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Potency and selectivity of a Urokinase-Activated recombinant
Anthrax Toxin to acute myeloid leukemia (AML) cells

By

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Potency and selectivity of a Urokinase-Activated recombinant Anthrax Toxin to acute myeloid leukemia (AML) cells

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ABSTRACT

Acute myeloid leukemia (AML) is a disorder characterized by uncontrolled mitogenetic activation and proliferation of hematopoietic precursor cells. Up to 30 % of adult patients with AML do not achieve complete remission when treated using chemotherapy. In addition, severe side effects usually limit the efficacy of available therapeutic approaches. As a result, novel tumor-selective therapeutics capable of targeting and eliminating AML blasts while sparing normal cells are urgently needed. In this study, we determine the potency and selectivity of an engineered, urokinase-activated recombinant anthrax fusion toxin (PrAgU₂/FP59) on a panel of 9 AML cell lines and on normal progenitor bone marrow blasts. All 9 AML cell lines tested were sensitive to PrAgU₂/FP59 while normal progenitor, bone marrow blasts were not, demonstrating the potency and selectivity of this tumor-targeted approach. Cytotoxicity of PrAgU₂/FP59 appeared to be non-apoptotic and is dependent on the expression of an active cell surface urokinase plasminogen activator system (uPA/uPAR) system. Targeting the urokinase system, a protease overexpressed on AML cells, appears to be a promising approach for the development of AML-specific therapeutics.

Keywords: AML, PrAgU₂/FP59, dual selectivity, apoptosis, anthrax toxin.

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List of Abbreviations

Abbreviation	Full Name
AKT	Protein Kinase B
AML	Acute Myeloid Leukemia
ANTXR _s	Anthrax Toxin Receptors
Ara-C	Cytosine arabinoside
CD87	Cluster of differentiation
CEBPA	CCAAT/ Enhancer Binding Protein Alpha
CMG-2	Capillary morphogenesis gene 2
DT	Diphtheria Toxin
eEF2	Eukaryotic elongation factor 2
EF	Edema Factor
ERK1/2	Extracellular Signal-Regulated Kinase 1 And 2
ITGB1	Integrin β 1
FITC	Fluorescein Isothiocyanate
FLT3	Fms-related Tyrosine Kinase 3
FP59	Pseudomonas Aeruginosa Exotoxin A
FTI	Farnesyltransferase Inhibitor
GCSF	Granulocyte Colony Stimulating Factor
GMSF	Granulocyte Macrophage Stimulating Factor
GPI	Glycosylphosphatidylinositol
HTLV-1	Human T-Lymphotropic Virus 1
IMDM	Iscove's Modified Dulbecco's Medium
ITD	Internal Tandem Duplication

LF	Lethal Factor
LT	Lethal toxin
LY294002	Potent inhibitor of PI3Ks
MAPK	Mitogen-Activated Protein Kinase
MAPKK	Mitogen-Activated Protein Kinase Kinase
MDM2	Mouse Double Minute 2
MEK1/2	Mitogen-Activated Protein Kinase Kinase
MFI	Mean Fluorescence Intensity
mTOR	Mammalian Target of Rapamycin
NPM1	Nucleophosmin 1
PAI	Plasminogen Activator Inhibitor
PI	Propidium Iodide
PrAg	Protective Antigen
PrAg/LF	Recombinant anthrax lethal toxin
PrAgU ₂	Binding moiety of LF
PrAgU ₂ /FP59	Urokinase-activated, recombinant Pseudomonas exotoxin A
PI3Ks	Phosphoinositide 3-kinases
RFI	Ratio of Fluorescence Intensity
RPMI	Roswell Park Memorial Institute
SCF	Stem Cell Factor
TEM-8	Tumor Endothelium Marker 8
uPA	Urokinase Plasminogen Activator
uPAR	Urokinase Plasminogen Activator Receptor
U0126	MEK1/2 inhibitor

CHAPTER ONE

INTRODUCTION

1.1 Acute Myeloid Leukemia

Acute myelogenous leukemia (AML) is the result of the constitutive mitogenetic activation of hematopoietic stem cells. According to the American Cancer Society, the expected number of new cases of leukemia is 54,270 in 2015 with 24,450 expected deaths from leukemia in the United States only. They also estimate about 10,460 deaths from AML specifically and almost all will be adults. It has been shown *in vitro* that multiple mutations must take place to end developing AML (Ferrara & Schiffer, 2013). This disorder leads to the disruption of normal differentiation and to the excessive proliferation of immature hematopoietic cells known as blasts (Perl & Carroll, 2007). More than 20% of blasts in the bone marrow are considered a characteristic of AML (Kumar, 2011). With time the increasing number of blasts will cause them to accumulate in the bone marrow, blood and organs. These alterations will interfere with normal production and differentiation of blood cells such as generating mature red blood cells, neutrophils, platelets and monocytes. These changes might cause anemia, neutropenia and thrombocytopenia which is characterized by bleeding (Ferrara & Schiffer, 2013; Villela & Bolaños-Meade, 2011). AML can also result from the exposure to DNA-

damaging reagents, such as chemotherapy, while treating other types of malignancies (Tawana et al., 2013). Treatment with cytotoxic agents increases the risk for developing AML in up to 15% of the patients usually treated for solid cancer. There are two types of genetic mutations correlated with chemotherapy which cause AML: drugs such as anthracyclines and epipodophyllotoxins that target topoisomerase II can cause cytogenetic abnormalities at the MLL gene locus at chromosome 11q23 within months to two years of treatment, while alkylator agent-induced disease cause myelodysplastic syndrome with complex deletions in chromosomes 5 and 7 might occur 5 to 6 years after exposure. These chemotherapy induced diseases are mostly observed in patients older than 65 who usually represent high comorbidities (Ferrara & Schiffer, 2013). AML progresses rapidly, it will cause fatal infection, bleeding or organ infiltration. Bone marrow failure is the most common cause of death, and its main symptom is infection, while organ infiltration fatality becomes more powerful as the disease progresses. Incidence of AML is expected to become higher with the coming years with the aging population: for instance there are 2000 new cases of AML every year in UK (Tawana et al., 2013), while in the US, the incidence is 3.5 cases per 100000 (Villela & Bolaños-Meade, 2011). The expected cure rate for AML is 30 to 40% depending on the biological characteristics of the leukemic clone (Villela & Bolaños-Meade, 2011).

1.2 Current therapies of AML

AML has a reduced prognosis and it is considered to be poorly curable due to the high percentage of secondary AML associated with multidrug resistance mechanisms.

Conventional treatment for AML includes two phases: induction and consolidation. While the purpose of induction is to achieve complete remission, the aim of consolidation is to eliminate all residual leukemia via stem cell transplantation or 1-4 cycles of chemotherapy using ara-C combined with anthracycline or anthracenedione (Ferrara & Schiffer, 2013; Roboz, 2011). Since all patients become pancytopenic and can have fever and infections after the first induction, only eligible patients can undergo a second course of induction on the fourteenth day. Usually, a second induction of drugs is favored in only some young patients due to the risk of severe pancytopenia (Ferrara & Schiffer, 2013). In a meta-analysis study, it has been shown that patients who are less than 60 years old have a long term survival rate with high ara-C and from doubling daunorubicin while patients older than 50 years do not benefit from these changes (Roboz, 2011). Blocking signaling pathways as therapy has been applied against specific receptors such as receptor tyrosine kinase or against intracellular pathways. As shown in figure 1, different drugs have been used to treat patients with AML. Tipifarnib acts as a nonpeptidomimetic farnesyltransferase inhibitor (FTI), Sorafenib is used against solid tumors and it inhibits many pathways especially Raf kinase, Lestaurtinib and Midostaurin inhibit FLT3 receptors, while Everolimus and cytarabine are thought to be capable of inhibiting the PI3K/AKT/mTOR pathway. And yet many patients relapsed after treatment or had complete remission without complete platelets recovery (Villela & Bolaños-Meade, 2011).

Figure 1.1: Drugs inhibiting at different stages the pathways leading to AML (Villela & Bolaños-Meade, 2011).

After complete remission is achieved, all patients will relapse without consolidation. The type of treatment following induction depends on the status of the organs of the patient. The high dose ara-C consolidation method is suggested in patients with NPM1 and CEBPA mutations in the absence of FLT3-ITD mutations (Roboz, 2011). Patients with KIT mutations are mostly considered for allogeneic stem cell transplant while patients with NPM1 mutation respond poorly to this type of therapy.

Autologous stem cell treatment is still not very successful most possibly due to graft contamination with residual leukemia. The relapse rate following stem cell transplant depends highly on the karyotype and on the type of mutation (Ferrara & Schiffer, 2013; figure 2).

Another novel therapy relies on epigenetic gene regulation which refers to the control of transcription that is not controlled by the DNA sequence but rather via DNA methylation or a variety of histones modifications such as acetylation, phosphorylation, and methylation. The use of hypomethylating or epigenetic agents such as decitabine and azacitidine have been adopted for the treatment of older patients with AML since they have a low chance of clinical benefit from extensive chemotherapy (Ungewickell, & Medeiros, 2012).

It has also been shown that patients with core binding factor AML including t(8;21) and abn16q22 have more than 60% chance of cure rate with high dose cytarabine however patients with other types of translocations such as t(6;9) respond poorly to chemotherapy (Ferrara & Schiffer, 2013). Up to 70% of these patients treated with araC + anthracycline induce remission of cancer after treatment due to the persistence of chemotherapy resistant blasts (Menezes et al., 2005). Patients who are less than 60 years old enter complete remission in more than 70% of the cases but more than half of them will relapse with a poor long term survival (Tawana et al., 2013). Administration of intensive induction therapy requires patients to be hospitalized for 4 to 5 weeks which increases the risk of infections especially due to the prolonged neutropenia. In addition, patients with AML develop adverse symptoms when treated with chemotherapy such as nausea, vomiting and diarrhea like any type of cancer treated as such.

Treatment with chemotherapy overall results have been very disappointing in showing a consistent improvement among most patients. *FLT3* is the only mutation that can be pharmacologically targeted (Ferrara & Schiffer, 2013). On the other hand, allogeneic stem cell transplantation is very limited to a certain type of patients. Therefore, a more efficient cure must be manufactured using advanced drugs that target the mutations within malicious cells without distressing the normal ones. Such definite drugs will minimize the disturbing impact caused by chemicals and harmful rays administered during chemotherapy and radiation (Kumar, 2011). More potent therapeutics must be manufactured in order to selectively eliminate AML cells without causing organ damage. One such class of therapeutics is recombinant toxins.

1.3 The recombinant toxins

1.3.1 Recombinant toxins

Recombinant toxins are genetically engineered proteins which consist of a toxin fused to a ligand that binds selectively to a target cell (Kreitman, 2003).

When the ligand is either an antibody or a growth factor it becomes a recombinant immunotoxin such as one recombinant toxin that contains the human interleukin-2 and the truncated diphtheria toxin has been approved for use in cutaneous T-cell lymphoma (Kreitman, 2009). Novel studies include the development of recombinant toxins vaccines such as a vaccine assembled from two recombinant fragments of toxin B produced by *Clostridium difficile* (Karczewski et al., 2014).

Recombinant toxins used for the treatment of cancer are expected to increase in number in the coming years. They are usually made of either a growth factor or a recombinant fragment of a monoclonal antibody fused to a truncated bacterial toxin, derived either from *Pseudomonas* exotoxin or from diphtheria toxin (Kreitman, 2003). A recombinant toxin targeted against adult T-cell leukemia consists of a fragment from *Pseudomonas* exotoxin (PE38) fused to CCR4 ligand, has shown high efficacy in eliminating infected cells. This recombinant toxin proved to vigorously control HTLV-1 infection in vitro and in vivo (Hiyoshi et al., 2015). Hence, AML could be also eliminated by using similar recombinant toxins. One such recombinant toxin has previously been developed to be able to recognize AML cells hence causing cell death to malignant cells only without affecting normal ones (Abi Habib et al., 2004).

1.3.2 Anthrax Lethal toxin (PrAg/LF)

The *Bacillus anthracis* is a spore forming gram-positive bacterium that secretes the anthrax toxin made of three polypeptides that comprise: protective antigen (PrAg) the binding component, and enzymatic moieties the edema factor (EF) and lethal factor (LF). PrAg, LF and EF act together to suppress the host's immune system (Wein et al., 2012). This toxin targets mammalian cells by originally binding on the tumor endothelial marker 8 (TEM-8), capillary morphogenesis gene 2 (CMG-2) and integrin $\beta 1$ (ITGB1) product cell surface receptors, through PrAg (Abi Habib et al., 2005; Martchenko et al., 2012). It is then proteolytically activated by cell surface furin which releases the amino-terminal 20 KDa peptide (PrAg20). At this moment the remaining carboxy-terminal

PrAg63 binds the cell to form a heptamer. Oligomerization of PrAg63 produces up to three binding sites for EF or LF, enzymatic moieties which cannot bind to the monomeric form of PrAg63, and induces their internalization into endosomes by endocytosis through a lipid raft-mediated clathrin-dependent process. Once the endosomes are acidic, the PrAg63 heptamer forms a channel through the endosomal membrane to allow the translocation of LF and EF into the cytosol. LF is a zinc-dependent metalloprotease that cuts mitogen-activated protein kinase kinases (MAPKKs) in their amino-terminal regions and causes cell cycle arrest and cell death. On the other hand, EF is a calcium and calmodulin-dependent adenylatecyclase that causes impairment of phagocytosis (Abi Habib et al., 2006; Liu et al., 2009). The MAPK pathway has been successfully targeted in AML using anthrax toxin. Previous studies had shown that anthrax lethal toxin is not sensitive to a majority of normal human cell lines such as cardiac microvascular endothelial cells, umbilical vein endothelial cells, cardiomyocytes, lung fibroblasts, astrocytes, hepatocytes, renal proximal tubule epithelial cells, renal cortical epithelial cells, skeletal muscle cells and lymphocytes. The few cells that were sensitive to the anthrax toxin were restricted to monocytes, coronary artery endothelial cells, pulmonary artery endothelial cells, lung microvascular endothelial cells, and renal mesangial cells (Abi-Habib et al., 2005). In another study, human melanocytes did not show cell death when incubated with anthrax lethal toxin for 8 days (Koo et al., 2002). The anthrax lethal toxin is, therefore, a specific toxin that only targets a limited number of normal human cells that depend solely on the MAPK pathway; therefore it can be considered an asset for treating selectively tumor cells such as AML cells. Furthermore, PrAg/LF can be modified by altering different sequences of its protective antigen moiety or by replacing the catalytic domain of lethal factor.

Figure 1.2: A model showing the mechanism of cell binding and internalization of anthrax lethal toxin.

1.3.3 PrAg/FP59

The amino terminal sequence of LF and the amino terminal sequence of EF constitute the PrAg heptamer-binding domain. The zinc metalloprotease domain of LF and the edema factor domain can be substituted with another bacterial toxin enzymatic domain as long as it contains the N-terminal 254 amino acid of anthrax toxin lethal factor (LFn). The bacterial catalytic domain that was used to replace the catalytic domain of LF is the catalytic domain of *Pseudomonas aeruginosa* exotoxin A, which ADP-ribosylates elongation factor 2 EF-2 leading to the inhibition of protein synthesis. The resulting fusion protein, termed FP59 (due to its molecular weight), is therefore, a fusion of the PrAg-binding domain of LF and the catalytic domain of *Pseudomonas aeruginosa* exotoxin A (Liu et al., 2009; Bachran et al., 2013). Once in the cytosol, the

catalytic domain of *Pseudomonas aeruginosa* exotoxin A (PEIII) will transfer ADP-ribose to eukaryotic elongation factor 2 (eEF2), leading to protein synthesis inhibition and subsequent cell death (Bachran et al., 2013). FP59 is a drug with a more potent and a wider range of activity than LF since, once inside the cytosol, it is cytotoxic to all cells (Abi-Habib et al., 2006). The PrAg/FP59 toxin is highly effective in specifically activating the cell surface PrAg and delivering of PEIII in the cytosol, it has been positively tested for a number of tumor types and it is expected to be active on most solid tumors (Bachran et al., 2014). Since FP59 has a much wider and potent toxicity than LF, therefore it is essential to make it more selective to AML cells hence reducing its effect on normal cells

1.3.4 The uPA/uPAR system

Several types of solid tumors overexpress the urokinase plasminogen activator (uPA), which increases their metastatic potential. However, the uPA system is rarely present on normal cells (Abi-Habib et al., 2006). AML blasts overexpress uPA and uPAR and have been successfully targeted using the recombinant diphtheria toxin (Abi Habib et al., 2004). Up to 73% of AML patients have been shown to express the uPA protease system (Atfy, Elissa & Salah, 2012). The uPA system is composed of a serine protease (uPA), its receptor, which is a urokinase-type plasminogen activator receptor-uPAR or CD87, and two main inhibitors, the plasminogen activator inhibitor 1 (PAI 1) and activator inhibitor 2 (PAI 2) (Béné et al., 2004). uPA is a protease that regulates the activation of plasminogen to the serine protease plasmin, causing the rearrangement of

the extracellular matrix and yielding to cell migration (Alfano et al., 2003). The uPAR is a highly glycosylated glycosyl-phosphatidyl inositol (GPI) that is anchored on the cell surface and binds to uPA which is generated endogenously or exogenously (Béné et al., 2004; Prager et al., 2004).

The activate uPA/uPAR system slices plasminogen into plasmin which causes the degradation of the extracellular matrix and subsequently tissue migration, invasiveness and metastasis. The inactive single chain precursor pro-uPA is made of two parts: an amino-terminal fragment and a carboxy-terminal catalytic domain (Alfano et al., 2003). uPA has proteolytic functions when it converts plasminogen and non-proteolytic functions when it binds to uPAR (Alfano et al., 2003). It all begins when the N-terminal domain of pro-uPA, binds to its receptor uPAR. This will cause pro-uPA to be activated and to be able to break down plasminogen into plasmin, which activates pro-uPA to uPA and prometalloproteinases to active metalloproteinases (figure 2) (Prager et al., 2004). The trimeric uPA/uPAR complex affects cell migration, adhesion, differentiation and proliferation (Alfano et al., 2003).

The inhibitors can bind to the active or inactive form of uPA. PAI1 acts by binding to uPAR and changes its characteristics, and then the uPA/uPAR bound to PAI1 becomes able to bind to alpha-2-macroglobulin which causes the internalization of the complex. The complex will then break down into two parts uPA-PAI1 and the receptor uPAR. The lysosomes will degrade uPA-PAI1 while the uPAR is recycled to the cell surface. Therefore, PAI1 regulates the activity of uPAR and its distribution on the cell surface. The uPA/uPAR complex is unaffected by the plasminogen activator inhibitor-1. Under normal conditions the uPA/uPAR system is only expressed when physiologic

processes need rapid division of cells such as wound healing and tissue remodeling (Béné et al., 2004; Abi Habib et al., 2006).

Figure 1.3: A model describing the mode of activation and action of the uPA/uPAR protease system (Alfano et al., 2003).

1.3.5 PrAgU₂/FP59

Since uPA and uPAR are poorly expressed in normal cells and overexpressed on cancer cells, particularly AML, we have replaced the furin activation site of PrAg ¹⁶⁴RKKR¹⁶⁷ with a urokinase activation site termed U2 ¹⁶³PGSGRSA¹⁶⁹. PrAgU₂ when

used in combination with FP59 constitutes a novel, potent and tumor selective toxin. PrAgU₂/FP59 binds to all cells through the ubiquitously expressed ANT XR s but is now only activated on AML cells by active uPA/uPAR (Liu et al., 2009). In normal cells, the expression of uPA and uPAR is induced in response to tissue injury, playing a major role in tissue repair and remodeling (Liu et al., 2003). uPA is initially secreted in a single chain inactive form, once cleaved by plasmin into a double chain it becomes active. Both the active and inactive form of these serine protease bind to uPAR. Potency of PrAgU₂/FP59, therefore, depends on the expression and activity of all the following markers; anthrax toxin receptors (ANT XR s), uPAR and uPA. The presence of ANT XR s is essential to the potency of PrAgU₂/FP59 independently of the other markers uPA and uPAR making ANT XR the primary binding site of PrAgU₂. In the absence of uPAR cell lines are not sensitive to the recombinant toxin PrAgU₂/FP59 even upon addition of excessive amount of exogenous pro-uPA (Abi Habib et al., 2004; Liu et al., 2009). In a previous paper it has been shown that PrAgU₂/FP59 is toxic to a wide range of tumors such as non-small cell lung tumor, prostate tumor, basal-like breast tumor, pancreatic tumor, and basal-like breast tumor. It has also been noted in this study that the tumor cell lines that did not respond to PrAgU₂/FP59 became sensitive upon adding exogenous pro-uPA. Therefore, this toxin is highly selective since most normal human cells tested were resistant (Abi Habib et al., 2004). In another study of B16 mice with melanoma were treated with high dose of PrAgU₂/FP59, these mice suffered from ulcerations of the anal region and edema of the small intestine since uPA and uPAR are expressed in the villi under normal conditions. No histologic marks of toxicity were observed in the lung, kidneys, liver, heart and spleen. Using an anti-inflammatory steroid drug such as dexamethasone, while administering PrAgU₂/FP59 alleviated the systemic toxicity

caused by the recombinant toxin and reduced mortality to 1%. In the same study, it was shown that PrAgU₂/FP59 has significant antitumor effect after intra-peritoneal administration in colon and breast cancer (Rono et al., 2006). In addition, some studies have shown the high potential and selectiveness of PrAgU₂/FP59 against tumors in vivo (Kassab et al., 2013). uPAR is highly expressed by macrophages, budding cancer cells in colon cancer, in squamous cell carcinomas, making these types of tumors such as lung, head, neck, cervical and esophageal squamous cell carcinomas a good candidate for PrAgU₂/FP59 based therapy aiming at selecting and treating the cancer cells directly (Rono et al. 2006). The purpose of the novel studies is to switch cancer treatment into becoming tumor-selective; such as targeting BCR-Abl mutants in chronic myeloid leukemia and targeting Her2-neu over-expression in breast cancer patients (Druker et al., 1996; Pegram et al., 1998). It is supposed that the use of such toxins will lead to a more specific treatment with a reduction of the adverse symptoms caused by traditional chemotherapy and radiation and a reduction in the relapse percentage among patients. In addition, such recombinant toxins can be modified to become more selective to AML cells; hence they can only be activated in hematopoietic cells harboring mutations leading to uncontrolled proliferation.

1.4 Cell death

Targeted therapies usually try to approach cell death in order to clear diseased cells. Cell death is mainly classified into different types based on morphological and biochemical changes occurring within the cell. Generally, the most important

mechanisms related to cell death are apoptosis, necrosis, and autophagy (figure 2). Apoptosis is an organized programmed cell death that is activated by series of caspases, a family of intracellular cysteine protease (Edinger & Thompson, 2004; Testa et al., 2007). Necrosis, on the other hand, is an unorganized type of cell death that induces inflammatory response (Edinger & Thompson, 2004). Moreover, autophagy is a process mediated by lysosomes, which is responsible for intracellular degradation (Zhang, 2015). Apoptosis is a major process that maintains tissue homeostasis, embryonic development and for the differentiation and function of hematopoietic and lymphoid cells (Testa et al., 2007). There are two types of caspases that control apoptosis: the initiator caspase, which act at the origin of the apoptosis process and the effector caspase, which act in cell death execution. There are two ways by which caspases are activated: the extrinsic pathway which is triggered by death receptors on the cell membrane and the intrinsic pathway which is activated by several intracellular and extracellular stresses such as growth factor deprivation, oncogene induction, DNA damage, hypoxia and cytotoxic drugs (Testa et al., 2007). The intrinsic factors induce biochemical events that lead to the degradation of the outer mitochondrial membrane, with the consequent release of cytochrome c and other pro-apoptotic molecules from the inner mitochondrial membrane into the cytosol. Factors leading to the deregulation of apoptosis disturb the complex balance between cell proliferation, cell survival and cell death initiating in most cases cancer and particularly leukemia. Furthermore, tumors caused by disruption of proteins involved in cell death signaling are frequently resistant to chemotherapy (Testa et al., 2007). In a recent study, Nutlins have been developed to target the overexpressed MDM2 in AML cells in order to inhibit p53 activation leading to the induction of apoptosis (Kojima et al., 2015). In another study, the combination of

sorafenib with cytarabine induced cell death in AML cell lines by lowering the apoptotic threshold of these cells making them more sensitive to the proapoptotic effect of other biological agents and chemotherapeutic (Zhang et al., 2008).

Figure 1.4: Main types of cell death, apoptosis, necrosis, and autophagy. The process of cell death is classified into three classes: stimulation, regulation, and degradation (Esteve & Knecht, 2011).

In previous studies, the use of PrAgU₂/FP59 indicated a high potential approach in targeting urokinase expressing solid tumors (Abi Habib et al., 2004). In addition, inducing apoptosis in AML cells through targeted therapy would be considered as a

reversible way to overcome leukemia. In the following study, we show the high selectivity and potency of the urokinase recombinant toxin PrAgU₂/FP59 in AML cell lines. We also investigated the mechanism of cell death induced in the AML cell lines upon treatment with the recombinant toxin.

CHAPTER TWO

MATERIALS AND METHODS

2.1 Expression and purification of PrAg, PrAgU₂ and FP59

Recombinant anthrax proteins, PrAg (activated by furin), PrAgU₂ (activated by the urokinase plasminogen activator), and FP59, were expressed and purified in the laboratory of Stephen H. Leppla at the National Institute of Allergies and Infectious Diseases (NIAID) of the National Institutes of Health (NIH) in Bethesda, MD, as described previously (Ramirez DM, 2002; Liu S., 2001).

2.2 Cells and cell lines

Human AML cell lines HL60, U937, ML1, ML2, Mono-Mac-1, Mono-Mac-6, TF1-vRaf, TF1-vSrc and TF1-HaRas were obtained from the American Type Culture Collection (ATCC) and grown as described previously in RPMI 1640 supplemented with 10% FBS and 5% penicillin/Streptomycin or in Iscove's Modified Dulbecco's Medium (IMDM) supplemented with 10% FBS and 5% penicillin/Streptomycin (ML1) at 37°C, 5% CO₂ (Pellizzari et al., 1999).

Human CD₃₄⁺ progenitor bone marrow blasts (PBMBs) were purchased from (Lonza, Basel, Switzerland) and grown in StemlineIITM 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 (GM-CSF), 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).

2.3 Proliferation inhibition assay (Cytotoxicity)

Sensitivity of human AML cell lines and Human CD₃₄⁺ progenitor bone marrow blasts to PrAgU₂/FP59 was determined using a proliferation inhibition assay (cytotoxicity) as described previously (Abi-Habib et al., 2005). We have also used a combination of the furin-activated PrAg with FP59 (PrAg/FP59) as a control for urokinase-independent cytotoxicity of FP59. Binding of FP59 to PrAg and its translocation into the cytosol are identical to those of PrAgU₂, however, it is activated by the ubiquitously-expressed furin protease instead of the AML-specific urokinase plasminogen activator. Hence PrAg/FP59 is cytotoxic to all cells, with no tumor selectivity, and serves as control for the potency of FP59. Briefly, aliquots of 10⁴ cells/well, in 100 µl cell culture medium, containing a fixed concentration of 10⁻⁹ M FP59 were plated in a flat-bottom 96-well plate (Corning Inc. Corning, NY). Then, 50 µl PrAgU₂ or PrAg in media were added to each well to yield concentrations ranging from 10⁻⁸ to 10⁻¹³ M. Following a 48 h incubation at 37°C/5% CO₂, 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_{50} 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_{50} .

2.4 Cell cycle analysis

The impact of treatment with PrAgU₂/FP59 on the cell cycle of AML cells was determined using Propidium Iodide (PI)-staining on flow cytometry. Briefly, cells incubated with PrAgU₂/FP59 (10^{-8} M), PrAg/FP59 (10^{-8} M) and FP59 alone (10^{-9} M) as a control in a 6-well plate (Corning Inc. Corning, NY) for 48 and 72 h at 37°C, 5% CO₂, were harvested and fixed in 70% ethanol for a minimum of 24 h, at -20°C. Cells were then incubated in 500 µl PI staining solution (50 µg/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 G0/G1, S and G2/M phase was determined in control cells and in cells treated with PrAgU₂/FP59 and with PrAg/FP59 following gating for the cell population on width versus forward scatter.

2.5 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 μ l media in a flat-bottom, 96-well plate and were incubated with either 100 μ l of medium alone (control cells) or medium containing three different concentrations of PrAgU₂/FP59 or PrAg/FP59 (as described above under cell cycle analysis) for 24 and 48 h at 37°C/5% CO₂. 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 μ 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.

2.6 Expression of uPAR

Expression of the urokinase plasminogen activator receptor (uPAR) on AML cell lines was determined using single cell staining with a FITC-conjugated anti-uPAR antibody on flow cytometry as described previously. Approximately 10^6 cells were incubated with a 1/100 dilution of a FITC-conjugated anti-uPAR mouse monoclonal antibody (Cell Signaling Technology, Danvers, MA) in antibody binding buffer for 1 h at 37°C. Cells stained only with a FITC-conjugated mouse IgG were used as isotypic control. Samples were then analyzed using a C6 flow cytometer (BD Accuri, Ann Arbor, MI). The expression of uPAR was analyzed on FL1-H and compared with that of the isotypic control. Positivity for the presence of uPAR was determined using the ratio of fluorescence intensity (RFI) between the mean fluorescence intensity (MFI) of the uPAR stained cells and the MFI of the isotypic control. $RFI > 2$ was considered positive. RFI between 1.5 and 2 were considered slightly positive and $RFI < 1.5$ was considered negative.

2.7 Blocking Assays

Blocking assays were performed to test the ability of specific anti-uPA antibodies to block the cytotoxicity of PrAgU₂/FP59 to uPAR-expressing AML cell lines. The same proliferation inhibition assays were performed as described earlier with the difference that in the blocking assays, 10 µg/mL monoclonal anti-uPA antibody (American Diagnostica) was added to a subset of cells upon plating. The rest of the assay proceeded as described above. Data was analyzed using GraphPad Prism V

software (GraphPad Software, San Diego, CA). The absorbance and the percent absorbance of controls were compared between the different treatment groups.

2.8 Effect of the addition of pro-uPA

This assay was performed to further demonstrate that the activity of PrAgU₂/FP59 was dependent on the activation of uPA. Aliquots of 10⁴ cells were incubated in 100 µL medium (same as that used to grow the cells) in Costar (Corning, NY) 96-well flat-bottomed plates in duplicate. Exogenous human pro-uPA (American Diagnostica, Stamford, CT) was added to 36 wells of each duplicate plate (100 ng/mL in each well). Then, 50 µL PrAgU₂ in medium was added to each column to yield concentrations ranging from 0.1 to 10 000 pM, and the cells were incubated at 37°C/5% CO₂ for 24 to 48 hours (Abi-Habib et al., 2004). Data was analyzed using GraphPad Prism V software (GraphPad Software, San Diego, CA). The absorbance and the percent absorbance of controls were compared between the different treatment groups.

CHAPTER THREE

RESULTS

3.1 Cell Sensitivity to PrAgU₂/FP59.

3.1.1 Potency of PrAgU₂/FP59 to AML Cell Lines

The potency of PrAgU₂/FP59 was determined on a panel of nine human AML cell lines using a proliferation inhibition assay (cytotoxicity). FP59 ADP-ribosylates elongation factor-2 (EF2) leading to the inhibition of protein synthesis and subsequent cell death; hence it is cytotoxic to all cells that express anthrax toxin receptors (ANTXRs) once it enters the cytosol. Potency of PrAgU₂/FP59 to AML cell lines will, therefore, depend on their cell surface uPA/uPAR activity and their ability to activate PrAgU₂ and deliver FP59 into the cytosol.

All 9 human AML cell lines were sensitive to PrAgU₂/FP59 with IC₅₀ values ranging from 2.0 to 414 pm and a more than 70% death at the highest concentration, indicating that AML cells do express uPA/uPAR and are effectively targeted by the urokinase-activated fusion toxin, PrAgU₂/FP59 (table 3.1 and figure 3.1.1). The range and potency of PrAgU₂/FP59 were similar to those of the furin-activated, non-tumor selective PrAg/FP59, which was used as a positive control for the potency of FP59 (table

3.1 and figure 3.1.1). Though the IC_{50} of PrAgU₂/FP59 to some of the cell lines tested (5 out of 9) was higher than that of PrAg/FP59, this data still demonstrates that the addition of a tumor-selectivity criterion, in the form of urokinase activation, did not affect the range and potency of this fusion toxin. This is expected because even though the expression and activity of the urokinase plasminogen activator protease system (uPA/uPAR) on AML cell lines is high, they may not reach the same levels as those of the furin protease system which is highly overexpressed and very active on all types of cells and tissues.

Figure 3.1.1: Cytotoxicity of PrAgU₂/FP59 and PrAg/FP59 to AML cell lines HL60 (A), Mono-Mac-6 (B), U937 (C), Mono-Mac-1 (D), ML-1 (E), TF1-vSrc (F), TF1-HaRas (G) and ML-2 (H) using a proliferation inhibition assay (cytotoxicity). As illustrated, PrAgU₂/FP59 is cytotoxic to all AML cell lines tested with the same potency (ML-2 and Mono-Mac-6) or a slightly lower one compared to the PrAg/FP59, the furin-activated positive control. X-axis represents the log of the molar drug concentration; Y-axis represents the cell viability expressed as optimal density at 450 nm.

3.1.2 Effect of PrAgU₂/FP59 on CD₃₄⁺ human progenitor bone marrow blasts.

In order to determine whether the addition of a urokinase activation site in PrAgU₂/FP59 increased its tumor selectivity we tested its cytotoxicity on CD₃₄⁺ human progenitor bone marrow blasts and compared it with that of PrAg/FP59. As illustrated in Figure 3.1.2, PrAgU₂/FP59 is not cytotoxic to CD₃₄⁺ progenitor bone marrow blasts with an IC₅₀ > 10,000 pM. In comparison, PrAg/FP59 was highly cytotoxic to CD₃₄⁺ blasts with an IC₅₀ value of approximately 10 pM (table 3.1 and figure 3.1.2). This demonstrates that the addition of a urokinase-activation site in PrAgU₂/FP59 drastically decreases its toxicity to normal, which, in turn, increases the tumor selectivity of this recombinant toxin.

Figure 3.1.2: Cytotoxicity of PrAgU₂/FP59 and PrAg/FP59 to CD_{34}^{+} progenitor bone marrow blasts using a proliferation-inhibition assay. As illustrated, CD_{34}^{+} cells are sensitive to PrAg/FP59 but not to the urokinase-activated PrAgU₂/FP59. On the X-axis represents the log of the molar drug concentration and the Y-axis represents cell viability expressed as optimal density at 450 nm.

Table 3.1 Sensitivity of AML cell lines and human CD₃₄⁺ progenitor bone marrow blasts (normal bone marrow cells) to PrAgU₂/FP59 and PrAg/FP59.

Cells	PrAgU ₂ /FP59 (IC ₅₀ ;pmol/L)	PrAg/FP59 (IC ₅₀ ;pmol/L)
AML Cell lines		
HL60	3.0	46.0
MonoMac-6	11.0	8.0
ML-1	26.0	7.0
ML-2	28.0	18.0
MonoMac-1	86.0	29.0
TF1-vRaf	134.4	9.0
U937	160.0	2.0
TF1-vSRC	359.0	11.0
TF1-HaRas	414.0	16.0
Normal Human Cells		
CD ₃₄ ⁺ Bone Marrow	2842.0	10.0
Blasts		

3.2 Cell Cycle Analysis.

In order to determine whether, in addition to cytotoxicity, treatment with PrAgU₂/FP59 induces cell cycle arrest in AML cell lines, we tested the cell cycle status of three AML cell lines ML-2, U937 and TF1-vRaf, at 48 hours following treatment, using flow cytometry. As expected, all the three cell lines had more than 45% of the cells in the G₀/G₁ phase, with 13 to 20% of the cells in the G₂ phase and less than 2% of the cells are in pre-G₀/G₁ (dead) in untreated cells. Following treatment with the recombinant toxin PrAgU₂/FP59, the percentage of living cells in both G₀/G₁ and G₂ phases decreased significantly to less than 13% of the cell population coupled to a dramatic increase in the percentage of cells in pre-G₀/G₁ ranging from 65% to 86% of the total cell population at both 24 and 48 hours post treatment (figure 3.2). This indicates that treatment with PrAgU₂/FP59 has no impact on the cell cycle of AML cell lines but rather leads to complete cell death with no or a very small proportion of surviving cells. This also further demonstrates the significant potency of the cytotoxic effects of PrAgU₂/FP59 to AML cell lines.

B)

C)

Figure 3.2: Flow cytometry analysis of the effect of PrAgU₂/FP59 on the cell cycle of three AML cells: ML-2 (A), TF1-vRaf (B), and U937 (C). The left panels show control cells and the right panels show cells treated with the recombinant toxin PrAgU₂/FP59. G0/G1 cells are gated M1, G2 cells are gated M2 and pre-G0/G1 M3. Cells were gated on width versus forward scatter.

3.3 Type of cell death following treatment with PrAgU₂/FP59.

In order to determine the mechanisms leading to cell death in AML cells treated with the recombinant toxin PrAgU₂/FP59, cells were stained with AnnexinV/PI and a cell permeable FITC-conjugated active caspase inhibitor and analyzed on flow cytometry. Treatment with different concentrations of PrAgU₂/FP59 led to an increase in the

percentage of cells stained positively for annexinV associated with a very small increase in PI staining, at both 24 and 48 hours post-treatment, indicating that treatment with PrAgU₂/FP59 induces apoptotic cell death in AML cell lines (figure 3.3). This was confirmed by AML cells staining positively with the FITC-labeled caspase inhibitor demonstrating the presence of active caspases in AML cells following treatment and further indicating that PrAgU₂/FP59 induces apoptotic cell death in AML cell lines.

Figure 3.3: AnnexinV (FL1-H)/PI (FL2-H) staining of two AML cell lines TF1-vRaf (A) and U937 (B) treated with PrAgU₂/FP59 for 48 hours and analyzed on flow cytometry. First panels from the left are control cells, second panels are cells treated with a low concentration (10^{-10} M) and the third panels are cells treated with a high concentration (10^{-8} M) of PrAgU₂/FP59. The last panels to the right show a histogram of control cell (black) and treated cells (red) stained with a FITC-labeled cell permeable active caspase inhibitor clearly showing increased presence of active caspases in treated cells. Cells were gated in width versus forward scatter.

3.4 uPAR expression

3.4.1 uPAR expression on AML cell lines

In order to further correlate the mechanisms of activation of PrAgU₂/FP59 in AML cells to the expression levels of the urokinase plasminogen activator receptor (uPAR) on cells, we determined by flow cytometry uPAR expression levels on the entire panel of AML cell lines using cell surface staining with a FITC-labeled anti-uPAR antibody. All stained AML cells were compared to the isotype control and expression levels were determined based on the ratio of fluorescence intensity (RFI) of stained and isotype control cells. All 9 AML cell lines stained positively for uPAR expression confirming that this cell surface serine protease is expressed on AML cells and is the underlying mechanism for the sensitivity of AML cells to the urokinase-activated PrAgU₂/FP59 (Figure 3.4.1 and Table 3.2). Of the 9 AML cell lines tested, 8 were found to highly express uPAR with a ratio of fluorescence intensity (RFI) ranging between 2.30 to 7.30 with only one cell line, MonoMac-6, being weakly positive for uPAR expression with an RFI = 1.82 (figure 3.4.1 and table 3.2). The fact that MonoMac-6 cells, though low uPAR expressers, are still sensitive to PrAgU₂/FP59 indicates that even low expression levels of uPAR are sufficient to generate enough urokinase activity to activate PrAgU₂/FP59 and to selectively kill AML cells.

E)

F)

G)

H)

Figure 3.4.1: Flow cytometry analysis of a panel of 9 AML cell lines ML1 (A), MonoMac-1 (B), HL60 (C), ML-2 (D), Monomac-6 (E), U937 (F), TF1-vRaf (G), TF1-vSrc (H) and TF1-HaRas (I) stained with a FITC-labeled anti-uPAR antibody and a FITC-labeled IgG isotype control. Left panels are cells stained with the isotypic control, middle panels are cells stained with anti-uPAR antibody, and right panels show a histogram of cells stained with the isotypic control (black) and cells stained with the anti-uPAR antibody (red). uPAR expression is considered positive when RFI > 1.5. Cells were gated on width versus forward scatter.

3.4.2 uPAR expression on CD₃₄⁺ cell line

As illustrated in figure 3.4.2 normal CD₃₄⁺ progenitor bone marrow blasts, when examined using cell surface staining on flow cytometry, do not express uPAR having an RFI value of 1.15 indicating negative staining for uPAR expression. This confirms that the lack of sensitivity of CD₃₄⁺ to PrAgU₂/FP59 is due to the absence of uPAR receptors on their cell membrane surface, hence the toxin is not activated in these cells and it is selective to the AML cells that overexpress uPAR (table 3.2 and figure 3.4.2).

Figure 3.4.2: Flow cytometry analysis of CD₃₄⁺ bone marrow blasts stained with a FITC-labeled anti-uPAR antibody and a FITC-labeled IgG isotype control. Left panels are cells stained with the isotypic control, middle panels are cells stained with anti-uPAR antibody, and right panels show a histogram of cells stained with the isotypic control (black) and cells stained with the anti-uPAR antibody (red). uPAR expression is considered positive when RFI > 1.5. Cells were gated on width versus forward scatter.

Table 3.2: uPAR expression on AML cells and normal CD₃₄⁺ bone marrow progenitor blasts.

Cells	uPAR expression level (RFI)	PrAgU ₂ /FP59 (IC ₅₀ ;pmol/L)
AML Cell Lines		
MonoMac-6	1.82 (+)	11.0
TF1-HaRas	2.30 (+)	414.0
MonoMac-1	2.41 (+)	86.0
TF1-vSRC	2.41 (+)	359.0
TF1-vRaf	2.76 (+)	134.4
ML-1	2.82 (+)	26.0
ML-2	3.10 (+)	28.0
HL60	3.25 (+)	3.0
U937	7.29 (++)	2.0
Normal Human		
Cells		
CD ₃₄ ⁺	1.15 (-)	3842.0

3.5 Blocking assays.

In order to demonstrate that the cell surface activity of the urokinase plasminogen activator system (uPA/uPAR) is essential for the activation and, subsequently, the potency of PrAgU₂/FP59, we examined the effect of the inhibition of the uPA/uPAR system, using a neutralizing anti-uPA antibody, on the potency of PrAgU₂/FP59 to AML cells. Three AML cell lines (Mono-Mac-1, Mono-Mac-6 and ML-2) were tested for their sensitivity to PrAgU₂/FP59 in the presence and absence of the anti-uPA antibody. As illustrated in figure 3.5, inhibition of the uPA/uPAR system greatly reduced the potency of PrAgU₂/FP59, with the reduction ranging from an increase in IC₅₀ of 100-fold (Mono-Mac-6) and a 1000-fold (ML-2) in the presence of the antibody to the complete inhibition of the potency of the toxin (Mono-Mac-1). The difference in the extent of inhibition between the different cell lines is most likely due to the different levels of uPA secreted by each of them. Nevertheless, these results show that the inhibition of the uPA/uPAR system decreases the ability of cells to activate PrAgU₂/FP59 and the subsequent potency of this toxin. This proves that an active uPA/uPAR system is essential for the potency of this urokinase-activated, tumor selective fusion toxin.

Figure 3.5: Cytotoxicity of PrAgU₂/FP59 in the presence and absence of an anti-uPA to 3 AML cell lines: MonoMac-1 (A), Monomac-6 (B) and ML-2 (C), using a proliferation inhibition assay. X-axis represents log of the molar drug concentration, Y-axis represents cell viability expressed as optimal density at 450 nm.

3.6 Addition of exogenous pro-uPA

In order to further confirm that the toxicity of PrAgU₂/FP59 relies solely on the activation of the urokinase plasminogen receptor we compared the potency of PrAgU₂/FP59 on U937 AML cell line in the presence and absence of exogenous pro-

uPA. The active uPA cleaves the proenzyme plasminogen into active plasmin, which in turns activates uPA in a positive feedback loop (Prager et al., 2004) resulting the activation of PrAgU₂/FP59 toxin. As shown in figure 3.6, the addition of exogenous pro-uPA increased the conversion of pro-uPA to active uPA hence increasing the activity of urokinase system. Now that PrAgU₂/FP59 is activated its toxicity level on U937 became higher. The presence of exogenous pro-uPA caused a 1000 folds reduction in IC₅₀. This confirms that the activation of the uPA/uPAR system has a direct positive impact on the potency of this urokinase-selective toxin on all AML cell lines.

Figure 3.6: Cytotoxicity of PrAgU₂/FP59 in the presence and absence of excess exogenous pro-uPA on U937 cells using a proliferation inhibition assay. X-axis represents log of the molar drug concentration, Y-axis represents cell viability expressed as optimal density at 450 nm.

CHAPTER FOUR

DISCUSSION

The uPA/uPAR system has been shown to be expressed in almost all types of human tumors such as carcinomas, sarcomas, monocytic and myelogenous leukemia. Since this particular system is poorly expressed in normal cells except in specific conditions such as tissue repair or remodeling, targeting the uPA/uPAR system could be a hallmark for different types of cancer (Liu et al., 2003). The poor prognosis of AML, the side effects of chemotherapeutic agents and the high mortality rates due to the development of resistance to the traditional chemotherapeutic drugs have led to identifying effective therapeutic drugs capable of targeting the resilient tumors without damaging normal tissue.

One such targeted therapeutic drug, is the urokinase activated anthrax fusion toxin PrAgU₂/FP59. This toxin is derived from anthrax lethal factor by first replacing the zinc metalloprotease domain of lethal factor on PrAg, the binding moiety of the anthrax lethal toxin, with a urokinase activation site. Also, the LF and EF binding site were substituted with the binding site of *Pseudomonas aeruginosa* exotoxin A, which is a protein synthesis inhibitor capable of causing cell death. By substituting its furin cleavage sequence with a urokinase specific cleavage sequence, the toxin PrAgU₂/FP59 becomes highly selective and potent (Abi-Habib et al., 2006). Targeting the urokinase system with fusion toxins is a novel approach in selectively targeting AML without

damaging DNA or blocking cell division, hence reducing drug side effect on normal organs (Wescott et al., 2004). A previous study was conducted to test the effect of recombinant toxins on AML. When diphtheria toxin furin cleavage site was replaced by a urokinase plasminogen activator cleavage site they obtained the DTU₂GMCSF recombinant toxin. DTU₂GMCSF has been shown to be highly potent against AML cells *in vitro* and highly selective since it did not induce liver damage in rats *in vivo* (Wescott et al., 2004). In a previous study we have shown that the fusion toxin PrAg/LF is highly cytotoxic against most AML cell lines through the LF-mediated inhibition of the MAPK pathway (Kassab et al., 2013). On the other hand, the potency and selectivity of PrAgU₂/FP59 were tested on many human tumor cell types: it was shown that many non-small cell lung cancer, pancreatic cancer, colon cancer, prostate cancer, and breast cancer cells all overexpress the urokinase plasminogen activator receptors on their cell surface hence these cell types were highly sensitive to the potent the drug PrAgU₂/FP59. In the same study, this potent drug was not cytotoxic to normal cells such as human lung fibroblasts, human cardiomyocytes, human cardiac microvascular endothelial cells, renal proximal tubule epithelial cells and renal cortical epithelial cells (Abi Habib et al., 2006). We also tested the pathogenicity of PrAg/LF and PrAgU₂/LF on Balb/c mice *in vivo* and found that the addition of the urokinase plasminogen activator system highly reduced the damaging effect of the toxin on internal organs. Since different experiments have shown that PrAgU₂/FP59 is capable of selectively successfully targeting the overexpressed urokinase system, and since recent studies have shown that more than 70% of AML patients express the uPA protease system, therefore this drug can be used for AML treatment.

In this study, we tested 9 AML cell lines' sensitivity to PrAgU₂/FP59 using a proliferation inhibition assay, and all cell lines were found to be sensitive (IC₅₀ < 400 pmol/L). Knowing that FP59 cannot inhibit protein synthesis unless the urokinase system has been activated, these results suggested that all tested AML cell lines express the uPA protease system. These results are in line with a previous study that shows that PrAgU₂/FP59 is highly activated in cancer cells since they overexpress the uPAR, thus this toxin can be used in targeted molecular therapy against AML. The human bone marrow progenitor blasts CD₃₄⁺ are very sensitive to PrAg/FP59, but when the drug was made more selective, this normal human cell line treated with PrAgU₂/FP59 using a proliferation inhibition assay was not sensitive to protein synthesis inhibition having an IC₅₀ 4X folds greater than the normal range (IC₅₀ >700 pmol/L). Moreover, in a previous study treating most normal human cell lines with this engineered recombinant toxin was proven to be very safe. We further examined the uPAR level of expression on the extracellular surface of cells and showed that it is highly expressed on AML cells but not on CD₃₄⁺. Upon adding anti-uPA to AML cell lines, they became resistant to the toxin confirming that uPAR is indispensable for the activation of PrAgU₂/FP59, and without it, the recombinant toxin is not toxic at all. This explains the sensitivity of CD₃₄⁺ to PrAg/FP59 and not to PrAgU₂/FP59, since the latter toxin was not activated due to the absence of sufficient uPA receptors on the cell membrane. Since we have shown that the lack of the recombinant toxin activation on CD₃₄⁺ cell line is due to the absence of urokinase receptor and in previous studies it has been shown that most human cell lines do not express the uPA protease system unless under specific conditions, we then assumed that different types of human cells are also resistant to the recombinant toxin PrAgU₂/FP59. This was shown with U937 AML cell line which exhibits a high increase

in sensitivity to PrAgU₂/FP59 upon addition of exogenous pro-uPA which highly increases the activity of uPA receptors on its cell surface. Therefore, any AML cell line that does not overexpress uPA will still be sensitive to the toxin by activating the uPA/uPAR system with the uPA secreted by neighboring leukemic cells. The selectivity of this toxin to AML cells only makes it a drug of choice since it will minimize all the side effects caused by current chemotherapeutic drugs, and since this targeted therapy relies on the overexpression of the uPA/uPAR system which is found on all AML cells, we suspect that the number of relapsing patients will reduce. Furthermore, we demonstrated that the reduction in proliferation of most AML cells is due to cell death rather than cell cycle arrest, using propidium iodide staining with flow cytometry. Additionally, we have revealed that the treated AML cells have an increase in annexin V staining, revealing apoptotic rather than necrotic cell death. PrAgU₂/FP59 has proved the ability to treat AML by triggering cell death without being able to induce the same mechanism in normal cells. Therefore, PrAgU₂/FP59 could be considered as a potential hallmark for the treatment of AML by selectively targeting the cancerous cells and initiating apoptosis without affecting normal cells.

In a previous *in vivo* study on Balb/c mice, we have shown that adding the urokinase moiety to the toxin PrAg/LF had a great impact on reducing its effect on normal cells even if used at high doses. Since FP59 is more potent than LF in causing cell degeneration, thus performing an *in vivo* study would probably have a selective effect on AML cells without causing organ deterioration because of the presence of the selective urokinase moiety in the toxin.

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