

# LEBANESE AMERICAN UNIVERSITY

Targeting human acute myeloid leukemia cells using human recombinant arginase I (Co)-PEG5000 [HuArgI (Co)-PEG5000]-induced arginine depletion

By

Rita Farid Tanios

A thesis

Submitted in partial fulfillment of the requirements  
for the degree of Master of Science in Molecular Biology

School of Arts and Sciences  
June 2013



LEBANESE AMERICAN UNIVERSITY  
School of Arts and Sciences - Byblos Campus

**Thesis Proposal Form**

Name of Student: Rita Tanios I.D.#: 201102100

Program / Department: M.S Molecular Biology / Natural sciences

On 03/06/13, has presented a thesis proposal entitled:

**“Targeting Human Acute Myeloid Leukemia Cells using Human Recombinant Arginase I (Co)-PEG5000 [HuArgI (Co)-PEG5000]-Induced Arginine Depletion”**

In the presence of the committee members and Thesis Advisor:

Thesis Advisor: Dr Ralph Abi Habib Signature :

Committee Member : Dr Mirvat El Sibai Signature :

Committee Member : Dr Roy Khalaf Signature :

Comments / Remarks / Conditions to Proposal Approval:

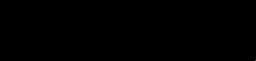
---

---

---

---

Date: 03/06/13

Acknowledged by:   
Dean, School of Arts and Sciences

cc: Department Chair  
School Dean  
Student  
Thesis Advisor



LEBANESE AMERICAN UNIVERSITY  
School of Arts and Sciences - Byblos Campus

**Thesis Defense Result Form**

Name of Student: Rita Tanios I.D.#: 201102100

Program / Department: M.S Molecular Biology / Natural sciences

Date of thesis defense: 03/06/2013

Thesis title: **“Targeting Human Acute Myeloid Leukemia Cells using Human Recombinant Arginase I (Co)-PEG5000 [HuArgI (Co)-PEG5000]-Induced Arginine Depletion”**

**Result of Thesis defense:**

- Thesis was successfully defended. Passing grade is granted
- Thesis is approved pending corrections. Passing grade to be granted upon review and approval by thesis Advisor
- Thesis is not approved. Grade NP is recorded

**Committee Members:**

Thesis Advisor:	Dr Ralph Abi Habib	Signature :	
Member :	Dr Mirvat El Sibai	Signature :	
Member :	Dr Roy Khalaf	Signature :	

Advisor's report on completion of corrections (if any):

Changes Approved by Thesis Advisor: Ralph Abi-Habib Signature:

Date: 23-7-2013 Acknowledge by Dean, School of Arts and Sciences

Cc: Registrar, Dean, Chair, Advisor, Student

**Thesis Approval Form**

Name of Student: Rita Tanios I.D.#: 201102100

Thesis title: **“Targeting Human Acute Myeloid Leukemia Cells using Human Recombinant Arginase I (Co)-PEG5000 [HuArgI (Co)-PEG5000]-Induced Arginine Depletion”**

Program : Master of science in Molecular Biology

Department : Natural sciences

School : **School of Arts and Sciences**

Approved by :

Thesis Advisor: Dr Ralph Abi Habib Signature

Member : Dr Mirvat El Sibai Signature

Member : Dr Roy Khalaf Signature

Date : 03/06/13

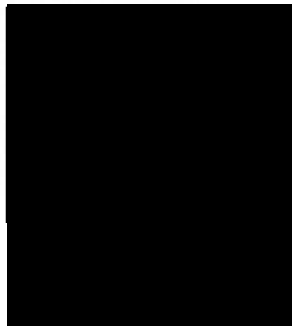
# THESIS COPYRIGHT RELEASE FORM

## LEBANESE AMERICAN UNIVERSITY NON-EXCLUSIVE DISTRIBUTION LICENSE

By signing and submitting this license, you (the author(s) or copyright owner) grants to Lebanese American University (LAU) the non-exclusive right to reproduce, translate (as defined below), and/or distribute your submission (including the abstract) worldwide in print and electronic format and in any medium, including but not limited to audio or video. You agree that LAU may, without changing the content, translate the submission to any medium or format for the purpose of preservation. You also agree that LAU may keep more than one copy of this submission for purposes of security, backup and preservation. You represent that the submission is your original work, and that you have the right to grant the rights contained in this license. You also represent that your submission does not, to the best of your knowledge, infringe upon anyone's copyright. If the submission contains material for which you do not hold copyright, you represent that you have obtained the unrestricted permission of the copyright owner to grant LAU the rights required by this license, and that such third-party owned material is clearly identified and acknowledged within the text or content of the submission. IF THE SUBMISSION IS BASED UPON WORK THAT HAS BEEN SPONSORED OR SUPPORTED BY AN AGENCY OR ORGANIZATION OTHER THAN LAU, YOU REPRESENT THAT YOU HAVE FULFILLED ANY RIGHT OF REVIEW OR OTHER OBLIGATIONS REQUIRED BY SUCH CONTRACT OR AGREEMENT. LAU will clearly identify your name(s) as the author(s) or owner(s) of the submission, and will not make any alteration, other than as allowed by this license, to your submission.

Name: Rita Farid Tanios

Signature:



Date: 03/06/2013

## PLAGIARISM POLICY COMPLIANCE STATEMENT

I certify that I have read and understood LAU's Plagiarism Policy. I understand that failure to comply with this Policy can lead to academic and disciplinary actions against me.

This work is substantially my own, and to the extent that any part of this work is not my own I have indicated that by acknowledging its sources.

Name: Rita Farid Tanios

Signature:



Date: 03/06/2013

# **Targeting Human Acute Myeloid Leukemia Cells Using Human Recombinant Arginase I (Co)-PEG5000 [HuArgI (Co)-PEG5000]-Induced Arginine Depletion**

**Rita Farid Tanios**

## **Abstract**

Acute myeloid leukemia (AML) is the most common leukemia in adults. With current chemotherapy protocols, patients have poor prognosis and more than 10,000 patients die yearly of AML. Therefore new treatment to improve survival rate are needed. In this study, we attempt to target AML cells using a pegylated recombinant human Arginase I cobalt [HuArgI (Co)-PEG5000].

HuArgI (Co)-PEG5000 was tested on ten human AML cell lines. All were sensitive to arginine depletion. Human peripheral blood mononuclear cells (PBMCs) and CD<sub>34</sub><sup>+</sup> bone marrow progenitor blasts (BMPBs) were not sensitive to HuArgI (Co)-PEG5000-mediated arginine depletion demonstrating that arginine deprivation selectively targets AML cells while sparing normal hematopoietic cells. Exogenous L-citrulline addition at low concentrations (1.14 to 114 μM) did not rescue cells from HuArgI (Co)-PEG5000 induced cytotoxicity. However only at the concentrations of 1.14 and 11.4 mM, the addition of exogenous L-citrulline reversed the sensitivity of five AML cell lines to HuArgI (Co)-PEG5000-induced arginine depletion. Furthermore, the sensitivity of AML cells to HuArgI (Co)-PEG5000-induced arginine depletion in the presence or absence of L-citrulline was reliant on the expression levels of argininosuccinate synthetase-1 (ASS1). The cell lines that did not express ASS1 were not rescued by L-citrulline. Addition of the autophagy inhibitor chloroquine increased the sensitivity of AML cell lines to HuArgI (Co)-PEG5000-mediated

arginine depletion indicating that autophagy is activated and plays a protective role in AML cells. Staining for annexin V/PI and active caspases showed an increase in cells stained positively with both annexin V and PI and the absence of caspase activation indicating that HuArgI (Co)-PEG5000-mediated arginine depletion in AML cells induces caspase-independent, nonapoptotic cell death.

In this study, we have demonstrated that AML cells lines are sensitive to arginine depletion and thus are auxotrophic for arginine and can be selectively treated by HuArgI (Co)-PEG5000 hence proving that L-Arginine deprivation is a potential selective and potent selective treatment for AML.

Keywords: HuArgI(Co)-PEG5000, AML, Arginine, Autophagy, ASS, Citrulline, Chloroquine, Auxotrophy



# TABLE OF CONTENTS

<b>I. Introduction.....</b>	<b>1</b>
1.1. The history of AML .....	1
1.2. Pathophysiology and clinical aspects of AML.....	2
1.3. The leukemia initiating cell.....	3
1.4. Molecular mechanism of AML .....	4
1.5. Conventional treatment of AML .....	6
1.6. Potential new therapy: Arginine auxotrophy .....	7
1.6.1. Arginine and urea cycle .....	7
1.6.2. Arginine depletion: ADI and arginase .....	9
1.6.3. Arginine depletion therapy and cancer .....	10
<b>II. Specific aims of the thesis .....</b>	<b>13</b>
<b>III. Materials and Methods: .....</b>	<b>14</b>
3.1. Expression and purification of human recombinant Arginase I cobalt [HuArgI (Co)-PEG5000].....	14
3.2. Cells and cell lines.....	14
3.3. Proliferation inhibition assay (cytotoxicity).....	15
3.4. Autophagy Assays.....	15
3.5. Cell cycle analysis .....	16
3.6. Intracellular Staining and Flow Cytometry Analysis .....	17
3.7. Analysis of Cell Cytotoxicity .....	17
<b>IV. Results .....</b>	<b>19</b>
4.1. Cytotoxicity of HuArgI (Co)-PEG5000.....	19
4.2. Cell cycle effect of HuArgI (Co)-PEG5000:.....	25
4.3. Autophagy: .....	28
4.4. Analysis of Argininosuccinate synthetase 1 (ASS1) levels: .....	30
4.5. Analysis of Cell Death: .....	33
<b>V. Discussion.....</b>	<b>37</b>
<b>VI. References:.....</b>	<b>40</b>

## LIST OF TABLES

Table 1. Prognosis and cytogenetics of AML.....	5
Table 2. Correlation of cytogenetic and molecular genetic data in AML .....	6
Table 3. Sensitivity of human cells to HuArgI (Co)-PEG5000 .....	19

## LIST OF FIGURES

Figure 1. The microscopic appearance of blood as seen by JH Bennett.....	1
Figure 2. Organization of normal and AML hematopoietic system. ....	3
Figure 3. Cytogenetic subgroups of AML - excluding AML-M3 .....	4
Figure 4. Molecular heterogeneity of cytogenetically normal AML. ....	5
Figure 5. Metabolic pathways of urea cycle . ....	8
Figure 6. Advantages of PEGylated proteins.....	10
Figure 7. Non-Linear regression curves of the cytotoxicity of HuArgI (Co)-PEG5000. ....	20
Figure 8. Cell cycle analysis of the different cell lines.....	26
Figure 9. Sensitivity of AML cell lines to HuArgI (Co)-PEG5000 and chloroquine.....	29
Figure 10. Intracellular staining of ASS-1.....	31
Figure 11. Analysis of annexin V/PI and active caspase. ....	34

## GLOSSARY OF TERMS

AA	Amino acid
ADI	Arginine deiminase
ALL	Acute lymphoblastic leukemia
AML	Acute Myeloid Leukemia
ara-C	Cytarabin
ASL	Argininosuccinate lyase
ASS	Argininosuccinate synthetase
ATP	Adenosine triphosphate
CD	Cluster of differentiation
CEBPA	CCAAT/enhancer binding protein alpha
CN-AML	Cytogenetically normal acute myeloid leukemia
Co	Cobalt
FAB	French-American-British
FDA	Food and drug administration
FITC	Fluorescein isothiocyanate
FLT3	fms-related tyrosine kinase 3
G-CSF	Granulocyte colony stimulating factor
GMCSF	Granulocyte macrophage colony stimulating factor
HCC	Hepatocellular carcinoma
HSC	Hematopoietic stem cell
HSCT	Hematopoietic stem cell transplantation
HuArgI	Human L-arginase I
HuArgI (Co)-PEG5000	Human Recombinant Arginase I (Co)-PEG5000

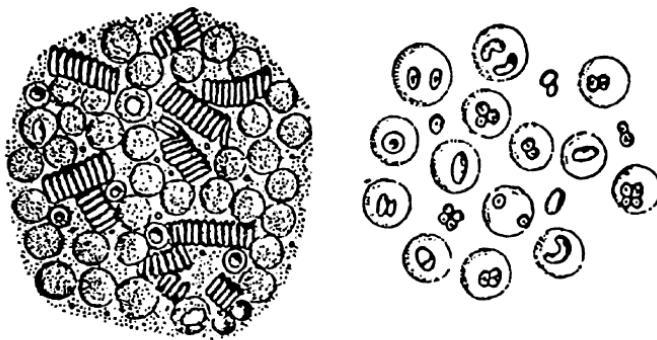
IC <sub>50</sub>	Half maximal inhibitory concentration
LIC	Leukemia initiating cells
MFI	Mean fluorescence intensity
MLL	Mixed-lineage leukemia
Mn <sup>2+</sup>	Manganese
MRC	Medical Research Council
NPM1	Nucleophosmin 1
NRAS	Neuroblastoma RAS viral oncogene homolog
OCT	Ornithine transcarbamyl transferase
PBMB	Progenitor bone marrow blasts
PBMC	peripheral blood mononuclear cell
PEG	Polyethylene glycol
PI	Propidium Iodide
RCC	Renal cell carcinoma
RFI	Ratio of fluorescence intensity
rhArg	Recombinant human arginase I
RUNX1	Runt-related transcription factor 1
SCF	Stem cell factor
WHO	World Health Organization
WT1	Wilms tumor 1

# CHAPTER ONE

## INTRODUCTION

### The history of AML

Leukemia was first published in 1827 when French physician Alfred-Armand-Louis-Marie Velpeau described a case of a 63-year-old male who developed an illness characterized by weakness, fever, hepatosplenomegaly and urinary stones (Wiernik, 2003). He subsequently speculated that the blood of his patient consists of white corpuscles. In 1845, Dr John Hughes Bennett concluded that transformation occurred in the series of patients who died with enlarged spleens and changes in the "colors and consistencies of their blood".<sup>2</sup> Microscopically, he saw round corpuscles which nucleus were generally composed of one large granule and two or three smaller granules (Bennett, 1845). His illustrations (Bennett, 1852) (Figure 1) were the first published drawings of a patient with leukemia (Pillar, 2001). Rudolf Virchow, a German pathologist, used for the first time the name 'leukemia' (Greek: "white blood") in 1847 (Virchow, 1847).



(left) Fresh blood x 250 diameters  
(right) The same after addition of  
acetic acid

**Figure 1.** The microscopic appearance of blood as seen by JH Bennett (Bennett, 1852).

In 1889, Wilhelm Ebstein differentiated the acute and rapidly progressive leukemia, from the more indolent chronic leukemia (Ebstein, 1889). Finally, in 1900, Naegeli described the myeloblast which he differentiated from the lymphoblast. He stated that the presence of myeloblasts or lymphoblasts in the circulating blood formed the diagnosis of leukemia (Naegeli, 1900).

Further understanding of AML occurred rapidly in the last century, and in 2008, AML became the first cancer genome to be fully sequenced (Ley et al., 2008).

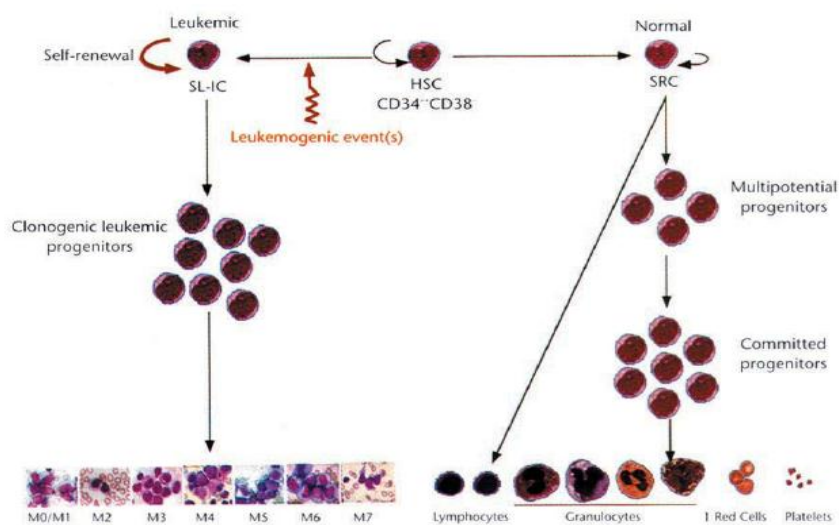
### **Pathophysiology and clinical aspects of AML**

AML is a malignant disease of the bone marrow. It is characterized by the rapid and abnormal growth of the myeloid line of blood cells. These factors lead to a maturational arrest of bone marrow cells in the earliest stages of differentiation resulting in the reduction of normal cell production, and the accumulation of poorly differentiated blasts in the bone marrow, liver or spleen (Estey & Dohner, 2006). Myelodysplastic syndromes, familial syndromes, environmental factors or drug exposure, and *de novo* mutations are implicated in the pathogenesis of AML. The symptoms of AML are related to bone marrow failure which gives rise to anemia, thrombocytopenia and neutropenia and from organ infiltration with leukemic white cells (Lowenberg, Downing, & Burnett, 1999). There are two main classifications for AML; the World Health Organization (WHO) classification where multiple factors are included such as morphology, cytogenetics, molecular genetics, and immunological markers (Vardiman, Harris, & Brunning, 2002) and the French-American-British (FAB) classification that divide AML based on the cell type and the level of maturation (Bennett et al., 1985).

AML is the most common acute leukemia in the adult population with estimated 14,590 new cases in the United States in 2013 (American Cancer Society, 2012). The 5-year survival rate is only 25%; this is mainly due to an increased rate of relapse secondary to the persistence of myeloblasts in the bone marrow. Adverse prognostic factors include increasing age, high white cell count at presentation, secondary AML and unfavorable cytogenetic abnormalities (Dohner et al., 2010).

## The leukemia initiating cell

Tumor cells, solid and leukemic, are clinically, genetically and phenotypically heterogeneous (Dick, 2008). Only a clone of cells, the leukemia initiating cells (LICs), can initiate and maintain tumor growth, while the rest of the tumor is non-tumorigenic and consists the bulk of the tumor (Bonnet & Dick, 1997). LICs are CD34<sup>+</sup> CD38<sup>-</sup> and constitute less than 1% of the total blasts (Lapidot et al., 1984). LICs possess the same fundamental characteristics as a normal hematopoietic stem cell (HSC), such as pluripotency and self-renewal (McCulloch, 1983). Therefore, leukemia is the consequence of a deregulation of HSC (Figure 2).

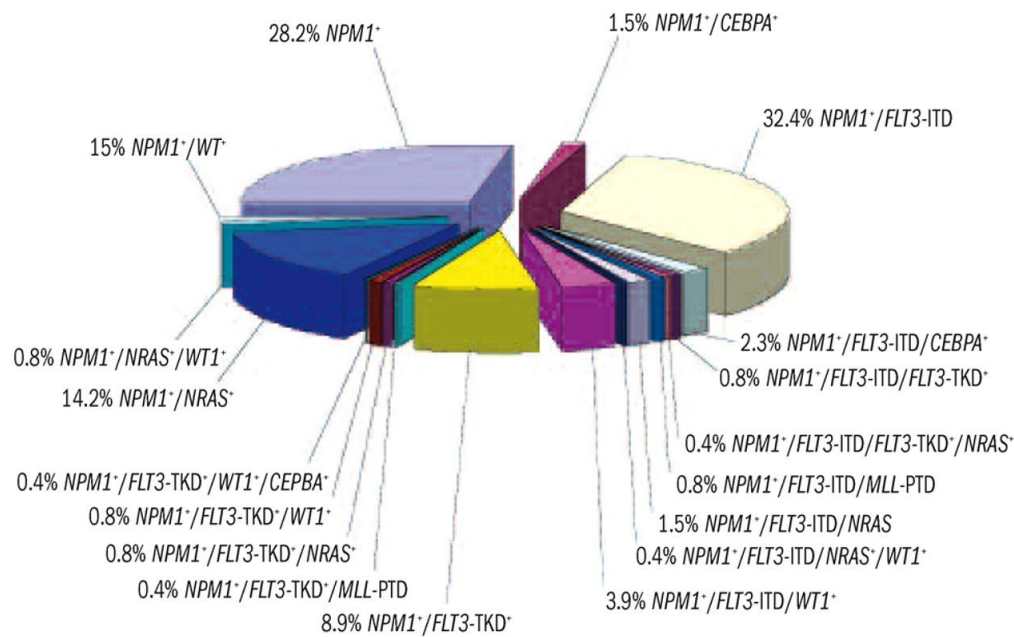


**Figure 2.** Organization of normal and AML hematopoietic system (Bonnet & Dick, 1997).

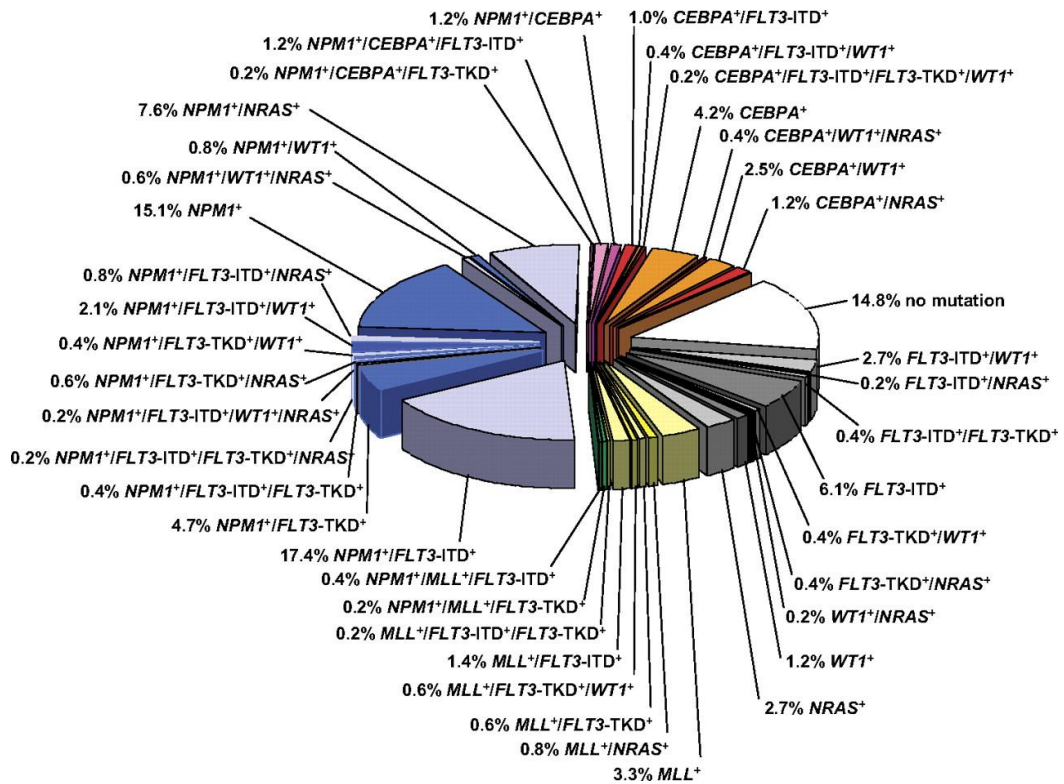


## Molecular mechanism of AML

Chromosome changes such as inversions, balanced translocations, monosomies, trisomies and deletions are detected around 55% of adults with AML. These changes constitute the most important diagnostic, therapeutic and prognostic factors (Mrózek, Heerema, & Bloomfield, 2004). Furthermore, current chemotherapeutic agents are being developed to target these mutations. About 50% of AML patients have a normal cytogenetic profile. This group is called cytogenetically normal acute myeloid leukemia (CN-AML). Several mutations make this group (Figure 3) such as the nucleophosmin 1 (NPM1) gene, the CCAAT/enhancer binding protein alpha (CEBPA) gene, the fms-related tyrosine kinase 3 (FLT3) gene, the mixed-lineage leukemia (MLL) gene, the Wilms tumor 1 (WT1) gene, the neuroblastoma RAS viral oncogene homolog (NRAS) gene, and the runt-related transcription factor 1 (RUNX1) gene (Dohner, 2007; Mrózek et al., 2007). These gene mutations also occur in patients with abnormal karyotypes.



**Figure 3.** Cytogenetic subgroups of AML - excluding AML-M3 (Dohner & Dohner, 2008)



**Figure 4.** Molecular heterogeneity of cytogenetically normal AML (Dohner et al., 2010).

The MRC trial in 1998 has categorized the prognosis of AML patients into 3 groups, good, intermediate and poor depending on cytogenetic abnormalities (Table 1). But recently, a fourth category and new genetic mutations were added (Table 2)

**Table 1.** Prognosis and cytogenetics of AML (Grimwade et al., 1998)

Risk Category	Abnormality
Good	t(8;21), t(15;17), inv(16)
Intermediate	Normal, +8, +21, +22, del(7q), del(9q), Abnormal 11q23, all other structural or numerical changes
Poor	-5, -7, del(5q), Abnormal 3q, Complex cytogenetics

**Table 2.** Correlation of cytogenetic and molecular genetic data in AML (Dohner et al., 2010)

Genetic group	Subsets
Favorable	t(8;21)(q22;q22); <i>RUNX1-RUNX1T1</i>
	inv(16)(p13.1q22) or t(16;16)(p13.1;q22); <i>CBFB-MYH11</i>
	Mutated <i>NPM1</i> without <i>FLT3</i> -ITD (normal karyotype)
	Mutated <i>CEBPA</i> (normal karyotype)
Intermediate-I	Mutated <i>NPM1</i> and <i>FLT3</i> -ITD (normal karyotype)
	Wild-type <i>NPM1</i> and <i>FLT3</i> -ITD (normal karyotype)
	Wild-type <i>NPM1</i> without <i>FLT3</i> -ITD (normal karyotype)
Intermediate-II	t(9;11)(p22;q23); <i>MLL3-MLL</i>
	Cytogenetic abnormalities not classified as favorable or adverse
Adverse	inv(3)(q21q26.2) or t(3;3)(q21;q26.2); <i>RPN1-EVII</i>
	t(6;9)(p23;q34); <i>DEK-NUP214</i>
	t(v;11)(v;q23); <i>MLL</i> rearranged
	-5 or del(5q); -7; abnl(17p); complex karyotype

## Conventional treatment of AML

The standard therapy for AML is divided into induction and consolidation. With induction chemotherapy, the number of leukemic cells is reduced and patients achieve a complete remission. The aim of consolidation therapy is to eradicate any residual disease and achieve a cure.

Cytarabine (ara-C) and an anthracycline are usually given during the induction phase (Dohner et al., 2010).

Consolidation therapy includes intensive chemotherapy, prolonged maintenance treatment, and autologous or allogeneic hematopoietic stem cell transplantation (HSCT) (Löwenberg, Griffin, & Tallman, 2003). Although treatment modalities have resulted in a high rate of complete remission, there are still an estimated 10,200 deaths yearly and a 5-year relative survival of 25% (American Cancer Society, 2012). Thus the need for alternative approaches using more selective treatment modalities for targeting AML myeloblasts. One such approach consists of targeting

potential arginine auxotrophy of AML cells by inducing arginine deprivation using a pegylated human recombinant Arginase I cobalt [HuArgI (Co)-PEG5000] (Stone et al., 2010).

## **Potential new therapy: Arginine auxotrophy**

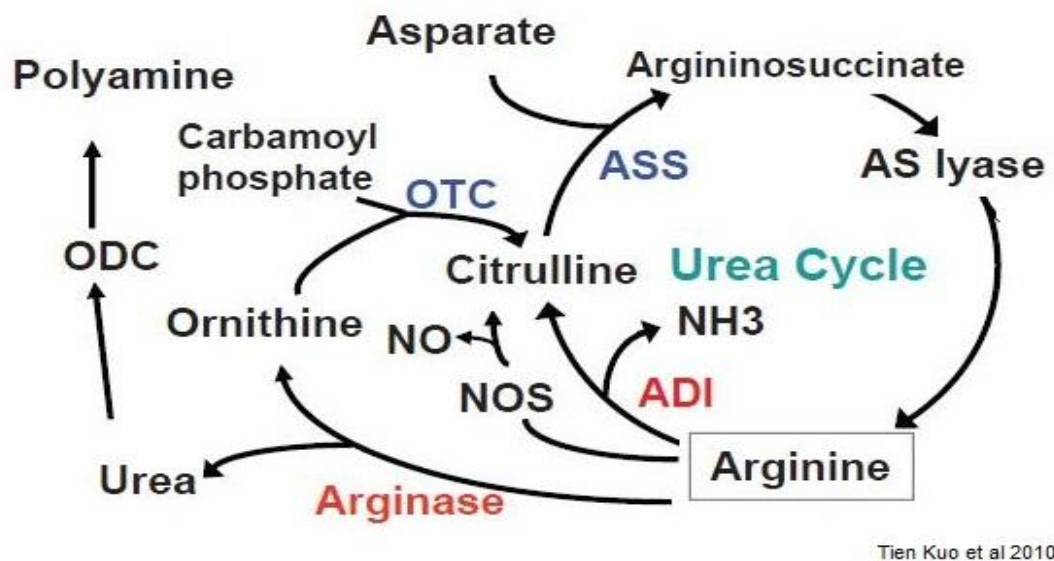
### **Arginine and urea cycle**

L-Arginine is a basic amino acid (AA). It is considered a semi-essential amino acid or conditionally essential amino-acid since its need and production varies depending on the developmental age and state of disease, stress, and proliferating cells (Beaumier, Castillo, Yu, Ajami, & Young, 1996). It is mainly a product of diet, turnover of proteins, and de-novo synthesis (Morris, 2004).

L-Arginine is involved in many important pathways such as nitric oxide and creatine production, nucleotides, proline and glutamate biosynthesis. It has as well an immunomodulatory effect including the stimulation of T and natural killer cell activity, it stimulates the release of insulin-like growth factor 1, growth hormone and prolactin (Feun et al, 2008).

In adults, de-novo synthesis of L-Arginine involves the intestinal-renal axis (Wu & Morris, 1998). Citrulline is synthesized from glutamine, glutamate and proline in the mitochondria of enterocytes. It is then released from the small intestine, and taken up by the proximal renal tubule for arginine production. L-arginine is synthesized from L-citrulline in two steps via the urea cycle. The argininosuccinate synthetase (ASS) catalyzes the conversion of aspartic acid and L-citrulline to argininosuccinate which is then further converted to L-arginine and fumaric acid by argininosuccinate lyase (ASL) (Kuo, Savaraj, & Feun, 2010). Citrulline is converted into Arginine in nearly all cells such as endothelial cells, macrophages, enterocytes, adipocytes and neurons

(Wu & Morris, 1998). L-arginine is then degraded to L-ornithine and urea by arginase and to citrulline and ammonium by arginine deiminase (ADI), a bacterial enzyme. L-ornithine is converted back to L-citrulline by ornithine transcarbamyl transferase (OTC) and recycled to arginine by ASS/ASL (Kuo et al., 2010).



**Figure 5.** Metabolic pathways of urea cycle relevant to Arg deprivation strategy using PEG-Arginase (Kuo et al., 2010).

ASS is ubiquitous in normal cells. However its expression differ depending on the cell type and extracellular factors such as cyclic AMP, IL-1, TNF- $\alpha$ , TGF- $\beta$ 1 and glutamate (Brasse-Lagnel et al., 2003; Brasse-Lagnel et al., 2005; Guei et al., 2008)

ASS levels vary significantly in tumors. High levels of ASS are found in colon, stomach and ovarian cancer, while undetectable levels are found in mesotheliomas, melanoma, renal cell carcinoma, and hepatocellular carcinoma (HCC). Arginine is considered an essential amino-acid in tumors that depend on an abundant arginine reserve thus ASS negative cancers cannot survive in the absence of arginine; hence they are called auxotrophic for arginine (Dillon et al, 2004).

Two proteins were developed to achieve arginine deprivation, ADI and arginase.

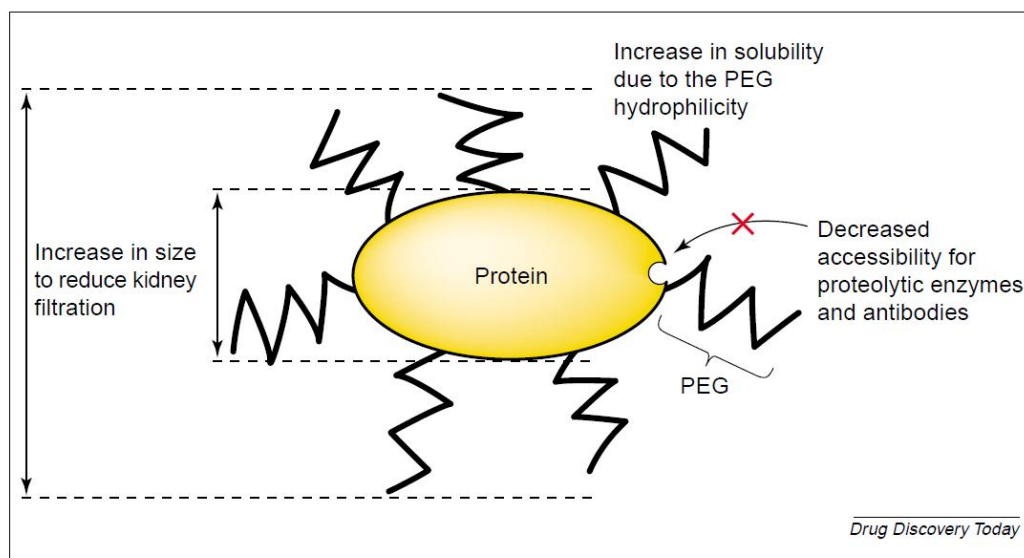
### **Arginine depletion: ADI and arginase**

Arginine depletion is achieved using two enzymes, arginine deiminase and arginase.

ADI is only secreted by microorganisms. It has two disadvantages; it is highly immunogenic in mammals especially after repeated injections and it has a short circulating half-life (Ensor, Holtsberg, Bomalaski, & Clark, 2002).

Arginase occurs in mammalian liver and other tissues. It degrades arginine in the urea cycle. Arginase has been described to have anti-tumor effect since the 1950s (Bach & Swaine, 1965). Arginase has, as well, several shortcomings such as short half-life, low affinity for arginine at physiologic pH, and an optimum pH of 9.6 (Savoca, Davis, van Es, McCoy, & Palczuk, 1984).

Because of the short half-life of native arginase (< 30 minutes), it is very difficult to achieve proper therapeutic concentrations in humans. Thus pegylation is needed to protect arginase from the degrading enzymes and to reduce its immunogenicity (Figure 6). First described in the 70s, pegylation modifies the protein extensively with maintenance of its activity. It is the process whereby polyethylene glycol chains are covalently attached to protein or peptide molecules. PEG is 20,000 MW, and it is approved by FDA. It is not toxic, not immunogenic, and is eliminated by the kidneys (Harris & Chess, 2003).



**Figure 6.** Advantages of PEGylated proteins (Veronese & Pasut, 2005).

Under physiological conditions, human arginase I (HuArgI), normally conjugated to two  $Mn^{2+}$  ions, has reduced activity because of a minimal catalytic activity due to low saturation constant (Glazer et al., 2011). Furthermore, in serum, the protein rapidly loses the  $Mn^{2+}$  ions, which inactivates the enzyme and results in a half-life of 4.8 hours (Stone et al., 2010). Replacing  $Mn^{2+}$  with  $Co^{2+}$  (Co-hArgI) lead to an increase in the enzyme activity and in serum stability (Stone et al., 2010).

### **Arginine depletion therapy and cancer**

Amino-acid depletion is now being used as treatment for some of cancers with L-asparaginase being an approved treatment for acute lymphoblastic leukemia (ALL) (Hawkins et al., 2004; Muller et al., 1998). Several other cancers such as HCC, RCC, melanoma, prostate and head and neck cancers are being evaluated for arginase and / or arginine deiminase therapy.

Leukemia

L-asparaginase is used for treatment of patients with ALL (Hawkins et al., 2004; Muller et al., 1998). However, arginine depleting therapy was found to be 100 times more potent than asparaginase (Gong et al., 2000). Recently, Hernandez et al. documented that pegylated arginase I (peg-Arg I) impairs the proliferation of malignant T cells (Hernandez et al., 2010).

## Melanoma

In 2002, Ensor et al. found that 23 melanoma cell lines were sensitive to ADI with an IC<sub>50</sub> ranging between < 0.01 and 0.3 ug/ml (Ensor, Holtsberg, Bomalaski, & Clark, 2002). In 2005, Ascierto et al. reported the results of phase I and phase II studies with 15 and 24 metastatic patients respectively (Ascierto et al., 2005). Six of 24 patients (25%) responded to treatment with five showing a partial response and one complete response. All six patients had prolonged survival. Human arginase was tested later by Lam et al. They found that recombinant human arginase I (rhArg) inhibits the growth of melanoma cell lines in vitro and in vivo (Lam et al., 2011).

## Hepatocellular carcinoma (HCC)

Similar to melanoma, ADI in HCC. Phase I/IIa trial demonstrated that PEG-ADI is effective in treating some patients with advanced HCC (Glazer et al., 2010). In addition to ADI, arginase also proved to be effective in inhibiting HCC in vitro and in vivo (Cheng et al., 2007; Tsui et al., 2009).

## Prostate cancer



Both ADI and arginase were found to cause a significant cytotoxic effect on prostate cancer (Hsueh et al., 2012; Kim et al., 2009).

#### Other types of cancers

In addition to melanoma, leukemia, HCC and prostate cancer, several other tumors were tested against arginine depletion therapy such as RCC, head and neck cancers and mesothelioma. Renal cell carcinoma was found to be highly sensitive to ADI (Yoon et al., 2004). A phase II study is planned to evaluate the effect of arginine depleting therapy on malignant mesothelioma. This clinical trial was planned after Szlosarek demonstrated the sensitivity of mesothelioma cell lines to arginine depletion treatment (Szlosarek et al., 2006). Finally, ADI treatment was used for head and neck cancers to which they were sensitive (Huang et al., 2012).

## **CHAPTER TWO**

### **SPECIFIC AIMS OF THE THESIS**

The aims of the study are to:

- ✓ Test potential arginine auxotrophy of AML cells using Human Recombinant Arginase I (Co)-PEG5000 [HuArgI (Co)-PEG5000]
- ✓ Characterize the response of AML cells to [HuArgI (Co)-PEG5000]-induced arginine depletion.
- ✓ Evaluate the combination therapy: HuArgI (Co)-PEG5000 with Cytarabine or Doxorubicin
- ✓ Check the effect of citrullin addition to cell lines
- ✓ Assess the effect of HuArgI (Co)-PEG5000 on cell cycle and the type of cell death
- ✓ Assess the level of ASS expression

## **CHAPTER THREE**

### **MATERIALS AND METHODS**

#### **Expression and purification of human recombinant Arginase I cobalt [HuArgI (Co)-PEG5000]**

Pegylated human recombinant Arginase I cobalt [HuArgI (Co)-PEG5000] was expressed and purified as described previously (Stone et al., 2006). Chloroquine, and L-citrulline were purchased from Sigma-Aldrich.

#### **Cells and cell lines**

Human AML cell lines HL60, U937, ML1, ML2, Mono-Mac-1, Mono-Mac-6, KG-1, TF1-vRaf, TF1-vSrc and TF1-HaRas were grown as described previously (Ramage et al., 2003).

Human CD<sub>34</sub><sup>+</sup> progenitor bone marrow blasts (PBMBs) were purchased from (Lonza, Basel, Switzerland) and grown in StemlineII<sup>TM</sup> hematopoietic stem cell expansion medium (Sigma-Aldrich) supplemented with 10 ng/ml IL-3, 5 ng/ml granulocyte colony stimulating factor (G-CSF), 5 ng/ml granulocyte macrophage colony stimulating factor (GMCSF), 10 ng/ml stem cell factor (SCF), 10 µg/ml insulin and 100 µg/ml transferrin (Sigma-Aldrich), as described previously (Blair, Hogge, & Sutherland, 1998).

Human peripheral blood mononuclear cells (PBMCs) were isolated using Ficoll-Paque gradient. Briefly, 10 ml of blood were diluted 3-fold in dilution buffer (phosphate buffered saline, 2 mM EDTA), layered, carefully, over 10 ml ficoll-paque and centrifuged at 4500 rpm for 20 min. The layer corresponding to PBMCs was

isolated, transferred to 45 ml of dilution buffer, centrifuged twice at 3000 rpm for 20 min at 20°C and the resulting pellet re-suspended in 10 ml growth media.

### **Proliferation inhibition assay (cytotoxicity)**

Sensitivity of AML cell lines, PBMCs and Human CD<sub>34</sub><sup>+</sup>PBMBs to HuArgI (Co)-PEG5000, in the presence and absence of excess exogenous L-citrulline, was determined using a proliferation inhibition assay as described previously (Abi-Habib et al., 2005). Briefly, aliquots of 10<sup>4</sup> cells/well (10<sup>5</sup> cells/well for PBMCs), in 100 µl cell culture medium, were plated in a flat-bottom 96-well plate (Corning Inc. Corning, NY). L-citrulline was added to cells at concentrations of 1.14 µM, 11.4 µM, 114 µM, 1.14 mM and 11.4 mM. This was followed by the addition of 50 µl HuArgI (Co)-PEG5000 in media to each well to yield concentrations ranging from 10<sup>-7</sup> to 10<sup>-13</sup> M. When Cytarabine or Doxorubicin was used, it was added as described for HuArgI (Co)-PEG5000 but in concentrations ranging from 10<sup>-3</sup> to 10<sup>-9</sup> M. Following a 24, 48 or 72 h incubation at 37°C/5% CO<sub>2</sub>, 50 µl of XTT cell proliferation reagent (Roche, Basel, Switzerland) were added to each well and the plates incubated for another 4 h. Absorbance was then read at 450 nm using a microplate reader (Thermo Fisher Scientific, Waltham, MA). Nominal absorbance and percent maximal absorbance were plotted against the log of concentration and a non-linear regression with a variable slope sigmoidal dose-response curve was generated along with IC<sub>50</sub> using GraphPad Prism 5 software (GraphPad Software, San Diego, CA). All assays were performed at least twice with an inter-assay range of 30% or less for IC<sub>50</sub>.

### **Autophagy Assays**

The potential role played by autophagy in arginine depletion-induced cytotoxicity of AML cells was tested by incubating AML cells with HuArgI (Co)-PEG5000 alone

and in combination with the autophagy inhibitor chloroquine. Briefly, aliquots of  $10^4$  cells/well, in 100  $\mu$ l cell culture medium, were plated in a flat-bottom 96-well plate (Corning Inc. Corning, NY). Chloroquine was added to a subset of wells at a concentration of 10  $\mu$ M and 100 mM, respectively. This was followed by the addition of 50  $\mu$ l HuArgI (Co)-PEG5000 in media to each well to yield concentrations ranging from  $10^{-7}$  to  $10^{-13}$  M and the plates were incubated for 48 h at 37°C/5% CO<sub>2</sub>. This was followed by the addition of 50  $\mu$ l XTT cell proliferation reagent (Roche, Basel, Switzerland) to each well and the plates were incubated for another 4 h. Absorbance was then read at 450 nm using a 96-well plate reader (Thermo Fisher Scientific, Waltham, MA). Data was analyzed using GraphPad Prism V software (GraphPad Software, San Diego, CA) and the IC<sub>50</sub> (inhibitory concentration 50) of HuArgI (Co)-PEG5000 alone or in the presence of chloroquine were compared.

### **Cell cycle analysis**

The impact of [HuArgI (Co)-PEG5000]-induced arginine depletion on the cell cycle of AML cells was determined using Propidium Iodide (PI)-staining on flow cytometry. Briefly, cells incubated with 3 different concentrations of HuArgI (Co)-PEG5000 (100000, 3000 and 45 pM) or media alone in flat-bottom 96-well plates (Corning Inc. Corning, NY) for 24 and 48 h at 37°C/5% CO<sub>2</sub>, were harvested and fixed in 70% ethanol for a minimum of 24 h, at -20°C. Cells were then incubated in 500  $\mu$ l PI staining solution (50  $\mu$ g/ml) for 40 min at 37°C. Samples were then read on a C6 flow cytometer (BD Accuri, Ann Arbor, MI) and total cell DNA content was measured on FL2-A. Percent of cells in G<sub>0</sub>/G<sub>1</sub>, S and G<sub>2</sub>/M phase was determined in control cells and in cells treated with the 3 different concentrations of HuArgI (Co)-

PEG5000 (100000, 3000 and 45 pM) following gating for the cell population on width versus forward scatter.

### **Intracellular Staining and Flow Cytometry Analysis**

Expression levels of Argininosuccinate Synthetase 1 (ASS-1) were assessed using flow cytometry as described previously (Kassab et al., 2013). Approximately  $3 \times 10^6$  cells were fixed in 70% ethanol for 15 min. Cells were then incubated with a 1/100 dilution of anti-ASS-1 mouse monoclonal antibodies (Sigma, Danvers, MA) in antibody binding buffer containing 0.05% Triton-X 100, for 1 h at 37°C, followed by a 30-minute incubation with a 1/100 dilution of a FITC-conjugated rabbit anti-mouse polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA). Isotypic control consisted of fixed cells incubated with a 1/100 dilution of mouse IgG (Sigma, Danvers, MA) and a FITC-conjugated rabbit anti-mouse polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA). Samples were then washed once, re-suspended in binding buffer and analyzed using a C6 flow cytometer (BD Accuri, Ann Arbor, MI). The presence of ASS-1 was analyzed and compared with that of the isotypic control. Positivity for the presence of ASS-1 was determined using the ratio of fluorescence intensity (RFI) between the mean fluorescence intensity (MFI) of the stained cells and the MFI of the isotypic control.  $RFI \geq 3.0$  was considered positive.

### **Analysis of Cell Cytotoxicity**

Determination of apoptotic versus non-apoptotic cell death was carried out using an Annexin V-fluorescein Isothiocyanate (Annexin V-FITC) and Propidium Iodide (PI) labeled apoptosis/necrosis detection kit (Abcam, Cambridge, MA) and a FITC-conjugated active caspase inhibitor (ApoStat Apoptosis Detection Kit, R&D Systems, Abingdon, England) on flow cytometry. Briefly,  $10^4$  cells/well were plated

in 100  $\mu$ l media in a flat-bottom, 96-well plate and were incubated with either 100  $\mu$ l of medium alone (control cells) or medium containing three different concentrations of HuArgI (Co)-PEG5000 (as described above under cell cycle analysis) for 24 and 48 h at 37°C/5% CO<sub>2</sub>. Cells were then harvested and incubated with a FITC-conjugated annexin V antibody (2.5 mg/ml and PI (5 mg/ml) in antibody binding buffer for 45 min at 37°C or incubated with 0.5  $\mu$ g/ml of apostat for 30 min then harvested. Cells were then read using a C6 flow cytometer. Annexin V/PI data was analyzed on FL1-H versus FL2-H scatter plot and active caspases were detected on FL1-H. Unstained cells were used as negative control. Cells had to show positive annexin V staining, negative PI staining and positive active caspase staining to be considered apoptotic, while cells positive for both annexin V and PI staining and negative for active caspase staining were considered non-apoptotic.

## CHAPTER FOUR

### RESULTS

#### Cytotoxicity of HuArgI (Co)-PEG5000

We tested the cytotoxicity of pegylated human recombinant Arginase I cobalt [HuArgI (Co)-PEG5000] on a panel of 10 human AML cell lines. All the AML cell lines tested were sensitive to the HuArgI (Co)-PEG5000-mediated arginine depletion with  $IC_{50} = 14-552$  pM and percent cell kill at highest concentration  $> 75\%$ , at 48 h post-treatment. (Table 3, Figure 7D).

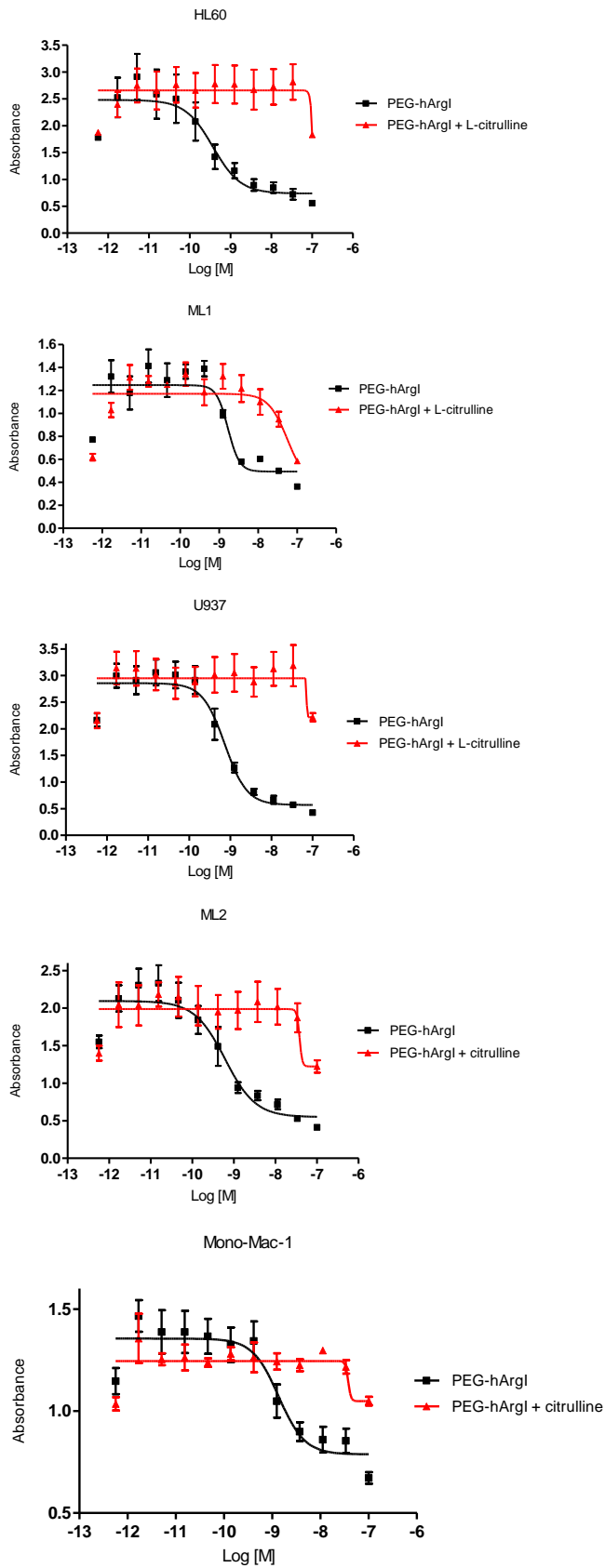
**Table 3.** Sensitivity of human AML cell lines, PBMCs and CD34+ bone marrow progenitor blasts (BMPBs) to HuArgI (Co)-PEG5000 in the presence and absence of exogenous L-citrulline (1.14 $\mu$ M and 11.4 mM).

Cells and cell lines	HuArgI (Co)- PEG5000	HuArgI (Co)- PEG5000 + L- cit (1.14 $\mu$ M)	HuArgI (Co)- PEG5000 + L- cit (11.4mM)
<b>Human AML cell lines</b>			
U937	58.0 pM	61.0 pM	>10,000 pM
ML1	200 pM	246 pM	>10,000 pM
TF1-VRaf	207 pM	260 pM	304 pM
TF1-HaRas	284pM	253 pM	248 pM
KG-1	320 pM	NA	NA
ML2	341 pM	335 pM	>10,000 pM
Mono-Mac-1	400 pM	413 pM	>10,000 pM
Mono-Mac-6	488 pM	432 pM	463 pM
TF1-VSrc	552 pM	586 pM	598 pM
HL60	722 pM	756 pM	>10,000 pM
<b>Normal human cells</b>			
Peripheral Blood Mononuclear Cells	>10,000 pM	>10,000 pM	NA
CD <sub>34</sub> <sup>+</sup> Progenitor Bone Marrow Blasts	>10,000 pM	> 10,000 pM	NA

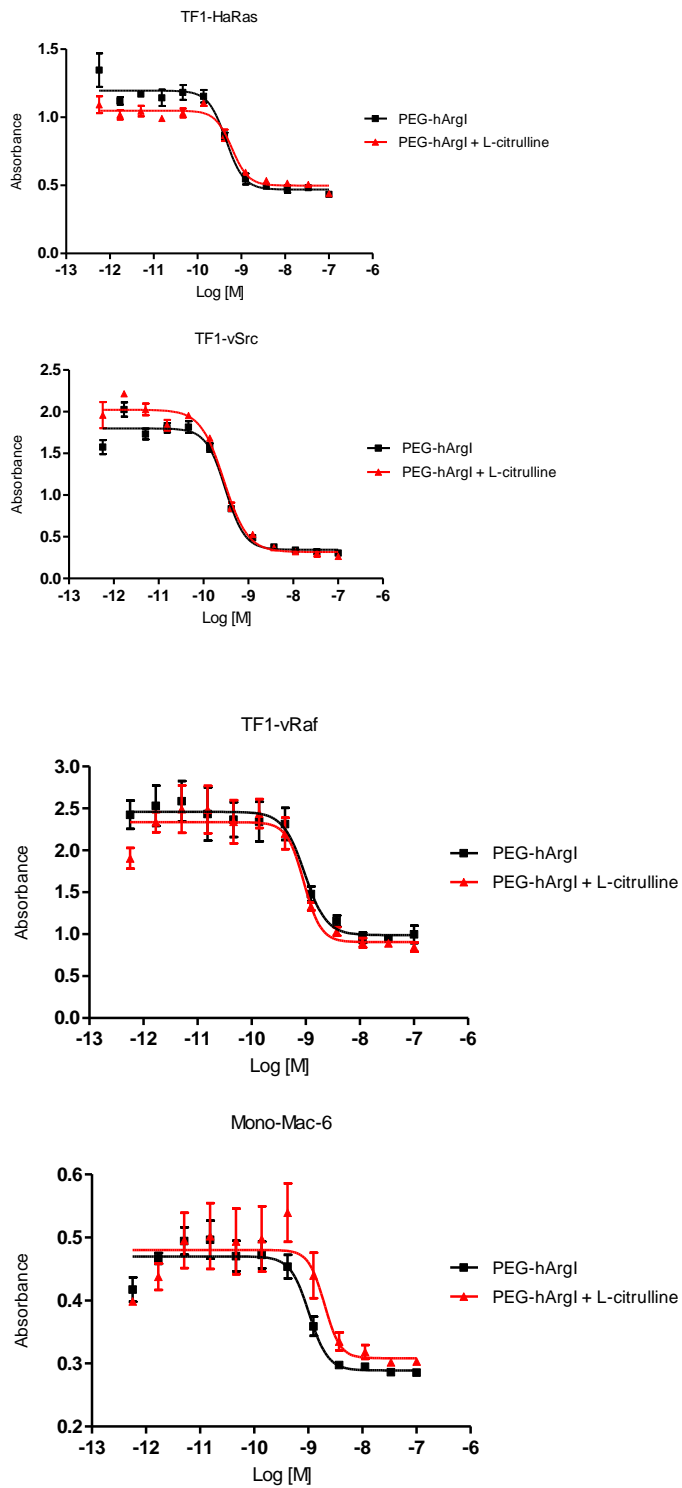
NA: Not available



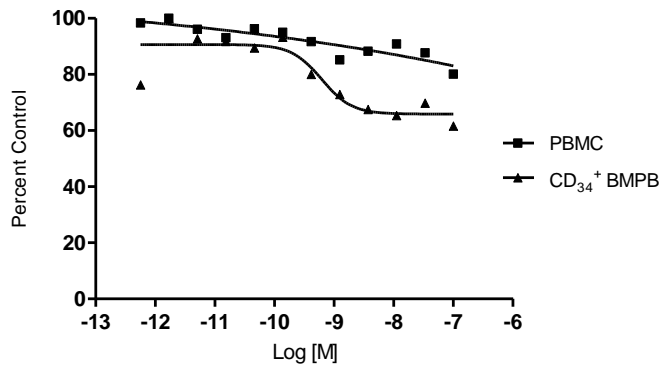
A)



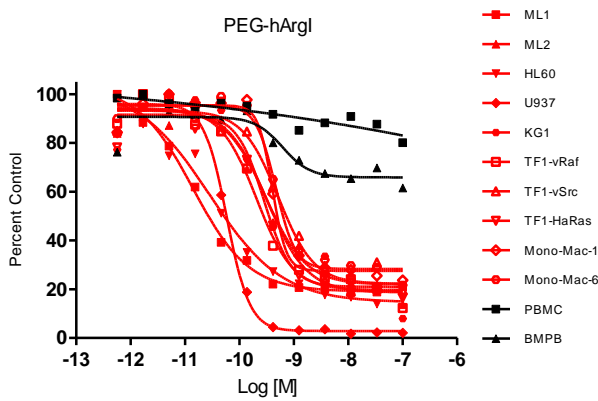
B)



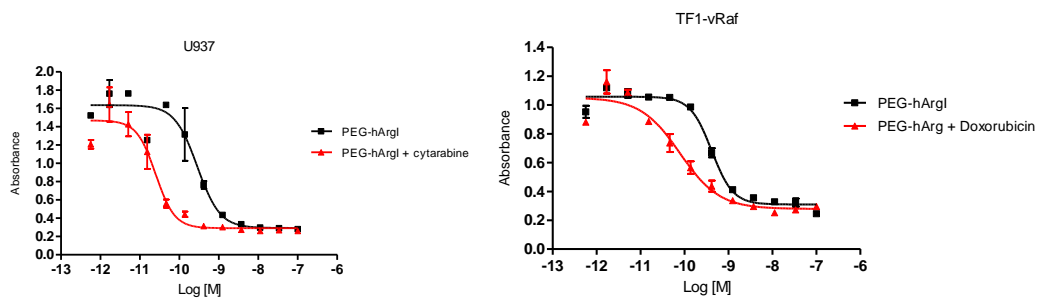
C)



D)



E)



**Figure 7.** Non-Linear regression curves of the cytotoxicity of HuArgI (Co)-PEG5000 (square) and HuArgI (Co)-PEG5000 + L-citrulline (11.4 mM) (triangle) on human AML cell lines HL60, Mono-Mac-1, U937, ML2 and ML1 (A) and TF1-HaRas, TF1-VRaf, TF1-vSrc and Mono-Mac-6 (B). C) Non-linear regression curves of the cytotoxicity of HuArgI (Co)-PEG5000 on human normal PBMCs (square) and on Human CD<sub>34</sub><sup>+</sup> bone marrow progenitor blasts (BMPBs) (triangle). D) Compilation of HuArgI (Co)-PEG5000 non-linear regression curves on all cells tested. E) Non-

linear regression curves of the combination of HuArgI (Co)-PEG5000 with cytarabine (U937) and doxorubicin (TF1-vRaf).

Incubation times of 24, 48 and 72 h were tested and revealed an increased sensitivity of AML cell lines to HuArgI (Co)-PEG5000 at 48 and 72 h incubation compared to 24 h incubation ( $IC_{50}$  lower by 5 to 10-fold) with no significant difference seen between 48 and 72 h (Data not shown). Several studies have shown that addition of excess exogenous L-citrulline rescues a number of cancer cell lines from arginine depletion-induced cytotoxicity by increasing flux through the urea cycle leading to an increase in intracellular arginine levels.<sup>23</sup> Therefore, the inability to rescue cells from arginine depletion-induced cytotoxicity by the addition of excess L-citrulline is considered a marker of irreversible arginine auxotrophy. Addition of L-citrulline, at concentrations of 1.14, 11.4 and 114  $\mu$ M, failed to rescue AML cells from arginine deprivation-induced cytotoxicity and did not affect the sensitivity of AML cell lines to HuArgI (Co)-PEG5000-induced arginine depletion with similar  $IC_{50}$  values observed in the presence and absence of L-citrulline (Table 3). However, increasing the concentration of exogenous L-citrulline to 1.14 and 11.4 mM, resulted in rescuing 5 out of the 9 cell lines tested (HL60, U937, ML1, ML2 and Mono-Mac-1) from arginine deprivation-induced cytotoxicity (Table 3, Figure 7A). The cytotoxic response of the remaining 4 cell lines (Mono-Mac-6, TF1-vRaf, TF1-HaRas and TF1-vSrc) to HuArgI (Co)-PEG5000-induced arginine depletion was not affected by the addition of 11.4 mM exogenous L-citrulline indicating complete arginine auxotrophy of these cell lines (Table 3, Figure 7B). These results demonstrate that all the AML cell lines tested show partial arginine auxotrophy, with a subset showing complete arginine auxotrophy, and are, subsequently, sensitive to the HuArgI (Co)-PEG5000-induced arginine depletion.

In order to determine whether HuArgI (Co)-PEG5000-induced arginine depletion selectively targets AML cells while sparing normal blasts, we tested the cytotoxicity of HuArgI (Co)-PEG5000 on both human peripheral blood mononuclear cells (PBMCs) and CD<sub>34</sub><sup>+</sup> bone marrow progenitor blasts (BMPBs). Both the PBMCs and the CD<sub>34</sub><sup>+</sup> BMPBs were resistant to the HuArgI (Co)-PEG5000-induced arginine depletion with an IC<sub>50</sub> > 10,000 pM and percent cell kill at highest concentration of 20% and 35% for PBMCs and CD<sub>34</sub><sup>+</sup> BMPBs, respectively. These findings demonstrate the selective cytotoxicity of HuArgI (Co)-PEG5000-induced arginine depletion to AML cell lines (Table 3, Figure 7C).

In order to investigate potential additive/synergistic effects of HuArgI (Co)-PEG5000-induced arginine depletion and standard chemotherapeutic agents in AML cells; we tested the cytotoxicity of a combination of HuArgI (Co)-PEG5000 and cytarabine or doxorubicin on our panel of AML cell lines. Three of the cell lines tested (TF1-vRaf, TF1-HaRas and TF1-vSrc) were not sensitive to cytarabine and were subsequently excluded from this analysis. Four out of the six remaining cell lines (HL60, U937, ML1 and ML2) showed additive/synergistic effects with IC<sub>50</sub> values of the combination of HuArgI (Co)-PEG5000 and cytarabine being 2.0 to 18-fold lower than those observed with HuArgI (Co)-PEG5000 alone (Figure 7E). The remaining two cell lines (Mono-Mac-1 and Mono-Mac-6) did not show any additive or synergistic effects for the combination of HuArgI (Co)-PEG5000 and cytarabine with similar IC<sub>50</sub> values observed for the combination compared to each treatment alone. For the doxorubicin combination, additive/synergistic effects were observed in 6 of the cell lines tested (Figure 7E). Four cell lines (HL60, U937, ML2 and TF1-vRaf) had IC<sub>50</sub> values for the combination of HuArgI (Co)-PEG5000 and doxorubicin that were 2.5 to 5 fold lower than those observed with HuArgI (Co)-

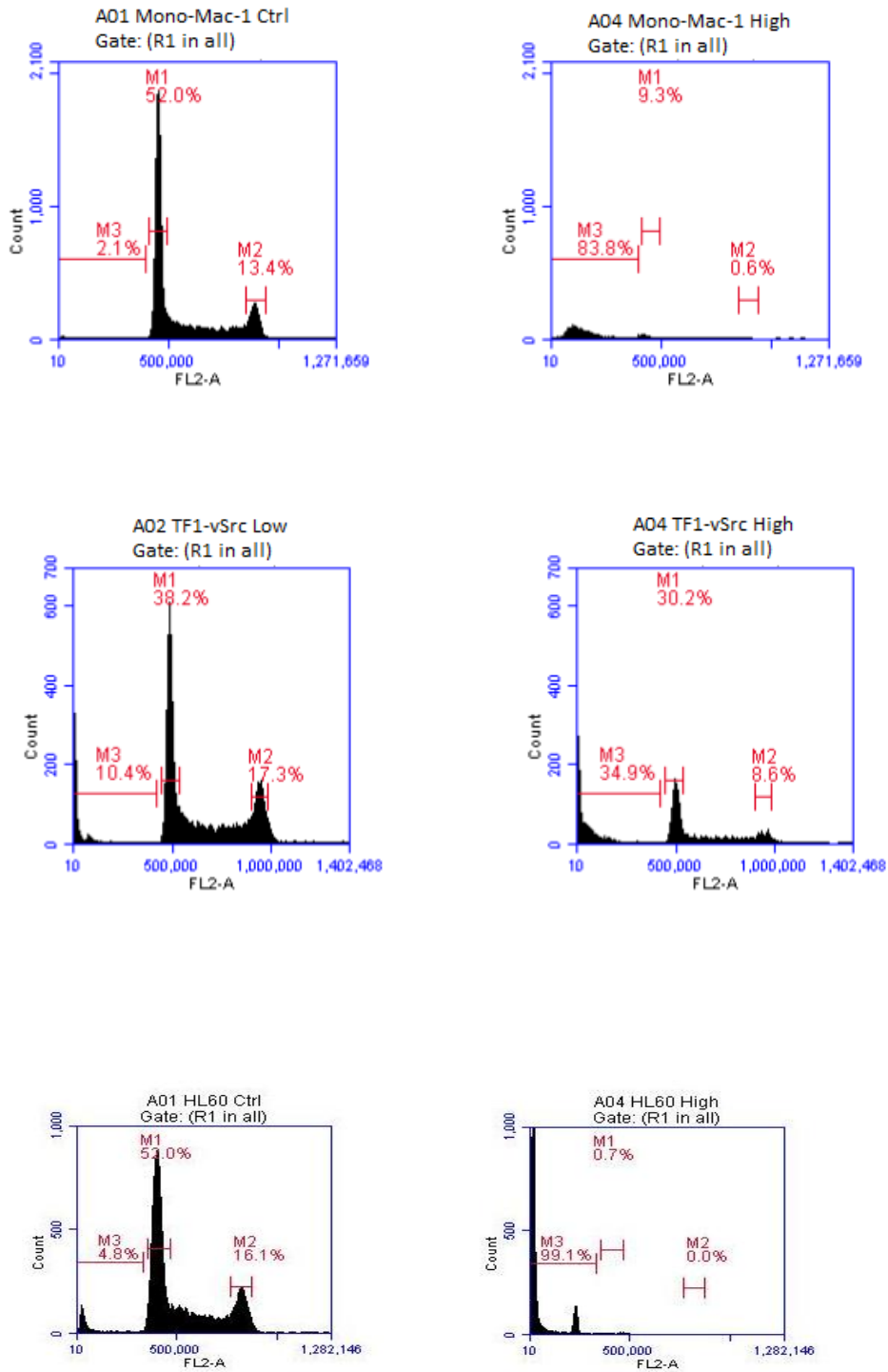
PEG5000 alone while another two cell lines (TF1-HaRas and TF1-vSrc) showed IC<sub>50</sub> values for the combination that were 4 to 5-fold lower than those observed with Doxorubicin alone. No additive/synergistic effects of the combination were observed in the remaining 3 cell lines (ML1, Mono-Mac-1 and Mono-Mac-6). This indicates that HuArgI (Co)-PEG5000-induced arginine depletion shows additive/synergistic effects with standard chemotherapeutic agents in a subset of AML cell lines.

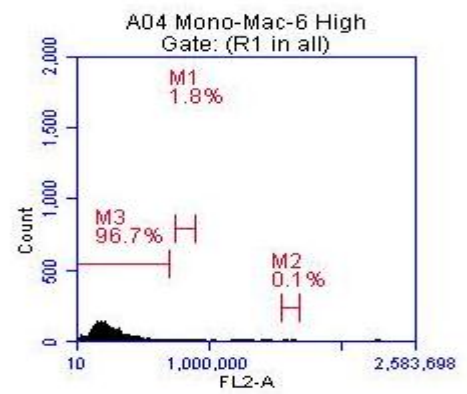
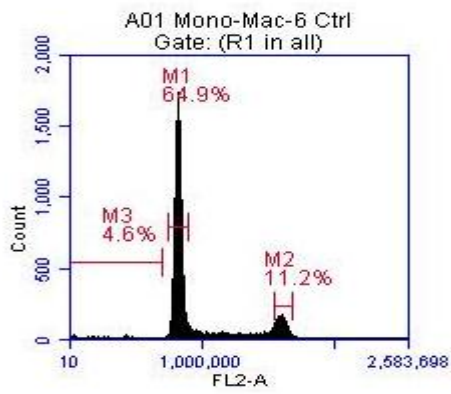
### **Cell cycle effect of HuArgI (Co)-PEG5000:**

In order to determine whether, in addition to cytotoxicity, HuArgI (Co)-PEG5000-induced arginine depletion causes cell cycle arrest in AML cells, we determined the cell cycle status of our panel of AML cell lines following 24 and 48 h incubation with three different concentrations of the HuArgI (Co)-PEG5000 (Fig. 8A and B). None of the cell lines tested showed any effect of HuArgI (Co)-PEG5000 treatment on cell cycle at any concentration at 48 h incubation (Fig. 8A). The fraction of cells in both the G<sub>0</sub>/G<sub>1</sub> and G<sub>2</sub>/M phases decreased with increasing concentrations of HuArgI (Co)-PEG5000. This was accompanied by an increase in the fraction of cells in the pre-G<sub>0</sub>/G<sub>1</sub> phase at the highest concentrations of HuArgI (Co)-PEG5000 at 48 h incubation, indicating that treatment induces complete cytotoxicity in the totality of treated cells (Fig. 8A). At 24 h incubation, 2 out of 10 cell lines (TF1-HaRas and TF1-vSrc) showed dose-dependent cell cycle arrest while the remaining cell lines did not show any effect of HuArgI (Co)-PEG5000 treatment on cell cycle (Fig. 8B). The fraction of cells in the G<sub>0</sub>/G<sub>1</sub> phase increased from approximately 42% and 38% of the total cell population in control cells to approximately 54% and 53% of the total cell population, for TF1-HaRas and TF1-vSrc respectively, following incubation with 100,000 pM of HuArgI (Co)-PEG5000

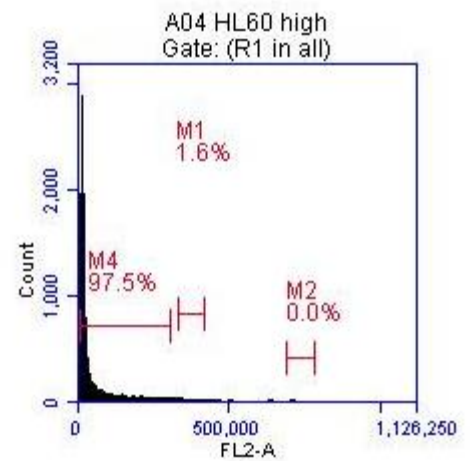
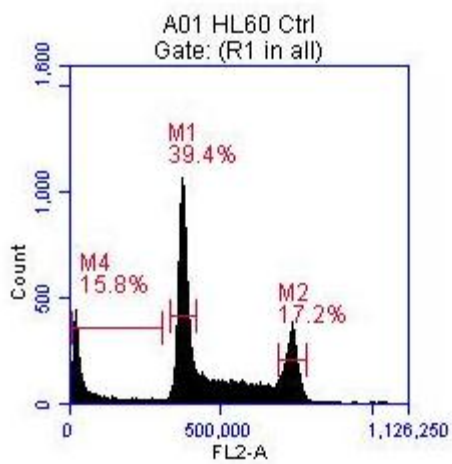
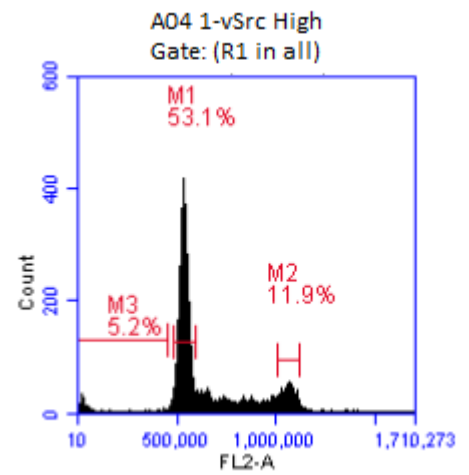
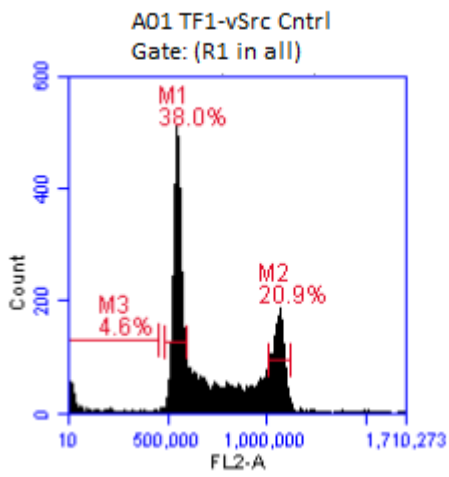
for 24 h. This was associated with a corresponding decrease in the percentage of cells in the G2/M phase.

A)

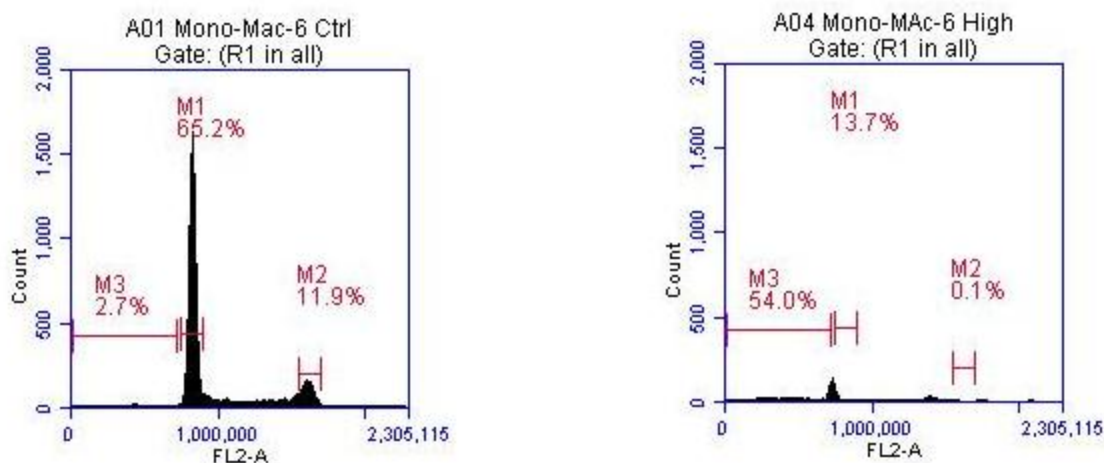




B)







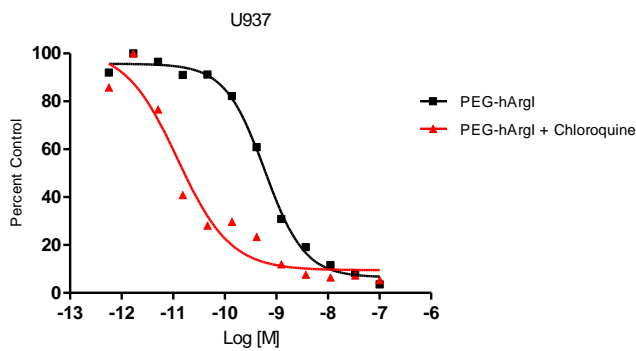
**Figure 8.** Cell cycle analysis of the Mono-Mac-1, TF1-vSrc, HL60 and Mono-Mac-6 (A) cell line following treatment with HuArgI (Co)-PEG5000 for 48 h and the TF1-vSrc, HL60 and Mono-Mac-6 cell line following treatment with HuArgI (Co)-PEG5000 for 24 h (B). Control cells are represented in the left panels and cells treated with 100 nM HuArgI (Co)-PEG5000 in the right panels. Cells are gated on width versus forward scatter (R1). Cells in G0/G1 are gated M1, G2/M are gated M2 and pre-G0/G1 (dead) are gated M3. Cell lines showed complete cell death following treatment, with no evidence of cell cycle arrest following 48 h of HuArgI (Co)-PEG5000-induced arginine depletion.

### Autophagy:

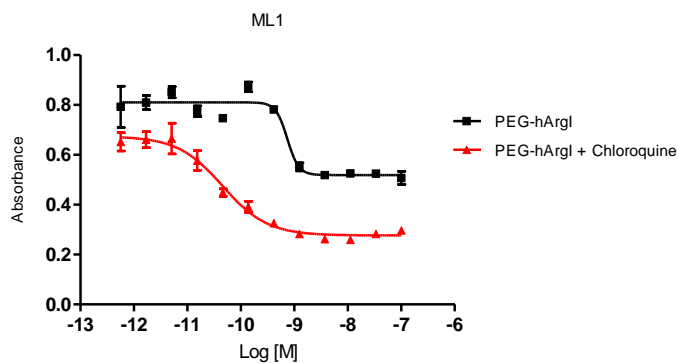
The potential role played by autophagy in the HuArgI (Co)-PEG5000-induced cytotoxicity of AML cells was investigated by co-incubating AML cells with HuArgI (Co)-PEG5000 and either the autophagy activator, trehalose or the autophagy inhibitor chloroquine. A subset of 3 AML cell lines was tested (TF1-vSrc, U937 and ML-1) and, in all three cell lines, the addition of chloroquine (10  $\mu$ M) induced a significant increase in the sensitivity of cells to HuArgI (Co)-PEG5000 at both 24 and 48 h incubation (Figure 9). The  $IC_{50}$  values obtained with the combination of HuArgI (Co)-PEG5000 and chloroquine were 4, 8 and 17-fold lower for TF1-vSrc, U937 and ML1, respectively, at 24 h and 12, 20 and 30-fold lower for TF1-vSrc, ML1 and U937, respectively, at 48 h, compared to  $IC_{50}$  values obtained with HuArgI (Co)-PEG5000 alone. Hence, inhibiting autophagy led to an increase in the

sensitivity of AML cells to arginine depletion, indicating that activation of autophagy, following arginine depletion, plays a protective role against the HuArgI (Co)-PEG5000-induced cell death of AML cells. Additional activation of autophagy through the addition of trehalose only led to a modest decrease in the sensitivity of AML cell lines to HuArgI (Co)-PEG5000 with  $IC_{50}$  values increasing by a maximum of 2-fold in the combination of HuArgI (Co)-PEG5000 and trehalose compared to HuArgI (Co)-PEG5000 alone. This indicates that HuArgI (Co)-PEG5000-induced arginine depletion leads to near maximal activation of autophagy, limiting the potential impact of the autophagy activator trehalose.

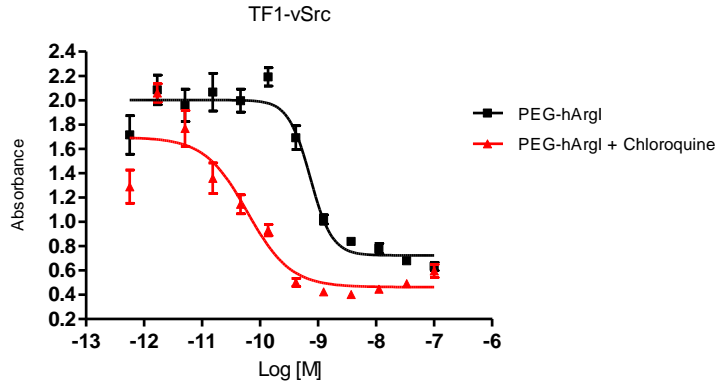
A)



B)



C)



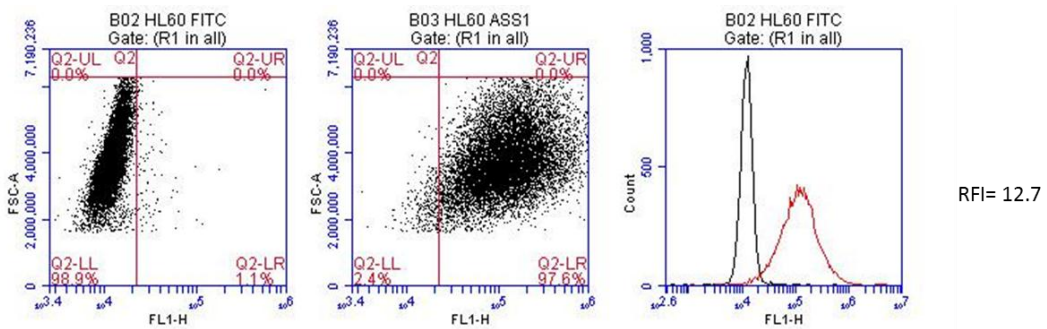
**Figure 9.** Sensitivity of AML cell lines to HuArgI (Co)-PEG5000 alone and in combination with the autophagy inhibitor chloroquine. Non-linear regression curves of U937 (48 h) (A), ML1 (24h) (B) and TF1-vSrc (48 h) (C) cells incubated with HuArgI (Co)-PEG5000 alone (square) or in combination with chloroquine (triangle). In the presence of chloroquine AML cell lines were significantly more sensitive to HuArgI (Co)-PEG5000, at both 24 and 48 h indicating the protective role played by chloroquine in arginine depletion-induced cell death.

### Analysis of Argininosuccinate synthetase 1 (ASS1) levels:

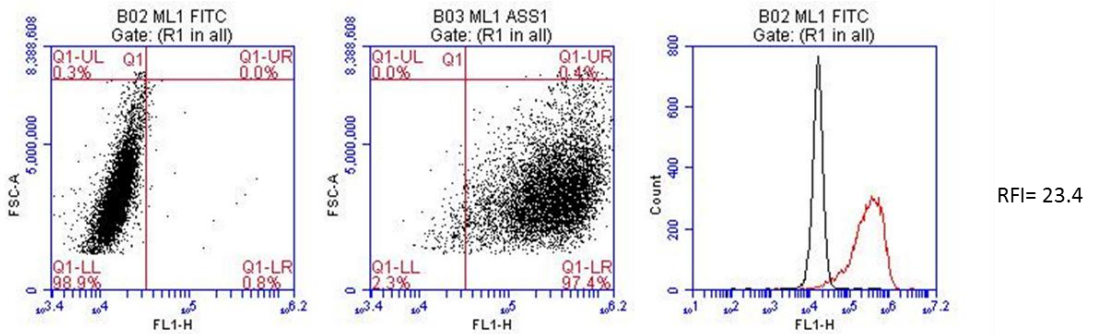
We determined the expression levels of argininosuccinate synthetase 1 (ASS1) in AML cell lines and in  $CD_{34}^{+}$  progenitor blasts using single-cell intracellular staining on flow cytometry. Four of the nine AML cell lines tested, ML1, ML2, U937 and HL60, were positive for the expression of ASS1, as evidenced by a ratio of fluorescence intensity (RFI) of 23.4, 3.5, 4.2 and 12.7, respectively (Fig. 10 A and B). The remaining five cell lines, on the other hand, Mono-Mac-1, Mono-Mac-6, TF1-vRaf, TF1-HaRas and TF1-vSrc, were negative for the expression of ASS1, as evidenced by RFI values of 1.88, 2.03, 2.63, 1.59 and 2.45, respectively (Fig. 10 C, D and E). Expression of ASS1 correlated with the sensitivity of cells to HuArgI (Co)-PEG5000-induced arginine depletion in the presence of excess (11.4 mM) exogenous L-citrulline in all but one cell line (Mono-Mac-1) which was rescued by the highest concentration of L-citrulline (11.4 mM) but was negative for ASS1 expression. The expression pattern of argininosuccinate synthetase in AML cells

confirms their arginine auxotrophy and represents the underlying mechanism for their sensitivity to HuArgI (Co)-PEG5000-induced arginine depletion, in the presence and absence of excess exogenous L-citrulline. Similarly, the resistance of CD<sub>34</sub><sup>+</sup> progenitor bone marrow blasts to the cytotoxicity of HuArgI (Co)-PEG5000-induced arginine depletion was supported by the expression of ASS1 in blast cells as evidenced by an RFI of 3.1 (Fig. 10F).

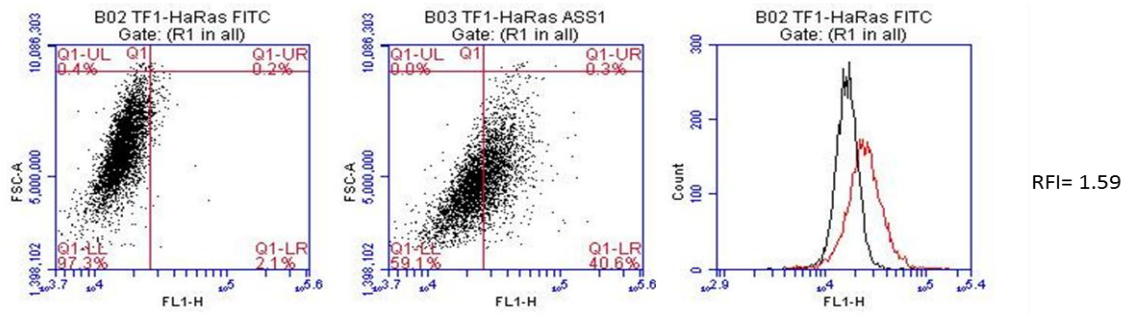
A)



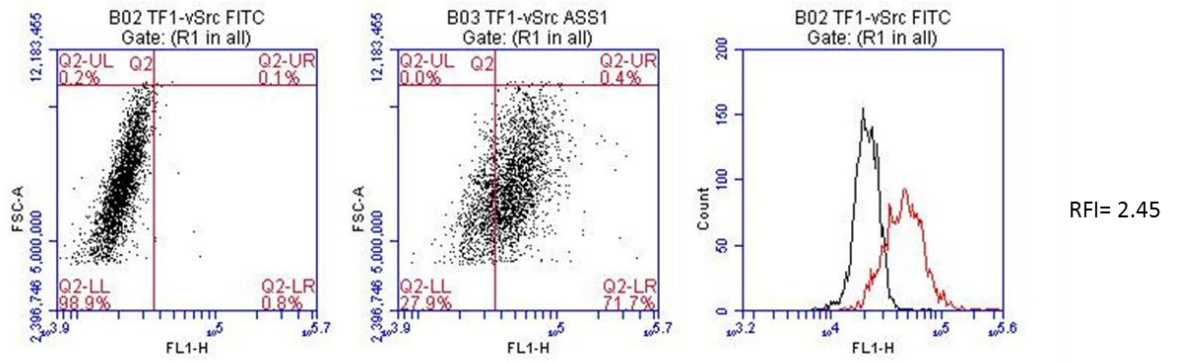
B)



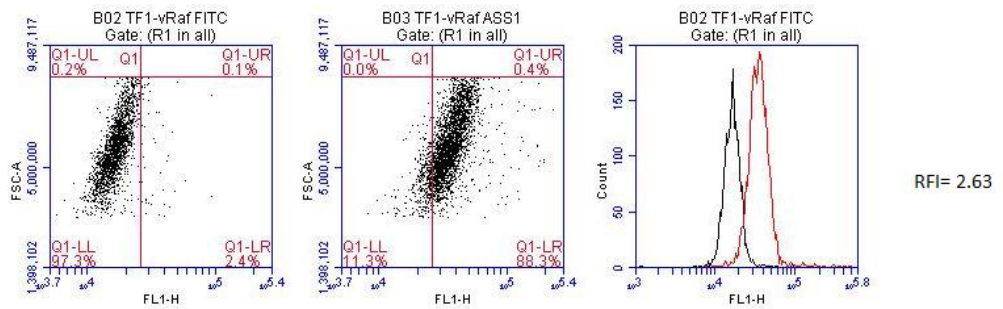
C)



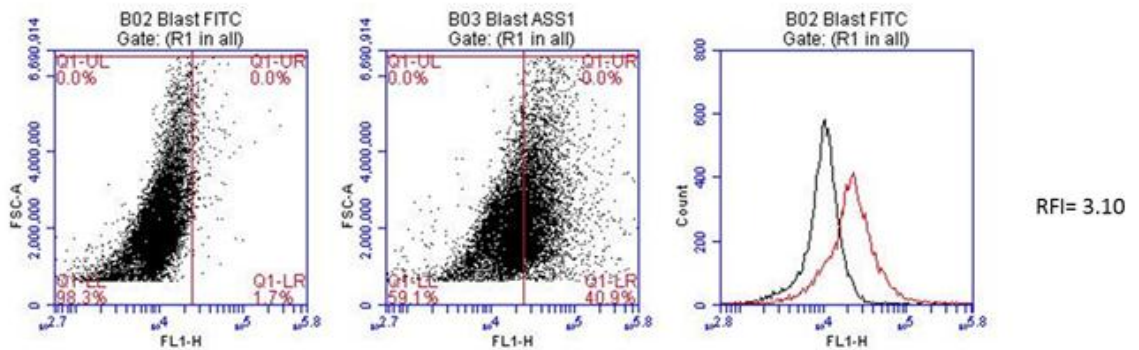
D)



E)



F)



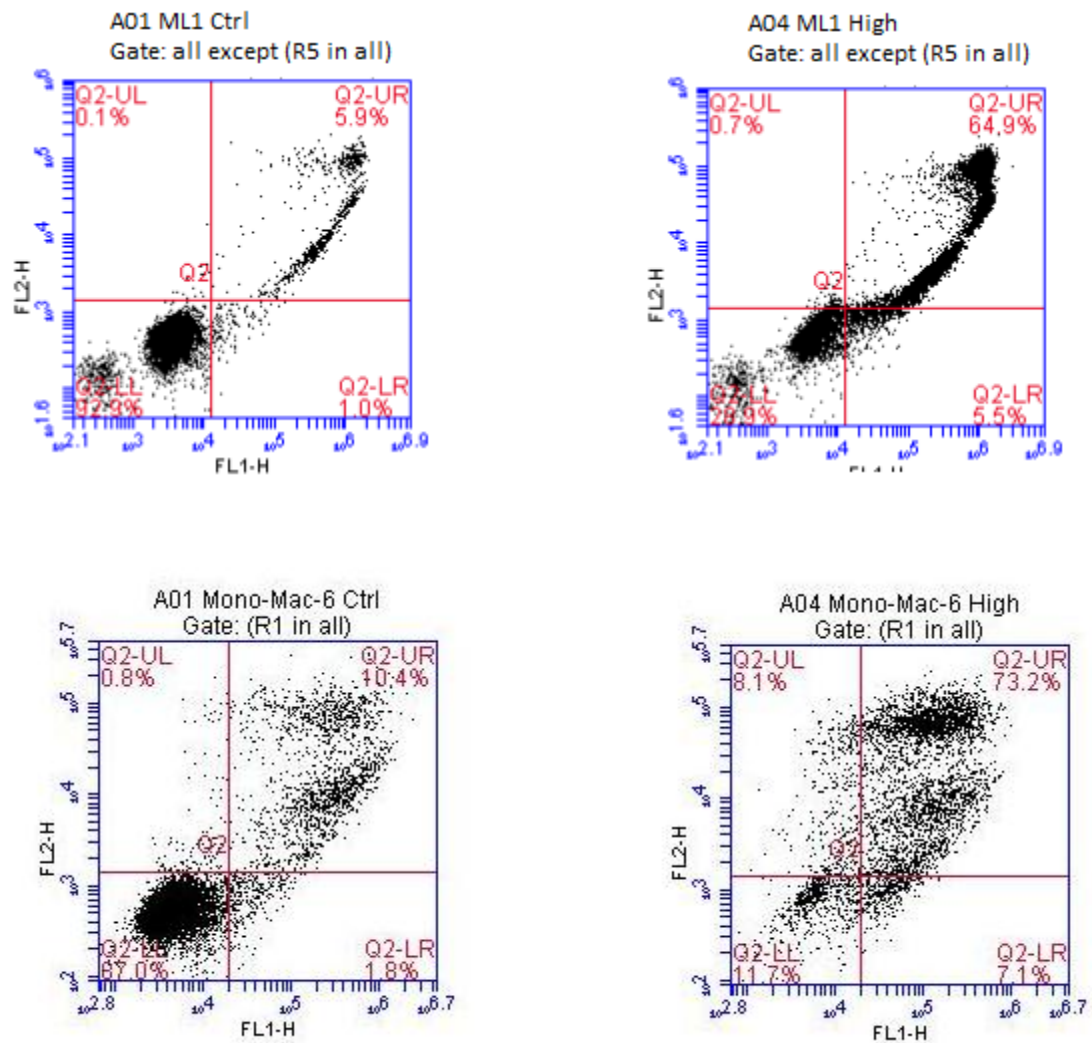
**Figure 10.** Single cell, intracellular staining of argininosuccinate synthetase-1 (ASS-1) in 4 AML cell lines, HL60 (A), ML1 (B), TF1-HaRas (C), TF1-vSrc (D), TF1-vRaf (E) and in human CD34<sup>+</sup> bone marrow progenitor blasts (HBMPBs) (F) using single-cell intracellular staining on flow cytometry. HL60, ML1 and CD34<sup>+</sup> HBMPBs were positive for the expression of ASS1 as evidenced by a ratio of fluorescence intensity (RFI) > 3 between cells stained TF1-HaRas and TF1-vSrc cells were negative for the expression of ASS1 with RFI = 1.58 and 2.45, respectively. Cells are gated on width versus forward scatter (R1).

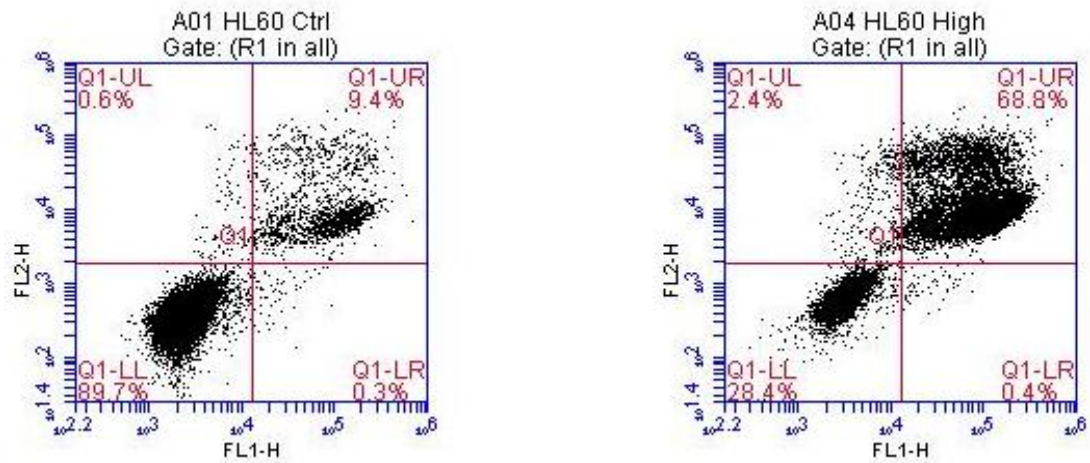
### Analysis of Cell Death:

To determine the mechanism of cell death observed following HuArgI (Co)-PEG5000-induced arginine depletion in AML cells, we tested for caspase activation and annexin V/PI staining in our panel of AML cell lines following treatment with three different concentrations of HuArgI (Co)-PEG5000 (100000, 3000 and 45 pM) for 24 and 48 h. Nine AML cell lines tested (TF1-vRaf, TF1-vSrc, HL60, U937, KG-1, Mono-Mac-1, Mono-Mac-6, ML1 and ML2), showed an increase in the percentage of cells stained with both annexin V and PI, at both 24 and 48 h, in cells treated at the highest concentration of HuArgI (Co)-PEG5000 (100 nM) compared to controls, indicating either necrotic or late-stage apoptotic cell death (Fig, 11A). However, staining for active caspases revealed a total absence of active caspases in all AML cell lines tested, following treatment with HuArgI (Co)-PEG5000 for 24

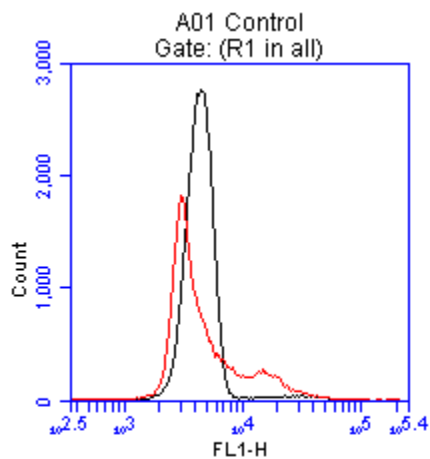
and 48 h (Fig. 11B). The absence of caspase activation, in addition to the loss of membrane integrity as evidenced by positive PI staining, indicate that arginine depletion-induced cytotoxicity in AML cells is mediated through caspase-independent, non-apoptotic mechanisms.

A)





B)



**Figure 11.** Analysis of the mechanism of HuArgI (Co)-PEG5000-mediated cytotoxicity in ML1, Mono-Mac-6 and HL60 cells using annexin V/PI (A) and active caspase staining on ML1(B). Cells incubated with 100 nM HuArgI (Co)-PEG5000 for 48 h (right panel) stained positively with both annexin V (FL1-H) and PI (FL2-H). Incubation of HuArgI (Co)-PEG5000-treated ML1 cells with a cell permeable, FITC-conjugated active caspase inhibitor revealed the absence of active caspases following incubation with HuArgI (Co)-PEG5000 (red).



## CHAPTER FIVE

### DISCUSSION

Over the last three decades advances in chemotherapy have resulted in a great improvement in survival for most types of leukemia. However, conventional chemotherapy for AML has not been successful. The 5 years survival rate is only 24% vs. 81% for CLL and 67% for ALL (American Cancer Society, 2012). Hence the necessity for a new treatment for AML. In our study we have demonstrated that AML cells responded well to arginine depletion, thus they are auxotrophic for Arginine.

All ten cell lines tested were sensitive to HuArgI (Co)-PEG5000 with IC50 values ranging between 78 pM for U937 and 722 pM for HL60, U937 being the most sensitive of all cell lines.

Several reports have proven the sensitivity of amino acid depletion for targeting cancers.

Asparagine depletion is an already approved treatment for acute lymphoblastic leukemia (Muller & Boos, 1998) and it is under investigation for acute myeloid leukemia (Agrawal et al., 2013), while renal cell carcinoma (RCC), hepatocellular carcinoma (HCC), head and neck cancer, melanoma, prostate cancer and T-ALL are being assessed for arginine depletion therapy by arginase and/or ADI (Ascierto et al., 2005; Cheng et al., 2007; Glazer et al., 2010; Hsueh et al., 2012; Huang et al., 2012; Kim et al., 2009; Lam et al., 2011; Szlosarek et al., 2006; Tsui et al., 2009; Yoon et al., 2007).

AML cells have not been previously investigated for Arginine auxotrophy, and the potential use of arginase for AML treatment has not been studied. In this study, we

have, for the first time, demonstrated that AML cell lines are auxotrophic for arginine, thus can be selectively treated with arginine depletion using a pegylated recombinant human arginase [HuArgI (Co)-PEG5000].

We have also demonstrated that human peripheral blood mononuclear cells (PBMCs) and human bone marrow progenitor blasts (BMPBs) were not sensitive to arginine depletion. This indicates the selectivity of HuArgI (Co)-PEG5000 to cancer cells while sparing normal cells.

Furthermore, adding exogenous L-citrulline at low concentrations, 1.14, 11.4 and 114  $\mu$ M did not rescue cells from arginine depletion-induced cytotoxicity. However when the concentration of L-citrulline was increased up to a concentration of 11.4 mM, 5 AML cell lines were rescued from the cytotoxic effects of HuArgI (Co)-PEG5000-induced arginine depletion. This indicates full arginine auxotrophy of a subset of AML cell lines and it confirms the possible use of HuArgI (Co)-PEG5000 for selectively targeting of AML.

We have also shown that arginine auxotrophy for AML cells range from partial auxotrophy in 5 cell lines to complete arginine auxotrophy in 4 cell lines. This is evidenced by the rescuing of AML cells with excess L-citrulline. Complete arginine auxotrophy seems to be secondary to the lack of ASS1 expression in AML cells, and the four cell lines that were not rescued by L-citrullin did not express ASS1. This can be compared to other tumors such as ALL, HCC and prostate cancer where the sensitivity of tumor cells was dependent on expression levels of ASS1 (Agrawal et al., 2013; Delage et al., 2010; Kim et al., 2009).

Moreover, we demonstrated that the addition of HuArgI (Co)-PEG5000 to regular AML chemotherapeutic agents such as doxorubicin and cytarabine resulted in signs of synergistic/additive effects in some of the cell lines. This shows that HuArgI (Co)-

PEG5000 may be used as a standalone treatment or in combination with standard chemotherapeutic agents.

Cell cycle analysis showed that treatment with HuArgI (Co)-PEG5000 resulted in complete cytotoxicity in all 10 AML cell lines at 48 hours. Only two cell lines had limited cell cycle arrest after 24 hours of arginine depletion. This proves that HuArgI (Co)-PEG5000-induced arginine depletion has a strong inhibitory effect and the cytotoxicity is only secondary to cell death and not to cell cycle arrest, thus the extreme potency of HuArgI (Co)-PEG5000 to AML cells.

Recently, autophagy has been extensively investigated as a response of tumor cells to several anti-cancer therapeutic drugs. However, its role in amino acid depletion has not been evaluated yet. For the first time, we show that autophagy is activated after treatment with HuArgI (Co)-PEG5000. Cell sensitivity to HuArgI (Co)-PEG5000 increased with the addition of chloroquine. Chloroquine is known to affect autophagolysosome thus inhibits autophagy (Periyasamy-Thandavan, Jiang, Schoenlein, & Dong, 2009). All this proves that inhibiting autophagy leads to cell death, thus demonstrating that autophagy has a protective role in acute myeloid leukemia cells after treatment with HuArgI (Co)-PEG5000.

In this study, we have demonstrated that AML cells can be selectively treated by HuArgI (Co)-PEG5000-induced arginine depletion, hence they are auxotrophic for arginine. We have as well shown that autophagy is activated after arginine depletion. This activation protects AML cell from cytotoxicity related to arginine deprivation. Thus, HuArgI (Co)-PEG5000 is a new and selective potential treatment for acute myeloid leukemia.

## REFERENCES

- Abi-Habib, R. J., Urieto, J. O., Liu, S., Leppla, S. H., Duesbery, N. S., & Frankel, A. E. (2005). BRAF status and mitogen-activated protein/extracellular signal-regulated kinase kinase 1/2 activity indicate sensitivity of melanoma cells to anthrax lethal toxin. *Molecular Cancer Therapeutics*, 4, 1303-1310.
- Agrawal, V., Woo, J. H., Borthakur, G., Kantarjian, H., & Frankel, A. E. (2013). Red blood cell-encapsulated L-asparaginase: Potential therapy of patients with asparagine synthetase deficient acute myeloid leukemia. *Protein & Peptide Letters*, 20(4), 392-402.
- American Cancer Society. (2013). *Cancer facts & figures*. Atlanta: American Cancer Society, Inc.
- Ascierto, P. A., Scala, S., Castello, G., Daponte, A., Simeone, E., Ottaiano, A., ... Logan, T. F. (2005). Pegylated arginine deiminase treatment of patients with metastatic melanoma: Results from phase I and II studies. *Journal of Clinical Oncology*, 23(30), 7660-7668.
- Bach, S. J., & Swaine, D. (1965). The effect of arginase on the retardation of tumour growth. *British Journal of Cancer*, 19, 379-386.
- Beaumier, L., Castillo, L., Yu, Y. M., Ajami, A. M., & Young, V. R. (1996). Arginine: New and exciting developments for an "old" amino acid. *Biomedical and Environmental Sciences*, 9, 296-315.
- Bennett, J. H. (1845). Case of hypertrophy of the spleen and liver in which death took place from suppuration of the blood. *Edinburgh Medical and Surgical Journal*, 64, 413-423.
- Bennett, J. H. (1852). Leucocythemia or white cell blood. *Edinburgh Medical and Surgical Journal*, 72, 7-82.

- Bennett, J. M., Catovsky, D., Daniel, M. T., Flandrin, G., Galton, D. A., & Gralnick, H. R. (1985). Proposed revised criteria for the classification of acute myeloid leukemia. A report of the French-American-British Cooperative Group. *Annals of Internal Medicine*, *103*(4), 620-625.
- Blair, A., Hogge, D. E., & Sutherland, H. J. (1998). Most acute myeloid leukemia cells with long-term proliferative ability in vitro and in vivo have the phenotype CD34+/CD71-/HLA-DR-. *Blood*, *92*(11), 4325-4335.
- Bonnet, D., & Dick, J. E. (1997). Human acute myeloid leukemia is organized as a hierarchy that originates from a primitive hematopoietic cell. *Nature Medicine*, *3*, 730-737.
- Brasse-Lagnel, C., Fairand, A., Lavoine, A., & Husson, A. (2003). Glutamine stimulates argininosuccinate synthetase gene expression through cytosolic O-glycosylation of Sp1 in Caco-2 cells. *Journal of Biological Chemistry*, *278*, 52504-52510.
- Brasse-Lagnel, C., Lavoine, A., Fairand, A., Vavasseur, K., & Husson, A. (2005). IL-1beta stimulates argininosuccinate synthetase gene expression through NF-kappaB in Caco-2 cells. *Biochimie*, *87*, 403-409.
- Cheng, P. N., Lam, T. L., Lam, W. M., Tsui, S. M., Cheng, A. W., Lo, W. H., ... Leung, Y. C. (2007). Pegylated recombinant human arginase (rhArg-peg5,000mw) inhibits the in vitro and in vivo proliferation of human hepatocellular carcinoma through arginine depletion. *Cancer Research*, *67*, 309-317.
- Delage, B., Fennell, D. A., Nicholson, L., McNeish, I., Lemoine, N. R., Crook, T., & Szlosarek, P. W. (2010). Arginine deprivation and argininosuccinate synthetase expression in the treatment of cancer. *International Journal of Cancer*, *126*(12), 2762-2772.
- Dick, J. E. (2008). Stem cell concepts renew cancer research. *Blood*, *112*, 4793-4807.

- Dillon, B. J., Prieto, V. G., Curley, S. A., Ensor, C. M., Holtsberg, F. W., Bomalaski, J. S., & Clark, M. A. (2004). Incidence and distribution of argininosuccinate synthetase deficiency in human cancers: A method for identifying cancers sensitive to arginine deprivation. *Cancer*, *100*(4), 826-833.
- Dohner, H. (2007). Implication of the molecular characterization of acute myeloid leukemia. *American Society of Hematology Education Program*, *1*, 412-419.
- Dohner, H., Estey, E. H., Amadori, S., Appelbaum, F. R., Büchner, T., Burnett, A. K., ... European LeukemiaNet. (2010). Diagnosis and management of acute myeloid leukemia in adults: Recommendations from an international expert panel, on behalf of the European LeukemiaNet. *Blood*, *115*(3), 453-474.
- Dohner, H., & Dohner, K. (2008). Molecular characterization of acute myeloid leukemia. *Haematologica*, *93*(7), 976-982.
- Ebstein, W. (1889). "Über die acute Leukämie und Pseudoleukämie". *Deutsches Archiv für klinische Medizin*, *44*, 343.
- Ensor, C. M., Holtsberg, F. W., Bomalaski, J. S., & Clark, M. A. (2002). Pegylated arginine deiminase (ADI-SS PEG20,000 mw) inhibits human melanomas and hepatocellular carcinomas in vitro and in vivo. *Cancer Research*, *62*(19), 5443-5450.
- Estey, E., & Dohner, H. Acute myeloid leukaemia. *Lancet*, *368*(9550), 1894-1907.
- Feun, L., You, M., Wu, C. J., Kuo, M. T., Wangpaichitr, M., Spector, S., & Savaraj, N. (2008). Arginine Deprivation as a Targeted Therapy for Cancer. *Current Pharmaceutical Design*, *14*(11), 1049-1057.
- Glazer, E. S., Stone, E. M., Zhu, C., Massey, K. L., Hamir, A. N., & Curley, S. A. (2011). Bioengineered human arginase I with enhanced activity and stability controls hepatocellular and pancreatic carcinoma xenografts. *Translational Oncology*, *4*(3), 138-146.

- Glazer, E. S., Piccirillo, M., Albino, V., Di Giacomo, R., Palaia, R., Mastro, A. A., ... Izzo, S. C. (2010). Phase II study of pegylated arginine deiminase for nonresectable and metastatic hepatocellular carcinoma. *Journal of Clinical Oncology*, 28(13), 2220-2226.
- Gong, H., Zolzer, F., von Recklinghausen, G., Havers, G., & Schweigerer, L. (2000). Arginine deiminase inhibits proliferation of human leukemia cells more potently than asparaginase by inducing cell cycle arrest and apoptosis. *Leukemia*, 14(5), 826-829.
- Grimwade, D., Walker, H., Oliver, F., Wheatley, K., Harrison, C., Harrison, G., ... Goldstone, A. (1998). "The importance of diagnostic cytogenetics on outcome in AML: Analysis of 1,612 patients entered into the MRC AML 10 trial. The Medical Research Council Adult and Children's Leukaemia Working Parties". *Blood*, 92(7), 2322-2333.
- Guei, T. R., Liu, M. C., Yang, C. P., & Su, T. S. (2008). Identification of a liver-specific cAMP response element in the human argininosuccinate synthetase gene. *Biochemical and Biophysical Research Communications*, 377, 257-261.
- Harris, J. M., & Chess, R. B. (2003). Effect of pegylation on pharmaceuticals. *Nature Reviews Drug Discovery*, 2(3), 214-221.
- Hawkins, D. S., Park, J. R., Thomson, B. G., Felgenhauer, J. L., Holcenberg, J. S., Panosyan, E. H., & Avramis, V. I. (2004). Asparaginase pharmacokinetics after intensive polyethylene glycol-conjugated L-asparaginase therapy for children with relapsed acute lymphoblastic leukemia. *Clinical Cancer Research*, 10(16), 5335-5341.
- Hernandez, C. P., Morrow, K., Lopez-Barcons, L. A., Zabaleta, J., Sierra, R., Velasco, C., ... Rodriguez, P. C. (2010). Pegylated arginase I: A potential therapeutic approach in T-ALL. *Blood*, 115(25), 5214-5221.
- Hsueh, E. C., Knebel, S. M., Lo, W. H., Leung, Y. C., Cheng, P. N., & Hsueh, C. T. (2012). Deprivation of arginine by recombinant human arginase in prostate cancer cells. *Journal of Hematology & Oncology*, 5, 17.

- Huang, C. C., Tsai, S. T., Kuo, C. C., Chang, J. S., Jin, Y. T., Chang, J. Y., & Hsiao, J. R. (2012). Arginine deprivation as a new treatment strategy for head and neck cancer. *Oral Oncology*, *48*(12), 1227-1235.
- Kassab, E., Darwish, M., Timsah, Z., Liu, S., Leppla, S. H., Frankel, A. E., & Abi-Habib, R. J. (2013). Cytotoxicity of anthrax lethal toxin to human acute myeloid leukemia cells is nonapoptotic and dependent on extracellular signal-regulated kinase 1/2 activity. *Translational Oncology*, *6*(1), 25-32.
- Kim, R. H., Coates, J. M., Bowles, T. L., McNerney, G. P., Sutcliffe, J., Jung, J. U., ... Kung, H. J. (2009). Arginine deiminase as a novel therapy for prostate cancer induces autophagy and caspase-independent apoptosis. *Cancer Research*, *69*(2), 700-708.
- Kuo, M. T., Savaraj, N., & Feun, L. G. (2010). Targeted cellular metabolism for cancer chemotherapy with recombinant arginine-degrading enzymes. *Oncotarget*, *1*(4), 246-251.
- Lam, T. L., Wong, G. K., Chow, H. Y., Chong, H. C., Chow, T. L., Kwok, S. Y., ... Leung, Y. C. (2011). Recombinant human arginase inhibits the in vitro and in vivo proliferation of human melanoma by inducing cell cycle arrest and apoptosis. *Pigment Cell & Melanoma Research*, *24*(2), 366-376.
- Lapidot, T., Sirard, C., Vormoor, J., Murdoch, B., Hoang, T., Caceres-Cortes, J., ... Dick, J. E. (1994). A cell initiating human acute myeloid leukaemia after transplantation into SCID mice. *Nature*, *367*(6464), 645-648.
- Ley, T. J., Mardis, E. R., Ding, L., Fulton, B., McLellan, M. D., Chen, K., ... Wilson, R. K. (2008). DNA sequencing of a cytogenetically normal acute myeloid leukaemia genome. *Nature*, *456*(7218), 66-72.
- Lowenberg, B., Downing, J. R., & Burnett, A. (1999). Acute myeloid leukemia. *New England Journal of Medicine*, *341*(14), 1051-1062.



- Lowenberg, B., Downing, J. R., & Burnett, A. (2003). Acute myeloid leukemia and acute promyelocytic leukemia. *American Society of Hematology Education Program, 1*, 82-101.
- McCulloch, E. A. (1983). Stem cells in normal and leukemic hemopoiesis. *Blood, 62*(1), 1-13.
- Morris, S. M. (2004). Enzymes of arginine metabolism. *Journal of Nutrition, 134*(10 Suppl), 2743S-2747S.
- Mrózek, K., Heerema, N. A., & Bloomfield, C. D. (2004). Cytogenetics in acute leukemia. *Blood Reviews, 18*, 115-136.
- Mrózek, K., Marcucci, G., Paschka, P., Whitman, S. P., & Bloomfield, C. D. (2007). Clinical relevance of mutations and gene expression changes in adult acute myeloid leukemia with normal cytogenetics: Are we ready for a prognostically prioritized molecular classification? *Blood, 109*, 431-448.
- Muller, H. J., & Boos, J. (1998). Use of L-asparaginase in childhood ALL. *Critical Reviews in Oncology/Hematology, 28*, 97-113.
- Naegeli, O. (1900). Uberrothes knochenmark und myeloblasten. *Deutsche Medizinische Wochenschrift, 18*, 287-290.
- Periyasamy-Thandavan, S., Jiang, M., Schoenlein, P., & Dong, Z. (2009). Autophagy: Molecular machinery, regulation, and implications for renal pathophysiology. *American Journal of Physiology: Renal Physiology, 297*(2), 244-256.
- Pillar, G. (2001). Leukaemia - A brief historical review from ancient times to 1950. *British Journal of Haematology, 112*(2), 282-292.
- Ramage, J., Vallera, D. A., Black, J., Aplan, P., Kees, U., & Frankel, A. E. (2003). The diphtheria toxin/urokinase fusion protein (DTAT) is selectively toxic to CD87 expressing leukemic cells. *Leukemia Research, 27*, 79-84.

- Savoca, K. V., Davis, F. F., van Es, T., McCoy, J. R., & Palczuk, N. C. (1984). Cancer therapy with chemically modified enzymes. II. The therapeutic effectiveness of arginase, and arginase modified by the covalent attachment of polyethylene glycol, on the taper liver tumor and the L5178Y murine leukemia. *Cancer biochemistry biophysics*, 7, 261-268.
- Stone, E. M., Glazer, E. S., Chantranupong, L., Cherukuri, P., Breece, R. M., Tierney, D. L., ... Georgiou, G. (2010). Replacing Mn(2+) with Co(2+) in human arginase I enhances cytotoxicity towards L-arginine auxotrophic cancer cell line. *ACS Chemical Biology*, 5(3), 333-342.
- Szlosarek, P. W., Klabatsa, A., Pallaska, A., Sheaff, M., Smith, P., Crook, T., ... Fennell, D. A. (2006). In vivo loss of expression of argininosuccinate synthetase in malignant pleural mesothelioma is a biomarker for susceptibility to arginine depletion. *Clinical Cancer Research*, 12(23), 7126-7131.
- Tsui, S. M., Lam, W. M., Lam, T. L., Chong, H. C., So, P. K., Kwok, S. Y., ... Leung, Y. C. (2009). Pegylated derivatives of recombinant human arginase (rhArg1) for sustained in vivo activity in cancer therapy: Preparation, characterization and analysis of their pharmacodynamics in vivo and in vitro. *Cancer Cell International*, 9, 9.
- Vardiman, J. W., Harris, N. L., & Brunning, R. D. (2002). The World Health Organization (WHO) classification of the myeloid neoplasms. *Blood*, 100(7), 2292-2302.
- Veronese, F. M., & Pasut, G. (2005). PEGylation, successful approach to drug delivery. *Drug Discovery Today*, 10(21), 1451-1458.
- Virchow, R. (1847). Zur pathologischen Physiologie des Blutes. II. Weisses Blut. *Archives of Pathology, Anatomy and Physiology*, 1, 563-572.
- Wiernik, P. H., Goldman, J. M., Dutcher, J. P., & Kyle, R. A. (2003). *Neoplastic diseases of the blood* (4th ed.). Cambridge: Cambridge University Press.

Wu, G., & Morris, S. M. (1998). Arginine metabolism: Nitric oxide and beyond. *Biochemistry*, 336, 1-17.

Yoon, C. Y., Shim, Y. J., Kim, E. H., Lee, J. H., Won, N. H., Kim, N. H., ... Min, B. H. (2007). Renal cell carcinoma does not express argininosuccinate synthetase and is highly sensitive to arginine deprivation via arginine deiminase. *International Journal of Cancer*, 120(4), 897-905.