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Targeting Arginine Auxotrophy in Pancreatic Cancer Cells using Human Recombinant Arginase I-Induced Arginine Deprivation

By

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Targeting Arginine Auxotrophy in Pancreatic Cancer Cells using Human Recombinant Arginase I-Induced Arginine Deprivation

Nathalie Khalil

ABSTRACT

Pancreatic cancer (PC) is a highly aggressive solid malignancy with no efficient therapy. It is one of the deadliest cancer types since its mortality rate per year is nearly equal to its incidence rate. Therefore, there's a need for more effective and selective therapeutic approaches for targeting PC. In this study, we examined the mechanisms of arginine auxotrophy in PC cell lines. Sensitivity to arginine depletion using HuArgI (Co)-PEG5000 was evident in all of the tested PC cell lines with IC_{50} values in the pM range at 72 hours following treatment. Normal cells, on the other hand, were resistant to HuArgI (Co)-PEG5000-induced arginine deprivation. Sensitivity to HuArgI (Co)-PEG5000-induced arginine deprivation was attributable to the complete dependence on exogenous arginine of two of the five PC cell lines tested and to the partial arginine auxotrophy in combination with fast proliferative requirements of the remaining three cell lines. The lack of ASS-1 expression was demonstrated to be the underlying cause behind complete arginine dependence. It is also important to mention that we noticed an increase in the percent of surviving cells among completely auxotrophic cell lines post-addition of citrulline at 72, 96 and 120 hours of drug incubation. This subpopulation of surviving cells was also determined to be ASS-1 positive hence partially auxotrophic,

despite the fact that the cell line was considered overall negative for ASS-1 expression and completely auxotrophic to arginine. This observation underlies the heterogeneity of PC cell lines in terms of ASS-1 expression and auxotrophy to arginine. All the PC cell lines investigated exhibited G0/G1 cell cycle arrest at 72 hours following arginine depletion treatment. Additionally, all five PC cell lines stained negative for annexin V and showed the lack of caspase activation following 24 and 48 hours of arginine deprivation. This absence of caspase activation was confirmed by a 72 hours cytotoxicity assay on Panc-1 cells, which revealed the failure of the pan-caspase inhibitor, Z-VAD, to reverse or decrease arginine depletion-triggered cell death. These findings indicate that HuArgI (Co)-PEG5000-induced arginine depletion results in non-apoptotic, caspase-independent cell death. Additionally, in this study, we are the first to examine the contribution of autophagy following arginine depletion therapy in PC. We have established that autophagy, a process of cellular destruction activated in response to arginine deprivation, can be the leading cause of cell death at late drug incubation times. Hence, our study demonstrates that HuArgI (Co)-PEG5000 is a novel, potent and selective potential therapeutic approach for PC.

Keywords: Pancreatic cancer, Arginine depletion/deprivation, HuArgI (Co)-PEG5000, Arginine auxotrophy/dependence, Cytotoxicity, Autophagy.

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GLOSSARY

ALL: acute lymphoblastic leukemia.

AML: acute myeloid leukemia.

APC: advanced pancreatic cancer.

ASL: argininosuccinate lyase.

ASS: argininosuccinate synthetase.

ADI: arginine deiminase.

ADI-PEG20: pegylated arginine deiminase.

CQ: chloroquine.

CRT: chemoradiation treatment.

CSCs: cancer stem cells.

DMEM: Dulbecco's modified Eagle's medium.

EGFR: epidermal growth factor receptor.

EMT: epithelial-mesenchymal transition.

FBS: fetal bovine serum.

FITC: fluorescin isothiocyanate.

5-FU: 5-fluorouracil.

GBM: Glioblastoma multiforme.

GTPases: guanosine triphosphatases.

hArgI: Human L-arginase I.

HCC: hepatocellular carcinoma.

HIFs: hypoxia-inducible factors.

HuArgI (Co)-PEG5000: pegylated cobalt-substituted recombinant human arginase.

IC_{50}: inhibitory concentration 50.

IPMN: intraductal papillary mucinous neoplasms.

3-MA: 3-methyladenine.

MCN: mucinous cystic neoplasms.

MDR: multidrug resistance.

MFI: mean fluorescence intensity.

miRNA: microRNA.

MM: malignant melanoma.

NF-κB: nuclear factor κB.

OTC: ornithine transcarbamylase.

PanIN: pancreatic intraepithelial neoplasia.

PARP: poly(ADP-ribose) polymerase.

PBS: phosphate buffered saline.

PC: pancreatic cancer.

PDAC: pancreatic ductal adenocarcinoma.

PI: propidium iodide.

RCC: renal cell carcinoma.

RFI: ratio of fluorescence intensity.

T-ALL: T-cell acute lymphoblastic leukemia.

TGF-β: transforming growth factor.

TICs: tumor initiating cells.

CHAPTER ONE

INTRODUCTION

1.1- Pancreatic Cancer, Precursor Lesions and Molecular Features

Pancreatic cancer (PC), lacking efficient therapy, is at present among the deadliest and most aggressive solid malignancies and its worldwide impact on cancer death keeps increasing (Wolfgang et al., 2013; American Cancer Society: Cancer facts & figures 2015). In spite of ranking thirteenth with respect to cancer incidence worldwide, PC ranks fourth with respect to mortality attributable to a cancer cause (Zhang et al., 2016). Moreover, it is expected to rank second among the main cancer types causing deaths within the coming decade in the United States (Rabih et al., 2014). The near equality between the number of deaths and the number of incidences of this cancer type per year along with a 6-7% survival rate at 5 years post-diagnosis highlight the high lethality of PC (Chand, O'Hayer, Blanco, Winter, & Brody, 2016; Liu et al., 2017; Zhang et al., 2016). The rates pertaining to incidence and mortality of PC are generally slightly greater in men than in women. Gender, in addition to other factors such as age, alcohol abuse, smoking habits, obesity, diabetes, physical activities, chronic pancreatitis, genetic alterations, vitamin D deficiency, and dietary factors represent potential risks for this cancer (Zhang et al., 2016). Early metastasis and late diagnosis characterize this cancer type, and conventional management approaches such as radiation therapy as well as chemotherapy fail to overcome its resistance; hence, all these factors culminate in an overall poor prognosis (Neureiter, Jäger, Ocker, & Kiesslich, 2014; Ni et al., 2012). In the rare cases where the existence of the disease is

confirmed at relatively early stages, surgery is suggested as the only potent cure (Chand et al., 2016). Nonetheless, less than 20% of patients who undergo pancreatic resection remain alive at 5 years post-surgery (Dreyer, Chang, Bailey, & Biankin, 2017). Malignant tumors affecting the pancreas are mainly categorized into two groups: those of the endocrine pancreas and those of the exocrine pancreas, and are commonly differentiated into three distinct types: acinar, ductal or endocrine. However, mixed pancreatic neoplasms that associate an exocrine part (acinar or ductal neoplasm) with a significant endocrine part (islet cell neoplasm) do occur but are very rare (Ballas et al., 2005). Within the pancreas, malignant tumors may appear in diverse forms; however, adenocarcinomas arising from cells of the epithelial lining of the ducts located in the exocrine pancreas represent the majority of these PCs (Harris, 2013). Approximately 90% of cancerous pancreatic neoplasms are classified based on histological examination as pancreatic ductal adenocarcinoma (PDAC) (Neoptolemos, Urrutia, Abbruzzese, & Büchler, 2010). PDAC is among the most lethal cancers, ranking fourth with respect to causes of cancer mortality in Western countries, though it's only responsible for 3% of the newly identified cancer cases every year (Jemal et al., 2009). Furthermore, diagnosis of this malignancy occurs at a median age of 71 years, and is rare among people below the age of 40 (Ryan, Hong, & Bardeesy, 2014).

Invasive PDAC arises from three distinct, non-invasive, premalignant precursor lesions that progress and evolve to result in PC: pancreatic ductal legions of microscopic dimensions called Pancreatic Intraepithelial Neoplasia (PanIN), less common Intraductal Papillary Mucinous Neoplasms (IPMN) and Mucinous Cystic Neoplasms (MCN), both lesions of macroscopic dimensions (Delpu et al., 2011). The incidence and death

attributed to pancreatic adenocarcinoma could be diminished by the early identification of such precursors. In fact, the initial detection of precursor lesions in the case of persons with significant incidence of PC in their family history was shown to be successful since these precursors may be identified by careful imaging of the pancreas followed by surgical resection (Omura & Goggins, 2009). Similarly to the stepwise evolution model observed in colon cancer from polyp stage to adenocarcinoma stage, the progression of the majority of PDAC cases is believed to evolve from PanINs that develop from being low-grade neoplasms to becoming high-grade neoplasms that will eventually result in invasive adenocarcinoma formation through sequences of alterations both on the genetic and epigenetic levels (Ryan et al., 2014; Omura & Goggins, 2009).

These genetic aberrations commonly involve many chromosomal gains and losses at specific loci and deletions/mutations of tumor suppressor genes and oncogenes (Yamano et al., 2000). Recently, a thorough genome project targeting PC identified 63 intragenic alterations (mutations or homozygous deletions/amplifications) assembled in 12 distinct signaling pathways (Jones et al., 2008). In general, suppressor genes are more frequently involved in PC than oncogenes (Neureiter et al., 2014). *K-RAS* (Kirsten rat sarcoma viral oncogene homolog) was recently shown to be the most commonly mutated (> 95%) oncogene, resulting in the constitutive signaling of cellular survival, proliferation, remodeling and motility (Hong, Park, Hruban, & Goggins, 2011). The main suppressor genes that are deregulated in PC include *TP53* (tumor protein p53), *CDKN2A*/*p16* (cyclin-dependent kinase inhibitor 2A) and *SMAD4*/*DPC4* (SMAD family member 4) which undergo inactivation. While *TP53* and *CDKN2A*/*p16* mostly function in the cell cycle as checkpoint arrest and control, *SMAD4*/*DPC4* is involved in signal

transduction of TGF-β (transforming growth factor) pathway, and also in proliferation (Neureiter et al., 2014). Overall, the events that summarize the conventional evolution of pancreatic carcinogenesis can be categorized into initial (telomere shortening and *KRAS* activation), midway (epigenetic silencing or inactivating genetic alterations of *CDKN2A2*) and late (mutations resulting in the inactivation of *TP53 and SMAD4*) modifications (Chand et al., 2016).

Furthermore, the development of human cancers fundamentally involves epigenetic mechanisms in addition to the frequent genetic aberrations (Feinberg, Ohlsson, & Henikoff, 2006; Herman, J. G., 2005). Mechanisms of epigenetic silencing can be mitotically transmissible; hence they can act as driving processes in neoplastic evolution and go through selective pressure similarly to genetic modifications (Omura & Goggins, 2009). Importantly, various epigenetic modifications can be depicted in PCs and these modifications can be detected in IPMNs and PanINs and the frequency of their occurrences becomes greater with more progressive lesion grade (Fukushima et al., 2002; Sato, et al., 2002; Sato, Fukushima, Hruban, & Goggins, 2008). Epigenetic alterations such as histone acetylation, DNA methylation or microRNAs (miRNAs) interactive regulation could principally be associated with diverse genetic and morphological alterations throughout pancreatic carcinogenesis. In addition, epigenetic modifications related to precursor legions of PC have been extensively investigated and such investigations suggest that epigenetic regulations are involved in the non-linked, varied, heterogeneous carcinogenesis pathways (Neureiter et al., 2014).

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1.2- Clinincal Aspects, Diagnosis and Staging

Nearly 78% of malignant cancers targeting the pancreas are situated in the head of the glandular organ, while 22% are equally spread in the other parts of the pancreas namely the tail and the body. The presenting symptoms and disease signs are associated with the location of the tumor (Sharma, Eltawil, Renfrew, Walsh, & Molinari, 2011). The diagnosis of approximately 50% of cases presenting PC happens following attendance to an emergency unit for jaundice or non-precise abdominal pain or both (Bond-Smith, Banga, Hammond, & Imber, 2012). Approximately 80% of affected patients present unresectable cancer when diagnosed (Sanjeevi et al., 2016). Patients suffering from PC very frequently present with asthenia, abdominal pain, weight loss, and anorexia (Porta et al., 2005). Additionally, diabetes exists in no less than 50% of PC suffering patients (Chari et al., 2005).

Following the detection of a pancreatic mass, imaging using mostly computed tomography (CT) of the abdominal region via venous as well as arterial phases generally allows the identification of the initial stage of the malignancy and treatment (Ryan et al., 2014). The classification of gastrointestinal tumors, including PC, is mostly based on TNM. The International Union Against Cancer and the American Joint Committee on Cancer established this staging system that associates the stage of the tumor with patient survival and allocates an independent classification score with respect to the primary tumor (T), nodal disease (N), and distant metastases (M) (Zeman et al., 1997) (table 1). Primarily, this cancer type metastasizes to the abdomen, liver, and lungs. Usually, pancreatic mass biopsy is conducted via endoscopic ultrasonography (Ryan et al, 2014).

Table 1. The American Joint Committee on Cancer (AJCC) 6th Edition TNM Staging System for PC.

Definitions of TNM				
1. * Denotes change in classification system from the 5th to 6th Edition AJCC *Cancer Staging Manual*.				
TX	Primary tumor cannot be assessed			
T0	No evidence of primary tumory			
Tis	Carcinoma in situ			
T1	Tumor limited to the pancreas, 2 cm or less in greatest diameter			
T2	Tumor limited to the pancreas, greater than 2 cm in greatest diameter			
T3*	Tumor extends beyond pancreas but no involvement of celiac axis or superior mestenteric artery			
T4*	Tumor involves the celiac axis or the superior mestenteric artery (unresectable)			
NX	Regional nodes cannot be assessed			
N0	No regional lymph node metastasis			
N1	Regional lymph node metastasis			
MX	Distant metastasis cannot be assessed			
M0	No distant metastasis			
M1	Distant metastasis			
Stage grouping				
Stage 0	Tis	N0	M0	Localized within pancreas

Definitions of TNM				
Stage IA*	T1	N0	M0	Localized within pancreas
Stage IB*	T2	N0	M0	Localized within pancreas
Stage IIA	T3	N0	M0	Locally invasive, resectable
Stage IIB*	T1,2, or 3	N1	M0	Locally invasive, resectable
Stage III*	T4	Any N	M0	Locally advanced, unresectable
Stage IV	Any T	Any N	M1	Distant metastases

Bilimoria et al., 2007

The five-year overall survival rate for this cancer type after diagnosis is approximately 6-7% and the median survival time for metastatic malignancy is approximately three to six months (Chand et al., 2016; Jemal et al., 2006; Liu et al., 2017). Due to the lack of early detection methods and due to the absence of early noticeable symptoms, the diagnosis of PC patients mostly occurs during their late cancer stages, following cancer metastasis to other organs (Wolfgang et al., 2013). More than 90% of diagnosed patients with identified PC die from the cancer. Nearly 70% of these 90% patients die due to widespread metastatic illness; the remaining 30% display restricted metastatic illness at their death time, nonetheless many among them display massive primary tumors (Iacobuzio-Donahue et al., 2009).

Many studies have depicted numerous drug resistance mechanisms in PC, including alterations in distinct signaling pathways or genes, and the impact of the microenvironment of the malignant tumor (Long et al., 2011). In fact, tumor progression

and the tumor microenvironment result in challenges that neoplastic cells need to face. Such challenges include depleted glucose supplies, oxidative stress, low oxygen levels, insufficient vascularization and minimal intratumoral perfusion. This hypoxic (oxygen poor) and nutrient poor microenvironment conveys a selective pressure in favor of the growth of the fittest, most aggressive PDAC cells. Hence chronic hypoxia induces the emergence of a resistant phenotype of PDAC cells which elude cytotoxicity by chemotherapeutic drugs (Chand et al., 2016). This happens through the stress-induced activation of hypoxia-inducible factors (HIFs; such as: HIF-1α, CAIX, PIM1, CCND1, PDK1), a major cellular reprogramming response which results in the activation of pathways accountable for the regulation of cell motility, angiogenesis, intracellular pH, cellular metabolism, mitochondrial function, cell survival, and DNA repair (Bertout, Patel, & Simon, 2008; Chand et al., 2016). In addition to the hypoxic microenvironment, the existence of a dense, barrier-like, unique desmoplastic stroma (which is not present in other metastatic growths, distal from PC) has been shown to greatly impact tumor progression and the delivery of chemotherapeutic agents in PC. It has been reported that stromal fibroblasts, which function in the production of the desmoplastic stroma, also generate secreted factors to stimulate pancreatic tumor development (proliferation, cell migration, basement membrane invasion, and formation of colonies) and to impair responsiveness to chemo- and radiation treatments (Long et al., 2011). Moreover, epithelial-mesenchymal transition (EMT), a mechanism which contributes to the spread, migration, and invasion of some PC cells (Panc-1, Colo-357, and IMIM-PC1), might as well be involved in drug resistance. This is due to the fact that mesenchymal PC cells frequently display resistance to gemcitabine (a chemotherapeutic drug) (Ellenrieder et al., 2001). Furthermore, there’s an established association between genetic and/or

epigenetic modifications and resistance to gemcitabine in PC. In addition to the genetic and epigenetic alterations that were previously described, it is important to highlight the overexpression of some pumps (such as members of the ATP-binding cassette, ABC, transporter superfamily), functioning in drug efflux, which leads to multidrug resistance (MDR) through reduced drug accumulation within the cell. MDR phenotype renders PC cells impervious to numerous therapeutic drugs and engenders ineffectiveness in chemotherapy for the management of PC (Long et al., 2011). It is also worth mentioning that deficiency in *BRCA1* or *BRCA2* makes cells more sensitive to treatment via poly(ADP-ribose) polymerase (PARP) inhibitors (Farmer et al., 2005). Hence synthetic lethality may be promising, since targeting a single gene in a cancer presenting a defective other gene results in the selective death of tumor cells with less toxicity to normal cells. However, Capan-1 PC cells have demonstrated their ability to acquire drug resistance and to display reversed sensitivity to PARP inhibition since they harbor a deletion of a mutation hence a gain-of-function alteration in *BRCA2* gene (Edwards et al., 2008). Besides abnormal gene expressions, inhibition or activation of transcription factors and important signaling pathways play a part as well in drug resistance. In PC, several signaling pathways have been shown to be contributing to drug resistance, such as Akt, nuclear factor κB (NF-κB) and Notch pathways. Resistance of PC cells to chemotherapy is essentially attributable to the constitutive activation of NF-κB, which is a key nuclear transcription factor responsible for the regulation of inflammation, apoptosis, various autoimmune disorders and tumorigenesis by monitoring the expression of a significant number of genes. Moreover, gamma irradiation and chemotherapeutic agents further activate NF-κB (Long et al., 2011).

Currently available therapies for patients suffering from PC aim to target and kill cancer cells that are differentiated as well as the tumor initiating cells (TICs) that are in quiescence (Yu, Zhang, Chen, Yao, & Li, 2010). TICs constitute a distinctive subpopulation of cells that are capable of initiating tumor development and sustaining self-renewal in addition to the ability to metastasize from the place of origin (Takaishi, Okumura, & Wang, 2008). Several studies refer to TICs as "tumorigenic cells" or "cancer stem cells (CSCs)" (Saikawa et al., 2010). These special populations of cells possess the ability of self-renewal and cellular differentiation hence they culminate in the development of heterogeneity in the progressing tumor (Caceres-Cortes, Mindeni, Patersoni, & Caligiuri, 1994). Furthermore, sub-sets of CSCs have been detected in the majority of tumors with PC being one of them, and the presence of these distinctive cellular populations holds clinical relevance. In fact, evidence concerning CSCs suggests that they contribute to migration/invasion, proliferation/cell growth, metastasis, and resistance to chemo-radio-therapeutic approaches, eventually supporting a poor overall clinical outcome (Bao et al., 2014).

1.3- Management

PC displays resistance to radiotherapy and standard chemotherapeutic regimens along with other properties such as aggressiveness, early metastasis, and local invasion (Jemal, Siegel, Xu, & Ward, 2010; Siegel, Ward, Brawley, & Jemal, 2011). Currently, the only therapy with a curative potential is surgical resection (Ryan et al., 2014). However, the optimal therapy primarily depends on precise cautious staging (Wolfgang et al., 2013). In the case of localized PC, the greatest chance of achieving long-term patient survival is by completely resecting the primary lesion. However, many factors

should be taken into consideration when making patients' selection based on who can most profit from undergoing surgery. Such factors include the effect of pancreatectomy on the patient's quality of life, the systemic cancer nature at diagnosis, and the low possibility of attaining long-term patient survival (Wolfgang et al., 2013).

Besides the stage of tumor progression, many factors must be examined in the selection process of patients for whom pancreatectomy may be most beneficial (Hsu et al., 2012). Such factors comprise the patients' tumor biology, their overall health, and the inclusion of neoadjuvant therapy in the treatment routine. These three considerations need to be collectively taken into account during the formulation of a management strategy since these factors can often influence one another (Pawlik et al., 2008). The management strategy includes, but is not limited to, determining the patients who meet the set of qualifications pertaining to curative surgery, assessing the need for preoperative (neoadjuvant) or postoperative (adjuvant) treatment, and choosing the proper palliation mode (Bond-Smith et al., 2012). As mentioned above, the assessment of tumor biology presents a considerable factor in the selection course of patients for resection. The "tumor biology" terminology refers to the relative tumor tendency toward local aggressiveness or metastatic spread. Due to the current lack of validated biomarkers for the prediction of clinical behavior, the characterization of tumor biology remains subjective. Overall, patients whose tumor biology is defined by aggressiveness manifest a low likelihood of benefiting from local therapy such as surgical resection even in the case where the patient's tumor is still at an early stage. Neoadjuvant therapy can possibly be beneficial in the case of patients with suspected aggressive yet localized tumors (Wolfgang et al., 2013). Patients with advanced age (older than 75 years) and co-

morbid health conditions displaying a poor general health status are unlikely to profit from pancreatectomy and may even become impaired by the additional immunosuppression and debilitation caused by such operations (Hsu et al., 2012). When surgical resection is not a viable option, patients are left with a few treatment alternatives such as image directed stereotactic radiosurgical methods (for instance CyberKnife), systemic chemotherapy, endoscopic gastrointestinal and biliary stenting, chemoradiotherapy, ablative therapies, and surgical bypass. These represent palliative measures that aim to improve the quality of life of suffering patients by easing the symptoms caused by the tumor (like pruritus and pain) but are in no case curative (Bond-Smith et al., 2012).

The characterization of resectability keeps on changing, and whatever was formerly thought of as unresectable ailment might now be seen as resectable (Wong & Raman, 2010). At present, conditions for resectability comprise the lack of distant metastases, the absence of primary tumor contribution from major arteries (superior mesenteric artery, celiac artery and hepatic artery), and the presence of a proper segment of superior mesenteric vein and portal vein, respectively below and above the site of venous invasion to make venous reconstruction possible in the case of venous involvement (Tseng, Tamm, Lee, Pisters, & Evans, 2006; Vauthey & Dixon, 2009). Hence the categorization of the localized tumors starting from the “resectable” disease to the locally advanced ailment described as “unresectable” is based on the detection of involved local vessels. However, there are tumors that evade this specific categorization; these became popularly known under the general terminology of “borderline resectable”. Following careful evaluation, merely 15 to 20% of patients can be thought of as

candidates for pancreatic surgical resection, and several among these candidates are considered to have extremely small optimistic margins by the time of surgery (Konstantinidis et al., 2013). Surgical resection followed by adjuvant treatment offers the greatest survival chances, however, only less than 20% of patients can benefit from such therapy (Vincent, Herman, Schulick, Hruban, & Goggins, 2011). Stage I/II diseased patients need to be treated by surgical resection plus adjuvant therapy. In the case of these patients, neoadjuvant therapy must be thought of, nonetheless, controversy still exists regarding this topic; whereas patients displaying borderline resectable disease at Stage III need neoadjuvant therapy before undergoing resection (Evans et al., 2008). Venous involvement currently characterizes T3 disease which displays a potential for resectability despite its local invasiveness, while involvement of the superior mesenteric artery or the celiac artery still characterizes T4 disease which is unresectable and locally advanced (Bilimoria et al., 2007; Katz, Hwang, Fleming, & Evans, 2008). At 5 years following resection, survival rate is roughly 12–20%, compared to a less than 1% rate for patients with unresectable cancer (Wong & Raman, 2010).

Local recurrence happens in the majority of cases in which patients opt for curative resection, either in the vicinity of the superior mesenteric artery or distantly (lung, liver, and peritoneum). Adjuvant therapy is consequently recommended to lessen the chance of metastatic and local/regional recurrence (Wolfgang et al., 2013). Adjuvant therapy comprises chemoradiotherapy to decrease the chance of local/regional failure and systemic chemotherapeutic treatment to decrease the chance of distant metastases (Ryan et al., 2014). This type of therapy usually begins after a recovery period of 1–2 months post-surgery (Wolfgang et al., 2013). It has been established by numerous

studies that 6 months of treatment using chemotherapy with 5-fluorouracil (5-FU) or gemcitabine, in comparison to observation, advances overall survival (Klinkenbijl et al., 1999). The incorporation of around 6 weeks of gemcitabine or 5-FU based chemoradiation treatment (CRT) throughout this six months therapy period represents an option and a preference in the case of R1 resections (microscopic residual tumor) and whenever the threat of local/regional recurrence seems high (Herman et al., 2008). Since more than 70% of cases show recurrence with distant ailment, systemic chemotherapy is commonly given in the beginning of the adjuvant therapy. Following the completion of chemotherapy, if there are no fears for metastatic recurrence based on clinical and radiographic evidence, CRT follows. However, the median overall survival pertaining to patients who underwent resection for PC remains around 20–22 months (Wolfgang et al., 2013).

Neoadjuvant therapy inclusion as part of a management plan for PC still holds controversy. However, this preoperative approach offers some benefits: it down-stages certain locally advanced cases and sterilizes the marginal hindrances of borderline resectable cases thus giving these patients the chance of undergoing surgery at a greater R0 resection (tumor negative, complete remission) possibility (Evans et al., 2008; Gillen, Schuster, Zum Büschenfelde, Friess, & Kleeff, 2010). Moreover, it is also advantageous as it can spare the 15–35% of cases who progress toward metastatic disease the hazards and tension of a major surgical process, since the development of metastases would eliminate the patients' candidacy for surgery (Katz et al., 2008; Laurence et al., 2011; White et al., 2001). Even though neoadjuvant therapy seems as an attractive option for the previously stated reasons, yet surgery represents a key

requirement for being cured and neoadjuvant therapy postpones patients' operation with its curative potential. Currently, resectable cases are frequently allowed for immediate surgery followed by adjuvant therapeutic management, while neoadjuvant therapy is commonly used in borderline resectable cases (Pawlik et al., 2008). Neoadjuvant therapy still lacks properly established protocols; nevertheless similar treatments, as in the case of locally advanced ailment (unresectable), are being used by most centers. The drug combination of leucovorin, 5-FU, oxaliplatin, and irinotecan (a regimen best known as FOLFIRINOX), the drug combination of capecitabine, gemcitabine, and docetaxel (a regimen best known as GTX), gemcitabine with nab-paclitaxel (paclitaxel particles which are albumin-bound), and gemcitabine on its own are likely usable chemotherapeutic treatments that are usually followed by uninterrupted infusion of gemcitabine, capecitabine, or 5-FU-based chemoradiation (Crane et al., 2011; De Jesus-Acosta et al., 2012; Faris et al., 2013; Gillen et al., 2010; Laurence et al., 2011; Le Scodan et al., 2009; Tempero et al., 2012). Surgery must be executed after finishing the neoadjuvant treatment regimen by 6–8 weeks (Wolfgang et al., 2013).

Greater than 80% of the newly identified PC cases display at the time of diagnosis either locally advanced (Stage III) or metastatic (Stage IV) ailment, both of which are unresectable and frequently labeled as advanced PC (APC) with significantly poor prognosis (Bond-Smith et al., 2012). Without chemotherapeutic management, patients suffering from APC barely have a median survival expectancy of 2–4 months (Cascinu, Graziano, & Catalan, 1999). Previous studies emphasized the importance of 5-FU as a radiation sensitizer (Abrams, 2003) as suggested in 1958 by Heidelberger et al (Heidelberger et al., 1958). In 1997, the Food and Drug Administration (FDA) declared

its approval of gemcitabine as the first-line drug for chemotherapeutic use by patients suffering from unresectable pancreatic adenocarcinoma either at its metastatic phase or at its locally advanced phase on the basis of gemcitabine versus 5-FU Phase III trial results, revealing a moderate survival advantage and reduced toxicity when using gemcitabine (Burris et al., 1997). Therefore, gemcitabine, a nucleoside analog, is considered as a standard effective APC therapy based on median overall survival and response rate. Nonetheless, the advantage it offers is modest, and its contribution to the enhancement of the dismal prognosis is minimal, with an overall median survival expectancy lesser than 6 months (Burris et al., 1997). Since approval of gemcitabine as first line therapy for APC, a number of trials have been carried out, all aiming to ameliorate the poor clinical results through combining additional cytotoxic drugs, for instance 5-FU, pemetrexed, irinotecan, exatecan, capecitabine, oxaliplatin, and cisplatin or targeted drugs, for instance bevacizumab with gemcitabine (Berlin, Catalano, Thomas, Kugler, & Haller, 2002; Colucci et al., 2002; Kindler et al., 2010). Still, most trials were unsuccessful in demonstrating valuable progress in response or survival rates in comparison to monotherapy using gemcitabine (Wang et al., 2016).

Accumulating evidence propose that the over-expression feature of epidermal growth factor receptor (EGFR) correlates with poor prognosis in the case of PC (Yang, Z. Y., et al., 2013). Therefore erlotinib, which acts as a tyrosine kinase inhibitor targeting EGFR, has been recently regarded as a promising drug for APC treatment (Lynch et al., 2007; Peréz-Soler & Saltz, 2005; Yang, Z. Y., et al., 2013). In 2007, Moore et al. initially showed considerably improved outcomes using gemcitabine in combination with erlotinib in comparison with gemcitabine on its own in their study

(Moore et al., 2007). This led to the combination of erlotinib and gemcitabine being approved by the US FDA as the front-line treatment regimen for APC. Subsequently, additional worldwide studies were carried on to gain knowledge concerning the safety and effectiveness of the erlotinib plus gemcitabine drug combination (Wang et al., 2016). In addition, FOLFIRINOX combination treatment was recently found to be superior to gemcitabine monotherapy in the management of PC at its metastatic stage (Conroy et al., 2011). Furthermore, nab-paclitaxel used along with gemcitabine was demonstrated to yield a more favorable therapeutic impact over gemcitabine monotherapy for PC at its metastatic stage based on overall survival (Goldstein et al., 2015). Consequently, the evaluation of gemcitabine plus nab-paclitaxel and FOLFIRINOX in locally advanced cases became of interest to researchers. Primary studies show that the possibility of a radiographic response to such therapies in cases of unresectable primary tumor is same as that induced in cases of metastatic disease (Faris et al., 2013; Ryan et al., 2014). This highlights the crucial need for more efficacious therapeutic drugs which hold the possibility of being used on their own as monotherapies or together along with present chemotherapies to promote the advancement of APC prognosis (Yang, Z. Y., et al., 2013).

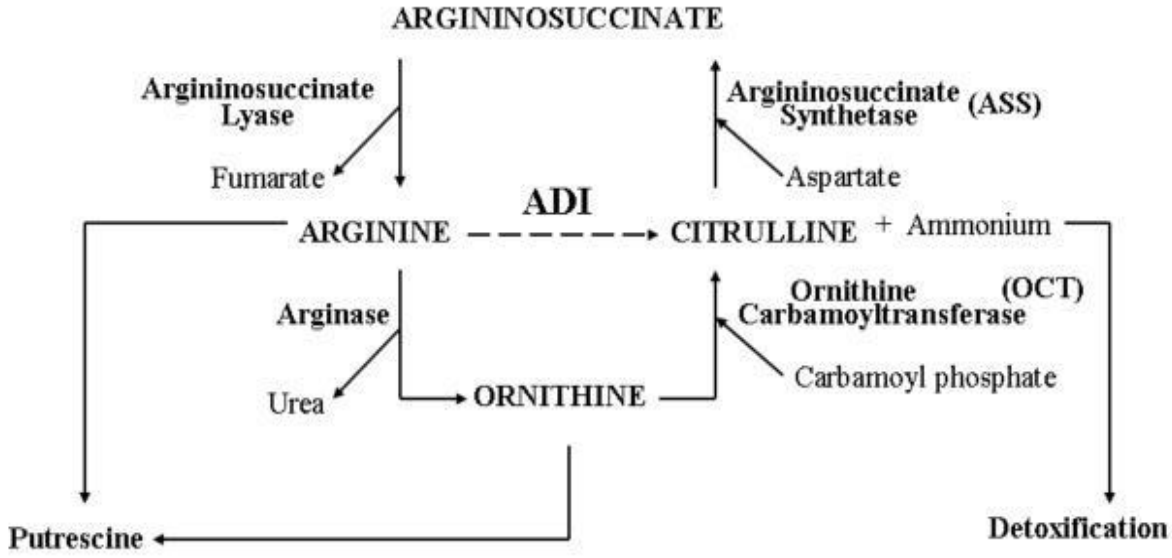
1.4- The Need for Innovative Targeted Treatments

Pancreatic carcinoma resists conventional therapies such as radiotherapy and chemotherapy and can only be potentially cured through surgical resection. However, late diagnosis characterizes this disease, thus most of the newly identified disease cases display unresectable therefore incurable ailment (locally advanced at Stage III or metastatic at Stage IV) at the time of diagnosis. Only a minor percentage of patients get

to be considered for surgical resection. Moreover, the majority of these patients show recurrence (Wolfgang et al., 2013). Hence PC is among the deadliest and most aggressive cancers. Such reasons obviously underlie the need for innovative, tumor-selective, and efficacious therapies to enhance the currently poor prognosis pertaining to PC and to offer better survival expectancies. We and many others have been studying the use of arginine deprivation, via a pegylated recombinant form of human arginase or the recombinant bacterial enzyme arginine deiminase, as a prospective targeted treatment against several tumor types, including acute myeloid leukemia (AML), glioblastoma multiforme (GBM), acute lymphoblastic leukemia (ALL), hepatocellular carcinoma (HCC), renal cell carcinoma (RCC), malignant melanoma (MM), prostate cancer, T-cell acute lymphoblastic leukemia (T-ALL) and some mesotheliomas (Khoury et al., 2015; Tanios et al., 2013; Wheatley & Campbell, 2003; Kim et al., 2009; Hernandez et al., 2010; Shen et al., 2003; Ensor et al., 2002; Savaraj et al., 2007). In this study, we attempt to use arginine deprivation through HuArgI (Co)-PEG5000 as a potential targeted therapeutic approach for PC cell lines presenting arginine auxotrophy.

1.5- Arginine Depletion as Targeted Treatment

Depriving tumor cells from key amino acids that they require for survival and proliferation is an effective targeted therapeutic approach that is being used to treat several cancer types (Cooney & Rosenbluth, 1975).



Feun et al., 2008

Figure 1. Enzymes and Intermediates included in the urea cycle. Arginine deiminase (ADI), expressed in bacterial cells, results in the extracellular cleavage of arginine to citrulline and ammonium while arginase, expressed in mammalian cells, is involved in the conversion of arginine to ornithine and urea.

A well-known example of such approaches would be the use of asparaginase to treat ALL by depleting the blood level of asparagine, a non-essential amino acid in humans (Müller & Boos, 1998). In adult humans, the synthesis of the amino acid L-arginine from L-citrulline is possible through the urea cycle where L-citrulline and aspartic acid get converted to argininosuccinate by argininosuccinate synthetase (ASS) and then argininosuccinate lyase (ASL) catalyzes argininosuccinate conversion to L-arginine and

fumarate (Shen, L. J., Beloussow, & Shen, W. C., 2006; Shen, L. J., Lin, Beloussow, & Shen, W. C., 2003). Hence, in addition to dietary consumption and muscle degradation, endogenous synthesis presents an important source of arginine in somatic cells (Manca et al., 2011). Nevertheless, this endogenous synthesis method is insufficient to meet the abundant arginine requirements of cells under stressful conditions and of cells that need to proliferate at higher than normal rates such as cancer cells. Hence, arginine is a semi-essential amino acid that can affect the proliferation rate and the growth of tumor cells and the regeneration of arginine from citrulline is dependent on the activity of the ASS and the ASL enzymes of the urea cycle (Lind, 2004; Shen et al., 2006; Shen et al., 2003). However, several tumor types such as HCC, RCC, MM, some mesotheliomas, AML and GBM have been demonstrated to lack ASS expression and thus to be auxotrophic for arginine, completely relying on extracellular arginine supplies for survival and proliferation (Khoury et al., 2015; Tanios et al., 2013; Wheatley & Campbell, 2003; Shen et al., 2003; Ensor, Holtsberg, Bomalaski, & Clark, 2002; Savaraj et al., 2007). Therefore, arginine depletion in the environment surrounding the tumor can induce cytotoxicity in ASS-1 deficient cancer cell lines that are completely auxotrophic for arginine. Moreover, ASS-1 positive cancer cell lines with rapid proliferation rates display partial arginine auxotrophy and are thus affected by arginine depletion-induced cytotoxicity. However, the partial arginine dependence of such cancer cells can be reversed through the addition of excessive, exogenous citrulline (Feun et al., 2008). Consequently, arginine restriction from the extracellular environment via arginine-catabolizing enzymes is a potentially selective antitumor strategy that targets the selective discrepancy in arginine requirement and in critical urea cycle enzyme

expression between tumor cell lines that display arginine auxotrophy and most somatic cell lines (figure 1).

Arginine degradation is mostly catalyzed by either arginine deiminase (ADI) or arginase. Among those two, only arginase is expressed in mammalian cells (Morris, 2007; Miyazaki et al., 1990). It catalyzes the conversion of arginine to ornithine and urea through the urea cycle. Citrulline can be regenerated from ornithine in the presence of ornithine transcarbamylase (OTC) and then arginine can be obtained from citrulline by the enzymes ASS/ASL (Feun et al., 2008). Besides its role as a house-keeping gene, *ASS-1* is in fact a rate-limiting catalyst for arginine biosynthesis in somatic cells, mainly in small bowel cells and in hepatocytes. Nevertheless, this critical urea cycle catalyst is absent in numerous tumors (Phillips, Sheaff, & Szlosarek, 2013). Hence, arginase-induced arginine depletion is a therapeutic approach that decreases the risk of normal tissue toxicity. However, arginase has a short half-life in blood circulation and requires an optimum pH of 9.6; therefore it displays low affinity for its substrate, arginine, at physiological pH (Savoca, Abuchowski, Van Es, Davis, & Palczuk, 1979). These shortcomings led to the failure of this therapy *in vivo* since the 1980s in spite of its promising *in vitro* anti-cancer characteristics. These early tests in experimental animals were carried using murine and bovine arginase enzymes, which display different biochemical characteristics than those of the human liver arginine-depleting enzyme. Recently, a recombinant version of human arginase, with far superior biochemical characteristics has been developed and tested in a number of pre-clinical tumor models. Human L-arginase I (hArgI), in its native form, contains two Mn^{2+} cofactor cations that get rapidly dissociated from its active site in serum thus leading to an inactivated

enzyme with minimal activity and a short half-life at physiological pH (Glazer et al., 2011). Substituting both manganese ions found within the active site of hArgI with cobalt cations (Co^{2+}) was shown to yield a 10-fold greater catalytic activity and a significantly enhanced structural stability of the protein in the human serum at approximately pH 7.4 (Stone et al., 2010). Moreover, pegylation was proven to be efficient in the amelioration of the biopharmaceutical characteristics of bioactive peptides hence resulting in enhanced solubility, stability, as well as immunological characteristics of the compounds of interest (Li et al., 2013). Therefore, the conjugation of HuArgI (Co) to 5-kDa polyethylene glycol (PEG) was performed in order to produce the Co^{2+}-substituted, pegylated recombinant human arginase [HuArgI (Co)-PEG5000] with improved persistence properties in circulation (Glazer et al., 2011). In fact, targeting arginine deprivation through the use of recombinant human arginase has demonstrated effective anti-cancer activity both *in vitro* and *in vivo* in T-ALL and AML and solid tumors (HCC, RCC, MM and prostate cancer) and is presently undergoing phase I clinical trials to depict its potential in the treatment of HCC and MM (Wang et al., 2014). Consequently, this therapy holds potential in the targeted treatment of pancreatic carcinoma. This study intends to depict the mechanisms of arginine auxotrophy in PC cell lines and to study its selective targeting through the use of HuArgI (Co)-PEG5000.

Another enzyme, ADI, isolated from *Mycoplasma*, catalyzes the degradation of arginine to citrulline and ammonia. Hence, cancer cell lines lacking ASS expression can also be sensitive to ADI therapy since they won't be able to convert intracellular citrulline to arginine unlike normal cells. Moreover, ADI has normal enzymatic activity at physiological pH and shows high affinity for the amino acid arginine. In addition,

ADI has been demonstrated to have *in vitro* and *in vivo* anti-tumor efficiency in the treatment of several arginine-auxotrophic cancer cells lines such as HCC and MM cell lines (Ensor et al., 2002; Savaraj et al., 2007; Gong, Zölzer, Von Recklinghausen, Havers, & Schweigerer, 2000; Shen, L. J., & Shen, W. C., 2006). Nonetheless, ADI is significantly antigenic and has a short half-life thus resulting in its rapid degradation in patients' sera. These drawbacks were avoided by the development of a pegylated type of ADI (ADI-PEG20) with a significantly increased half-life and decreased antigenicity that was clinically tested on MM and HCC patients following extensive studies both *in vitro* and in-vivo (Holtsberg, Ensor, Steiner, Bomalaski, & Clark, 2002; Bowles et al., 2008; Ensor et al., 2002; Shen, L. J., & Shen, W. C., 2006). However, the issue of immunogenicity continues to plague arginine deiminase, rendering recombinant human arginase, the best potential candidate for achieving arginine deprivation in human tumors.

1.6- Autophagy and its Role in Cancer Therapeutics

Autophagy is a survival mechanism used by cells in a state of metabolic stress, which makes use of bulk degradation. However, if performed to completion, it can result in cell death (Mathew et al., 2007). Such homeostatic process is also evolutionary conserved, and functions by degrading proteins and cellular organelles, along with preserving cellular biosynthesis in the conditions of metabolic stress and nutrient scarcity (Degenhardt et al., 2006). It starts with the formation of autophagosomes, which are vesicles surrounded by a double-membrane that engulf cytoplasmic components. These autophagosomes, along with their segregated contents, then combine with

lysosomes to initiate the degradation of the isolated contents and their subsequent recycling (Degenhardt et al., 2006). Such mechanism is under the control of many different kinases, phosphatases, and guanosine triphosphatases (GTPases) and culminates in the relief from distinctive circumstances of cellular stress (Klionsky & Emr, 2000; Yang, Z., & Klionsky, 2009). Autophagy also has significant roles throughout the development of cells and their differentiation, in adaptive and innate immunological defenses, in addition to others (Yang, Z., & Klionsky, 2009).

Moreover, autophagy assumes dual functions in cancer, operating as both a tool for cell survival which assists in tumor growth, and as a tumor suppressor by inhibiting the buildup of damaged organelles and proteins (Yang, Z. J., Chee, Huang, & Sinicrope, 2011). Depicting the mechanisms used by autophagy to impact responses to anti-cancer treatments and tumorigenesis is of critical importance since autophagy was revealed to be activated by several cancer treatment approaches, although the significance of triggering autophagy in this particular context remains contentious (Yang, Z. J., et al., 2011). The association of autophagy deficiencies with predisposition to genomic damage, metabolic stress, as well as tumorigenesis in mice can best elucidate the tumor suppressor behavior of autophagy (Qu et al., 2003). Moreover, monoallelic loss of *Beclin 1* which is a vital autophagy gene, has been established in 40 to 75% of ovarian, prostate, and breast cancer cases (Qu et al., 2003), emphasizing that autophagy may contribute to the prevention of such tumors (Yang, Z. J., et al., 2011). Although autophagy may behave as tumor suppressor, it does confer upon tumor cells the ability to tolerate stress and thus survive under hostile conditions (Degenhardt et al., 2006). Furthermore, tumor cells face added stress due to the great metabolic demand created by

the need for fast proliferation. Autophagy induction due to stressful conditions can result in therapeutic resistance and tumor latency eventually leading to tumor advancement and regrowth (Lu et al., 2008). Preclinical studies showed that pharmacological and genetic inhibition of protective autophagy kills cancer cells and elicits cell death through apoptosis (Degenhardt et al., 2006; Amaravadi et al., 2007; Carew et al., 2007). The protective and pro-survival contribution of autophagy in response to most anti-cancer medications can be a key hurdle to effective cancer cure and hence represents an innovative treatment target (Yang, Z. J., et al., 2011). However, autophagy resembles a double-edge sword: in certain cancer cells, excessive or persistent autophagy can possibly be pro-death, predominantly in the case of apoptosis-defective cells (Maiuri, Zalckvar, Kimchi, & Kroemer, 2007).

Whether autophagy assumes a protective function that hinders apoptosis, or whether it represents an essential precursor of apoptosis resulting from metabolic stress remains an intricate topic (Gozuacik & Kimchi, 2004). Abedin et al. stated that autophagy inhibits apoptosis which is triggered by chemotherapy, whereas Cui et al. stated that oridonin-mediated apoptosis is triggered by autophagy in breast cancer (Cui, Tashiro, Onodera, Minami, & Ikejima, 2007; Abedin, Wang, McDonnell, Lehmann, & Kelekar, 2007). However, Bowles et al. proposed that the recycling mechanism of bulk proteins may halt apoptosis under comparatively minor insult circumstances, while apoptosis may be induced by more offensive distortions of energy homeostasis within cells (Bowles et al., 2008). Arginine deprivation, and amino acid deprivation in general, are known activators of autophagy. However, the contribution of autophagy to tumor cell response to arginine deprivation remains elusive. A crucial aspect of understanding

the mechanisms of arginine deprivation-induced targeting of pancreatic carcinoma cells consists of understanding the exact contribution of autophagy to the response of cancer cells.

CHAPTER TWO

MATERIALS AND METHODS

2.1- Cell Lines and Reagents

To test the effectiveness of HuArgI (Co)-PEG5000, a panel of 5 human PC cell lines was used: Panc-1, Capan-1, Hs 766T, Panc 04.03, and Panc 10.05. The normal human brain cell line, SVG p12 was also used to test for the selectivity of HuArgI (Co)-PEG5000. All cells were purchased from the American Type Culture Collection (ATCC). Panc-1 and Hs 766T cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% heat inactivated fetal bovine serum (FBS) and 1% Penicillin/Streptomycin at 37°C and 5% CO2. Capan-1 cells were cultured in DMEM with 20% FBS and 1% Penicillin/Streptomycin at 37°C and 5% CO2 while Panc 04.03 and Panc 10.05 cells were cultured in DMEM with 15% FBS, human insulin at a concentration of 0.01 mg/ml, and 1% Penicillin/Streptomycin at 37°C and 5% CO2. Moreover, SVG p12 cells were cultured in Eagle's Minimum Essential Medium with 10% FBS and 1% Penicillin/Streptomycin at 37°C and 5% CO2.

Human Arginase I cobalt [HuArgI (Co)] combined with polyethylene glycol 5000 [HuArgI (Co)-PEG5000] was synthetized by George Georgiou and Everett Stone at the University of Texas in Austin, TX, USA, as described previously (Zakalskiy et al., 2012).

2.2- Proliferation Inhibition Assay (cytotoxicity)

The proliferation inhibition assay was performed to determine the sensitivity of PC cells to HuArgI (Co)-PEG5000, in the absence or presence of excess exogenous L-citrulline, as detailed previously (Khoury et al., 2015). Briefly, aliquots of 10^4 cells containing exogenous citrulline at a concentration of 11.4 mM were plated in 100 µl DMEM in Costar flat-bottomed 96-well plates (Corning Inc. Corning, NY). 50 µl of HuArgI (Co)-PEG5000 was added to each well such as concentrations ranging from 10^{-7} to 10^{-13} mol/L were obtained then the plates were incubated at 37°C/5% CO2 for 24, 48, 72, 96 and 120 hours. In addition, a 72 hours assay was carried where 20 µM Z-VAD, a cell-permeable pan-caspase inhibitor, was added in addition to HuArgI (Co)-PEG5000 in a subset of wells. Following these incubation times, 50 µl of XTT cell proliferation reagent (Roche, Basel, Switzerland) were added to each well and the plates incubated for an additional 3 hours. Then a microplate reader was used to determine the absorbance at 450 nm (Thermo Fisher Scientific, Waltham, MA). Plots showing the nominal and percent average absorbance versus the log of the drug concentration were consequently generated. GraphPad Prism software was used to produce nonlinear regression with a variable slope sigmoidal dose-response curve in addition to the IC_{50} (GraphPad Software, San Diego, CA). The experiments were performed in triplicates and all three replicate values were entered and used to calculate the mean values that were plotted. Bars displaying the standard error of the mean (SEM) were included in the plots.

2.3- Cell Cycle Analysis

The effect of HuArgI (Co)-PEG5000 treatment on the cell cycle of PC cells was investigated using Propidium Iodide (PI)-staining on flow cytometry as described previously (Khoury et al., 2015). In short, cells (10^5 cells/well in 2 ml growth medium) incubated with the high concentration of HuArgI (Co)-PEG5000 (10^{-7} mol/L) or with media alone (control) in flat-bottom 6-well plates (Corning Inc. Corning, NY) for 72 hours at 37ºC/5% CO2, were harvested and fixed in 70% ethanol for a minimum of 24 hours at -20ºC. Following fixation, cells were incubated in 500 µl PI staining solution (50 µg/ml) for 10 minutes at 37ºC. A reading of all samples was then performed using a C6 flow cytometer (BD Accuri, Ann Arbor, MI) and cell DNA content was measured on FL2-A. The target cell population was gated on width versus forward scatter. Percent of cells in G0/G1, S, G2/M and pre-G0/G1 phase (dead cells) was determined in cells treated with the high concentration of HuArgI (Co)-PEG5000 and in control cells in the different cell lines.

2.4- Analysis of Cell Death

Fluorescin Isothiocyanate (FITC)-conjugated Annexin V antibody- (Annexin V-FITC) and PI staining (apoptosis detection kit, Abcam, Cambridge, MA) were used to determine the type of cell death as described previously (Khoury et al., 2015). In addition, a cell permeable, FITC-conjugated active caspase inhibitor (R&D systems) was used on flow cytometry to detect the presence of active caspases in cells cultured for 24 and 48 hours with or without HuArgI (Co)-PEG5000 as described previously (Khoury et al., 2015). Briefly, cells (10^5 cells/well in 2 ml growth medium) were plated

in flat-bottom 6-well plates and incubated either with the high concentration of HuArgI (Co)- PEG5000 (10^{-7} mol/L) or with media alone (control) for 24 and 48 hours at 37ºC/5% CO2. Afterwards, cells were either harvested then incubated with FITC-conjugated annexin V antibody (2.5 mg/ml) and PI (5 mg/ml) in antibody binding buffer for 5 minutes at 37ºC in the dark, or incubated with a FITC-conjugated active caspase inhibitor (ApoStat Apoptosis Detection Kit, R&D Systems, Abingdon, England) for 60 minutes and then harvested. A C6 flow cytometer was then used to read the samples (BD Accuri, Ann Arbor, MI). Annexin V/PI data was examined on FL1-H versus FL2-H scatter plot and caspase activation was spotted on FL1-H. Unstained cells were used as negative control. After gating the cells on width versus forward scatter, analysis was performed: only the cells positive for both annexin V staining and active caspase staining while being negative for PI staining were designated apoptotic, whereas cells positive for both annexin V and PI staining while being negative for active caspase staining were regarded as non-apoptotic/necrotic.

2.5- Autophagy Assays

To test for the possible role played by autophagy in arginine deprivation-induced cytotoxicity of PC cells, cells were incubated with HuArgI (Co)-PEG5000 alone and in combination with an autophagy inhibitor which is either chloroquine (CQ) or 3-methyladenine (3-MA). Aliquots of 10^4 cells in 100 µl DMEM/well, were plated in a flat-bottom 96-well plate (Corning Inc. Corning, NY). In a subset of wells containing cells and culture media, 100 µM chloroquine or 500 mM 3-methyladenine was added, followed by the addition of 50 µl HuArgI (Co)-PEG5000 to each well at concentrations ranging from 10^{-7} to 10^{-13} M. This was followed by incubation of the plates for 24, 48,

72 and 96 hours at 37ºC/5% CO2. After these incubation times, 50 µl XTT cell proliferation reagent (Roche, Basel, Switzerland) was added to each well and the plates were incubated for additional 3 hours. Absorbance values were then obtained at 450 nm using a 96-well plate reader (Thermo Fisher Scientific, Waltham, MA). GraphPad Prism V software was used for data analysis (GraphPad Software, San Diego, CA) and comparison between the IC_{50} (inhibitory concentration 50) of HuArgI (Co)-PEG5000 alone and in the presence of chloroquine or 3-methyladenine was performed.

2.6- Intracellular Staining and Flow Cytometry Analysis

In order to assess the expression levels of Argininosuccinate Synthetase 1 (ASS-1), intracellular staining on flow cytometry was used as described previously (Kassab et al., 2013). Approximately, $3x10^6$ cells were collected and fixed in 70% ethanol for 15 min. Afterwards, cells were incubated for 1 hour at 37ºC in the dark in antibody binding buffer comprising 0.05% Triton-X 100 along with a 1/100 dilution of anti-ASS-1 mouse monoclonal antibody (Sigma, Danvers, MA), followed by incubation with a 1/100 dilution of a FITC-conjugated rabbit anti-mouse polyclonal antibody for 30 minutes (Santa Cruz Biotechnology, Santa Cruz, CA). Fixed cells incubated with a 1/100 dilution of a mouse IgG and a FITC-conjugated rabbit anti-mouse polyclonal antibody served as isotypic control. A one-time phosphate buffered saline (PBS) wash was then performed on all samples, followed by re-suspension in binding buffer and analysis via a C6 flow cytometer (BD Accuri, Ann Arbor, MI). Cells were gated on width versus forward scatter and ASS-1 staining was spotted on FL1-H. The existence of ASS-1 was examined in comparison with that of the isotypic control. The ratio of fluorescence intensity (RFI) between the mean fluorescence intensity (MFI) of the stained cells and

the mean fluorescence intensity of the isotypic control cells was used to conclude on positivity for the presence of ASS-1. Positivity for ASS-1 expression was defined by an RFI > 2 whereas negativity was determined by an RFI ≤ 2. Furthermore, analysis of cells based on forward scatter versus FL1-H was used to find the percentage of cells displaying ASS-1 positivity.

CHAPTER THREE
RESULTS

3.1- Proliferation Inhibition Assay (cytotoxicity)

Five human PC cell lines were used in order to test the cytotoxic effect of HuArgI (Co)-PEG5000. All the tested PC cell lines were sensitive to HuArgI (Co)-PEG5000-induced arginine deprivation with IC_{50} values ranging from 88 to 1480 pM at 72 hours following treatment (Figure 2, Table 2). Moreover, HuArgI (Co)-PEG5000-triggered arginine deprivation demonstrated potent cytotoxicity on PC cell lines resulting in a percent cell death ranging between 50% and 76% at highest toxin concentration at 72 hours following treatment (Percent cell death of 64%, 76%, 76%, 50% and 75% for Capan-1, Panc-1. Hs 766T, Panc 10.05 and Panc 04.03, respectively).

Since the cytotoxic effects of arginine depletion may be time dependent, the cytotoxicity of HuArgI (Co)-PEG5000 was tested for longer time points (96 and 120 hours) on all five cell lines. Two of the cell lines (Panc-1 and Panc 10.05) showed a decrease in IC_{50} values with increasing incubation time while the remaining three cell lines did not (Table 2, Figure 2). Moreover, the percent cell death at the highest concentration increased for the same two cell lines at the longer incubation times (from 76% at 72 hours to 95% at 96 hours and 94% at 120 hours for Panc-1 and from 50% at 72 hours to 71% at 96 hours and 88% at 120 hours for Panc 10.05) while remaining relatively unchanged for the rest of the cell lines (Capan-1, HS 766T and Pan 04.03). This decrease in cell survival further highlights the time-dependent cytotoxic effect of HuArgI (Co)-PEG5000 on these two cell lines. For comparison purposes, the 72 hours'

time point was considered the benchmark to compare the different cell lines and to determine the responses of cell lines to excess citrulline.

Table 2. Sensitivity of PC cell lines and normal human cells to HuArgI (Co)-PEG5000 both in the absence and presence of 11.4 mM of exogenous L-citrulline at 72 h, and in the absence of citrulline at 96 and 120 h, in addition to positivity or negativity for ASS-1 expression with ratio of fluorescence intensity (RFI) values.

Cells and cell lines	HuArgI (Co)-PEG5000 (72 h)	HuArgI (Co)-PEG5000 (72 h) + L-cit	HuArgI (Co)-PEG5000 (96 h)	HuArgI (Co)-PEG5000 (120 h)	ASS-1 expression (RFI)
Pancreatic Cancer cell lines					
Panc-1	306 pM	436 pM	97 pM	48 pM	1.79 (-)
Capan-1	1480 pM	>10,000 pM	1331 pM	460 pM	4.42 (+)
Hs 766T	230 pM	>10,000 pM	136 pM	156 pM	3.01 (+)
Panc 04-03	88 pM	>10,000 pM	193 pM	227 pM	2.34 (+)
Panc 10-05	217 pM	362 pM	166 pM	52 pM	1.21 (-)
Normal human cells					
SVG p12	>10,000 pM	>10,000 pM	>10,000 pM		9.46 (+)

In order to determine the level of arginine auxotrophy and differentiate between cells that are completely auxotrophic and cells that are partially auxotrophic for arginase we added excess amounts of L-citrulline and observed the effect of such addition on HuArgI (Co)-PEG5000-induced cytotoxicity. Successful rescuing by excess L-citrulline of tumor cells from cytotoxicity due to deprivation from arginine supplies denotes partial arginine auxotrophy.

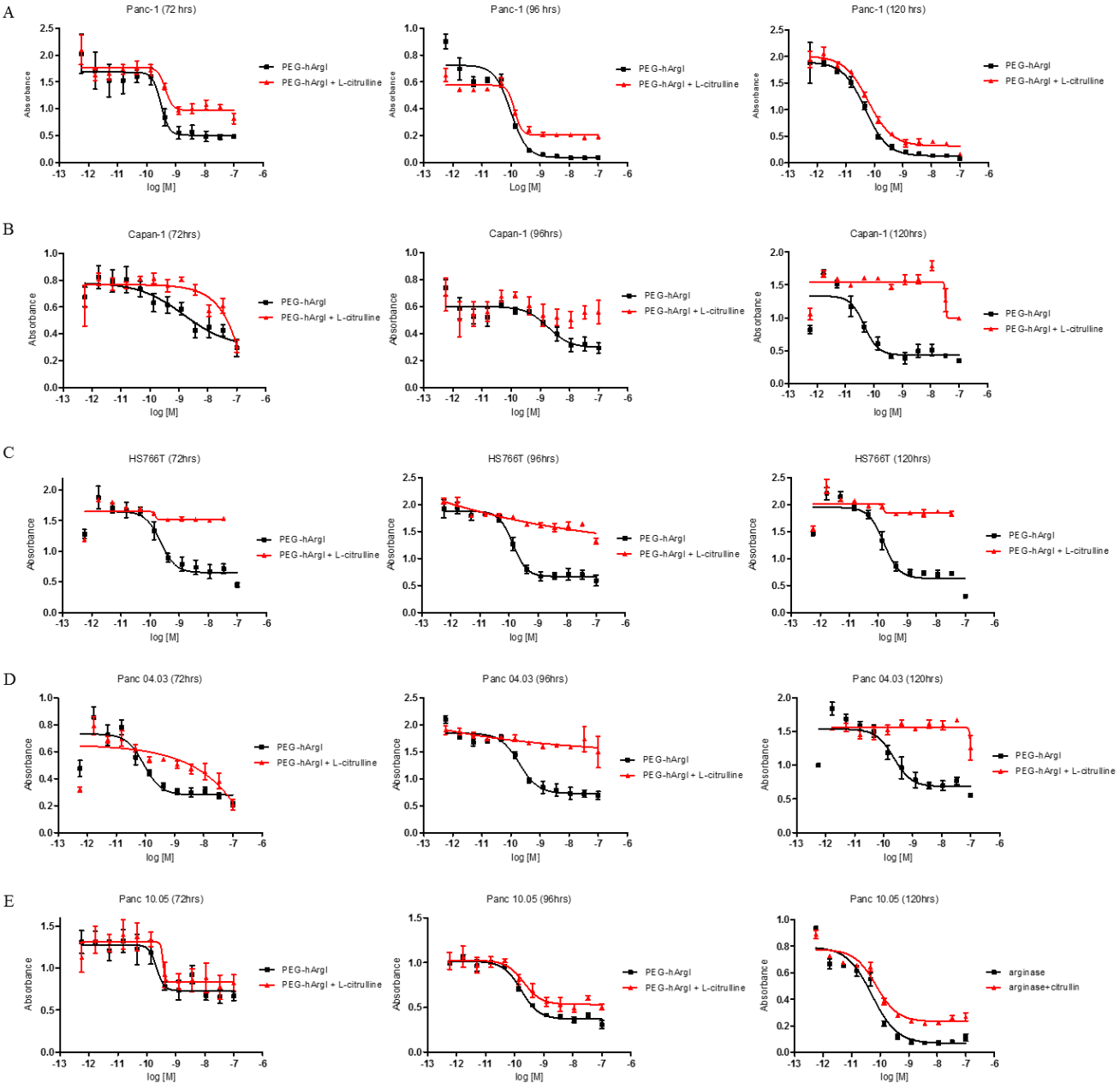


Figure 2. Non-Linear regression curves of the cytotoxicity of HuArgI (Co)- PEG5000 (black square) and HuArgI (Co)-PEG5000 + L-citrulline (red triangle) on human PC cell lines Panc-1 (A), Capan-1 (B), Hs 766T (C), Panc 04.03 (D) and Panc 10.05 (E) at 72, 96 and 120 h post-treatment.

Addition of 11.4 mM L-citrulline completely rescued Hs 766T, Capan-1 and Panc 04.03 cells from arginine depletion-mediated cytotoxicity (IC_{50} >10,000 pM) (Table 2, Figure 2). Therefore, those three cell lines display partial arginine auxotrophy since they can replenish some arginine from the addition of L-citrulline. However, the response of Panc-1 and Panc 10.05 cells to HuArgI (Co)-PEG5000-induced cytotoxicity did not change following the addition of exogenous L-citrulline, thus denoting the complete arginine auxotrophy of these cell lines (Table 2, Figure 2).

Even though Panc-1 and Panc 10.05 were not rescued by the addition of citrulline, an increase in the percent of surviving cells was observed whenever citrulline was supplied. Following the addition of citrulline, the percent of surviving Panc 1 cells increased by approximately 15% at 72 hours, 25% at 96 hours and 12% at 120 hours (Figure 2 A). Similarly, the percent of surviving Panc 10.05 cells increased by approximately 10% at 72 hours and 18% at 96 and 120 hours following the addition of L-citrulline (Figure 2 E). Hence indicating that within the completely auxotrophic cell lines a small percentage of cells may be partially auxotrophic to arginine, underlying the heterogeneity of PC cell lines.

These results show that although all PC cell lines studied display arginine auxotrophy and show sensitivity towards HuArgI (Co)-PEG5000-mediated arginine depletion, the majority of these PC cell lines are partially auxotrophic for arginine, with a subgroup of cell lines demonstrating complete arginine auxotrophy.

3.2- Cell Cycle Analysis

Cytotoxicity data show the presence of a certain percentage of surviving cells following arginine deprivation. In order to determine whether these surviving cells are fully proliferating or whether they are in cell cycle arrest and in order to study the effect of HuArgI (Co)-PEG5000-mediated arginine deprivation on the cell cycle status of PC cell lines, PI-staining was used on flow cytometry after a 72 hours-incubation period with the high concentration of HuArgI (Co)-PEG5000 (10^{-7} M). All the PC cell lines tested showed a G0/G1 cell cycle arrest at 72 hours post-treatment with HuArgI (Co)-PEG5000. The percentages of cell cycle arrest ranged between 7% and 18%, depending on the cell line. The percent cells in G0/G1 increased from approximately 60% , 63%, 58% and 56% of the total population of cells in control samples to approximately 69%, 70%, 76% and 68% of the surviving cell fraction, for Panc-1, Hs 766T, Panc 04.03 and Panc 10.05 cell lines, respectively, after incubation with 10^{-7} M of HuArgI (Co)-PEG5000 for 72 hours (Figure 3).

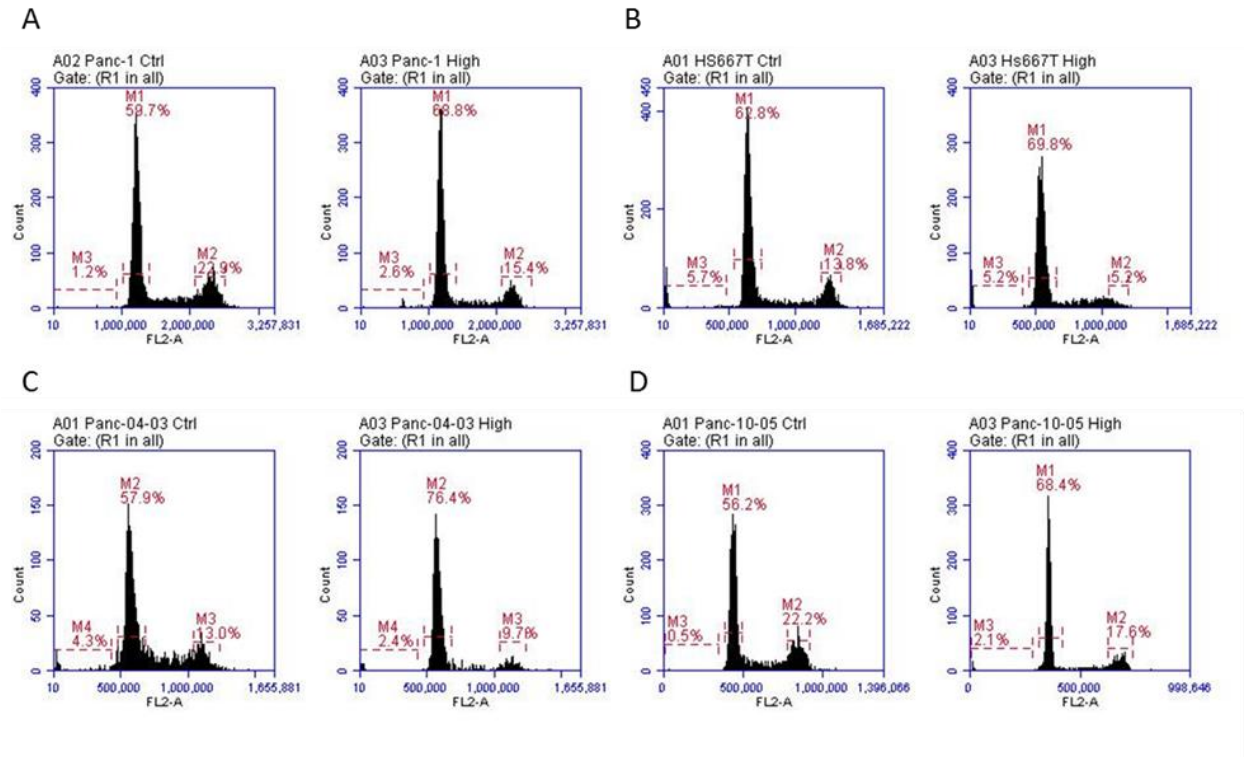


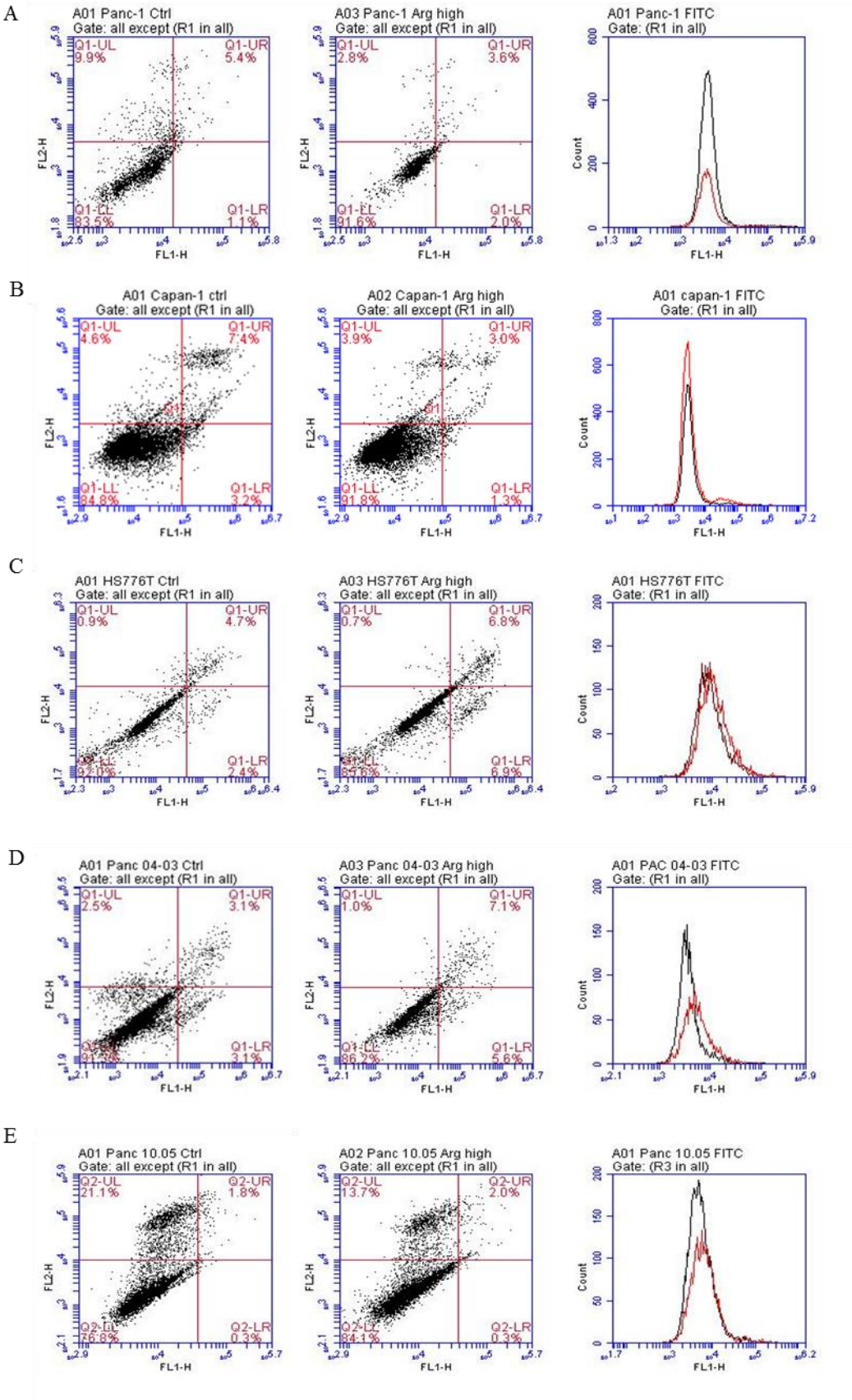
Figure 3. Cell cycle analysis of Panc-1 (A), Hs 766T (B), Panc 04.03 (C) and Panc 10.05 (D) cell lines after treatment with HuArgI (Co)-PEG5000 for 72 h. Control samples are shown to the left and samples treated with 10^{-7} M HuArgI (Co)-PEG5000 are shown to the right. The width versus forward scatter gating (R1) was used on all cells. M1 is used to gate G0/G1 cells, M2 is used to gate G2/M cells and M3 is used to gate pre-G0/G1 (dead) cells. All cell lines demonstrated cell cycle arrest following 72 h of HuArgI (Co)-PEG5000-induced arginine deprivation.

3.3- Analysis of Cell Death

In order to determine the type of cell death seen after HuArgI (Co)-PEG5000-induced arginine deprivation in PC cells, we performed annexin V/PI staining and detection assays for active caspases on PC cell lines after 24 and 48 hours of incubation with the high concentration of HuArgI (Co)-PEG5000 (10^{-7} M) and then analyzed by flow cytometry. The five tested PC cell lines (Panc 04.03, Capan-1, Hs 766T, Panc-1 and Panc 10.05) showed no signs of apoptosis. There was no increase in the percent of cells stained for annexin V (FL1-H), at either 24 or 48 hours after treatment with the high concentration of HuArgI (Co)-PEG5000 (10^{-7} M) in comparison with control cells

in all tested cell lines. Moreover, staining for active caspases showed no sign of caspase activation following treatment in all tested cell lines (Figure 4). In addition, data from the 72 hours proliferation inhibition assay that was performed on Panc-1 cells using HuArgI (Co)-PEG5000 and 20 μM Z-VAD, a cell-permeable pan-caspase inhibitor, demonstrated as well the lack of caspase activation in HuArgI (Co)-PEG5000-induced cell death. The sensitivity of Panc-1 cells to HuArgI (Co)-PEG5000-mediated cytotoxicity remained the same following the addition of 20 μM Z-VAD thus indicating that HuArgI (Co)-PEG5000-triggered cell death is caspase-independent and occurs through a non-apoptotic mechanism (Figure 5).

The lack of caspase activation and the lack of difference in annexin V staining between treated cells and control cells imply that arginine depletion-mediated cytotoxicity and cell death happen through caspase-independent, non-apoptotic mechanisms in all the five tested PC cell lines.


A
A01 Panc-1 Ctrl
Gate: all except (R1 in all)
Q1-UL 9.9%
Q1-UR 5.4%
Q1-LL 83.5%
Q1-LR 1.1%
A03 Panc-1 Arg high
Gate: all except (R1 in all)
Q1-UL 2.8%
Q1-UR 3.6%
Q1-LL 91.6%
Q1-LR 2.0%
A01 Panc-1 FITC
Gate: (R1 in all)
B
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Gate: all except (R1 in all)
Q1-UL 4.6%
Q1-UR 7.4%
Q1-LL 84.8%
Q1-LR 3.2%
A02 Capan-1 Arg high
Gate: all except (R1 in all)
Q1-UL 3.9%
Q1-UR 3.0%
Q1-LL 91.8%
Q1-LR 1.3%
A01 capan-1 FITC
Gate: (R1 in all)
C
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Q1-LR 3.1%
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Q2-LR 0.3%
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Gate: (R3 in all)
FL1-H
FL2-H
Count

Figure 4. Study of the type of cell death following arginine deprivation due to HuArgI (Co)-PEG5000 treatment in PC cell lines by testing for annexin V/PI (represented by both panels to the left) and active caspase staining (represented by the panel to the right) in Panc-1 (A), Capan-1 (B), Hs 766T (C), Panc 04.03 (D) and Panc 10.05 (E) cell lines. All of the tested PC cells that were incubated with 10^{-7} M HuArgI (Co)-PEG5000 for 24 and 48 h showed negative staining with both annexin V (FL1-H) and PI (FL2-H) compared to control cells. Following incubation of the treated and control PC cells with a cell permeable, FITC-conjugated active caspase inhibitor, lack of active caspases was revealed in all HuArgI (Co)-PEG5000 treated cells (red line) in comparison with non-treated cells (black line).

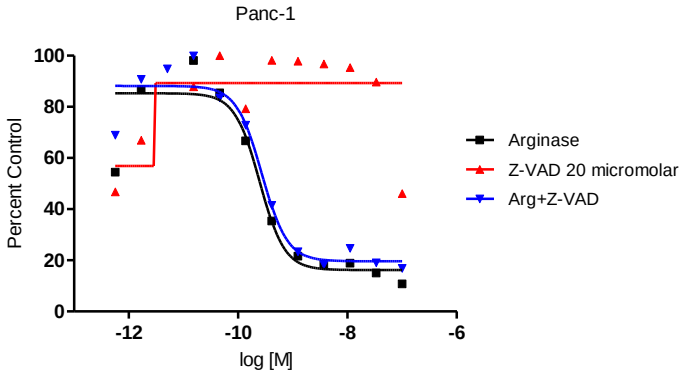


Figure 5. Non-Linear regression curves of the cytotoxicity of HuArgI (Co)-PEG5000 (black square), HuArgI (Co)-PEG5000 + 20 µM Z-VAD (blue triangle) and 20 µM Z-VAD alone (red triangle) on Panc-1 at 72 h. These curves were generated by plotting the percent average absorbance versus the log of the drug concentration.

3.4- Autophagy Assay

The impact of autophagy on HuArgI (Co)-PEG5000-mediated cytotoxicity of PC cells was studied through the use of autophagy inhibitors at early and late stages of the process on Panc-1 and Capan-1 cells. Two autophagy inhibitors were used in our study: chloroquine (CQ), a late stage inhibitor of autophagy which impedes the fusion of autophagosomes with lysosomes, and 3-methyladenine (3-MA), an early stage inhibitor of autophagy which hinders the formation of autophagosomes (Verschooten et al.,

2012). Panc-1 cells were co-incubated with either CQ (100 μM) or 3-MA (500 mM) and with HuArgI (Co)-PEG5000. Treatment with 3-MA didn't have any impact on the response of the cells to arginine deprivation (Figure 6 C). Chloroquine addition resulted in an increased sensitivity to HuArgI (Co)-PEG5000 at an early time point (following incubation for 24 hours). However, the addition of 100 μM chloroquine resulted in a decreased sensitivity to HuArgI (Co)-PEG5000 at later time points (48, 72 and 96 hours) (Figure 6 A). In fact, the percent of surviving Panc-1 cells increased from 37% with HuArgI (Co)-PEG5000 alone to 59% following the addition of chloroquine at 48 hours (Figure 6 A). Moreover, the percent of surviving Panc-1 cells increased from 30% with HuArgI (Co)-PEG5000 alone to 62% following the addition of chloroquine at 72 hours (Figure 6 A). Capan-1 cells were tested for sensitivity to HuArgI (Co)-PEG5000-mediated arginine deprivation in the presence or absence of 10 μM chloroquine at 72 hours. The addition of chloroquine (10 μM) resulted in a decrease with respect to the sensitivity of Capan-1 cells to HuArgI (Co)-PEG5000 at 72 hours incubation: the percent of surviving Capan-1 cells increased from 60% with HuArgI (Co)-PEG5000 alone to 85% following the addition of 10 μM chloroquine at 72 hours (Figure 6 B). Therefore, autophagy was demonstrated to play a protective role, at an early time point, against HuArgI (Co)-PEG5000-mediated cell death in Panc-1 cells since the inhibition of autophagy increased the sensitivity of Panc-1 to arginine deprivation. However, autophagy was shown to be contributing to the cell death induced by arginine depletion at later time points (48, 72 and 96 hours) in this PC cell line since the inhibition of autophagy at these later time points resulted in a decreased sensitivity of Panc-1 cells to HuArgI (Co)-PEG5000-induced arginine depletion.

These results show that arginine deprivation, resulting from the treatment of PC cell lines with HuArgI (Co)-PEG5000, prompts the activation of autophagy, which appears to be playing a protective role at early time points yet at late time points, it seems to be contributing to cell death from arginine starvation-induced cytotoxicity.

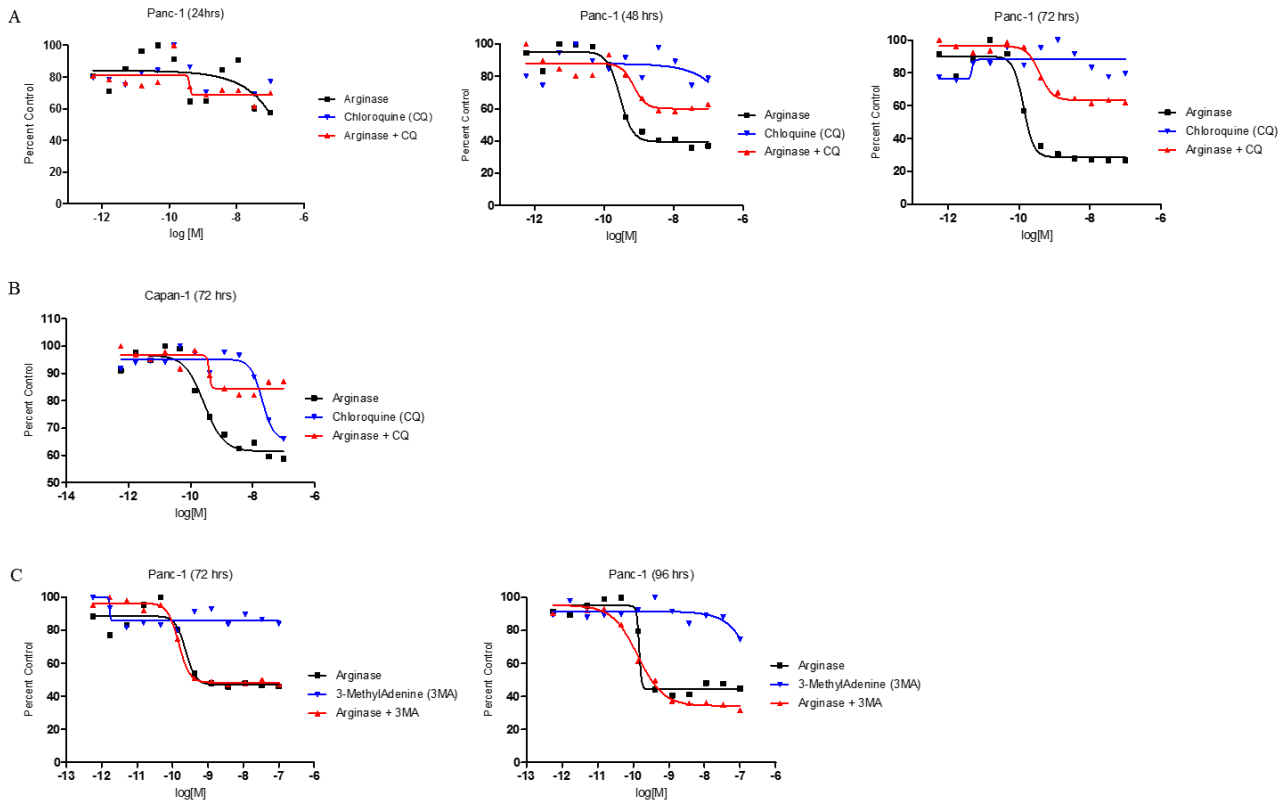


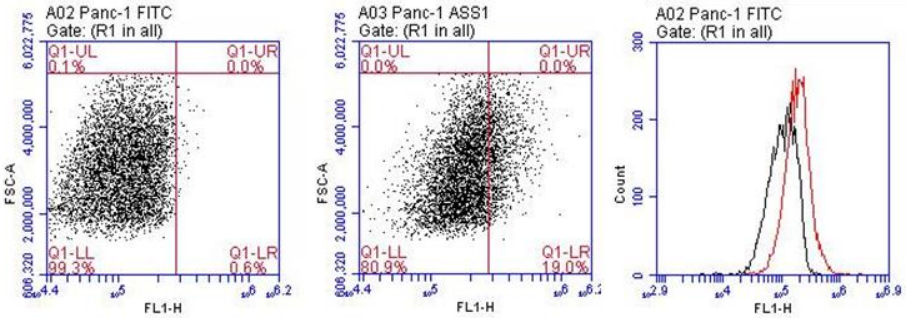
Figure 6. Sensitivity of Panc-1 cells to HuArgI (Co)-PEG5000 in the presence and absence of 100 µM of the autophagy inhibitor chloroquine at 24, 48 and 72 h incubation times (A), and in the presence or absence of 500 mM of the autophagy inhibitor 3-methyladenine at 72 and 96 h incubation times (C). Sensitivity of Capan-1 cells to HuArgI (Co)-PEG5000 in the presence and absence of 10 µM chloroquine at 72 h (B). Non-linear regression curves are shown with HuArgI (Co)-PEG5000 alone (back square) or in combination with chloroquine or 3-methyladenine (red triangle). The cytotoxic effects of chloroquine alone or 3-methyladenine alone are represented by blue triangles.

3.5- Intracellular Staining and Flow Cytometry Analysis

In order to determine the underlying mechanisms that are causing arginine auxotrophy in PC cell lines and that are resulting in their sensitivity to HuArgI (Co)-PEG5000-prompted arginine deprivation, we studied the expression levels of Argininosuccinate Synthetase-1 (ASS-1) in all five tested PC cell lines through the use of intracellular staining and single-cell analysis by flow cytometry. Three out of five PC cell lines, Capan-1, Hs 766T and Panc 04.03, were found positive for ASS-1 expression (Figure 7 B, C and D) with RFI (Ratio of Fluorescence Intensity) values of 4.42, 3.01 and 2.34, respectively (Table 2), confirming the partial arginine auxotrophy observed in these cell lines. The remaining two cell lines, Panc-1 and Panc 10.05, were found negative for ASS-1 expression (Figure 7 A and E), as demonstrated by RFI values of 1.79 and 1.21, respectively, confirming the complete arginine auxotrophy of the cells (Table 2).

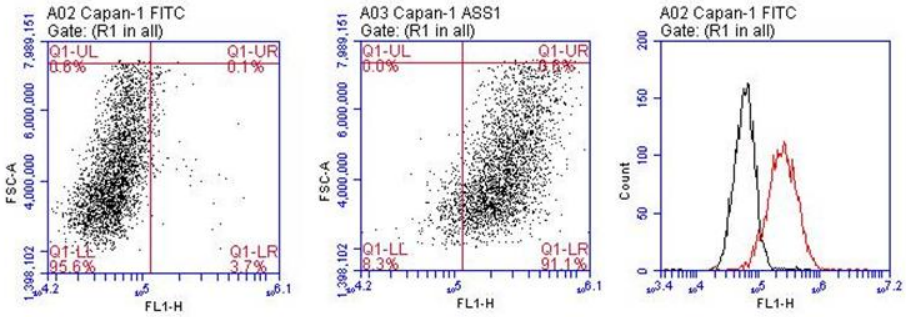
Examination of the percentage of cells that are ASS-1 positive in the panel of PC cell lines (in plots of forward scatter versus FL1-H) showed that 10 to 19% of the cell population in total was positive for ASS-1 expression in the two cell lines that were found to be negative for ASS-1 expression (Panc-1 and Panc 10.05), demonstrating the heterogeneity of these cells and underlying the increase in cell survival following the addition of L-citrulline (Figure 7 A and E). In the three PC cell lines that were found positive for ASS-1 expression, the percentage of cells that are ASS-1 positive ranged from 45% to 91% of the cell population in total (Figure 7 B, C and D).

A



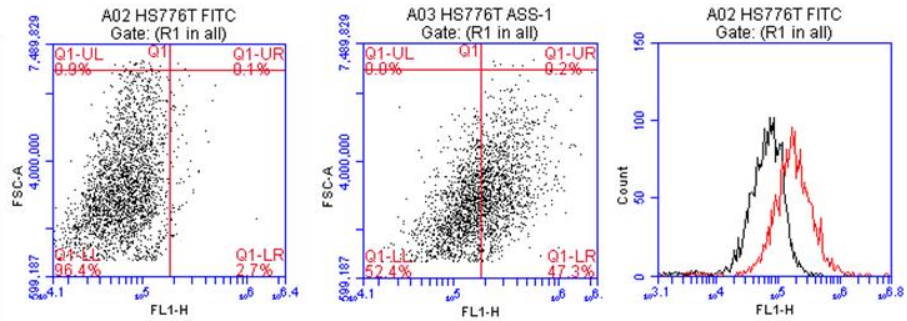
RFI= 1.79

B



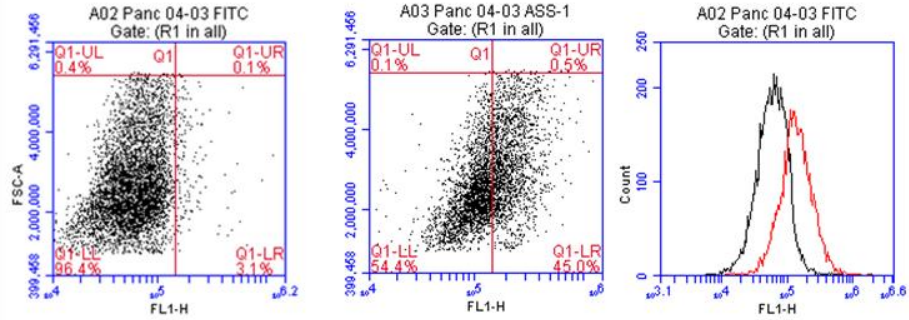
RFI= 4.42

C



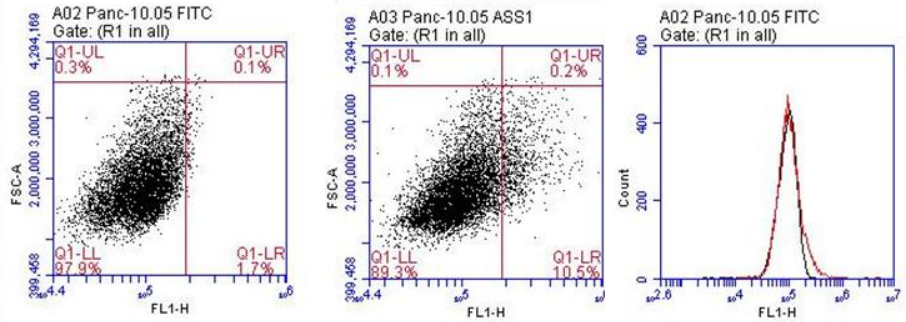
RFI= 3.01

D



RFI= 2.34

E



RFI= 1.21

Figure 7. Flow cytometry data obtained from the single-cell intracellular staining of Argininosuccinate Synthetase-1 (ASS-1) in five PC cell lines, Panc-1 (A), Capan-1 (B), Hs 766T (C), Panc 04.03 (D) and Panc 10.05 (E). Panc-1 and Panc 10.05 were ASS-1 negative as indicated by RFI (Ration of Fluorescence Intensity) values of 1.79 and 1.21, respectively, among cells stained for ASS-1 (black line in the left panels) and cells incubated with an isotype control (red line in the left panels). Capan-1, Hs 766T and Panc 04.03 were positive for the presence of ASS-1 with RFI values > 2. R1 represents the gating of cells on width versus forward scatter.

The three cell lines that were rescued following the addition of 11.4 mM L-citrulline, Capan-1, Hs 766T and Panc 04.03, were all positive for ASS-1 expression, underlying their partial auxotrophy. The expression of ASS-1 in these cell lines implies that the fundamental mechanism behind their sensitivity to HuArgI (Co)-PEG5000-induced arginine deprivation is due, mostly, to their elevated replication rate. The two cell lines that were not rescued by addition of L-citrulline, Panc-1 and Panc 10.05, did not express ASS-1 and were completely auxotrophic for arginine, hence, their sensitivity to HuArgI (Co)-PEG5000-induced arginine deprivation is independent of their replication rate.

CHAPTER FOUR
DISCUSSION

PC is one of the most aggressive types of solid malignancies with pancreatic ductal adenocarcinoma (PDAC) being its most common subtype. The five-year overall survival rate for PDAC following diagnosis is of 6-7% (Chand et al., 2016, Liu et al., 2017). Moreover, PDAC is estimated to rank as the second principal cause of cancer death within the coming decade in the United States (Rabih et al., 2014). There is no efficient therapy for PDAC and surgical resection presents the only hope for a cure. However, most PDAC display distant metastasis or/and local invasion at diagnosis which renders them unresectable (Liu et al., 2017). The median survival time pertaining to metastatic PC is three to six months (Jemal et al., 2006). The asymptomatic nature of the disease at early stages results in late diagnosis. In addition to late diagnosis, this cancer type displays early metastasis, local invasion and resistance to radiotherapy and standard chemotherapeutic regimens. All these properties culminate in an overall dismal prognosis for PC (Jemal et al., 2010; Siegel et al., 2011). Therefore, there's a need to investigate and develop more selective and effective strategies for targeting PC.

In normal human cells, arginine is a non-essential amino acid since normal cells are capable of producing arginine from citrulline via the catalytic activity of ASS and ASL (argininosuccinate lyase) in the urea cycle (Liu et al., 2017). Many studies have established that in normal cells, *ASS-1* is a house-keeping gene and a rate-limiting catalyst for biosynthesis in endothelial cells and hepatocytes, yet in numerous tumors it is absent (Phillips, Sheaff, & Szlosarek, 2013). Moreover, the selectivity of arginine

depletion via HuArgI (Co)-PEG5000 to tumor cells and its prospective use as targeted cancer treatment were highlighted by previous studies which showed the lack of arginine dependence and the resistance to arginine depletion of a subset of normal cells (Agrawal et al, 2012). The potential auxotrophy for arginine of PC cells and the prospective targeting of these PC cells through arginine depletion have not been thoroughly examined yet. The use of bioengineered arginase I in the treatment and control of pancreatic carcinoma xenografts was studied in 2011 by Glazer et al. and the potential use of arginine deprivation by arginine deiminase for the treatment of PC cell lines was studied in 2008 by Bowles et al. Nevertheless, as far as we know, this is the first study to thoroughly investigate the mechanisms of arginine auxotrophy in PC cell lines and the selective targeting of this auxotrophy by HuArgI (Co)-PEG5000.

In this study, we have demonstrated that PC cells are auxotrophic for arginine, hence sensitive to arginine depletion via HuArgI (Co)-PEG5000. All the PC cell lines tested showed sensitivity to HuArgI (Co)-PEG5000 and resulted in IC_{50} values in the pM range, hence denoting the potency of arginine depletion in targeting PC cell lines. The potent cytotoxic effect of HuArgI (Co)-PEG5000 on the panel of PC cell lines was further highlighted by the high percent cell kill ranging between 50% and 76% that was obtained at highest toxin concentration at 72 hours post-treatment. This percent cell kill at highest drug concentration at 72 hours kept increasing as incubation periods using HuArgI (Co)-PEG5000 increased in the completely auxotrophic cell lines, Panc-1 and Panc 10.05 which are ASS-1 deficient, reaching almost complete cell death at 120 hours. A decrease in IC_{50} values was also observed with increasing incubation time in these two completely auxotrophic cell lines. Therefore the potency of arginine deprivation was

found to be time-dependent since increased cytotoxicity was depicted with longer drug incubation times in most of the cell lines, which matches the mechanism of action of arginine deprivation-induced cytotoxicity. A similar pattern was detected in our preliminary findings in AML and GBM, though longer periods of arginine deprivation were not tested in these tumor types.

Furthermore, the cytotoxic effect of arginine deprivation can result from complete arginine auxotrophy of cancer cell lines or from the coupling of partial arginine auxotrophy with fast proliferative rates. Hence, we needed to define the level of auxotrophy and the extent of arginine dependence since tumor cells with partial arginine auxotrophy and rapid proliferation needs might be rescued from arginine deprivation-triggered cytotoxicity through the addition of excessive amounts of exogenous L-citrulline which can replenish the depleted arginine levels inside tumor cells via the urea cycle (Feun et al., 2008). We also had to study the expression levels of ASS-1 in PC cell lines in an attempt to further uncover the underlying mechanisms of arginine auxotrophy (whether partial or complete) which results in sensitivity of PC cells to HuArgI (Co)-PEG5000-triggered arginine depletion. Conventionally, cells totally lacking ASS-1 expression would fail to be rescued by the addition of L-citrulline, therefore they'll be displaying complete arginine auxotrophy. Partial arginine dependence was demonstrated in three out of the five PC cell lines as Hs 766T, Capan-1 and Panc 04.03 cells were completely rescued from arginine depletion-mediated cytotoxicity following the addition of 11.4 mM exogenous L-citrulline. Consequently, those three cell lines showed partial arginine auxotrophy since they could replenish some arginine from the added L-citrulline. This was confirmed by the fact that all three cell lines expressed ASS-1 (RFI > 2). On the other hand, compete arginine auxotrophy was seen in the remaining two cell

lines as Panc-1 and Panc 10.05 cells were not rescued from HuArgI (Co)-PEG5000-induced cytotoxicity following the addition of exogenous L-citrulline at a concentration of 11.4 mM. These completely auxotrophic cell lines revealed negative ASS-1 expression (RFI $\leq$ 2). Hence the absence of ASS-1 expression is in fact the underlying cause for complete arginine auxotrophy which results in the failure of excess L-citrulline in reversing cytotoxicity in the completely arginine-dependent PC cell lines. Therefore, complete arginine auxotrophy is the underlying mechanism of the sensitivity of these cell lines to HuArgI (Co)-PEG5000-induced arginine deprivation. However, partial arginine auxotrophy is attributable to ASS-1 expression and can be illustrated by the ability of excess L-citrulline to rescue the partially auxotrophic PC cell lines. These cell lines that were rescued from arginine depletion-triggered cytotoxicity by additional L-citrulline show sensitivity to HuArgI (Co)-PEG5000-prompted arginine depletion due to their partial arginine dependence in combination with fast replication rates. These results detailed above are significant since so far no real treatment for PC has been identified. They are in fact matching to what our lab has previously reported in acute myeloid leukemia (AML) (Tanios et al., 2013) and in glioblastoma multiforme (GBM) (Khoury et al., 2015). They match as well what other people have published since arginine auxotrophy and sensitivity to arginine depletion have been revealed in numerous tumor types comprising GBM, AML, HCC, RCC, MM, prostate cancer, T-ALL and some mesotheliomas (Wheatley & Campbell, 2003; Kim et al., 2009; Hernandez et al., 2010; Shen et al., 2003; Ensor et al., 2002; Savaraj et al., 2007). Several studies have demonstrated that these cancer types show sensitivity to arginine depletion-triggered cytotoxicity post-treatment with either human arginase or with bacterial arginine deiminase hence proving that arginine depletion in auxotrophic cancers is a novel

targeted therapeutic approach (Khoury et al., 2015; Tanios et al., 2013; Wheatley & Campbell, 2003; Kim et al., 2009; Hernandez et al., 2010; Shen et al., 2003; Ensor et al., 2002; Savaraj et al., 2007). In this study, we examined the mechanisms of arginine auxotrophy in PC cell lines in an attempt to investigate the usefulness of arginine deprivation through HuArgI (Co)-PEG5000 as a potential targeted therapy for PC cell lines presenting arginine auxotrophy.

It is also important to note that we detected an increase in the percent of surviving cells following the addition of citrulline in cell lines that weren't rescued by this supplementation (Panc-1 and Panc 10.05) at 72, 96 and 120 hours incubation times. This observation denotes the heterogeneous nature of PC cell lines as a small percentage of partially auxotrophic cells might be located within cell lines demonstrating complete arginine auxotrophy. This heterogeneity among PC cell lines was further highlighted by the results that we obtained from intracellular staining for ASS-1 and analysis by flow cytometry. Within cell lines that were considered as ASS-1 negative based on RFI values (Panc-1 and Panc 10.05), we found a subpopulation of cells that expressed ASS-1. This ASS-1 positive subpopulation of cells might in fact account for that small percentage of surviving cells that was noticed in cytotoxicity assays of cell lines displaying complete arginine auxotrophy.

Moreover, the role of *ASS-1* as a tumor suppressor was revealed by recent studies that were carried on bladder cancer and sarcoma cell lines (Kobayashi et al., 2010; Allen et al., 2014). In these studies, the tumor suppressor function of *ASS-1* was underlined by its ability to decrease colony forming capability, proliferative and invasive properties of tumor cells and by abolishing the in-vivo growth of tumor xenografts. Actually, it seems that the reprogramming of arginine metabolic pathways in tumors through the

deactivation of *ASS-1* results in added aggressiveness to the tumor phenotype that is driven via exogenous arginine. Hence these recent findings further accentuate that arginine deprivation holds the potential to ameliorate the management of many cancers presenting treatment difficulties by taking advantage of differential expression of *ASS-1* and *ASL*, key enzymes in the urea cycle (Phillips et al., 2013).

Furthermore, we have investigated whether surviving cells from arginine deprivation were still proliferating or whether they have entered cell cycle arrest. A higher fraction of surviving cells was shown in partially auxotrophic cell lines. All the PC cell lines examined displayed G0/G1 cell cycle arrest at 72 hours following incubation with the high concentration of HuArgI (Co)-PEG5000 (10^{-7} M). The percentages of the increase in arrested cells among the surviving populations compared to controls ranged between 7% and 18% in the examined cell lines. Consequently, the impact of HuArgI (Co)-PEG5000-induced arginine deprivation is primarily cytotoxic, however, in the fraction of surviving cells, arginine deprivation does also cause cell cycle arrest.

Our findings with respect to the type of cell death following HuArgI (Co)-PEG5000-prompted arginine depletion revealed seemingly caspase-independent, non-apoptotic cell death mechanisms in all the five studied PC cell lines. All the tested cell lines showed no signs of apoptosis: after 24 and 48 hours of incubation with the high concentration of HuArgI (Co)-PEG5000, they were all negative for annexin V staining and they all showed absence of caspase activation. The lack of caspase activation was further highlighted by results obtained from the 72 hours proliferation inhibition assay that we performed on Panc-1 cells using HuArgI (Co)-PEG5000 and the pan-caspase inhibitor, Z-VAD, whose addition failed to block or decrease arginine deprivation-

induced cell death in these cells. An elevated fragmentation rate among the PC cell lines tested rendered our flow cytometry analysis complicated. We previously obtained and published similar results in AML (Tanios et al., 2013) and GBM (Khoury et al., 2015) cell lines. However, these findings contradict what other people have described concerning the type of cell death observed following arginine depletion in other tumor types (such as hepatocellular carcinoma) or even in the same tumor type since they have attributed the cytotoxicity of arginine depletion to apoptotic mechanisms of cell death (Glazer et al., 2011). We will further investigate in more details the type of cell death in PC cells in response to HuArgI (Co)-PEG5000-prompted arginine deprivation in the future. That being said, our data so far indicated that the type of cell death is not apoptotic.

Additionally, in this study, we are the first to investigate the contribution of autophagy following arginine depletion therapy in PC. Autophagy, a mechanism of cellular destruction based on lysosomal activity, presents a normal cell response to arginine deficiency (Bowles et al., 2008). Therefore, arginine deprivation by HuArgI (Co)-PEG5000 definitely leads to the activation of autophagy. In order to determine whether activated autophagy plays a protective role against drug–induced cytotoxicity or whether it contributes to drug-prompted cell death, we investigated the effect of the inhibition of autophagy on cell response to arginine deprivation at different time points. Hindrance of the fusion of autophagsomes with lysosomes and the resulting inhibition of autophagy in PC cell lines, following the addition of chloroquine, led to a decrease in the sensitivity of PC cells to HuArgI (Co)-PEG5000-triggered arginine depletion at late incubation time points (48, 72 and 96 hours). Hence, autophagy was shown to be

contributing to the cell death prompted by arginine deprivation at later drug incubation time points in the tested PC cell line (Panc-1). Our findings propose that autophagy is activated in response to HuArgI (Co)-PEG5000-triggered arginine depletion in PC cells and that it plays a pro-death role with increased drug incubation times.

We need to further investigate the mechanisms of cell death by autophagy in PC cells following arginine depletion in future experiments. We need to target the autophagy signaling pathway later on and in particular, its critical regulator mTOR. We also need to monitor autophagy through the detection of the protein LC3 by immunofluorescence and immunoblotting. Moreover, we can perform immunostaining and immunofluorescence experiments to track the accumulation of autophagolysosomes under various conditions and at different time points.

To conclude with, in this study, we have demonstrated the dependence of PC cell lines on exogenous arginine and we have highlighted the heterogeneity of these cell lines regarding the degree of arginine auxotrophy and the correlated, differential ASS-1 expression. Consequently, we have shown the potential use of these features to target PC via HuArgI (Co)-PEG5000-triggered arginine deprivation. Moreover, we have revealed cell cycle arrest and non-apoptotic, caspase-independent cell death in PC cell lines as the display of HuArgI (Co)-PEG5000-induced cytotoxicity. We have also demonstrated that autophagy, which is activated following arginine depletion, can be the main cause of cell death at late incubation time points with HuArgI (Co)-PEG5000. That being said, HuArgI (Co)-PEG5000 presents a potential novel, selective and potent treatment for PC.

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