		TOC + non-abd RT (n = 9)	0 (0%)	
TOC alone (n = 20)	8 (40%)	.198	TOC alone (n = 20)	0 (0%)	1
Abd RT alone (n = 30)	6 (20%)		Abd RT alone (n = 30)	0 (0%)	
TOC + abd RT (n = 19)	11 (57.9%)	**.004**	TOC + abd RT (n = 19)	6 (31.6%)	.136
TOC + non-abd RT (n = 9)	0 (0%)		TOC + non-abd RT (n = 9)	0 (0%)	
TOC + abd RT (n = 19)	11 (57.9%)	**.013**	TOC + abd RT (n = 19)	6 (31.6%)	**.002**
Abd RT alone (n = 30)	6 (20%)		Abd RT alone (n = 30)	0 (0%)	
TOC + non-abd RT (n = 9)	0 (0%)	.306	TOC + non-abd RT (n = 9)	0 (0%)	1
Abd RT alone (n = 30)	6 (20%)		Abd RT alone (n = 30)	0 (0%)	
Any grade vomiting			Severe vomiting		
TOC alone (n = 20)	2 (10%)	**.006**	TOC alone (n = 20)	0 (0%)	.231
TOC + abd RT (n = 19)	10 (52.6%)		TOC + abd RT (n = 19)	2 (10.5%)	
TOC alone (n = 20)	2 (10%)	.568	TOC alone (n = 20)	0 (0%)	1
TOC + non-abd RT (n = 9)	2 (22%)		TOC + non-abd RT (n = 9)	0 (0%)	
TOC alone (n = 20)	2 (10%)	.999	TOC alone (n = 20)	0 (0%)	1
Abd RT alone (n = 30)	4 (13.3%)		Abd RT alone (n = 30)	0 (0%)	
TOC + abd RT (n = 19)	10 (52.6%)	.223	TOC + abd RT (n = 19)	2 (10.5%)	1
TOC + non-abd RT (n = 9)	2 (22%)		TOC + non-abd RT (n = 9)	0 (0%)	
TOC + abd RT (n = 19)	10 (52.6%)	**.008**	TOC + abd RT (n = 19)	2 (10.5%)	.145
Abd RT alone (n = 30)	4 (13.3%)		Abd RT alone (n = 30)	0 (0%)	
TOC + non-abd RT (n = 9)	2 (22%)	.607	TOC + non-abd RT (n = 9)	0 (0%)	1
Abd RT alone (n = 30)	4 (13.3%)		Abd RT alone (n = 30)	0 (0%)	

was no significant difference in the development of severe diarrhea across the treatment groups ($p = 0.067$). Severe hyporexia was only seen in patients receiving TOC and abdominal RT (n = 6/19, 31.6%) which resulted in a significant difference across the treatment groups ($p = 0.003$). There was a significant increase in the incidence of severe hyporexia when comparing the patients who received TOC and abdominal RT to those receiving either abdominal RT alone ($p = 0.002$) or TOC alone ($p = 0.008$). When comparing the TOC and abdominal RT group to the TOC and non-abdominal RT group, there was not a significant difference in the incidence of severe hyporexia ($p = 0.136$), despite a notable difference in the rate of severe hyporexia between the groups (31.6% of TOC and abdominal RT vs 0% in the TOC and non-abdominal RT). The lack of statistical significance of this effect may arise due to the relatively small sample size in the TOC and non-abdominal RT group (n = 9) compared to the number of dogs in the TOC and abdominal RT group (n = 19). Severe vomiting was only seen in patients receiving TOC and abdominal RT (10.5%, n = 2/19). This was not significant ($p = 0.126$) when compared across the treatment groups nor when comparing the cohorts individually to each other. These results are presented in **Figure 2** and **Table 5**.

Of note, one patient in the TOC and abdominal RT group was reported as developing clinically mild GI toxicity in the categories of hyporexia (Grade 2) and vomiting (Grade 2) one week following the last dose of RT. However, at this time, the patient was diagnosed with gastric perforation via ultrasonographic evaluation. Gastric perforation is considered a severe adverse effect in VCOG scoring. While this severe effect is recognized and reported for this patient, the Grade 2 hyporexia and vomiting which was recorded at the time of this event was used for statistical analyses of the treatment-associated GI toxicity in this case.

We analyzed whether there were differences in the percentage of patients receiving corticosteroids, H2 antagonists, or proton-pump inhibitors across the treatment groups. The rate of

administration of these medications is summarized in **Table 2.** No significant differences were found across the groups for treatment with proton pump inhibitors (p = 0.12) or H2 agonists (p = 0.18); however, a significant difference was documented with respect to corticosteroid administration (p = 0.00004), as all patients in the TOC and non-abdominal RT group (100%, n = 9) received corticosteroids during their RT protocols. We also analyzed whether treatment with these medications corresponded with differences in the rates of GI toxicities compared to patients in the same treatment group who were not receiving these medications and there were no significant differences.

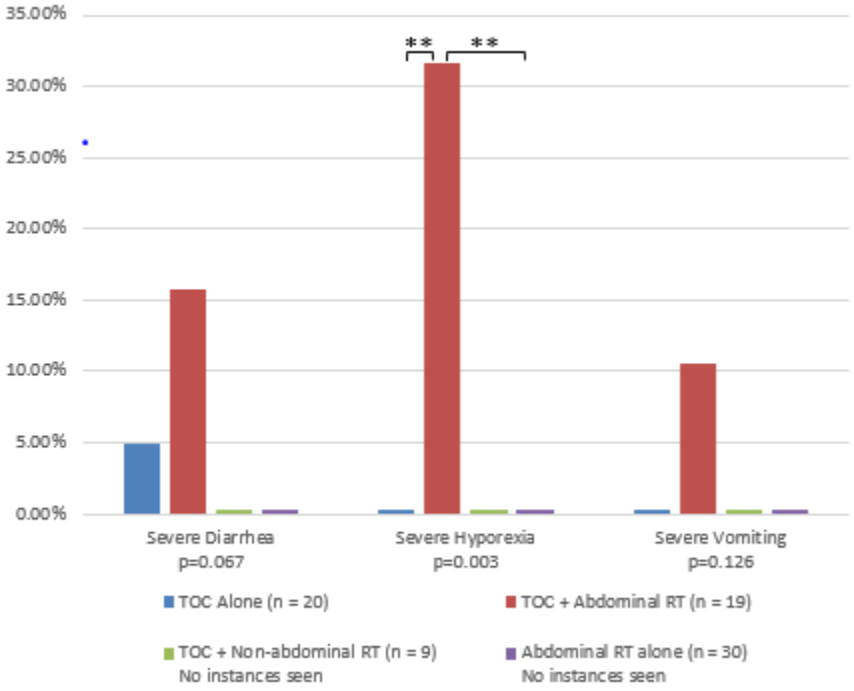

*Figure 2: Severe (grades 3-4) gastrointestinal toxicities of canine patients receiving hypofractionated abdominal radiation therapy with or without toceranib, toceranib alone, or non-abdominal hypofractionated radiation therapy with toceranib. (*p<0.05; **p<0.01; ***p<0.001) RT, radiation therapy.*

The rate of GI adverse effects was compared between dogs in the two groups receiving concurrent TOC and RT with respect to patients starting TOC prior to RT compared to those starting TOC

on the day of the first fraction of RT. There were no significant changes in rate of GI adverse effects, in any category of toxicity. Within the TOC and abdominal RT group, no significant differences in GI toxicity were found between dogs treated with TOC prior to RT compared to those dogs treated with TOC at the start of RT and with respect to diarrhea (p = 0.47), hyporexia (p = 0.34), and vomiting (p = 0.22). A similar lack of significant differences was seen within the TOC and non-abdominal RT group between dogs treated with TOC prior to RT compared to those dogs treated with TOC at the start of RT with respect to diarrhea (p = 0.42) and vomiting (p = 0.59). There were no instances of hyporexia for this group. These results are summarized in **Table 6**.

Table 6: Timeline of initiation of toceranib treatment and radiation therapy and development of gastrointestinal toxicities among canine patients receiving hypofractionated abdominal radiation therapy with toceranib compared to those receiving non-abdominal hypofractionated radiation therapy with toceranib. Abbreviations: TOC, toceranib; Abd, abdominal; AE, Adverse Event; RT, radiation therapy.

TOC + Abd RT (n=19)	**Exhibited AE**	**p value**	**TOC + Non abd RT (n=9)**	**Exhibited AE**	**p value**
	n (%)			n (%)	
Any Grade Diarrhea			**Any Grade Diarrhea**		
TOC started prior to RT (n=12)	7 (58%)	0.47	TOC started prior to RT (n=7)	3 (42%)	0.42
TOC started with RT (n=7)	5 (71%)		TOC started with RT (n=2)	0 (0%)	
Any Grade Hyporexia			**Any Grade Hyporexia**		
TOC started prior to RT (n=12)	6 (50%)	0.34	TOC started prior to RT (n=7)	0 (0%)	N/A
TOC with RT (n=7)	5 (71%)		TOC started with RT (n=2)	0 (0%)	
Any Grade Vomiting			**Any Grade Vomiting**		
TOC started prior to RT (n=12)	5 (55.5%)	0.22	TOC started prior to RT (n=7)	2 (28.5%)	0.59
TOC started with RT (n=7)	5 (71%)		TOC started with RT (n=2)	0 (0%)	

There were four patients (21%, 4/19) in the TOC and abdominal RT group that had one-week TOC holidays; two (11%) at 7 days after their first fraction of RT, one patient (5%) 28 days after the first fraction of RT, and one (5%) 4 weeks after RT was completed. One patient (5%) in this group had a TOC dose reduction 21 days after the first fraction of RT due to GI toxicity. Two patients (22%, 2/9) in the TOC and non-abdominal RT group required a one-week TOC holiday due to GI adverse effects at 9 and

21 days after their first fraction of RT, respectively. One patient (11%) in the TOC and non-abdominal RT group had a TOC dose reduction 3 weeks after completing RT. In the TOC alone group, five patients (25%, 5/20) had dose reductions during the 3-month period reviewed: three patients (15%) at 3 weeks after starting TOC, one (5%) at 4 weeks, and one (5%) at 6 weeks. One patient (5%) in this group was discontinued from treatment with TOC after 6 weeks and two patients (10%) had a one-week TOC holiday due to GI adverse effects, both occurring 4 weeks after starting TOC.

The timing of the development of GI toxicity relative to the start of RT was evaluated between the treatment groups. GI toxicity was seen most frequently 7 days after the first fraction of RT for patients in the two groups treated with concurrent RT and TOC. For patients in the TOC and abdominal RT group, 84% (n = 16/19) developed GI side effects, and, of these cases with GI toxicity, 56% (n = 9/16) were seen at day 7 after starting RT. For patients in the TOC and non-abdominal RT group, 44% (n = 4/9) of patients developed GI side effects and, of these cases with GI toxicity, 75% (n = 3/4) were seen at day 7 after starting RT. Patients in the abdominal RT alone group that developed GI side effects (26%, n = 8/30) were most likely to do so 14 days after starting RT (38%, n = 3/8); however, there was a range of timing for the development of GI toxicity spanning day 7 (n = 1), day 21 (n = 1), day 28 (n = 1), and day 35 (n = 1) from the start of RT.

We examined the frequency RT treatment and the number of anesthetic events for effects on the development of any grade of GI toxicity. In the abdominal RT alone group, those treated with daily RT protocols, either on two consecutive days (n = 3) or five consecutive days (n = 2), no toxicity was seen; those treated with 6 weekly anesthesia events (n = 6) or 5 weekly anesthesia events (n = 2) anesthetic events did not develop GI toxicity. For those with four weekly anesthesia events (n = 15), 46.7% (n = 7/15) had GI toxicities. Of those treated with three weekly anesthesia events (n = 3), 33.3% (n = 1) developed GI toxicities. The TOC and non-abdominal RT group all had 3 weekly anesthesia events (n = 9) with 44.4% (n = 4) exhibiting GI toxicity. In the TOC and abdominal RT group (n = 19), 84% (n =

16/19) developed GI side effects. Within the TOC and abdominal RT group, those with six weekly anesthesia events (n = 2) exhibited no GI toxicity; those with 4 weekly anesthesia events (n = 13), 92.3% (n = 12/13) exhibited GI side effects. The remaining patients in this group were treated with three weekly anesthesia events (n = 4) and 50% (n = 2) developed GI side effects. We compared the incidence of GI toxicities for dogs in the abdominal RT alone and the TOC and abdominal RT groups for those receiving $\leq$3 or >3 fractions of RT. There was no significant difference in GI toxicity for dogs in the abdominal RT group ($p = 0.59$) nor the TOC and abdominal RT group ($p = 0.1$) when comparing those receiving protocols with $\leq$3 or >3 fractions of RT. This comparison was not possible for dogs in the TOC and non-abdominal RT groups, as they all received a 3 weekly fraction protocol.

Abdomen in the RT Field

We analyzed the incidence and severity of each of the GI adverse effects for the groups treated with abdominal RT with respect to characteristics of the RT field. The locations of the targeted lesions were characterized as external abdomen, internal abdomen, or both external and internal abdominal targets. The group receiving abdominal RT alone consisted of 76.7% (n = 23/30) external abdominal targets, 20% (n = 6/30) internal abdominal targets, and 3.3% (n = 1/30) had targets encompassing both. The group receiving concurrent abdominal RT and TOC consisted of 78.9% (n = 15/19) external abdominal targets, 5.3% (n = 1/19) internal abdominal targets, and 15.8% (n = 3/19) with targets that included both. No significance was seen when comparing the distribution of RT targets between these groups for external abdominal targets ($p = 0.57$), internal abdominal targets ($p = 0.16$), or for targets including both locations ($p = 0.2$).

The equivalent square calculated for each RT field was used to compare the area of the treatment fields utilized for patients. The average equivalent square of the RT treatment fields was 16.95 cm^2 (range: 6.9 cm^2 to 35.6 cm^2). The average equivalent square field of the abdominal RT alone

group was 16 cm^2 (6.9 – 35.6 cm^2) and 18.5 cm^2 (9.6 – 33.4 cm^2) for the TOC and abdominal RT group. This difference in equivalent square field size was not significantly different (p = 0.169).

The portion of abdomen included in the RT field, <25% or ≥25%, was determined via review of corresponding portal imaging. The majority of patients in the TOC and abdominal RT treatment group were treated with <25% of the abdomen in the RT field (63%, n = 12), with 37% (n = 7) of dogs treated with ≥25% of the abdomen in the RT field. Similarly, the majority of patients in the abdominal RT alone group were treated with <25% of the abdomen in the RT field (60%, n = 18), and 40% (n = 12) of dogs treated with ≥25% of the abdomen in the field. When looking at any grade of GI toxicities in the categories of diarrhea, hyporexia, or vomiting, we found no significant difference between patients receiving abdominal RT alone to <25% of the abdomen when compared to those receiving abdominal RT alone to ≥25% of the abdomen (p = 0.5478 for any grade diarrhea, p = 0.6599 for any grade hyporexia, and p > 0.9999 for any grade vomiting). There was also no significant difference seen for patients receiving TOC and abdominal RT for any grade toxicity (p > 0.9999 for any grade diarrhea, p > 0.9999 for any grade hyporexia, and p = 0.3498 for any grade vomiting) when percent of abdomen in the field was considered. There were no instances (0%) of severe (grade 3 or 4) adverse effects in any GI category for patients receiving abdominal RT alone regardless of the percent of abdomen in the field. Further, no significant difference was found for severe adverse effects in patients receiving TOC and abdominal RT when percent of abdomen in the field was considered (p > 0.9999 for severe diarrhea, p > 0.9999 for severe vomiting, and p = 0.6027 for severe hyporexia).

We did attempt to evaluate whether there were differences in GI toxicity according to anatomic region of the abdominal treatment field. However, most patients (96%, n = 47) were treated with RT fields in the caudal abdomen, one patient (2%) was identified as receiving RT to the cranial abdomen, and one patient (2%) had a treatment field that extended across both cranial and caudal parts of the

abdomen. This did not provide enough data to draw statistical conclusions regarding incidence of GI toxicity between the radiation fields of the cranial versus caudal portion of the abdomen.

Discussion

The results of this retrospective study demonstrate that there was an increased incidence of GI toxicities in dogs treated with TOC and abdominal RT compared to dogs treated with abdominal RT alone, TOC alone, or TOC combined with non-abdominal RT. Specifically, when the TOC and abdominal RT group was compared to abdominal RT alone, significant increases in any grade of vomiting ($p = 0.008$), any grade of diarrhea ($p = 0.0002$), and severe hyporexia ($p = 0.002$) were reported. When compared to dogs treated with TOC alone, dogs in the TOC abdominal RT group had significantly increased incidences of any grade of vomiting ($p = 0.006$) and severe hyporexia ($p = 0.008$). Those dogs receiving TOC and abdominal RT had significantly increased incidences in any grade hyporexia when compared to those receiving TOC and non-abdominal RT ($p = 0.004$). Radiation exposure to the GI tract is known to cause symptoms of acute GI toxicity across species.[17,20] This toxicity is due to damage to the crypts of the lumen of the intestines through cellular apoptosis as well as through mucosal thinning and villi degeneration.[17] The cells of the GI tract lining have a short lifespan and rapid turnover, making them especially sensitive to radiation. GI side effects can be caused by cellular damage or death, resulting in loss of epithelial integrity, or from inflammation of the epithelium as a response to radiation exposure. GI side effects are seen in the days to weeks following radiation and can manifest as varying degrees of nausea, hyporexia, diarrhea, GI bleeding, or GI ulceration.[17] In this study, 27% dogs treated with abdominal RT alone developed some type of GI toxicity, however, they were all categorized as mild (Grade 1-2).

TOC is known to cause GI adverse effects in approximately one third of canine patients, with reported rates of any grade diarrhea being 46%, hyporexia 39%, and vomiting 32%.[83] The incidence of GI

side effects associated with patients receiving TOC alone in our study population was similar to what has been previously reported with any grade diarrhea being 55%, hyporexia 40%, and vomiting 10%. The exact mechanism of action of TOC-associated GI toxicity is not known but is suspected to be due in part to mucosal damage. This mucosal damage could be the result of disruption in blood supply caused by the blockage of several TK receptors, including VEGFR2 which is highly expressed in tissues with rich blood supply and rapid cellular turnover, such as the epithelial lining of the GI tract.[83] Another suspected mechanism of action is damage to the interstitial cells of Cajal. These cells maintain proper GI motility and are KIT dependent.[83] When KIT is inhibited by TKIs such as TOC, GI hypermotility results which can then lead to diarrhea.[83]

We hypothesize that the combination of TOC and abdominal RT causes an increase in GI toxicity due to VEGFR2 inhibition by TOC and the radiation sensitivity of the highly vascular GI epithelial lining. Blocking VEGFR2 leads to increased tumor control by decreasing blood vessel growth to the tumor, but the systemic nature of oral TOC can cause a decrease in blood supply to normal tissues as well, which are then more susceptible to damage induced by RT. This susceptibility may manifest as an increase in toxicities in the GI tract when the abdomen is exposed to radiation while the patient is receiving concurrent TOC therapy. The effect of VEGFR2 suppression by TKIs used in human medicine has been suspected of causing increased GI bleeding[74], GI perforation[98], and potentially radiosensitization.[71]

This retrospective study reveals an increased incidence of both mild and severe GI toxicity when abdominal RT is combined with TOC in canine patients, which may reinforce the clinical concerns for increased normal tissue toxicity noted in reports from human patients when TKIs are combined with hypofractionated RT and should be further investigated.

In this study, we reviewed our cases retrospectively to evaluate the incidence of GI toxicity in dogs which received TOC concurrently with hypofractionated abdominal RT, after it became apparent

that this combination could be associated with increased normal tissue complications. The dogs included in this study were treated with manually calculated, isocentric RT fields, delivered with 6MV photons. Based on clinical experience, and supported here by the low toxicity profile associated with the abdominal RT treatment group in this study, the photon-based parallel-opposed radiotherapy planning approach and protocols described herein are generally well-tolerated by dogs undergoing hypofractionated radiotherapy with treatment fields which include the abdomen. The patients in this study were treated with what was considered conservative, palliative radiotherapy that did not seemingly require advanced imaging for computer-based planning. However, after completing this project, we recognize the potential for increased GI toxicity in canine patients undergoing hypofractionated abdominal RT and concurrently treated with TOC. Moving forward, when canine patients are presented for RT and concurrently receiving TOC, computer-based radiation planning may be considered for tumor targets in anatomic locations associated with the abdomen to allow administration of more targeted, conformal radiotherapy; however, in clinical reports from human patients, the combination of computer based planning and concurrent TKI administration may still increase gastrointestinal toxicity.[60,71,74] Alternatively, avoidance of concurrent administration of TOC with hypofractionated abdominal radiation may be considered.

Limitations of this study include the retrospective nature of data collection and analysis, with grading and interpretation of adverse effects being subject to possible bias and error. Toxicity reporting depended on owner communication at weekly appointments, or by phone between appointments, and there was no standardized questionnaire or timing for the reporting of these toxicities. Low grade toxicities that occurred between appointments at the hospital may be underreported. The study is also limited by the number of patients included in each treatment category, with a noted small group size for those canine patients who received TOC combined with non-abdominal RT (n = 9). Additionally, there is variability between the treatment groups with respect to in the distribution of disease, tumor types

being treated, and differences in RT protocols and corresponding number of anesthetic events. With respect to RT protocols, all dogs which received daily RT were in the abdominal RT treatment group. Comparison of daily and weekly RT protocols using biologically effective dose (BED) calculations is challenging due to the influence of time and cellular repair between treatments; however, we acknowledge there is a potential for variation in the degree of GI injury induced by daily RT compared to weekly RT protocols in this study. We recognize that the patients in the TOC only treatment group were not subject to repeated anesthesia events which is a limitation in comparing these dogs those undergoing anesthesia for RT. Further, dogs in the TOC only group may not have been returning to the hospital for evaluation at the same interval as patients in the other three RT treatment groups. Also, we recognize that patients with MCT could be at risk of higher rates of GI clinical signs related to their disease process, which may lead to a perceived increase in treatment-related GI toxicities; however, by excluding patients exhibiting any signs of GI abnormalities at the start of TOC and/or RT, we tried to reduce the impact of this variable. Finally, we recognize that the TOC and non-abdominal RT treatment group did not experience GI side effects at the expected rate of patients receiving TOC. Again, with reported rates of any grade diarrhea being 46%, hyporexia 39%, and vomiting 32% for dogs treated with TOC[83], the dogs in the non-abdominal RT and TOC treated group had reduced reported rates of any grade diarrhea 33%, hyporexia 0%, and vomiting 22%. We suspect these results are influenced by the small number of dogs evaluated (n = 9) and not indicative of an effect whereby non-abdominal RT would reduce GI toxicity associated with TOC.

Despite these limitations, we report the first evidence of increased incidences of GI toxicity in canine cancer patients when TOC is combined with hypofractionated RT in areas involving the abdomen. Future directions will include *in vitro* and *in vivo* laboratory studies to elucidate the mechanisms underlying the GI toxicity associated with this combination therapy. Additionally, prospective canine

trials may be designed to define an optimal dosing protocol for TOC and RT when the abdomen is included in the radiation field.

CHAPTER 3

INVESTIGATION OF GASTROINTESTINAL TOXICITIES ASSOCIATED WITH CONCURRENT ABDOMINAL RADIATION THERAPY AND THE TYROSINE KINASE INHIBITOR SUNITINIB IN A MOUSE MODEL

Introduction

Radiation therapy (RT) is a mainstay of cancer treatment. In the process of targeting and killing cancer cells, RT can lead to normal tissue damage, the severity of which depends on dose, fractionation, and the organs at risk in the treatment field.[84] Abdominal RT can lead to gastrointestinal (GI) mucositis which can cause adverse GI side effects such as nausea, vomiting, and diarrhea.[99] Radiation-induced damage to epithelial stem cells located in the base of intestinal crypts can result in clonogenic cell death due to apoptosis of crypt epithelial cells.[13] RT can also induce apoptosis of irradiated GI microvascular endothelial cells which release cytokines, chemokines, and growth factors as they apoptose.[6,20] Additionally, RT administered to the GI tract causes inflammation as local immune cells infiltrate the area and release cytokines and chemokines as part of the inflammatory response.[6,20,24,26,99]

Another method of treating cancer that emerged over two decades ago is through the inhibition of naturally occurring tyrosine kinases. Tyrosine kinases are enzymes which are often overexpressed in cancers and activate many proteins.[35,100] This activation occurs when a phosphate group is transferred from ATP to the tyrosine group on the receptor tyrosine kinase (RTK).[35] RTKs span the cell membrane with extracellular and intracellular domains connected by a hydrophobic transmembrane domain.[35] RTK activation induces signaling of pathways associated with proliferation, such as Ras, Raf, MEK, mTOR, and MAPK, as well as angiogenesis pathways such as those associated with vascular endothelial growth

factor (VEGF).[36,60,101] These cellular proliferation and angiogenesis pathways play an important role in wound healing in normal tissues, but, in tumor microenvironments, can lead to tumor growth and metastasis.[59,100,102] Tyrosine kinase inhibitors (TKIs) compete with the ATP, preventing phosphorylation, and act as an off-switch to prevent proliferation and growth factor signaling.[35] The TKI sunitinib specifically targets the endothelial growth factors VEGFR1, VEGFR2, and VEGFR3, platelet derived growth factors (PDGF) PDGFRa and PDGFRb, as well as KIT, FLT3, and CSF1R.[91,101,103,104]

In cancer therapy, TKIs can be used as a single agent or in conjunction with RT to enhance tumor control, both locally and systemically, due to anticancer and antiangiogenic effects.[36,59,89] TKIs are an appealing cancer treatment because formulations can be chosen to target a particular pathway of interest, allowing for selective inhibition of proteins found specifically in a certain cancer; or a more broadly targeted formulation may be chosen for selective inhibition of many pathways, potentially decreasing risk of developing resistance.[59] When anti-angiogenic TKIs were first developed, it was hoped that they could improve the efficacy of RT due to normalization of vasculature in solid tumors.[58,59] Tumor types explored for such treatments included rectal carcinoma, glioblastoma, hepatocellular carcinoma, and lung cancers.[57,74] These early concurrent treatment protocols used conventionally fractionated RT, and more recent protocols have utilized stereotactic body radiation therapy (SBRT) and whole brain radiation therapy (WBRT).[58,60,62,71,74] When TKIs are administered in combination with RT, favorable tumor response and progression-free survival times have been reported in human and veterinary clinical trials.[63–65,67,68,105] Tumors treated in these studies in which the investigational therapeutic protocol involved some combination of TKI and RT have included intracranial metastatic non-small cell lung cancer (NSCLC)[64], primary NSCLC[63], metastatic renal cell carcinoma[105], and head and neck squamous cell carcinoma[65] in human cancer patients, and inflammatory mammary carcinoma[67], cutaneous mast cell tumors[68], and nasal carcinoma[106] in canine cancer patients. More recent publications support the use of concurrent fractionated RT, WBRT, or stereotactic radiosurgery with TKI

to treat primary and metastatic NSCLC.[107,108] While the previously mentioned studies conclude that this combination is effective with respect to tumor response and survival times, there have also been reports raising concern for unanticipated toxicities. Pneumonitis has been reported in human medicine when thoracic RT was combined with EGFR-TKIs.[63,107] Significantly increased rates of radiation necrosis have been seen in treatment of brain metastases with SRS when combined with VEGF TKIs or EGFR TKIs.[109] Increased GI toxicities, including bowel perforation, have been seen with SBRT involving the abdomen when administered in combination with TKIs.[60,71,84,98] Notably, a phase I study investigating the combination of the TKI sorafenib with SBRT for treatment of hepatocellular carcinoma concluded that this treatment combination should not be used concurrently due to significant toxicities, including death. [74] In veterinary medicine, the TKI toceranib is commonly used to treat mast cell tumors (MCT) and is also used off-label for treatment of various other tumor types including sarcomas and carcinomas, sometimes in combination with RT.[66–69,110] At our institution, we suspected an increased incidence of GI toxicity when toceranib was administered to canine patients that were receiving concurrent abdominal RT. Upon retrospective review of canine cancer cases, we found significant increases in rates of diarrhea, vomiting, and hyporexia with this combination when compared to dogs treated with toceranib alone, abdominal RT alone, or RT administered to sites outside of the abdomen.[70] Although the GI toxicity associated with abdominal RT and concurrent TKI administration has now been documented in human and canine cancer patients, the underlying biological and pathological processes of this toxicity remain unknown.

Both TKIs and RT affect the vasculature of normal tissues and alter endothelial cell proliferation, which impacts angiogenesis and wound healing.[13,33,36,111,112] Abdominal radiation causes vascular permeability within 24 hours, with this acute phase predominated by endothelial apoptosis.[10,113,114] After irradiation, an inflammatory response is mounted and endothelial proliferation is initiated, leading to a vascular response and the creation of new capillaries that are irregular in shape and diameter.[112] An

increase in VEGF expression, an indicator of angiogenesis, has been shown in rectal biopsies taken 1-3 days following completion of 72-74 Gy total dose fractionated 3D conformal RT in human prostate cancer patients.[29] With respect to TKIs, the lining of the GI tract is sensitive to TKI inhibition of endothelial growth factors, such as those in the VEGF family, due to their direct impact on endothelial cells and indirect impact on epithelial stem cells in intestinal crypts.[20,32] TKIs can inhibit angiogenesis,[36] which can lead to increased healing times due to lack of vascular formation in response to wound development.[33,60,115] Signaling pathways that lead to proliferation, such as Ras, Raf, MEK, mTOR, and MAPK, targeted by TKIs, play an important role in the resolution of inflammation, re-epithelialization, and healing.[102] Therefore, we hypothesized that abdominal RT in combination with the TKI sunitinib would lead to increased GI toxicities due to compromised intestinal healing caused by inhibition of both vascular repair and proliferation pathways in the irradiated normal GI tissues. In this study, we explore the normal tissue changes underlying gastrointestinal toxicity associated with abdominal RT and concurrent treatment with the tyrosine kinase inhibitor, sunitinib, in a preclinical mouse model.

Materials and Methods

Animal husbandry

Six- to 14-week-old female and male CD-1 outbred mice (Envigo, Indianapolis, IN) were used in this study. Mice were housed in accordance with animal welfare standards of the laboratory animal facilities at Colorado State University (CSU) (Fort Collins, CO). Mice were provided with food and water *ad libitum* and exposed to a 12:12 h light-dark schedule. The experimental protocol was reviewed and approved by the CSU Institutional Animal Care and Use Committee.

Sunitinib (SUN) preparation

The aqueous vehicle for sunitinib administration was a carboxymethylcellulose (CMC) formulation containing: 0.5% CMC, 1.8% sodium chloride, 0.4% Tween-80, and 0.9% benzyl alcohol dissolved in reverse osmosis deionized water. Sunitinib malate powder (LC Laboratories, Woburn, MA)

was added to vehicle at concentration of 12 mg/mL and suspended via vortex. Vehicle and suspension were created weekly and stored in the dark at 4° C.

Irradiation

Abdominal Irradiation. Six-week-old mice were randomized to treatment groups (n = 2/group). Mice were anesthetized with 2% isoflurane gas mixed with oxygen in an induction chamber and maintained via nosecone with the same. They were placed in an X-RAD SmART irradiator (Precision X-ray, Inc., North Branford, CT) in sternal recumbency. A 40x40mm square collimating cone was used to target the beam to the area of the abdomen from the diaphragm through pelvis (**Figure 3**), which was verified by fluoroscopy at 40 kVp and 2.5 mA with a 2mm aluminum filter for proper alignment. Mice were irradiated with parallel-opposed fields at 90° and 270° with a single fraction of 4, 6, 8, 10, 11, or 13 Gy with a dose rate of 386 cGy/min at target depth using SSD calculations with 225 kVp and 13 mA and a 0.3 mm copper filter. Control mice (0 Gy) were anesthetized for the same amount of time but did not receive irradiation. Mice were irradiated on day 0 and then monitored and weighed daily for 7 days. On day 7 mice were euthanized and GI tissues were collected.

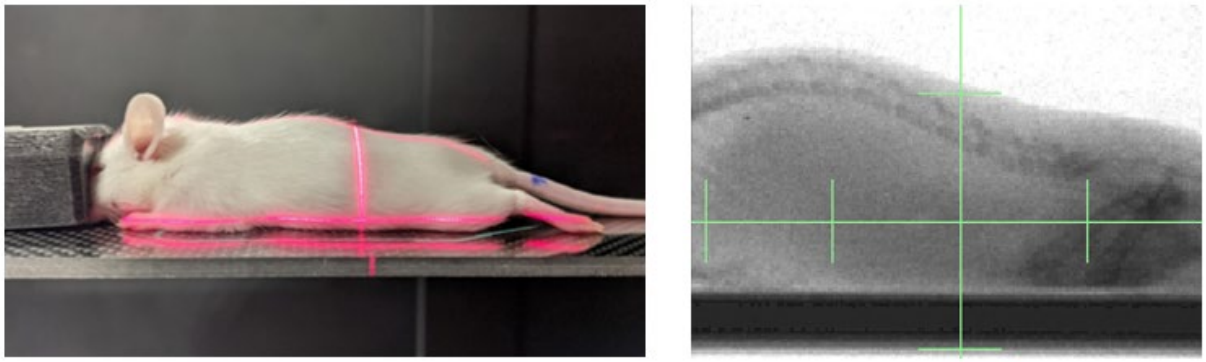

Figure 3: Mouse abdominal RT. Targeted treatment field spans from the diaphragm through the pelvis.

Abdominal Irradiation +/- Sunitinib. Fourteen-week-old mice were randomized to treatment groups of control (n = 5), sunitinib alone (n = 7), RT alone (n = 6), or RT + sunitinib (n = 7). Sunitinib suspension was delivered via oral gavage at a reported therapeutic dosage (40 mg/kg/day)[104] On day 0 daily oral gavage (equivalent volume of vehicle control or sunitinib) was initiated and continued for 14 days. On day 7 mice were irradiated with sham RT or a single fraction of 12 Gy in the manner described above. Mice in control or sunitinib-alone groups were anesthetized for the same duration but did not receive irradiation. On day 14 mice were euthanized and GI tissues were collected.

Experimental endpoints

Mice were evaluated and weighed daily. Mice were either euthanized according to IACUC-approved morbidity criteria, which included greater than 20% loss in body weight, or euthanized at a fixed end point of 14 days. Euthanasia was carried out via isoflurane gas anesthesia followed by cervical dislocation.

Tissue Collection

Small intestine, cecum, and colon were harvested after euthanasia. Intestinal sections were flushed with 4% paraformaldehyde (PFA) to speed fixation and were then gently rolled. Tissues were stored in PFA for 24 hours prior to being transferred to 70% ethanol to be stored until processed for histology.

Histopathological Evaluation and Quantitative and Semiquantitative Scoring of Injury

Fixed GI sections were embedded in paraffin, sectioned 5 µM, and stained with hematoxylin and eosin (H&E). Prepared slides were imaged using an Olympus VS120 scanning microscope and analyzed using OlyVIA (Olympus, Waltham, Massachusetts) software. Each section was blindly reviewed and evaluated by the first author (AR) and a veterinary pathologist (DR).

Thirty randomly selected small intestinal villi per mouse were measured for length using OlyVIA software. A measurement was taken from the mucosal/submucosal junction to the tip of each villus.

Thirty colonic crypts per mouse were randomly selected and crypt area was calculated using OlyVIA software. A measurement was taken from the crypt base to the lumen for length and perpendicular to this line at the midpoint for width. The product of these two measurements was used to approximate an area for each crypt. All measurements were obtained and calculated with the evaluator blinded to the treatment group from which the tissue sample was obtained.

Semiquantitative scoring of GI injury was performed by a pathologist in the same blinded fashion. SI and colon were scored for five parameters: inflammation, crypt density/crypt loss, crypt abscesses, ulceration, and crypt hyperplasia/regeneration. Within the parameter of inflammation, a score was generated by examining the extent of separation/effacement of crypts and the presence of inflammatory infiltrates. The inflammatory cells included in this evaluation were polymorphonuclear neutrophils (PMNs), lymphocytes, plasma cells, macrophages, and giant cells. (**Table 7**).

Table 7: Semiquantitative scoring rubric for gastrointestinal tissues.

	Score				
Parameter	0	1	2	3	4
Inflammation	none	1: minimal inflammation with minimal to no separation of crypts (generally focal affecting <10% of mucosa)	2: mild inflammation with mild separation of crypts (generally affecting 11%–25% of mucosa or mild, diffuse inflammatory infiltrates with minimal separation of crypts)	3: moderate inflammation with separation of crypts, with or without focal effacement of crypts (generally affecting 26%–75% of mucosa or moderate, diffuse separation of crypts);	5: diffuse inflammation with marked separation and effacement of crypts (generally affecting >75% of mucosa
Crypt density/crypt loss	normal/none	minimal; decreased by <10%	mild; decreased by 11-25%	moderate; decreased by 25-75%	severe; diffuse loss/effacement of mucosal architecture
Crypt abscesses	absent	-	-	-	present
Ulceration	absent	-	-	-	present
Crypt hyperplasia/regeneration	normal	marked; >10 regenerating crypts per 20x/1.1mm FOV	moderate; 5-10 regenerating crypts per 20x/1.1mm FOV	mild; 1-4 regenerating crypts per 20x/1.1mm FOV	none

Ki67 and CD31 Immunostaining

Formalin-fixed paraffin embedded tissue sections were processed for immunohistochemical (IHC) staining for Ki67 to assess cell proliferation and CD31, also known as PECAM-1, to assess vascularity of tissues (University of Colorado Research Histology Section (Aurora, CO)). Four-micron thick

paraffin sections were prepared for immunodetection of Ki-67 (Neomarkers/Thermo Scientific, Waltham, MA; clone SP6; 1:250) and CD31 (Cell Signaling, Danvers, MA; clone D8V9E; 1:100). Antigens were revealed in pH 9.5 BORG solution (Biocare Medical, Concord, CA) for 10 minutes at 110°C (NxGen Decloaker, Biocare) with a 10-minute ambient cool down. Immunodetection of was performed on the Benchmark XT stainer (Ventana/Roche, Indianapolis, IN) with primary incubations for 32 minutes at 37°C (Ventana/Roche). Antibodies were detected with a modified I-VIEW detection kit (Ventana/Roche) where the secondary antibody and streptavidin HRP were replaced with Rabbit ImmPress (Vector Laboratories, Burlingame, CA). The secondary antibody was replaced with full strength Rabbit ImmPress and the streptavidin-HRP was replaced with half strength Rabbit ImmPress diluted in PBS pH 7.6. Antibody-antigen complexes were visualized with diaminobenzidine from the I-VIEW kit. All sections were counterstained in Harris hematoxylin for 2 minutes, blued in 1% ammonium hydroxide, dehydrated in graded alcohols, cleared in xylene and coverglass mounted using synthetic resin. Negative controls to confirm the specificity of the immunostaining included omission of the primary antibody incubation step in the IHC protocol, substitution of the primary antibody diluent. Prepared slides were imaged using an Olympus VS120 scanning microscope and analyzed for density of positive cells according to quantified count of vessels per mm^2 (using Visiopharm. (Visiopharm Corporation, Wesminster, CO) software. Tissues analyzed were entire harvested SI, LI, and cecum.

Statistical Analysis

Statistical Analysis was performed using GraphPad Prism 9.3.1 (La Jolla, CA). All data are reported as mean ± standard error of the mean (SEM). Comparisons of effects across treatment groups were examined for significance using Fisher's exact test (categorical data), Ordinary one-way ANOVA (continuous data), or two-way ANOVA (weight comparisons between groups over time). Multiple comparisons were made using Tukey's post-hoc multiple comparisons test. Differences were considered significant for $p \leq 0.05$.

Results

Defining Abdominal RT Dose

The purpose of this step was to identify an optimal abdominal RT dose for experimentation in CD-1 mice. Our aim was to ascertain a dose that led to mild weight loss, from which mice were recovering by day 7, as well as mild GI inflammation and mild crypt loss seen histologically. The percent weight change seen for each mouse from day 0 to day 7 post-RT are as follows: 0 Gy (103%, 104%), 4 Gy (107%, 104%), 6 Gy (105%, 102%), and 8 Gy (102%, 102%). (**Figure 4A**) We saw that mice exposed to 0, 4, 6, or 8 Gy abdominal RT had stable weights and no histological changes were seen in any part of the intestinal tract at the time euthanasia. Mice that were exposed to 11 Gy abdominal RT maintained stable weights (95%, 100%) and mild histologic changes were seen in the LI. Those that were exposed to 13 Gy had moderate weight loss (88%, 86%) and moderate histologic changes in the LI, which included blunted villi, moderate inflammation, and decreased crypt mucosal architecture. Mice that received either 14 or 15 Gy had severe weight loss (80%, 83%) and were euthanized on day 5 due to unacceptable weight loss and morbidity scores. Histology revealed severe GI tissue destruction, which were progressive changes from what was noted in tissues exposed to 11 and 13 Gy. (**Figure 4B**) Based on the evidence of very mild weight loss and mild histologic changes seen at 11 Gy and more moderate weight loss and histologic changes at 13 Gy, we selected 12 Gy for the RT arms of the study to test our hypothesis that adding sunitinib would lead to increased toxicities.

Radiation therapy + Sunitinib

Weights. The mean weight and 95% CI for each group over the 14-day protocol is as follows: Control 32.8 g (28.9-36.6 g), SUN alone 32.8 g (28.8-36.8 g), RT Alone 33.9 g (28.6-39.3 g), and RT+SUN 31.6 g (28.0-35.2 g). The mean percent weight change among mice in control or sunitinib alone groups were stable (97% and 100%, respectively). Both groups that received abdominal RT lost weight, with those receiving RT+SUN losing more (88%) than those receiving RT alone (92%). There was a statistical

difference in weight loss across the treatment groups (p = 0.0001), and upon multiple comparisons, statistically significant differences were seen, as the control group lost more weight than the SUN group (p < 0.0001), the RT group lost more weight than SUN (p < 0.0001), RT+SUN lost more weight than SUN (p < 0.0001), and RT+SUN lost more weight than RT (p = 0.0258). (**Figure 5**)

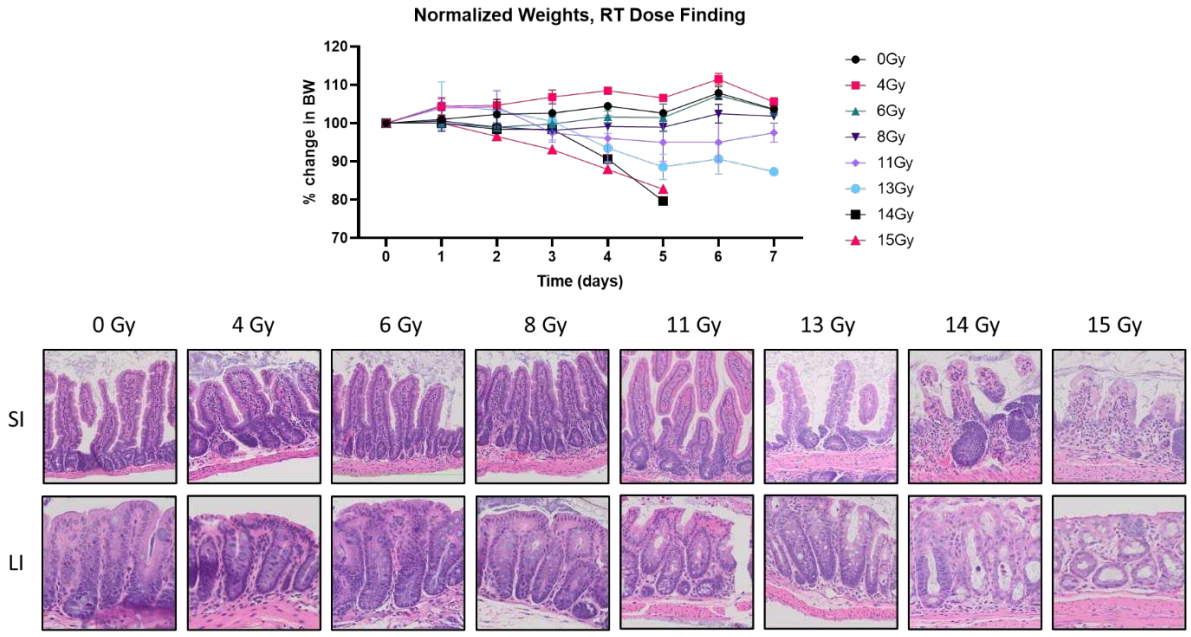

Figure 4A: Change in body weight relative to Day 0 in mice exposed to abdominal RT. Figure 4B: Representative H&E-stained photomicrographs of tissue sections of SI and LI five to seven days following exposure to various doses of abdominal RT. 20x magnification. RT, radiation therapy; SI, small intestine; LI, large intestine.

Quantitative Histology. SI villi lengths and colon crypt area were measured as an assessment of repair response after RT, which has been shown to be impacted by microvascular function and the loss of crypt cell proliferation.[116,117] When exposed to RT, SI villi are expected to be shortened between 36 hours and 3.5 days after RT, as the crypts fail to replace older cells sloughing from the tips of the villi.[19] The difference in SI villi length across groups at Day 14, seven days post-abdominal RT or sham RT, was not significant (p = 0.605) (**Figure 6**)**.**The difference in colonic crypt area compared across the treatment groups was highly significant (p < 0.0001). No significant difference in crypt area was found between the two groups that did not receive RT (Control, SUN) (p = 0.9997) and there was no significant difference

between the two groups exposed to abdominal RT (RT, RT+SUN) (p = 0.9935). There was however significance with every comparison between a group not receiving RT with a group that did receive RT (Control vs RT, p = 0.0003; Control vs RT+SUN, p = 0.0003; SUN vs RT, p = 0.0001; SUN vs RT+SUN, p = 0.0001), as crypt area increased in the irradiated tissues (**Figure 7**).

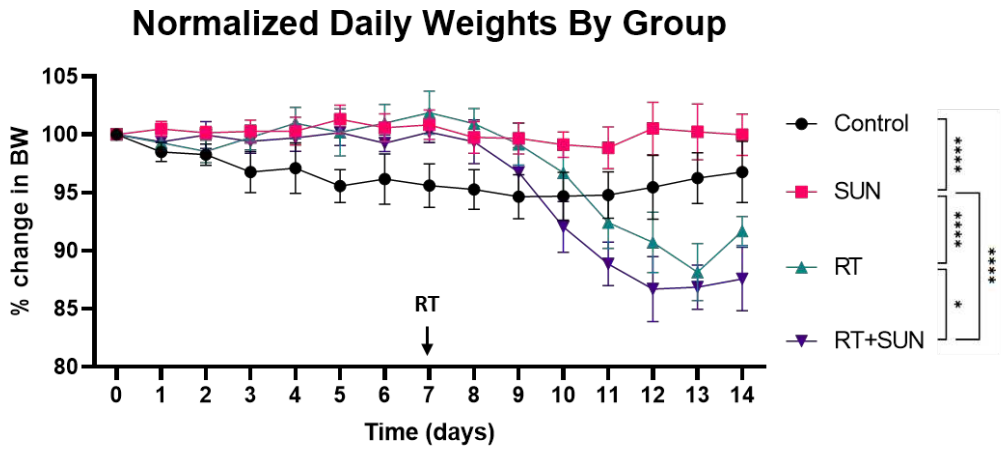

Figure 5: Change in body weight relative to Day 0. Oral treatment initiated on Day 0, and abdominal or sham RT occurred on day 7. (p < 0.0001) (p < 0.05; **** p < 0.0001). RT, radiation therapy, SUN, sunitinib.*

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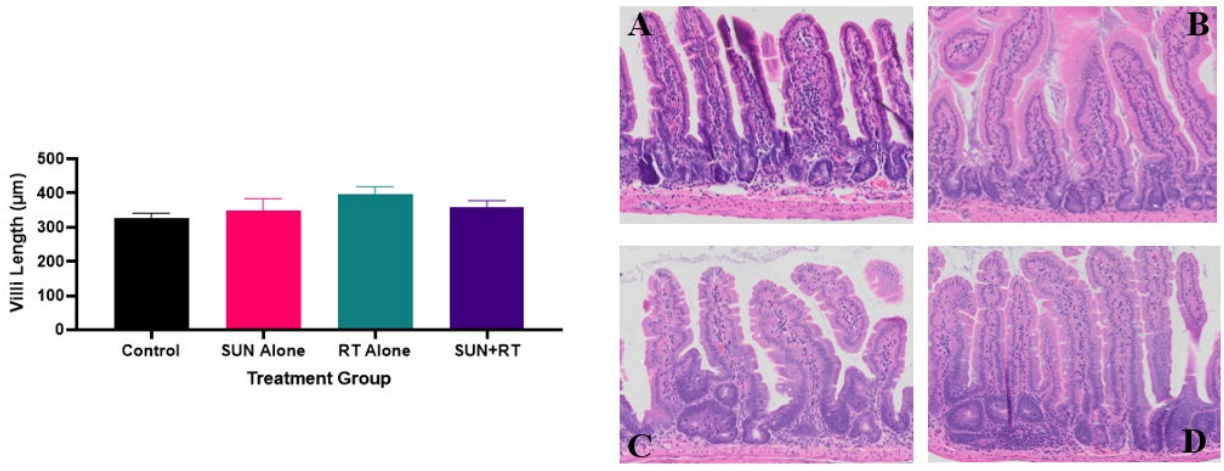

Figure 6: Mean (+SEM) of SI villi length for each treatment group. Photomicrographs of histological sections of colon from each of the four groups: Control (A), Sunitinib alone (B), RT alone (C), and RT + Sunitinib (D). Magnification 20x. SI, small intestine; RT, radiation therapy

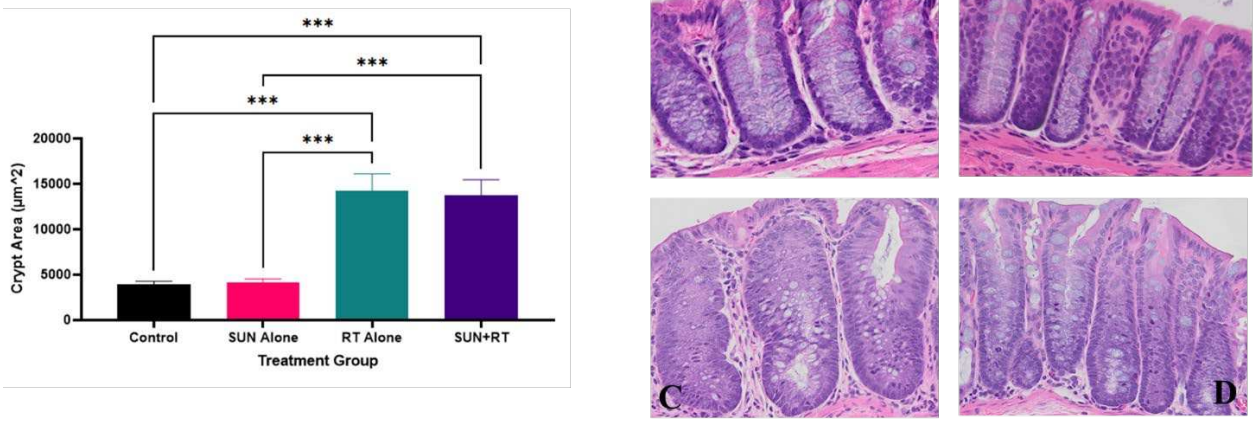

*Figure 7: Mean (+SEM) of LI crypt area for each treatment group. (p < 0.0001) Photomicrographs of histological sections of colon from each of the four groups: Control (A), Sunitinib alone (B), RT alone (C), and RT + Sunitinib (D). Magnification 20x. (*** p < 0.001). LI, large intestine; RT, radiation therapy.*

Semiquantitative Histopathologic Scoring of Gastrointestinal Toxicity. There was an absence of inflammatory response throughout the GI tract of mice in control or SUN groups (all Inflammation Score: 0), while mice in both groups receiving RT (RT, RT+SUN) had the presence of PMNs, lymphocytes, plasma cells, macrophages, and giant cells to varying degrees throughout the GI tract (Inflammation Score: RT (1-3), RT+SUN (1-3) (**Table 8**). This inflammation score was highly significant between groups (p<0.0001); however, on multiple comparisons, the significant differences emerged when groups not receiving RT (control, SUN) were compared to groups that did receive RT (Control vs RT, p = 0.0043; Control vs RT+SUN, p = 0.0025; SUN vs RT, p = 0.0006; SUN vs RT+SUN, p = 0.0003), with groups receiving RT having more inflammation than those receiving sham irradiation. The results for crypt density/loss was highly significant between groups (p < 0.0001). This consisted of zero scores for control and SUN groups, and on multiple comparisons, showed significance when groups not receiving RT (Control, SUN) were compared to groups that did receive RT (Control vs RT, p = 0.0043; Control vs RT+SUN, p = 0.0013; SUN

vs RT, p = 0.0006; SUN vs RT+SUN, p < 0.0006). The presence of crypt ulceration was not significantly different across groups (p = 0.4552). The presence of crypt abscessation was significantly different

Table 8: Semiquantitative scoring of GI inflammation for each animal organized by treatment group. GI, gastrointestinal; SUN, sunitinib; RT, radiation therapy.

Treatment Group	Animal ID	PMNs	Lymphs	Plasma cells	Mφ	Giant cells	Cumulative inflammation score	Crypt density/crypt loss	Crypt abscess	Ulceration	Crypt Hyperplasia/ regeneration
Control	1	0	0	0	0	0	0	0	0	0	0
	2	0	0	0	0	0	0	0	0	0	0
	3	0	0	0	0	0	0	0	0	0	0
	4	0	0	0	0	0	0	0	0	0	0
	5	0	0	0	0	0	0	0	0	0	0
SUN Alone	1	0	0	0	0	0	0	0	0	0	0
	2	0	0	0	0	0	0	0	0	0	0
	3	0	0	0	0	0	0	0	0	0	0
	4	0	0	0	0	0	0	0	0	0	0
	5	0	0	0	0	0	0	0	0	0	0
	6	0	0	0	0	0	0	0	0	0	0
	7	0	0	0	0	0	0	0	0	0	0
RT Alone	1	1	2	1	1	0	3	3	4	4	3
	2	1	1	0	1	0	2	2	4	0	2
	3	1	1	0	1	0	1	1	0	0	0
	4	1	1	1	1	0	2	2	0	0	2
	5	1	1	0	1	0	1	1	0	0	2
	6	1	1	0	0	0	1	1	0	0	1
RT + SUN	1	1	1	0	1	0	2	2	4	0	3
	2	1	1	0	1	0	2	1	4	0	3
	3	1	1	1	1	0	3	3	4	4	2
	4	1	1	0	0	0	1	1	0	0	0
	5	1	1	0	1	0	2	2	4	0	3
	6	1	1	0	1	0	2	2	4	4	2
	7	1	1	0	0	0	1	1	4	0	1

across groups (p = 0.0009), and when compared between groups, RT+SUN had significantly increased levels of abscessation when compared to groups not receiving RT (RT+SUN vs. Control, p = 0.0076; vs. SUN, p = 0.0023). RT+SUN had more abscessation than RT alone, but did not reach significance (p = 0.0862). There was a statistically significant difference in the score of crypt hyperplasia/regeneration between groups (p = 0.0018). Control and SUN groups had scores of 0 and both groups receiving RT had scores of 0 to 3. On multiple comparisons, there was significance only when groups not receiving RT (control, SUN) were compared to groups that did receive RT (Control vs RT, p = 0.0411; Control vs

RT+SUN, p = 0.0223; SUN vs RT, p = 0.0047; SUN vs RT+SUN, p < 0.0023). Groups receiving RT had less hyperplasia/regeneration than those that did not. These results are summarized in **Figure 8**.

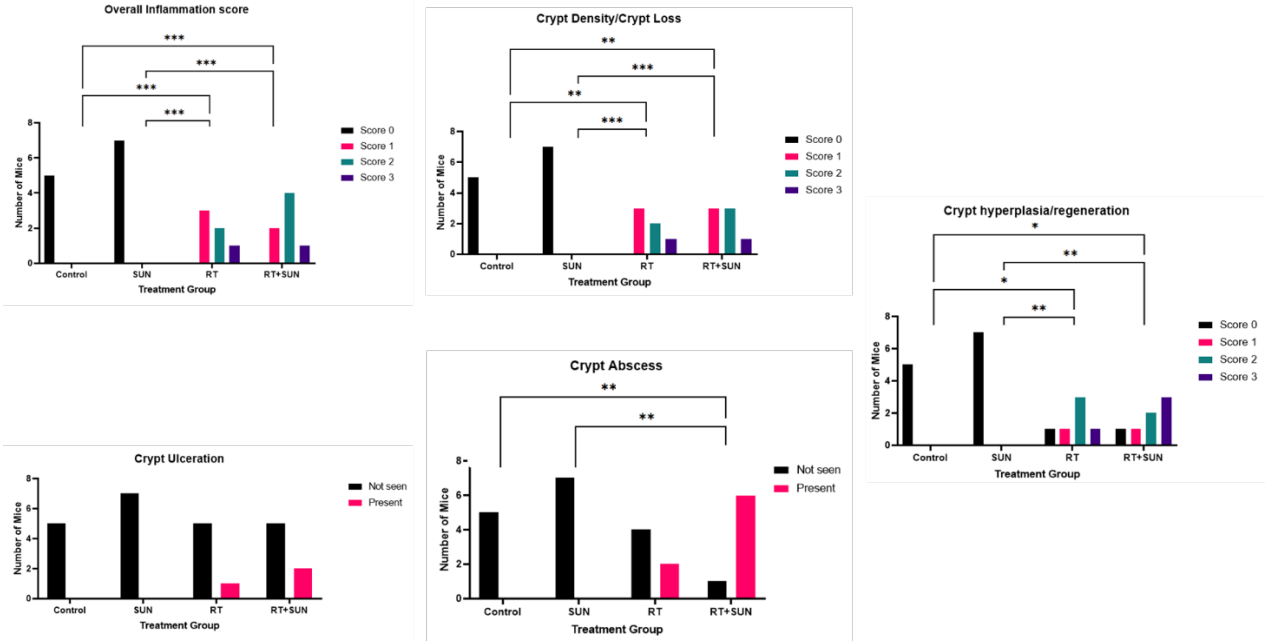

Figure 8: Summary of semiquantitative scoring of GI inflammation parameters. (p < 0.05; ** p < 0.01; *** p < 0.001; **** p < 0.0001). GI, gastrointestinal; SUN, sunitinib; RT, radiation therapy*

Immunohistochemistry. The density of CD31+ cells within the GI tract was significantly different across groups (p = 0.0235). On multiple comparisons, the RT alone group had an increased in CD31+ expression compared to the SUN group (p = 0.0252). The density of Ki67+ cells within the GI tract was not significantly different across groups (p = 0.1937). (**Figure 9**) However, on review of the tissues, it was observed that the Ki67+ cells were distributed differently throughout the GI tract when compared between the treatment groups. We used semiquantitative scoring to assess the presence of Ki67+ cells within the SI villi and LI crypts (**Table 9**)**.** In the SI, each sample was scored for the frequency Ki67+ cells were seen within the villi. (**Figure 10A**) When unblinded, the distribution of Ki67+ cells in the SI from the mice in treatment groups that did not receive RT were contained primarily in the crypts of the villi,

whereas the mice in groups exposed to abdominal RT had Ki67+ cells dispersed throughout the length of the villi. The scoring showed significant difference across the groups ($p < 0.0001$). There was no difference in Ki67+ cells of the villi when non-irradiated groups were compared

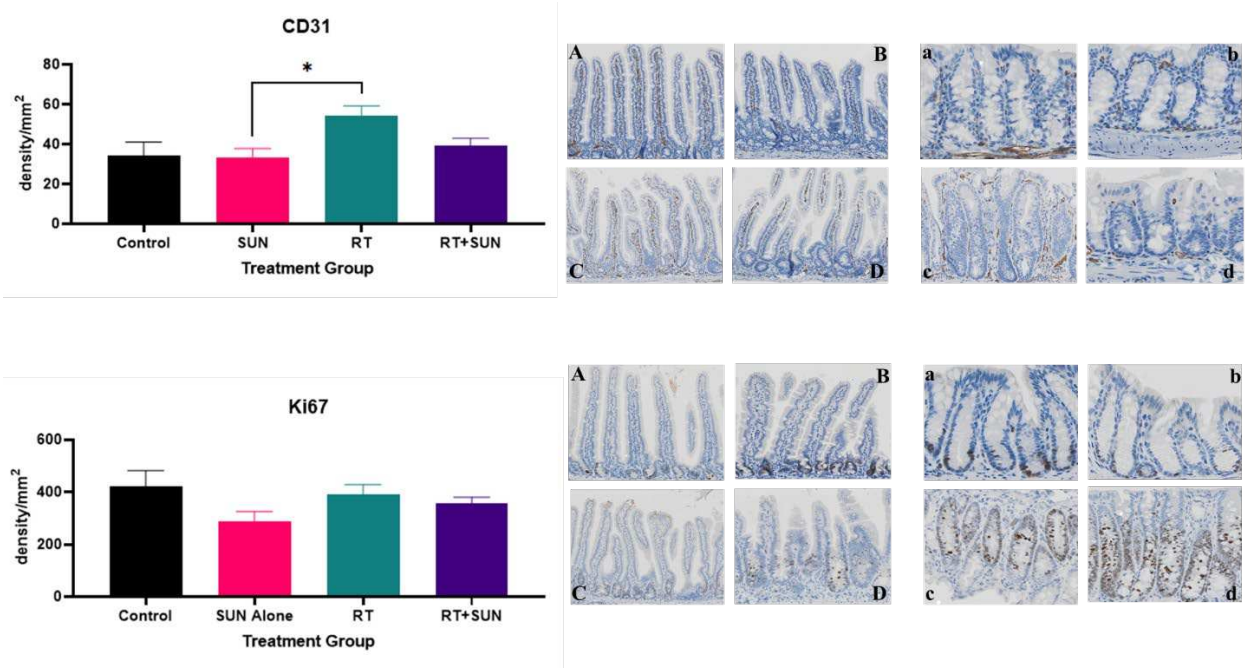

Figure 9: Mean (+SEM) density of positively stained cells per mm2 for CD31 and Ki67 for each group with representative photomicrographs. SI villi, Control (A), Sunitinib alone (B), RT alone (C), and RT + Sunitinib (D); LI crypts, Control (a), Sunitinib alone (b), RT alone (c), and RT + Sunitinib (d). Magnification 20x. ($p < 0.05$). SI, small intestine; LI, large intestine; RT, radiation therapy; SUN, sunitinib.*

Table 9: Scoring rubric for location of Ki67+ cells as represented by frequency of + cells within the SI villi and extent of + cells around circumference of LI crypt. SI, small intestine; LI, large intestine.

	Score		
Parameter	**0**	**1**	**2**
Ki67+ Within Villi	Never	Rarely (present in < 10% of villi)	Occasionally (present in 10-30% of villi)
Ki67+ colonic crypt circumference	<50%	>50%	

(Control vs. SUN, p = 0.5581), when the two groups exposed to abdominal RT were compared (RT vs RT+SUN, p = 0.4615), or for Control vs RT+SUN (p = 0.0530). However, there was significance seen in the following comparisons of a non-irradiated group to one exposed to abdominal RT, with RT increasing the incidence of Ki67+ cells in the SI villi (Control vs RT, p < 0.0022; SUN vs RT, p = 0.0006; SUN vs RT+SUN, p = 0.0035). In the LI, colonic crypts were scored as having Ki67+ cells confined to the periphery of the crypt closest to the basement membrane (score 0) or extending beyond halfway around the crypt (score 1) (**Figure 10B**). All crypts analyzed in non-irradiated groups scored a 0, with Ki67+ cells confined to the lower half of the crypt. In groups receiving abdominal RT, all crypts analyzed contained scored a 1, with Ki67+ cells present throughout the full circumference of small crypts and spanning the lower half-to-two thirds of larger crypts. The overall differences were highly significant (p < 0.0001); however, this quantification showed no difference in location of Ki67+ cells between the two non-irradiated groups (p > 0.9999) nor between the two groups that received abdominal RT (p > 0.9999). Significant differences were seen when either group receiving abdominal RT was compared to either non-irradiated group (p < 0.0001 for all comparisons).

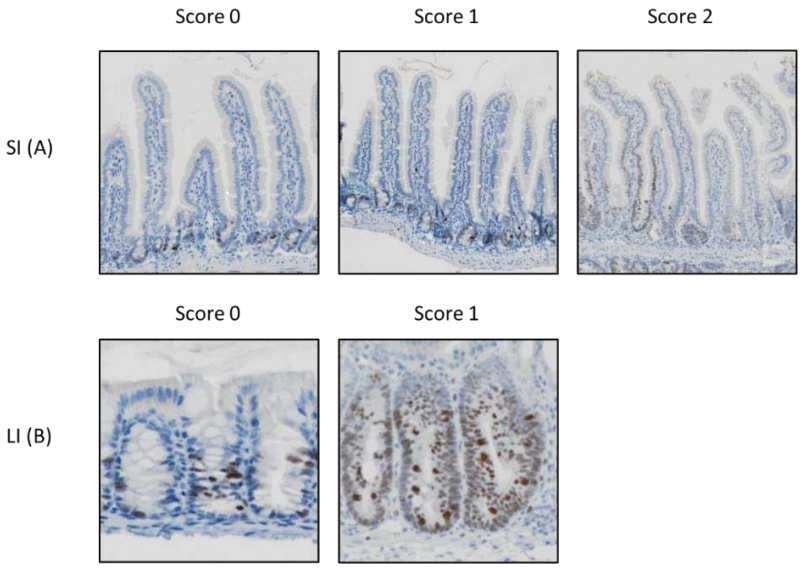

*Figure 10: Photomicrographs showing an example of each semiquantitative score indicating Ki67+ cell distribution in SI villi (**A**) and LI crypts (**B**). Magnification 20x. SI, small intestine; LI, large intestine.*

Discussion

In this study, we aimed to characterize the underlying normal tissue changes associated with GI toxicity when abdominal RT is combined with the TKI sunitinib in a mouse model. Our results identified significant differences between the group receiving RT+SUN when compared against SUN or RT groups in the area of weight loss, which could be interpreted as preclinical evidence of GI pathologic changes leading to morbidity. We identified a significant increase in crypt abscessation when RT+SUN was compared against vehicle control or sunitinib alone; the increase in crypt abscessation approached significance when compared to RT alone. The findings of increase crypt abscessation in mice treated with RT+SUN suggests a histopathologic effect of compromised healing with this treatment combination. We also investigated the microvascular density and cellular proliferation throughout the GI tract following treatment. Mice treated with abdominal RT developed significantly increased GI microvascular density following treatment, whereas this affect was not seen in mice treated with RT+SUN nor in mice in the control or SUN treatment groups.

Differences in weight loss were statistically significant across the four treatment groups. Interestingly, the control group lost significantly more weight than the SUN group, and there was no difference in weight loss seen between control and the two RT groups. The mice in groups treated with abdominal RT lost the most weight. Both groups receiving abdominal RT lost significantly more weight than the SUN group; this abdominal RT-associated weight loss in mice treated with 12 Gy was expected due to the mild GI side effects documented in mice treated with 11-13 Gy in the dose-response experiment. However, the group of mice receiving concurrent abdominal RT+SUN lost significantly more weight than the RT alone group, which may align with the clinical toxicities seen in our prior retrospective study of canine cancer patients receiving concurrent abdominal RT and the TKI toceranib.[70]

We saw no difference in SI villi lengths between all four of the treatment groups. The villi are expected to be blunted 3.5 days after RT due to normal tissue damage.[19] As such, our results were

somewhat unexpected, but this lack of shortening could be due to the SI villi having fully repaired by Day 7 post-RT, indicating the repair process was not hindered or slowed by the addition of sunitinib. Colonic crypt enlargement was identified in mice receiving abdominal RT when compared non-irradiated mice. This enlargement of crypts after abdominal RT was not unexpected based on the work of Withers showing colonic crypts are regenerated and enlarged 5.5 days after irradiation;[19] however, the lack of significant difference between the two RT groups indicates that the addition of sunitinib did not exacerbate or hinder this normal response to RT.

Semiquantitative scoring of GI injury revealed differences when tissues from the two non-irradiated groups were compared to those receiving abdominal RT. The inflammatory response did not show significant differences between RT and RT+SUN for any parameters but approached significance for the presence of crypt abscessation. Crypt abscessation occurs when inflammatory cells infiltrate the lamina propria surrounding the crypts[118] and has been documented in the normal rectal mucosa of human cancer patients two weeks after starting fractionated RT for non-gastrointestinal cancers with a radiation field which included the rectum.[16] Crypt abscessation was also identified in rats 5 days after single fraction 10 Gy abdominal RT.[119] Crypt abscessation has been seen in human cancer patients who report GI side effects while receiving TKI therapy.[120] It has been reported that radiation-induced crypt abscessation in rats correlates with clinical signs of diarrhea and weight loss.[118,119] In human cancer patients, histologic changes, including crypt abscess, have been associated with loose stools.[16,121] In our study, we used weight loss as a surrogate marker for GI clinical signs. Given the evidence of correlation between abscessation and clinical signs in rats and human cancer patients, we might consider the significant weight loss seen in mice receiving RT+SUN when compared to RT to be correlated with their increased rates of crypt abscessation.

There was a significant difference in the microvascular density of the GI tissues between SUN and RT groups. The mice exposed to abdominal RT had significantly increased density of CD31+ cells

compared to those treated with SUN. The results from the mice exposed to abdominal RT are consistent with an increased vascular response to RT. This vascular response was seen only in the mice receiving RT, not in mice treated with RT+SUN. This normal vascular response seems to have been suppressed by the presence of SUN in the RT+SUN group. Following GI irradiation, normal tissues undergo an inflammatory response which includes increased expression of CD31.[13] The link between post-RT GI vascularization and inflammation has been made in previous studies based on increased numbers of CD31+ cells seen in rectal biopsies from human patients treated with RT for prostate cancer[29] and irradiated rectal tissues in mice.[122] Currently, there is a gap in knowledge of whether CD31+ expression is altered in normal GI tissues after administration of sunitinib or other TKIs and this is an area of potential future investigation. If the increase in vascularity seen in the group treated with RT alone was a response to abdominal irradiation, we would have expected to see increased vascularization in the GI tract in both groups receiving abdominal RT. The normal radiation response of tissues in the GI tract is an activation of vascular repair and angiogenic pathways to begin the processes of wound repair and healing; an absence of this response in the RT+SUN group could indicate that at least one of these pathways has been altered and may be associated with the increased GI side effects that have been reported following concurrent abdominal RT with TKI administration in human and veterinary medicine. Alternatively, it could be argued that the GI vascular response following abdominal RT is linked with radiation-induced GI toxicity, and, as there is a lack of angiogenic response in the RT+SUN group, perhaps sunitinib is suppressing the tissue irradiation damage and associated RT-induced GI toxicity. However, this is not supported by the results of this study which demonstrate significantly increased weight loss seen in the RT+SUN group and equivalent levels of GI inflammation seen in both groups of mice receiving abdominal RT. Further, when comparing the GI toxicities reported in our retrospective canine study, concurrent abdominal RT with TKI resulted in significantly increased GI toxicity compared to dogs treated with abdominal RT alone, not a protective effect.[70]

Ki67 stains proliferating cells and is an indicator of regeneration.[123–125] Epithelial stem cells, located within the base of SI and LI crypts, divide asymmetrically and produce one daughter stem cell which remains in the crypt and one daughter cell that proliferates and differentiates at it moves up and out of the crypt.[8] The normal epithelial differentiation and migration of intestinal stem cells takes approximately 3-4 days.[11] The differentiating daughter cell moves higher up in the crypt and into the villus (SI), losing its stem cell characteristics as it progresses and matures, eventually reaching senescence and being sloughed off into the intestinal lumen.[8,11] Ki67+ cells are known to migrate within the crypts of both the SI and LI as a response to radiation.[126] The onset of epithelial regeneration is seen in the SI of mice 60 hours after irradiation and at 3.5 days in the LI.[19] Otsuka and Suzuki (2016) showed a significant reduction in Ki67+ cells in the lower region of ileal and duodenal crypts in the SI at five timepoints, up to 72 hours, after abdominal irradiation with 1 and 4 Gy and stable numbers of Ki67+ cells in the upper region at these doses. They did not see changes at a dose of 0.1 Gy. They noted no significant changes in overall number of Ki67+ cells present in the crypts. In our study, the density of Ki67+ cells throughout the GI tract of treated mice was not significantly different between the groups. What was different, however, was the location of the Ki67+ cells themselves when non-irradiated groups were compared to those that received abdominal RT. In non-irradiated GI tissues, these cells were confined to the crypts of the villi in the SI and the lower half of the perimeter of colonic crypts. In irradiated tissues the Ki67+ cells had moved out of the villi crypts and into the villi themselves in the SI and in the LI had moved to greater than 50% of the periphery of the colon crypts. There was no significant difference in location between the two groups receiving RT, indicating that the addition of sunitinib did not affect this migration. In the SI, we examined the location of Ki67+ and graded them as confined to the crypts at the base of the villi or extending into the villi. Otsuka and Suzuki did not comment on Ki67+ cells within the villi, however they did see the Ki67+ moving to the portion of the villi crypts closest to the villi with all doses. They also documented similar results in the crypts of the colon

over the same doses and time, with Ki67+ cells not occurring in the upper most area of the crypts of non-irradiated tissues, but, having a significantly higher percentage of Ki67+ cells in this location at all timepoints up to 72 hours for tissues receiving 4 Gy. We observed something similar for colonic crypts receiving 12 Gy harvested at 7 days after abdominal RT. The findings of Otsuka and Suzuki's study are similar to our findings where we saw no significance in the percent-stained area between our 4 treatment groups, indicating stable numbers of proliferating cells, but did have significant differences in the location of the Ki67+ cells when non-irradiated groups were compared to those receiving abdominal RT. To our knowledge, there are no known studies reporting the location of Ki67+ cells in mouse GI tissues 7 days after RT, which we are reporting here.

This study has several limitations. Our results are limited by the single endpoint and timing of tissue harvesting post-treatment. Tissues harvested at earlier times after RT likely would have shown different states of vascular repair and proliferation. Alternatively, had we chosen a later time-point we may have seen more significance in weight loss between groups and/or differences in the time to weight loss recovery. We chose 7 days post-abdominal RT or sham irradiation as our endpoint in an effort to balance the desire for evaluating histological GI changes with biologic changes in weight. We also recognize that different RT fractionation and dosing could have given different results than our single dose of 12 Gy. We targeted the whole abdomen but recognize that a smaller field or targeted RT could have also produced different results. We identified two IHC markers to investigate; this could be expanded into other markers of angiogenesis and proliferation pathways such as VEGF and co-receptors, CD34, CD105, proliferating cell nuclear antigen (PCNA), Lgr5, and MCM2.[29,30,58,127] Apoptotic markers such as TUNEL and p53 could also be considered.[6,90] This study is exploratory and descriptive at this point; future mechanistic studies could further define the drivers of the effects and toxicities seen.

Our study has translational potential as TKIs are commonly used in human medicine as a powerful treatment option for cancers with an overexpression of tyrosine kinases.[35] Concerns have been raised

about the combination of TKIs and concurrent abdominal RT; however, this combination has also shown improved outcomes over either therapy used alone in primary and metastatic NSCLC, head and neck squamous cell carcinoma, and metastatic renal cell carcinoma in human cancer patients, and cutaneous mast cell tumors and inflammatory mammary carcinoma in canine cancer patients. In order to capitalize on this increased tumor control, it will be important to identify safe approaches to combining RT with TKIs. Further investigation is warranted into the clinical use of RT and TKIs to optimize timing of administration, dose of either modality, or fractionation of RT.

In conclusion, we report on increased weight loss and histopathologic changes throughout the GI associated with the combination of abdominal RT and the TKI sunitinib in a mouse model. We found a significant increase in weight loss in mice receiving SUN+RT when compared to mice receiving abdominal RT alone or sunitinib alone. We report a lack of GI vascular response following RT in mice receiving RT+SUN, indicating that the typical GI radiation-associated angiogenic response may have been hindered by the addition of sunitinib. We also quantified an increase in the incidence of crypt abscessation in mice treated with RT+SUN, and, while not statistically significant compared to RT, reveals a trend that suggests compromised healing of GI tissues in mice treated with RT+SUN. It is possible that clinical toxicities which have been reported with simultaneous abdominal RT and TKI administration could be attributed to an impaired tissue response and healing in irradiated normal tissues of the GI tract. Further study is recommended to determine whether approaches to minimize GI toxicity associated with abdominal RT and TKI treatment combinations are effective, while also preserving the beneficial biological therapeutic effects of concurrent RT and TKI.

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