Sensitivity of Acute Myeloid Leukemia Cell Lines to the Dual Targeting of the Urokinase System and the MAPK Pathway by Modified Anthrax Toxins

By

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ABSTRACT

Acute myeloid leukemia (AML) is a hematological malignancy characterized by the rapid expansion of immature clonal myeloid cells, which leads to the failure of normal hematopoiesis. The remission rate in AML patients, following induction therapy, is 50 to 70% but most patients who undergo complete remission, recur later on with a poor prognosis. Thus leukemic relapse in AML remains a major therapeutic problem with the need of developing novel, tumor specific AML therapies. We aim to selectively target AML cells using a recombinant anthrax lethal toxin (LeTx) and a urokinase-activated recombinant anthrax toxin (PrAgU2/LF).

LeTx is a binary toxin consisting of 2 proteins, protective antigen (PrAg), and lethal factor (LF). PrAg binds cells through the anthrax toxin receptors (ANTXRs) and is cleaved by cell surface furin proteases leading to the release of a 20 kDa fragment and the generation of an activated 63 kDa PrAg protein. Active PrAg forms heptamers, binds 3 molecules of LF and undergoes receptor-mediated endocytosis followed by release of LF into the cytosol. LF is a zinc metalloprotease that cleaves and inactivates mitogen-activated protein kinase (MAPK) kinases leading to the inactivation of the MAPK pathway. A modified, urokinase-activated version of anthrax lethal toxin (PrAgU2/LF) in which the furin cleavage sequence of PrAg is substituted with a sequence cleaved by the urokinase plasminogen activator (uPA/uPAR) protease system was generated.

The MAPK pathway and the uPA/uPAR system have been shown to be active in a number of tumors, including AML, rendering AML cells potential targets for both LeTx (PrAg/LF) and the dual-specific, urokinase-activated PrAgU2/LF.

In this study, we tested the sensitivity of a panel of AML cell lines to LeTx (PrAg/LF), PrAgU2/LF, PrAg/FP59 and PrAgU2/FP59 using a proliferation inhibition (cytotoxicity) assay. In addition, we targeted the PI3K/AKT pathway using the PI3K inhibitor LY294002 and the Ras-Raf-MEK1/2-ERK1/2 pathway using the MEK1/2 inhibitor U0126.

The majority of AML cell lines (6 out of 9) were sensitive to LeTx (PrAg/LF) with IC_{50} values ranging from 14 to 94 pM with a third of LeTx-sensitive cell lines (2 out of 6) being also sensitive to the urokinase-activated PrAgU2/LF with IC_{50} values of 56 and

151 pM. Furthermore, all 9 cell lines were sensitive to PrAgU2/FP59 with IC_{50} values ranging from 2.9 pM to 318 pM, indicating the expression of active uPA/uPAR on the surface of AML cells. In addition, treatment of LeTx-sensitive AML cells with the MEK1/2 inhibitor U0126, replicated the cytotoxicity of LeTx indicating that the cytotoxicity of LeTx (PrAg/LF) may be mediated through the inhibition of the Ras-Raf-MEK1/2-ERK1/2 branch of the MAPK pathway. AML cells that were not sensitive to LeTx were sensitive to co-incubation with the PI3K (Phosphatidylinositol 3-kinase) inhibitor LY294002, indicating that dual inhibition of both the MAPK and PI3K/AKT pathway is necessary to target AML cells that are resistant to the inhibition of the MAPK pathway alone.

These results indicate that a majority of AML cell lines are sensitive to the inhibition of the MAPK pathway and do express an active urokinase protease system rendering both these pathways attractive targets for the selective treatment of AML.

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List of Abbreviation

Abbreviation	Full Name
AML	Acute myeloid leukemia
LeTx	Anthrax lethal toxin
PrAg	Protective antigen
LF	Lethal factor
ANTXRs	Anthrax toxin receptors
TEM-8	Tumor endothelium marker 8
CMG-2	Capillary Morphogenesis Gene 2
MAPK	Mitogen-activated protein kinase
uPA	Urokinase plasminogen activator
uPAR	Urokinase plasminogen activator receptor

JNK	C-Jun NH2-terminal kinase
Gab1	Grb2-associated binder 1
PH	Plekstrin Homology
TNF-α	Tumor necrosis factor- α
MEK	Mitogen-activated protein kinase kinase
ERK	Extracellular signal-regulated kinase
MLK	Mixed-lineage kinase
ASK1	Apoptosis signal-regulating kinase 1
PAI-1	Plasminogen activator inhibitor-1
MMP	Metalloprotease
FP59	Fusion protein 59
EF-2	Elongation factor 2
Ras	Rat-adeno-sarcoma
RTK	Receptor tyrosine kinase
GRB2	Growth factor receptor-bound protein 2
SOS	Son of Sevenless
GEF	Guanine nucleotide exchange factor

PI3K	Phosphatidylinositol 3-kinase
PI(4,5)P2	Phosphatidylinositol 4,5-bisphosphate
PDK1	3-Phosphoinositide-dependent protein kinase-1

Acknowledgement

I would like to thank Dr. Ralph Abi-habib for being a great mentor and a patient advisor. The high standards set by Dr. Abi-habib were hard to keep up with, but the outcome, and achievement were gratifying. Thank you for giving me the incentive to pursue a PhD in cancer biology, and most importantly thanks for everything I learned from you!

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Last but not least, I want to thank God for blessing me with a family, without whom I would never have been able to achieve so much. I can't thank them enough for their endless love, support, and constant motivation.

Chapter 1

INTRODUCTION

1.1. Acute Myeloid Leukemia

Acute myeloid leukemia (AML) is a hematologic malignancy characterized by the rapid proliferation of immature myeloid precursors in the bone marrow and blood with simultaneous reduction of hematopoiesis. The development of symptomatic marrow failure over weeks to a few months characterizes the clinical presentation, which includes symptoms of fatigue, recurrent infections, and unprovoked bleeding and bruising (Rowe *et al.* 2004).

AML induction therapy, which is the first stage of chemotherapy treatment that aims to restore normal blood production, 50% to 70% of newly diagnosed patients enter complete remission (no signs of leukemia cells) when treated with induction therapy, with variation in this result based upon patient age and cytogenetic risk profile (Rowe *et al.* 2004). Standard treatment for patients diagnosed with AML, is chemotherapy with cytotoxic agents. Cytarabine is an agent active against AML and is included in most chemotherapy regimens in combination with an anthracycline such as idarubicin (Coutsouvelis and Carallo 2009). Despite the relative success of induction therapy, many patients relapse and eventually succumb to their disease, hence the importance of developing novel, tumor-specific, AML therapeutics (Slovak *et al.* 2000). Devising novel, AML specific therapeutics can be achieved through targeting of specific signaling pathways such as the MAPK (mitogen activated protein kinase) pathway or the PI3K/AKT pathway, and/or cell surface proteases, such as the urokinase system or matrix metalloproteases, which are active in AML cells but not in normal cells and tissues (Gossage and Eisen 2010, Perl and Carrol 2007).

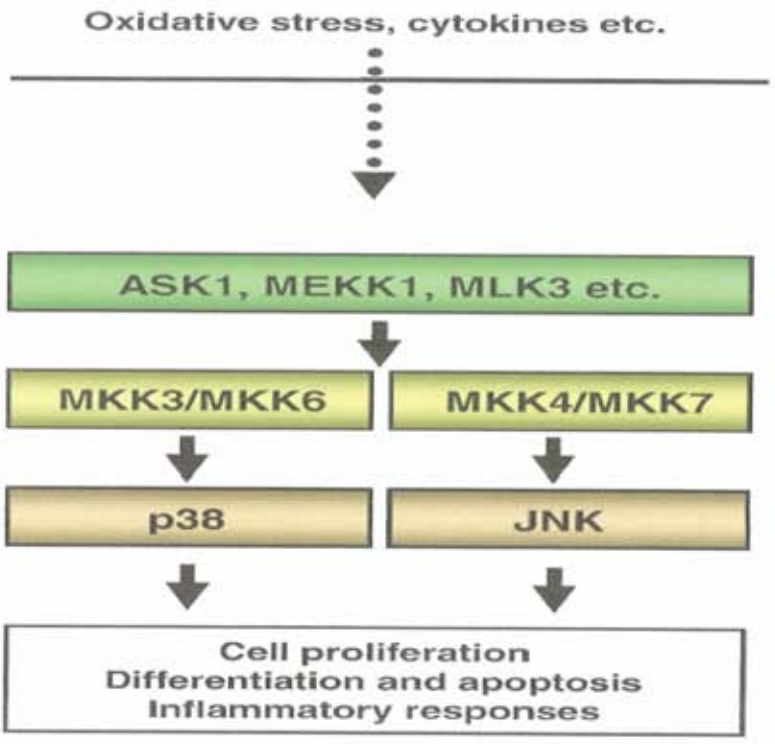
1.2. The MAPK pathway

The MAPK signal transduction pathway is involved in many aspects of normal cell function such as cell cycle progression and cell differentiation. The MAPK pathway

consists of three main branches, the JNK branch, the p38 MAPK branch, and the Ras/Raf/MEK1/2/ERK1/2 branch (Ding *et al.* 2008).

The JNK (c-Jun NH2-terminal kinase) and p38 signaling pathways are activated by pro-inflammatory cytokines such as TNF-α (tumor necrosis factor- α) or in response to cellular stresses such osmotic, or oxidative stress (Fig 1.1) (Kim and Choi 2010).

Figure 1.1: The JNK and p38 signaling pathways



From Kim and Choi 2010

In the Ras/Raf/MEK1/2 (mitogen-activated kinase1/2) /ERK1/2 (extracellular signal-regulated kinase 1/2) branch, an activated receptor tyrosine kinase (RTK) autophosphorylates tyrosine residues and causes the relocalization of the GRB2 (growth factor receptor-bound protein 2)–SOS (Son of Sevenless) complex, which causes the conversion of Ras-GDP into Ras-GTP (Baccarini 2005). Ras-GTP recruits Raf to the membrane, promoting its activation. Activated Raf phosphorylates and activates MEK1/2, which, in turn, stimulates ERK1/2 activity. ERK1/2 have several

1.3. The PI3 kinase/AKT pathway

PI3K (phosphatidylinositol 3-kinase) is a heterodimer composed of a regulatory p85 subunit and a 110-kDa catalytic subunit. Upon stimulation of appropriate cells with RTK ligands, Gab1 (Grb2-associated binder 1), becomes tyrosyl phosphorylated and binds multiple signal relay molecules, including the p85 subunit of PI3K (Zhang *et al.* 2002, Kunigal *et al.* 2006).

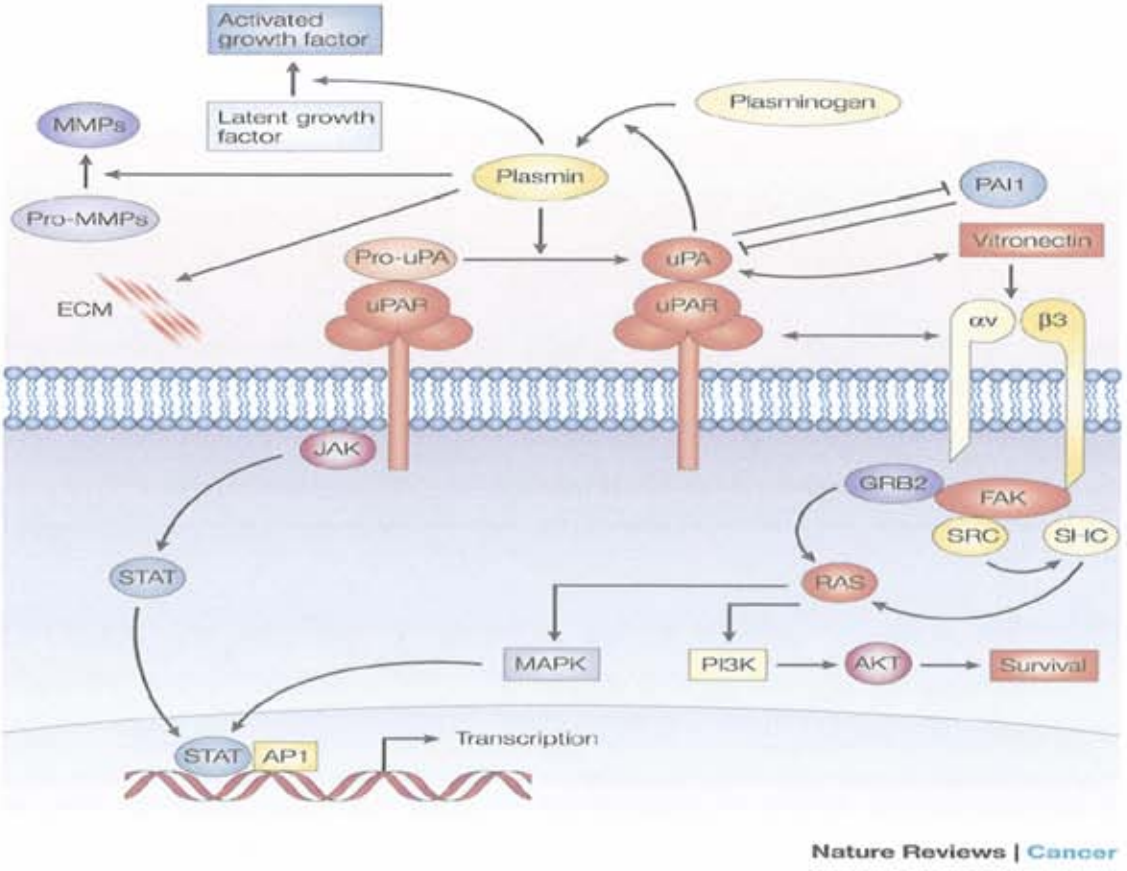
Once activated, PI3 kinase phosphorylates phosphatidylinositol 4,5-bisphosphate (PI(4,5)P2), converting PI(4,5)P2 to PI(3,4,5)P3, which is a major lipid second messenger. PI(3,4,5)P3 binds to AKT, also known as protein kinase B (PKB), a significant downstream effecter of the PI3-kinase pathway, and stimulates a conformational change in AKT. This process allows phosphorylation of AKT at Thr308 by 3-phosphoinositide-dependent protein kinase-1 (PDK1) (Hayashi *et al.* 2008). AKT phosphorylates several substrates that are involved in different cellular functions such as cell proliferation, survival, and motility (Hayashi *et al.* 2008).

1.4. The urokinase plasminogen activator protease system

A large number of solid and hematological malignancies including AML have been shown to over express extracellular protease systems. One of the major proteases over expressed on such tumors is the Urokinase-type plasminogen activator (uPA) (Ehnman *et al.* 2009). uPA is a serine protease that is secreted in a single-chain inactive form (sc-uPA), that is then cleaved by plasmin into a double-chain active uPA . The uPA receptor (uPAR) then binds uPA in both its active and inactive forms and shields it from inhibition by the plasminogen activator inhibitor- 1 (PAI-1) (Be´ne´ *et al.* 2004). This uPA-uPAR complex is a multifunctional system that is usually absent on normal cells and is up-regulated only during specific physiologic processes, such as wound healing and tissue remodeling (Abi-Habib *et al.* 2004).

Generally, uPA has a localized activity at the invasive edge of tumors and plays a critical role in tissue invasion and metastasis of tumors. The active uPA/uPAR complex cleaves plasminogen into plasmin, thus leading to extracellular matrix degradation, activation of latent matrix metalloproteases (MMPs) and tissue invasiveness that ultimately leads to metastasis (Fig 1.3) (Rao 2003). Even though a number of inhibitors of the uPA/uPAR system have been studied as potential cancer therapies, such inhibitors have proven to be more tumor static than cytotoxic (Abi-Habib *et al.* 2004, Liu *et al.* 2003). We, therefore, favor the approach that takes advantage of the overexpression of the uPA/uPAR system on tumors to specifically target them with urokinase-activated cytotoxic molecules, such as modified toxins.

Figure 1.3: uPA/uPAR system



From Rao 2003

1.5. Fusion toxins

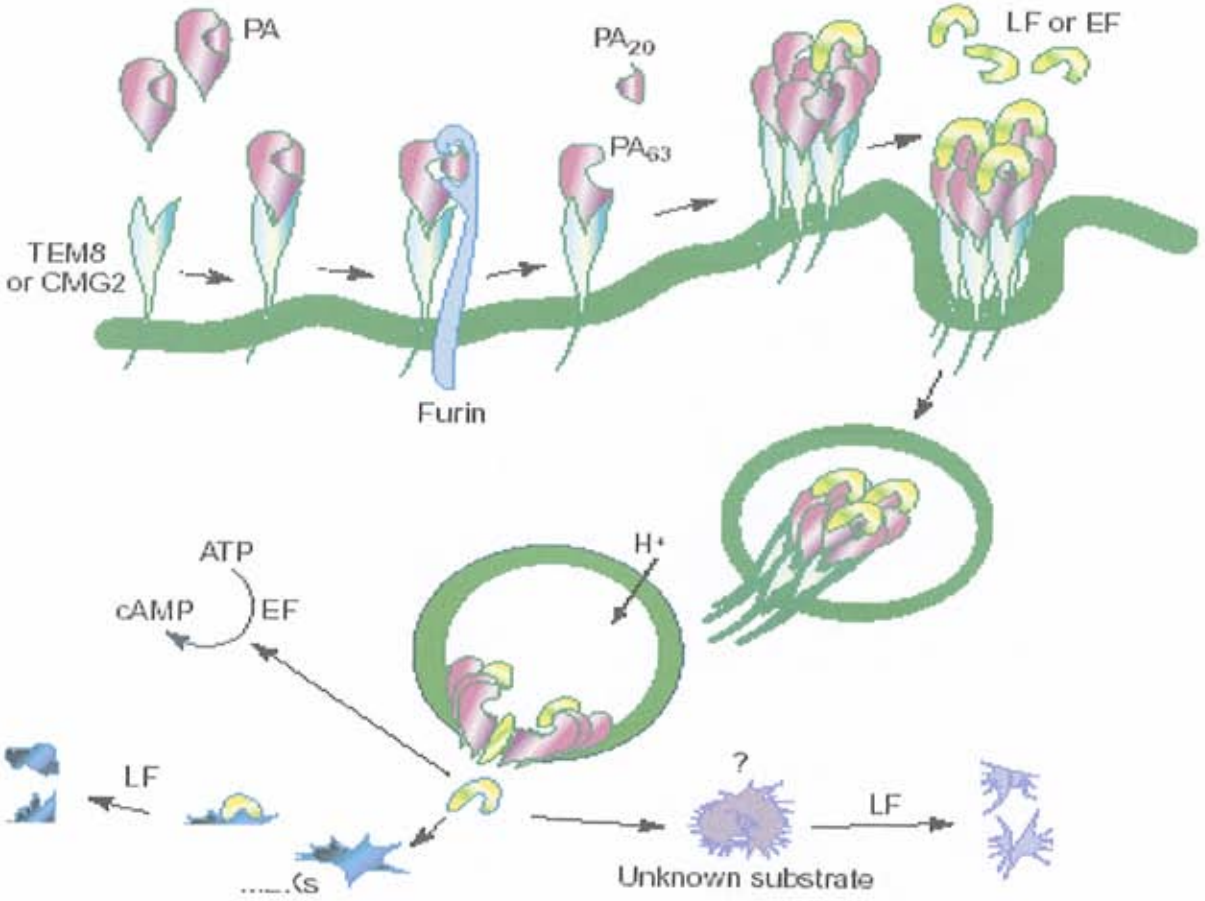
Fusion toxins consist of recombinant bacterial or plant protein toxins genetically modified to specifically target tumor cells (Kreitman 2009). They are designed for the selective and potent targeting of tumor cells with no or minimal targeting of normal cells. There are 3 general classes of fusion toxins based on their selectivity mechanisms. The first class includes toxins whose selectivity is cell-targeting derived and that generally consist of the catalytic domain of a potent toxin such as Diphtheria toxin or *Pseudomonas aeruginosa* exotoxin A fused to a ligand that targets a receptor overexpressed on a particular tumor. Examples of these toxins include DTIL2, DTIL3 and DTGMCSF (Westcott *et al,*2004, Horita *et al.* 2008). The second class is comprised of fusion toxins whose selectivity is activation- derived, (i.e. specific activation on tumor cells,) and that generally consist of toxins whose furin activation sequence was modified into a urokinase or matrix metalloprotease (MMP) activation sequence (Alfano *et al.* 2010). The third class encompasses toxins whose selectivity is catalytically-derived (i.e. toxins that inhibit a specific signaling pathway and are, therefore, specifically toxic to cells that rely on that pathway). An example of such toxins is LeTx whose toxicity is specific to cells relying on the MAPK pathway for survival (Bodart *et al.* 2002). Combining two of the above-mentioned selectivity criteria leads to the generation of dual specific fusion toxins that possess two overlapping selectivity criteria, e.g. DTU2GMCSF (both cell targeting specific and activation specific) and PrAgU2/LF (both activation specific and catalytically specific) (Abi-Habib *et al.* 2004).

One fusion toxin that falls into the category of catalytically selective toxins is recombinant anthrax lethal toxin (LeTx). Anthrax toxin secreted by *Bacillus anthracis* is a tripartite toxin, composed of a receptor binding/ pore-forming moiety, protective antigen (PrAg, 83 kDa) and two catalytic moieties, edema factor (EF, 90 kDa) and lethal factor (LF, 90 kDa) (Table1.1) (Abi Habib *et al.* 2005, Sun and Collier 2010). Anthrax lethal toxin (LeTx) refers only to the combination of PrAg and LF. PrAg binds to the ubiquitously expressed cell-surface anthrax toxin receptors (ANTXRs), and is then

cleaved at the sequence, 164RKKR167, by cell-surface furin-like proteases (Abi-Habib *et al.* 2005). Two cellular receptors for PrAg have been identified: tumor endothelial marker 8 (TEM-8) and capillary morphogenesis gene 2 (CMG-2). Both receptors are ubiquitously expressed type-I transmembrane proteins, which reveal a high degree of similarity; each encompassing an extracellular domain (ectodomain), a single-pass transmembrane domain, and a cytoplasmic domain. The active, 63-kDa fragment $PrAg_{63}$ then oligomerizes into a heptameric or octameric, receptor- bound pre pore,that contains high-affinity binding sites for 3 molecules of LF (Sun and Collier 2010).

Once the toxin-receptor complexes are internalized by receptor-mediated endocytosis, the pre-pore moiety undergoes an acidic pH-dependent conformational rearrangement within the endosome to form a cation-selective, transmembrane pore (Fig 1.4) (Moayeri and Leppla 2004). The PrAg pore mediates translocation of LF across the endosomal membrane into the cytosol, where, LF, a zinc metalloprotease, cleaves and inactivates MAPK kinases (Sun and Collier 2010). Hence, LeTx constitutes an attractive means for the specific targeting of the MAPK pathway in tumors carrying oncogenic mutations in this pathway such as AML (Abi-Habib *et al.* 2005).

Figure 1.4: Mechanism of action of Anthrax toxin



From Moayeri and Leppla 2004

In order to specifically target the uPA/uPAR system in AML cells, and enhance the tumor-specificity of LeTx, we substituted the furin cleavage sequence of PrAg 164RKKR167 with a urokinase-specific cleavage sequence 163PGSGRSA169 termed U2 (Table 1.1) (Abi Habib *et al.* 2006).

The resulting urokinase-activated recombinant anthrax toxin PrAgU2/LF binds to all cells through the ubiquitously expressed ANTXRs but is only activated on AML cells expressing an active uPA/uPAR system and is toxic only to those cells that are sensitive to the inhibition of the MAPK pathway.

Additionally, a more potent, wider range, MAPK-independent anthrax recombinant toxin, termed (PrAg/FP59), was generated in which the zinc metalloprotease domain of LF was substituted with the more potent, protein synthesis inhibitor, *Pseudomonas aeruginosa* exotoxin A (FP59), (Liu *et al.* 2001). In this case, LF residues 1 to 254, which bind the $PrAg_{63}$ receptor, were fused with the 38-kDa ADP-ribosylation catalytic domain of *Pseudomonas aeruginosa* exotoxin A which ADP-ribosylates elongation factor 2 (EF-2) leading to the inhibition of protein synthesis and subsequent cell death (Su *et al.* 2007). Hence this toxin binds to all cells through ANTXRs, is ubiquitously activated by furin proteases and induces cytotoxicity through protein synthesis inhibition and is, therefore, devoid of any selectivity criteria.

Table 1.1: Description of all the toxins used and specifies their selectivity mechanisms.

Recombinant Toxin	Mechanisms of action and selectivity
PrAg/LF (LeTx)	• Cell binding ubiquitous • Cell activation ubiquitous (furin proteases) • Cleaves and inhibits the MAPK pathway • Catalytically specific to MAPK dependent cells
PrAgU2/LF	• Cell binding ubiquitous • Cell activation specific to cells expressing an active uPA/uPAR system • Catalytically specific to MAPK dependent cells
PrAg/ FP59	• Cell binding ubiquitous • Sequence cell activation ubiquitous (furin proteases) • Inhibits protein synthesis • No catalytic specifcity
PrAgU2/FP59	• Cell binding ubiquitous • Cell activation specific to cells expressing an active uPA/uPAR system • Inhibits protein synthesis • No catalytic specifcity

In this study, we sought to test for the possibility of selective treatment of AML cells through targeting of the MAPK pathway and the urokinase plasminogen activator system using modified anthrax toxins. We, therefore, tested for the sensitivity of a panel of AML cells to the inhibition of the MAPK pathway by LeTx, and for the dual targeting of the urokinase and MAPK pathway in AML by the dual-specific, urokinase-activated toxin, PrAgU2/LF. We also attempted to decipher the molecular mechanisms underlying the response to the LeTx-mediated inhibition of the MAPK pathway in AML cells.

Chapter 2

MATERIALS AND METHODS

2.1. Toxins and Inhibitors

Recombinant PrAg, PrAgU2, LF, and FP59 were prepared by genetic modification of LeTx (Liu *et al.* 2001). These recombinant toxins were provided by our collaborators at the National Institute of Allergies and Infectious Diseases (NIAID), the National Institutes of Health (NIH), Bethesda, Maryland, USA. PrAg and PrAgU2 (generated by the substitution of the furin cleavage sequence of PrAg 164RKKR167 with a urokinase-specific cleavage sequence 163PGSGRSA169 termed U2) have a molecular weight of 83 kDa, whereas the molecular weight of FP59 (generated by the substitution of the zinc metalloprotease domain of LF with the more potent, protein synthesis inhibitor, *Pseudomonas aeruginosa* exotoxin A) is 59 kDa and that of LF is 90 kDa. The specific MEK1/2 inhibitor U0126, and the specific PI3K inhibitor LY294002 were purchased from Cell Signaling Technology (Danvers, MA).

2.2. Cells and Cell Lines

A panel of 9 AML cell lines, TF1-vSrc, TF1- HaRas, TF1- vRaf, HL60, U937, ML-2, KG-1, Mono Mac-1, and Mono Mac-6 was used in this study. These cells were provided by our collaborators at the Cancer Research Institute of Scott & White Memorial Hospital in Temple, Texas, USA. Cells were continuously maintained by splitting them according to their density or count number that we determined using a haemocytometer. Cells were cultured as recommended by the American Type Culture Collection (ATCC) in RPMI 1640 (LONZA, Switzerland) containing 10% heat inactivated fetal bovine serum (FBS), L-Glutamine, and Penicillin-Streptomycin. Cells were grown at 37°C and 5% CO_2.

2.3. Cell proliferation/cytotoxicity Assay

Aliquots of 10^4 cells/well were incubated with 10^{-9} mol/L FP59 or 10^{-9} mol/L LF 100 µL medium in Costar (Corning,NY) 96-well flat-bottomed plates. 50 µL of wild-type PrAg or PrAgU2 in medium were added to cells to yield concentrations ranging from 10^{-8} to 10^{-13}

mol/L. LY294002 (PI3 Kinase Inhibitor) was added to cells at a constant concentration of 20 µM and U0126 (MEK1/2 Inhibitor) was added in 50 µL of media generating concentrations ranging from 100µM to 1nM. Cells were then incubated at 37°C/ 5% CO_2 for 48 hours, which was followed by an incubation with 50 µL/well of XTT labeling mixture for 4 hours at 37°C/ 5%CO_2. XTT is a nonradioactive cell proliferation reagent used for the analysis of the effect of cytotoxic anti-cancer drugs on cell viability. The assay is based on the cleavage of the tetrazolium salt XTT in the presence of an electron-coupling reagent, producing a soluble formazan salt. The cleavage of the yellow tetrazolium salt into the orange colored formazan salt only occurs in viable cells via the succinatetetrazolium reductase system in the mitochondria of these metabolically active cells. After this incubation period, the formazan dye formed was quantitated using a scanning multi-well spectrophotometer (ELISA reader) and the absorbance was read at 450 nm.

2.4. Statistical Analysis:

The absorbance read in each well directly correlates to the number of viable cells. The absorbance was plotted versus the log of the toxin concentration, and nonlinear regression with a variable slope sigmoidal dose-response curve was generated along with IC_{50} (half maximum inhibitory concentration), using GraphPad Prism software (GraphPad Software, San Diego, CA). The IC_{50} (inhibitory concentration 50) value represents the concentration of a cytotoxic molecule that inhibits cell viability by 50%. It is a subset of the more general EC_{50} (effective concentration 50) value, which represents the concentration of a molecule that achieves 50% of the effect of that molecule. The IC_{50} is, therefore, a specific and widely accepted expression of the potency of cytotoxic drugs and molecules. A lower IC_{50} value indicates a higher potency and vice versa. The IC_{50} value cannot be calculated in the absence of the hill-slope of the non-linear regression curve and if the maximum cell death at the highest concentration of drug used does not reach at least 50%. Though it is a widely accepted

expression of the potency of cytotoxic molecules, the IC_{50} does not provide any information regarding the extent of cell death at the maximal concentration used, a factor with important implications in cancer drug development.

Chapter 3

RESULTS

In this study, we tested a panel of 9 AML cell lines for sensitivity to the inhibition of the MAPK pathway by LeTx (PrAg/LF) and the urokinase-activated PrAgU2/LF (Table 3.1). To control for the expression of ANTXRs, AML cell lines were also tested for sensitivity to PrAg/FP59.

All cell lines tested were sensitive to PrAg/FP59 with IC_{50} values ranging from 0.4 to 12.7 pM (Table 3.1). This indicates that all the AML cell lines used in this study do express high levels of ANTXRs and active cell surface furin proteases to bind and activate PrAg. Hence, the potential cytotoxic effects of LeTx (PrAg/LF) or PrAgU2/LF on these cell lines would be due to their response to the LF-mediated inhibition of the MAPK pathway and to the expression of active uPA/uPAR on the cell surface.

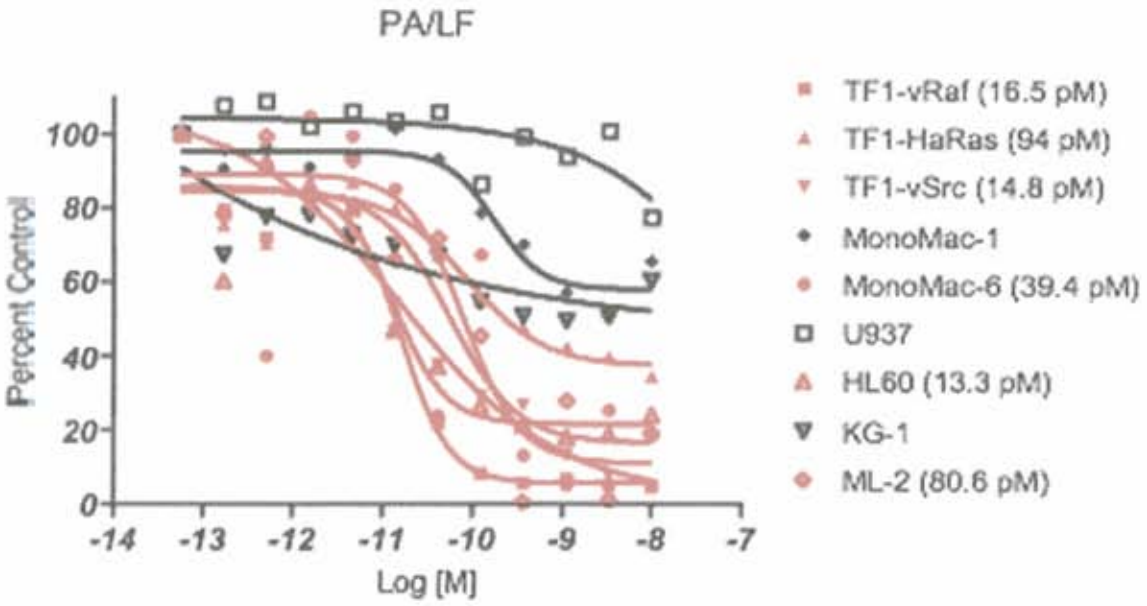
Six out of 9 AML cell lines (HL60, MonoMac-6, TF1-HaRas, TF1-vRaf, TF1-vSrc, and ML-2) were highly sensitive to LeTx (PrAg/LF) with IC_{50} values ranging from 13 pM (MonoMac-6) to 94 pM (TF1-HaRas) (Fig 3.1, Table 3.1).

Additionally, 2 out of the 6 LeTx-sensitive cell lines (HL60 and ML-2) were sensitive to the urokinase-activated PrAgU2/LF with IC_{50} values of 56 pM (Table 3.1, Fig 3.2a) and 151 pM, respectively, indicating that they, in addition to relying on the MAPK pathway express high levels of active uPA/uPAR (Table 3.1). The remaining LeTx-sensitive cell lines were not sensitive to PrAgU2/LF with IC_{50} values > 4000 pM (Table 3.1, Fig 3.2b).

Table 3.1: Sensitivity of AML cell lines to the recombinant anthrax toxins

Cell line	PrAg/FP59 (IC_{50};pmol/L)	PrAg/LF (IC_{50};pmol/L)	PrAgU2/LF (IC_{50};pmol/L)
HL60	0.7	13.3	56.7
TF1-vSrc	3.4	14.8	> 4000
TF1-vRaf	3.8	16.5	> 4000
MonoMac-6	12.7	39.4	> 4000
ML-2	6.6	80.6	151
TF1-HaRas	0.4	94	> 4000
U937	0.87	> 4000	> 4000
KG-1	1.5	> 4000	> 4000
MonoMac-1	2.1	> 4000	> 4000

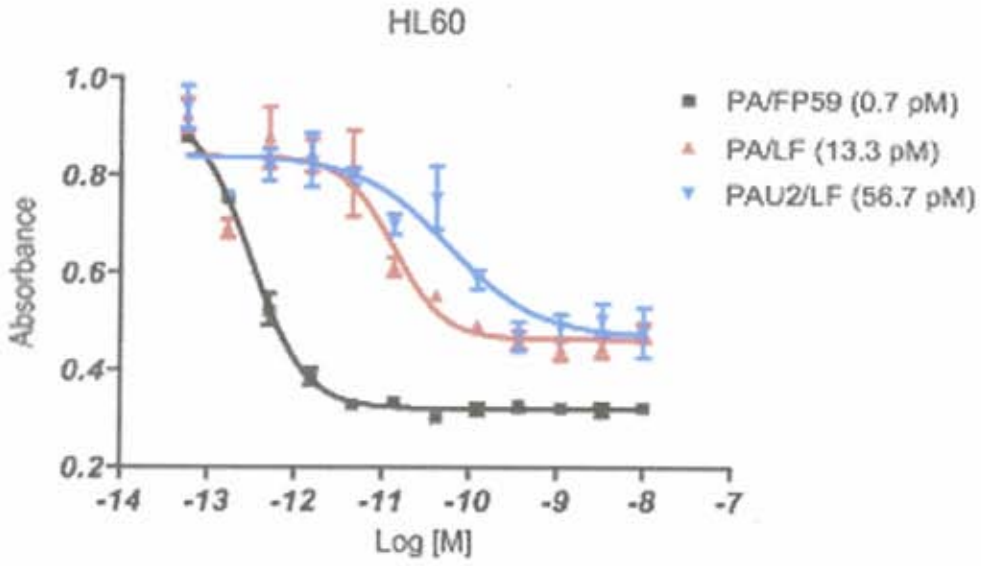
Figure 3.1: Cytotoxicity of LeTx (PrAg/LF) to a panel of AML cell lines.



Non-linear regression curves of 9 AML cell lines treated with increasing concentrations of LeTx (PrAg/LF) in an XTT-based cell proliferation (cytotoxicity) assay. Cells that are sensitive to LeTx are depicted in red while those resistant to LeTx are depicted in black. The name of each cell line and its IC_{50} are listed next to the symbols.

Figure 3.2: PrAg/FP59, PrAg/LF, and PrAgU2/FP59 cytotoxicity to the a) HL60, b) TF1-vRaf, and c) U937 AML cell line

a)



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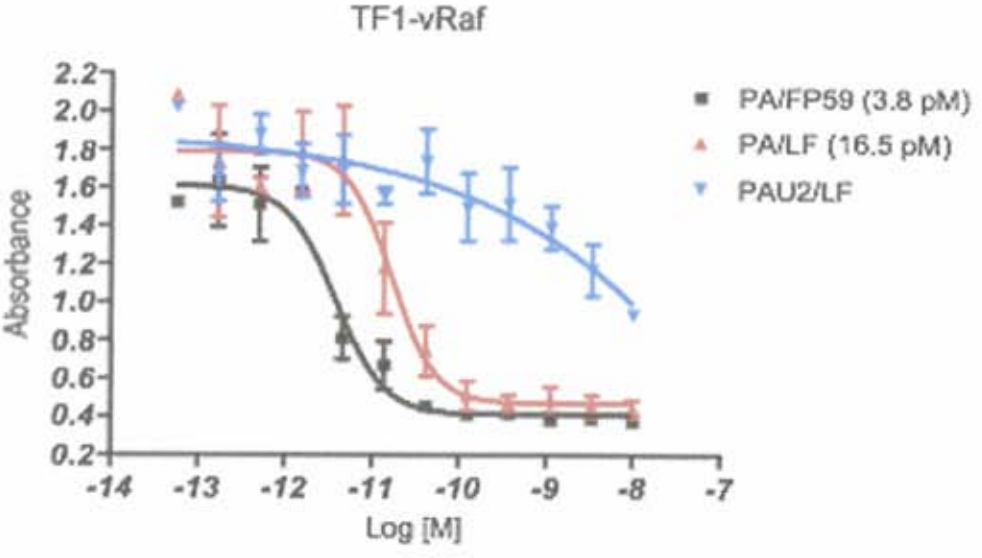

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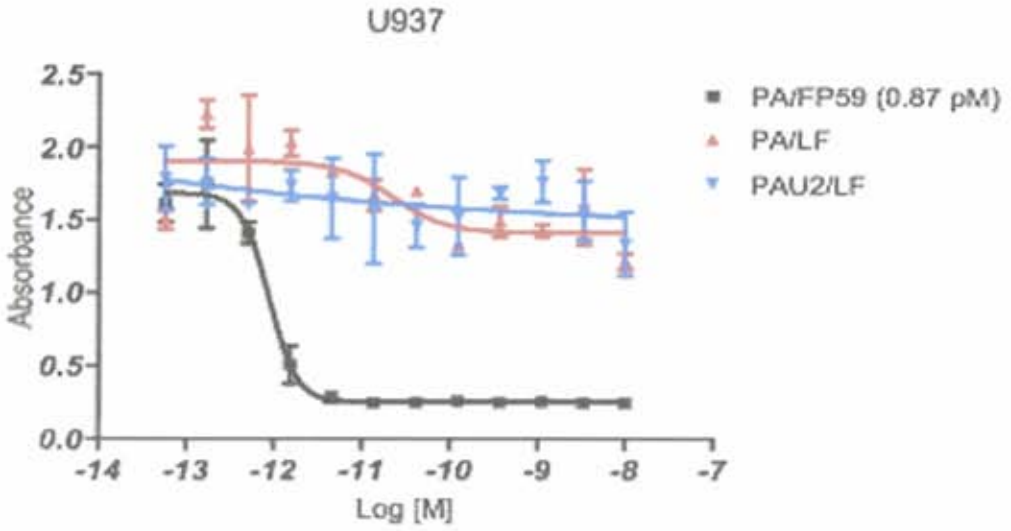


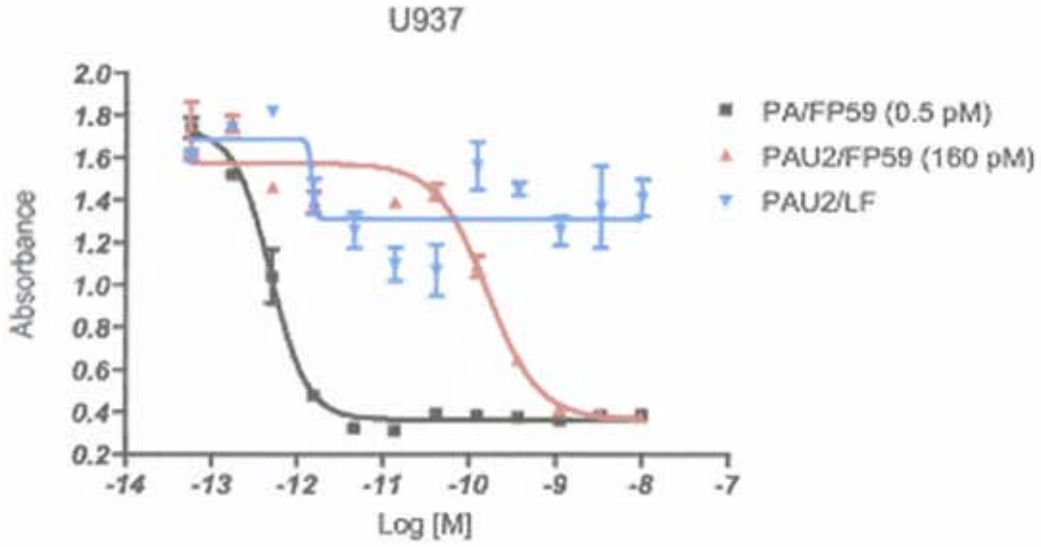
In order to investigate the expression and activity levels of the urokinase system on AML cells, independently of the cells response to the inhibition of the MAPK pathway, we tested the panel of AML cell lines, including the 3 that were not sensitive to LeTx (U937, KG-1 and Mono-Mac-1), for sensitivity to the urokinase-activated, MAPK-independent toxin PrAgU2/FP59. All 9 AML cell lines were sensitive to PrAgU2/FP59 with IC_{50} values ranging from 2.9 pM to 318 pM, indicating that all AML cell lines tested express an active urokinase system (Table 3.2, Fig 3.3a, b and c).

Table 3.2: Sensitivity of AML cell lines to PrAg/FP59, PrAgU2/LF and PrAgU2/FP59

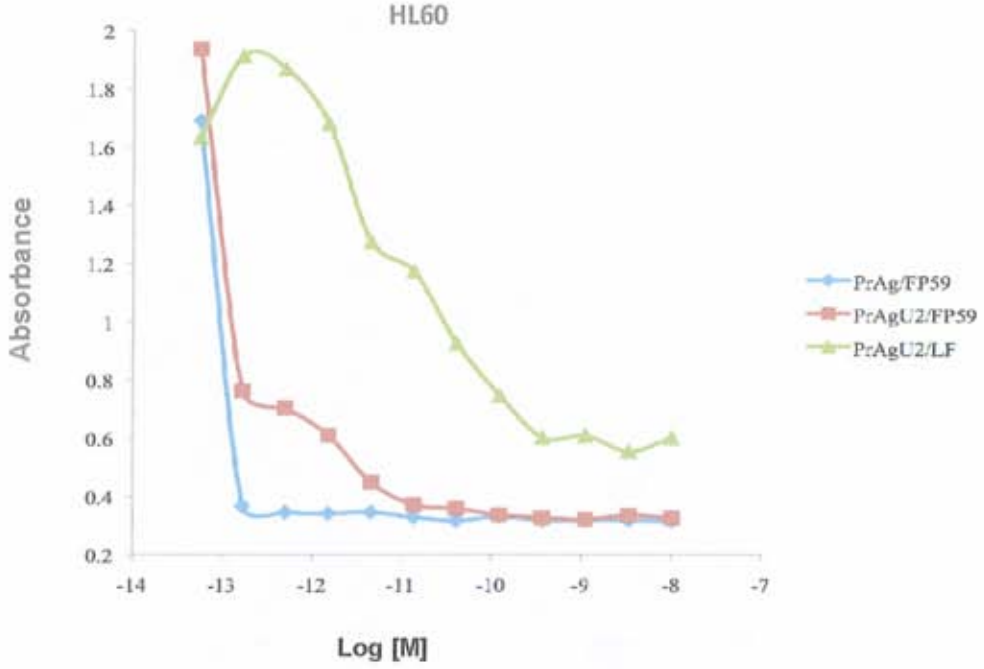
Cell line	PrAg/FP59 (IC_{50};pmol/L)	PrAgU2/LF (IC_{50};pmol/L)	PrAgU2/FP59 (IC_{50};pmol/L)
HL60	0.7	56.7	2.9
TF1-vSrc	3.4	> 4000	318
TF1-vRaf	3.8	> 4000	4.1
MonoMac-6	12.7	> 4000	130
ML-2	6.6	151	71
TF1-HaRas	0.4	> 4000	59
U937	0.87	> 4000	160
KG-1	1.5	> 4000	134
MonoMac-1	2.1	> 4000	256

Figure 3.3: PrAg/FP59, PrAgU2/FP59, and PrAgU2/LF cytotoxicity to a) U937, b) HL60 and c) TF1-vRaf AML cell lines

a)

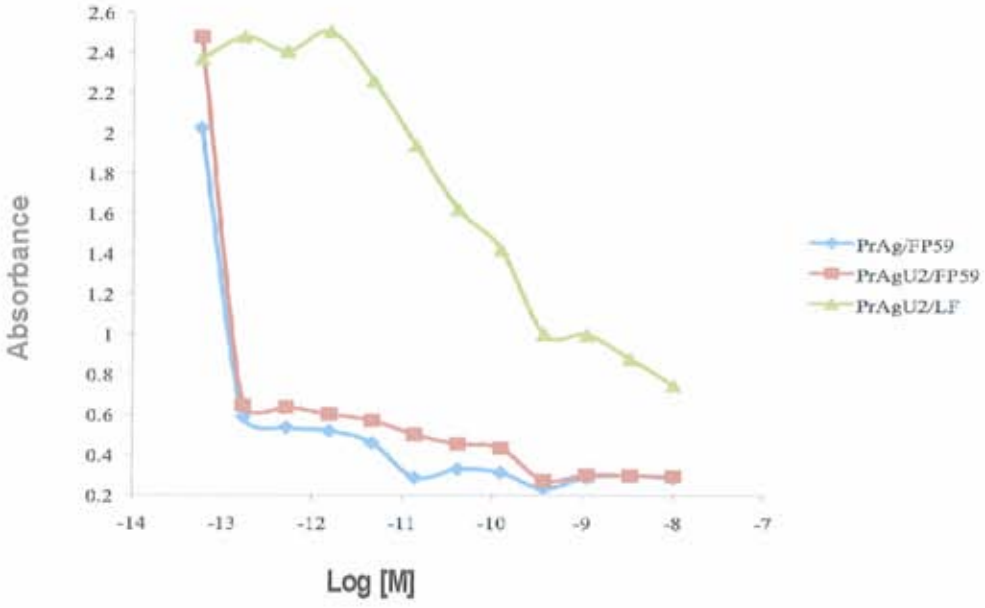


b)



TF1-vRaf

c)



In order to investigate the contribution of the different pathways to the observed response of each cell line to the LeTx-mediated inhibition of the MAPK pathway, we used the small molecular weight inhibitors U0126 and LY294002 to specifically target MEK1/2 and PI3K, respectively. Each inhibitor was used alone and in combination with LeTx.

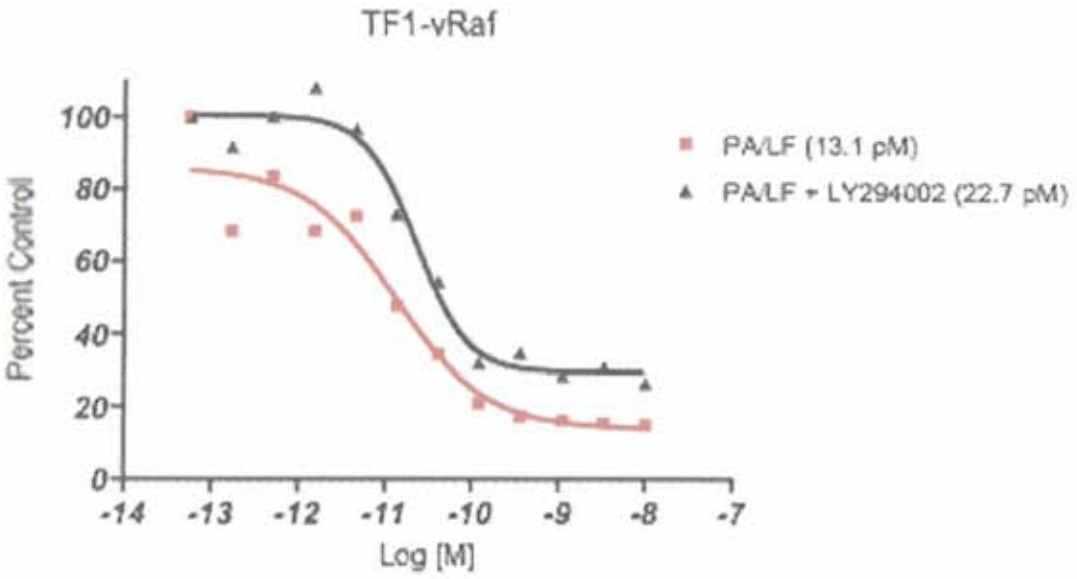
In cell lines that were sensitive to LeTx such as TF1-vRaf, and HL60 co-incubation of the cells with LeTx (PrAg/LF) and LY294002 (a highly selective, small molecular weight PI3 Kinase inhibitor) did not enhance the cytotoxicity of LeTx alone with IC_{50} values in the case of TF1-vRaf of 13.1pM and 22.7 pM for LeTx and LeTx + LY294002, respectively (Fig 3.4a and b). This indicates that, for AML cells sensitive to the inhibition of the MAPK pathway, the additional inhibition of the PI3K/AKT pathway does not add to the observed cytotoxicity.

Cell lines that were not sensitive to LeTx, such as Mono-Mac-1, were sensitive to the co-incubation with LeTx (PrAg/LF), and LY294002. The IC_{50} of Mono-Mac-1 cells to the

co-incubation with LeTx and LY294002 was 1.1 pM versus > 4000 pM for LeTx alone (Fig 3.5 a and b). These results indicate that, in AML cells resistant to the inhibition of the MAPK pathway, dual targeting of both the MAPK pathway and the PI3K pathway is necessary to achieve cell death.

Figure 3.4: PrAg/LF, and PrAg/LF+ LY294002 cytotoxicity to a) TF1-vRaf b) HL60 AML cell line

a)



b)

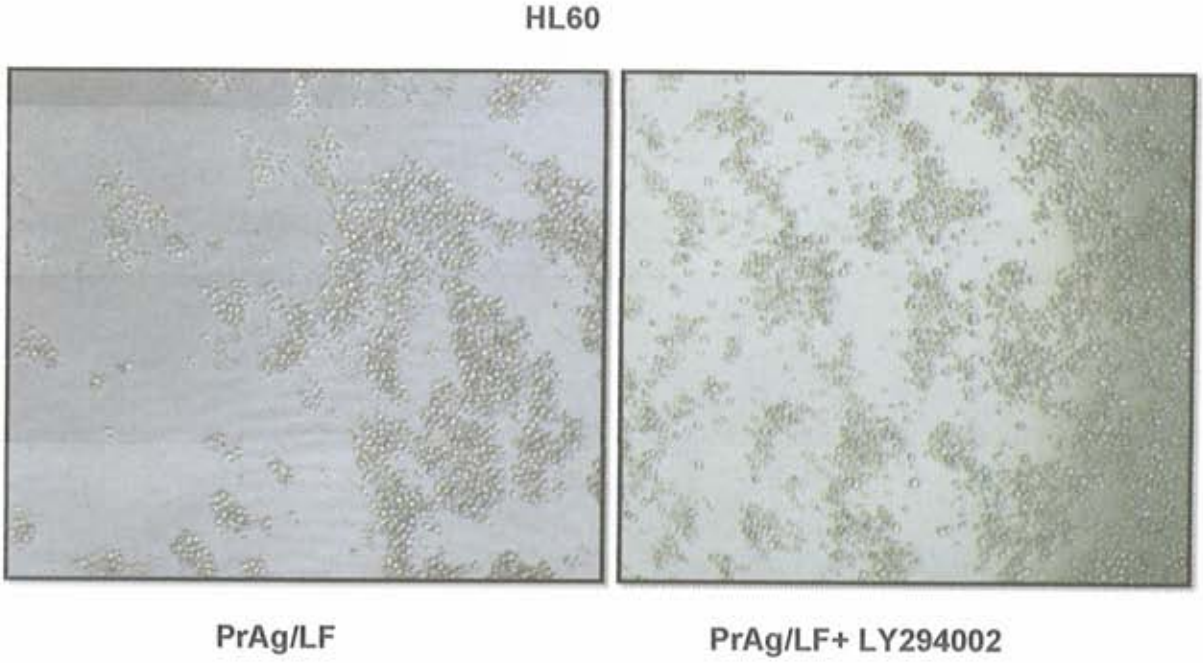
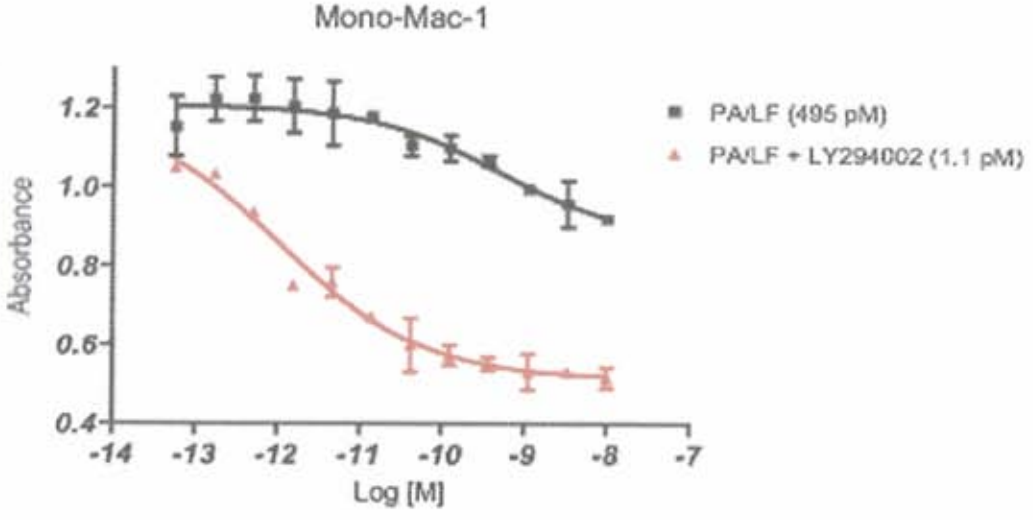


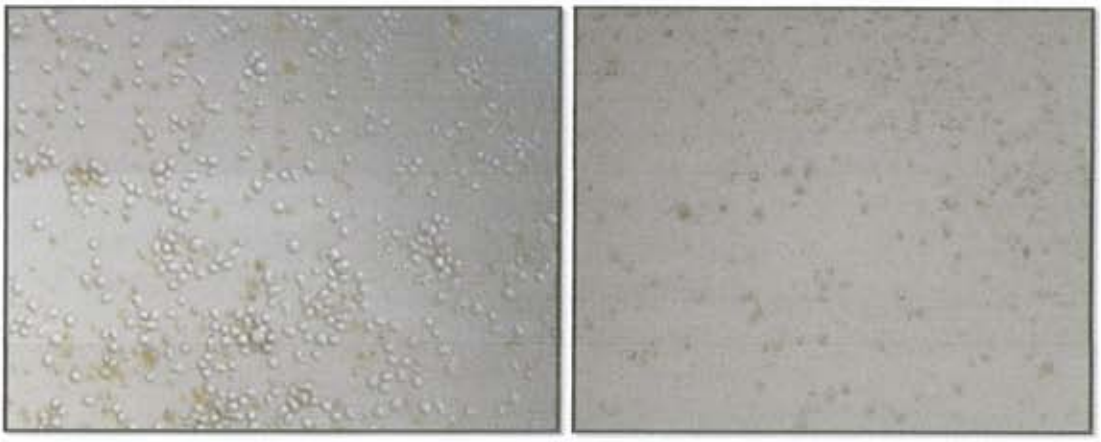
Figure 3.5: PrAg/LF, and PrAg/LF+ LY294002 cytotoxicity to the Mono-Mac-1 AML cell line

a)



b)

Mono-Mac-1

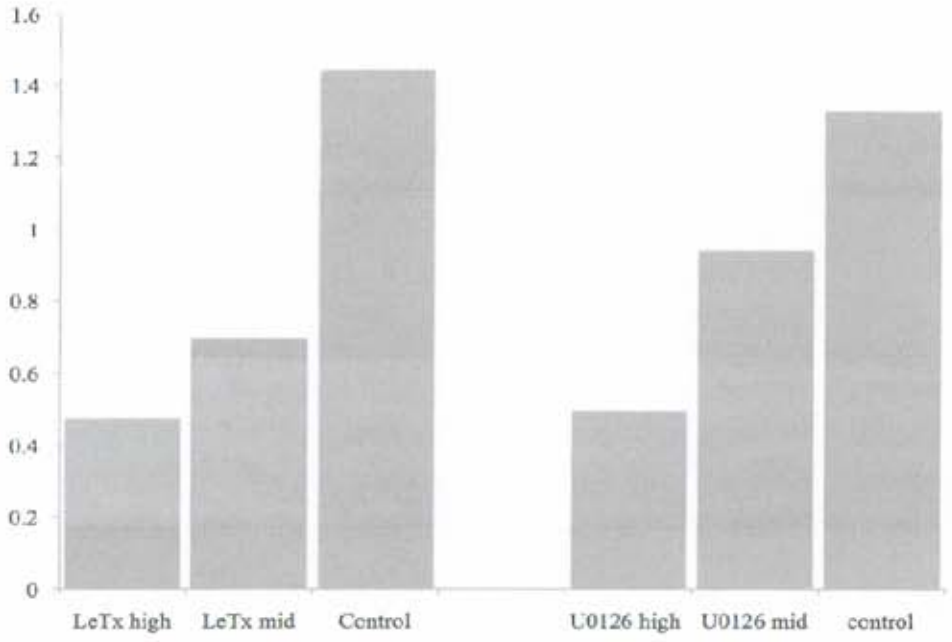


PrAg/LF **PrAg/LF+ LY294002**

Treatment of LeTx-sensitive AML cells with the specific MEK1/2 inhibitor U0126 replicated the response seen with the broad MAPK inhibitor LeTx, with all 6 LeTx-sensitive AML cell lines showing a similar pattern of cytotoxicity with U0126 (Fig 3.6 a and b). This indicates that, though LF cleaves all MEKs and inhibits all 3 branches of the MAPK pathway, the cytotoxicity of LeTx is specifically due to the LF-mediated inhibition of MEK1/2 and the Ras-Raf-MEK1/2-ERK1/2 pathway.

Fig 3.6: Sensitivity of a) TF1-vRaf , and b) HL60 AML cells to LeTx and U0126

a)



Cytotoxic effects of 2 different concentrations of LeTx and U0126 on the TF1-vRaf AML cell line. Control columns refer to cells grown in the absence of any toxin or inhibitor. LeTx high refers to the highest concentration of LeTx used (10^{-8} M) while U0126 high refers to the highest concentration of U0126 used (10^{-3} M). The mid concentrations for LeTx and U0126 are $4.1x10^{-11}$ and $4.1x10^{-6}$ M, respectively.

b)

HL60

Chapter 4

DISCUSSION and CONCLUSION

Devising novel, tumor-selective treatments for human malignancies, both solid and hematologic, is the main focus of current advances in cancer research and cancer drug development. Studies are uncovering an ever-increasing number of molecular targets that can be used for the selective targeting of different tumors. The ultimate objective of these developments is achieving personalized cancer therapy in which patients with a particular cancer would be treated with one of a battery of molecular therapeutics depending on the molecular characteristics of their tumor (Singh *et al.* 2007).

Fusion toxins and tumor protease-activated fusion toxins provide excellent means for the selective targeting of tumors. These therapeutics take advantage of the over-expression of certain receptors on tumor cells, such as GMCSF receptors on AML, and the reliance of a tumor on a particular signaling pathway, such as the MAPK pathway in melanoma. They also exploit the over-expression of specific tumor proteases, such as the urokinase plasminogen activator (uPA) in non-small cell lung cancer, or a combination of the above-mentioned factors, to specifically target highly potent bacterial or plant toxins to tumor cells (Westcott *et al.* 2004). One example of a fusion toxin used as first line therapy is Ontak (DTIL2) used for the treatment of lymphoid malignancies that over-express IL-2 receptors. Other fusion toxins such as PSA-PAH1, a urokinase-activate proaerolysin toxin for the treatment of prostate cancer, and IL4-PE, fusion of IL-4 and Pseudomonas aeroginosa exotoxin A for the treatment of glioblastoma, are in different phases of clinical development. Other fusion toxins such as DTGMCSF, a fusion of diphtheria toxin and the granulocyte macrophage colony stimmulating factor for the treatment of AML, DTU2GMCF, a urokinase-activated version of DTGMCSF for the selective targeting of AML, are still in the pre-clinical stage of development (Abi-Habib *et al.* 2005, Abi-Habib *et al.* 2006)

Previous studies have indicated that the dual-specificity fusion toxin DTU2GMCSF may be a selective and potent agent for targeting AML cells (Abi-Habib *et al.* 2004). Similar studies have shown that PrAgU2/FP59 can specifically target uPA-expressing tumor cell lines (non-small cell lung cancer, pancreatic cancer, and basal-like breast cancer cell lines), independently of the tissue of origin of these cells. These studies have also

identified anthrax toxin receptors, uPA, and uPA receptor (uPAR), that can be used as predictors of tumor cell sensitivity to PrAgU2/FP59 (Abi-Habib *et al.* 2006).

In this study we test for the sensitivity of AML cells to the inhibition of the MAPK pathway by LeTx, and for the dual targeting of the urokinase and MAPK pathways by the urokinase-activated prAgU2/LF. A panel of 9 AML cell lines was targeted with LeTx. 6 AML cell lines (HL60, MonoMac-6, TF1-HaRas, TF1-vRaf, TF1-vSrc, and ML-2) were highly sensitive to LeTx (PrAg/LF). This indicates that they express high levels of ANTXRs and active cell surface furin proteases that bind and activate PrAg, and, most importantly, that these AML cells depend on the MAPK pathway for survival, since inhibition of this pathway led to tumor cell death.

One third of the LeTx-sensitive AML cells (HL60 and ML-2) were also sensitive to the dual specific, urokinase-activated recombinant anthrax toxin (PrAgU2/LF) which is activation specific and catalytically specific, demonstrating that these cells, in addition to relying on the MAPK pathway express high levels of active uPA/uPAR allowing for the selective activation of the toxin.

Moreover, all 9 AML cell lines (the LeTx-resistant and the LeTx-sensitive cells) were sensitive to PrAgU2/FP59 indicating the presence of an active urokinase system on all these AML cell lines. However, out of the 6 LeTx-sensitive cell lines, only 2 were sensitive to PrAgU2/LF, with the remaining 4 cell lines showing no response to the urokinase activated PrAgU2/LF. The fact that all cell lines were sensitive to PrAgU2/FP59 and only 2 were sensitive to PrAgU2/LF indicates that the cells response to a urokinase-activated toxin depends on the catalytic activity of the toxin itself. In this case, FP59 (protein synthesis inhibitor) is at least 100-fold more potent than LF (MAPK inhibitor) and is, therefore, cytotoxic at lower intracellular concentrations. Hence a lower level of urokinase activity is required to activate enough toxin to reach cytotoxic levels of FP59, while higher levels of urokinase activity are required to reach cytotoxic levels of LF.

In targeting the MAPK pathway in LeTx-sensitive AML cells, the cytotoxicity of LeTx was not enhanced by the addition of LY294002 (a selective PI3 Kinase inhibitor), which

possibly suggests reliance of these cell lines on the MAPK pathway alone. In LeTx-resistant cell lines, the addition of LY294002 (specific PI3K inhibitor) sensitized the cells to the targeting of the MAPK pathway using LeTx. This indicates that these cell lines may depend on both the MAPK and the PI3K /AKT pathways for survival. Hence, dual targeting of both the MAPK pathway and the PI3K pathway is necessary to achieve cell death.

Treatment of the 6 LeTx-sensitive AML cells with U0126 (specific MEK1/2 inhibitor) simulated the response seen with LeTx (wide range MAPK inhibitor), showing a similar pattern of cytotoxicity. This signifies that, though LF cleaves all MEKs and inhibits all 3 branches of the MAPK pathway (JNK branch, the p38 MAPK branch, and the Ras/Raf/MEK1/2/ERK1/2 branch), the cytotoxicity of LeTx is due to the LF-mediated inhibition of MEK1/2 and the Ras-Raf-MEK1/2-ERK1/2 pathway. Thus, targeting MEK1/2 and not all three branches may be sufficient to cytotoxically target AML cells.

In conclusion, we have provided evidence that a majority of AML cell lines are sensitive to the inhibition of the MAPK pathway, and thus susceptible to the targeting of the MAPK pathway which has significant implications for the development of fusion toxin derived AML drugs that can be clinically beneficial to patients with chemotherapy refractory AML. Thus, specific targeting of the MAPK pathway in AML cell lines deserves further investigation and development. The dual targeting of the MAPK and urokinase pathways, also represents an attractive target that warrants further in-depth studies. Such studies are likely to contribute towards the development of molecularly-driven targeted therapies in the near future.

Chapter 5

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