THE ROLE OF CD44V9/XCT AS A PROTECTIVE MECHANISM AGAINST ROS-INDUCED CHEMOTHERAPY IN GASTRIC CANCER

by

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ABSTRACT:

Gastric cancer remains a public health concern all over the world and specifically in South America and Eastern Asia. The 5-year survival rate for GC remains dismal and this could be attributed to diagnosis at a late stage. The GC tumor has been observed to possess a unique subpopulation of cells known as cancer stem cells (CSCs). Furthermore, these stem cells have been shown to be identified through a marker known as cluster-of-differentiation 44 (CD44). It has been reported that CD44+ cells possess a higher ability for defense against reactive oxygen species (ROS) and this has been attributed to its role in stabilizing xCT on the surface of the cancer stem cells (CSCs). The goal of our work was to utilize an in vitro and in vivo model to identify the role of the CD44v9-xCT mechanism and how it may be contributing to resistance through defense against ROS. Overall, our work showed that when CD44+ xCT knockdown MSI-H IM95 cells were treated with optimal dose of sulfasalazine alongside varying concentrations of cisplatin, they were sensitized to the treatment. This was also visualized in the patient-derived gastric cancer organoid model, which inhibited the xCT transporter with sulfasalazine treatment. In conclusion, our findings indicate that CD44 stabilized xCT transporter on CSCs is contributing to ROS defense and is allowing for persistence of the tumor. Furthermore, our findings elucidate that CD44-xCT system may be a potential therapeutic target to allow for sensitization to treatment.
CHAPTER ONE: INTRODUCTION

Overview of the Gastrointestinal System

The digestive system is a complex system that is comprised of multiple vital organs that together function to break down food, absorb nutrients and excrete waste from the body (Treuting et al., 2018). Beginning at the mouth, there is a continuous sequence of tube-shaped organs which eventually lead to the anus and together make up the alimentary canal (National Institute of Diabetes and Digestive and Kidney Diseases, 2004). This organ pipeline is made up of the mouth, esophagus, stomach, small intestine, large intestine, anal canal and anus. This entire tract is composed of 4 layers from innermost mucosa, submucosa, tunica muscularis, to the outermost serosa (Daniels and Allum, 2005; Treuting et al., 2018).

The mouth functions to mechanically breakdown food and form the bolus. Once the bolus has formed, it travels through the esophagus, which is a hollow tube that allows for the completion of swallowing and aids in delivering the bolus to the stomach. The stomach acts as a short-lived reservoir for food. It functions to mix food with water and gastric enzymes to aid with further breakdown to form a liquid substance known as chyme. After the formation of chyme, it passes through the pyloric sphincter and into the first portion of the small intestine. As a whole, the small intestine, which is divided into duodenum, jejunum, and ileum, functions to further digest and absorb nutrients from the food that has been previously broken down. Once the chyme reaches the large intestine, a majority of the nutrients have already been absorbed. The large intestine is divided into ascending, descending, transverse, and sigmoid. This organ functions for electrolyte and water absorption from the chyme. After the chyme passes through the large intestine, it reaches the last portion, the sigmoid colon, and is now formed feces. The
fecal matter will pass through the rectum and through the anal canal, eventually being excreted into the environment through the anus.

The three accessory organs that are a part of the digestive system are the liver, gallbladder and pancreas. The liver is situated in the right upper quadrant of the abdomen, and it has several digestive functions. Some of its main functions include bile formation and it serves as a major site for vitamin and mineral storage. The gallbladder is the storage organ for the bile that is produced by the liver and it secretes it into the duodenum as needed. The other vital accessory organ is the pancreas, which is located in the upper right quadrant of the abdomen, and it serves both exocrine and endocrine functions. The most important role in regard to digestion is the production of various enzymes to aid in the digestive process (Guyton and Hall Textbook of Medical Physiology., 2015).

**Gastric Anatomy and Physiology**

The stomach is a vital organ that functions to breakdown food, with the use of acids, to form a liquid substance called chyme, that the duodenum will absorb nutrients from (Daniels and Allum., 2005). Anatomically, the stomach is situated in the upper left quadrant of the abdomen below the esophagus and above the first part of the small intestine. Macroscopically the stomach has a J like shape to it, with two bends known as the greater and lesser curvatures (Rao and Wang., 2010). The stomach contains two openings called the gastroesophageal junction and the gastroduodenal junction. These openings are controlled by their respective sphincters, which control the passage of food into the next portion of the digestive system (Daniels and Allum., 2005).
The stomach can be divided into four main sections depending on which anatomical site they occupy (Rao and Wang., 2010). The cardia is the area distal to the gastroesophageal junction and is located in the upper portion of the stomach (Rao and Wang., 2010). The fundus and the body are below this and are considered proximal. The area that makes up the distal portion of the stomach is the pylorus. Each of these sections are comprised of different cell types that secrete various substances to aid in breakdown of food. Microscopically, the stomach wall is composed of several layers which are the innermost mucosa, submucosa, muscularis externa, and adventitia/serosa (Hsu et al., 2020; Treuting et al., 2018). The gastric mucosa can be further subdivided into 3 distinct layers. Beginning from the outermost to the innermost, the layers are the epithelium, a connective tissue lamina propria, and the inner most musculature layer called muscularis propria (Hsu et al., 2020). The presence of pits and glands within the gastric mucosa is due to the gastric epithelium invaginating into the lamina propria.

Within the gastric layers, there are differing cell types present that carry out different functions. The main 4 cell types of importance in the stomach are chief cells, mucus secreting cells, parietal cells, and G cells (Hsu et al., 2020). The chief cells and mucus secreting cells are mainly located in the fundus and body of the stomach. The other two cell types, parietal and G cells, tend to be located in the pylorus region mainly. The chief cells are a specialized cell type of the stomach that functions to secrete an enzyme precursor, pepsinogen, which will become pepsin in the presence of gastric juices (Reed and Wickham., 2009; Chaudhry and Shazia., 2020). The mucus secreting cells are present throughout the fundus and the body of the stomach and function to secrete mucus to aid in protection (Reed and Wickham., 2009). The parietal cells, which are mainly found in the pylorus, aid in the secretion of hydrochloric acid and intrinsic factor (Chaudhry and Shazia., 2020). They are stimulated for release of their hormones
by the G cells. The pyloric cells secretin of HCl allows for the stomach to maintain its highly acidic pH of about 1.5-2.0 and allows for defense against most microbes because they cannot thrive in such an environment. Intrinsic factor is essential for proper vitamin B12 absorption in the small intestine (Ogobuiro et al., 2020). Lastly, the G cells are present in the pylorus and they secrete gastrin which functions to stimulate the parietal and chief cells for secretion (Ogobuiro et al., 2020). Overall, the stomach is a vital organ that is highly compartmentalized and is able to carry out various functions.

Table 1. Main Cell Types of the Stomach and their Key Characteristics

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>Location</th>
<th>Secretion</th>
<th>Stimulated by</th>
<th>Physiological function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chief cells</td>
<td>Fundus and body</td>
<td>Pepsinogen</td>
<td>Gastrin from G cells</td>
<td>Digestion</td>
</tr>
<tr>
<td>Mucus secreting cells</td>
<td>Fundus and body</td>
<td>Mucus</td>
<td>Prostaglandins (i.e prostacyclin)</td>
<td>Protection from bacteria and maintenance of acidic environment of stomach</td>
</tr>
<tr>
<td>Parietal cells</td>
<td>Pylorus</td>
<td>HCl</td>
<td>Gastrin from G cells</td>
<td>Protection and vitamin B12 absorption</td>
</tr>
<tr>
<td>G cells</td>
<td>Pylorus</td>
<td>Gastrin</td>
<td>Acetylcholine</td>
<td>Stimulation of parietal and chief cells</td>
</tr>
</tbody>
</table>

Gastric Cancer Pathogenesis and Associated Risk Factors

Gastric cancer is a complex disease that continues to rank as the fifth most common malignancy in the world (Figuieredo et al., 2013). There is no exact pathogenic mechanism that
has been described in the literature, but it is an assortment of factors that all play a contributory role in the pathogenesis of gastric cancer. The gastric cancer stem cells (GCSC) are a subset of cells that are present in the gastric glands and have been hypothesized to play a major role in the development of the disease (Singh, S., 2013). The CSCs are a unique population of cells present within the tumor microenvironment that exhibit similar qualities as stem cells, like self-renewal properties and ability proliferate into new tumors (Zhang et al., 2019; Singh, S., 2013). These cells have been thought to directly contribute to the tumor heterogeneity and thus alter the response of the tumor to different treatment modalities, ultimately leading to increased resistance (Zhang et al., 2011). It continues to remain unclear why this subpopulation seems to persist within the tumor microenvironment of GC patients, but it is pertinent that these cells be targeted in order to have effective regression of tumor size in response to standard-of-care treatment (Zavros, Y., 2017).

A specific subtype of CSCs has been identified through a cell surface adhesion molecule known as cluster-of-differentiation 44 (CD44). Interestingly, it has been seen that those cell types that express CD44, have a higher rate of becoming malignant and giving rise to GC. Additionally, a study conducted in 2009 reported by Takaishi et al, showed that the CSCs that were CD44+ were able to form spheroids in vitro and when the cells were orthotopically transplanted into severe combined immunodeficient (SCID) gamma mice, they formed tumors (Takaishi et al., 2009). Furthermore, when CD44 was knocked down, with the use of short hair pin RNA, the tumorigenic ability dwindled (Takaishi et al., 2009). Moreover, CD44 is able to generate multiple isoforms through alternative RNA splicing of its 6-15 exons, most commonly referenced are variants 1-10, but the number of variants generated could be well into the 100’s (Jang et al., 2015). These various isoforms have varying levels of expression when analyzed by
RT-PCR. The variant that has been most strongly correlated as a gastric cancer stem cell marker is CD44 variant isoform 9 (CD44v9). The exact role of CD44 and its isoforms remains unclear, but it continues to be a major area of research as it has the capacity to become a predictive biomarker for determining response to treatment of GC.

*Helicobacter pylori* is a Gram-negative spirochete that is able to colonize the stomach and affects about 50% of the population worldwide (Marshall and Warren., 1984). The route of transmission is not exactly clear, but it is thought that person-to-person transmission is the most probable (Stefano et al., 2018). *H. pylori* has the ability to colonize the stomach and induce several diseases, but most commonly leads to gastritis (Kusters et al., 2006). Previous work has shown that the stem cells in the stomach may be altered during bacterial infection to become malignant and become tumor initiating cells (Zavros, Y., 2017). Infection with *H. pylori* is a well-established risk factor for development of GC and may be responsible for the alteration of the CSCs present in the stomach glands. In the normal human stomach, there is a rich layer of mucus present that functions in protection from pathogens, but *H. pylori* favors this and is able to colonize in the mucus lining (Penta et al., 2005). The initial infection with *H. pylori* results in an extensive immune response, which establishes the initial gastritis (Penta et al., 2005). This pathogen has a variety of virulence factors that heighten its pathogenicity, which allows it to thrive in low pH environments, like the stomach and further proliferate. The main virulence factors of importance are urease, cytotoxin-associated gene A (cagA), heat shock B protein and vacuolating cytotoxin A (VacA) (Penta et al., 2005). The secretion of urease allows *H. pylori* to increase the acidic pH of the stomach by converting urea to ammonia and carbon dioxide (De Falco et al., 2015). This mechanism allows the pathogen to persist in the mucus lining of the stomach, decrease the amount of stomach acid present and maintain the inflammatory state. The
virulence factor, cagA, functions on the epithelial cells present within the stomach and it allows for persistent infection and deregulation of vital cellular pathways. Some of its functions during *H. pylori* infection are to prevent epithelial cell apoptosis and to alter the cell polarity (Amieva and Peek., 2016). These mutations are what eventually leads the cells to start becoming metaplastic (De Falco et al., 2015). Additionally, studies have shown that *H. pylori* strains that are CagA+, tend to be more virulent and are more likely to develop into GC (Jang et al., 2015). Another protein involved in the pathogenicity of *H. pylori*, is the heat shock b protein (HSpB). It has been shown that *H. pylori* secretes this protein, and it functions to allow bacteria to further proliferate despite the temperature it is present in (De Luca et al., 2008; Iaquinto et al., 2000).

Furthermore, it has been seen that *H. pylori* strains that secrete this tend to lead to development of GC more than other strains (De Luca et al., 2008). The pathogenic roles of VacA are to induce vacuolization in gastric cells and to inhibit the G1 phase of the cell cycle, which inhibits growth and leads to apoptosis (De Falco et al., 2015). It has also been shown that VacA can alter the epidermal growth factor signaling cascade and this can lead to poor ulcer healing (Penta et al., 2005).

Gastric cancer is a complex multifactorial disease that has been shown to have several associated risk factors. The most common type of GC are adenocarcinomas, and they are usually classified as cardia or non-cardia type. There are several risk factors that have been associated with the development of GC, but the most defined link is *Helicobacter pylori*, which has been classified as a Group 1 carcinogen and it has been observed to increase the incidence of GC, more specifically the non-cardia type (Park et al., 2018; Bornschein et al., 2011). Furthermore, in parts of the world like East Asia, East Europe, and South America the incidence of GC remains high when compared to other countries like North America (Karimi et al., 2014). It is thought
that *H. pylori*, is better controlled in the Western world, therefore lower incidence of non-cardia GC has been observed. The challenging aspect of this infection is that it rarely causes symptoms, which makes it difficult to diagnose (Khatoon et al., 2016).

Several other risk factors have been associated with this disease like diet, age, and cigarette smoking. These factors have been linked to be associated with GC of both the cardia region and non-cardia region (Karimi et al., 2014). Interestingly, the risk factors for the disease vary depending on the country where they are assessing for the disease. In North America, the incidence of cardia associated has been on the rise, whereas in other countries the rates of non-cardia are higher (Karimi et al., 2014). The reason for this may be attributed to the Western diet which may be contributing to the rates of obesity seen in the United States, which is a risk factor for cardia associated GC. More specifically, the incorporation of high salt foods into one’s diet has been seen to increase the risk of being diagnosed with gastric cancer, both the cardia and non-cardia types, in all parts of the world. It is hypothesized that salt may contribute to the development of gastritis through injury of the gastric mucosa (Karimi et al., 2014). Age has been seen to be a non-modifiable risk factor that increases the incidence of GC for both females and males. Interestingly, cigarette smoking has been shown to increase GC risk (both cardia and non-cardia) for males more than females. Overall, there are a wide range of modifiable and non-modifiable risk factors that have been seen to increase the risk of developing GC.

**Gastric Cancer Classification and Staging**

Over the years, the various classification systems for GC have evolved as researchers continue to uncover more knowledge of the disease. To classify the macroscopic appearance of gastric tumors, the Borrmann’s classification system describes 4 main types of tumors based on
their macroscopic growth patterns. Type I is polypoid growth, type II is fungating, type III is ulcerating, and type IV is diffuse infiltrating (Hu et al., 2012). The American Joint Committee on Cancer developed the tumor, node, metastasis (TNM) classification system, which has been widely accepted for staging GC. The tumor, T, is regarding the primary tumor and how far along it has spread throughout the stomach or nearby organs. Next, the regional lymph nodes (N) of the stomach are assessed and this is useful to identify the extent of metastasis if at all present. If metastasis (M) is present, it is vital to determine if it is local or distant (Shamudheen, R., 2019).

An average of 90% of gastric cancers are diagnosed as adenocarcinomas (Jang et al., 2015). For patients with gastric carcinomas that are undergoing a gastrectomy the most commonly used histological classification system is Lauren’s classification, which was established in the 1960’s (Chen et al., 2015; Lauren, P., 1965). Lauren’s classification system states that gastric adenocarcinomas can be characterized as diffuse or intestinal types (Jang et al., 2015). If the patient exhibits patterns of intestinal and diffuse, these are denoted as mixed type, which isn’t very common. Additionally, intestinal type has been described as well differentiated and tends to have a better prognosis. Alternatively, the diffuse type has been described as poorly differentiated and has been correlated with a lower OS (Jang et al., 2015).

In 2019, the World Health Organization has established a classification system that is all encompassing and aims to combine molecular and histopathological characteristics for tumors of the digestive system. More specifically, according to this system the exact mechanism for GC development remains unclear. However, it has become evident that chronic inflammation, as seen with *H. pylori* infection, is a key contributor for development of the disease (Nagtegaal et al., 2019). In addition to chronic inflammation, environmental factors, diet, smoking, and alcohol
consumption are all thought to play an important role as well, but the exact molecular mechanism is still unknown.

**Clinical Aspects of Gastric Cancer**

It has been shown that GC follows the model proposed by Correa and others, which outlines the progression of the disease (Correa and Piazuelo., 2012). A majority of GC’s manifest due to infection with *Helicobacter pylori* and subsequently developing superficial gastritis (Sandoval-Bórquez., 2015). These types of GC tend to be classified as intestinal type, according the Lauren’s classification system. The sequence of pathologic events that take place prior to the diagnosis of GC are known collectively as the precancerous cascade. After infection with *H. pylori*, there is development of superficial gastritis, which establishes a chronic inflammatory state. The length of time that this chronic inflammatory state takes to lead to the next step in the precancerous cascade depends on the level of virulence the strain of *H. pylori* is that caused the initial infection (Sandoval-Bórquez., 2015).

According to histologic classification, there are two main classifications of gastritis known as atrophic or non-atrophic (Hunt et al., 20xx). Due to this chronic inflammatory state, the gastritis becomes non-atrophic, but once the mucosa starts to lose its normal glandular architecture it becomes classified as multifocal atrophic gastritis (Correa and Piazuelo., 2012). At this point in the cascade, the chronic inflammation could still be reversed if there is successful treatment of the causative agent, *H. pylori* (Sandoval-Bórquez., 2015). Continuing with the cascade, the next changes start to affect the mucosal lining of the stomach and the epithelium starts to become metaplastic. The phenotypic changes associated with this type of metaplasia make the gastric epithelium resemble the intestine due to the presence of microvilli and goblet
cells, which is why this process is called intestinal metaplasia (Correa and Piazuelo., 2012).

Eventually, this inflammatory state leads to development of dysplastic cells that eventually become malignant and progress into GC (Sandoval-Bórquez., 2015).

Throughout the progression of disease, patients may experience a wide range of symptoms, but may not attribute them to cancer. One of the reasons that makes GC so difficult to diagnose, is that the symptoms that arise are very non-specific. The symptoms associated with GC do not manifest until the diseases has become more advanced or even metastasized to nearby organs. With advanced stage GC, the symptoms overlap with that of other diseases and some patients may not develop any or may not attribute them to GC (Mansfield et al., 2003).

According to the American Cancer Society, the most common symptoms are weight loss, nausea, vomiting, heartburn, feeling full after small meals, and blood in the stool (American Cancer Society, n.d.). It is common for doctors and patients to overlook a lot of these symptoms; thus, GC does not become diagnosed until it has become advanced (Mansfield et al., 2003).

Unfortunately, once GC becomes advanced the treatment options are less effective and overall survival (OS) decreases.

**Standard-of-Care Treatments for Gastric Cancer**

The standard-of-care treatments for GC are ever evolving as researchers aim to learn more about the disease. The treatment plans for GC are largely dependent on the stage of the cancer at the time of diagnosis and if the patient is a candidate for surgical resection of the tumor. The main treatment options can be classified as surgery, adjuvant, or neo-adjuvant (perioperative) chemotherapy or chemoradiation (Orditura et al., 2014).
Since GC is typically diagnosed when it has advanced to later stages, curative-intent surgical resection is not always the best course of action, because metastasis to other organs complicates removal and leads to poorer overall survival (Leiting and Grotz., 2019). However, if a patient is a candidate for surgical resection, this is typically the first line treatment (Koeda et al., 2011 Curative-intent gastrectomies can become complicated because the complex architecture of the stomach contains numerous lymph nodes; therefore, patients typically undergo a lymphadenectomy of all the nearby lymph nodes as well (Chen et al., 2015; Leiting and Grotz., 2019). Moreover, the resected lymph nodes have been shown to act as a prognostic marker, thus the higher amount of resected lymph nodes the lower incidence of death (Leiting and Grotz., 2019). Minimally invasive surgical techniques for the treatment of GC are not common. According to a Surveillance, Epidemiology, and End Results analysis conducted in the United States from 2008-2013, around 8% of gastrectomies conducted were laparoscopic and only 2% were conducted with robotic assistance (Glenn et al., 2015). This could be attributed to GC being diagnosed at more advanced stages, whereas minimally invasive techniques are favored for early GC. In all, curative-intent surgical resection continues to prove as the most effective standard of care procedure for GC, but not all patients may be eligible.

Around the time of removal of the tumor or the period before, patients may be treated with a combination regimen of standard-of-care chemotherapeutics epirubicin, cisplatin, and 5-fluorouracil (ECF) (David Ilson., 2018). These chemotherapeutics are used as first-line treatment for patients who have non-metastatic stage II GC or higher (Leiting and Grotz., 2019). According to the National Cancer Comprehensive Network, if a patient is medically fit, treatment with perioperative 5-fluorouracil, leucovorin, oxaliplatin, and docetaxel (FLOT) is standard-of-care. Alternatively, in patients with a low performance status (PS) or are medically
unfit, perioperative treatment with oxaliplatin and a fluoropyrimidine is recommended (Ajan et al., 2013).

Over the past few years, several researchers have been investigating the efficacy of adjuvant treatments for GC versus surgical treatment alone. Several meta-analysis studies have reported that there is a statistically significant benefit to adjuvant chemotherapy treatment, when compared to surgical resection alone. Some of the commonly used adjuvant chemotherapeutics are regimens containing 5-fluoruracil, capecitabine, or oxaliplatin. In a phase III clinical trial conducted by Bang et al, adjuvant treatment with oxaliplatin and capecitabine was administered after gastrectomy. Their results showed that the 3-year survival rate for the surgery and adjuvant treatment was 74% compared to 59% with surgery alone (Bang et al., 2012).

**Role of Transporter xCT and CD44 Variant Isoform 9**

Gastric cancer continues to be a global problem especially in Eastern Asia and South and Central America (Chakrabarti et al., 2018). The five-year survival rate of this disease remains low at only 29% (Steele et al., 2019). It has been shown that the cancer stem cells present within the tumor allow for the resistance to treatment that is observed in GC patients. The GCSCs are a sub-population of cells that are present within the tumor that exhibit stem cell like characteristics, like the ability to initiate and maintain tumor growth (Zavros, Y., 2017). Furthermore, research has shown that through differentiation, the CSCs are responsible for producing a majority of the non-tumorigenic cell population, present within the tumor (Ishimoto et al., 2011). A well-established marker for these cells is known as the cluster of differentiation 44. CD44 is classified as a cell surface adhesion molecule that functions as a hyaluronic acid cell surface receptor (Jang et al., 2011). Normally, CD44’s are present in hematopoietic cell
populations or in normal epithelial cells (Ishimoto et al., 2011). However, the CD44+
subpopulation of stem cells are able to generate variants through alternative mRNA splicing of
its exons 6-15 (Jang et al., 2015). The CD44 variants have been noted to be expressed mainly in
epithelial type carcinomas (Jang et al., 2015). More specifically, the variant that has been
associated as a marker for GCSCs is CD44v9. The exact role of CD44v9 has not been well
studied in GC, but it is hypothesized that it may contribute to the highly resistant phenotype that
is characteristic of GC. Moreover, it has been seen that CD44v9 functions to stabilize the
cystine/glutamate antiporter known as xCT on the surface of the cancer stem cells.

The cystine/glutamate xC- system is composed of two subunits, a light-chain xCT
SLC7A11 and a heavy-chain CD98hc, SLC3A2 (Ishimoto et al., 2011). This transporter is
present on the surface of cells and functions as a cystine-glutamate antiporter (Miyoshi et al.,
2018). The functional portion of the system is xCT and it has been observed to contribute to
 glutathione (GSH) synthesis and redox balance, through exchange of extracellular cystine for
intracellular glutamate (Chen et al., 2009). This exchange leads to the reduction of cystine to
cysteine, which leads to GSH production in the cell (Chen et al., 2009). GSH acts as an
antioxidant within the cell and is able to reduce the amount of reactive oxygen species (ROS)
present, which leads to the inhibition of the p38mapk pathway (Chen et al., 2009; Ishimoto et al.,
2011). Without proper levels of ROS intracellularly its downstream target, p38mapk, will not be
activated, therefore its function of inhibiting carcinogenesis is no longer carried out (Ishimoto et
al., 2009). In a study conducted by Miyoshi et al, where xCT was pharmacologically inhibited
with sulfasalazine, showed reduced tumor formation in mice treated with cisplatin (Miyoshi et
al., 2018).
An analysis of ROS levels in CD44+ CSCs of the breast showed that this subset of cells expresses lower levels of reactive oxygen species (ROS), meaning that they have higher defense systems for ROS, leading to increased resistance to standard-of-care chemotherapeutics (Diehn et al., 2009). This defense mechanism against ROS is thought to be due to the relationship between CD44v9 stabilizing the $\chi$CT transporter on the surface of the CSCs and has also been observed in GC (Ishimoto et al., 2011). Furthermore, data from our lab has shown that patient-derived gastric cancer organoids that were CD44+ displayed resistance to treatment with cisplatin (Zavros, Y., 2017). These findings support the need for further investigation into the hypothesis that CD44v9 and $\chi$CT are contributing to ROS defense. Overall, the aim of my work was to identify the potential role of CD44v9 stabilized by transporter $\chi$CT as a mechanism that the tumor may use to avoid sensitization to ROS inducing chemotherapeutics.
CHAPTER TWO: MATERIALS AND METHODS

Generation of Human Resected Tumor-Derived Organoids

Tumor tissue was also obtained from patients undergoing surgical resection for gastric cancer (IRB protocol number: 2015-5537, University of Cincinnati; or IRB protocol number: 1912208231R001, University of Arizona Human Subjects Protection Program; IRB protocol number: 1099985869R001, University of Arizona Human Subjects Protection Program TARGHETS) after voluntary consent for tissue and blood collection.

With the use of a previously published protocol, human derived gastric organoids were generated from various resected tumor tissues of gastric cancer patients (Steele et al., 2019). First, the tumor tissue was obtained and washed in cold Dulbecco’s phosphate buffered saline (DPBS Fisher Scientific, 21-040-CV) without calcium or magnesium and supplemented with various antibiotics (1% penicillin/ streptomycin, 1% kanamycin and Amphotericin B (0.25 mg/ml)/Gentamicin (10 mg/ml)). The tumor tissue was minced into small fragments and then placed into 10mL of a pre-warmed EDTA stripping buffer (HBSS (14175-095) with 20% FCS, 1% Pen/Step (30-002-CI), HEPES and EDTA). These were placed in a rotating incubator for 10 minutes at 37°C and then after replacement with fresh EDTA buffer, another five minutes of incubation. The tissue fragments were washed two times with Dulbecco’s Modified Eagle Medium (DMEM) with the addition of the following antibiotics, 1% penicillin/streptomycin, 1% kanamycin and Amphotericin B (0.25 mg/ml)/Gentamicin (10 mg/ml) (Thermofisher Scientific R-01510) and then incubated for 15-30 minutes with 10mL of previously warmed collagenase A (C9891, Sigma) hyaluronic acid (Sigma, H3884) buffer at 37°C in a rotating shaker. Once digested, the tissue was filtered through a 40 μm filter. The filtered cells were centrifuged for 5 minutes at 400xg and then washed with DPBS (Fisher Scientific, 21-040-CV), then centrifuged
again for another 5 minutes at 400xg. After discarding the supernatant, the remaining pellet was resuspended in the desired amount of Matrigel™ (Corning CB40230C), with the addition of 1% Kanamycin and 1% Penicillin/streptomycin, and seeded onto culture plates that had been overlaid with gastric organic media beforehand ((Advanced DMEM/F12 (Thermofisher Scientific 12634010) supplemented with 10 mM HEPES (Thermofisher Scientific BP299-100), 1X Glutamax (Thermofisher Scientific 350-50-061), 1% Pen/Strep (Thermofisher Scientific), 1X N2 (Thermofisher Scientific 17502048), 1X B27 (Thermofisher Scientific 12587010), 10mM Nicotinamide (Sigma-Aldrich N0636), 1 mM N-Acetylcyctine (Sigma-Aldrich A7250), 50 ng/mL EGF (Peprotech 315-09), 100 ng/mL Noggin (Peprotech 250-38), 10% R-Spondin Conditioned Media, 50% Wnt Conditioned Media, 200 ng/mL FGF10 (Peprotech 100-26), 1 nM Gastrin (Tocris Bioscience 30061), 10 uM Y-27632 (Sigma-Aldrich Y0503). These were incubated at 37°C in a CO2 incubator.

**Culture of MSI-H IM95 Cells**

To achieve a culture of the MSI-H IM95 gastric cancer cell line (JCRB Cell Bank, JCRB1075.0), the cells were thawed quickly in a water bath. Following this, 1 mL of pre-warmed Dulbecco's modified Eagle's medium with high concentration of glucose, 10% fetal calf serum (R&D Systems S12450H) and 10mg/L insulin (Tocris, 3435) was added. The cells were placed into a 15mL Falcon tube and centrifuged for 5 minutes at 4°C at a speed of 400 x g. The supernatant was discarded, and the pellet was resuspended in 1mL of pre-warmed Dulbecco's modified Eagle's medium with high concentration of glucose, 10% fetal calf serum and 10mg/L insulin. After resuspension, the cells were seeded onto a 6 well plate. The growth was monitored, and media was changed every 2-3 days. Once high confluency was achieved, the cells were sub
cultured weekly with the use of 0.25% trypsin. For freezing, the cells were trypsinized, harvested and resuspended in freezing media, containing 80% complete growth media supplemented with 10% FCS and 10% DMSO. The cells were aliquoted into 2mL cryovials and then transferred to Mr. Frosty containing isopropanol and stored in -80°C overnight to slowly freeze and then followed by final storing at liquid nitrogen.

**Knocking Down of XCT Expression in MSI-H IM95 Cell line**

The IM95 cells were washed in DPBS and then incubated with 1 milliliter of trypsin at 37°C for 8 minutes. Following this, cells were harvested using 2 milliliters of complete growth media and transferred to a 15mL Falcon tube and centrifuged for 5 minutes at 400 x g. Once the supernatant was discarded, the pellet was resuspended in 1mL of trypsin and then incubated at 37°C for 8 minutes. Following this, 2 milliliters of complete growth media was added to the tube and centrifuged for 5 minutes at 400 x g. The pellet was resuspended in 1mL of IM95 media (Dulbecco's modified Eagle's medium with high concentration of glucose, 10% fetal calf serum (R&D Systems S12450H) and 10mg/L insulin). The empty vector (EV) or short hairpin RNA lentiviral particles of XCT were added to the cells using their respective MOI unit as well as 8mg/ml of hexadimethrine bromide. The cells were incubated for 48 hours at 37°C. After the incubation period, 2mL of complete growth media was added to each tube and centrifuged at 400 x g for 5 minutes. The cells were resuspended in complete growth media and seeded into 12 well culture plates. After 72 hours, the cells were selected with the addition of 1μg/mL puromycin (Sigma, P9620) within the culture. The control (EV) and knockdown (KD) cells were grown for 7-10 days.
Establishment of MSI-H IM95 spheroids using AggreWell™ Microwell Plates

In order to be able to generate cell spheroids, also known as aggregates, an Aggrewell 800 microwell plate was used because each well of this plate contains an array of 300 microwells that are 800 μm in size, which allow for the growth of the spheroids. Based on the manufacturer’s instructions, the MSI-H spheroids were generated. First, 500mL of Anti-adherence rinsing solution (Stemcell Technologies, CAT07010) was added to each well and then it was centrifuged at 1300 x g for 5 minutes in a swinging bucket rotor. Following this, each well was rinsed with 2mL of pre-warmed basal medium and then 1mL of complete medium was added to each well. The fully grown MSI-H gastric cancer cells were harvested with the use of pre-warmed DPBS and trypsin, then centrifuged at 400 x g for 5 minutes. The supernatant was removed, and the pellets were resuspended in 4mL complete media. Then 500μl cells were added to each well respectively. The Aggrewell plate was immediately centrifuged at 100 x g for 3 minutes. The culture was maintained for 72 hours. The spheroids were imaged at 0 and 48 hours using brightfield microscopy (Nikon Spinning disk confocal microscope). The changes in area for each experimental condition per timepoint were measured by utilizing Nikon Element software and manually drawing ROIs around individual spheroids from a single microwell. The summarized area was calculated and plotted as a histogram using commercially available Graphpad Prism software (GraphPad Software, San Diego, CA). A statistical significance was determined when the p-value was less than 0.05.

Immunofluorescence Staining of MSI-H IM95 Cells

The cells were fixed by adding 200 uL/well of 3.7% paraformaldehyde for 15 minutes at room temperature. Following this, the organoids were washed with 200 uL/well of DPBS (Fisher Scientific, 21-040-CV) for a total of 5 minutes, at room temperature. In order to permeabilize the
cells, 200 uL/well of 0.5% Triton X-100 in PBS (PBST) was added and allowed to incubate for 20 minutes at room temperature. The cells were then washed with 200 uL/well 0.01% PBST for 5 minutes at room temperature. Cells were blocked with the addition of 200 uL/well of 2% normal donkey serum (Jackson Immuno Research #017-000-121) in 0.01% PBST for 1 hour at room temperature. After 1 hour, blocking was removed, and the cells were incubated with primary antibody diluted in 0.01% PBST overnight at 4°C. The cells were washed with 0.01% PBST for 5 minutes at room temperature. After washing, they were incubated with secondary antibody and Hoechst (diluted 1:1000 in 0.01% PBST), diluted in 0.01% PBST placed in the dark for 1 hour at room temperature. Lastly, the organoids were washed with 0.01% PBST for 5 minutes at room temperature and were stored in DPBS (Fisher Scientific, 21-040-CV). The following primary antibodies and dilutions were used: 1:100 human and mouse specific rabbit anti xCT (Novus #NB300-318) and 1:1000 rat anti CD44v9 (Cosmo Bio #LKG-M001).

**Patient Derived Xenografts (PDX)**

The mouse xenograft assay was conducted by injecting 20 NOD scid gamma mice subcutaneously in the right flank with either 1000 gastric tumor derived organoids or 50,000 IM95 cells. Tumor growth was visualized at day 15 and treatment was begun with alternating side intraperitoneal injection of vehicle or cisplatin at a dose of 3mg/kg of body weight. The treatment was conducted every day for a period of 30 days. The tumor dimensions were measured every 3-7 days for the duration of the assay.

**Drug Assay in MSI-H IM95 Cell Line**
The IM95 cells and organoids were seeded on a 96 well plate. Then, the cells were treated with either vehicle or cisplatin (Selleckchem, S1166) at concentrations of 0, 0.5, 1, 5, 10, 50, 100, and 200 μmol/L in combination with varying concentrations of sulfasalazine from 0, 0.5, 1, 5, 10, 50, and 100 μmol/L for a total of 48 hours (SSZ, Selleckchem, 50883-10G). After the 48-hour period, the cell proliferation rate was measured with the use of an MTS assay (Promega 93582). Additionally, dose-response curves were calculated on the basis of the absorbance readings acquired from the MTS assay relative to the drug concentration used. The absorbance was normalized to the vehicle controls, and the drug concentration were converted to logarithms with the use of commercially available GraphPad Prism (GraphPad Software, San Diego, CA).

**Western blot**

Organoids were harvested after treatment using cold DPBS and then centrifuged for 5 minutes at 400 x g. The supernatants were decanted, and the pellets were resuspended in phosphate free lysis buffer. Following this, measurement of protein concentration was conducted using a Bradford kit (Thermofisher Scientific, 1856207) and the cell lysates were diluted in 4x Laemmli loading buffer (Bio-Rad Laboratories, 1610737) with β-mercaptoethanol (Bio-Rad Laboratories, 1610710). Then, the samples were loaded onto 4-20% Tris-Glycine Gradient Gels (Thermofisher Scientific, XV04205) and were run at 80 volts for around 3 hours. They were then transferred onto nitrocellulose membranes at 105 volts for 1.5 hours at a temperature of 4°C. The membranes were blocked at room temperature for a total of 1 hour utilizing the KPL Detector Block Solution (Sara-Care, 5920-0004). Incubation of membranes was done overnight at 4°C with either 1:2000 dilution of mouse anti-GAPDH (Millipore, MAB374) antibodies or
1:1000 anti-rabbit p38^{mapk} (Thermofisher Scientific, 9212S) and 1:1000 anti-rabbit phospho-p38^{mapk} (Thermofisher Scientific 4631S) by 1 hour incubation with a 1:1000 dilution anti-mouse, or rabbit Alexa Fluor 680 (Thermofisher Scientific). Once incubation time was completed, the blots were imaged with the use of a scanning densitometer along with analysis software (Odyssey Infrared Imaging Software System).

**Statistical Analyses**

The significance of the results was tested with the use of a two-way analysis of variance or a student’s t-test. These were completed using GraphPad Prism (GraphPad Software, San Diego, CA). A p-value less than 0.05 was established as significant.
CHAPTER THREE: RESULTS

The approach we took to test my hypothesis was to use an established protocol in the lab that derives organoids from resected tumor tissue or from biopsies collected from patients that have been diagnosed with gastric cancer. In order to analyze for expression of CD44v9 and xCT, an immunofluorescence stain was used. It was observed that there was high co-expression of both CD44v9 and xCT among all the lines (Figure 1A-E). Next, to assess for expression of phosphorylated p38mapk (phospho-p38mapk), a western blot was conducted, and the lines were treated with vehicle, cisplatin (Cis), or Cis and sulfasalazine (SSZ) with and without the co-treatment of N-Acetylcysteine (NAC). It is clear that only when the combination therapy is used there is expression of phospho-p38mapk (Figure 1F). It has been observed that organoids maintain similar characteristics to the parent tumor from which they are derived from. This was verified following immunofluorescent staining of the patient tumor from which TGO2 was derived, we observed similar co-expression of CD44v9 and xCT as seen with the huTGO2 line (Figure 1G). To analyze for the presence of reactive oxygen species (ROS), the huTGO1 line was stained with dihydroethidium (Thermofisher Scientific, D11347). High expression of ROS was visualized (Figure 2A). In order to confirm the presence of CD44v9 and xCT, immunofluorescence was conducted (Figure 2B). Collectively this data shows that when xCT is inhibited through knockdown or by SSZ treatment, it is allowing for increased expression of phosphor- p38mapk, which is responsible for inhibiting carcinogenesis.

Based on these results, we wanted to design an in vivo model to see if sensitization to cisplatin treatment could be achieved, after sulfasalazine inhibition of the xCT transporter, in a human-derived gastric cancer organoid line. A xenograft experiment was conducted with patient-derived gastric cancer organoids and the NSG mice were treated with vehicle, cisplatin,
sulfasalazine, or cisplatin and sulfasalazine. We observed that the tumor volume decreased the most with the combination treatment (Figure 3A). This is attributed to successful inhibition of xCT with sulfasalazine, resulting in tumors that are susceptible to treatment (Figure 3B). Immunofluorescence for expression of CD44v9 and phospho-p38mapk was conducted on the tumor tissue, within the various treatment groups of vehicle, cisplatin, or cisplatin with sulfasalazine. The results showed that the combination treated group expresses the activated form of p38mapk, indicative of effective xCT inhibition (Figure 3C). To further verify expression of phospho-p38mapk, western blot analysis was conducted within the various treatment groups (vehicle, cisplatin, cisplatin and sulfasalazine). Once again, expression of activated p38mapk was only visualized in the combination treated group (Figure 3D). Expression of CD44v9 was analyzed with a western blot assay, within the same treatment groups, and expression of variant 9 was lost in the combination treated group, further supporting the effective inhibition of xCT.

In order to examine the potential for xCT to be protective against ROS inducing chemotherapeutics, we conducted a xenograft experiment using an MSI-H IM95 cell line, with xCT knockdown through short hair pin RNA. The data showed that xCT does play a role in protecting the tumor from cell death, but there was a lack of complete regression in tumor volume, despite having treated and knocked down xCT (Figure 4A). In order to ensure successful knockdown of xCT, immunofluorescence was conducted for xCT and CD44v9 in both the empty vector (EV) and xCT knockdown (KD) lines. CD44v9 expression was maintained in both the empty vector and knockdown lines, but expression of xCT was diminished in the xCT knockdown group (Figure 4B).

Additionally, we wanted to investigate the efficacy of combination therapy, of SSZ and Cis, in an in vitro model utilizing the MSI-H IM95 GC cell line. With the use of a 96-well plate
we determined the best IC50 of sulfasalazine, using 6 different concentrations (0, 1, 5, 10, 50, and 100), along with 8 varying concentrations of cisplatin (0, 0.5, 1, 5, 10, 50, 100, 200) (Figure 5A). Once the optimal dose was established for both the EV and KD lines, we wanted to test the efficacy of the dose. With the use of an AggreWell™ 800 plate and the optimal dose of SSZ, we showed the changes in area and morphology at varying concentrations of cisplatin. The results were analyzed, and the area was normalized based on the 0-hour time point, and these were plotted on a histogram (Figure 6A-B). From this data we can visualize that after addition of SSZ, cell death begins to increase, meaning the MSI-H IM95 cell lines have become sensitized to treatment (Figure 6C-D).
CHAPTER FOUR: DISCUSSION AND FUTURE DIRECTIONS

Our data showed that CD44v9 and xCT are co-expressed within patient derived gastric cancer organoid lines. Previous work conducted by Ishimoto et al (Ishimoto et al., 2011) showed expression of CD44+ variants 8-10 in various human gastrointestinal cell lines, when analyzed through RT-PCR. Furthermore, their group measured the protein level of xCT in a colorectal cancer line expression CD44 shRNA, then treated with a protein synthesis inhibitor, to see the effect in xCT expression. From this experiment they concluded that CD44 is responsible for stabilizing xCT (Ishimoto et al., 2011). Our group analyzed the co-expression of CD44v9 and xCT in gastric cancer but with the use of an advanced model system of patient derived gastric cancer organoids, which retain tumor characteristics. From our experiments, we visualized co-expression of xCT and CD44v9 in 5 patient-derived gastric cancer organoid lines. We then analyzed the expression of xCT and CD44v9 from patient tissue from which huTGO2 was derived from, we saw similar expression. Our findings exemplify that CD44v9 and xCT co-localize within patient derived gastric cancer organoids. Thus, organoids recapitulate similar characteristics as the patient and may serve as a superior model system over cell lines, which tend to lack tumor heterogeneity.

The resistant phenotype of cancer stem cells within the gastric cancer organoids to standard-of-care chemotherapy was attributed to the expression of xCT. Specifically, xCT when stabilized by CD44v9 on the surface of CSCs, facilitates resistance to ROS-induced cancer cell death. Several studies have reported that a main contributing factor GC resistance is due to the cancer stem cells present within the tumor (Miyoshi et al., 2018 Singh et al., 2013). A recent study conducted by Miyoshi et al (Miyoshi et al., 2018), showed that gastric cancer cells were sensitized to 5-fluorouracil treatment, following xCT inhibition. We show that combination
treated (SSZ and Cis) patient-derived gastric cancer organoids expressed activated p38\textsuperscript{mapk}. Therefore, inhibiting the function of xCT to defend against ROS. This data supports the use of patient-derived organoids to effectively study drug responses in gastric cancer and potential therapeutic targets to aid in tumor sensitization in patients with GC.

Resistance to cisplatin was sensitized with sulfasalazine. This drug has been widely used to treat patients with rheumatoid arthritis and other inflammatory conditions (Okazaki et al., 2018). The mechanism for which sulfasalazine is able to sensitize the tumor treatment is through inhibition of the xCT transporter (Miyoshi et al., 2018; Ishimoto et al., 2011). Several studies have shown that sulfasalazine is a potent xCT inhibitor and has been able to reduce the amount of CD44v\textsuperscript{+} cancer stem cells \textit{in vitro} and \textit{in vivo} (Miyoshi et al., 2018; Shitara et al., 2017; Okazaki et al., 2018). According to a phase I clinical trial in patients with advanced staged gastric cancer, they showed that 4 of the 8 patients enrolled demonstrated a decrease in CD44v\textsuperscript{+} CSCs following treatment with sulfasalazine (8-12 g/day) (Shitara et al., 2017). In our \textit{in vitro} system, sulfasalazine sensitized CD44v9\textsuperscript{+} cells and patient-derived gastric cancer organoids to cisplatin. Our data suggests that sulfasalazine is effective at inhibiting the function of xCT, stabilized by CD44v9. Therefore, CD44v9 may be considered as a potential therapeutic target to aid with tumor sensitization in patients with GC.

In conclusion, I find that patient-derived organoids exemplify and recapitulate characteristics from the patient’s tumor. Thus, this culture system serves as a model to further study the xCT-CD44v9 mechanism and other related mechanisms relevant to the tumor microenvironment. Future studies aim to use a patient derived organoid lines and knockdown xCT to recreate the xenograft experiment conducted with the MSI-H IM95 cell line, as a model for analyzing drug responsiveness. Additionally, we aim to study drug response, similarly to the
Aggrewell™ 800 experiment, with the use of an xCT knockdown patient-derived gastric organoid line. The AggreWell™ format may be used to screen for candidate drugs that sensitize cancer cells to chemotherapies. It is also crucial to consider the patient’s immune system within the tumor microenvironment, thus the contribution of immune suppressive cells can be studied using co-cultures and humanized mice (Holokai et al., 2020; Bertaux-Skeirik et al., 2017).

Given that patient-derived gastric organoids lack accounting for the influence of the patient’s immune response, which may play a significant role in drug responsiveness, humanized mice may be used. Typically, NRG-SGM3 mice are injected with blood from the patient in order to allow for the mice to produce human immune cells, which allows for a response that accounts for the patient’s immune system versus a patient-derived organoid model that lacks this (Bertaux-Skeirik et al., 2017). Once presence of the immune cells is verified within the mice, they can be orthotopically transplanted with patient-derived gastric cancer organoids (Bertaux-Skeirik et al., 2017). Therefore, a future approach would be to utilize the humanized NSG-SGM3 mice to study the response of combination treatment with sulfasalazine and cisplatin, in order to better determine how the immune response will affect efficacy of cisplatin treatment, in patients with gastric cancer.
Figure 1. Expression of p38\textsuperscript{mapk} activity in huTGOs \textit{in vitro} in response to \textit{xCT} knock down. Immunofluorescence of CD44v9 (green) and xCT (red) expression in (A) huTGO1, (B) huTGO2, (C) huTGO4, (D) huTGO5, (E) huTGO6. (F) Western blot analysis for p38\textsuperscript{mapk} expression using huTGO1, huTGO2, huTGO3, and huTGO5, treated with vehicle, cisplatin, or cisplatin and sulfasalazine with and without N-Acetylcysteine. (G) Immunofluorescence of CD44v9 (green), xCT (red), and cell nuclei (blue) in patient tumor tissue from which huTGO2 was derived.

Figure 2. (Expression of ROS, CD44v9 and \textit{xCT} in human-derived gastric cancer organoid line. Immunofluorescence for reactive oxygen species (red) in the huTGO1 line. (B) Immunofluorescence for expression of CD44v9 (green) and xCT (red) and cell nuclei (blue).
Figure 3. Expression of p38\textsuperscript{mapk} activity in MSI-H (IM95) cell lines \textit{in vivo} in response to xCT knockdown. (A) Tumor volume over time of nod scid gamma (NSG) mice treated with vehicle, cisplatin (cis), sulfasalazine (SSZ), or Cis and SSZ for a period of 30 days. (B) Images of gross measurements of tumor volume from NSG mice. (C) Immunofluorescence for expression of CD44v9 (green) and phospho-p38\textsuperscript{mapk} (red) and cell nuclei (blue). (D) Western blot analysis for expression of total and phospho-p38\textsuperscript{mapk} or expression of (E) CD44v9 and GAPDH in NSG mice treated with vehicle, Cis, or Cis and SSZ.
Figure 4. Expression of \( p38_{\text{mapk}} \) activity in huTGOs \emph{in vivo} in response to \emph{xCT} knockdown.

(A) Tumor volume over time of nod scid gamma (NSG) mice treated with cisplatin in empty vector (EV) or \emph{xCT} knockdown lines for a period of 28 days. (B) Immunofluorescence of CD44v9 (green), \emph{xCT} (red) and cell nuclei (blue) in IM95 cell line.
Figure 5. Dose-response curves of cisplatin and sulfasalazine in MSI-H IM95 Cells. Dose response curves generated by using (A) empty vector and (B) xCT knockdown IM95 cell lines in response to 6 concentrations of sulfasalazine and 8 concentrations of cisplatin.
Figure 6. Response to cisplatin in IM95 cells in combination with sulfasalazine. Changes in area over time of MSI-H IM95 (A) empty vector or (B) xCT knockdown. Morphological changes visualized in MSI-H IM95 spheroid aggregates (C) empty vector and (D) xCT knockdown over time points of 0 and 48 hours.
References


