Human recombinant arginase I (Co)-PEG5000 [HuArgI (Co)-PEG5000]-induced arginine depletion selectively inhibits colon cancer cell migration and invasion

By
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A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Molecular Biology

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May 2018
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Dedication Page

To my supportive and loving Family
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First and foremost, I thank God for giving me the strength and dedication to complete this work in the time I had.

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Human recombinant arginase I (Co)-PEG5000 [HuArgI (Co)-PEG5000]-induced arginine depletion selectively inhibits colon cancer cell migration and invasion

Houssam Khaled Al Koussa

Abstract

Many arginine deprivation studies have been done on different cancer cell lines to understand the complete mechanisms of HuArgI(Co)-PEG5000. Colorectal cancer is the third most common type of cancer worldwide, and it represents over half of all gastrointestinal cancer death. Therefore, the first purpose of this study is to examine the cytotoxic effect of HuArgI(Co)-PEG5000 on colorectal cancer cell lines (HT-29, Caco-2, Sw837, Sw1116, SKco-1). The second aim is to investigate the effect of arginase depletion on colorectal cancer cell line Caco-2 metastatic and invasive abilities. This is achieved by performing cytotoxicity, 2D and 3D migration assays, western immunoblotting, immunostaining, and Förster Resonance energy transfer. Analysis of the results show that HuArgI(Co)-PEG5000 downregulates ASS1 and RhoA expression levels while it also downregulates cell migration, adhesion, and invasion in Caco-2 cell lines. However, L-citrulline can significantly restore arginine levels and hence counter the effect of HuArgI(Co)-PEG5000. Therefore, we can conclude that colorectal cancer is partial auxotrophic to arginine depletion and that arginine depletion plays an important role in regulating cancer cells motility and invasion.

Keywords: Colorectal cancer, HuArgI(Co)-PEG5000, L-citrulline, RhoA, Focal adhesion, metalloproteinase
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LIST OF ABBREVIATIONS

ADI: Arginine deiminase
ADI-PEG: Pegylated arginine deiminase
AML: Acute myeloid leukemia
APC: Adenomatous polyposis coli
Arg1: Arginase 1
ASL: Arginosuccinate lyase
ASS: Arginosuccinate synthetase
BSA: Bovine serum albumin
CPS: Carbamoyl phosphate synthetase
CRC: Colorectal cancer
CSR: SEER Cancer statistics review
CXCL12: CXC-motif chemokine 12
CXCR4: CXC-chemokine receptor type 4
DMEM: Dulbecco’s modified eagle media
ECM: Extracellular matrix
FA: Focal adhesions
FAK: Focal adhesion kinases
FRET: Förster resonance energy transfer
FBS: Fetal bovine serum
HCC: hepatocellular carcinoma
HNPCC: hereditary nonpolyposis colon cancer
HNPS: Hereditary mixed polyposis syndrome
HuArg1: human pegylated arginase 1
HuArgI(Co)-PEG5000: Human recombinant arginase I cobalt PEG 5000

L-Cit: L-citrulline

MEL: melanoma

MMP: Matrix metalloproteinase

MSI: Microsatellite instability

NPS: National Polyps study

OTC: Ornithine transcarbamoylase

PANC: Pancreatic cancer

PBS: Phosphate-buffered saline

PEG-Arg: pegylated arginase 1

PEG-BCT-100: Mutant human recombinant arginase I PEG 5000

PLR-3: Phosphatase of regenerating liver 3

ROCK: Rho associated protein kinase

SEER: Surveillance epidemiology and end results

TIMP: Tissue inhibitor of metalloproteinase

WHO: World health organization
Chapter 1

Literature Review

1.1 Cancer Overview

The term “cancer” is associated with a collection of related diseases that are characterized by uncontrollable cell divisions. This unusual action is due to accumulation of genetic mutations, and it has the potential to spread into surrounding tissues (Alberts et al., 2002). Once this abnormal proliferation of cells occurs, a tumor is formed that could be classified as benign or malignant. It is significant to distinguish between those two types, for a benign tumor is immobile and has neither the ability to invade nor spread to neighboring tissues while the malignant tumor can migrate to different parts of the body and is referred to as “cancer”-the perilous tumor (Cooper, 2000). Cancer is labeled as metastatic cancer or stage four cancer when cancer cells spread via circulation, a process known as metastasis (“Metastatic Cancer,” 2017.).

The development of cancer is not a quick nor a simple process, for the stages of tumor development consist of more than one step. It begins with mutation on the genetic level, then it involves cell proliferation, survival, invasion, and finally metastasis. Tumor initiation is when genetic modifications occur continuously provoking the cell to undergo abnormal proliferation, giving rise to a population of
tumor cells with the same mutations. Then, tumor progression transpires which is the additional mutations that happen within the tumor cells. Referring to figure 1, this allows the cells to gain selective advantages, like the ability to undergo rapid growth, that will then be transmitted to their progenies and become dominant within the population of existing cells (Cooper, 2000). This process is known as clonal evolution, and it continues throughout the entire tumor progression phase, giving the tumor more tools to grow and evolve (Greaves & Maley, 2012).

Figure 1: tumor progression. Mutation in cells give rise to abnormal cells with increase in the ability to proliferate. Additional mutations result in further advantages like increased cell divisions, decreased apoptosis, and evasion of the immune system. (Dean, Fojo, & Bates, 2005)
Hanahan and Weinberg (2000) suggested a model that represents the hallmarks of cancer. Their model consists of six biological capabilities gained by the cancer cell and establishes a common ground for scientists to be able to comprehend the complexities of neoplastic disease. The hallmarks include Sustaining proliferative signaling, evading growth suppressors, resisting cell death, enabling replicative immortality, inducing angiogenesis, and activating invasion and metastasis (Hanahan & Weinberg, 2000). Later in 2011, Hanahan and Weinberg’s model got revised and four more traits were further included: evading the immune response, reprogramming metabolic energetics, initiating tumor-associated inflammation, and having genomic instability (Hanahan & Weinberg, 2011).

1.2 Colorectal Cancer

1.2.1 Colorectal Cancer definition

Colon cancer and rectal cancer start in the colon and rectum respectively, as their names imply. However, since the aforementioned types have common features, they are grouped together as colorectal cancer (“Colon Cancer Treatment,” 2018). A model known as adenoma-carcinoma sequence is made to link the genetic alteration that happens to the normal colonic mucosa which causes an intraepithelial neoplasia and the appearance of adenomatous growth (figure 2). This model shows the different stages of tumor development leading to the stage of having CRC and whether or not it will progress to the invasive type of cancer (Hagland et al., 2013). The majority of CRC develop from a small mass of cells in the inner lining of the colon or the rectum.
This mass of cells develops further to become a lump known as polyps that are of two types: the non-neoplastic type, known as hyperplastic polyps, and the pre-cancerous neoplastic polyps, known as adenomatous polyps (Colucci, Yale, & Rall, 2003). The National Polyps Study suggests that majority of the CRC initiate from the former type of poly that is also known as adenomas. (Winawer, 1999).

**Figure 2: The Adenoma-carcinoma sequence model.** Represents the development of CRC from stem cells and the set of complicated mechanisms that lead to metastasis and to the mucosal membrane. It also shows several hallmarks that are involved. (Hagland et al., 2013)
1.2.2 Colorectal Cancer classification

Many view CRC as a single disease, but in fact it is a family of diseases that originate from different precursor lesions. Knowing that CRC has a potential of high malignancy, the focus should be on the polyp’s type, size, and degree of dysplasia. For the adenoma CRC, there are different degrees of dysplasia that range from low gradient to high gradient. The high gradient of dysplasia is associated with increasing percentage of villous tissue incorporated with the polyps; once the polyps’ size becomes greater than 1cm in diameter, the risk of being malignant increases drastically. At this point, the CRC cells would have reached the submucosa layer by penetrating the muscularis mucosal layer (Colucci et al., 2003). Table 1 represents the different types of polyps and assesses their ability to be malignant.

Table 1: Classification of Colorectal Polyps. Source: (Colucci et al., 2003)

<table>
<thead>
<tr>
<th>Classification of Colorectal Polyps.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Histological Classification</strong></td>
</tr>
<tr>
<td>Non-neoplastic</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>Neoplastic (adenomas)</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
</tbody>
</table>
Dysplasia is the presence of abnormal cells within a tissue, and it highlights the transition of normal mucosa to adenoma and ending up as carcinoma through the pathway of carcinogenesis. Traditionally, the adenoma development starts by the deactivation of the two-adenomatous polyposis coli gene, a type of tumor suppressor, followed by the overactivation of oncogenes such a k-Ras in one of the colorectal epithelial cell (Colucci et al., 2003; Langner, 2015).

According to WHO, there are other significant pathways, including microsatellite instability (MSI) and Epigenetic silencing, that can drive the cell into carcinogenesis as represented previously in figure 2 (Hagland et al., 2013). The MSI is caused by a defect in the cell repair mechanism after DNA replication and thus a sequence of mistakes accumulates. This causes the number of inherited microsatellites, a short-repeated sequence of DNA, to change, affecting the overall DNA sequence. Examples of CRC’s that can take the MSI path include hereditary nonpolyposis colon cancer and sporadic carcinomas (Aust, 2011; Langner, 2015).

The third hallmark (figure 2) includes the epigenetic silencing, which is mediated by CpG island methylation that influences many genes, involving cell cycle control, signaling apoptosis, and DNA repair, by inactivating their transcription, and this plays an important role in tumor development. This proves that the colorectal carcinogenic process is a heterogenous process involving more than one precursor lesion (Hagland et al., 2013; Teodoridis et al., 2004). Table 2 represents other cancer hallmarks that are involved in CRC development while table 3 represents the histological classification of the CRCs by the WHO.
Table 2: Cancer hallmarks in relation to CRC. Source: (Hagland et al., 2013)

<table>
<thead>
<tr>
<th>Cancer hallmarks</th>
<th>Examples of involving factors in CRC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Growth signal autonomy</td>
<td>EGFR, KRAS, BRAF</td>
</tr>
<tr>
<td>Insensitivity to antiproliferative signals</td>
<td>P53, PTEN, APC</td>
</tr>
<tr>
<td>Unlimited replicative potential</td>
<td>TERT</td>
</tr>
<tr>
<td>Angiogenesis</td>
<td>VEGF</td>
</tr>
<tr>
<td>Escaping apoptosis</td>
<td>P53, MLH1</td>
</tr>
<tr>
<td>Invasion and metastasis</td>
<td>Cdc-42, RhoA GTPase</td>
</tr>
<tr>
<td>Reprogramming of cell metabolism</td>
<td>PI3K, AKT, c-MYC</td>
</tr>
<tr>
<td>Evading immune destruction</td>
<td>IL-8</td>
</tr>
</tbody>
</table>

PTEN = Phosphatase and tensin homolog; TERT = telomerase reverse transcriptase; VEGF = vascular endothelial growth factor; IL-8 = interleukin-8.
### Table 3: WHO histological classification of tumors of the colon and rectum. Source: (Bosman et al., 2000.)

<table>
<thead>
<tr>
<th>Epithelial tumours</th>
<th>Non-epithelial tumours</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Adenoma</strong></td>
<td><strong>Lipoma</strong></td>
</tr>
<tr>
<td>8140/0</td>
<td>8850/0</td>
</tr>
<tr>
<td><strong>Tubular</strong></td>
<td><strong>Leiomyoma</strong></td>
</tr>
<tr>
<td>8211/0</td>
<td>8990/0</td>
</tr>
<tr>
<td><strong>Villous</strong></td>
<td><strong>Gastrointestinal stromal tumour</strong></td>
</tr>
<tr>
<td>8261/0</td>
<td>8536/1</td>
</tr>
<tr>
<td><strong>Tubulovillous</strong></td>
<td><strong>Leiomyosarcoma</strong></td>
</tr>
<tr>
<td>8263/0</td>
<td>8890/3</td>
</tr>
<tr>
<td><strong>Serrated</strong></td>
<td><strong>Angiosarcoma</strong></td>
</tr>
<tr>
<td>8293/0</td>
<td>9120/3</td>
</tr>
<tr>
<td><strong>Intraepithelial neoplasia</strong> (dysplasia)</td>
<td><strong>Kaposi sarcoma</strong></td>
</tr>
<tr>
<td>associated with chronic inflammatory diseases</td>
<td>9140/3</td>
</tr>
<tr>
<td><strong>Low-grade glandular intraepithelial neoplasia</strong></td>
<td><strong>Malignant melanoma</strong></td>
</tr>
<tr>
<td><strong>High-grade glandular intraepithelial neoplasia</strong></td>
<td>8720/3</td>
</tr>
<tr>
<td><strong>Carcinoma</strong></td>
<td><strong>Others</strong></td>
</tr>
<tr>
<td><strong>Adenoarcinoma</strong></td>
<td><strong>Malignant lymphomas</strong></td>
</tr>
<tr>
<td>8140/3</td>
<td>9699/3</td>
</tr>
<tr>
<td><strong>Mucinous adenocarcinoma</strong></td>
<td><strong>Marginal zone B-cell lymphoma of MALT Type</strong></td>
</tr>
<tr>
<td>8480/3</td>
<td>9673/3</td>
</tr>
<tr>
<td><strong>Signet-ring cell carcinoma</strong></td>
<td><strong>Diffuse large B-cell lymphoma</strong></td>
</tr>
<tr>
<td>8490/3</td>
<td>9690/3</td>
</tr>
<tr>
<td><strong>Small cell carcinoma</strong></td>
<td><strong>Burkitt lymphoma</strong></td>
</tr>
<tr>
<td>8041/3</td>
<td>9887/3</td>
</tr>
<tr>
<td><strong>Squamous cell carcinoma</strong></td>
<td><strong>Burkitt-like / atypical Burkitt lymphoma</strong></td>
</tr>
<tr>
<td>8070/3</td>
<td>9087/3</td>
</tr>
<tr>
<td><strong>Adenosquamous carcinoma</strong></td>
<td><strong>Others</strong></td>
</tr>
<tr>
<td>8560/3</td>
<td></td>
</tr>
<tr>
<td><strong>Medullary carcinoma</strong></td>
<td><strong>Secondary tumours</strong></td>
</tr>
<tr>
<td>8510/3</td>
<td></td>
</tr>
<tr>
<td><strong>Undifferentiated carcinoma</strong></td>
<td><strong>Polyps</strong></td>
</tr>
<tr>
<td>8020/3</td>
<td><strong>Hyperplastic (metaplastic)</strong></td>
</tr>
<tr>
<td><strong>Carcinoid (well differentiated endocrine neoplasm)</strong></td>
<td><strong>Peutz-Jeghers</strong></td>
</tr>
<tr>
<td>8240/3</td>
<td><strong>Juvenile</strong></td>
</tr>
<tr>
<td><strong>EC-cell, serotonin-producing neoplasm</strong></td>
<td></td>
</tr>
<tr>
<td>8241/3</td>
<td></td>
</tr>
<tr>
<td><strong>L-cell glucagon-like peptide and PP/PYY producing tumour</strong></td>
<td></td>
</tr>
<tr>
<td><strong>Others</strong></td>
<td></td>
</tr>
<tr>
<td><strong>Mixed carcinoid-adenocarcinoma</strong></td>
<td></td>
</tr>
<tr>
<td>8244/3</td>
<td></td>
</tr>
<tr>
<td><strong>Others</strong></td>
<td></td>
</tr>
</tbody>
</table>

1. This classification is modified from the previous WHO histological classification of tumors (845) taking into account changes in our understanding of these lesions. In the case of endocrine neoplasms, it is based on the recent WHO classification (1784) but has been simplified to be of more practical utility in morphological classification.

2. Morphology code of the International Classification of Diseases for Oncology (ICD-0) (542) and the Systematized Nomenclature of Medicine (http://snomed.org). Behaviour is coded /0 for benign tumours, /3 for malignant tumours, /2 for in situ carcinomas and grade III intraepithelial neoplasia, and /1 for unspecified, borderline or uncertain behaviour. Intraepithelial neoplasia does not have a generic code in ICD-0. ICD-0 codes are available only for lesions categorized as glandular intraepithelial neoplasia grade III (8140/2), and adenocarcinoma in situ (8140/2).
1.2.3 Colorectal Cancer statistics and epidemiology

Being the third most common type of cancer worldwide, the CRC accounts for roughly around 600,000 annual deaths with about 1.2 million new cases emerging every year. Even with the advancement in treatments and surgical managements, it still represents over half of all gastrointestinal cancer deaths (Brenner et al., 2014; Hagland et al., 2013). In Europe, the chances of having CRC in a person’s lifetime is about five to six percent (Kuipers et al., 2015). Moreover, patients with CRC are more prone to die with the cancer metastasized to other organs especially the liver (Ferlay et al., 2010).

The median age of diagnosis of CRC in developed countries is above 70 years old. However, the chances of a person to be diagnosed with CRC are proportional to his/her age unless this individual has genetic predispositions whereby he/she has inherited specific genetic variations which will highly increase the risk of developing it (Colucci et al., 2003; Hagland et al., 2013). Another interesting fact reported in SEER cancer statistic reviewers is that the rate of CRC adenocarcinomas is higher in males than in females especial between the years of 1973 – 1983 (Noone et al, 2017).

The irregular increase in percentage of having CRC is majorly due to lifestyle factors, which include increasing aging population, adopting an unhealthy diet, constant smoking, and most importantly low physical activity (Bosman et al., 2000; Hagland et al., 2013).
1.3 Cell motility, invasion, and adhesion

1.3.1 Cell motility

Cell migration is an important process, for it plays an important role in the growth and development of many organisms. It takes place normally during essential physiological events such as in embryogenesis, inflammatory response, and wound healing. However, cell migration can also be a key factor in the progression of many human diseases such as cancer metastasis (Ananthakrishnan & Ehrlicher, 2007; Friedl & Wolf, 2009).

A key component of cell metastasis is the cytoskeleton that is defined by a polymer network composed of actin, microtubules, and intermediate filaments. It plays a crucial role in making the motility-driving forces and coordinating the entire process of movement. Thus, for the cancer cell to metastasize it must extend protrusions towards the direction of the chemoattractant which takes place by initiating the polymerization of new actin filaments that will be used as adhesins to the cells substratum. This will allow the cell to generate a mechanical force strong enough to drag itself forward towards the direction of the chemoattractant; this is also coupled with the disassemble of adhesins that are found at the rear and the withdraw at the cell’s tail (Ananthakrishnan & Ehrlicher, 2007; Hanna & El-Sibai, 2013).

Contact inhibition is a process used by the cell to stop the migration process, exhibiting how significant the interaction between cells is in regulating cells’ movement. After the cells interact, they either choose to form cell to cell adhesion, or
they will be forced to change their direction, which leads to the spreading of the cells in vivo (Ridley, 2015).

1.3.2 CRC metastasis to the liver

Metastasis is a complex process of correlated sequential steps undergone by the primary tumors to establish a secondary lesion in distant organs. However, metastasis is not as random as it may seem. As an overview, the outcome of metastasis consists of various interactions between the tumor cells and their microenvironment, which will dictate the survival of the tumor in different organs. Based on previous studies, clinicians noticed that specific primary tumors tend to metastasize to specific organs. For instance, the CRC predominantly metastasizes to the liver and not the brain nor the bone despite its ability to metastasize to different organs, and this is clearly demonstrated in the pie charts of Figure 3. Moreover, it is highlighted that liver is one of the predominant causes of mortality in patients suffering from CRC (Hess et al., 2006; JIN et al., 2012).
Figure 3: Represents metastatic sites for different primary tumors. Each color represents a different metastatic site. CRC preferred target metastatic site is the liver. Source: (Hess et al., 2006).

There are many factors that are suspected to govern the site where the tumor is going to spread. These factors can generally include the tumor’s origin, its inherited properties, the tissue’s affinities, and the circulation patterns. In case of the CRC, one of the factors responsible for the high levels of liver metastasis is venous drainage of the gastrointestinal tract straight into the liver using the portal vein. The liver’s microvessel structure has an incomplete layer of hepatic endothelial cells, circulating tumor cells can easily access its extracellular membrane where they can stabilize and adhere (Fidler, 1990; JIN et al., 2012).
Chemoattractants also play a role in this process, whereby they tend to attract the cancer cells to the site where they are being expressed. This gives chemokines and their receptors a crucial role in regulating organ selection. Previous studies demonstrate the importance of CXC-chemokine receptor type 4 in the spreading of CRC to the liver, noting that the liver produces CXC motif chemokine 12 and that CXCR4 is its alpha chemokine receptor. The CXCL12 is a strong chemotactic factor for lymphocytes and has a crucial role in angiogenesis. The studies highlight that the CXCR4 is not only involved in organ specific metastatic distribution of CRC to the liver but its level of expression in metastatic CRC has a significant effect on the overall survival of patients (Bleul et al., 1996; JIN et al., 2012).

On another note, mutation in some genes boosts the cancer cell metastatic abilities. For example, in CRC, the tyrosine phosphatase PRL-3 is considered as a valued marker for liver metastasis because its overexpression boosts the metastatic abilities of CRC, and it’s up-regulation in colon cancer enhances the tumor’s progression substantially. The study shows that the metastatic CRC tumors express PRL-3 more than fifty percent higher than that of primary CRC tumor regardless of the metastatic site (Al-Aidaroos & Zeng, 2010; Jiang et al., 2011; JIN et al., 2012).
1.3.3 Rho A and focal adhesions

Rho A is a member of the Rho GTPases family that consists of 23 members and is divided into six subgroups. The family belongs to the Ras-like protein superfamily, and its members are identified by their Rho-specific insert domain and exist in two states: an active GTP-bound state or an inactive guanosine diphosphate (GDP)-bound state. Recent studies show that Rho GTPases contribute significantly in cell migration (Hanna & El-Sibai, 2013; Ridley, 2015).

Rho A prevails in the cytoplasm at the plasma membrane and plays a defining role in the formation of focal adhesions and stress fibers by prompting mDia dependent actin nucleation and inducing interaction with Rho-associated protein kinase for cell contraction (Hanna & El-Sibai, 2013; Ridley, 2015; Wojciak-Stothard & Ridley, 2003). Moreover, Rho A is found to be very analogous to RhoB and RhoC that tend to bind to same effectors but with different affinities (Wojciak-Stothard & Ridley, 2003).

Cell survival, differentiation, migration, and proliferation are partly regulated by cellular adhesion, which is made possible by the interaction between FA and integrin. FAs need certain tyrosine kinases to convey the signal to the cell, and the cell relies on its main protein receptor for binding and interacting with the ECM, integrin. However, integrin does not have an enzymatic activity, so FA kinases and Src cytoplasmic tyrosine kinases will drive the integrin-mediated signaling cascade by phosphorylating multiple integrin-associated proteins. Hence, at some point, FAK and
Src are molecular switches capable of triggering a diversity of cellular responses (McDonald et al., 2008; Mitra, Hanson, & Schlaeffer, 2005).

During migration, the cell depends on FAs to produce the force needed for the cell to move forward. Nevertheless, moving means that the cell has to attach and then detach itself from the ECM (Ezratty et al., 2009; Nagano et al., 2012). The latter is dependent on the interference of microtubules extensions that stimulate the FAs’ disassembly and the internalization of integrin from the surface in a dynamic-dependent manner (Ezratty et al., 2005; Zaidel-Bar et al., 2007). Integrin is internalized by regulatory GTPase called dynamin, which is recruited by FAK. Hence, FAK, Src, and dynamin work on FA disassembly and tail detachment (Cox et al., 2001). This detachment and the recycling of integrin assist the migration toward the chemoattractant (Tang et al., 2008).

1.3.4 Metalloproteinase and tumor progression

Matrix metalloproteinase has a central role in numerous physiological processes which include tissue remodeling, organ development, inflammation regulation, and cancer cell’s enhanced invasiveness. MMP plays an important role in the process of tumor spreading to another metastatic site. MMPs are a family of extracellular zinc-dependent proteinases that target ECM components, and their activity can enable tumor cell metastasis, invasion, and survival by distinct mechanisms. These enzymes work on adjusting different physiological processes and signaling events, leading to the establishment of molecular communication between tumor cells and the host tissue stroma. There are different types of MMPs by which they are produced in their inactive
form and require proteolytic remodeling (Kleiner & Stetler-Stevenson, 1999; Stamenkovic, 2000).

MMPs proteinase activity allows it to degrade the ECM macromolecules such as proteoglycan, collagen, and laminins, eliminating the physical barriers that hinders tumor cells from invasion and development. Another feature of MMPs is their ability to induce some biological activities that favor the tumor’s development. For instance, MMP-2 can degrade laminin-5 to release soluble chemotactic fragments. Other ways through which MMPs can aid tumor cells include facilitating the intravasation of the tumor into the blood stream, assisting the tumor stelae on the hosts’ tissue stroma after extravasation, cleaving latent cytokines, and providing growth factors that could support the tumors survival. Hence, MMPs are multifunctional enzymes that helps the tumor migrate to other organs in the body, but its function in vivo rely on its balance with its physiological inhibitors the tissue inhibitors of metalloproteinases (Kessenbrock et al., 2010; Stamenkovic, 2000). Figure 4 shows different types of MMPs and how they assist the tumor.
Figure 4: Modulation of tumor environment by MMPs. This picture summarizes various processes that are affected by MMPs’ activity in tumor microenvironment. The given examples of MMPs and ADAMs, metalloprotease like enzymes, could promote (pro) or suppress (anti) these processes. Source: (Kessenbrock et al., 2010).
1.4 Amino acid and Arginine deprivation

1.4.1 Amino Acid deprivation

Persistent genetic mutations cause cancer cells to undertake drastic changes in their cell cycle, making them proliferate at a higher rate compared to the normal counterpart. This increase in proliferation and metabolic rate will drive the cancer cell to a higher nutritional need, forcing itself to up-regulate the glucose and amino acids transporters on the cell membrane to attain the excess required nutrients from the circulation (Fung & Chan, 2017).

The cancer cell goes to an extent to rewire its metabolic pathways to upgrade their glucose uptake and ferment glucose to lactose just to increase the rate of ATP production. More ATP means that the cancer cells can promote growth, survival, proliferation and long-term maintenance. This phenomenon is known as Warburg effect (Liberti & Locasale, 2016; Fung & Chan, 2017).

In the case of nonessential amino acids, under normal conditions, they are synthesized, de novo, by the cell unlike essential amino acids that are obtained only by the diet. However, many nonessential amino acids become essential when the cells exceed their capability of synthesizing these amino acids for example, in the case of stress, illness, trauma, or cancer. Then, those amino acids will be called conditionally essential, something that happens in some types of physiological diseases. In other cases, the cell undergoes mutations to a point that it loses the ability to synthesize this nonessential amino acid, which then becomes essential, for it can only be obtained
from the environment. Hence, in both cases, the cell becomes auxotrophic to this amino acid (Fung & Chan, 2017; Morris et al., 2017). Auxotroph refers to a mutant organism the has an additional nutritional requirement essential for survival compared to the original phenotype (Low, 2001).

Scientists are looking into different non-essential amino acids to find a specific cancer targeting mechanisms while trying to minimize the damage to normal cells. These anti-cancerous drugs include anti-metabolites that tends to interrupt the synthesis of biochemical substances like nitrogenous bases, nucleotides, and amino acids. In cancer cells, a pathway suffering from mutations will affect the metabolism, making the cancer cell auxotrophic to one of the mentioned biochemical substances. In case of an amino acid, this will make the cancer cell susceptible to amino acid starvation treatments (Geck & Toker, 2016; Morris et al., 2017).

1.4.2 Arginine synthesis and urea cycle

Arginine is a non-essential amino acid, but it becomes conditionally essential when the endogenous bio-synthesis is no longer capable of supplying the cell with adequate amounts of arginase. Thus, the dietary intake will still be the major source of arginine. Arginine metabolism is complex and is involved in different pathways. For example, it serves as a substrate for protein structures, a precursor for signaling molecules, an intermediate in the urea cycle, and an intermediate in the tricarboxylic acid cycle (Geck & Toker, 2016; Morris et al., 2017).
Cases when the Arginine becomes essential include hemolytic anemia, asthma, pregnancy, and critical illness (like sepsis, trauma, and cancer). As mentioned previously, Arginine serves as a substrate for protein synthesis, for example it is a precursor for nitric oxide, polyamines, proline, glutamate, creatine, and agmatine (Haines et al., 2010). Additionally, arginine can induce the secretion of hormones, such as insulin, growth hormone, glucagon, and prolactin (Morris et al., 2017). The significance of maintaining arginine homeostasis is crucial, for arginine deficiency has the potential to disrupt many cellular and organ functions (Fung & Chan, 2017; Morris et al., 2017).

Approximately, eighty percent of the body’s arginine supply is from dietary protein intake and the rest relies on de novo production by the cell. After the body breaks down the proteins, arginine is absorbed by the small intestine and transported to the liver. Dietary intake of glutamine is also important in arginine synthesis, for most of the body’s citrulline originate from glutamine, and it is known that arginine, de novo, synthesis is primarily from citrulline. The, de novo, synthesis of arginine takes place primarily in the proximal renal tubule of the kidneys through the urea cycle by using citrulline as the primary substrate. However, other cells in the body have the capability of synthesizing arginine as well (Feun et al., 2015; Morris et al., 2017).

The urea cycle, also known as the Krebs urea cycle or ornithine cycle, is summarized in figure 5. It involves the functions of five different enzymes. The first two enzymes, carbamoyl phosphate synthetase I (CPS) and ornithine transcarbamoylase (OTC), are found in the mitochondria while the remaining three enzymes are in the cytoplasm. CPS and OTC will lead to the production of citrulline.
From citrulline arginine is synthesized using the arginosuccinate synthetase (ASS) and the arginosuccinate lyase (ASL). ASS catalyzes the conversion of L-citrulline and aspartic acid to arginosuccinate, while ASL will do the rest by converting arginosuccinate to L-arginine and fumaric acid. Finally, L-arginine will be converted to L-ornithine and urea by arginase, which is the final enzyme of the urea cycle. The cycle starts all over when urea is excreted, and L-ornithine is transported to the mitochondria to be converted back to L-citrulline by OCT (Haines et al., 2010; Feun et al., 2015; Fung & Chan, 2017). The ability to recycle arginine from citrulline depends on the amount and the activity of ASS and ASL enzymes. Several studies pointed out that those two enzymes play a crucial role in the regulation of arginine synthesis (Shen et al., 2006; Feun et al., 2008).

**Figure 5: summarizes the Krebs urea cycle.** It shows how L-citrulline gets converted to L-Arginine. Source: (Feun et al., 2008)
1.4.3 Arginine deprivation

Tumors that cannot express the ASS or ASL enzymes will be unable to synthesize Arginine. These types of tumors are known as arginine auxotrophic tumors because they are susceptible to arginine deprivation therapy. Example of known arginine auxotrophs include ovarian cancer, glioblastoma, melanomas, and the majority of acute myeloid leukemias (Miraki-Moud et al., 2015; Feun et al., 2008; Fultang et al., 2016). As mentioned previously, cancer cells have a higher nutritional need as compared to normal cells, so targeting tumors using arginine deprivation therapy is a reasonable approach to treat different arginine auxotrophic tumors. Over seventy percent of CRC tissues express high amounts of arginine transporters, which highlights the need of CRCs for arginine abundance (Fultang et al., 2016).

Two types of arginine depleting agents have been developed and tested on a set of different types of tumors. They are pegylated arginine deiminase (ADI) and pegylated arginase 1. ADI is an extracellular enzyme that converts arginine to citrulline and ammonia. It has not been found in humans nor in any other mammal, and it is derived from a gram-negative bacterium, Mycoplasma arginine (Miyazaki et al., 1990; Feun et al., 2008).

ADI is a foreign protein which makes it highly antigenic, so it will face problems of its own. The solution was to make a pegylated form of ADI known as ADI-PEG which will reduce the proteins immunogenicity and increase its half-life. ADI-PEG does not require ASS-1 downregulation since it targets arginine directly, decreasing its amount and causing arginine starvation. ADI-PEG20 is the industrial version of this protein, and it has been reported to have antiproliferation and anti-angiogenesis
activity. Preclinical studies highlight that it induces apoptosis and autophagy in different types of cancer cells. Some studies reported synergism by combining ADI-PEG20 with chloroquine in sarcoma cancer cells (Kim et al., 2009; Feun et al., 2015; Fung & Chan, 2017).

It was noted that cancer cells resistance to ADI-PEG20 could be due to the ASS and OTC secretion because of their ability to restore the arginine pool in the cell to a certain extent (Ensor et al., 2002; Fung & Chan, 2017).

1.5 Human recombinant Arginase I Cobalt PEG 5000

Another method of arginine deprivation is using arginase. Arginase unlike ADI is found in humans and other mammals, which is an advantage over ADI, for it minimizes host immunogenic responses. Arginase tends to metabolize arginine to give L-ornithine and urea as shown in figure 5. Two subtypes of arginase exist in humans with a close amino acid sequence similarity. Arginase-1 is usually found in the cytoplasm of the hepatocytes, and it plays an important role in ammonia detoxification in the liver. Arginase-2 is found in the mitochondria of cells found in the kidney, brain, prostate, small intestine, and skeletal muscles to provide L-ornithine. Studies suggest that the native human Arginase-1 (HuArg-1) has been assessed as an anticancer drug with short term successes. The HuArg-1 is conjugated to two manganese ions as its cofactors that are rapidly lost in the serum, which leads to inactivation of the enzyme giving the enzyme a short life span, approximately four and a half hours. It also has an optimal PH of 9.6, so it will not be active in an environment with a neutral PH like
the plasma whose PH is around 7.4. (Dillon et al., & Clark, 2002; Hsueh et al., 2012). Thus, to achieve arginine deprivation using HuArg1, it must undergo several changes.

The Native HuArg needed several adjustments to become an effective anticancer drug. One of the adjustment done on HuArg was replacing two Mn$^{2+}$ ions with two Cobalt ions. Co$^{2+}$ coupled with Arg will shift the optimal PH to 7.5 making it compatible with that of the plasma with 7.4 PH. This will increase the overall catalytic activity of the enzyme around tenfold higher and increase its serum stability around 5-folds more than with the native cofactor (Stone & Chantranupong, 2010; Stone & Glazer, 2010; Fultang et al., 2016). Another adjustment included the pegylation of HuArg, which means attaching polyethylene glycol chains to the enzyme. This increased the molecular mass of the enzyme and simultaneously shielded it from proteolytic cleavages. Thus, pegylation enhanced pharmacokinetics of the drug and increased the HuArg half-life and further increased the drug’s serum stability (Roberts et al., 2002; Harris & Chess, 2003; Yau et al., 2015). Cobalt coupled PEG-Arg known as HuArgI(Co)-PEG5000 is more suitable and effective for cancer treatment with an improved life span (Hsueh et al., 2012). Pre-clinical trials proved that it inhibited proliferation of ASS1 negative tumors like hepatocellular carcinoma, melanoma, acute myeloid leukemia, glioblastoma, and pancreatic cancer. Also, it was noted that some in-vitro and in-vivo studies suggested that HuArgI(Co)-PEG5000 induced apoptosis in MEL and HCC cells (Fultang et al., 2016).
1.6 Purpose of the study

The purpose of this study is to see the effect of arginine depletion on different types of colon cancer cell lines by using the arginine depletion agent HuArg1 (CO)-PEG 5000. Many arginine deprivation studies have been done on different cancer cell lines to understand the complete mechanisms of this drug.

Therefore, the aim of this study is first to test the cytotoxicity of HuArg1(CO)-PEG 5000 on colon cancer cell lines HT-29, Caco-2 and Sw837, while adding L-citrulline as a supplement and to determine if the cell lines are Auxotrophic or partially auxotrophic to the deprivation of Arginine. It is hypothesized that if the study cell lines expressed the ASS1 protein then they will be rescued by L-citrulline to produce L-arginine using the Urea cycle and hence they will be considered partially auxotrophic. The effect of Arg1 combined with L-citrulline on the cell lines’ motility, adhesion, and invasiveness will also be studied by doing two-dimension motility assay and three-dimension invasion assays. The effect of the drug on the level of focal adhesion and metalloproteinase expression are also examined by doing immunostaining and Western blot respectively. Finally, the effect of Arg1 combined with L-citrulline on the expression levels of RhoA are elucidated by doing Förster Resonance energy transfer.
Chapter 2

Materials and methods

2.1 Cell cultures

Human epithelia colon carcinoma (HT-29), heterogeneous human epithelial colorectum adenocarcinoma (Caco-2), human epithelial colorectal adenocarcinoma (Sw837), human epithelial colorectal adenocarcinoma (SKCO-1), and human epithelial colon carcinoma cell (Sw116) were cultured in DMEM medium supplemented with 10% FBS and 100 U penicillin/streptomycin at 37°C and 5% CO2 in a humidified chamber.

2.2 Expression and Purification of HuArg1(CO)-PEG 5000

Pegylated human recombinant Arginase I cobalt [HuArg1 (Co)-PEG5000] was expressed and purified as described by Stone et al. (Stone, Glazer, et al., 2010).

2.3 Proliferation inhibition Assay (cytotoxicity)

Sensitivity of the CRC cell line to HuArg1 (Co)-PEG5000, in the presence and absence of excess L-citrulline was determined using a proliferation inhibition assay.

Briefly, aliquots of $10^4$ cells/well in 100µL cell culture medium, were plated in a flat-bottom 96-well plate (Corning Inc. Corning, NY). When used, L-citrulline was added at a concentration of 11.4 mM. This was followed by the addition of 50 µL
HuArgI (Co)-PEG5000 in media to each well from a round-bottom 96-well plate (Corning Inc. Corning, NY) to yield concentrations ranging from $10^{-7}$ to $10^{-13}$ M. Following a 48, 72, 96 hours incubation at $37^\circ C/5\%$ CO$_2$, 50µl of XTT cell proliferation reagent (Roche, Basel, Switzerland) were added to each well and the plates incubated for 4h. Absorbance was then read at 450 nm using a Varioskan Flash plate reader (Thermo Fisher Scientific, Waltham, MA). Nominal absorbance and percent maximal absorbance were plotted against the log of concentration and a non-linear regression with a variable slope sigmoidal dose response curve was generated along with IC$_{50}$ using GraphPad Prism 5 software (GraphPad Software, San Diego, CA) and the IC$_{50}$ (inhibitory concentration 50) of HuArgI (Co)-PEG5000 alone or in the presence of L-citrulline were compared.

### 2.4 Antibodies and reagents

The following primary antibodies were used in this study: Rabbit polyclonal anti-beta Actin, Mouse monoclonal anti- ASS1[2B10], Mouse monoclonal anti-MMP2[6E3F8], Rabbit polyclonal anti-MMP9, and Mouse monoclonal anti-Vinculin [VIN-54] (Abcam Inc., Cambridge, UK).

The following secondary antibodies were used in this study: Alexa Fluor 488 Goat anti-Mouse IgG (H+L) was obtained from Invitrogen). Anti-rabbit and anti-mouse HRP-conjugated secondary antibodies were obtained from promega (Promega Co., Wisconsin).
2.5 Western immunoblotting

Whole cell lysates were prepared by scraping the cells with laemmli sample buffer containing 4% SDS, 20% glycerol, 10% β mercaptoethanol, 0.004% bromophenol blue and 0.125M Tris HCL (pH 6.8). SDS-PAGE was carried out under standard conditions and proteins were blotted onto a PVDF membrane. The membranes were then blocked with 5% bovine serum albumin for 1 hour and then incubated overnight at 4°C with either primary antibody against ASS1 (abcam, 1:1000 dilution), MMP2 (abcam,1:1000 dilution), MMP9 (abcam, 1:1000), actin (abcam, 1:2500). After the incubation with the primary antibody, the membranes were washed and incubated with secondary antibody at a concentration of 1:1000 for 1 hour at room temperature. The membranes were then washed, and the bands visualized by treating the membranes with western blotting chemiluminescent reagent ECL (GE Healthcare). The levels of protein expression were compared by densitometry using the ImageJ software.

2.6 Fixed Wound Healing Assay

Cells were grown to confluence on culture plates while incubated with to HuArgI (Co)-PEG5000, in the presence and absence of excess L-citrulline. Then after 24 hours a wound was made in the monolayer with a sterile pipette tip. Cells were then washed twice with PBS to remove debris and new medium was added. Phase-contrast images of the wounded area were captured at 0 and 24 h after wounding. Wound widths were measured at 11 different points for each wound, and the average rate of wound closure was calculated (in μm/h).
2.7 Live Wound Healing Assay

Cells were plated on a 35mm petri dish while incubated with to HuArgI (Co)-PEG5000, in the presence and absence of excess L-citrulline. Then after 24 hours a wound was made in the monolayer with a capillary pipette tip. Cells were then washed twice with PBS to remove debris and new medium was added. Cells were buffered using HEPES and overlaid with mineral oil on a 37°C stage. Images were collected every 120 seconds for 6 hours using a 20X objective lens on Zeiss Observer Z1 microscope. Images of the wounded area were captured at 0 and 6 h after wounding. Wound widths were measured at 11 different points for each wound, and the average rate of wound closure was calculated (in μm/h).

2.8 Immunostaining

Cells were plated on glass coverslips while incubated with to HuArgI (Co)-PEG5000, in the presence and absence of excess L-citrulline. After 72 hours the cells were fixed with 4% paraformaldehyde for 10 minutes at 37°C, and permeabilized with 0.5% Triton-X 100 for 15 minutes on ice. For blocking, cells were incubated with 1% filtered BSA in PBS for 1 hour. Samples were then stained with primary antibodies overnight at 4°C and with fluorophore-conjugated secondary antibodies for 1 hour. Fluorescent images were taken using a 63X objective lens on Zeiss Observer Z1 microscope.
2.9 Adhesion assay

96-well plates were coated with collagen using Collagen Solution, Type I from rat tail (Sigma) overnight at 37 °C then washed with washing buffer (0.1% BSA in DMEM). The plates were then blocked with 0.5% BSA in DMEM at 37 °C in a CO2 incubator for 1 hour. This was followed by washing the plates and chilling them on ice. Meanwhile, the cells were trypsinized and counted to $4 \times 10^5$ cell/ml. 50 μl of cells were added in each well and incubated at 37°C in a CO2 incubator for 30 minutes. The plates were then shaken and washed 3 times. Cells were then fixed with 4% paraformaldehyde at room temperature for 10 minutes, washed, and stained with crystal violet (5 mg/ml in 2% ethanol) for 10 minutes. Following the staining with crystal violet, the plates were washed extensively with water, and left to dry completely. Crystal violet was solubilized by incubating the cells with 2% SDS for 30 minutes. The absorption of the plates was read at 550 nm using an Thermo scientific Varioskan Flash Multimode reader (Thermo fisher scientific, USA).

2.10 Invasion Assay

Cells were grown to confluence on culture plates while incubated with to HuArgI (Co)-PEG5000, in the presence and absence of excess L-citrulline. Invasion assay was performed 72hrs following treatment period using the Matrigel-based invasion assay (Millipore) according to manufacturer’s instructions. Cells were harvested, centrifuged and then resuspended in quenching medium (without serum). Cells were then brought to a concentration of $1 \times 10^6$ cells/ml. In the meantime, inserts were pre-
warmed with 300μl of serum free medium for 30min at room temperature (Corning, New York). After rehydration, 250μl of media was removed from inserts and 250μl of cell suspension was added. Inserts were then placed in a 24-well plate, and 500μl of complete media (with 10% serum) was added to the lower wells. Plates were incubated for 24hrs at 37°C in a CO₂ incubator. Following 48 hours of incubation period, inserts were stained for 20min at room temperature with 400μl of cell stain provided with the kit. Stain was then extracted with extraction buffer (also provided with the kit).100ul of extracted stain was then transferred to a 96-well plate suitable for colorimetric measurement using a plate reader. Optical Density was then measured at 560μm.

2.11 Förster Resonance energy transfer (FRET)

Caco-2 cells were plated on a 35mm petri dish while incubated with to HuArgI (Co)-PEG5000, in the presence and absence of excess L-citrulline with a confluency of 4x10⁵ cell/ml. Cells were transfected with 2.5 μg of the RhoA fluorescence resonance energy transfer (FRET)-based biosensor plasmid. The RhoA biosensor consists of (from the N-terminus) the Rho binding domain (RBD) of the effector Rhotekin, a cyan fluorescent protein (CFP), a protease resistant 17-mer unstructured linker, a yellow fluorescent protein (YFP) domain, and a full length (Pertz, Hodgson, Klemke, & Hahn, 2006). Figure 6 represents the RhoA biosensor used on Caco-2 cell line.
**Figure 6: The RhoA FRET biosensor.** The biosensor consists of (from the N-terminus) the Rho binding domain (RBD) of the effector Rhotekin, a cyan fluorescent protein (CFP), a protease resistant 17mer unstructured linker, a yellow fluorescent protein (Citrine) domain, and a full length RhoA (Pertz et al., 2006)

### 2.12 Quantitation of focal adhesions

Image J was used to quantitate focal adhesions. Briefly, two main plugins were used to quantitate focal adhesions, these two plugins are CLAHE and Log3D. CLAHE enhances the local contrast of the image and Log3D filters the image based on user predefined parameters which will allow us to detect and analyze focal adhesions (Horzum, Ozdil, & Pesen-Okvur, 2014).
Chapter 3

Results

3.1 Cytotoxic effect of HuArgI (Co)-PEG5000 on CRC cell lines

The study of the cytotoxic effect of pegylated human recombinant Arginase I cobalt [HuArgI (Co)-PEG5000] on CRC cells was done. Cell viability assay on a time gradient to test the effect of HuArgI (Co)-PEG5000 with or without L-citrulline on HT-29 cell line was performed. Referring to figure 7, the time gradient started from 48 till 96 hours, while table 4 represents the half maximum inhibitory concentration, IC50, values. Then based on the results we decided to test the duration of cytotoxicity for 72 hours on the other cell lines with HuArgI (Co)-PEG5000 with or without L-citrulline. Figure 8 represents the IC50 of Sw1116, Sw837, SKCO-1, and Caco-2, while the IC50 values are shown in table 5.
Table 4. Sensitivity of the HT-29 cell line to HuArgI(Co)-PEG5000 at different periods of treatment. IC50: half maximal inhibitory concentration.

<table>
<thead>
<tr>
<th>Time (hours)</th>
<th>IC50 (PM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>48</td>
<td>170</td>
</tr>
<tr>
<td>72</td>
<td>100</td>
</tr>
<tr>
<td>96</td>
<td>83</td>
</tr>
<tr>
<td>144</td>
<td>80</td>
</tr>
</tbody>
</table>

Figure 7. Cytotoxicity of HuArgI(Co)-PEG5000 alone and with L-citrulline (11.4mM) to H-29 at 48, 72, 96, 144 hours
Table 5. Sensitivity of Sw1116, Sw837, SkCo-1, and Caco-2 cell line to HuArgI(Co)-PEG5000 at 72 hours. IC50: half maximal inhibitory concentration.

<table>
<thead>
<tr>
<th>Cell lines</th>
<th>IC50 (PM)/72hrs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sw1116</td>
<td>1000</td>
</tr>
<tr>
<td>Sw837</td>
<td>531</td>
</tr>
<tr>
<td>SkCo-1</td>
<td>256</td>
</tr>
<tr>
<td>Caco-2</td>
<td>200</td>
</tr>
</tbody>
</table>

Figure 8. Cytotoxicity of HuArgI(Co)-PEG5000 alone and with L-citrulline (11.4mM) to Sw1116, Sw837, SK-Co1, and Caco-2 at 72 hours
3.2 Effect of HuArgI (Co)-PEG5000 on Caco-2 ASS1 gene expression

The effect of HuArgI (Co)-PEG5000 on CRC cell lines gene expression of ASS1 was examined. To confirm the cytotoxicity results and to make sure that the cell lines are partially auxotrophic due to ASS1 expression and not something else, western immunoblotting for Caco-2 cell line treated with HuArgI (Co)-PEG5000 with or without L-citrulline was performed. Western immunoblotting results showed a decrease of fifty percent in ASS1 expression when treated with HuArgI (Co)-PEG5000. Important to note that when treated with HuArgI (Co)-PEG5000 in the presence of L-citrulline, nearly a full restoration of ASS1 expression represented in figure 9A and B was observed.

Figure 9: HuArgI (Co)-PEG5000 downregulated ASS1 gene expression while L-citrulline restores expression on Caco-2 cell line. Caco-2 cells where treated with HuArgI (Co)-PEG5000 with or without L-citrulline for 72 hours. (A) ASS1 expression. (B) quantitation of (A).
3.3 HuArgI (Co)-PEG5000 downregulates 2D motility in CRC cell lines

3.3.1. HuArgI (Co)-PEG5000 decreases Caco-2, HT-29, and Sw837 Cell migration.

To further study the effect of HuArgI(Co)-PEG5000 on CRC, cells behavior in 2D motility was observed. First, fixed and live wound healing assays were done to see the effect of the drug on cell migration.

For the fixed wound healing assay, HT-29, Caco-2, and Sw837 cell lines were treated by HuArgI(Co)-PEG5000 with or without L-citrulline on represented in Figure 10, 11, and 12 respectively. The rate of wound closure was calculated before and after inserting the wound. HuArgI(Co)-PEG5000 without L-citrulline caused a decrease in the rate of wound closure compared to the control and to that with L-citrulline as represented in figure 10B, 11B, and 12B.

To verify that the wound closed due to cell migration and not replication in the fixed wound healing assay, the live wound healing assay was done as an alternative to the time laps assay. Caco-2 cell line was plated on a 35mm petri dish and was filmed for 6 hours using a 20X objective lens on Zeiss Observer Z1 microscope. The live wound healing showed a clear wound closure due to cell migration and not cell replication as represented in figure 13.
Figure 10. (A) **Fixed wound healing assay.** HT-29 cell line was treated by HuArgI(Co)-PEG5000 with or without L-citrulline for 72 hours. (B) Quantitation of (A). L-Cit: L-citrulline

Figure 11. (A) **Fixed wound healing assay.** Caco-2 cell line was treated by HuArgI(Co)-PEG5000 with or without L-citrulline for 72 hours. (B) Quantitation of (A). L-Cit: L-citrulline
Figure 12. (A) **Fixed wound healing assay**. Sw837 cell line was treated by HuArgI(Co)-PEG5000 with or without L-citrulline for 72 hours. (B) Quantitation of (A). L-Cit: L-citrulline

Figure 13. **Live wound healing assay done on Caco-2 cell line**. The movie duration is 6 hours having a picture taken every 120 seconds. ImageJ RIO manager was used to ensure that both magnified images where take at the same location and same size.
3.3.2. HuArgI (Co)-PEG5000 decreases adhesion to collagen human epithelial colorecta adenocarcinoma Caco-2 cell line

The effect of the drug on cellular adhesions was examined by performing cell adhesion assay. Caco-2 cell line were plated on collagen and then treated by HuArgI(Co)-PEG5000 with or without L-citrulline. Results suggest that cells incubated with arginase show around fifty percent decrease in cellular adhesion when compared to the control. The L-citrulline was not able to significantly restore cell adhesion compared to the control (Figure 14).

Figure 14: HuArgI(Co)-PEG5000 demotes cell adhesion to collagen in Caco-2 cells. A) Representative micrographs of Caco-2 cells plated at different conditions control (Upper lane left), Caco-2 plated with HuArgI(Co)-PEG5000 (Upper lane right), HuArgI(Co)-PEG5000 and L-citrulline (lower lane left), fixed and stained with crystal violet (as described in methods. B) Quantitation of solubilized Crystal violet from Caco-2 plates and the absorption of the plates were measured at 550 nm using an ELISA plate reader. Data were measured in fold change of adhesion normalized to the control.
3.3.3. **HuArgI (Co)-PEG5000 decreases focal adhesion formation in human epithelial colorectal adenocarcinoma Caco-2 cell line**

To confirm the cell adhesion assay results done on human epithelial colorectal adenocarcinoma Caco-2 cell line, the mediators of the adhesion process Focal Adhesions (FA) were targeted. Caco-2 cells were treated by HuArgI(Co)-PEG5000 with or without L-citrulline and stained for vinculin, a component of the FA structures (Humphries et al., 2007). The focal adhesions were quantitated with a specific macro according to the manufacturer’s instructions using ImageJ (Horzum et al., 2014). The presence of HuArgI(Co)-PEG5000 with or without L-citrulline decrease the number of FA structures by 21% and 32% respectively as represented in figure 15A and 15B. On the other hand, the presence of HuArgI(Co)-PEG5000 increased the number of focal adhesin like structures roughly by 44.5% and 37.5 % respectively as represented in figure 15C.
Figure 15: HuArgI (Co)-PEG5000 decreases focal adhesion formation in human epithelial colorectal adenocarcinoma Caco-2 cell line. A) Representative micrographs of (Caco-2) control (upper line), HuArgI (Co)-PEG5000 without L-citrulline (second lane), HuArgI (Co)-PEG5000 with L-citrulline (third lane), and siRhoA/RhoC (Lower lane) that were fixed and stained with anti-vinculin. Cells were imaged using a 60x objective. B) Quantitation of areas of focal adhesions (Left figure). C) Quantitation of number of focal adhesions (Right figure) upon treatment with HuArgI (Co)-PEG5000 in the absence of presence of L-citrulline.
3.4 HuArgI (Co)-PEG5000 downregulates 3D motility in CRC cell lines

3.4.1. HuArgI (Co)-PEG5000 decreases cellular invasion of Caco-2 Cell line

HuArgI (Co)-PEG5000 had a substantial effect on Caco-2 cells 2D motility, so the role of this drug on 3D invasion using in-vitro Matrigel based invasion assay with FBS as a chemoattractant was studied. The trans-well chambers were filled with serum free media and used as a negative control. Cellular invasion was dropped around 40% and 18% when treated by HuArgI(Co)-PEG5000 without or with L-citrulline respectively based on figure 16A and B.

Figure 16: HuArgI (Co)-PEG5000 decrees 3D invasion in human epithelial colorectal adenocarcinoma Caco-2 cell line. Caco-2 cells were treated by HuArgI(Co)-PEG5000 without or with L-citrulline. After 72 hours cell that invaded the basement side of the Matrigel basement membrane towards the FBS were stained with a cell invasion cell dye take from cell invasion kit. After that the stain was extracted and absorbance was measured under 560 nm. A) represents the micrographs of Caco-2 invading cells. B) Quantitation of (A) represented in fold change.
3.4.2. HuArgI (Co)-PEG5000 decreases MMPs expression in Caco-2 Cell line

To confirm the cell migration assay results done on human epithelial colorectal adenocarcinoma Caco-2 cell line, the effect of HuArgI(Co)-PEG5000 on the expression level of metalloprotease MMP9 was studied. The Caco-2 cells treated with HuArgI(Co)-PEG5000 were plated with or without L-citrulline. Then, western immunoblotting was performed, and the results (figure 17) show a decrease in MMP9 expression levels when treated by HuArgI(Co)-PEG5000.

Figure 17: HuArgI (Co)-PEG5000 downregulated MMP9 gene expression while L-citrulline restores partially expression on Caco-2 cell line. Caco-2 cells where treated with HuArgI (Co)-PEG5000 with or without L-citrulline for 72 hours. (A) MMP9 expression. (B) quantitation of (A).
3.5 HuArgI (Co)-PEG5000 downregulates RhoA expression in Caco-2 as shown by FRET Analysis

After doing 2D and 3D motility in Caco-2 cell line, the effect of HuArgI(Co)-PEG5000 on RhoA was monitored. Rho A is a member of Rho-GTPase with wide implication on cell 2D and 3D motility, and it plays an important role in the formation of focal adhesions for cell adhesions and the release of MMPs for cell migration. Thus, the activation levels of RhoA were of great interest and were studies using FRET. Caco-2 cells were treated with HuArgI(Co)-PEG5000 with or without L-citrulline for 72 hours. The Caco-2 cells were transfected with RhoA biosensor to do FRET. The results represented in figure 18 show a decrease in RhoA activation when the cells were treated with HuArgI(Co)-PEG5000 with or without citrulline compared to the control.
Figure 18: HuArgI(Co)-PEG5000 downregulates the activation of RhoA. A) Representative micrographs of Caco-2 cells (Left image), HuArgI(Co)-PEG5000 without L-citrulline (middle image), and HuArgI(Co)-PEG5000 with L-citrulline (lower image), fixed, and transfected with the FRET RhoA biosensor. B) Quantitation of (A). The cells were imaged via using the FRET channel and the raw FRET images were normalized to the CFP images. The images reflect the FRET ratio = Raw FRET image/CFP
Chapter 4
Discussion

Many clinical investigations are being done on arginine deprivation therapies that target arginine auxotrophic malignancies. In this study, arginine deprivation using HuArgI(Co)-PEG5000 showed a strong cytotoxic effect on different CRC cell lines. Previous studies in our lab showed that cytotoxic effects of HuArgI(Co)-PEG5000 on different types of cancer cells including AML and glioblastoma cells while highlighting the potential use of arginase depletion as a method of treating different types of cancer cells (Khoury et al., 2015; Tanios et al., 2013).

With the increase of the incubation period, the drug exhibits positive feedback by resulting in a higher cytotoxic effect as shown on HT-29 cell lines but to a certain extent. Based on the results, the incubation period for the drug should be 72 hours at 100pM because after this period is exceeded, no significant improvement in the cytotoxicity effect was observed. The IC50 was constantly decreasing as the incubation period increased to reach 80pM at a time of 144 hours. 72 hours was taken as a standard incubation period for the rest of the CRC cell lines whereby the IC50 of Caco-2, Skco1, Sw837, and Sw1116 were 200, 256, 531, and 1000pM respectively. The addition of L-citrulline to the CRC cells lead to rescuing the cells. This might indicate that the CRC cell lines used were partially auxotrophic to HuArgI(Co)-PEG5000 and that arginine deprivation could be reversible if the cells were able to utilize L-citrulline as an alternative. To support the importance of L-citrulline a study
was done, in-vivo, on mice with hepatocellular carcinoma. In this study, an adequate
decrease in HuArgI(Co)-PEG5000 toxicity was observed when L-citrulline
supplement were administer (Mauldin et al., 2012). Another study done on HT-29 also
highlighted the importance of L-citrulline in rescuing the cells from arginine
departvation. By doing metabolic studies, it was found that the cell has the equipment
to convert L-citrulline to L-arginine. This further stresses the importance of L-
citrulline as a precursor for L-arginine and L-ornithine synthesis (Selamnia et al.,
1998).

L-citrulline works by being a substrate for L-arginine synthesis in the urea cycle,
which involves ASS1 and ASL enzymes. First, the L-citrulline must be converted to
arginosuccinate by ASS1, and then arginosuccinate is converted to L-arginine by ASL.
The western blot that revealed that CRC cells were being rescued by L-citrulline. This
technique helped us examine ASS1 gene expression in the Caco-2 cell line treated by
HuArgI(Co)-PEG5000 with or without L-citrulline. After quantitating the results, it
was clear that the ASS1 gene expression in HuArgI(Co)-PEG5000 with L-citrulline
was almost like that of the control. This indicated that ASS1 expression was high and
that the ASS1 enzyme was acting on L-citrulline to compensate for the lack of L-
arginine.

The second step was to see the effect of arginine depletion on cell motility,
adhesion, and invasion. Fixed wound healing assay, one of the 2D migration assays,
was done on Caco-2, HT-29, and Sw837 cell lines. Those cell lines were treated by
HuArgI(Co)-PEG5000 with or without L-citrulline for 72 hours. The results of Ht-29,
Caco-2, and Sw837 wound closure were shown in figure 11, 12, and 13 respectively.
The rate of wound closure drastically decreased compared to control in all the cell
lines when they were treated with HuArgI(Co)-PEG5000. This decrease was significant in HT-29 cell line, for the results showed that the rate of migration with the drug was one quarter the rate of migration of the control. It is noteworthy that RhoA is a key component of cell migration because it stimulates integrin adhesions and actomyosin-based contractility to pull the rear end of the cell for the retraction of the tail (Trepat, Chen, & Jacobson, 2012; Worthylake, Lemoine, Watson, & Burridge, 2001). A study showed that inhibiting p160ROCK, Rho binding protein, by using a Rho-Kinase inhibitor, Y-27632, inhibited the tail retraction of prostate cancer cell (Somlyo et al., 2000). This highlighted the importance of p160ROCK in tail retraction by being downstream of RhoA (Ishizaki et al., 1997; Tominaga et al., 1998).

To eliminate the possibility of cell replication in closing the wound, live wound healing assay was performed. The live wound healing experiment confirmed that the cause of wound closure was due to cell migration and not proliferation because when comparing results at time zero and at 6 hours, movement was observed.

Next, the effect of HuArgI (Co)-PEG5000 on cell adhesion needed to be observed. The results of the cell adhesion assay showed that it down regulated cell adhesion. The results were further verified by doing vinculin immunostaining, which was also performed on the Caco-2 cell line. Moreover, Vinculin immunostaining results showed that when treated with HuArgI(Co)-PEG5000, the focal adhesion was reduced but with the appearance of flecked-like structures known as focal contacts that indicated an early adhesion and the initiation of a motility cycle. RhoA activation was necessary for maturation of focal contacts to become focal adhesions (DePasquale & Izzard, 1987; Guo et al., 2006). This was supported by a previous study done in our lab on CRC which demonstrated that the Knockdown of RhoA downregulates cellular
adhesion (Nasrallah et al., 2014). This aligned with the results observed with the HuArgI(Co)-PEG5000 treatment of Caco-2 cells, in which focal contacts were perceived instead of focal adhesions.

After the role of HuArgI(Co)-PEG5000 on Caco-2 cells 2D motility was identified, the effect of arginine depletion on 3D invasion needed to be determined. Thus, the invasion assay using Matrigel was performed on the Caco-2 cell line. Results indicated that cell invasion decreased significantly upon treatment with HuArgI(Co)-PEG5000. Next, to investigate further on the effect of the drug on invasion, western blot was done and the expression of MMP9 was determined. These metalloproteins degraded the ECM to help the cell invade, so they are important cell invasion markers. Results showed that the MMPs expression decreased when the cell was treated with HuArgI(Co)-PEG5000. This explains the decrease in cellular invasion after the cells were treated with arginase.

Finally, to confirm that arginine depletion downregulates RhoA expression, FRET was used. The Caco-2 cells treated with HuArgI(Co)-PEG5000 were transfected with RhoA biosensor, and the analysis of the results showed a decrease in the RhoA activation with the depletion of Arginine.
Chapter 5

Conclusions

This study aimed to highlight the effect of arginine deprivation done by HuArgI(Co)-PEG5000 on CRC’s activity. Results showed that the depletion of Arginine, an important nutrient, had a drastic effect on the cancer cell’s viability, motility, adhesion and invasion. In this study, it was established that CRCs are partially auxotrophic, meaning that they had a functional ASS1 and can use L-citrulline to produce arginine by the urea cycle. All experiments done in this study showed that the L-citrulline at a certain extent was able to counter the effect of HuArgI(Co)-PEG5000 on the cell and was able to restore most of the cancer cell’s metastatic and invasive functions. Furthermore, Arginine deprivation using HuArgI(Co)-PEG5000 demonstrated a down regulation of the ASS1 gene expression level and simultaneously downregulated the RhoA gene expression. Knowing that RhoA plays an important role in the cell’s metastatic and invasive capabilities, further studies should examine the crosstalk between ASS1 and RhoA or other members of Rho-GTPases such as Rac-1 and CDC42. In future studies the effect of HuArgI(Co)-PEG5000 should be examined on a larger panel of CRC cell lines.
Bibliography


