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**Sensitivity of Acute Myeloid Leukemia Cells to the Dual
Targeting of the Urokinase Plasminogen Activator and the
MAPK Pathway by a Urokinase-Activated Anthrax Lethal
Toxin**

By

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Sensitivity of Acute Myeloid Leukemia Cells to the Dual Targeting of the Urokinase Plasminogen Activator and the MAPK Pathway by a Urokinase-Activated Anthrax Lethal Toxin

Amira Mohammad Khalid Bekdash

Abstract

Acute myeloid leukemia (AML) is a hematopoietic malignancy characterized by abnormal proliferation of myeloid progenitor cells. Around 15,000 new case of AML are diagnosed each year with a fatality rate of 65%. Conventional treatment consists of two phases, an induction phase and a consolidation phase. Though standard treatment yields a high remission rate, the majority of patients eventually relapse due to the proliferation of drug-resistant leukemic blasts in the bone marrow, hence the need for alternative approaches employing novel, more selective mechanisms for targeting AML blasts. In this study, we attempt to target both the MAPK pathway and the uPA/uPAR protease system in AML cells using a dual-selective, urokinase-activated recombinant anthrax lethal toxin (PrAgU2/LF).

Anthrax lethal toxin (PrAg/LF) is a binary toxin that consists of two separate proteins, a protective antigen (PrAg) and a lethal factor (LF). PrAg binds cells, is cleaved by furin, oligomerizes, binds three to four molecules of LF, and undergoes endocytosis, releasing

LF into the cytosol. LF cleaves MAPK kinases, inhibiting the MAPK pathway. We have generated a modified anthrax lethal toxin in which the furin activation site was replaced with uPA/uPAR activation site generating a urokinase-targeted anthrax lethal toxin, PrAgU2/LF.

The MAPK pathway is a conserved pathway that regulates growth, proliferation, differentiation, and death. Its constitutive activation promotes proliferation and survival of most human cancer cells. The urokinase plasminogen activator is a serine protease consisting of the urokinase plasminogen activator (uPA) and its receptor (uPAR) and it has been shown to be overexpressed in a wide array of solid and hematological malignancies including AML.

We tested the potency and selectivity of PrAgU2/LF on a panel of 11 human AML cell lines and two normal hematopoietic cells, peripheral mononuclear cells and CD34⁺ progenitor bone marrow blasts. Five AML cell lines showed cytotoxic responses to PrAgU2/LF while none of the normal cells tested were targeted by this toxin indicating potency and selectivity of this dual-selective molecule in AML. Further analysis revealed that, out of the 6 AML cell lines that did not respond to PrAgU2/LF, 4 were also not responsive to the wild-type PrAg/LF, indicating resistance to the inhibition of the MAPK pathway, while the remaining 2 cell lines did not express the uPA/uPAR system. Furthermore, sensitivity of cells to PrAgU2/LF was linked to the expression levels of uPAR and was reversed through the inhibition of the uPA/uPAR system. Finally, a dose escalation study in mice showed that the maximal tolerated dose (MTD) of PrAgU2/LF is more than 4-fold higher than that of the wild-type PrAg/LF. Hence, introduction of the urokinase-activation sequence generated a dual-selective molecule

whose activity requires both the expression of the uPA/uPAR system and the dependence to the MAPK pathway by the targeted cells.

In this study, we have shown that the Urokinase-activated anthrax lethal toxin (PrAgU2/LF) selectively targets AML cells while sparing normal blasts and is a novel, dual selective molecule for the potential targeting of AML.

Keywords: AML, Anthrax lethal toxin, Urokinase, MAPK, Dual-targeting

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List of Abbreviation

Abbreviation	Full Name
AML	Acute Myeloid Leukemia
ANTXR_s	Anthrax Toxin Receptors
CMG-2	Capillary Morphogenesis Gene 2
ERK1/2	Extracellular Signal-Regulated Kinase 1 And 2
FP59	<i>Pseudomonas Aeruginosa</i> Exotoxin A
GDP	Guanosinediphosphate
GPI	Glycosyl-Phosphatidyl Inositol
GTP	Guanosine Triphosphate
HSC	Hematopoietic Stem Cell
JNK	C-Jun NH ₂ -Terminal Kinase
LeTx	Anthrax Lethal Toxin
LF	Lethal Factor
MAPK	Mitogen-Activated Protein Kinase
MAPKK	Mitogen-Activated Protein Kinase Kinase

MAPKKK	Mitogen-Activated Protein Kinase Kinase kinase
MEK1/2	Mitogen-Activated Protein Kinase Kinase
MKK	Mitogen-Activated Protein Kinase Kinase
MMP	Metalloprotease
MTT	Maximal Tolerated Dose
PAI-1	Plasminogen Activator Inhibitor- 1
PBMB	Progenitor Bone Marrow Blasts
PrAg	Protective Antigen
RFI	Ratio of Fluorescence Intensity
TEM-8	Tumor Endothelium Marker 8
uPA	Urokinase Plasminogen Activator
uPAR	Urokinase Plasminogen Activator Receptor

CHAPTER ONE

INTRODUCTION

1.1 -Acute Myeloid Leukemia

Acute myeloid leukemia (AML) is a hematopoietic malignancy characterized by abnormal proliferation of myeloid progenitor cells along with a permanent block in cellular differentiation. Bone marrow infiltration of immature leukemic myeloblasts to the peripheral blood leads to abnormal survival of leukemic cells (Weisberg et al., 2009). The loss of differentiation and proliferation of myeloid progenitor cells can provoke symptoms ranging from fatal infection to bleeding and organ infiltration, especially in the absence of treatment (Estey & Döhner, 2006).

It is estimated that the long-term survival rates in AML patients younger than 60 years ranges from 25%-70%; however, older patients have survival rates of only 5 to 15% (Kindler, Lipka, & Fischer, 2010). The survival rates depend on many factors such as the type of treatment, age, patient's performance status, organ dysfunction, and biological characteristics of AML (such as cytogenetics and mutations) (Ravandi, 2012).

Currently, the conventional treatment of AML involves two phases, an induction phase and a consolidation phase (Ferrara & Schiffer, 2013). Both phases employ chemotherapy, as a main option, and hematopoietic stem cell (HSC) transplantation, if

possible. The aim of the induction regimen, which includes the use of anthracycline (daunorubicin) and cytarabine, is to achieve clinical response; and the aim of the consolidation regimen, which includes the use of cytarabine or combination of other chemotherapeutic drugs, is to eliminate the presence of leukemic cells in the bone marrow and peripheral blood (Ferrara & Schiffer, 2013). Unfortunately, there is currently no well-defined treatment protocol for AML patients. Current treatment approaches are yielding high remission rates around 85%; however, they are also associated with a high relapse rate with 55% to 75% of patients going into relapse after a short period of time (Ravandi, 2012). Relapse is due to the proliferation of drug-resistant leukemic blasts that remain in the bone marrow after treatment (Ferrara & Schiffer, 2013). Thus, the need for the development of novel, tumor-targeted, and selective strategies for the treatment of acute myeloid leukemia cells is essential. For this reason, over the past two decades, extensive studies were done to understand the pathogenesis and the pathomechanisms of AML, including the mutations involved and the cytogenetics of the diseases in an attempt to increase the knowledge base that may allow the development of such novel, effective and selective therapies (Graubert & Mardis, 2011).

1.2 - The MAPK pathway

Studies showed that the Mitogen-Activated Protein Kinase (MAPK) pathway is constitutively activated in approximately 74% of AML cases, especially in myeloid cells (Brown & Sacks, 2008). The most important three MAPK families are the

extracellular signal-regulated kinases 1 and 2 (ERK1/2), the c-Jun N-terminal kinase (JNK (1–3)) and the p38 (Raman, Chen, & Cobb, 2007). The MAPK signal transduction pathways consist of three major consecutive protein kinases termed MAPK kinase kinase (MAPKKK), MAPK kinase (MAPKK), and MAPK, where phosphorylation is a key element throughout this flow (Krishna & Narang, 2008). The MAPK cascade is an important signaling pathway that is activated by several extracellular stimuli to contribute in regulating cellular responses such as apoptosis, survival, proliferation, and differentiation (Bermudez, Pagès, & Gimond, 2010). Activation of the signaling cascade in the MAPK pathway is initiated with small G proteins of the Ras family that are present at the cell membrane's inner surface; a stimuli will transform RAS-GDP (inactive form) into RAS-GTP (active form) (Shaul & Seger, 2007). Active RAS interacts with various effectors such as the RAF Kinase family, leading to its activation. Active RAF will phosphorylate and activate MEK1/2, which in turn will activate ERK1/2 to accomplish the role of the MAPK pathway (Zebisch et al., 2007).

The continuous activation of the signal transduction Ras/Raf/MEK1/2ERK1/2 cascade, in a myeloid cell, is due to constitutive mutation in a member of this pathway leading to uncontrolled cell proliferation and survival; thus transforming normal myeloid cells into leukemic clones (Shaul & Seger, 2007). Most frequently, deregulation in the MAPK pathway can lead to AML formation, hence the potential of targeting the MAPK pathway, in AML cells.

Several previous studies were able to show that targeting the MAPK pathway through inhibition was enough to kill different tumor types. A study done by Huang et

al. (2008) showed that renal cell carcinoma having high expression levels of MKK1 and ERK2 were sensitive to the fusion anthrax lethal toxin due to its capability of inhibiting the MAPK pathway leading to cell death. An alternative way of targeting tumor cells that are dependent on the MAPK pathway is through using the anthrax lethal toxin PrAg/LF. Kassab et al. (2013) showed that targeting human AML cell lines with PrAg/LF, was enough to kill cells that are dependent on the MAPK pathway for survival.

1.3 -The urokinase plasminogen activator protease system

In addition to the constitutive expression of the MAPK pathway, an important hallmark of AML is the significant overexpression on AML cells of a mainly tumor-specific serine protease, the urokinase plasminogen activator (uPA) (Béné et al., 2004). Approximately 73% of AML patients have been shown to express the uPA protease system (Atfy, Eissa, & Salah, 2012). The urokinase plasminogen activator system is composed of the proteinase (uPA), its receptor (the urokinase-type plasminogen activator receptor – uPAR or CD87), and two major inhibitors, the plasminogen activator inhibitor 1 (PAI 1) and 2 (PAI 2) (Béné et al., 2004). uPA is a serine protease that catalyses the conversion and digestion of extracellular plasminogen to plasmin, thus inducing migration, cell invasion and metastasis (Graf et al., 2005). uPAR is a highly glycosylated glycosyl-phosphatidyl inositol (GPI) -anchored cell-surface receptor that binds uPA, which is generated endogenously or exogenously, in order to convert plasminogen to plasmin (Béné et al., 2004). The process starts by binding the catalytic

C-terminal domain of (pro)-uPA to the N-terminal domain of uPAR. This connection between uPAR and (pro)-uPA will lead to the enzymatic activation of (pro)-uPA into uPA. Activated uPA will consequently degrade plasminogen into plasmin (Figure 1.1).

Figure 1.1: Mechanism of action of the urokinase plasminogen activator system (Duffy & Duggan, 2004).

The uPA system is regulated by two major specific inhibitors, PAI 1 and PAI 2. These act directly on uPA either in its free state or in its bound state to the receptor uPAR. The PAI 1 operates by binding to uPA that is already bound to uPAR, thus

forming the uPAR–uPA–PAI 1 complex. This complex will change the properties of the receptor uPAR leading to its internalization. The complex will then break down into two parts the UPA-PAI1 and the receptor uPAR. The uPA-PAI1 will head into the lysosome for degradation and the receptor uPAR will be recycled to the cell surface (Figure 1.2). As a result, PAI 1 shows a great importance due to its role in regulating the activity of uPAR and maintaining the distribution of uPAR on the cell surface (Figure 1.2).

Figure 1.2: Mechanism of regulation of uPAR expression through PAI-1 (Dass et al., 2008)

The uPA system is not expressed on healthy cells under normal conditions, however, certain physiological processes, such as wound healing or tissue remodeling,

can cause the up-regulation of the uPA system (Abi-Habib, Liu, Bugge, Leppla, & Frankel, 2004). Leukemic cells such as AML over express the uPA system and as mentioned above, approximately 73% of AML patients express this protease system. Observation suggests that the expression of the uPA system in AML patient is correlated with bad prognosis (Sidenius & Blasi, 2003). Hence, the opportunity of taking advantage of the overexpression of the uPA/uPAR system to selectively target AML cells.

One example that shows the use of the expression of the uPA/uPAR system on cancer cells as a selection tool is the study done by Ramage et al. (2003) on AML cell lines. This study showing the ability of targeting specific leukemic cells expressing the uPA/uPAR system through the diphtheria toxin/urokinase fusion protein (DATA), which leads to cell cytotoxicity. Another study done by Abi-Habib et al. (2006) demonstrated the advantage of the urokinase system that is present on different types of tumor cell lines, such as non-small cell lung cancer, pancreatic cancer, colon cancer and prostate cancer, as a selection mechanism in order to target those cell lines with the recombinant anthrax toxin PrAgU2/FP59.

In this study, we attempt to target both the MPAK pathway and the urokinase plasminogen activator protease system in AML cells using a urokinase-activated, recombinant anthrax lethal toxin (PrAgU2/LF).

1.4-Fusion toxins

Fusion toxins are genetically modified recombinant proteins consisting, traditionally, of two parts, the target moiety and the bacterial or plant protein toxin (Kreitman, 2009). The fusion of those two parts will result in a protein conjugate equipped with cytotoxic agents (the toxin) capable of selectively targeting tumor-specific markers, receptors or pathways (Chandramohan, Sampson, Pastan, & Bigner, 2012). Fusion toxins consist of a catalytic domain of a toxin, such as Diphtheria toxin or *Pseudomonas aeruginosa* exotoxin A, fused to a binding domain, such as an overexpressed receptor on the cell-surface of a particular tumor. Examples of class I toxins include DTGMCSF (Diphtheria toxin granulocyte-macrophage colony-stimulating factor), DTIL2 (Diphtheria toxin interleukin 2), and DTIL3 (Diphtheria toxin interleukin 3) (Horita, Frankel, & Thorburn, 2008). The toxic effect of the fusion toxin on the target cell is due to the enzymatic activity of the catalytic domain leading to the inhibition of protein synthesis. Novel classes of recombinant toxins target either tumor-selective cell surface proteases such as matrix metalloproteases (MMPs) and the urokinase plasminogen activator (UPA) or signaling pathways excessively activated in tumor cells such as the MAPK pathway. Examples of tumor protease targeting include a urokinase-activated fusion of Diphtheria toxin and GMCSF (DTU2GMCSF) and a MMP-activated version of anthrax lethal toxin (PrAgU2/LF). Anthrax lethal toxin (LeTx), is an example of a recombinant toxin that targets a signaling pathway on which cancer cells are highly dependent, namely the MAPK pathway.

The urokinase-activated anthrax lethal toxin (PrAgU2/LF) is one example of a recombinant toxin that combines two different tumor-targeting mechanisms in the form of urokinase activation and inhibition of the MAPK pathway. PrAgU2/LF is a potentially dual selective recombinant toxin that requires both overexpression of the urokinase system and dependence on the MAPK pathway to be active (Abi-Habib et al., 2004).

1.5 – Anthrax Lethal Toxin

Anthrax toxin is a major virulence factor secreted by *Bacillus anthracis* that is a gram-negative bacterium. It consists of three separate proteins: the protective antigen (PrAg, 83 kDa), the edema factor (EF, 90 kDa), and the lethal factor (LF, 90 kDa) (Liu, Bugge, Frankel, & Leppla, 2009). The protective antigen (PrAg), the cell binding and internalization moiety, is responsible for cell binding and translocation of LF or EF into the cell (Abi-Habib et al., 2005). The edema factor (EF), one of the catalytic moieties, is capable of cell damage due to its intracellular adenylate cyclase activity (Koo et al. 2002). The lethal factor (LF), the other catalytic moiety, is a Zn^{2+} metalloprotease that specifically cleaves and inactivates all MEKs except MKK5 (Duesbery et al., 1998; Pellizzari et al., 1999). Those three proteins alone are non-toxic, but when combined they can form the anthrax toxin (Bann, 2011). The term anthrax lethal toxin (LeTx) refers to the combination of PrAg and LF and has been used to target the MAPK pathway in a number of tumors.

Figure 1.3: Mechanism of action and the pathway of the anthrax lethal toxins (LeTx) (Rainey & Young, 2004)

PrAg binds to the anthrax toxins receptors (ANTXR_s), tumor endothelial marker 8 (TEM8), and capillary morphogenesis gene 2 (CMG2), that are present on the cell surface of all cells and tissues. PrAg is then cleaved at a specific sequence 164RK_{KKR}167 by cell surface furin-like proteases, resulting in the release of a 20 kDa fragment and the activation of PrAg (Figure 1.3). The resulting fragments of active, receptor-bound, PrAg₆₃, oligomerize, bind three to four molecules of LF before undergoing receptor-mediated endocytosis by acidic lipid rafts (Liu et al., 2009). Upon acidification of the endosome, PrAg oligomers undergo a conformational change to be

able to form insert in the endosomal membrane and mediate the escape of LF from the endosome (Melnik & Collier, 2006). In the cytosol LF, a zinc metalloprotease, cleaves and inhibits the mitogen-activated protein kinase kinases (MAPKK) through binding to the N-terminus of most isoforms of the MAPK kinase (Tonello & Montecucco, 2009). Thus, cancer cells that have a dependence on the MAPK pathway for survival may be targeted by LF causing its cleavage and leading to cell cycle arrest and cell death (Koo et al., 2002).

1.6 – The Urokinase-Activated Anthrax Lethal Toxin (PrAgU2/LF)

In order to selectively target both the urokinase plasminogen activator system (uPA/uPAR) and the MAPK pathway in AML cells, we have modified the activation step of anthrax lethal toxin to substitute activation by the tumor-specific urokinase system to the activation by the ubiquitously expressed furin protease system. The furin cleavage and activation site of PrAg164RKKR167 was, therefore, replaced by a urokinase-specific cleavage sequence 163PGSGRSA169 called U2 (Abi-Habib et al., 2006). Hence, PrAgU2/LF is the resulting urokinase-activated recombinant anthrax lethal toxin. In this case, the recombinant toxin specifically targets two different tumor-specific markers; the MAPK pathway, on the activity of which many tumor types are dependent, including AML, and the urokinase plasminogen activator system (uPA/uPAR), a tumor-specific cell surface protease overexpressed on a number of tumor types, including AML (Abi-Habib et al., 2006). PrAgU2/LF is, therefore, a dual-selective fusion toxin that targets two separate tumor specific markers.

In this study, we attempt to target both the MAPK pathway and the urokinase plasminogen activator protease system in AML cells lines using a urokinase-activated, recombinant anthrax lethal toxin (PrAgU2/LF).

CHAPTER TWO

Materials and Methods

2.1 - Expression and purification of PrAg, PrAgU2, LF, and FP59:

Recombinant anthrax toxin proteins, PrAgU2 (activated by the urokinase plasminogen activator), PrAg (activated by furin) and LF, as well as FP59 (fusion of the PrAg binding domain of LF and the catalytic domain of *Pseudomonas aeruginosa* exotoxin A) were expressed and purified, in the lab of Stephen Leppla at the National Institute of Health (NIH), as described previously (Oka et al., 1995; Duesbery et al., 1998).

2.2 - Cells and cell lines:

Human AML cell lines HL60, U937, ML1, ML2, Mono-Mac-1, Mono-Mac-6, KG-1, SigM5, 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).

Human peripheral blood mononuclear cells (PBMCs) were isolated using Ficoll-Paque gradient as described previously (Blair, Hogge, & Sutherland, 1998). 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.

2.3 - Animals:

Female Balb/c mice, 8 to 10 weeks old, were obtained from the animal facility of the Lebanese American University. Food and water were provided ad libitum. The mice were allowed to adjust to their environment for 1 week.

2.4 - Proliferation inhibition assay (cytotoxicity):

Sensitivity of AML cell lines, PBMCs and Human CD₃₄⁺ PBMBs to PrAgU2/LF and PrAg/LF was determined using a proliferation inhibition assay as described previously (Abi-Habib et al., 2005). We have also used a recombinant protein, termed FP59, consisting of the PrAg binding domain of LF fused to the catalytic domain of *Pseudomonas aeruginosa* exotoxin A. Binding of FP59 to PrAg or PrAgU2 and its translocation into the cytosol are identical to LF, however, it does not target the MAPK pathway but rather ADP-ribosylates elongation factor 2 (EF-2) leading to inhibition of protein synthesis and cell death. A combination of PrAg and FP59 was used as a control for catalytic domain entry into the cytosol of AML cells while a combination of PrAgU2 and FP59 was used as a control for the cell surface activity of the urokinase plasminogen activator independently of cell sensitivity to the inhibition of the MAPK pathway. Briefly, aliquots of 10⁴ cells/well, in 100 µl cell culture medium, containing a fixed concentration of 10⁻⁹ M LF or FP59 and a concentration of 100 ng/mL exogenous human pro-uPA (American Diagnostica, Stamford, CT), when added, 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. When U0126 was used, it was added as described above for PrAgU2 but in concentrations ranging from 10⁻⁴ to 10⁻⁹ 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} .

2.5 - Blocking Assays:

Blocking assays were performed to test the ability of specific anti-uPA antibodies to block the killing of the sensitive cell lines by PrAgU2/LF and PrAgU2/FP59. The same proliferation inhibition assays were performed as described earlier but without the addition of pro-uPA. In the blocking assays, 10 μ g/mL monoclonal anti-uPA antibody (American Diagnostica) was added to the 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.

2.6 - uPAR expression:

Expression of the urokinase plasminogen activator receptor (uPAR) on AML cell lines and CD₃₄⁺ progenitor bone marrow blasts (PBMBs) was determined using single cell staining with a FITC-conjugated anti-uPAR antibody on flow cytometry as described previously. Approximately 3x10⁶ 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 isotopic 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. $RFI \geq 2$ was considered positive.

2.7–Soluble uPAR, uPA, and PAI-1 levels:

The uPA, plasminogen activator inhibitor 1 (PAI-1), and soluble uPAR levels were determined using enzyme-linked immunosorbent assay (ELISA) kits (American Diagnostica), and the assays were done according to the description provided in each assay kit. For each cell line 100 µL cell culture supernatant (cell density >10⁶ cells/mL)

was used, in duplicate, in each ELISA. Both assays detect total levels of uPA, PAI-1, and soluble uPAR.

2.8-In vivo toxicity studies:

Balb/c mice (5 to 10 mice per group) were injected i.p. with 200 μ l of either vehicle alone (Phosphate buffered saline) or increasing doses of PrAg/LF or PrAgU2/LF (5:1 ratio of PA or PrAgU2 to LF, given simultaneously) every other day for a total of three injections. Both toxins were administered at a 5:1 ratio of PrAg or PrAgU2 to LF. Anthrax lethal toxin (PrAg/LF) was administered at doses of 15 μ g PrAg/ 3 μ g LF, 20 μ g PrAg/ 4 μ g LF, 25 μ g PrAg/ 5 μ g LF, 30 μ g PrAg/ 6 μ g LF and 35 μ g PrAg/ 7 μ g LF (which corresponds to cumulative doses of 9, 12, 15, 18 and 21 μ g total LF, respectively) while the urokinase-activated anthrax lethal toxin (PrAgU2/LF) was administered at doses of 35 μ g PrAgU2/ 7 μ g LF, 50 μ g PrAgU2/ 10 μ g LF, 60 μ g PrAgU2/ 12 μ g LF, 75 μ g PrAgU2/ 15 μ g LF and 85 μ g PrAgU2/17 μ g LF (which corresponds to cumulative doses of 21, 30, 36, 45 and 51 μ g total LF, respectively). Mice were monitored twice daily for signs of toxicity. Mice that presented with dehydration, hypothermia, and/or dyspnea were considered moribund and were euthanized by standard CO₂ asphyxiation, consistent with the recommendations of the Panel on Euthanasia of the American Veterinary Medical Association. Samples from major organs, including heart, lungs, liver, spleen, duodenum, colon, and kidneys, were

removed, fixed in 10% buffered formaldehyde, dehydrated, and embedded in paraffin. Sections were stained with H&E and examined under the microscope. All surviving mice were euthanized at day 15 post-injection.

CHAPTER THREE

Results

3.1-Cytotoxicity

3.1.1 – Cytotoxicity of PrAg/LF (LeTx) to AML cell lines

A panel of 11 human AML cell lines was used in order to test first for sensitivity to the inhibition of the MAPK pathway by PrAg/LF (LeTx). The cytotoxic effect was monitored by XTT, which detects the proliferation of viable cells. The PrAg/LF toxin is activated by furin and leads to the inhibition of the MAPK pathway. 7 out of the 11 AML cell lines showed sensitivity to PrAg/LF, with an IC_{50} values ranging from 13 to 94 pM and percent cell kill at highest concentration >75% (Table 3.1). As shown in figure 3.1, the sensitive cell lines to the inhibition of MAPK pathway through the PrAg/LF toxin were TF1-vRaf, TF1-vSrc, TF1-HaRas, Mono-Mac-6, HL-60, ML-2, and Sig-M5. The remaining 4 cell lines that were resistant to the inhibition of the MAPK pathway had an IC_{50} > 10,000 pM and percent cell kill at highest concentration $\leq$ 40%) (Table 3.1 and Figure 3.1). Those cell lines are Mono-Mac-1, U937, KG-1, and ML-1.

Figure 3.1: Cytotoxicity of PrAg/LF to a panel of 11 human AML cell lines using a proliferation-inhibition assay. X-axis, log of the molar concentration of PrAg/LF, Y-axis cell viability expressed as percent control.

The sensitivity of a majority of AML cell lines tested to the LF-mediated inhibition of the MAPK demonstrates the potential for targeting the MAPK pathway in AML and supports the possibility of targeting both the MAPK pathway and the uPA/uPAR system in AML cells.

3.1.2 – Cytotoxicity of PrAgU2/LF to AML cell lines

The 7 AML cell lines that showed sensitivity to the inhibition of the MAPK pathway through the PrAg/LF toxin were tested with the dual targeted toxin PrAgU2/LF

that targets both the MAPK pathway and the urokinase system. Using XTT, the proliferation-inhibition assay showed that 5 out of 7 cell lines, namely TF1-vRaf, TF1-vSrc, HL-60, ML-2, and Sig-M5, were sensitive to the dual targeted toxin PrAgU2/LF (Figure 3.2, A). Those sensitive cell lines had an IC_{50} values ranging from 12.0 to 151 pM. The 4 cell lines that were not sensitive to the PrAg/LF-mediated inhibition of the MAPK pathway were also tested for sensitivity to the urokinase-activated PrAgU2/LF. As expected, these cell lines were also resistant to PrAgU2/LF due to their resistance to the inhibition of the MAPK pathway (Figure 3.2, D).

B)

C)

D)

E)

Figure 3.2: Cytotoxicity of PrAgU2/LF to AML cell lines using a proliferation inhibition assay. A) Response of the 7 PrAg/LF-sensitive human AML cell lines to PrAgU2/LF. HL60 and ML2 (B and C) are cells sensitive to both PrAg/LF and PrAgU2/LF. TF1-HaRas (E) is sensitive to PrAg/LF but not to PrAgU2/LF and U937 (D) is not sensitive to either toxin. X-axis, log of the molar drug concentration, Y-axis, cell viability.

3.1.3 – Cytotoxicity of PrAg/FP59 and PrAgU2/FP59 to AML cell lines

Since resistance of some AML cell lines to PrAg/LF and PrAgU2/LF can be due to the absence of the anthrax toxin receptors (ANTXRs) or to inefficient internalization and cytosolic release of LF, we tested the sensitivity of our panel of 11 AML human cell lines using a combination of PrAg and FP59 (PrAg/FP59), instead of LF. FP59 is a variant of the lethal factor (LF) that is independent on MAPK inhibition but dependent on protein synthesis inhibition leading to cell death. FP59 was manufactured by replacing the zinc metalloprotease domain of LF with the more potent ADP-ribosylation domain of *Pseudomonas aeruginosa* exotoxin A, which ADP ribosylates elongation factor 2 (EF2) leading to the inhibition of protein synthesis and subsequent cell death. FP59 is, therefore, capable of killing any cell expressing ANTXR receptor that binds PrAg (Lui et al. 2001). As a result, cell sensitivity to PrAg/FP59 is an indicator of successful cell binding and internalization of the toxin. Furthermore, in order to determine urokinase activity on AML cells and their ability to activated a urokinase-targeted toxin independently of the inhibition of the MAPK pathway, we tested the sensitivity of our panel of AML cell lines to the combination of PrAgU2 and FP59 (PrAgU2/FP59) instead of LF. Sensitivity to this toxin depends solely on the activity of the uPA/uPAR system.

Figure 3.3: Cytotoxicity of PrAgU2/FP59 to a panel of 10 human AML cell lines using a proliferation-inhibition assay. X-axis, log of the molar concentration of PrAgU2/FP59, Y-axis cell viability expressed as percent control.

As illustrated in Table 3.1, all the cell lines tested were sensitive to PrAg/FP59 (IC_{50} ranging from 0.7 to 17 pM), indicating the four PrAg/LF-resistant cell lines were capable of binding and internalizing the toxin but were resistant to the inhibition of the MAPK pathway. Furthermore, proliferation inhibition assays carried out on the panel of AML cell lines using PrAgU2/FP59 revealed that all the AML cell lines tested were sensitive to PrAgU2/FP59 with IC_{50} values ranging from 1.0 to 415 pM and a percent cell death $\geq 70\%$ (Table 3.1, Figure 3.3). These results indicate that all AML cell lines express uPA/uPAR at a level that allows them to effectively activate PrAgU2. Moreover, this confirms that in four of the cell lines that were not sensitive to PrAgU2/LF, resistance was due to their lack of sensitivity to the inhibition of the MAPK pathway confirming the dual-selectivity of PrAgU2/LF.

Table 3.1 Sensitivity of human AML cell lines to PrAg/LF, PrAgU2/LF, PrAg/FP59, and PrAgU2/FP59. PrAg/FP59 is a control for cell binding and internalization and PrAgU2/FP59 a control of urokinase activity.

Cell line	LeTx (PrAg/LF) (IC₅₀;pmol/L)	PrAgU2/LF (IC₅₀;pmol/L)	PrAg/FP59 (IC₅₀;pmol/L)	PrAgU2/FP59 (IC₅₀;pmol/L)
HL60	13	56	0.7	1.0
TF1-VSrc	15	12	3.0	8.0
TF1-VRaf	16	46	4.0	32
Mono-Mac-6	39	>10000	13	27
SigM5	40	94	17	19
ML-2	81	151	7.0	28
TF1-HaRas	94	>10000	0.4	415
ML-1	> 10000	>10000	6.0	33
U937	> 10000	>10000	1.0	108
KG-1	> 10000	>10000	1.0	134
Mono-Mac-1	> 10000	>10000	2.0	116

3.1.4– Effect of the addition of exogenous pro-uPA on urokinase activated toxin

In order to determine whether the lack of sensitivity to PrAgU2/LF of the two cell lines that were sensitive to the LF-mediated inhibition of the MAPK pathway (Mono-Mac-6 and TF1-HaRas) can be reversed by the addition of exogenous pro-uPA, we compared the cytotoxicity of PrAgU2/LF, and PrAgU2/FP59, in the presence and absence of excess, exogenous pro-uPA, the inactive form of uPA. As illustrated in Figure 3.4 A, the addition of excess pro-uPA did not reverse the resistance of Mono-Mac-6 to PrAgU2/LF. On the other hand, addition of exogenous pro-uPA to cell lines sensitive to PrAgU2/LF and PrAGU2/FP59 (U937 and Mono-Mac-1) led to a noticeable increase in sensitivity as illustrated by a decrease in IC_{50} values (Figure 3.4, B and C).

A)

B)

Figure 3.4: Cytotoxicity of PrAgU2/LF and PrAgU2/FP59 to AML cell lines in the presence of excess exogenous pro-uPA. A) Mono-Mac-6 cells were not sensitive to PrAgU2/LF even after the addition of exogenous pro-uPA. Sensitivity of U937 and Mono-Mac-1 (B and C) to PrAgU2/FP59 was increased by the addition of exogenous pro-uPA. X-axis, log of the molar drug concentration, Y-axis cell viability.

The inability of excess pro-uPA to reverse resistance to urokinase-activated toxins along with its ability to increase the sensitivity of responsive cells indicates that the underlying mechanism of resistance to the urokinase-activated toxin is not uPA-dependent but may depend on other components of the uPA/uPAR protease system.

3.2 -Selectivity of PrAgU2/LF

3.2.1 – Cytotoxicity of PrAgU2/LF to normal Monocytes

In order to determine whether the cytotoxicity of PrAgU2/LF is selective to AML cells, we tested the sensitivity of human peripheral monocytes to PrAgU2/LF and compared their response to PrAg/LF. As illustrated in Figure 3.5, normal monocytes were highly sensitive to PrAg/LF with an IC_{50} =15 pM but significantly less sensitive to PrAgU2/LF with an IC_{50} =2661 pM. Normal human monocytes were, therefore, 177.4 fold less sensitive to PrAgU2/LF compared to PrAg/LF indicating that the addition of a second tumor-selectivity criterion (urokinase activation) in the dual-selective PrAgU2/LF led to a significant increase in the selectivity of the molecule compared to PrAg/LF, a single-selectivity molecule (MAPK targeting).

Figure 3.5: Cytotoxicity of PrAg/LF and PrAgU2/LF to normal human monocytes using a proliferation-inhibition assay. X-axis, log of the molar concentration of PrAg/LF and PrAgU2/LF, Y-axis cell viability expressed as percent control.

3.2.2 – Cytotoxicity of PrAgU2/LF on normal CD34⁺ progenitor Bone Marrow Blasts

In addition, to normal peripheral monocytes, the selectivity of PrAgU2/LF was tested on normal human CD34⁺ progenitor bone marrow blasts. As shown in figure 3.6 A, CD34⁺ progenitor bone marrow blasts were not sensitive to PrAgU2/LF. Furthermore, CD34⁺ progenitor bone marrow blasts were also not sensitive to PrAg/LF, indicating that these cells are resistant to the LF-mediated inhibition of the MAPK pathway. Moreover, CD34⁺ progenitor bone marrow blasts were not sensitive to PrAgU2/FP59 (Figure 3.6 B) indicating their lack of expression of an active urokinase

plasminogen activator system and further confirming the tumor selectivity of the dual-selective PrAgU2/LF.

A)

B)

Figure 3.6: Cytotoxicity of PrAgU2/LF (A) and PrAgU2/FP59 (B) to CD_{34}^{+} PBMBs using a proliferation-inhibition assay. X-axis, log of the molar concentration of PrAg/LF and PrAgU2/LF, Y-axis cell viability expressed as percent control.

3.3 - Blocking Assays:

In order to demonstrate whether the sensitivity of AML cells to PrAgU2/LF is dependent on the expression of an active uPA/uPAR protease system, we tested for the ability of an anti-uPA blocking antibody to reverse the sensitivity of cells to PrAgU2/LF. As illustrated in Figure 3.7, addition of a blocking anti-uPA antibody reversed the sensitivity of AML cells to PrAgU2/LF with an increase in IC_{50} from 27 and 29 pM for PrAgU2/LF alone to 602 and 4,000 pM in the presence of anti-uPA blocking antibody in HL60 and ML2 cells, respectively (Figure 3.7 A and B).

The ability of the inhibition of the urokinase plasminogen activator system to block the cytotoxicity of urokinase-activated toxins was also confirmed on PrAgU2/FP59. Addition of a blocking anti-uPA antibody reversed or significantly decreased the cytotoxicity of PrAgU2/FP59 on all the AML cell lines tested with IC_{50} values increasing by several dozen folds in the presence of the anti-uPA antibody (Figure 3.7, C, D, E and F).

A)

B)

C)

D)

Figure 3.7: The Cytotoxicity effect of PrAgU2/LF to A) HL-60 and B) ML2 was reduced in the presence of a blocking anti-uPA antibody. Cytotoxicity effect of PrAgU2/FP59 to C) Mono-Mac-1, D) ML2, E) Mono-Mac-6 and F) U937 was also reversed when a blocking anti-uPA antibody was added. X axis, log of the molar drug concentration. Y-axis, cell viability.

These findings demonstrate that the cytotoxicity of PrAgU2/LF is dependent on the expression of an active uPA/uPAR protease system on the surface of target cells. These results, added to the already demonstrated MAPK targeting of the LF catalytic moiety, demonstrate that PrAgU2/LF is a dual-selective toxin whose cytotoxicity is underlined by both dependence on the MAPK pathway and expression of active uPA/uPAR.

3.4–Expression of uPAR:

In order to further investigate the underlying mechanisms of the cytotoxicity of PrAgU2/LF to AML cells, we determined the expression levels of the urokinase plasminogen activator receptor (uPAR) on cells both directly, using cell surface staining on flow cytometry, and indirectly, by determining levels of soluble uPAR (s-uPAR) using an anti-uPAR ELISA.

Cell surface staining and flow cytometry analysis that revealed the expression of uPAR levels in all but one AML cell lines with a ratio of fluorescence intensity (RFI) values ranging from 2.30 to 6.11 (Figure 3.8, Table 3.2). A RFI > 2 is considered positive for the expression of uPAR since it indicates more than 2-fold higher mean fluorescence intensity (MFI) of the uPAR stained cells compared to the MFI of the isotypic control cells. The only cell line that was negative for uPAR expression was Mono-Mac-6 (RFI = 1.82), one of two cell lines that were sensitive to PrAg/LF but not

to PrAgU2/LF. The other cell line, TF1-HaRas was positive for the expression of uPAR but had the lowest RFI (2.30). Moreover, analysis of soluble uPAR (s-uPAR) levels revealed that both Mono-Mac-6 and TF1-HaRas had undetectable levels of s-uPAR, whereas the majority of the remaining cell lines that were positive for the expression of uPAR were also positive for the presence of s-uPAR (Table 3.2).

Normal CD₃₄⁺ progenitor bone marrow blasts, on the other hand, were negative for the expression of cell surface uPAR (RFI= 1.15), a result that matches their observed resistance to PrAgU2/FP59 and that confirms the absence of an active uPA/uPAR system on normal progenitor blasts (Table 3.2, Figure 3.8 J).

E **F** **G** **H**

Figure 3.8: Flow cytometric analysis of uPAR expression levels in a panel of 9 AML cell lines; ML1 (A), ML2 (B), Mono-Mac-1 (C), Mono-Mac-6 (D), U937 (E), TF1-vRaf (F), TF1-vSrc (G), TF1-HaRas (H), HL-60 (I), and normal CD34⁺ progenitor bone marrow blasts (J). Left panels are cells stained with the isotypic control, middle panels are cells stained with Anti-uPAR, and right panels show a histogram of cells stained with both the isotypic control (black) and with anti-uPAR antibody (red). Positivity for the expression of uPAR was considered having RFI>2. Cells are gated on width versus forward scatter (R1). RFI results are listed on Table 3.2.

Table 3.2. uPAR expression and soluble uPAR (s-uPAR) levels on AML cells and normal CD₃₄⁺ bone marrow progenitor blasts.

Cell line	Flow cytometry (RFI)	s-uPAR (ng/ml)	PrAgU2/LF (IC₅₀;pmol/L)
HL-60	2.58 (+)	NA	56
TF1-VSrc	2.41 (+)	1.42	12
TF1-VRaf	2.71 (+)	ND	46
Mono-Mac-6	1.82 (-)	ND	>10000
ML-2	3.10 (+)	0.55	151
TF1-HaRas	2.30 (+)	ND	>10000
ML-1	2.82 (+)	0.16	>10000
U937	6.11 (+)	3.11	>10000
Mono-Mac-1	2.41 (+)	0.84	>10000
CD₃₄⁺ PBMB	1.15 (-)	NA	>10000

NA: Not available

ND: Not Detectable

The pattern of uPAR expression on AML cells matched their sensitivity to PrAgU2/LF with the 2 out of 7 PrAg/LF sensitive cell lines (Mono-Mac-6 and TF1-HaRas) that were resistant to PrAgU2/LF showing no expression of uPAR by either one or both of the uPAR detection methods used. This demonstrates that uPAR expression is an essential underlying mechanism of the cytotoxicity of PrAgU2/LF. Furthermore, these results show that the failure to reverse resistance of Mono-Mac-6 to PrAgU2/LF, following the addition of excess pro-uPA, was due to the lack of uPAR expression of these cells.

3.5 - uPA and PAI-1 levels:

In order to gain a better understanding of the properties of the Urokinase plasminogen activator system in AML cells we tested the expression levels of both uPA and PAI-1 in our panel of AML cell lines. The levels of both uPA and PAI-1 in the supernatants of AML cells varied over a large range of values, particularly for PAI-1, and did not correlate with the sensitivity of AML cells to PrAgU2/LF.

Table 3.3. uPA and PAI-1 expression levels in the supernatants of AML cell lines.

Cells	uPA (ng/ml)	PAI-1 (ng/ml)
HL-60	NA	NA
TF1-VSrc	ND	0.46
TF1-VRaf	0.22	11.55
Mono-Mac-6	0.45	1.14
ML-2	0.27	1.81
TF1-HaRas	ND	0.03
ML-1	0.4	1.17
U937	1.0	1.24
Mono-Mac-1	0.67	0.81
CD₃₄⁺ PBMB	NA	NA

NA: Not available
ND: Not detectable

3.6 - In vivo toxicity studies:

In order to determine the *in vivo* safety and selectivity of the dual-selective PrAgU2/LF and to compare it to the safety of PrAg/LF, we carried out a dose-escalation, safety study in female Balb/c mice. As illustrated in the Kaplan-Meyer curve (Figure 3.9), a mortality rate of 10% was observed with PrAg/LF starting at a dose of 20 µg PrAg/ 4 µg LF, which corresponds to a cumulative dose of 12 µg LF. Mortality rate increased with increasing doses to reach 100% of treated animals at the highest dose tested of 35 µg PrAg/ 7 µg LF. The only dose at which no mortality was observed was the lowest dose used of 15 µg PrAg/ 3 µg LF, which corresponds to a cumulative dose of 9 µg LF. Hence for PrAg/LF the 20 µg PrAg/ 4 µg LF was estimated to be the maximal tolerated dose (MTD) since it was the highest dose that did not lead to more than 10% mortality. For PrAgU2/LF, on the other hand, no mortality was observed at any of the doses that correspond to those of PrAg/LF and dose escalation continued to a maximal dose of 85 µg PrAgU2/ 17 µg LF, which corresponds to a cumulative dose of 51 µg LF, without causing any mortality. Hence, the MTD for PrAgU2/LF was not reached in this study. Histological analysis of a wide array of organs revealed necrotic damage and inflammation in the livers of mice in the PrAg/LF treatment groups starting at a dose of 20 µg PrAg/ 4 µg LF and also in the livers of mice in the PrAgU2/LF treatment groups, but to a much lesser extent, at the two highest doses tested 75 µg PrAgU2/ 15 µg LF and 85 µg PrAgU2/ 17 µg LF. No sign of damage in any other organ was detected in this study.

Figure 3.9: Kaplan-Mayer curve of the dose escalation study of PrAg/LF and PrAgU2/LF in Balb/c mice. Mice were injected i.p. with different doses of a 5 to 1 ratio of PrAg or PrAgU2 and LF, every other day for a total of 3 injections.

These results confirm that the dual-selective toxin PrAgU2/LF is highly selective to tumor cells with its safety profile being significantly enhanced compared to the single-selective toxin PrAg/LF. The MTD of PrAgU2/LF was not reached and is, therefore, more than 4-fold higher than that of PrAg/LF.

Chapter 4

Discussion& Conclusion

The importance of the uPA system was discovered in 1976 for its high activity in cancer cells (Astedt & Holmberg, 1976). The overexpression of uPA leads to the activation of cell proliferation, invasion, and metastasis following the degradation of the extracellular matrix (ECM). Studies have shown that the expression of high levels of uPA or soluble uPAR in cancer patients, including AML patients, is correlated with bad prognosis (Dano et al., 2005). Thus, attempts are being made in order to inhibit the uPA/uPAR or take advantage of its overexpression to selectively target tumor cells. Our interest in AML is due to the failure of conventional treatments, which are associated with high mortality rates. In addition, according to Atfy (2010) 72.7% of AML patients express the uPA/uPAR system, which is one the markers of poor prognosis in this disease. Our approach is to selectively target AML cell lines that express the uPA/uPAR system using a urokinase-activated anthrax lethal toxin. Therefore, the aim of this study is to target both the uPA/uPAR system and the MAPK pathway in AML cell lines through the dual-selective, recombinant urokinase-activated anthrax lethal toxin (PrAgU2/LF).

A panel of 11 human AML cell lines was tested for sensitivity to PrAg/LF, which targets AML cell lines through the inhibition of the MAPK pathway. 7 out of the 11 AML cell lines tested were sensitive to the LF-mediated inhibition of the MAPK pathway with IC₅₀ values in the pM range. This indicates that the majority of AML cell

lines is dependent on the MAPK pathway for survival and is sensitive to the inhibition of this pathway. Furthermore, these findings provide the opportunity for targeting those MAPK-dependent AML cells with a dual-selective, urokinase-activated anthrax lethal toxin, PrAgU2/LF.

The majority of the cell lines sensitive to the inhibition of the MAPK pathway (5 out of 7) were also sensitive to the dual targeted, urokinase-activated version of the toxin, PrAgU2/LF. The cytotoxicity of PrAgU2/LF to AML cell lines showed IC_{50} values also in the pM range, indicating that PrAgU2/LF is as potent as PrAg/LF to AML cell lines. As expected, the 4 AML cell lines that were not sensitive to the LF-mediated inhibition of the MAPK pathway were also resistant to PrAgU2/LF. However, all 4 cell lines were sensitive to the MAPK-independent PrAgU2/F59, a protein synthesis inhibitor resulting from the replacement of the catalytic domain of LF with the catalytic domain of *Pseudomonas aeruginosa* exotoxin A, indicating that these 4 cell lines do express an active uPA/uPAR system and demonstrating that their resistance to PrAgU2/LF was solely due to their lack of dependence on the MAPK pathway for survival. Furthermore, inhibition of the uPA/uPAR system using a blocking anti-uPA antibody reversed or greatly reduced the sensitivity of AML cells to both PrAgU2/LF and PrAgU2/FP59 demonstrating the lack of activity of these toxins in the absence of an active uPA/uPAR system. Hence, introducing a urokinase-activation step into anthrax lethal toxin did not affect the potency of the molecule and resulted in the production of a highly potent toxin that targets both the MAPK pathway and the urokinase plasminogen activator system in AML cells. Therefore, in order for cells to be sensitive to PrAgU2/LF they must simultaneously express the uPA/uPAR system and be dependent on the MAPK

pathway for survival. The absence of any one of these tumor-specific markers leads to total resistance or at least to greatly reduced sensitivity to PrAgU2/LF.

In order to demonstrate that the introduction of a second tumor specific marker increased the selectivity of anthrax lethal toxin to AML cells, we tested the cytotoxicity of the urokinase-activated toxin, PrAgU2/LF, on both human peripheral mononuclear cells (monocytes) and human CD₃₄⁺ Progenitor bone marrow blasts. Human monocytes were sensitive to the PrAg/LF-mediated inhibition of the MAPK pathway but were approximately 177-fold less sensitive to the urokinase-activated PrAgU2/LF, demonstrating that the introduction of the second tumor specific marker (urokinase activation) greatly reduced the toxicity of the molecule to normal cells. CD₃₄⁺ progenitor bone marrow blasts, on the other hand, were not resistant to both PrAg/LF and PrAgU2/LF indicating that they were resistant to the inhibition of the MAPK pathway. However, these normal progenitor blasts were also resistant to PrAgU2/FP59 indicating the lack of expression of an active uPA/uPAR system on these cells. These findings demonstrate the tumor-selectivity of PrAgU2/LF and its lack of toxicity to normal hematological cells that either lack the uPA/uPAR system or are not dependent on the MAPK pathway. Hence, introducing the urokinase-activation sequence in PrAgU2/LF generated a potent and selective dual-targeted toxin.

PrAgU2/LF is the first dual-selective toxin that targets both a tumor cell surface protease system (uPA/uPAR) and a signaling pathway critical to tumor cells (MAPK). The only other dual-selective fusion toxin investigated to date, DTU2GMCSF, is a urokinase-activated fusion of diphtheria toxin (DT) and the granulocyte macrophage colony stimulating factor (GMCSF), which targets both a tumor cell surface protease

(uPA/uPAR) and a tumor cell surface marker (GMCSFR) (Abi-Habib et al., 2004). Moreover, DTU2GMCSF only targets AML cells, whereas, PrAgU2/LF is capable of potentially targeting any tumor type that shows MAPK pathway dependence and overexpression of uPA/uPAR and, hence, potentially possesses a much wider range.

We then investigated the underlying mechanisms of the cytotoxicity of PrAgU2/LF to AML cells by determining their expression levels of the urokinase system components uPAR, uPA, and PAI-1 and by investigating the effects of the addition of exogenous pro-uPA on the sensitivity of AML cells to PrAgU2/LF. Addition of excess exogenous pro-uPA failed to reverse resistance of the 2 AML cell lines that were not responsive to PrAgU2/LF while being sensitive to the inhibition of the MAPK pathway. This indicated that sensitivity of AML cells to PrAgU2/LF was not dependent on uPA but on other components of the urokinase system. When uPAR expression levels were determined, both cell lines were negative for the expression of uPAR indicating that sensitivity to PrAgU2/LF was dependent on uPAR expression rather than uPA levels. This is line with findings from other studies targeting the urokinase system, showing that uPAR expression is the primordial factor for urokinase activity since in the absence of uPAR, pro-uPA cannot be effectively activated and is inhibited by PAI-1 (Abi-Habib et al., 2004). This further confirms the selectivity of PrAgU2/LF, which requires both dependence on the MAPK pathway and expression of uPAR (necessary for the maintenance of active uPA) to be active.

Finally, we determined the *in vivo* safety of PrAgU2/LF in a dose-escalation mouse model. The MTD for PrAgU2/LF was not reached in this study indicating that PrAgU2/LF is a safe and selective toxin. Furthermore, the MTD for PrAg/LF was more

than 4-fold lower than the higher dose of PrAgU2/LF used demonstrating that the introduction of the second tumor-selective factor, in the form of urokinase activation, greatly increased the selectivity and, subsequently, the *in vivo* safety of this toxin.

As a conclusion, PrAgU2/LF is a highly selective and highly potent dual-selective toxin that simultaneously targets both the uPA/uPAR protease system and the MAPK pathway in AML cells. Our results demonstrate the potency and selectivity of this molecule and provide a strong justification for its further development for the treatment of AML and for investigating its potential usefulness in other tumor types. Thus, future work needs to be further done on different tumor cells, on AML patient's cells, and animal models. This would allow us to better understand the dual targeted toxin, PrAgU2/LF, just before transferring it later on from the bench side to the bedside.

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