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Characterization of the potency and mechanism of action of a novel dual-selective intermolecularly complemented version of Anthrax Lethal Toxin (PAU2R200A- PAL1I207R/FP59) in Acute myeloid leukemia

By
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Characterization of the potency and mechanism of action of a novel dual-selective intermolecularly complemented version of Anthrax Lethal Toxin (PAU2R200A- PAL1I207R/FP59) in Acute Myeloid Leukemia

Mirna Farhat

Abstract

Anthrax lethal toxin (PrAg/LF) is a binary toxin consisting of protective antigen (PrAg), the cell binding moiety, and lethal factor (LF), the catalytic moiety. Inside the cell, LF cleaves MEKs and subsequently leads to the inhibition of the MAPK pathway causing cell death. Due to its off-target toxicity, a more selective generation of PrAg/LF was re-engineered by making its activation dependent on cleavage by tumor specific cell surface proteases enriched on the surface of tumor cells and not normal cells. This resulted in PrAgU2 and PrAgL1 variants that mandate activation by either uPA/uPAR or MMPs tumor specific proteases, respectively. Both variants proved to be highly potent against tumors while having an enhanced selectivity and as such paved the way to create a modified intermolecularly complementing (IMC), PAU2R200A-PAL1I207R, version of PrAg that requires activation by both uPA/uPAR and MMPs proteases, simultaneously. IMC combined with LF or FP59, an inhibitor of protein synthesis, is thought to be highly selective requiring two distinct proteolytic activities overexpressed by tumor tissues for its activation. In this study we tested the potency of IMC/FP59 and IMC/LF on a panel of AML cell lines. IMC/LF treatment didn't show any signs of cytotoxicity to AML cells, but induced cell cycle arrest in a subset of these cells. On the other hand, IMC/FP59 displayed potency on AML cells with four levels of sensitivity seen; high sensitivity, moderate sensitivity, mild sensitivity, and no sensitivity. We showed evidence that PAU2R200A/FP59, from the IMC variant, induced a cytotoxic response that matched the pattern of IMC/FP59, as such indicating that uPA/uPAR is the rate limiting factor in the activation of IMC/FP59.In addition, staining for Annexin V/PI post IMC/FP59 treatment showed an increase in double positive cells indicating non-apoptotic cell death. The sensitivity of AML cells to IMC/FP59 did not depend on the basal levels of expression of uPAR and MMPs (MMP2 and MMP9). However, given that MMP9 was found not to be expressed in the sensitive cell lines, we were able to exclude its

expression as a requirement for cytotoxicity. The absence of dependence indicated that cytotoxicity levels seen may depend on the activity levels of these proteases, rather than on their expression levels. Finally, while IMC/LF treatment did not affect the expression of any of the proteases tested (uPAR, MMP2, and MMP9), treatment with IMC/FP59 did affect their expression with significant cytotoxic responses seen only in cells whose uPAR expression was not affected at any time point post-treatment.

In this study, we showed for the first time the potency of IMC PrAg variant on AML cell lines, its mode of action, as well as its mechanism of cell death.

Keywords: Acute Myeloid Leukemia, Anthrax Lethal Toxin, Mitogen Activated Protein Kinase Pathway (MAPK), Intermolecularly Complementing, Targeted Therapeutics, Cytotoxicity

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LIST OF ABBREVIATIONS

AML	Acute Myeloid Leukemia
ANTXRs	Anthrax Toxin Receptors
BSA	Bovine Serum Albumin
CMG-2	Capillary Morphogenesis Gene-2
ECL	Enhanced Chemiluminescence
EF-2	Elongation Factor 2
EGF	Epidermal Growth Factor
EGFR	Epidermal Growth Factor Receptor
ERK	Extracellular Signal-Regulated Kinase
FAB	French-American-British
FISH	Fluorescence In Situ Hybridization
FP59	Fusion Protein
GO	Gemtuzumab Ozogomycin
GRB2	Growth Factor Receptor-Bound Protein 2
IC_{50}	Inhibitory Concentration 50
IMC	Intermolecularly Complementing Version
Jnk1/2/3	Jun Amino-Terminal Kinases
LF	Lethal Factor
MAPK	Mitogen-Activated Protein Kinase
MEK	Mitogen-Activated Protein Kinase Kinase
MMP	Matrix Metalloproteinase
MMP-2	Gelatinase A
MMP-9	Gelatinase B
MT1-MMP	Membrane-Type 1 MMP
NCI	National Cancer Institute
NIH	National Institutes of Health
PA20	20-Kda PrAg Fragment

PA63	63-Kda PrAg Fragment
PAL1/LF	Matrix Metalloprotease-Activated Anthrax Lethal Toxin
PAU2/LF	Urokinase-Activated Anthrax Lethal Toxin
PAU2R200A-PAL1I207R/LF	Urokinase And Matrix Metalloprotease-Activated Anthrax Lethal Toxin
PI	Propidium Iodide
PrAg	Protective Antigen
PrAg/LF	Furin-Activated Anthrax Lethal Toxin
RPMI	Roswell Park Memorial Institute
RTK	Receptor Tyrosine Kinase
SOS	Son Of Sevenless
TEM-8	Tumor Endothelial Marker-8
uPA	Urokinase Plasminogen Activator
uPAR	Urokinase Plasminogen Activator Receptor
WHO	World Health Organization

CHAPTER ONE

INTRODUCTION

1.1. Acute Myeloid Leukemia (AML)

1.1.1. Acute Myeloid Leukemia

The World Health Organization (WHO) defines cancer as: "uncontrolled proliferation of abnormal cells that surpass their physiological boundaries". With an inclination to manifest in almost any part of the body, cancer that starts in the blood-forming tissues, such as the bone marrow and lymphatic system, is termed leukemia. Several types of leukemia exist depending on whether it is acute (fast growing), or chronic (slow growing), and whether it gets initiated in myeloid cells or lymphoid cells (American Cancer Society, 2018).

One such type is Acute Myeloid Leukemia (AML). AML begins in the bone marrow, which is the spongy tissue inside bones and the location of blood cell formation. It affects the myeloid line of blood cells, where aberrant proliferation and lack of differentiation of myeloid stem cells lead to disturbances in normal blood cell production (Figure 1) (De Kouchkovsky & Abdul-Hay, 2016).

Once these early hematopoietic progenitors experience a differentiation block, the ensuing defective cancerous cells become known as blast cells (Gocek & Marcinkowska, 2011). Such abnormal cells start to build up in the bone marrow, eventually moving into the blood and can even spread to distinct parts of the body, such as the lymph nodes, liver, spleen, central nervous system, and testicles. (American Cancer Society, 2018).

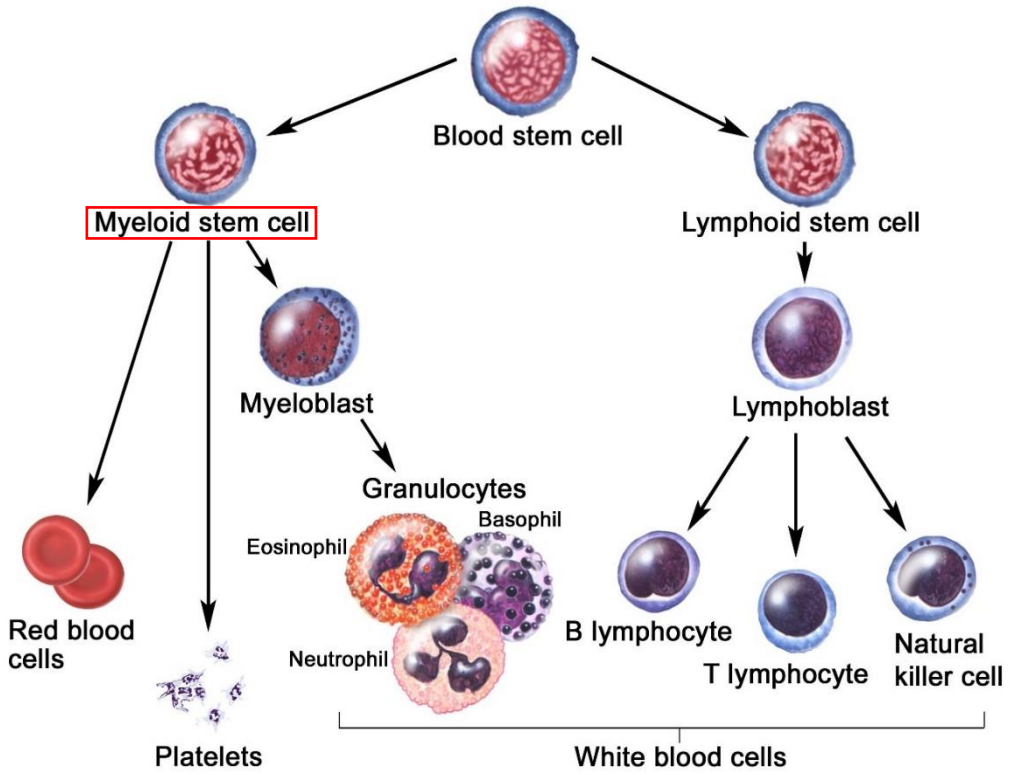


***Figure 1: Blood cell formation**. Hematopoietic stem cells will differentiate and grow into different types of blood cells, including red blood cells, white blood cells, and platelets. (Outlined in red) (Modified from: National Cancer Institute, 2023)*

1.1.2. Statistics and Epidemiology

AML is one of the most prevalent types of leukemia in adults, accounting for approximately 1% of all cancers (American Cancer Society, 2023).

It is uncommon in children, while on the other hand it accounts for 80–85% of acute leukemia cases in adults, indicating that AML is predominantly a disease of older adults (Gocek et al., 2011).

According to the American Cancer Society, it is estimated that there will be approximately 20,380 emerging cases of AML and 11,310 deaths from AML in the United States in 2023, with the majority occurring in adults (American Cancer Society, 2023). Moreover, it has been reported that men are more likely than women to develop AML (Shallis et al., 2019). Additionally, adults with AML have a 5-year survival rate of only around 24%. This rate has increased slightly with advances in therapeutic strategies, however further improvements are still needed (Deschler et al., 2006).

1.1.3. Clinical Presentation and Diagnosis

AML imposes physical and mental burdens on patients, causing them to experience general symptoms that hinder their lives such as weight loss, fatigue, fever, night sweats, and loss of appetite. In addition, by affecting the normal production of blood cells, anemia, thrombocytopenia, and leukocytosis are common within patients. These as well lead to headaches, lightheadedness, excessive bleeding, and frequent infections which complicates and increases the risk on the patients (De Kouchkovsky et al., 2016).

To confirm AML, several tests need to be performed, most notably bone marrow and blood tests that include complete blood counts and differential counts, bone marrow aspiration, immunophenotyping, cytogenetic analysis and screening (Döhner et al., 2010)

First, blood and bone marrow samples are collected and examined. The presence of 20% or more blasts in the bone marrow or peripheral blood confirms the diagnosis of AML (De Kouchkovsky et al., 2016)

AML is then further diagnosed by establishing the myeloid origin of the cells which is done by immunophenotyping, that quantifies the expression patterns of a number of surface and cytoplasmic antigens and is important for lineage assignment (Döhner et al., 2010).

In addition, the diagnostic assessment of a patient with suspected acute leukemia must include conventional cytogenetic analysis, since chromosomal abnormalities, such as translocations and inversions, are very common in AML cases and are detected in approximately 55% of adult AML (Döhner et al., 2010). Reverse transcriptase polymerase chain reaction (RT-PCR) and fluorescence in situ hybridization (FISH) can be used to detect these rearrangements (Döhner et al., 2010).

Furthermore, AML shows somatically acquired mutations that have been found in multiple genes, such as FLT3 and NPM1. Screening for such mutations can add an additional layer of confirmation to the diagnosis of AML (Döhner et al., 2010).

1.1.4. Classifications of AML

The French-American-British (FAB) and the more recent World Health Organization (WHO) classifications are two of the primary approaches that have been used to divide AML into subtypes. In 1976, the French-American-British classification scheme was the first effort to categorize various AML subtypes based on the morphology of leukemic cells (De Kouchkovsky & Abdul-Hay, 2016). Eight subtypes of AML were identified, M0 through M7:

A. M0 Undifferentiated acute myeloblastic leukemia

B. M1 Acute myeloblastic leukemia with minimal maturation

C. M2 Acute myeloblastic leukemia with maturation

D. M3 Acute promyelocytic leukemia (APL)

E. M4 Acute myelomonocytic leukemia

F. M5 Acute monocytic leukemia

G. M6 Acute erythroid leukemia

H. M7 Acute Megakaryoblastic leukemia

Subtypes M0 through M5 originate in immature white blood cells. M6 AML emerges from immature red blood cells, whereas M7 AML arises from immature cells that form platelets (American Cancer Society, 2018).

With advances made in understanding AML, a new method of AML classification was introduced by the World Health Organization (WHO) in 2016. This classification identifies six main AML subtypes that are not only based on the morphology of the cells but also incorporate features such as genetic abnormalities and data, immunophenotype, and clinical presentation of the patients. The six AML subtypes were as follows:

A. AML with recurrent genetic abnormalities (i.e., gene or chromosome changes), accounts for about 20–30% of patients with AML.
B. AML with myelodysplasia-related features.
C. Therapy-related AML, accounts for 10–20% of all cases of AML.

D. AML not otherwise specified, includes cases of AML that are not classified into any of the mentioned categories.
E. Myeloid sarcoma.
F. Myeloid proliferation related to Down syndrome.

Identifying the subtype of AML might be crucial in assisting in determining a patient's prognosis and treatment (Hwang, 2020).

1.1.5. Physiopathology

AML is a disease in which differentiation is suppressed and proliferation is boosted. It has been found that chromosomal rearrangements and many gene alterations, mostly affecting epigenetic regulators and transcription factors/activators, can hinder this hematopoietic differentiation while providing a proliferative and survival advantage (Lagunas-Rangel et al., 2017).

Chromosomal abnormalities and translocations are commonly observed in patients with AML. Some of them include t (15;17) (q22; q12), inv (16) (p13; q22) or t (16;16) (p13; q22), t (8;21) (q22; q22) and t (9;11) (p22; q23). These will lead to the formation of fusion proteins such as PML-RARA, MYH11-CBFB, RUNX1-RUNX1T1, KMT2A-MLLT3, that will play a major role in disturbing hematopoiesis contributing to AML formation (Wang et al., 2017).

However, nearly 50% of AML samples have a normal karyotype and many of these genomes lack structural abnormalities. Therefore, some genetic mutations were found to attribute as well in the manifestation of AML (The Cancer Genome Atlas Research Network, 2013).

In 2002, Gilliland proposed a two-hit model (Gilliland, 2002). This model speculates that AML can result from the added effect of two classes of mutations: Class I

mutations that confer proliferative and survival advantages to hematopoietic progenitors, and Class II mutations that halt the processes of hematopoietic differentiation and apoptosis (Gilliland, 2002) (Lagunas-Rangel et al., 2017)(Grove & Vassiliou, 2014). In addition, mutations that do not fit into these two classes are being identified in recent studies (Lagunas-Rangel et al., 2017).

Being a heterogeneous disease, AML has no single prevalent somatic mutation that is present in all cases. However, several recurrent mutations have been identified using sequencing techniques. These include mutations in FLT3 (in approximately one-third of AML patients), NPM1, KIT, DNMT3A, IDH1/2, TET2, AML1, TP53, NRAS, CEBPA, and WT1 (Gruszka et al., 2017). These mutations can lead to aberrant signal transduction and alterations in multiple intracellular pathways, such as the mitogen-activated protein kinase (MAPK) and PI3K/AKT cascades, promoting the survival and growth of hematopoietic progenitor cells. Consequently, these pathways represent interesting targets for applicable therapeutic strategies (Stefan Fröhling, 2008).

1.1.6. Treatment of AML

The goal of AML therapies is to put leukemia into complete remission, which usually indicates that the bone marrow contains less than 5% blast cells and cell counts return to normal (American Cancer Society, 2018).

Eligible cases first receive induction chemotherapy, known as the 7+3 regimen, to try achieving complete remission (De Kouchkovsky et al., 2016). In this case, intensive chemotherapy with cytarabine and anthracyclines, as well as other agents is induced. This involves an induction of cytarabine for 7 days, accompanied by an anthracycline, , typically daunorubicin , for 3 days (Lagunas-Rangel et al., 2017). However, minimal residual disease persists in complete remission, meaning that small numbers of

leukemic cells remain in patients during remission, which represents a major cause of relapse in leukemia. Therefore, to increase the chance of complete-long term remission and to prevent relapse, induction therapy is followed by consolidation (post remission) chemotherapy with the hopes to get rid of any remaining leukemia cells (De Kouchkovsky et al., 2016).

This regimen did report improved survival mostly in patients that are young and that have favorable prognosis, but relapses are not out of the picture. On the other hand, elderly patients are more likely to have unfavorable chromosome abnormalities, as well as little tolerance for intensive chemotherapy and are susceptible to treatment related toxicities. Hence, lower doses are administered but are not optimal. Understanding how to approach such cases is still in the works (Lagunas-Rangel et al., 2017) .

Many prognostic factors that explain the reason some patients have a better outlook than others, correlate with AML. Such factors include a person's age, having certain gene or chromosome changes, previous cytotoxic therapy for another disorder as well as the subtype of AML. These all play a role in depicting the response of leukemic cells to the available therapeutic strategies (Döhner et al., 2010).

These factors will help stratify patients into groups, and accordingly choosing between standard or more intense treatment based on the severity of their AML prognostic factors (De Kouchkovsky et al., 2016).

Although treatment has been optimized by advances in prognostic risk stratification, AML continues to have a poor long-term survival rate (De Kouchkovsky et al., 2016) , with the National Cancer Institute (NCI) reporting a 5-year overall survival rate of only 29.5% (National Cancer Institute, 2020). Furthermore, the remission rate for most types of AML remains around 67% (American Cancer Society) and as such

a large percentage of AML patients fail to achieve complete remission, particularly elderly patients who account for most newly diagnosed cases and cannot receive aggressive treatment regimens (De Kouchkovsky et al., 2016). Therefore, the 7+3 treatment may be ineffective and result in hospitalization, systematic toxicity, and fatal side effects (Eleni et al., 2010).

All in all, this highlights the importance of advancing in therapeutic strategies, reaching a new era in the treatment of AML that can achieve enhanced responses with better survival rates, especially for the elderly population. Here comes the role of novel targeted therapeutics, that introduce effective anti-leukemic activity with minimized toxicity from off-target effects (De Kouchkovsky et al., 2016).

1.2. Targeted therapeutics

In recent years, there have been substantial changes in the way that cancer is treated due to the extraordinary advances in understanding the molecular nature of cancer. Targeted therapeutics include a wide range of molecules such as low–molecular-weight inhibitors drugs, monoclonal antibodies, and bacterial toxins. Rather than generally producing cytotoxicity, such as conventional chemotherapeutics, these targeted therapeutics are directed medications that selectively and specifically block one of the biochemical targets that drive tumor formation within cancer cells. Hence limiting the adverse effects on healthy tissues that are off target, increasing the tolerance to such therapy. Nowadays, these molecules combined with chemotherapy and other approaches are used for the treatment of many prevalent cancers such as breast, lung, lymphoma, as well as leukemia (Gerber, 2008).

The abnormal biological state of cancer comes from malfunctioning signaling proteins that are members of pro-survival, pro-proliferation, and anti-apoptotic pathways. Hence, by inhibiting such proteins or even their downstream effectors, the oncogenic program can be halted. As such, these targeted cancer therapies aim to stop proliferation and growth of tumor cells, improve control over the cell cycle, and encourage programmed cell death via apoptosis or autophagy, which all in all can lead to long term remission (Padma, 2015).

The path to reach targeted therapeutics was paved first after the knowledge gathered the past few decades on the factors that underline and drive tumor development, such as oncogenes, which in turn provided a variety of possible molecular targets in cancer. In addition, these molecular targets, although present in both normal and cancer cells, represent a variable between the two. In cancer cells, they either carry mutations that cause gain of function or are overexpressed. Hence, drugs can be developed to only selectively bind the mutant oncogene, and not the protooncogene, or to unique cell surface markers of cancer cells inhibiting its activity and blocking the oncogenic pathway. Therefore, improving the selectivity of such methods and possibly reducing toxicities for patients (Padma, 2015).

In some groups of AML patients, the addition of different targeted medications to the standard 7+3 induction chemotherapy led to an improved outcome. Gemtuzumab ozogomycin (GO), represents one of those targeted therapeutics in AML. It is a monoclonal antibody against CD-33, which is a protein expressed by AML cells. In certain groups of patients, namely those with favorable and intermediate risk, introducing GO to standard chemotherapy lowers the risk of relapse and, in some trials, increases the overall survival of patients (Pelcovits et al., 2020).

Another example is demonstrated with the oral tyrosine kinase inhibitor, midostaurin. It is an effective inhibitor of FLT3, which is highly mutated in AML patients with about 25–30% of AML patients having FLT3 mutations that start oncogenic signal transduction. Combining midostaurin with the standard 7+3 chemotherapy boosted the survival, from a median of 25 to 74 months (Pelcovits et al., 2020).

The availability of treatments for older patients who are more at risk of mortality from conventional chemotherapy regimens will be expanded by the introduction of well-tolerated targeted therapeutics (De Kouchkovsky et al., 2016).

Yet, the genetic complexity of AML makes it unlikely that targeted therapeutic will act as a single "magic bullet" treatment that can offer a cure. Instead, gradual improvements in remission and survival can be anticipated as a result of the development of such targeted therapeutics, in conjunction with enhanced genetic screening and risk stratification, which has also made it possible to assess each patient's tumor and customize the therapy accordingly. (De Kouchkovsky et al., 2016).

Hence, targeted therapeutics can inhibit the hyperactive oncoproteins, or its signal-transducing proteins downstream of it, that drive tumor progression causing the tumor growth program to collapse. The Mitogen-activated protein kinase (MAPK) pathway is a pro-survival pathway that is mutated in a big percentage of AML patients. As such, its protein components represent interesting targets when aiming for therapy.

1.3. Mitogen-activated protein kinase (MAPK)

1.3.1. The RAS-RAF-MEK-ERK Pathway

Mitogen-activated protein kinase (MAPK) cascades are intracellular signaling pathways that are essential for transducing extracellular stimuli to the nucleus

affecting gene expression and causing a change in a cell's behavior (Zhang & Liu, 2002).

Three MAPK families have been clearly characterized in mammalian cells: the extracellular signal-regulated kinases (Erk1/2), Jun amino-terminal kinases (Jnk1/2/3), and p38-MAPK (Zhang & Liu, 2002). In such pathways, a series of protein kinase cascades get activated upon tyrosine and threonine phosphorylation (Katz et al., 2007). In turn, this will relay, amplify, and integrate signals from a variety of stimuli to then promote cellular proliferation, differentiation, development, inflammatory responses, and apoptosis in mammalian cells, among other physiological responses (Zhang et al., 2002).

In these cascades, some enzymes including a MAPK kinase kinase (MAPKKK), a MAPK kinase (MAPKK) and a MAP kinase (MAPK), get activated in series (Zhang & Liu, 2002).

The ERK/MAPK pathway branch is an evolutionarily conserved, very well characterized, pathway that is also known as the RAS–RAF–MEK–ERK/MAPK pathway (Kolch, 2005) (Braicu et al., 2019). As shown in Figure 2, activation of this pathway starts with the binding of a ligand, primarily a growth factor such as epidermal growth factor (EGF), to the extracellular domain of a membrane bound receptor belonging to the family of receptor tyrosine kinase (RTKs), such as the epidermal growth factor receptor (EGFR) (Katz et al., 2007).

Each RTK has an extracellular ligand-binding domain, a single transmembrane domain, and an intracellular region with a tyrosine kinase domain which can undergo auto- or trans-phosphorylation(Katz et al., 2007).

Ligand binding drive the dimerization of two subunits of the RTK, accordingly leading to the activation of the intracellular intrinsic tyrosine kinase domain, that in turn will

catalyze auto-phosphorylation of itself and trans-phosphorylation of the other subunit. Activated RTK represents a docking and binding site that allows for the recruitment of adaptor protein GRB2 (Growth factor receptor-bound protein 2), which sequentially interacts with the guanine nucleotide exchange factor, SOS (son of sevenless) (Katz et al., 2007).

SOS is consequently drawn to the proximity of the plasma membrane, where it interacts with a small GTPase named RAS and induces it to undergo nucleotide exchange of GDP to GTP. Hence, allowing RAS to switch from the inactive GDP bound form, to the active GTP bound form. In this active conformation, RAS then attaches and activates the downstream RAF protein kinase, the MAPKKK protein (Katz et al., 2007). Consequently, RAF induces the phosphorylation of the MAPK kinases, MEK1 and MEK2 (MAPKK), switching them on. In turn, MEKs phosphorylate the MAPK proteins ERK1 and ERK2, on tyrosine and threonine residues thus activating them. Active ERKs will then phosphorylate several proteins, and will as well translocate to the nucleus where it activates various transcription factors, affecting gene expression and as such controlling various cellular processes such as proliferation, development, and differentiation (Katz et al., 2007). Playing such a crucial role, any dysregulation in RAS-RAF-MEK-ERK signaling pathway will have drastic effects and can lead to pathological behaviors (Li et al., 2019).

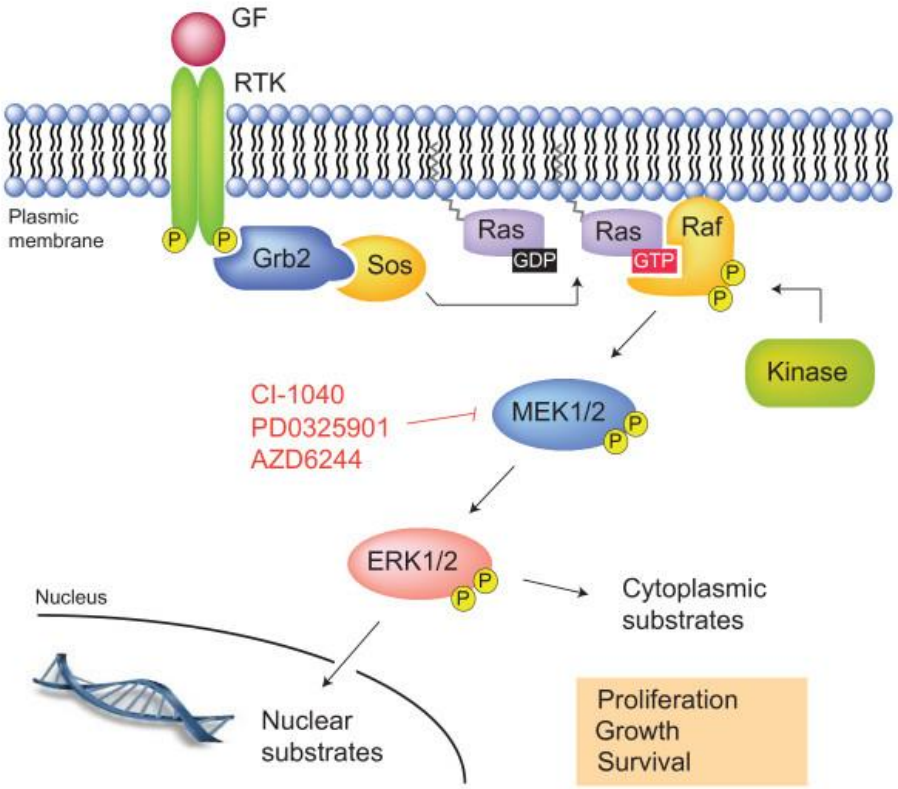


Figure 2: The RAS-RAF-MEK-ERK Pathway. *A series of phosphorylation reactions transmitting the signal from the cell surface to promote proliferation, differentiation, and survival. Source:(Frémin et al.,2010)*

1.3.2. Mutations in the RAS-RAF-MEK-ERK pathway

The MAPK is activated in a pulsatory manner where signaling can be halted at any time, since it is closely regulated and controlled by feedback loops at several levels (Li et al., 2019). Any event that affects the members of the MAPK pathway causing them to be constitutively active, will in turn promote constant cell proliferation and growth, eventually leading to tumorigenesis (Katz et al., 2007).

One such event is aberrations in RTKs, where they have frequently been discovered to be either mutant or overexpressed in human cancers, and as such leading to constitutively activated tyrosine kinase receptors that boost MAPK activation (Katz et al., 2007). One example, highly common in AML blasts, is aberrant FLT3. FLT3 was found to be overexpressed in a large percentage of AML patients. In addition, somatic mutations that activate the *FLT3* gene are among the most prevalent genetic

abnormalities in AML and have a major bearing on prognosis. As such, *FLT3* gene was shown to be mutated in about 30% of cases of AML at diagnosis (Grafone et al., 2012).

Furthermore, another way could be by co-expression of RTKs and their respective ligands together, which leads to the activation of an autocrine loop, that sustains MAPK activation causing uncontrolled cell growth (Katz et al., 2007)

Other events, that could lead to permanent activation independently of any upstream growth signals, include mutations in each and every member of the MAPK cascade, namely *RAS*, *RAF*, *MEK*, and *ERK* genes (Katz et al., 2007)

K-Ras, H-Ras and N-Ras, which are three members of the RAS family encoded by potent proto-oncogenes (Kolch, 2005) , have shown to be mutated in about 20% of all AMLs (Towatari et al., 1997), with K-Ras acquiring the most mutations (Kolch, 2005). Such alterations reduce the GTPase activity of RAS, leading to the accumulation of activated RAS-GTP that is unable to switch back off to the GDP bound form, sustaining the activation of the MAPK pathway (Katz et al., 2007)

RAF can also harbor mutation, with the gene encoding B-Raf showing mutations in many cancers, mainly in melanoma and cancers of the thyroid, colon, and ovaries (Kolch, 2005). Of these mutations, V600E is the most common, in which a valine is replaced by glutamic acid. Accordingly such mutation will constitutively activate RAF and its downstream effector (White et al., 2018).

Furthermore, MEK and ERK aberrations do exist as well, where for example ERK is shown to be prevalently overexpressed in a major percentage of AML cases, which contributes to the abnormal cell proliferation and growth in AMLs (Platanias, 2003).

Altogether, these studies have shown the involvement of the RAS-RAF-MEK-ERK pathway in the pathogenesis of many cancers, including AML (Platanias, 2003).

Hence, members of this pathway represent interesting and major targets for new therapeutic approaches, promoting to pharmacologically inhibit and manipulate this signaling pathway in a selective manner and successfully fight cancer (Katz et al., 2007).

1.4. Anthrax Lethal Toxin (PrAg/LF)

Infectious bacteria often produce A-B protein toxins to exert virulence. Such toxins consist of subunit "A", the enzymatic moiety that promotes the toxic activity by targeting host proteins, and subunit "B", the binding component that targets the toxin to a specific receptor on host cells. These toxins target very-well defined substrates in their hosts, usually intracellular proteins, giving them a selective and specific mode of action. As such, bacterial toxins can be helpful in targeted therapy (Ghazaei, 2022).

Bacillus anthracis, a Gram-positive rod-shaped bacterium, is the causative agent anthrax. It is characterized by the production of an exotoxin called Anthrax Lethal Toxin (PrAg/LF) which represents an important developing targeted therapeutic to selectively target cancer cells (Lowe et al., 2012).

1.4.1. Mode of action of Anthrax Lethal Toxin (PrAg/LF)

PrAg/LF is an A-B binary toxin composed of two distinct proteins: the protective antigen (PrAg) which is the cell binding and internalization moiety, and the lethal factor (LF) which is the catalytic moiety that exerts its toxic activity (Klimpel et al., 1994) (Kassab et al., 2013). PrAg (83 kDa) represents subunit "B", while LF (90 kDa) represents subunit "A", together making PrAg/LF (Friebe et al., 2016).

PrAg binds cells through ubiquitously expressed cell surface receptors, tumor endothelial marker-8 (TEM-8) and capillary morphogenesis gene-2 (CMG-2), together

referred to as anthrax toxin receptors (ANTXRs) (Bradley et al., 2001) (Liu et al., 2009) (Abi-Habib et al., 2006). Following its binding, PrAg is cleaved at the sequence ^{164}RKKR^{167} by cell surface furin-like proteases leading to the release of a 20 kDa fragment, and the production of an active 63kDa fragment ($PrAg_{63}$) (Gordon et al., 1995) (Escuyer et al.,1991) (Abi-Habib et al., 2006). The resulting 63 kDa PrAg fragments then assemble into heptamers, after which three molecules of LF will bind, and the whole complex will be internalized by receptor mediated endocytosis (Abi-Habib et al., 2006). In acidic endosomes, the PrAg complexes undergo a conformational change which leads to the formation of pores, allowing LF to reach the cytosol (Figure 3 and 4) (Melnyk & Collier, 2006). As such, the zinc metalloprotease LF cleaves and inactivates MEKs, subsequently leading to MAPK pathway inhibition which in turn causes growth inhibition and cell death (Figure 3) (Chopra et al., 2003) (Kassab et al., 2013).

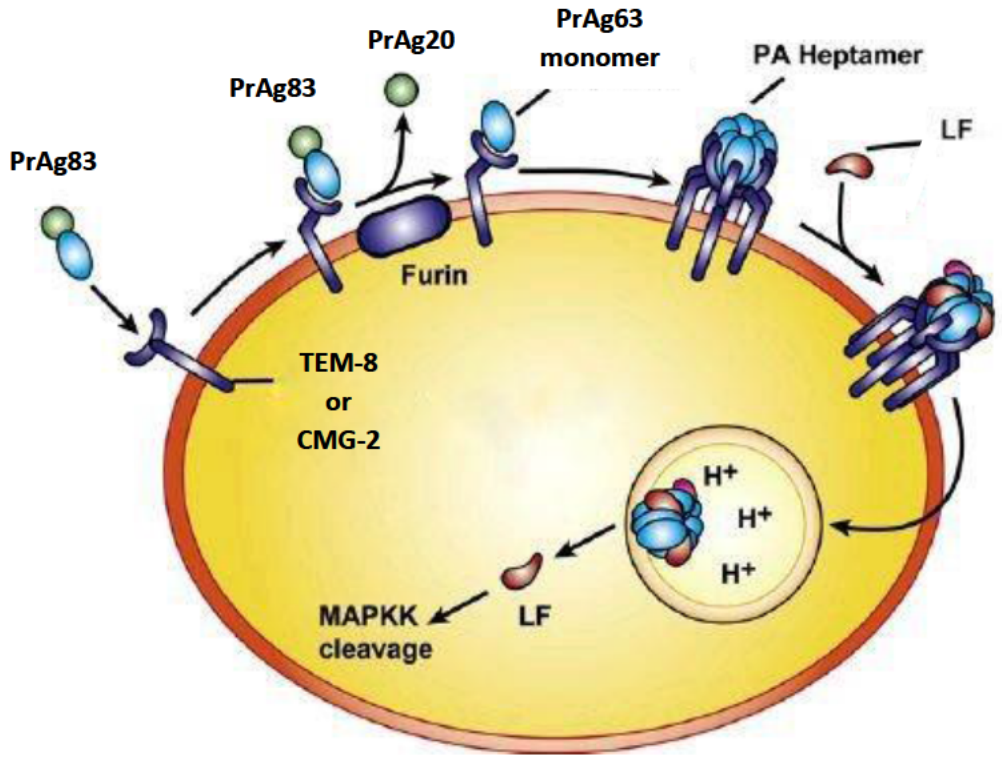


Figure 3:Mode of action of PrAg/LF. *PrAg binds the cell surface and is cleaved. Upon its heptamerization, LF binds, and the complex is internalized. (Modified from: (Sweeney et al., 2011)*

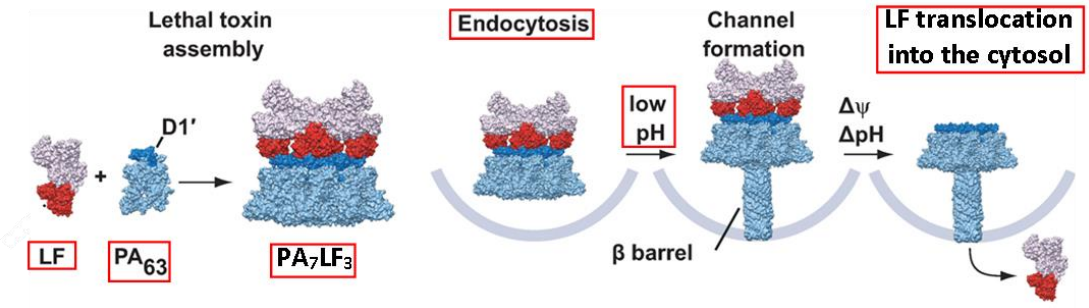


Figure 4: PrAg/LF binding, assembly and LF translocation into the cytosol. *(Outlined in red) (Modified from: Feld et al., 2010).*

1.4.2. FP59

FP59 is a fusion protein of LF residues 1–254 and the catalytic ADP-ribosylation domain *of Pseudomonas aeruginosa* exotoxin A (Liu et al., 2005). Anthrax Lethal Toxin, being a binary toxin, allows the possibility for each moiety to be taken on its own. PrAg/FP59 represents the fusion of PrAg, the binding domain of anthrax lethal toxin, with FP59 (Liu et al., 2001) (Abi-Habib et al., 2006) (Kassab et al., 2013).

PrAg/FP59 moves into the cytosol of the cell identically like PrAg/LF. However, FP59 does not affect the MAPK pathway, it instead leads to ADP-ribosylation of the elongation factor 2 (EF-2), which prevents protein synthesis and causes cell death. Therefore, when PrAg and FP59 are combined, all cells that express the anthrax toxin receptors experience different levels of cytotoxicity that is MAPK-independent, making it a more potent toxin with a wider range of toxicity (Abi-Habib et al., 2006) (Kassab et al., 2013).

1.4.3. Previous work done with PrAg/LF and PrAg/FP59

Given the significance of MEK signaling in tumorigenesis, PrAg/LF has demonstrated effectiveness as a potential targeted therapeutic in a number of human cancers, including first and foremost melanoma as well as acute myeloid leukemia (Abi-Habib et al., 2005) (Kassab et al., 2013). As such, it was shown *in vitro* that PrAg/LF is effectively cytotoxic to most melanoma cell lines that depend on the MAPK pathway for survival, and its specificity was demonstrated by showing that most normal cell types were not sensitive to PrAg/LF treatment, since they can survive the blockage of the MAPK pathway, with only a small minority showing cytotoxicity. Hence, PrAg/LF can be a useful therapeutic for melanoma patients (Abi-Habib et al., 2005). In addition, *in vivo* systemic PrAg/LF treatment of athymic nude mice harboring melanoma tumors, showed partial and complete regression with only minor toxicity to mice, demonstrating PrAg/LF to be a promising potent anti-melanoma agent (Abi-Habib et al., 2006).

Moreover, a study by Huang et al., demonstrated that PrAg/LF can suppress renal cell carcinoma tumor growth *in vivo*, as well as inducing necrosis and decreased tumor neovascularization, showing that it exhibits significant anti-tumor activity in renal cell carcinoma (Huang et al., 2008).

Another study showed that most AML cell lines are sensitive to the inhibition of the MAPK pathway by LF, demonstrating the ability of selectively targeting this pathway in these cells. Moreover, aside from cytotoxicity, PrAg/LF induces arrest of cell cycle in a subset of AML cell lines (Kassab et al., 2013). Furthermore, testing for annexin V/PI staining and for caspase activation demonstrated that the mechanism of death observed in sensitive AML cells is occurring via caspase-independent, non-apoptotic

mechanisms (Kassab et al., 2013). In this study, the control used, PrAg/FP59, showed cytotoxicity to all the tested AML cell lines, both PrAg/LF –sensitive and PrAg/LF – resistant, with a percent cell death >90% (Kassab et al., 2013)..

All in all, these studies show that PrAg/LF is an effective potential targeted therapeutic in a number of human cancers. It is highly cytotoxic to cells that depend on the MAPK pathway for growth and survival, making addiction to the MAPK pathway the first criteria for sensitive cells(Kassab et al., 2013). PrAg/LF targeted a limited number of normal tissues, showing signs of selectivity (Abi-Habib et al., 2005). However, some *in vivo* toxicity was demonstrated indicating that its tumor selectivity remains limited (Liu et al., 2009) since some normal cells are unable to survive the treatment (Abi-Habib et al., 2005). In the effort to increase the selectivity of this binary toxin towards cancer cells, and to eradicate its cytotoxicity on normal cell, variants of PrAg/LF were generated.

1.5. Anthrax Lethal Toxin (PrAg/LF) variants

1.5.1. PrAgU2 and PrAgL1

The first generation of more selective anthrax lethal toxin (PrAg/LF) variants are termed PrAgU2 and PrAgL1. The particular requirement for PrAg to be activated on the cell surface by furin-like proteases presents a chance to modify this protein to make its activation reliant on cleavage by proteases enriched on the surface of tumor cells and not normal cells, targeting the cytotoxicity towards tumor cells (Liu et al., 2005).

Tumors have been shown to overexpress extracellular proteases that are rarely present on normal cells under physiologic conditions (Abi-Habib et al., 2006). Two such tumor-specific surface proteases include the urokinase plasminogen activator (uPA)

and matrix metalloproteinases (MMPs), both of which increase the tissue invasiveness and the metastatic potential of cancer cells (Wein et al., 2015) (Abi-Habib et al., 2006).

Urokinase plasminogen activator (uPA) consists of uPA, which is a serine protease, and its uPA receptor (uPAR) that binds it, together forming the active uPA/uPAR complex, a potent protease system (Abi-Habib, Singh, Liu, et al., 2006).

Matrix metalloproteinases (MMPs), another tumor specific protease, constitute a family of zinc-dependent endopeptidases. Of these MMPs, MMP-2 (gelatinase A), MMP-9 (gelatinase B), and membrane-type 1 MMP (MT1-MMP) are found to be most related to invasion and metastasis in a variety of human cancers(Liu et al., 2000)

Taking advantage of these specific tumor-expressed proteases, two PrAg variants, PrAgU2 and PrAgL1 were generated.

In PrAgU2 and PrAgL1, the furin cleavage sequence of PrAg ^{164}RKKR^{167} was replaced by sequences susceptible to cleavage by either uPA/uPAR complex or MMPs, respectively (Liu et al., 2001) (Alfano et al., 2009) (Bekdash et al., 2015) (Liu et al., 2000). If combined with LF, PrAgU2/LF and PrAgL1/LF will bind all cells through ANTXRs, including normal cells, but PrAgU2/LF will only be activated on tumor cells overexpressing the uPA/uPAR system while PrAgL1/LF will be activated on tumor cells highly expressing MMPs. With normal cells lacking such tumor-specific proteases, these variants will not be activated, and no cytotoxicity would be manifested (Liu et al., 2003) (Abi-Habib et al., 2006)

As such, PrAgU2/LF and PrAgL1/LF are considered dual-selective toxins, where they target two distinct tumor-specific markers: the uPA/ uPAR system or MMPs expression as well as reliance on the MAPK pathway for survival (Bekdash et al., 2015) (Liu et al., 2000). Making them two variants of the anthrax lethal toxin with an

added layer of selectivity that encourages them to be preferentially activated on tumor cells.

1.5.2. Previous work done on PrAgU2 and PrAgL1

PrAgU2 and PrAgL1 combined with either LF or FP59 have shown selective targeting of several cancer cell lines.

PAU2/LF showed effective cytotoxicity against AML cells where five out of nine AML cell lines showed sensitivity to its treatment. These cells expressed uPAR along with significant levels of phosphorylated MEK1/2, demonstrating that an active uPA/uPAR system and the reliance on the MAPK pathway for survival are two fundamental requirements for the sensitivity of AML cells to PAU2/LF (Bekdash et al., 2015) . The mechanism of cell death showed to be nonapoptotic and was associated with MAPK dependence and uPA/uPAR activity since all cell lines that showed sensitivity demonstrated both significant levels of MEK1/2 phosphorylation and uPAR expression. PrAgU2/LF also induced arrest in the cell cycle of some AML cell lines. Moreover, cells that resisted PrAgU2/LF treatment showed expression of uPAR which indicates that their resistance is not due to their inability to activate it, but is a result of the absence of dependence on the MAPK pathway for survival in these cells (Bekdash et al., 2015).

PAU2/LF also showed high cytotoxicity when tested on primary blasts that were obtained from AML patients, where it was cytotoxic to the majority of AML blasts, including one that carried aberrant FLT3 (Bekdash et al., 2015).

PAU2/LF cytotoxicity was then tested on normal cells, such as normal peripheral mononuclear cells, which demonstrated to be sensitive to the cytotoxicity of PrAg/LF but showed no cytotoxicity with PAU2/LF, demonstrating that the added requirement

of activation by uPA/uPAR reduced the toxicity of PAU2/LF to normal cells, since they lack active uPA system on their cell surface, hence increasing its tumor selectivity (Bekdash et al., 2015).

In vivo safety testing of PrAgU2/LF in mice demonstrated that its maximal tolerated dose was several folds higher than that of the wild-type PrAg/LF, indicating its enhanced safety and tumor selectivity (Bekdash et al., 2015).

In addition, the combination of PAU2 with FP59 showed a high cytotoxic potency to a wide range of tumor cell lines, including non–small cell lung cancer, pancreatic cancer, and prostate cancer cell lines (Abi-Habib et al., 2006). Furthermore, Normal human cell types were several folds less sensitive to PAU2/FP59 than tumor cells, while maintaining the same sensitivity to the furin-activated PrAg/FP59, demonstrating the selectivity of PAU2/FP59 for tumor cells (Abi-Habib et al., 2006).

In vivo studies showed that the native PrAg/ FP59 was extremely toxic where mice became terminally ill with demonstrated widespread organ damage. In contrast, PrAgU2/FP59, displayed highly attenuated toxicity to mice and they displayed no outward or histological signs of toxicity (Liu et al., 2003).

Furthermore, PrAgL1 /FP59 treatment killed tumor cells while sparing normal nontumorigenic cells which demonstrated its enhanced selectivity (Liu et al., 2000).

All in all, this demonstrates that the PrAgU2 and PrAgL1 variants combined with either LF or FP59 maintain the potency observed with the wild type PrAg, while having an enhanced specificity towards cancer cells, adding another criteria and requirement for their activity.

1.5.3. The intermolecularly complemented (PAU2R200A-PAL1I207R) version of Anthrax Lethal Toxin

A novel and improved generation of Anthrax Lethal Toxin's PrAg that is immensely more selective is termed PAU2R200A/PAL1I207R (Wein et al., 2015). The previously discussed PrAgU2 version required activation by the uPA/uPAR tumor specific protease system (Liu et al., 2003), while the PrAgL1 version required activation by the tumor expressed MMPs (Liu et al., 2000). Expanding on these findings, a novel concept emerged by creating a reengineered and modified intermolecularly complementing PAU2R200A-PAL1I207R version of PrAg that necessitates activation by both the uPA/uPAR system and the MMPs simultaneously (Liu et al., 2005). This approach restricts the activation of this new version to cancerous cells, thereby improving its selectivity by mandating two distinct proteolytic activities overexpressed by tumor tissues for its activation and as such diminishing off-target cytotoxicity (Liu et al., 2005).

1.5.4. $PrAg_{63}$ heptamerization and LF binding

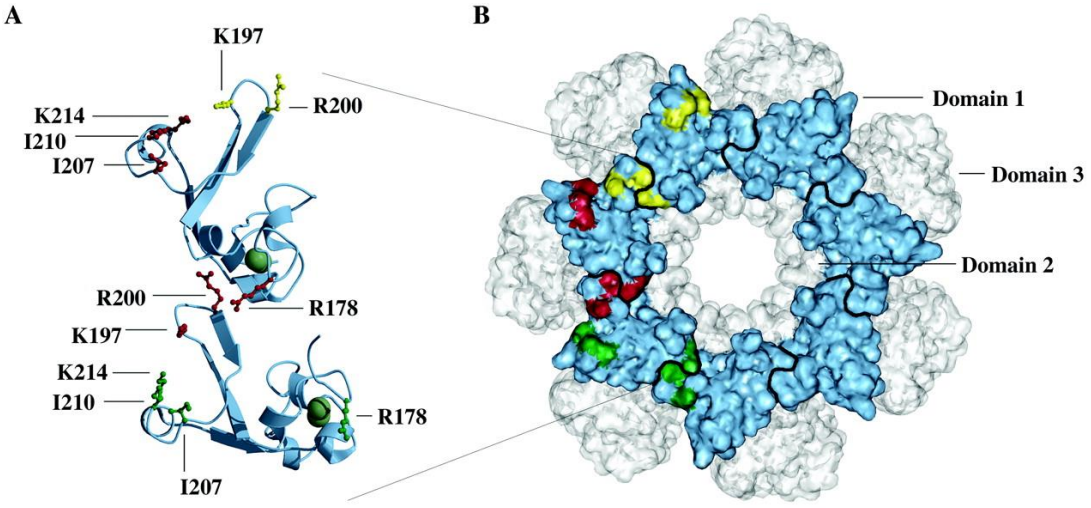


Figure 5: Ligand sites on heptameric PA63. *(A) The important subsites involved in LF binding. (B) The heptameric PA63 (Cunningham et al., 2002).*

As described previously, once PrAg binds its receptors, it is cleaved by cell-associated proteases. This subsequently dissociates the smaller 20-kDa fragment (PA20) into the medium, allowing the larger, 63-kDa fragment (PA63) to self-associate into a ring-shaped heptamer (Figure 3 and 6 A) (Klimpel et al., 1992). This heptamer can then bind up to three LF molecules, and the whole complex will be internalized by receptor mediated endocytosis (Abi-Habib et al., 2006).

Studies conducted recently have demonstrated that LF exclusively binds to the oligomeric ring of PA63 (Mogridge et al., 2002), and that its binding site spans two adjacent PrAg monomers (Liu et al., 2005). Through mutagenesis studies, specific amino acid residues of PrAg that play a role in LF binding have been identified and categorized into three subsites: subsite I (Arg178), subsite III (Ile207, Ile210, and Lys214) present in the clockwise (right-hand) subunit, and subsite II (Lys197 and Arg200) present in the counterclockwise (left-hand) subunit. (Figure 5 A and 5 B). (Cunningham et al., 2002). These subsites together comprise one functional binding site for LF as seen in Figure 6 A (Liu et al., 2005).

1.5.5. Mechanism of PAU2R200A-PAL1I207R intermolecular complementation

As previously discussed, three subsites of PrAg have been identified to be critical for LF binding. Hence mutations at these sites disrupt LF binding and result in diminished toxicity (Wein et al., 2015).

PAU2R200A-PAL1I207R intermolecular complementation is based on the fact that each LF-binding site is made by subsites from two adjacent PrAg monomers (Figure 6 A) (Liu et al., 2005).

PAU2R200A is a modified version of PrAgU2 that is activated by uPA/uPAR, but also harbors an additional mutation at Arg200 of subsite II where arginine is replaced by alanine. As seen in Figure 6 C, binding of PAU2R200A to cells expressing the uPA/uPAR protease system leads to the assembly of heptamers that contain the inactivating subsite II mutation in every LF binding site (Liu et al., 2005) (Schafer et al., 2011).

PAL1I207R requires activation by MMPs, but has an additional mutation at I207 of subsite III, where isoleucine is replaced by arginine and disrupts LF binding. As such, heptamers of PAL1I207R harbor disrupted subsite III in every LF binding site (Figure 6 B) (Liu et al., 2005) (Schafer et al., 2011).

Accordingly, heptamers consisting solely of PAU2R200A or PAL1I207R have impaired LF binding sites and are unable to bind and facilitate its transportation to the cytoplasm (Figure 6) (Schafer et al., 2011).

However, adding a mixture of PAU2R200A and PAL1I207R to cells with both uPA/uPAR system and MMPs, would generate randomly assembled heterogenous heptamers with functional LF binding sites due to intermolecular complementation between the subsites of PAU2R200A and PAL1I207R (Figure 6 D) (Figure 7) (Liu et al., 2005) (Schafer et al., 2011).

Therefore, PAU2R200A and PAL1I207R depend on their intermolecular complementation to create functional LF binding sites with all three active subsites (Wein et al., 2015). This process necessitates the presence of uPA/uPAR system and the MMPs simultaneously (Figure 7) (Liu et al., 2005). Thus, restricting and targeting the toxin activation to cancerous cells by demanding two distinct proteolytic activities

overexpressed on such cells, thereby achieving high tumor selectivity which will minimize the side effects that have limited previous treatments (Liu et al., 2005).

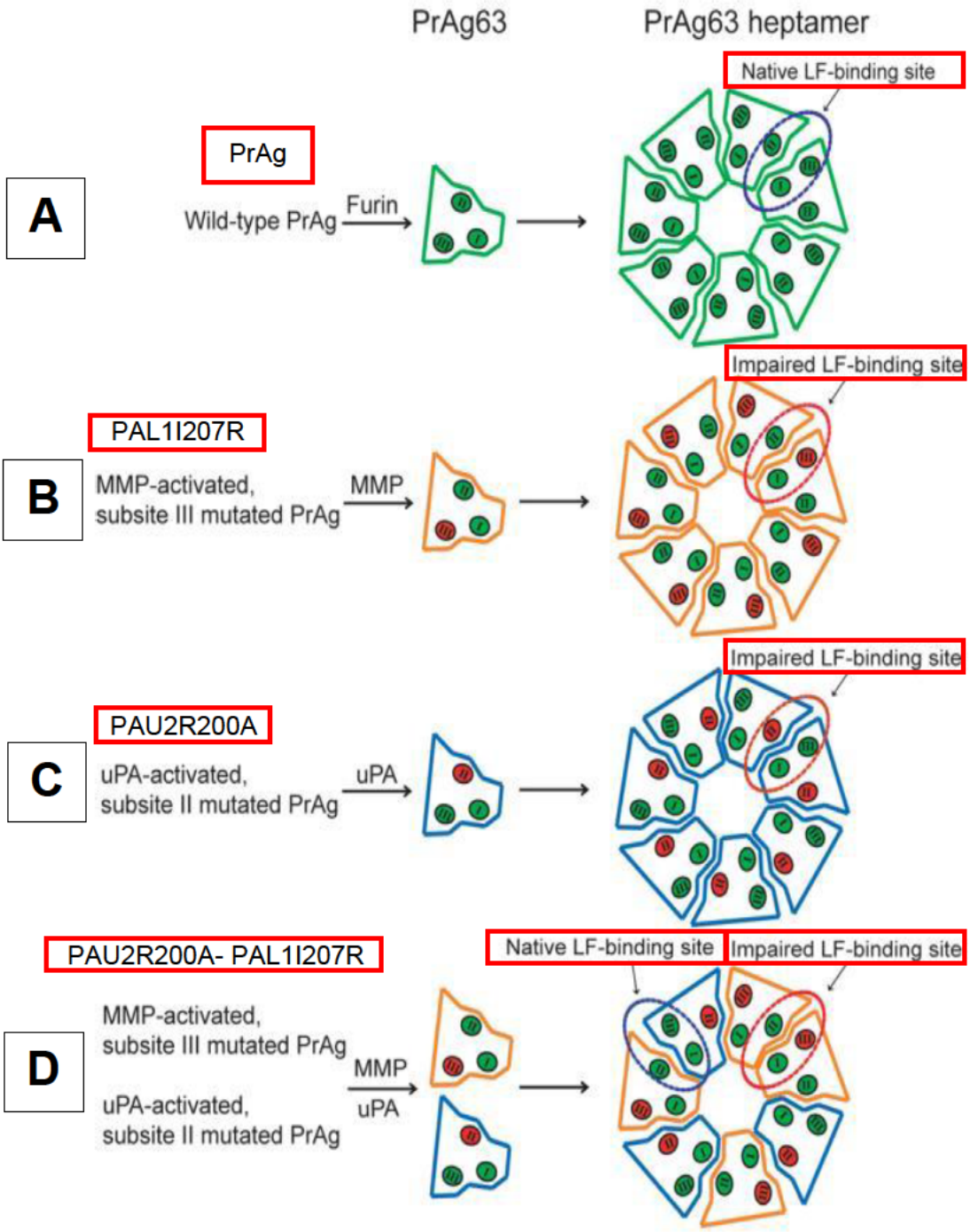


Figure 6: Schematic representation of heptamer formation and intermolecular complementation by mutated PrAg proteins. *Subsites I, II, and III are shown in green, while mutations in any of these subsites are shown in red (Outlined in red) (Modified from: Liu et al., 2005).*

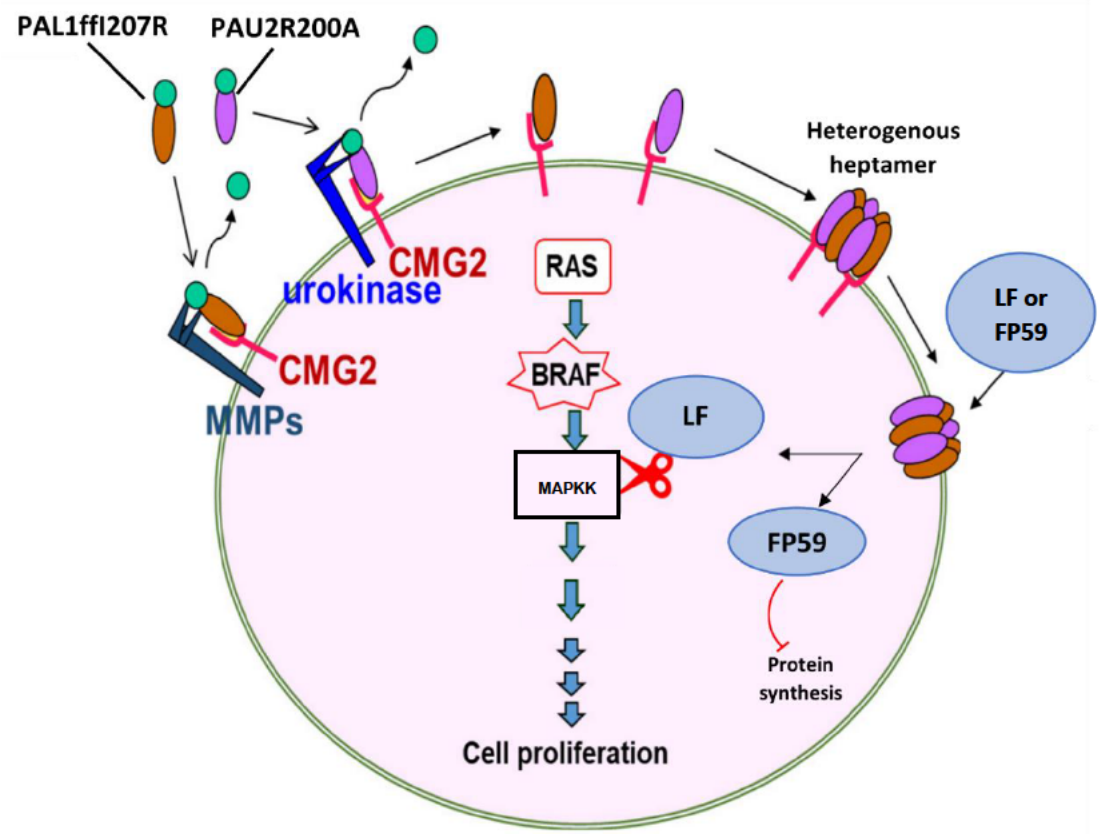


Figure 7: Mode of action of the reengineered PAU2R200A-PAL1I207R variant. *PrAg mutants will bind the cell surface and become activated by the uPA/uPAR system and the MMPs, simultaneously. Heterogenous heptamers form that can bind LF or FP59. (Modified from: Zuo et al., 2022)*

1.5.6. Triple versus dual selectivity of PAU2R200A-PAL1I207R with LF and FP59

A unique characteristic of this toxin is the multiple layers of selectivity that lead to preferential tumor targeting. Combining PAU2R200A-PAL1I207R with the catalytic partner LF, that cleaves and inactivates MEKs of the MAPK, results in the formation of a triple selective anthrax lethal toxin. This version requires three specific criteria for its activity that include: the cell surface uPA/uPAR system activity, cell surface MMPs activity, combined with selective toxicity to cells dependent on the MAPK pathway for survival (Figure 7) (Kassab et al., 2013) (Liu et al., 2005) . On the other hand, combining PAU2R200A-PAL1I207R with FP59, results in the formation of a dual selective toxin (Schafer et al., 2011). The selective activation of PAU2R200A-

PAL1I207R/FP59 necessitates no less than two criteria which are two distinct proteolytic activities overexpressed by tumor tissues: the uPA/uPAR system, and the MMPs. This is due to the ability of the catalytic moiety FP59 to prevents protein synthesis in the cells instead of cleaving MEKs of the MAPK pathway, and as such leading to cell death (Figure 7) (Schafer et al., 2011).

1.5.7. Previous work done on the intermolecularly complementing version of anthrax lethal toxin

1.5.7.1. Selection of R200A and I207R mutations

Mutations at subsites important for LF binding had to be created in a way to generate PrAg variants with activities that were strictly dependent on intermolecular complementation. Such mutations had to make the heptamers made by each variant on its own unable to bind LF, but heterogenous heptamers from both variants able to bind it (Wein et al., 2015).

In a study carried out by Cunningham et al., a PrAg variant library was purified and screened for its ability to bind LF. Alanine substitutions for residues R200, I207, I210 among others, caused 90% loss of LF binding. This showcased those substitutions in R200, I207, and I210 residues are deleterious (Cunningham et al., 2002).

In another study by Liu et al., heptamers of PrAg-200A alone or PrAg-I210A alone had significantly decreased LF binding as seen in Figure 8 (Liu et al., 2005). However, the application of PrAg-200A and PrAg-I210A together led to restoration of LF binding ability. Therefore, R200A and I210A mutations display intermolecular complementation in the formation of LF-binding PrAg heptamers (Liu et al., 2005).

The original versions of the intermolecular complementing PrAg had the subsite III PrAg-I210A substitution instead of PrAg-I207R (Liu et al., 2005). Afterwards, PrAg-I207R was found to behave better in complementing with PrAg-R200A, by displaying

no cytotoxicity towards cells when used singly with LF or FP59, which is not the case for PrAg-I210A (Wein et al., 2015). As such, PrAg-I207R was identified as an improved variant for intermolecularly complementing with PrAg-R200A (Wein et al., 2015).

Consequently, the R200A and I207R mutations of PrAg are selected as the candidates for intermolecular complementation, showing low LF binding and cytotoxicity when used solely, but on the other hand able to intermolecularly complement each other and lead to cytotoxicity (Liu et al., 2005) (Wein et al., 2015).

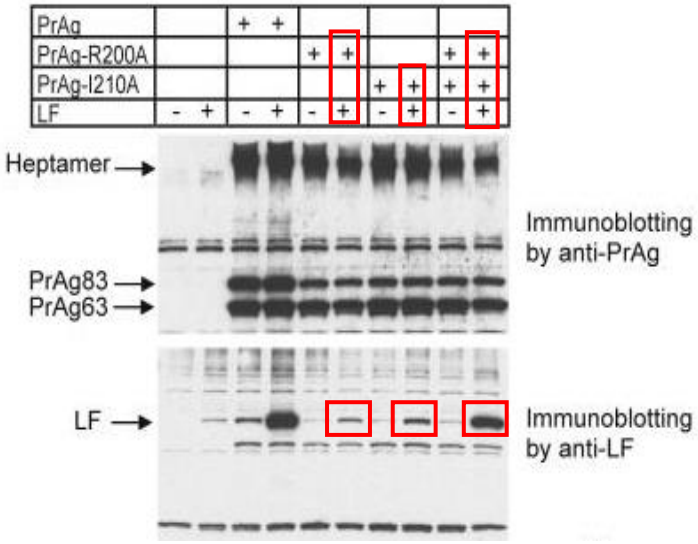


Figure 8: Intermolecular complementation and LF binding. *Heptamers of PrAg-200A alone or PrAg-I210A alone have low LF binding. Combining PrAg-200A and PrAg-I210A together restores the LF binding ability (Outlined in red) (Modified from: Liu et al., 2005).*

1.5.7.2. Potency and selectivity of the intermolecularly complementing version of anthrax lethal toxin

A study by Liu et al., highlighted the potency and the importance of intermolecular complementation for the tumoricidal activity of such PrAg variants where mice bearing intradermal tumor nodules were treated with PBS, PrAgU2R200A,

PrAgL1I210A, or a combination of both, all with FP59. The combination of PrAgU2-R200A and PrAg-L1-I210A significantly reduced tumor size, with a 94% reduction observed for B16-BL6 melanoma. In contrast, individual PrAgU2R200A or PrAgL1I210A had little or no effects on tumor size (Liu et al., 2005).

In another study, it was demonstrated that PAU2R200A-PAL1I210A/LF treatment efficiently treated mice with solid tumors, showing cases where 40% of the mice remained tumor-free for up to a year after treatment. These variants were well tolerated as only 2 out of 40 (5%) treated mice died during treatment. As such further emphasizing on the efficacy and safety of intermolecular complementation (Schafer et al., 2011).

Further investigation by Wein et al., compared the effectiveness of the new PAU2R200A-PAL1I207R combination and the original PAU2R200A-PAL1I210A combination in treatment of melanoma tumors in mice. Both combinations showed significant anti-tumor activity when coupled with LF, but PAU2R200A-PAL1I207R/LF was more effective and safer than the original combination, with only 10% mortality compared to 30% in the original combination, illustrating that the newer PAU2R200A-PAL1I207R intermolecular complementation is an improved, more potent and safer version (Wein et al., 2015).

1.6. The aim of this study

The story started with the unmodified wild type PrAg containing anthrax lethal toxin that showed a limited selectivity due to some toxicity towards normal cells, both *in vitro* and *in vivo*. Subsequently, the first generation of more selective PrAg of the anthrax lethal toxin was generated, termed PrAgU2 and PrAgL1. PrAgU2 demands activation by the uPA/uPAR tumor specific protease system, while the PrAgL1 requires activation by the tumor expressed MMPs, both of which are overexpressed on tumor cells. These variants proved to be novel strategies and as such paved the way for the construction of an intermolecularly complemented version of the anthrax lethal toxin that targets two distinct proteolytic activities overexpressed by tumor tissues, both the uPA/uPAR system and MMPs simultaneously.

Our initial objective in this study was to test the intermolecularly complemented form of PrAg conjugated to LF (PAU2R200A-PAL1I207R/LF), the triple selective version. However, it resulted in no cytotoxicity even on cell lines that were originally sensitive to PrAg/LF, the unmodified cytotoxic anthrax lethal toxin. This indicated that the cells may not have enough uPA/uPAR and MMPs simultaneous activity to sufficiently activate this form and translocate enough LF, hence we switched to conjugating PAU2R200A-PAL1I207R to FP59, which is a more potent version with a wider range of toxicity due to inhibition of protein synthesis in cells.

As such, in this study, we conduct the first ever worldwide testing of PAU2R200A-PAL1I207R/FP59, the dual selective intermolecularly complementing version combined with FP59, on AML cells.

We aim to test the potency of PAU2R200A-PAL1I207R/FP59 on 7 different AML cell lines while determining the IC_{50}. We also desire to investigate if this intermolecularly complemented version, aside from causing cytotoxicity, will induce cell cycle arrest in the AML cells, and if PAU2R200A-PAL1I207R/LF, even though it didn't show any clear cytotoxicity, will provoke a cytostatic effect by arresting the cell cycle. We as well aspire to dive deeper and reveal the mechanism of cell death mediated by this version, whether it is apoptotic or non-apoptotic. Finally, we seek to examine the basal levels of expression of uPAR, and MMPs (mainly MMP2 and MMP9) and to investigate if treatment with PAU2R200A-PAL1I207R conjugated to LF or FP59 affects their expression, in order to explore whether the sensitivity of AML cells depends on the levels of expression of uPAR and MMPs.

CHAPTER TWO

MATERIALS AND METHODS

2.1. Expression and Purification of PrAg, PAU2R200A, PAL1I207R, LF and FP59

Wild type PrAg, the intermolecularly complementing PAU2R200A and PAL1I207R, LF as well as FP59 were expressed and purified, in the laboratory of Stephen H. Leppla at the National Institute of Allergies and Infectious Diseases (NIAID) of the National Institutes of Health (NIH) in Bethesda, MD. , as previously described (Ramirez et al., 2002) (Liu et al., 2001).

2.2. Cell Lines and Cell Culture

A panel of 7 human AML cell lines was tested: ML1, ML2, MM6, U937, TF1-vSrc, TF1-vRaf, and TF1-HaRas. Cells were obtained from the American Type Culture Collection (ATCC) and cultured in RPMI 1640 (Sigma-Aldrich) supplemented with 10% Fetal Bovine Serum (heat inactivated, Sigma- Aldrich) and 1% Penicillin/Streptomycin (Biowest). The cultured cells were incubated at 37°C and 5% CO_2 in a humidified chamber.

2.3. Proliferation Inhibition Assay (Cytotoxicity)

The potency of PAU2R200A-PAL1I207R conjugated to LF or FP59 on AML cells was determined using a proliferation inhibition assay. PrAg/FP59 was used as a control for catalytic domain entry into the cytosol of AML cells. Briefly, aliquots of 10^4 cells/well in 100 µl of cell culture medium, containing a fixed concentration of 10^{-9} M FP59 or LF, were plated into two flat-bottom 96-well plates (Corning Inc.). 50 µl of either PrAg, PAU2R200A only, PAL1I207R only, or PAU2R200A-PAL1I207R combined, in media were added to each column to yield concentrations ranging from 10^{-8} to 10^{-13} M. Triplicates of each condition were made. Cells were then incubated for 24, 48, 72, 96, and 120 hours at 37ºC and 5% CO_2. At the end of each time point, the viability of cells was assessed using fluorescence after adding 30 µl of Cell Titer Blue (Promega) to each of the 96 wells. After incubating the plates for 4 hours, fluorescence at 590 nm was measured using a Varioskan Flash plate reader (Thermo Fisher Scientific, Waltham, MA). Nominal fluorescence and percent maximal fluorescence were plotted against the log of concentration and a non-linear regression with a variable slope sigmoidal dose-response curve was generated along with inhibitory concentration 50 (IC_{50}) using GraphPad Prism 5 software (GraphPad Software, San Diego, CA).

2.4. Cell cycle analysis

The effect of PAU2R200A-PAL1I207R in conjugation with LF or FP59 on the cell cycle of AML cells was determined using propidium iodide (PI) staining on flow cytometry. Briefly, $1x10^6$ cells/well were plated in flat-bottom 6-well plate (Corning Inc.) in 2 mL of cell culture medium. The cells were treated with either media alone (control cells), PrAg coupled with LF or FP59, PAU2R200A-PAL1I207R coupled with LF or FP59. PrAg and PAU2R200A-PAL1I207R were used with a concentration of 10^{-8} M, while LF and FP59 had a concentration of 10^{-9} M. The cells were then incubated at 37°C and 5% CO_2 for 24, 48, and 72 hours. Afterwards, they were harvested and fixed in 70% ethanol for a minimum of 24 hours, at −20°C. Cells were then incubated in 500 µl of PI staining solution (Sigma-Aldrich, 50 µg/ml), supplemented with RNase (Roche,100 µg/ml), for 40 minutes at 37°C in the dark. Samples were then read on a C6 flow cytometer (BD Accuri, Ann Arbor, MI) and total cell DNA content was measured on FL2-A. The target cell population was identified using width versus forward scatter gating. The percentage of cells in different phases (G0/G1, S, G2/M), as well as the percentage of cells in the pre-G0/G1 phase (dead cells), was determined for both control cells and cells treated with the three different conditions.

2.5. Mechanism of cell death analysis

Determination of apoptotic versus nonapoptotic cell death was carried out using an Annexin V–FITC and Propidium iodide (PI)-labeled apoptosis/ necrosis detection kit (Abcam, Cambridge, MA, ab14085) on flow cytometry. Briefly, $1x10^6$ cells/well were plated in flat-bottom 6-well plate (Corning Inc.) in 2 mL of cell culture medium. The cells were treated with either: medium alone (control cells), PrAg/FP59, or PAU2R200A-PAL1I207R/FP59. PrAg and PAU2R200A-PAL1I207R were utilized

with a concentration of 10^{-8} M, while FP59 was used at a 20 pM concentration for all time points, and at the highest concentration of 10^{-9} M for some time points. After incubating for 24, 48 and 72 hours at 37°C and 5% CO_2, the cells were then harvested and re-suspended in 500µl binding buffer, then incubated with a FITC-conjugated annexin V (2.5 mg/ml) and PI (5 mg/ml) for 5 mins in the dark. Following that, cells were then read using a C6 flow cytometer (BD Accuri, Ann Arbor, MI). Annexin V/PI data were analyzed on FL1-H versus FL2-H scatter plot and the cells were gated on width versus forward scatter. For cells to be considered apoptotic, they had to show positive annexin V staining and negative PI staining, whereas cells positive for both annexin V and PI staining were considered non-apoptotic.

2.6. Antibodies

Rabbit monoclonal anti- uPAR (12713S), Rabbit monoclonal anti-MMP-2 (40994S), Rabbit monoclonal anti-MMP-9 (13667S), and Rabbit monoclonal anti-β -actin (4970S) were all acquired from Cell Signaling Technology (USA). Anti-Rabbit IgG (H+L), HRP Conjugate secondary antibodies (W401B) were obtained from Promega (Promega, Madison, WI, USA).

2.7. Protein expression Analysis

2.7.1. Protein extraction and quantification

$1x10^6$ cells/well were plated in flat-bottom 6-well plate (Corning Inc.) in 2 mL of cell culture medium. Cells were treated with either: medium alone (control cells), or FP59 (20 pM) coupled with PrAg (10^{-8} M) or PAU2R200A/PAL1I207R (10^{-8} M). Cells were then harvested at three time points 24, 48, and 72 hours. Following that, cells were lysed on ice in 1x RIPA buffer (Millipore, RIPA buffer) supplemented with 1x

protease inhibitor cocktail (Sigma-Aldrich). The supernatant was collected, boiled at 95 °C for 5 mins, and the protein concentration of lysates was measured using DC protein assay (500–0112; Bio-Rad Laboratories).

2.7.2. Western blot

Protein samples were separated by 10 % SDS-PAGE gels with equal amounts of proteins (20–50 µg/well), alongside 7-10 µl of Precision Plus Kaleidoscope Prestained Protein Standards (Bio-Rad) in the first well. Proteins were then transferred to PVDF membranes (Bio-Rad), that were then blocked with 5% Bovine Serum Albumin (BSA) (Sigma-Aldrich #A2153) for 1 hour at room temperature. Afterwards, the membranes were incubated overnight at 4°C or for 2 hours at room temperature accompanied by gentle shaking with primary Rabbit monoclonal antibodies against uPAR (1:1000), MMP2 (1:1000), MMP9 (1:1000), or β-actin (1:1000) (Cell Signaling Technology).Following that, the membranes were subjected to serial washes and subsequently incubated with Anti-Rabbit IgG HRP Conjugate secondary antibodies (1:2500) (Promega) for 1 hour at room temperature accompanied by gentle shaking. After serially washing the membranes, the bands were visualized by treating with enhanced chemiluminescence western-blotting detection reagents (ECL, Bio-Rad). Development was done using Bio-Rad Molecular Imager® (ChemiDoc™XRS+) Imaging System. The levels of protein expression were compared by densitometry using the ImageJ software.

CHAPTER THREE

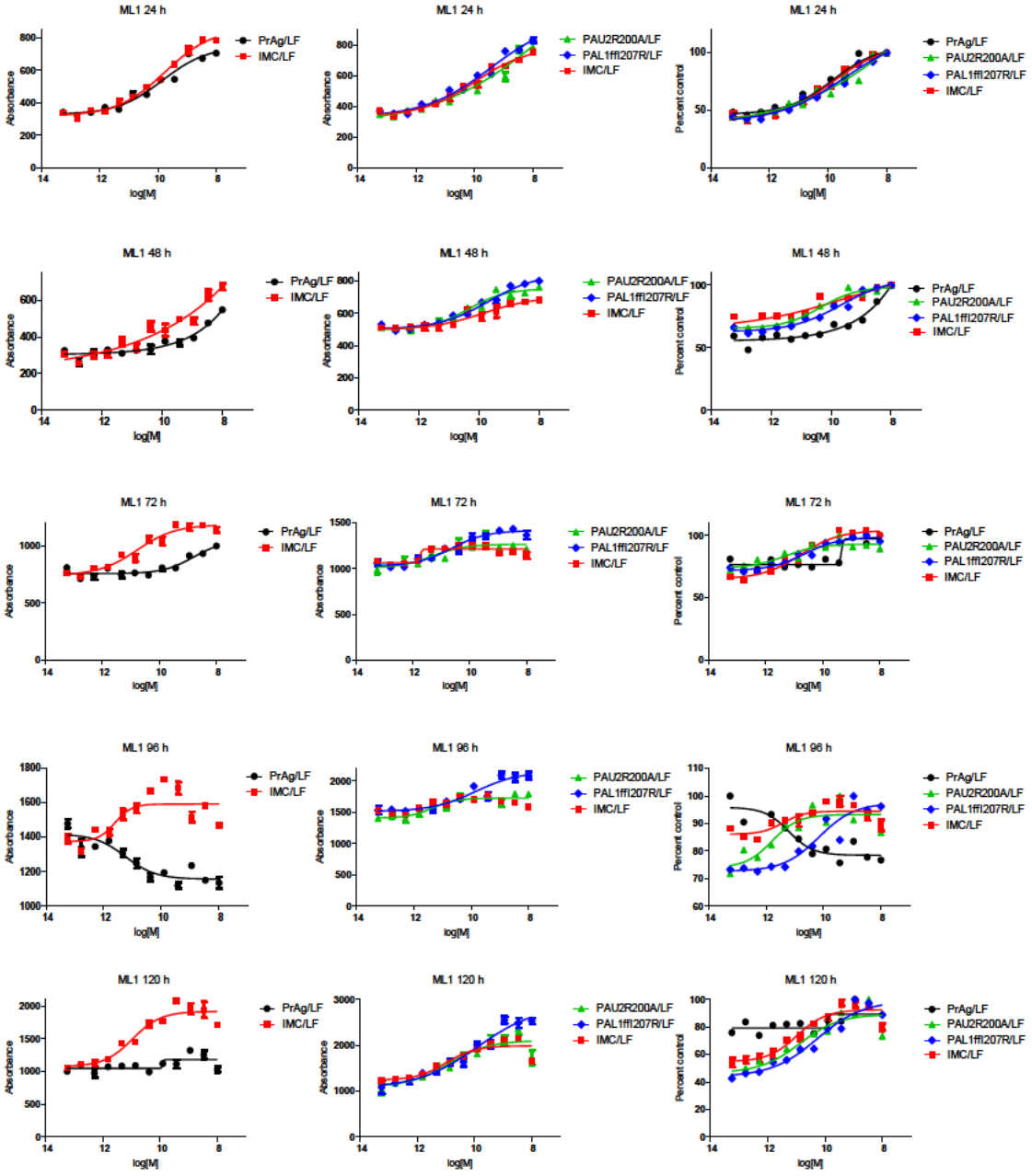
RESULTS

3.1. Cytotoxicity of the intermolecularly complementing PAU2R200A-PAL1I207R/FP59 on AML cells

We first started testing the cytotoxicity of the triple selective intermolecularly complementing version (IMC), PAU2R200A- PAL1I207R combined with LF, on ML1 and ML2 AML cell lines, while using PrAg/LF as a positive control for toxin translocation into the cytosol.

IMC/LF showed no signs of cytotoxicity on any of the cell lines, even at the highest concentration used, while PrAg/LF was cytotoxic especially at later time points (Figures 9 A and B). This suggests that the cells may not have sufficient simultaneous activity levels of uPA/uPAR and MMPs to abundantly activate IMC/LF and translocate enough LF into the cytosol to inhibit the MAPK and cause cytotoxicity.

A


ML1 24 h
ML1 48 h
ML1 72 h
ML1 96 h
ML1 120 h
Absorbance
Percent control
log[M]
PrAg/LF
IMC/LF
PAU2R200A/LF
PAL1ffl207R/LF

B

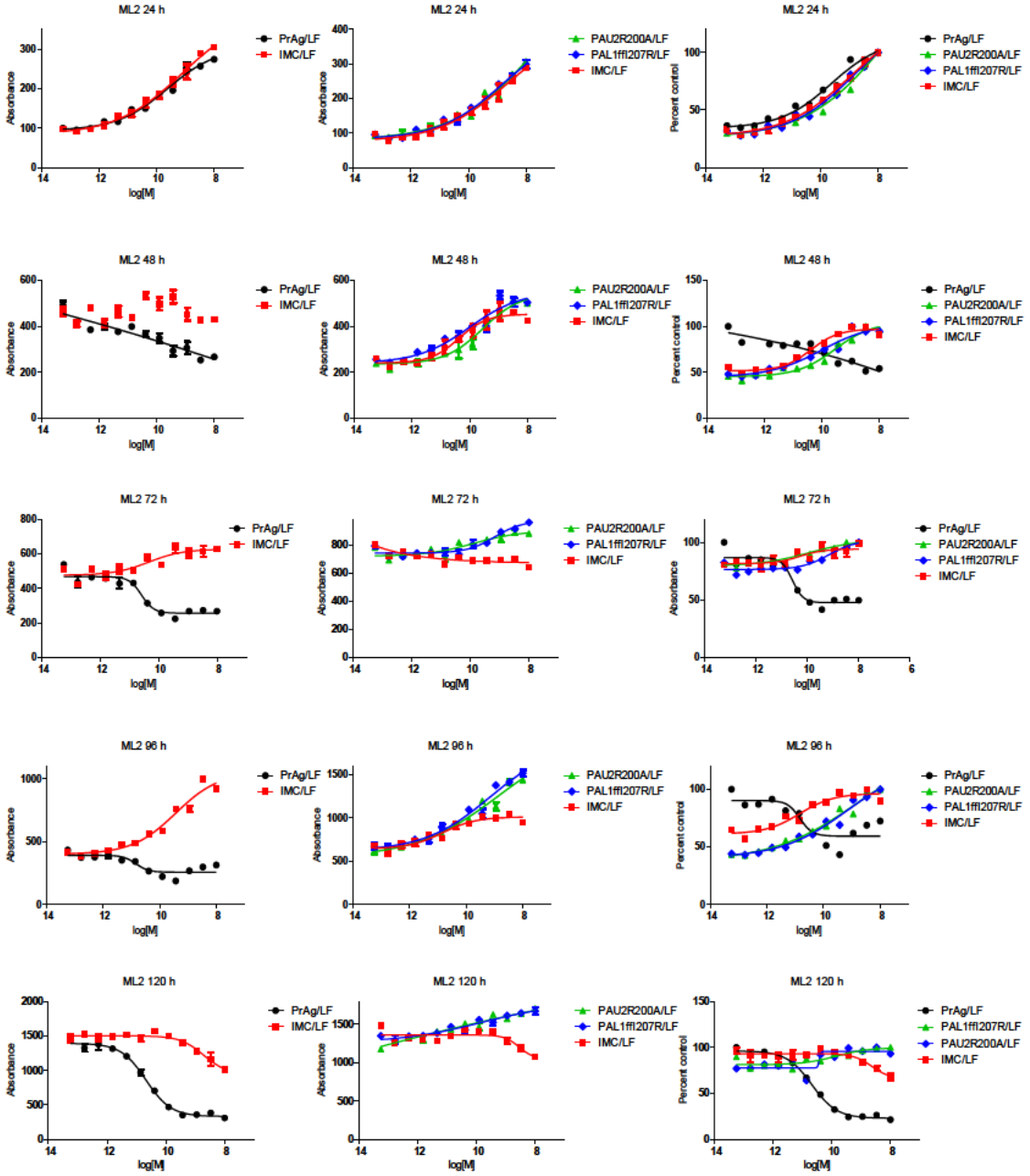


Figure 9: Cytotoxicity of the PrAg variants in combination with LF to ML1 and ML2. *(A and B) Non-Linear regression curves showing the cytotoxicity of PrAg/LF (●), IMC/LF(■), PAU2R200A/LF alone(▲), and PAL1I207R/LF alone(◆) on ML1and ML2 acute myeloid leukemia cell line. The viability of the cells was assessed at 24 h, 48 h, 72 h, 96 h, and 120 h. X axis represents the log of the molar protective antigen versions concentration (ranging from 10^{-8} to 10^{-13} M); Y axis indicates the cell viability expressed as absorbance and as percent control. LF was used at a fixed concentration of 10^{-9} M. No sensitive response towards treatment with IMC/LF or each of the mutants alone was demonstrated.*

As a result of IMC/LF having no clear cytotoxic effect, we opted to conjugate the IMC version to FP59, which has a wider range of toxicity and is more potent due to the inhibition of protein synthesis in cells.

We tested the cytotoxicity of PAU2R200A- PAL1I207R/FP59 (IMC/FP59) on a panel of 7 AML cell lines ML1, ML2, MM6, U937, TF1-vSrc, TF1-vRaf, and TF1-HaRas using a Cell Titer Blue proliferation inhibition assay. PrAg/FP59 was used as a positive control for cell sensitivity and the cells were tested against either IMC/FP59, PAU2R200A/FP59 alone or PAL1I207R/FP59 alone at 5 different time points 24 h, 48 h, 72 h, 96 h, and 120 h.

Four groups of cell lines with different types of responses were identified. First, the highly sensitive cell lines, ML1 and MM6, with IMC/FP59 IC_{50} values ranging from 23.5 pM to 287.7 pM and 20.6 pM to 68.0 pM, respectively (Table 1). These cell lines showed sensitivity to IMC/FP59 even at 24 h time points, with similar responses to the positive control PrAg/FP59. PAU2R200A/FP59 alone showed cytotoxicity that is comparable to IMC/FP59 with IC_{50} values ranging from 20.3 pM to 483.0 pM for both ML1 and MM6, while PAL1I207R/FP59 had no effect (IC_{50}:>10,000 pM) (Figures 10 A and B, Table 1).

Second, the moderately sensitive cell lines, ML2 and U937, showed a medium level of sensitivity to IMC/FP59 with IC_{50} values ranging from 166.2 pM to 770.9 pM and 171.0 pM to 1034.6 pM, respectively (Table 1). Treatment with PAU2R200A/FP59 alone showed a minor response at higher time points (IC_{50}:>10,000 pM), while PAL1I207R/FP59 had no effect (IC_{50}:>10,000 pM) (Figures 10 C and D, Table 1).

Third, the mildly sensitive cell line, but only at later time points (72 h, 96 h ,120 h), is TF1-vSrc as seen in Figure 10 E. IC_{50} values for IMC/FP59 ranged from 290.8 pM to

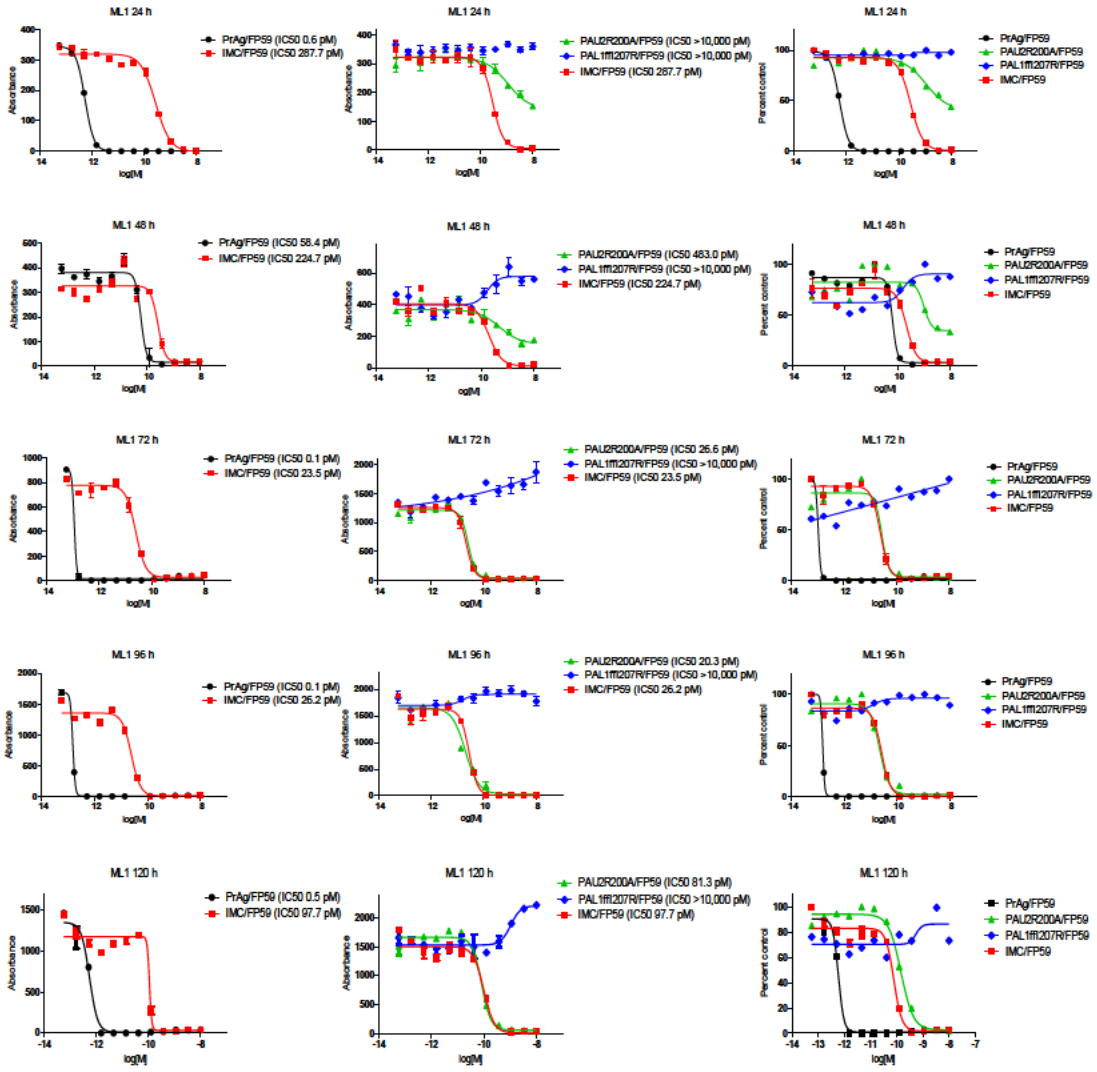
10,000 pM, indicating a mild response (Table 1), on the other hand both treatments with PAU2R200A/FP59 alone or PAL1I207R/FP59 alone had no effect with IC_{50} values > 10,000 pM at all time points (Figure 10 E, Table 1).

Finally, we identified TF1-vRaf and TF1-HaRas to be resistant to the treatment of IMC/FP59 (IC_{50}>10,000 pM), with TF1-vRaf even showing a minor sensitivity to PrAg/FP59. Neither treatments with PAU2R200A/FP59 alone nor PAL1I207R/FP59 alone showed any effect with IC_{50} values exceeding 10,000 pM (Figures 10 F and G, Table 1).

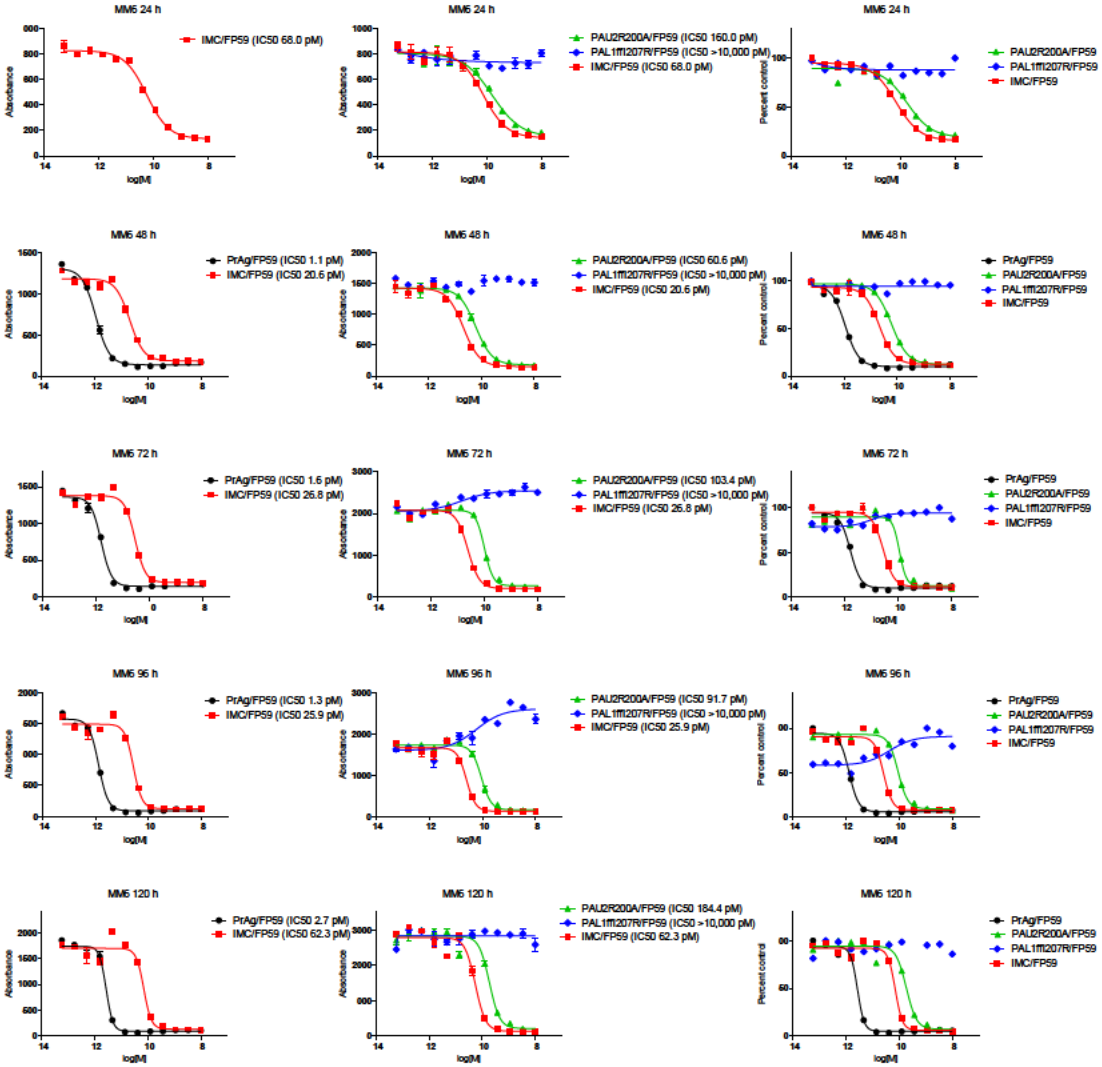
A pattern can be identified in these different responses, whereby the IMC/FP59 highly sensitive cell lines demonstrated similar sensitivity to the treatment of PAU2R200A/FP59 alone, the moderately sensitive cell lines showed a minor response to PAU2R200A/FP59 alone, while the resistant cell lines had no response to the treatment of PAU2R200A/FP59 alone. Treatments of PAL1I207R/FP59 alone had no effect in all these cases. As such, this highlights that the response to IMC/FP59 matches the sensitivity of cells to PAU2R200A/FP59 alone, which may indicate that the rate limiting factor in the activation of the IMC/FP59 in cells is the uPA/uPAR protease system.

These results demonstrate that two cell lines are highly sensitive to IMC/FP59 treatment (ML1 and MM6), two other cell lines are moderately sensitive (ML2 and U937), one is mildly sensitive but only at later time points (TF1-vSrc), and two cell lines are resistant to the IMC/FP59 treatment (TF1-vRaf and TF1-HaRas). These levels of sensitivity depend on whether the cells are sensitive to PAU2R200A/FP59 alone, indicating that the uPA/uPAR system might be the rate limiting factor in the activation of the intermolecularly complementing PAU2R200A- PAL1I207R/FP59 version (IMC/FP59).

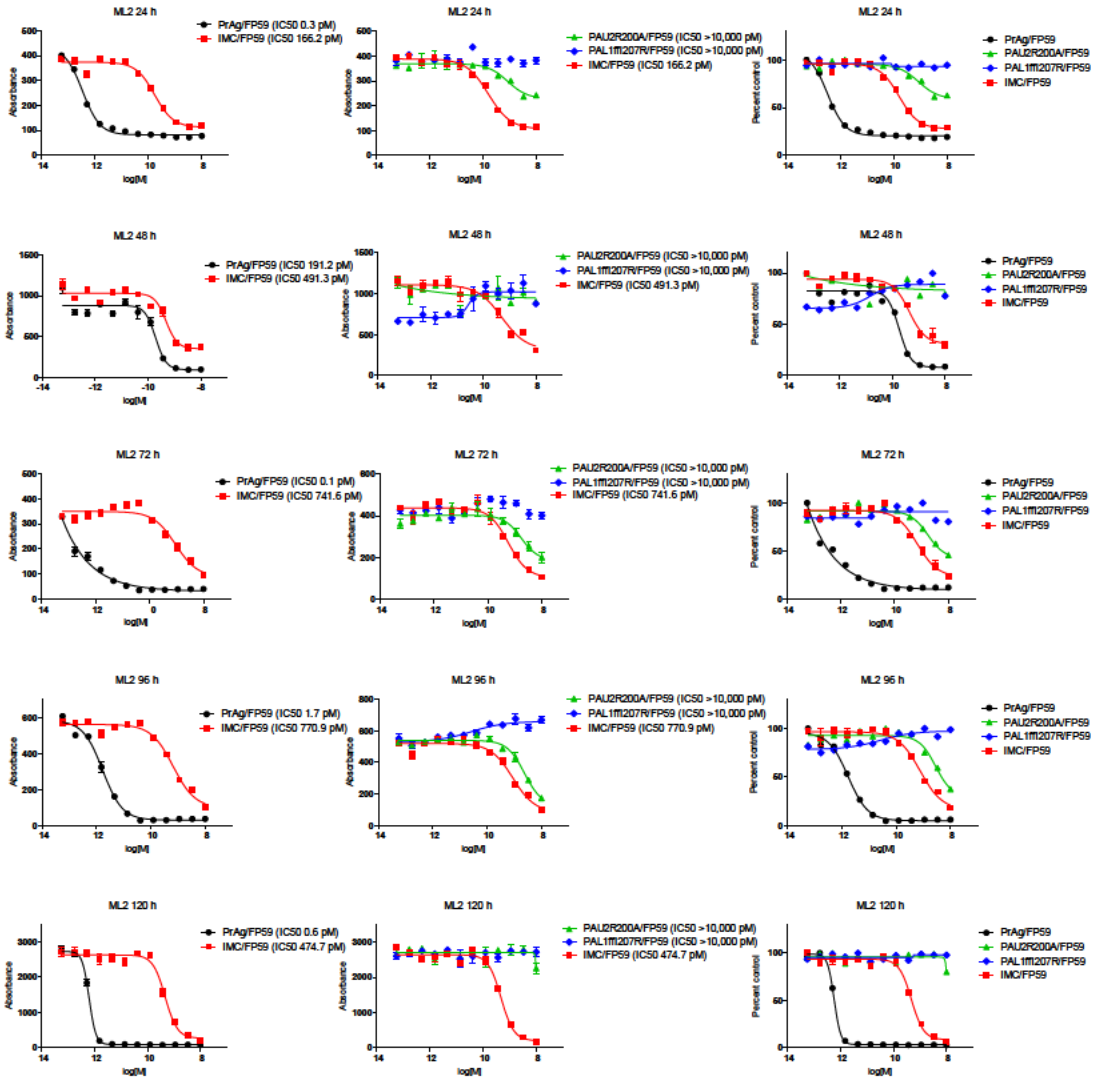
A


ML1 24 h
PrAg/FP59 (IC50 0.6 pM)
IMC/FP59 (IC50 287.7 pM)
PAL2R200A/FP59 (IC50 >10,000 pM)
PAL1ff1207R/FP59 (IC50 >10,000 pM)
IMC/FP59 (IC50 287.7 pM)
PrAg/FP59
PAL2R200A/FP59
PAL1ff1207R/FP59
IMC/FP59
ML1 48 h
PrAg/FP59 (IC50 58.4 pM)
IMC/FP59 (IC50 224.7 pM)
PAL2R200A/FP59 (IC50 483.0 pM)
PAL1ff1207R/FP59 (IC50 >10,000 pM)
IMC/FP59 (IC50 224.7 pM)
ML1 72 h
PrAg/FP59 (IC50 0.1 pM)
IMC/FP59 (IC50 23.5 pM)
PAL2R200A/FP59 (IC50 26.6 pM)
PAL1ff1207R/FP59 (IC50 >10,000 pM)
IMC/FP59 (IC50 23.5 pM)
ML1 96 h
PrAg/FP59 (IC50 0.1 pM)
IMC/FP59 (IC50 26.2 pM)
PAL2R200A/FP59 (IC50 20.3 pM)
PAL1ff1207R/FP59 (IC50 >10,000 pM)
IMC/FP59 (IC50 26.2 pM)
ML1 120 h
PrAg/FP59 (IC50 0.5 pM)
IMC/FP59 (IC50 97.7 pM)
PAL2R200A/FP59 (IC50 81.3 pM)
PAL1ff1207R/FP59 (IC50 >10,000 pM)
IMC/FP59 (IC50 97.7 pM)
Absorbance
Percent control
log[M]

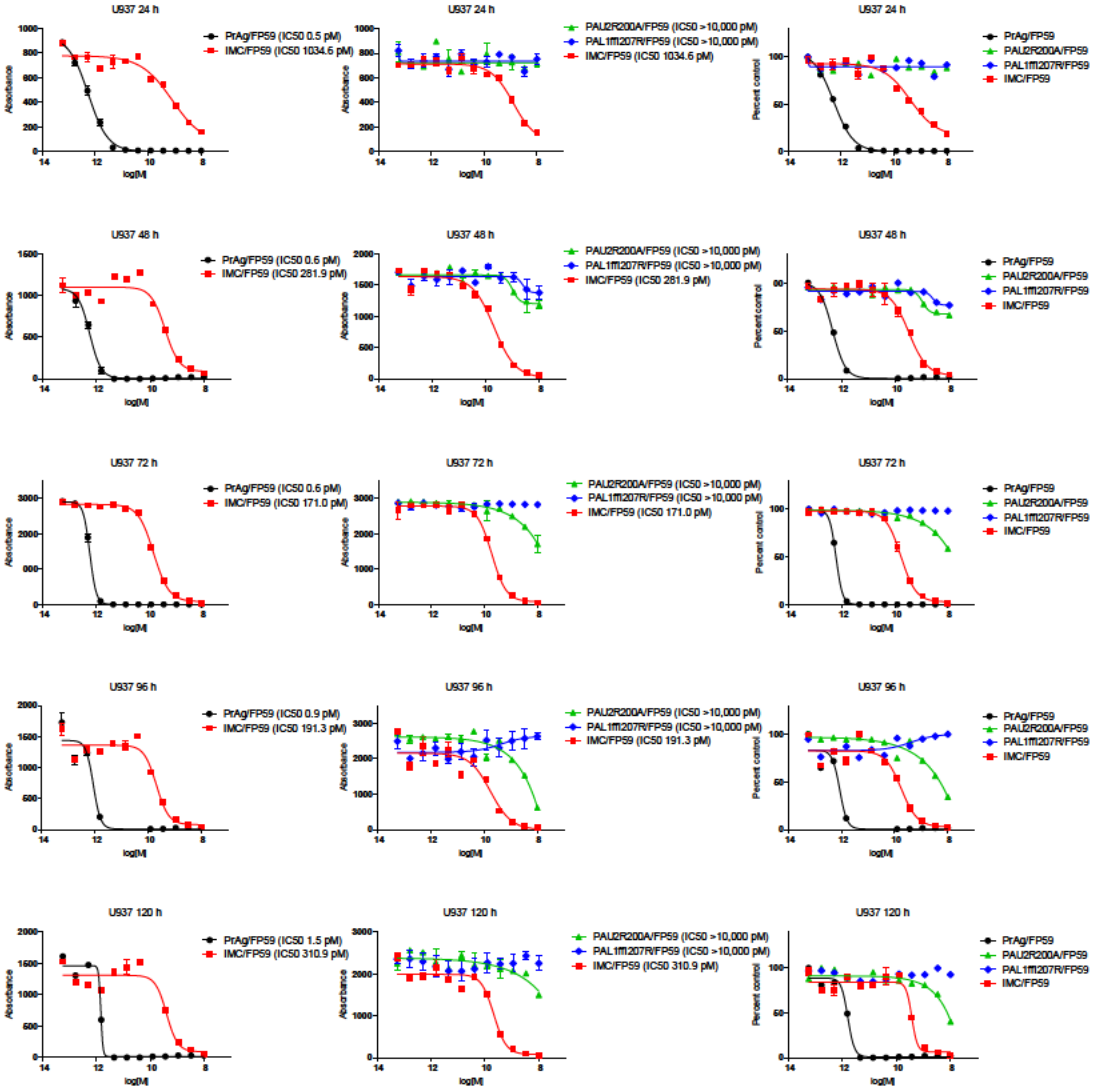
B


MM6 24 h
IMC/FP59 (IC50 68.0 pM)
PAL2R200A/FP59 (IC50 160.0 pM)
PAL1ff1207R/FP59 (IC50 >10,000 pM)
IMC/FP59 (IC50 68.0 pM)
PAL2R200A/FP59
PAL1ff1207R/FP59
IMC/FP59
MM6 48 h
PrAg/FP59 (IC50 1.1 pM)
IMC/FP59 (IC50 20.6 pM)
PAL2R200A/FP59 (IC50 60.6 pM)
PAL1ff1207R/FP59 (IC50 >10,000 pM)
IMC/FP59 (IC50 20.6 pM)
PrAg/FP59
PAL2R200A/FP59
PAL1ff1207R/FP59
IMC/FP59
MM6 72 h
PrAg/FP59 (IC50 1.6 pM)
IMC/FP59 (IC50 26.8 pM)
PAL2R200A/FP59 (IC50 103.4 pM)
PAL1ff1207R/FP59 (IC50 >10,000 pM)
IMC/FP59 (IC50 26.8 pM)
PrAg/FP59
PAL2R200A/FP59
PAL1ff1207R/FP59
IMC/FP59
MM6 96 h
PrAg/FP59 (IC50 1.3 pM)
IMC/FP59 (IC50 25.9 pM)
PAL2R200A/FP59 (IC50 91.7 pM)
PAL1ff1207R/FP59 (IC50 >10,000 pM)
IMC/FP59 (IC50 25.9 pM)
PrAg/FP59
PAL2R200A/FP59
PAL1ff1207R/FP59
IMC/FP59
MM6 120 h
PrAg/FP59 (IC50 2.7 pM)
IMC/FP59 (IC50 62.3 pM)
PAL2R200A/FP59 (IC50 184.4 pM)
PAL1ff1207R/FP59 (IC50 >10,000 pM)
IMC/FP59 (IC50 62.3 pM)
PrAg/FP59
PAL2R200A/FP59
PAL1ff1207R/FP59
IMC/FP59
Absorbance
Percent control
log[M]

C


ML2 24 h
PrAg/FP59 (IC50 0.3 pM)
IMC/FP59 (IC50 166.2 pM)
PALI2R200A/FP59 (IC50 >10,000 pM)
PAL1ffI207R/FP59 (IC50 >10,000 pM)
IMC/FP59 (IC50 166.2 pM)
PrAg/FP59
PALI2R200A/FP59
PAL1ffI207R/FP59
IMC/FP59
ML2 48 h
PrAg/FP59 (IC50 191.2 pM)
IMC/FP59 (IC50 491.3 pM)
PALI2R200A/FP59 (IC50 >10,000 pM)
PAL1ffI207R/FP59 (IC50 >10,000 pM)
IMC/FP59 (IC50 491.3 pM)
ML2 72 h
PrAg/FP59 (IC50 0.1 pM)
IMC/FP59 (IC50 741.6 pM)
PALI2R200A/FP59 (IC50 >10,000 pM)
PAL1ffI207R/FP59 (IC50 >10,000 pM)
IMC/FP59 (IC50 741.6 pM)
ML2 96 h
PrAg/FP59 (IC50 1.7 pM)
IMC/FP59 (IC50 770.9 pM)
PALI2R200A/FP59 (IC50 >10,000 pM)
PAL1ffI207R/FP59 (IC50 >10,000 pM)
IMC/FP59 (IC50 770.9 pM)
ML2 120 h
PrAg/FP59 (IC50 0.6 pM)
IMC/FP59 (IC50 474.7 pM)
PALI2R200A/FP59 (IC50 >10,000 pM)
PAL1ffI207R/FP59 (IC50 >10,000 pM)
IMC/FP59 (IC50 474.7 pM)
Absorbance
Percent control
log[M]

D


U937 24 h
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Absorbance
log[M]
U937 24 h
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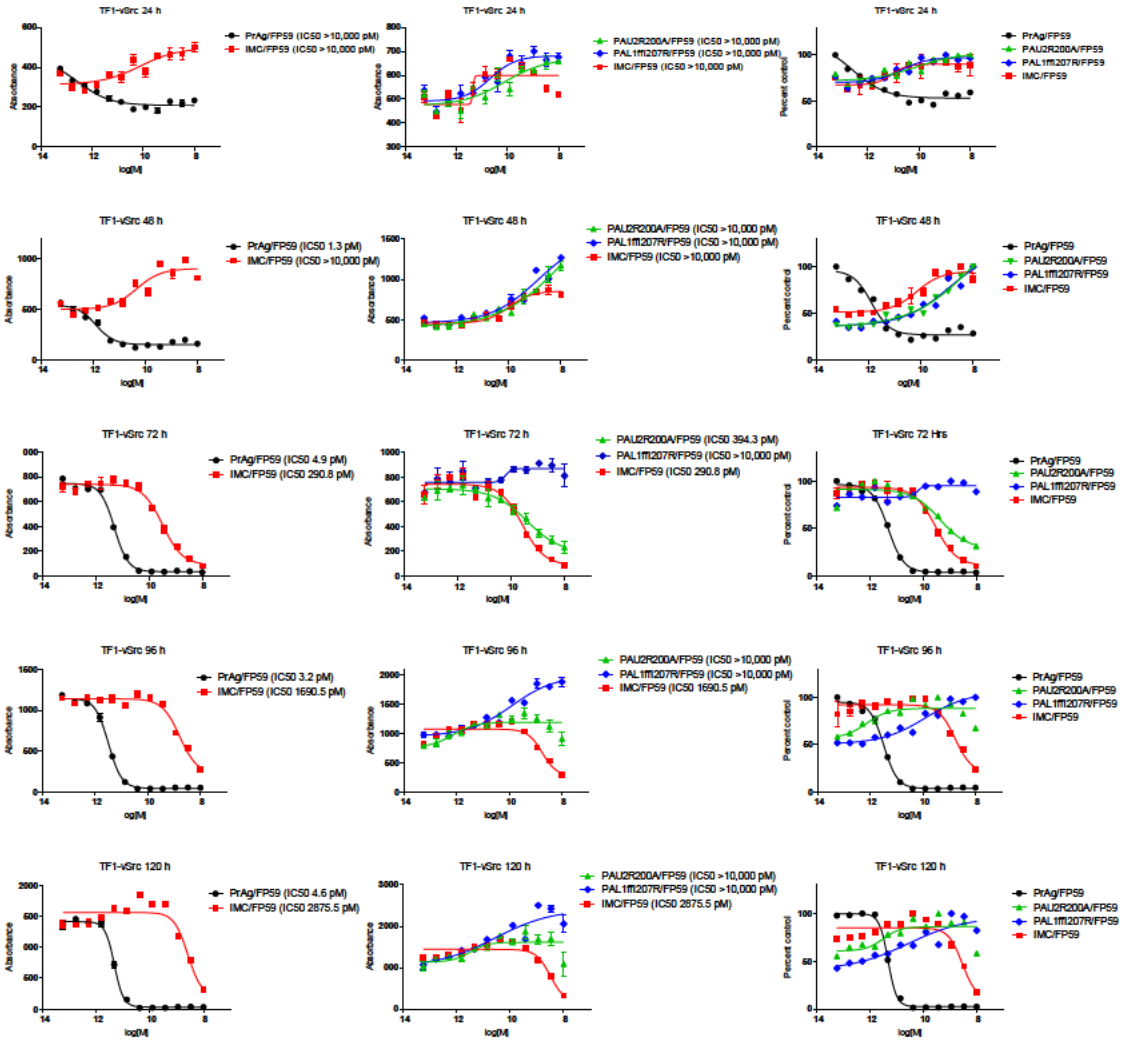
PAL1ff1207R/FP59 (IC50 >10,000 pM)
IMC/FP59 (IC50 1034.6 pM)
Absorbance
log[M]
U937 24 h
PrAg/FP59
PAL2R200A/FP59
PAL1ff1207R/FP59
IMC/FP59
Percent control
log[M]
U937 48 h
PrAg/FP59 (IC50 0.6 pM)
IMC/FP59 (IC50 281.9 pM)
Absorbance
log[M]
U937 48 h
PAL2R200A/FP59 (IC50 >10,000 pM)
PAL1ff1207R/FP59 (IC50 >10,000 pM)
IMC/FP59 (IC50 281.9 pM)
Absorbance
log[M]
U937 48 h
PrAg/FP59
PAL2R200A/FP59
PAL1ff1207R/FP59
IMC/FP59
Percent control
log[M]
U937 72 h
PrAg/FP59 (IC50 0.6 pM)
IMC/FP59 (IC50 171.0 pM)
Absorbance
log[M]
U937 72 h
PAL2R200A/FP59 (IC50 >10,000 pM)
PAL1ff1207R/FP59 (IC50 >10,000 pM)
IMC/FP59 (IC50 171.0 pM)
Absorbance
log[M]
U937 72 h
PrAg/FP59
PAL2R200A/FP59
PAL1ff1207R/FP59
IMC/FP59
Percent control
log[M]
U937 96 h
PrAg/FP59 (IC50 0.9 pM)
IMC/FP59 (IC50 191.3 pM)
Absorbance
log[M]
U937 96 h
PAL2R200A/FP59 (IC50 >10,000 pM)
PAL1ff1207R/FP59 (IC50 >10,000 pM)
IMC/FP59 (IC50 191.3 pM)
Absorbance
log[M]
U937 96 h
PrAg/FP59
PAL2R200A/FP59
PAL1ff1207R/FP59
IMC/FP59
Percent control
log[M]
U937 120 h
PrAg/FP59 (IC50 1.5 pM)
IMC/FP59 (IC50 310.9 pM)
Absorbance
log[M]
U937 120 h
PAL2R200A/FP59 (IC50 >10,000 pM)
PAL1ff1207R/FP59 (IC50 >10,000 pM)
IMC/FP59 (IC50 310.9 pM)
Absorbance
log[M]
U937 120 h
PrAg/FP59
PAL2R200A/FP59
PAL1ff1207R/FP59
IMC/FP59
Percent control
log[M]

E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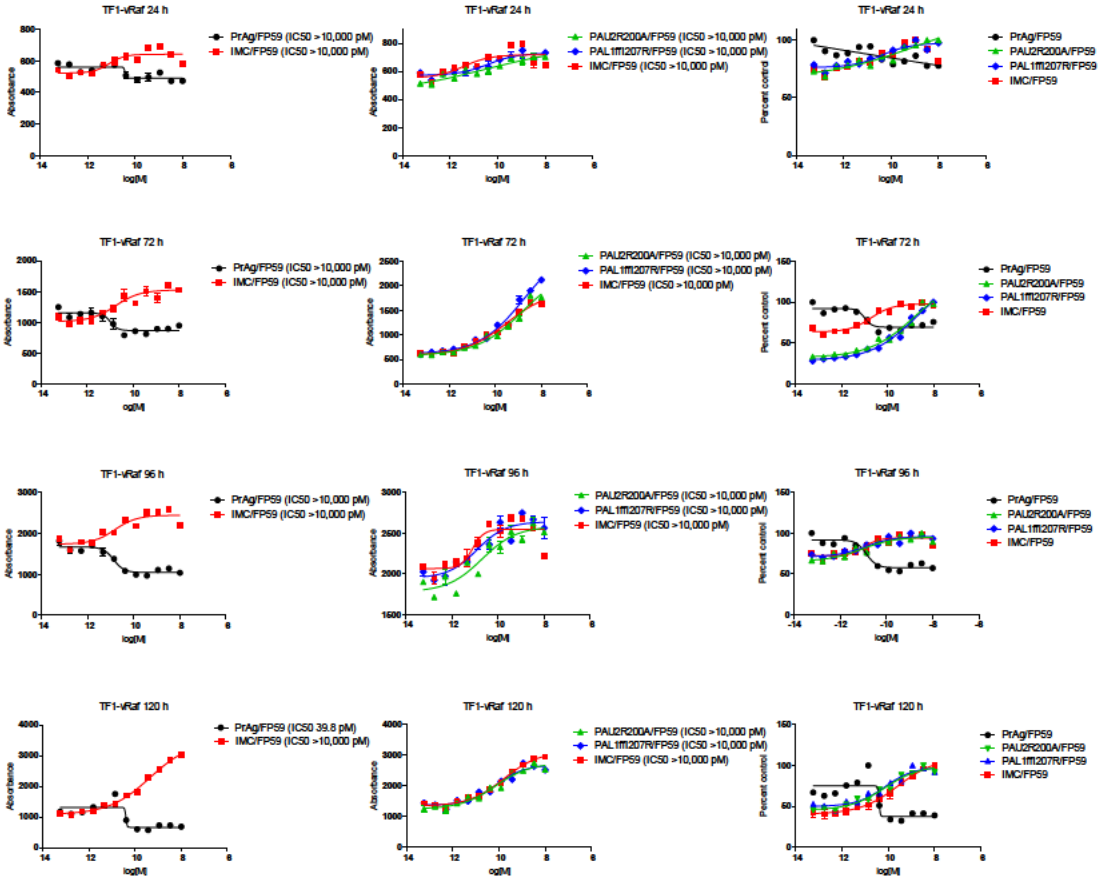
PAU2R200A/FP59 (IC50 >10,000 pM)
PAL1ff1207R/FP59 (IC50 >10,000 pM)
IMC/FP59 (IC50 >10,000 pM)
PrAg/FP59
PAU2R200A/FP59
PAL1ff1207R/FP59
IMC/FP59
TF1-vSrc 48 h
PrAg/FP59 (IC50 1.3 pM)
IMC/FP59 (IC50 >10,000 pM)
PAU2R200A/FP59 (IC50 >10,000 pM)
PAL1ff1207R/FP59 (IC50 >10,000 pM)
IMC/FP59 (IC50 >10,000 pM)
TF1-vSrc 72 h
PrAg/FP59 (IC50 4.9 pM)
IMC/FP59 (IC50 290.8 pM)
PAU2R200A/FP59 (IC50 394.3 pM)
PAL1ff1207R/FP59 (IC50 >10,000 pM)
IMC/FP59 (IC50 290.8 pM)
TF1-vSrc 72 Hrs
TF1-vSrc 96 h
PrAg/FP59 (IC50 3.2 pM)
IMC/FP59 (IC50 1690.5 pM)
PAU2R200A/FP59 (IC50 >10,000 pM)
PAL1ff1207R/FP59 (IC50 >10,000 pM)
IMC/FP59 (IC50 1690.5 pM)
TF1-vSrc 120 h
PrAg/FP59 (IC50 4.6 pM)
IMC/FP59 (IC50 2875.5 pM)
PAU2R200A/FP59 (IC50 >10,000 pM)
PAL1ff1207R/FP59 (IC50 >10,000 pM)
IMC/FP59 (IC50 2875.5 pM)
Absorbance
Percent control
log[M]

F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PrAg/FP59 (IC50 >10,000 pM)
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Absorbance
log[M]
TF1-vRaf 24 h
PAL2R200A/FP59 (IC50 >10,000 pM)
PAL1ff1207R/FP59 (IC50 >10,000 pM)
IMC/FP59 (IC50 >10,000 pM)
Absorbance
log[M]
TF1-vRaf 24 h
PrAg/FP59
PAL2R200A/FP59
PAL1ff1207R/FP59
IMC/FP59
Percent control
log[M]
TF1-vRaf 72 h
PrAg/FP59 (IC50 >10,000 pM)
IMC/FP59 (IC50 >10,000 pM)
Absorbance
log[M]
TF1-vRaf 72 h
PAL2R200A/FP59 (IC50 >10,000 pM)
PAL1ff1207R/FP59 (IC50 >10,000 pM)
IMC/FP59 (IC50 >10,000 pM)
Absorbance
log[M]
TF1-vRaf 72 h
PrAg/FP59
PAL2R200A/FP59
PAL1ff1207R/FP59
IMC/FP59
Percent control
log[M]
TF1-vRaf 96 h
PrAg/FP59 (IC50 >10,000 pM)
IMC/FP59 (IC50 >10,000 pM)
Absorbance
log[M]
TF1-vRaf 96 h
PAL2R200A/FP59 (IC50 >10,000 pM)
PAL1ff1207R/FP59 (IC50 >10,000 pM)
IMC/FP59 (IC50 >10,000 pM)
Absorbance
log[M]
TF1-vRaf 96 h
PrAg/FP59
PAL2R200A/FP59
PAL1ff1207R/FP59
IMC/FP59
Percent control
log[M]
TF1-vRaf 120 h
PrAg/FP59 (IC50 39.8 pM)
IMC/FP59 (IC50 >10,000 pM)
Absorbance
log[M]
TF1-vRaf 120 h
PAL2R200A/FP59 (IC50 >10,000 pM)
PAL1ff1207R/FP59 (IC50 >10,000 pM)
IMC/FP59 (IC50 >10,000 pM)
Absorbance
log[M]
TF1-vRaf 120 h
PrAg/FP59
PAL2R200A/FP59
PAL1ff1207R/FP59
IMC/FP59
Percent control
log[M]

G

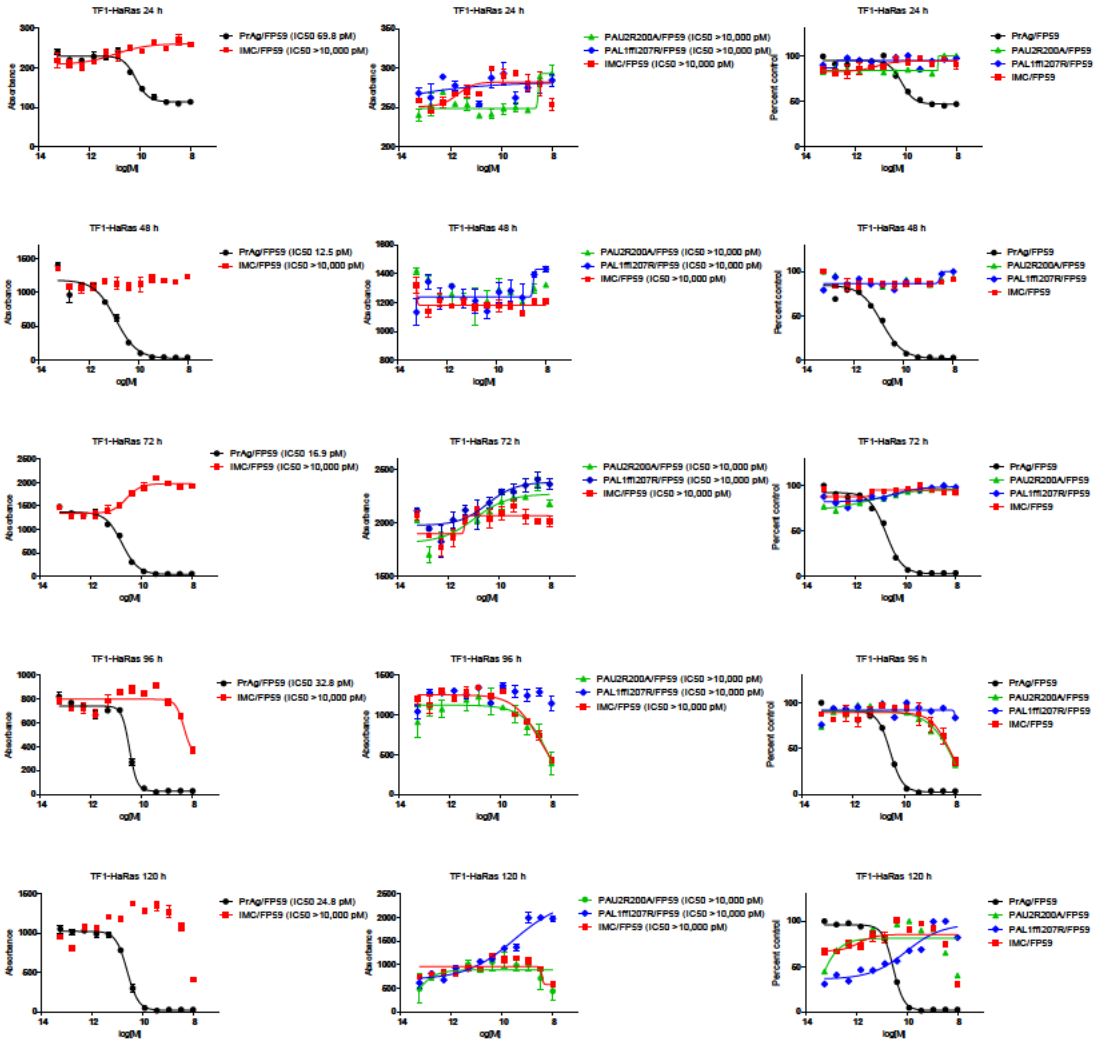


***Figure 10**: **Cytotoxicity of the PrAg variants in combination with FP59 to AML cell lines.** Non-Linear regression curves showing the cytotoxicity of PrAg/FP59 (●), IMC/FP59 (■), PAU2R200A/FP59 alone (▲), and PAL1I207R/FP59 alone (◆) on ML1(A), MM6 (B), ML2 (C), U937 (D), TF1-vSrc (E), TF1-vRaf (F), and TF1-HaRas (G) acute myeloid leukemia cell lines along with their respective IC50 values. Data for TF1-vRaf at 48h is not available along with MM6 response to PrAg/FP59 at 24 h. The viability of the cells was assessed at 24 h, 48 h, 72 h, 96 h, and 120 h. X axis represents the log of the molar protective antigen versions concentration (ranging from 10^{-8} to 10^{-13} M); Y axis indicates the cell viability expressed as absorbance or as percent control. FP59 was used at a fixed concentration of 10^{-9} M.*

Table 1: IC_{50} values reflecting the sensitivity of AML cells to the different PrAg variants in combination with FP59. *IC_{50} values in pM determined in 7 human AML cell lines after treatments with PrAg/FP59, IMC/FP59, PAU2R200A/FP59 alone, and PAL1I207R/FP59 alone at the different time points. (N/A: not available)*

Cell Line	Variants	IC_{50} (pM)				
		24h	**48h**	**72h**	**96h**	**120h**
ML1	IMC/FP59	288	224.7	23.5	26.2	97.7
	PrAg/FP59	0.6	58.4	0.1	0.1	0.5
	PAU2R200A/FP59	>10,000	483.0	26.6	20.3	81.3
	PAL1ffI207R/FP59	>10,000	>10,000	>10,000	>10,000	>10,000
MM6	IMC/FP59	68.0	20.6	26.8	25.9	62.3
	PrAg/FP59	N/A	1.1	1.6	1.3	2.7
	PAU2R200A/FP59	160.0	60.6	103.4	91.7	184.4
	PAL1ffI207R/FP59	>10,000	>10,000	>10,000	>10,000	>10,000
ML-2	IMC/FP59	166.2	491.3	741.6	770.9	474.7
	PrAg/FP59	0.3	191.2	0.1	1.7	0.6
	PAU2R200A/FP59	>10,000	>10,000	>10,000	>10,000	>10,000
	PAL1ffI207R/FP59	>10,000	>10,000	>10,000	>10,000	>10,000
U937	IMC/FP59	1034.6	281.9	171.0	191.3	310.9
	PrAg/FP59	0.5	0.6	0.6	0.9	1.5
	PAU2R200A/FP59	>10,000	>10,000	>10,000	>10,000	>10,000
	PAL1ffI207R/FP59	>10,000	>10,000	>10,000	>10,000	>10,000
TF1-vSrc	IMC/FP59	>10,000	>10,000	290.8	1690.5	2875.5
	PrAg/FP59	>10,000	1.3	4.9	3.2	4.6
	PAU2R200A/FP59	>10,000	>10,000	394.3	>10,000	>10,000
	PAL1ffI207R/FP59	>10,000	>10,000	>10,000	>10,000	>10,000
TF1-vRaf	IMC/FP59	>10,000	N/A	>10,000	>10,000	>10,000
	PrAg/FP59	>10,000	N/A	>10,000	>10,000	39.8
	PAU2R200A/FP59	>10,000	N/A	>10,000	>10,000	>10,000
	PAL1ffI207R/FP59	>10,000	N/A	>10,000	>10,000	>10,000
TF1-HaRas	IMC/FP59	>10,000	>10,000	>10,000	>10,000	>10,000
	PrAg/FP59	69.8	12.5	16.9	32.8	24.8
	PAU2R200A/FP59	>10,000	>10,000	>10,000	>10,000	>10,000
	PAL1ffI207R/FP59	>10,000	>10,000	>10,000	>10,000	>10,000

3.2. Impact of IMC/LF and IMC/FP59 on cell cycle progression

We then set out to explore whether IMC/LF, even though it did not cause cytotoxicity and inhibition of cell viability, will provoke cell cycle arrest. In addition, to investigate whether the intermolecularly complemented version combined with FP59 (IMC/FP59), aside from causing cytotoxicity, will induce cell cycle arrest in AML cells.

To determine their effect on cell cycle progression, we analyzed the cell cycle status by performing PI staining of the surviving fraction of cells following treatment with IMC/LF, PrAg/LF, IMC/FP59 or PrAg/FP59, on a panel of five AML cell lines. PrAg/LF and PrAg/FP59 were used as positive controls and the examination took place at 24 h, 48 h, and 72 h post treatment, while no surviving cells remained to be analyzed at 96 h and 120 h since the conjunction with FP59 for such time points is too potent, leaving behind only a minor population to be analyzed.

Three out of the five cell lines tested (ML1, ML2 and U937) showed a significant G0/G1 cell cycle arrest post-treatment with PrAg/LF and IMC/LF at 24 h, 48 h, and 72 h (Figure 11 A, B, and C). For example, ML2 cells after 72 h of treatment with PrAg/LF and IMC/LF had the percentage of cells in the G0/G1 phase increase significantly from 52.9% of the total cell population in the control untreated cells to 73.5% and 71.9%, respectively. This was accompanied by a respective decrease in the percentage of cells in the G2/M phase from 8.6% to 0.6% and 2.7% demonstrating their ability to induce cell cycle arrest (Figure 11 B).

Treatments of these cells with PrAg/P59 and IMC/FP59 showed that the percentage of cells in the G0/G1 and G2/M phases was reduced while it was accompanied by an increase in the percentage of cells in the pre-G0/G1 phase, indicating that treatment

with PrAg/FP59 and IMC/FP59 induces complete cytotoxicity and as such the majority of the cells were dead (Figures 11 A, B, and C).

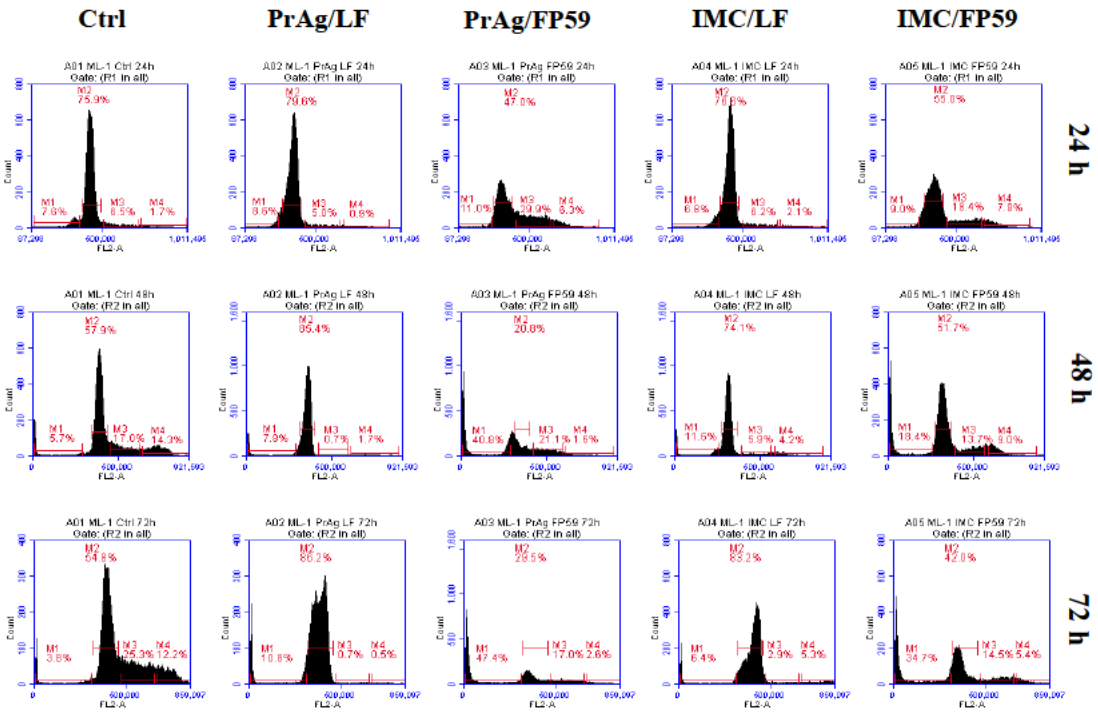
Neither PrAg/LF, PrAg/FP59, IMC/LF nor IMC/FP59 had any effect on the cell cycle of TF1-vSrc (Figure 11 D). In addition, the fraction of cells in the G0/G1 phase of TF1-HaRas was not affected by PrAg/LF, IMC/LF, or IMC/FP59 at 72 h, indicating the absence of cell cycle arrest, while PrAg/FP59 caused cell death indicated by the accumulation of cells in the pre-G0/G1 phase (Figure 11 E). This matches its response previously seen in the proliferation inhibition assay, where it was sensitive to PrAg/FP59 treatment, but not to IMC/FP59.

PI staining revealed that, in addition to cytotoxicity, PrAg/LF induces cell cycle arrest in the surviving cell fraction of ML1, ML2 and U937 cells, while PrAg/FP59 and IMC/FP59 mainly have a cytotoxic effect causing major levels of cell death (Table 2).

Whereas IMC/LF, even though it did not show any clear signs of cytotoxicity and inhibition of cell viability, is significantly inducing cell cycle arrest in ML1, ML2 and U937 cell lines. Since these three cell lines were highly or moderately sensitive to IMC/FP59 cytotoxicity, this indicates that the activation level of IMC/LF might not be high enough to allow sufficient LF translocation into the cells to inhibit the MAPK pathway and induce cytotoxicity but is high enough to cause cell cycle arrest in ML1, ML2 and U937, rendering its effect as cytostatic.

The examined cell cycle arrest pattern, to a certain extent, did correlate with that of cytotoxicity, whereby IMC/LF arrested the cell cycle of three cell lines belonging to the group that demonstrated a cytotoxic response with IMC/FP59 (ML1, ML2 and U937), while the two other cells lines (TF1-vSrc and TF1-HaRas), that belong to the groups that showed mild or no sensitivity, had their cell cycle unaffected.

A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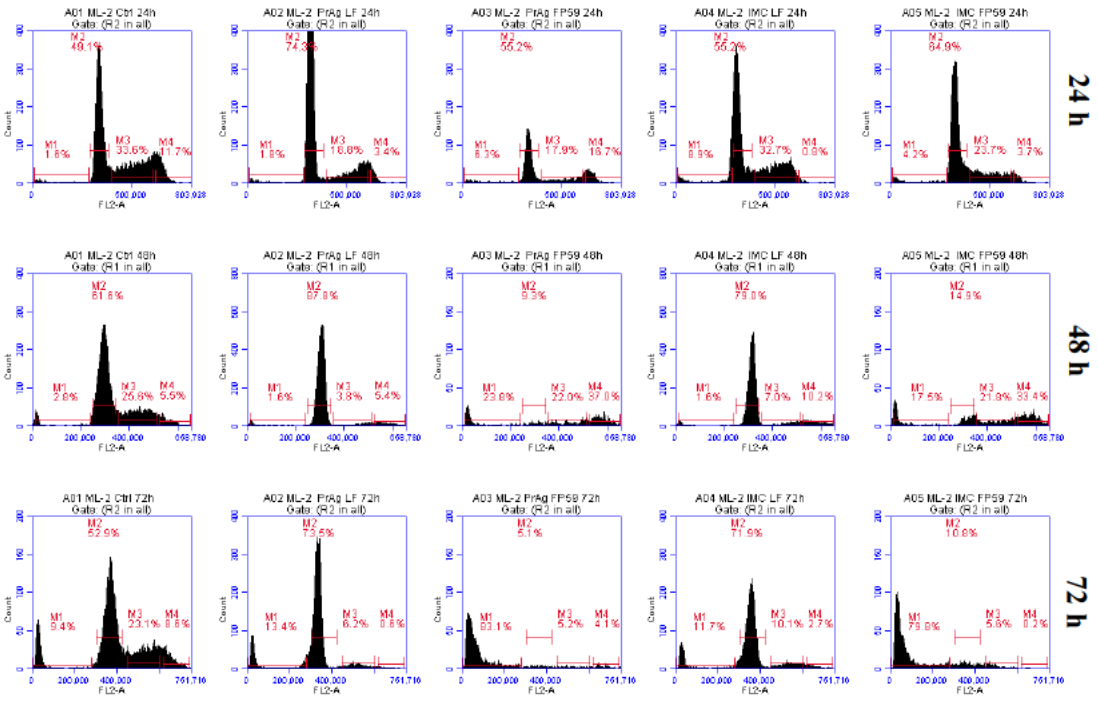
24 h
48 h
72 h

B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48 h
72 h

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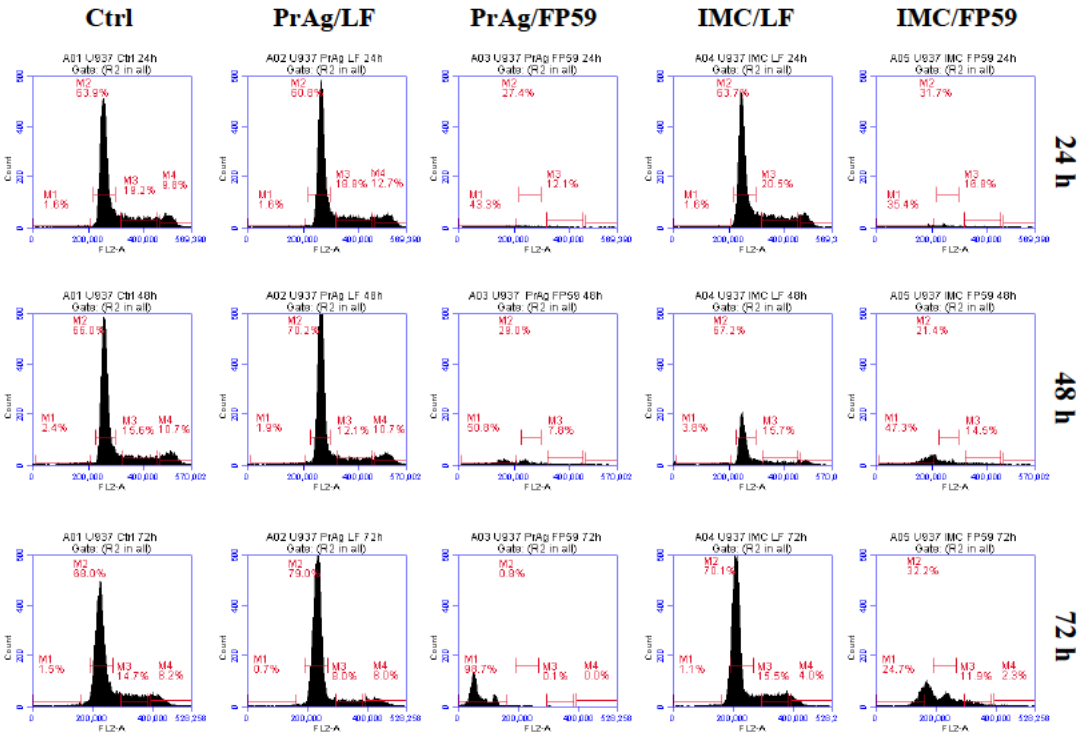
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PrAg/FP59
IMC/LF
IMC/FP59
24 h
48 h
72 h

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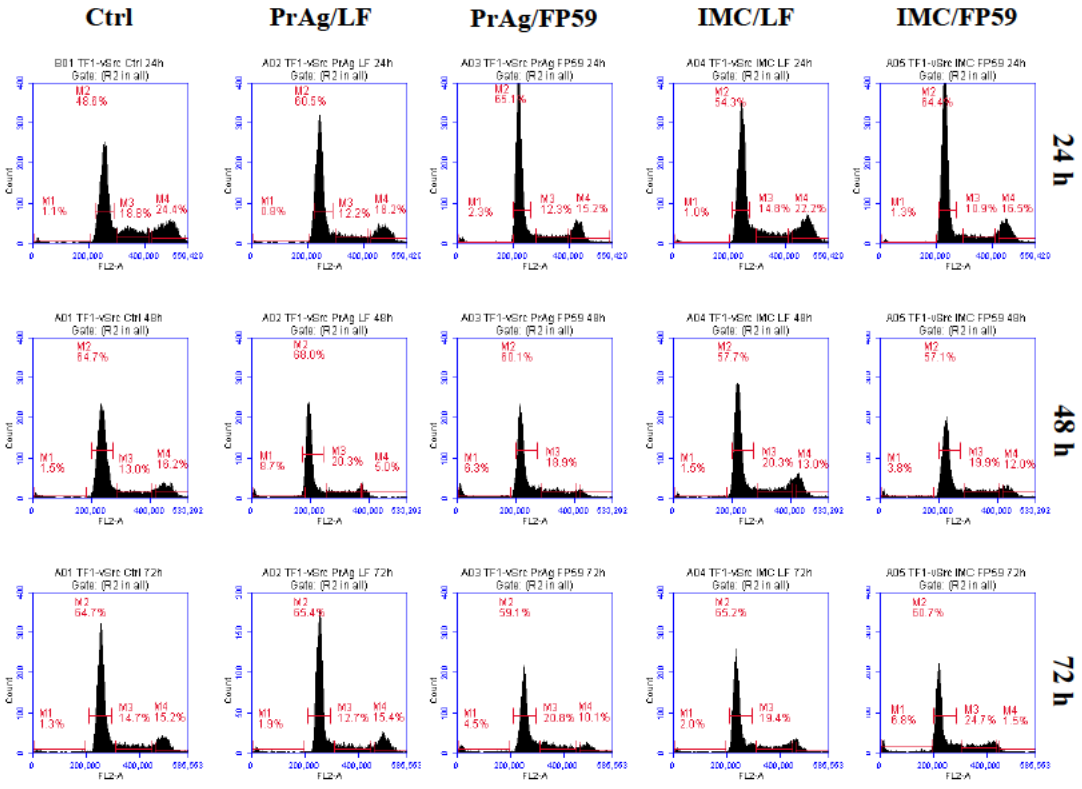
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24 h
48 h
72 h

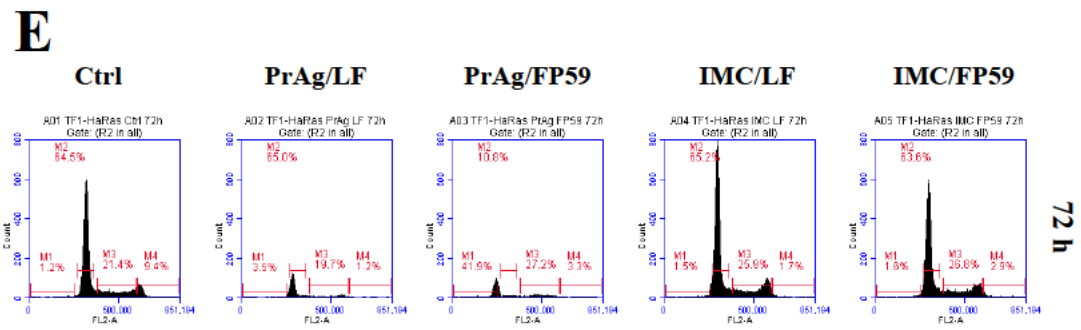

Figure 11: Cell cycle analysis of AML cells following treatments with the different PrAg variants in combination to both LF and FP59. *Cell cycle analysis of ML1(A), ML2(B), U937 (C), TF1-vSrc (D), and TF1-HaRas (E) cell lines following treatment with PrAg/LF, PrAg/FP59, IMC/LF and IMC/FP59 at 24 h, 48 h, and 72 h. Control cells are represented in the left panels. Cells are gated on width versus forward scatter (R1 and R2). Cells in pre-G0/G1 (dead) are gated M1, G0/G1 are gated M2, S are gated M3, and G2/M are gated M4.*

Table 2:Summarizing the effect of PrAg/LF, PrAg/FP59, IMC/LF and IMC/FP59 on cell cycle progression of five AML cell lines.

Cell lines	Impact on cell cycle			
	PrAg/LF	PrAg/FP59	IMC/LF	IMC/FP59
ML1	Arrest	Cell death	Arrest	Cell death
ML2	Arrest	Cell death	Arrest	Cell death
U937	Arrest	Cell death	Arrest	Cell death
TF1-vSrc	No effect	No effect	No effect	No effect
TF1-HaRas	No effect	Cell death	No effect	No effect

3.3. Mechanism of cell death induced by IMC/FP59

To demonstrate the mechanism of cell death induced by IMC/FP59, we tested for annexin V/PI staining in an AML panel consisting of ML1, ML2, U937, TF1-vSrc, and TF1-HaRas, both IMC/FP59 sensitive and resistant cell lines. We treated the cells with PrAg/FP59 or IMC/P59 for 24 h, 48 h, 72 h, and in some cases 96 h. FP59 was first used at the highest concentration of 10^{-9} M which was too potent leaving a small

population to be analyzed, hence we switched to a concentration of 20 pM for most of the analysis.

Three cell lines, ML1, ML2, and U937 that demonstrated a cytotoxic response to IMC/FP59, showed an increase in the percentage of cells stained with both annexin V and PI at all time points following treatment with IMC/FP59 compared to the control untreated cells, as such showing evidence of a non-apoptotic mechanism of cell death (Figure 12 A, B, and C).

ML2 additionally showed a shift towards the lower right quadrant where cells were stained with annexin V only and not PI, following treatment with PrAg/FP59 or IMC/FP59. Demonstrating that multiple mechanisms of cell death, apoptotic or non-apoptotic, might be involved for this cell line (Figure 12 B).

In addition, U937 cells that were treated with a concentration of 20 pM of FP59, showed an increase of annexin V/PI staining at 72 h, from 7.9% in the control untreated cells to 97.4% with PrAg/FP59, and 44.6% with IMC/FP59, Furthermore, cells treated with PrAg/FP59 had only 1.7% of the population still alive, while those treated with IMC/FP59 had 51.6%. As such the higher percentage of the living population of IMC/FP59 correlates with its higher IC_{50} values identified with the proliferation inhibition assay (Figure 12 C, Table 1).

Moreover, with 20 pM of FP59 at 24 h, 48 h and 72 h, both TF1-vSrc and TF1-HaRas, that manifested a mild or no cytotoxic response to the IMC/FP59 treatment, showed no increase in the percentage of cells stained with both annexin V and PI compared to the untreated controls, validating the absence of cell death after treatment with IMC/FP59 (Figure 12 D and E).

However, TF1-vSrc, the cell line that is mildly sensitive only at later time points, demonstrated a shift towards the upper right quadrant only with 10^{-9} M of FP59 and at higher time points of 72 h and 96h indicating double positivity for annexin V and PI staining (Figure 12 D). Which further confirms the mild sensitivity of TF1-vSrc that only manifests at later time points.

As a result, we have demonstrated the double positivity for annexin V and PI staining in the IMC/FP59 responsive cell lines, showing evidence that the mechanism of cell death induced by this variant might be non-apoptotic. However, further investigation needs to be done to rule out the involvement of apoptosis. In addition to this double positivity, only ML2 demonstrated a simultaneous shift towards the lower right quadrant, indicating some death via apoptosis as well. TF1-vSrc matched its cytotoxic response where no double positivity staining was detected before reaching late time points with the highest FP59 concentration of 10^{-9} M. The non-responsive cell line TF1-HaRas, lacked annexin V and PI positive staining which further indicates the absence of cell death following treatment with IMC/FP59. Furthermore, the demonstrated evidence of double positivity with IMC/FP59 was lower compared to PrAg/FP59 which correlates with its higher IC_{50}.

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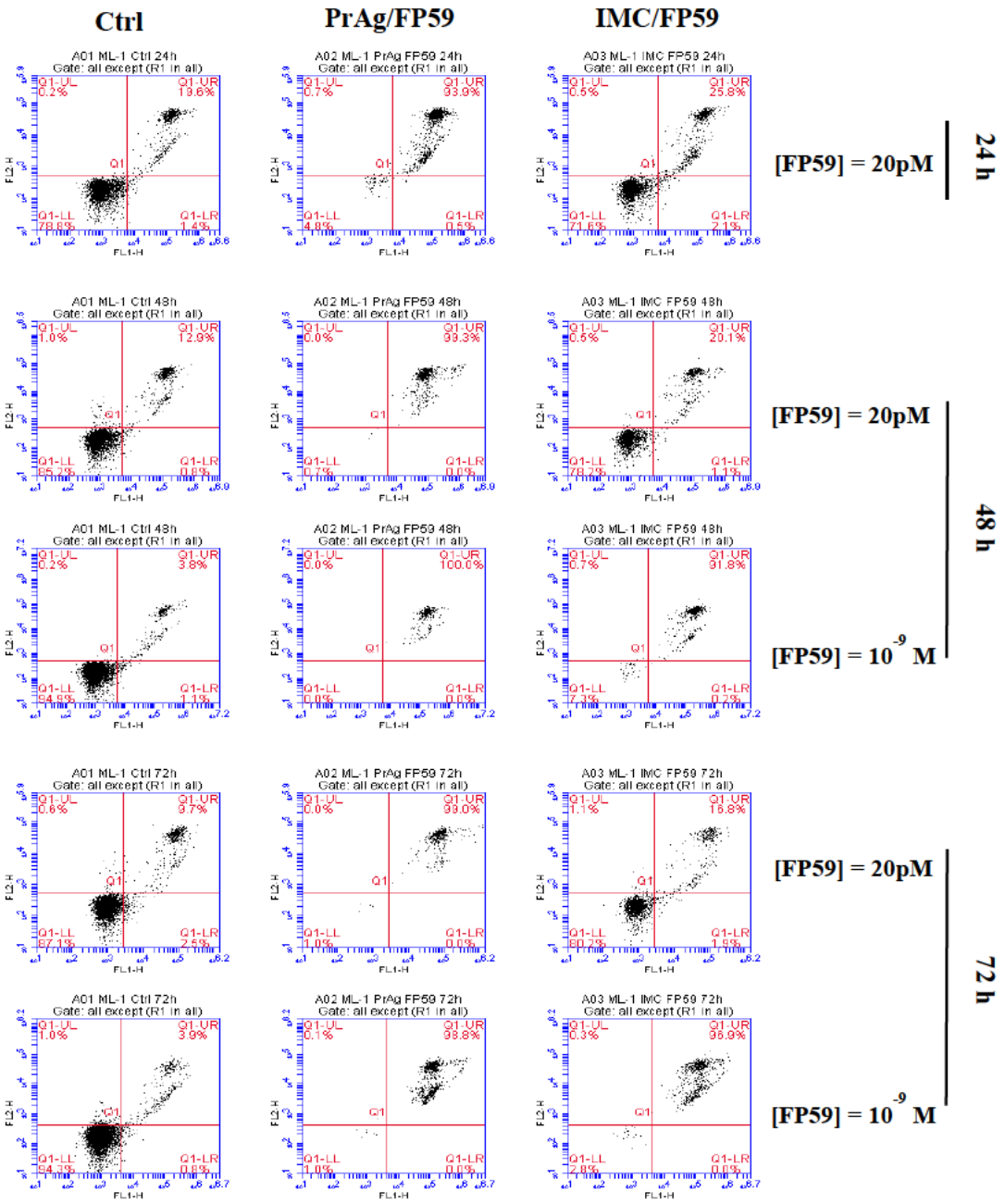
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IMC/FP59
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A03 ML-1 IMC FP59 24h
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Q1-UR 19.6%
Q1-LL 79.6%
Q1-LR 1.4%
Q1-UL 0.7%
Q1-UR 99.9%
Q1-LL 4.0%
Q1-LR 0.5%
Q1-UL 0.5%
Q1-UR 25.8%
Q1-LL 71.6%
Q1-LR 2.1%
A01 ML-1 Ctrl 48h
A02 ML-1 PrAg FP59 48h
A03 ML-1 IMC FP59 48h
Q1-UL 1.0%
Q1-UR 12.9%
Q1-LL 85.7%
Q1-LR 0.5%
Q1-UL 0.0%
Q1-UR 99.3%
Q1-LL 0.7%
Q1-LR 0.0%
Q1-UL 0.5%
Q1-UR 20.1%
Q1-LL 78.2%
Q1-LR 1.1%
A01 ML-1 Ctrl 48h
A02 ML-1 PrAg FP59 48h
A03 ML-1 IMC FP59 48h
Q1-UL 0.2%
Q1-UR 3.8%
Q1-LL 94.8%
Q1-LR 1.1%
Q1-UL 0.0%
Q1-UR 100.0%
Q1-LL 0.0%
Q1-LR 0.0%
Q1-UL 0.7%
Q1-UR 91.8%
Q1-LL 7.3%
Q1-LR 0.2%
A01 ML-1 Ctrl 72h
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Q1-LL 94.3%
Q1-LR 0.8%
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Q1-LL 1.0%
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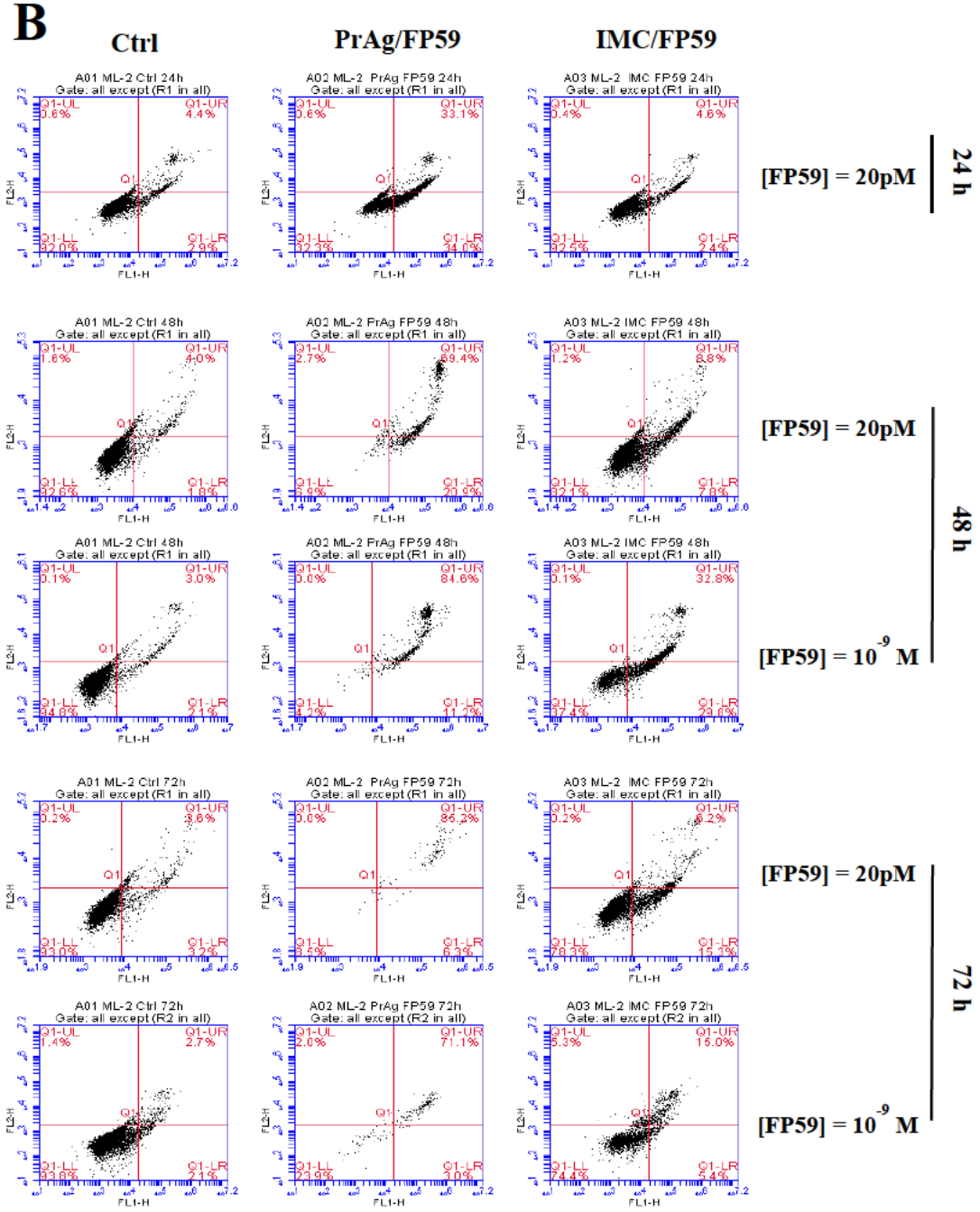
Q1-UR 96.9%
Q1-LL 2.8%
Q1-LR 0.0%
FL2-H
FL1-H
[FP59] = 20pM
24 h
[FP59] = 20pM
[FP59] = 10^-9 M
48 h
[FP59] = 20pM
[FP59] = 10^-9 M
72 h

B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Q1-LL 92.0%
Q1-LR 2.9%
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Q1-UR 33.1%
Q1-LL 32.3%
Q1-LR 34.0%
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Q1-UR 4.6%
Q1-LL 92.5%
Q1-LR 2.4%
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24 h
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Q1-LL 92.6%
Q1-LR 1.8%
A02 ML-2 PrAg FP59 48h
Gate: all except (R1 in all)
Q1-UL 2.7%
Q1-UR 69.4%
Q1-LL 7.9%
Q1-LR 20.0%
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Q1-UL 1.2%
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Q1-LL 82.1%
Q1-LR 7.8%
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Q1-UR 3.0%
Q1-LL 94.8%
Q1-LR 2.1%
A02 ML-2 PrAg FP59 48h
Gate: all except (R1 in all)
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Q1-UR 84.6%
Q1-LL 4.2%
Q1-LR 11.2%
A03 ML-2 IMC FP59 48h
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Q1-UR 32.8%
Q1-LL 57.4%
Q1-LR 9.8%
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Q1-UR 3.6%
Q1-LL 93.0%
Q1-LR 3.2%
A02 ML-2 PrAg FP59 72h
Gate: all except (R1 in all)
Q1-UL 0.0%
Q1-UR 86.2%
Q1-LL 3.5%
Q1-LR 6.3%
A03 ML-2 IMC FP59 72h
Gate: all except (R1 in all)
Q1-UL 0.2%
Q1-UR 6.2%
Q1-LL 76.3%
Q1-LR 15.3%
[FP59] = 20pM
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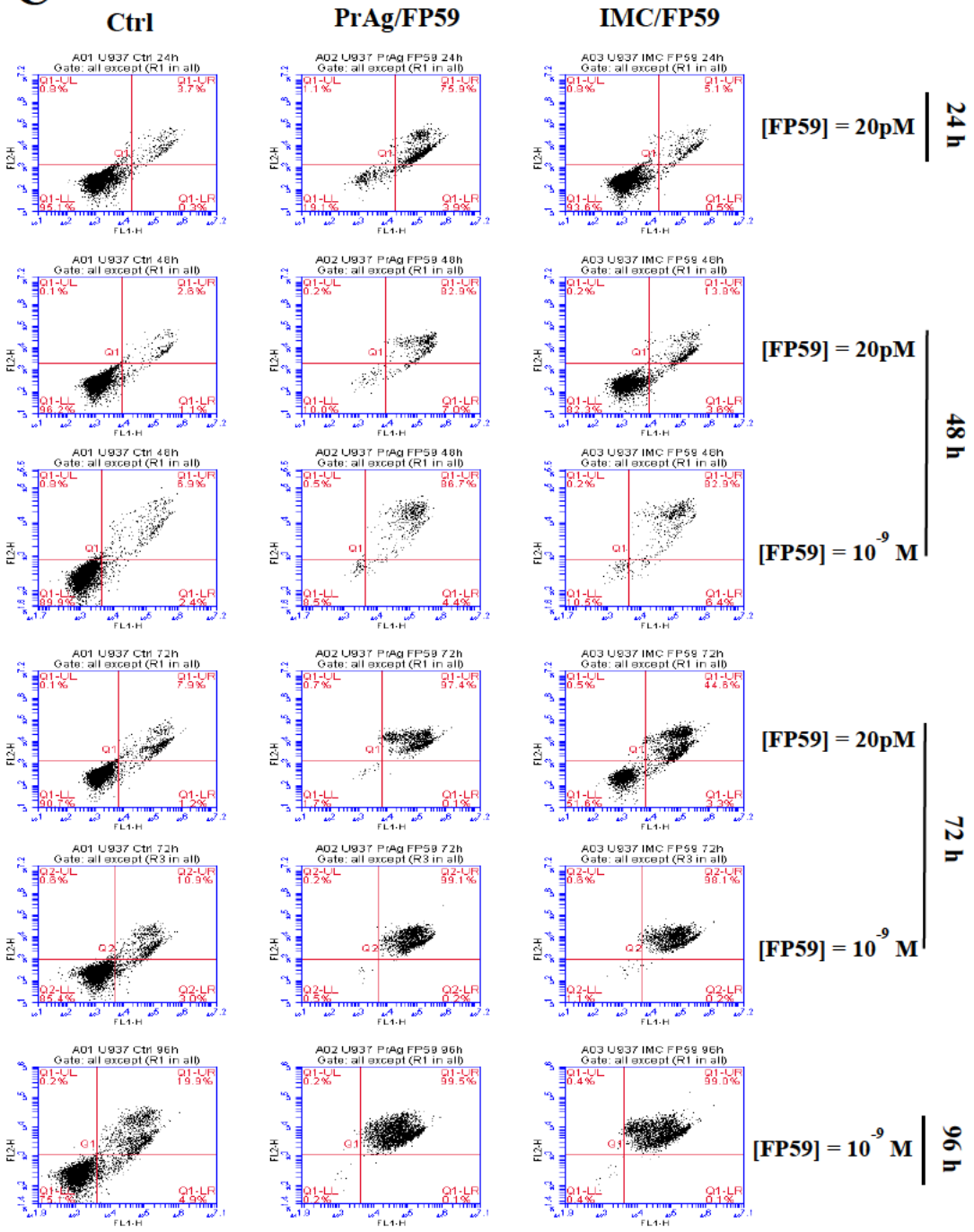
Gate: all except (R2 in all)
Q1-UL 1.4%
Q1-UR 2.7%
Q1-LL 93.8%
Q1-LR 2.1%
A02 ML-2 PrAg FP59 72h
Gate: all except (R2 in all)
Q1-UL 2.0%
Q1-UR 71.1%
Q1-LL 23.9%
Q1-LR 3.0%
A03 ML-2 IMC FP59 72h
Gate: all except (R2 in all)
Q1-UL 5.3%
Q1-UR 15.0%
Q1-LL 74.4%
Q1-LR 5.4%
[FP59] = 10-9 M
72 h
FL1-H
FL2-H

C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IMC/FP59
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24 h
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72 h
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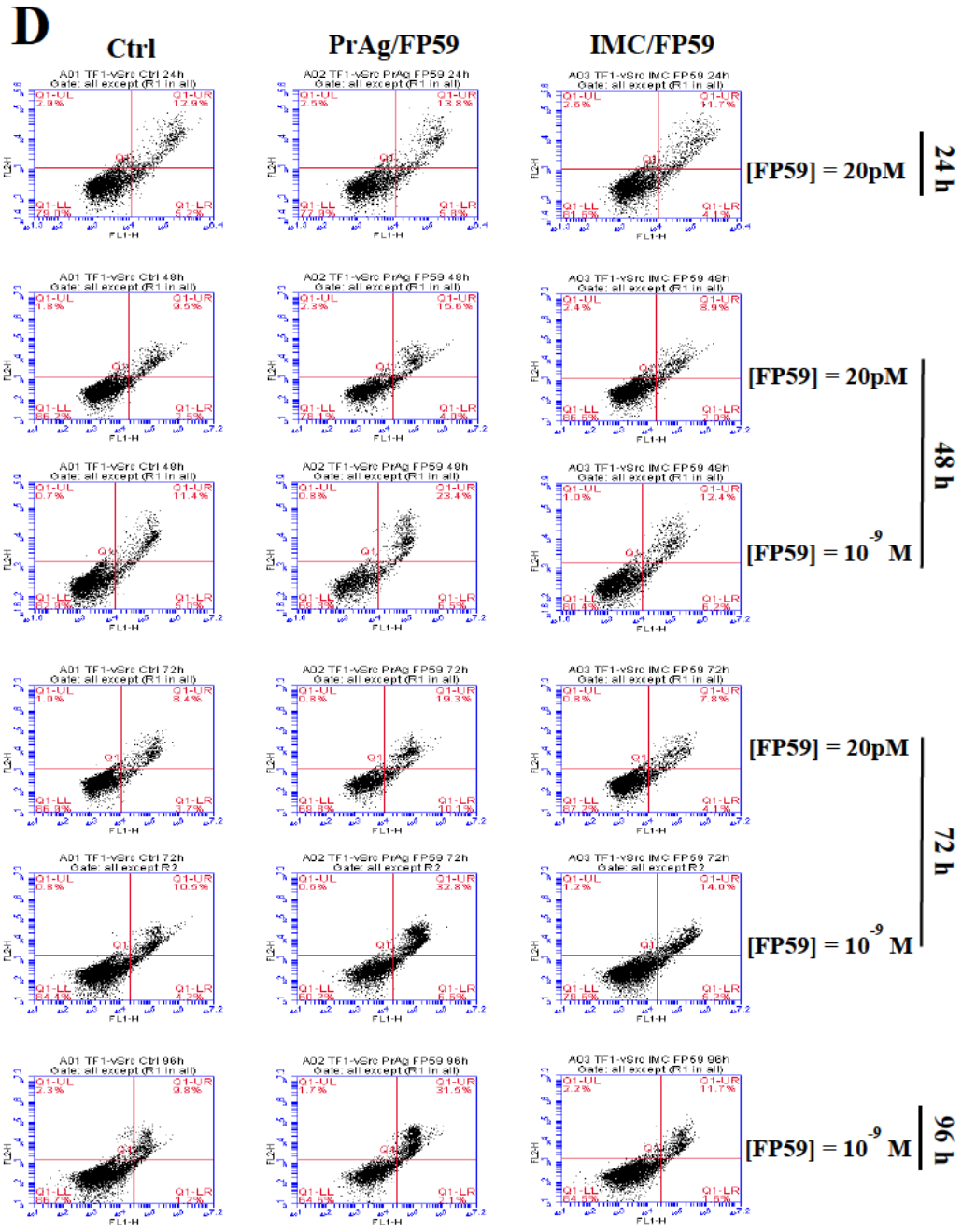
96 h


D
Ctrl
PrAg/FP59
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96 h

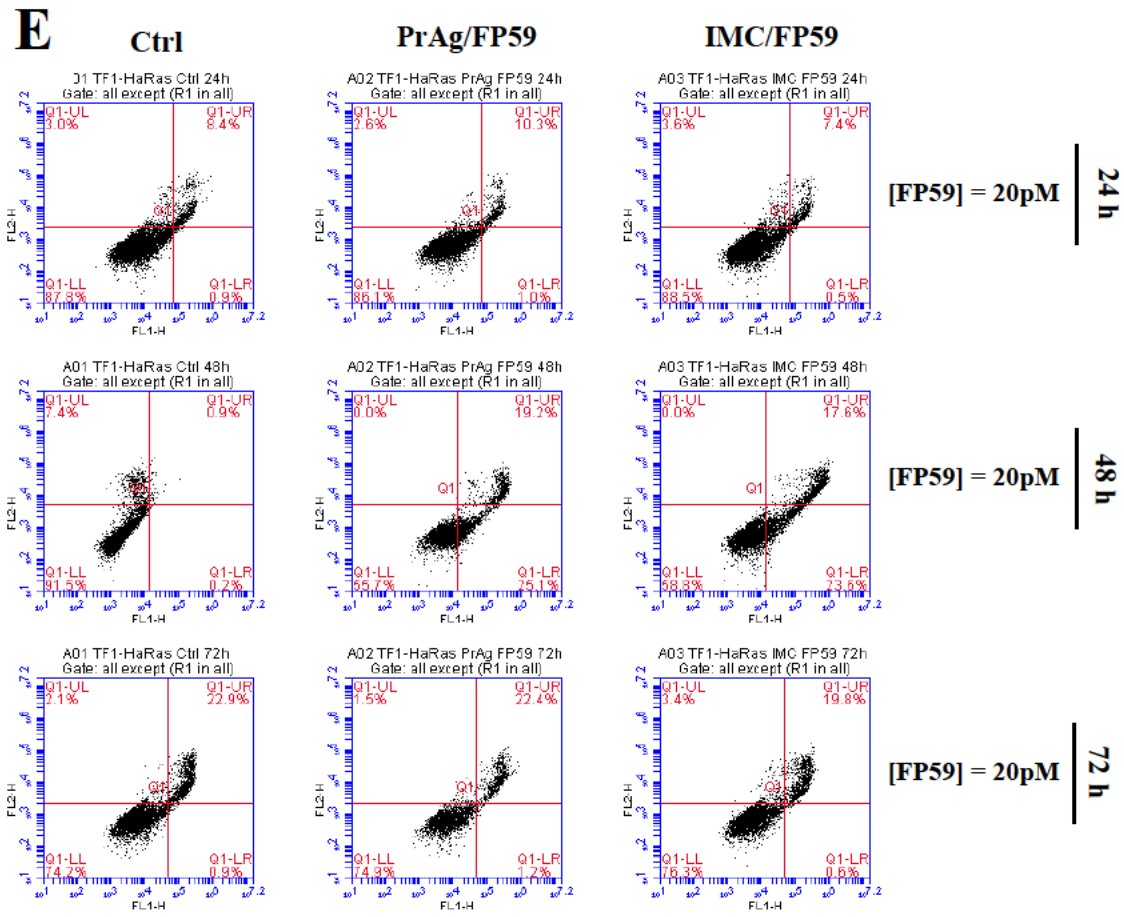


Figure 12: Analysis of the mechanism of IMC/FP59 -mediated cell death in AML cell lines. *Annexin V/PI staining of ML1 (A), ML2 (B), U937 (C), TF1-vSrc (D), and TF1-HaRas (E) cells following treatment with PrAg/FP59 or IMC/FP59 at 24 h, 48 h, and 72 h using annexin. The Cells were treated with 10^{-8} M of either PrAg or PAU2R200A-PAL1I207R, in combination with 20 pM of FP59 at all the time points, or 10^{-9} M of FP59 at some of the time points. Cells were gated on width versus forward scatter, and the dot plot of annexin-V (FL1-H)/PI (FL2-H) staining was generated. The lower left quadrant compromises the unstained viable cells, the lower right quadrant contains early apoptotic cells stained by annexin-V, the upper right quadrant stained by annexin-V and PI indicates late apoptotic cells and the upper left quadrant are necrotic cells stained by PI only.*

3.4. MMP2, MMP9 and uPAR expression levels

3.4.1 Comparing the basal expression level of MMP2, MMP9 and uPAR between the AML cell lines

We then wanted to study the expression of MMP2, MMP9 and uPAR as they are simultaneously crucial for the activation of the IMC version of anthrax lethal toxin. In addition, we wanted to investigate the uPA/uPAR protease system to see whether its expression correlates with the responses to treatments, as it's shown to be the rate limiting factor.

We first started by comparing the basal level of expression of MMP2, MMP9 and uPAR between ML1, ML2 U937, TF1-vSrc, TF1-HaRas and TF1-vRaf AML cell lines.

Western blot analysis along with its quantification demonstrated that all tested cell lines express MMP2 but to different extents. U937, the IMC/FP59 moderately sensitive cell line, was determined to have the highest level of MMP2 among all cell lines. IMC/FP59 resistant cell lines, TF1-HaRas and TF1-vRaf express MMP2 but at a lower level compared to U937, with TF1-vRaf having the lowest expression **(**Figure 13 A and A', and Table 3).

MMP9 on the other hand, is not expressed in all cell lines. ML1, ML2, and U937 lack basal levels of expression of MMP9 even though they showed high to moderate sensitivity to the IMC/FP59 treatments. While TF1-vSrc, TF1-HaRas, and TF1-vRaf, the mildly sensitive or resistant cell lines harbor significant levels with TF1-HaRas having the highest expression of MMP9 (Figure 13 A, A', and Table 3).

uPAR, the receptor that binds to uPA in the uPA/uPAR system, demonstrated to be basely expressed in all cell lines tested, with the IMC/FP59 moderately sensitive cell

line, U937, having the most abundant expression. Notably, TF1-HaRas that showed no response to the IMC/FP59 treatment harbors a significant basal expression of uPAR (Figure 13 A, A', and Table 3).

As a result, we demonstrated that MMP2 and uPAR have basal levels of expression in both sensitive and resistant cell lines tested, with U937 having the highest levels of both. Notably, IMC/FP59 sensitive cell lines ML1, ML2, and U937 lack the basal expression of MMP9 that was exclusively expressed in cell lines that showed mild to no sensitivity, which indicates that MMP9 expression might not be critical for the sensitivity of cells.

After the proliferation inhibition assay, ML1, ML2 and U937, the highly and moderately IMC/FP59 sensitive cell lines, demonstrated that their sensitivity to IMC/FP59 matched that of PAU2R200A/FP59, and as such the uPA/uPAR protease system is thought to be the rate limiting step. In the effort of linking these basal levels of MMP2, MMP9 and especially uPAR to the response of AML cell lines to IMC/FP59 sensitivity, no correlation seems to be demonstrated. ML1, the highly sensitive cell line, shows moderate MMP2 and uPAR basal expression, while it lacked MMP9 expression. Resistant cell lines such as TF1-HaRas, demonstrated to have low levels of MMP2 but significant levels of MMP9 and uPAR. The absence of correlation, especially between uPAR expression and the sensitivity of AML cells to IMC/FP59, suggests that the response of AML cells might not be depicted by the basal levels of uPAR, but by the activity of the uPA/uPAR protease system.

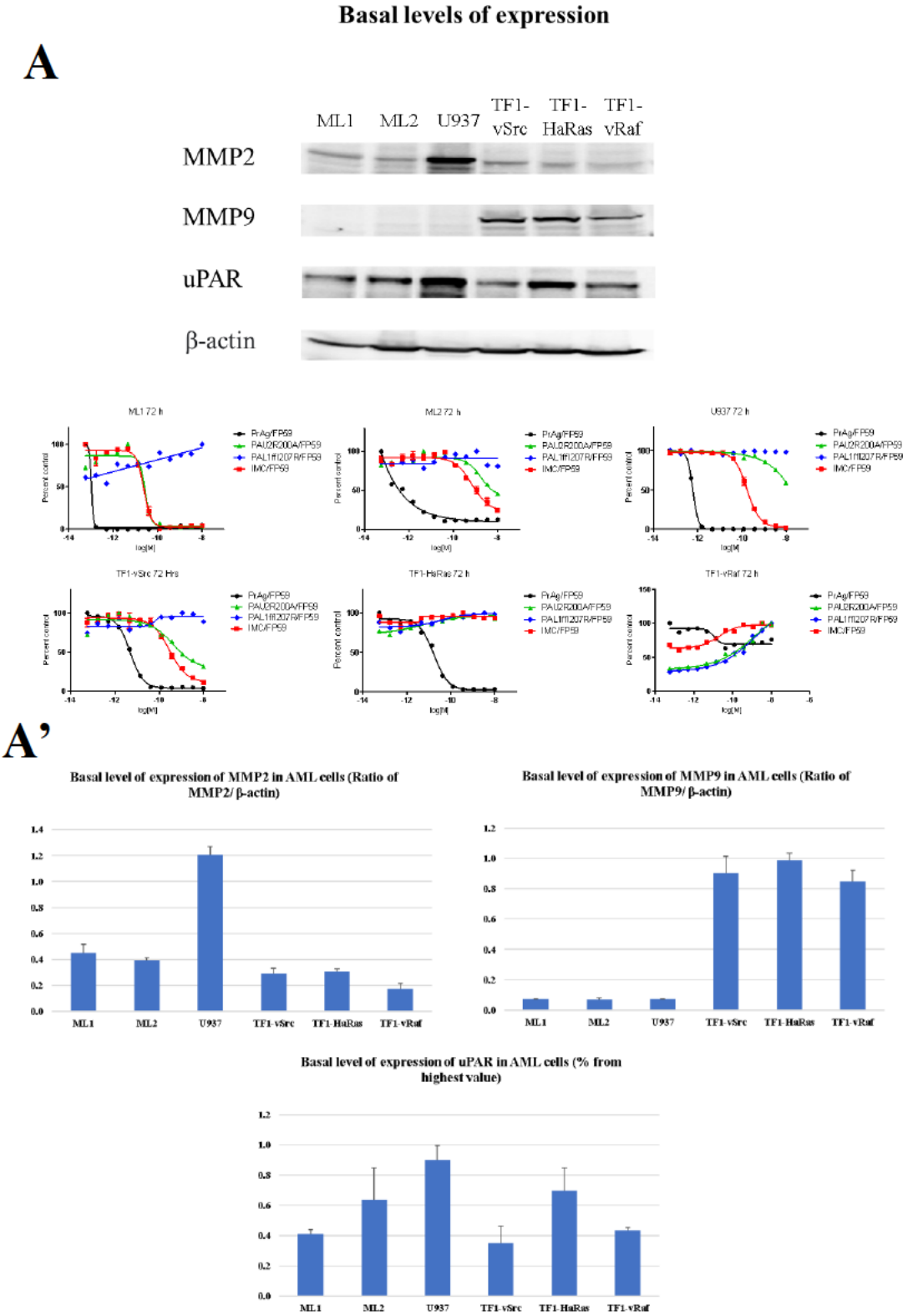


Figure 13: Basal expression levels of MMP2, MMP9 and uPAR in AML cell lines. *(A) Western blots comparing the basal expression level of MMP2, MMP9, and uPAR between untreated ML1, ML2, U937, TF1-vSrc, TF1-HaRas, and TF1-vRaf cell lines, along with the Non-Linear regression curves that demonstrate the response of cells to PrAg/FP59, IMC/FP59, PAU2R200A/FP59 alone, and PAL1I207R/FP59 alone. β-actin was used as a loading control (lower gel). (A') The representative quantification graphs of the basal levels of MMP2, MMP9 and uPAR expressed as a ratio of the protease to β-actin. The values are an average of 2 trials done for each protein in all the cell lines.*

Table 3: The basal levels of MMP2, MMP9, and uPAR expression in AML cell lines expressed as a ratio to β-actin*. The values are an average of 2 trials done for each protein in all the cell lines.*

Cell line	Basal level of expression		
	Ratio MMP2/β-actin	Ratio MMP9/β-actin	Ratio uPAR/β-actin
ML1	0.5	0.1	0.4
ML2	0.4	0.1	0.6
U937	1.2	0.1	0.9
TF1-vSrc	0.3	0.9	0.4
TF1-HaRas	0.3	1.0	0.7
TF1-vRaf	0.2	0.9	0.4

The next step was to demonstrate whether treatment with PrAg/LF, PrAg/FP59, IMC/LF and IMC/FP59 will affect the expression of MMP2, MMP9 and uPAR in the AML cell lines after 24 h, 48 h, and 72 h. Later time points of 96 h and 120 h were not investigated since FP59 was too potent at those time points, leaving no cells to be analyzed.

3.4.2. The effect of PrAg/LF and IMC/LF on the level of expression

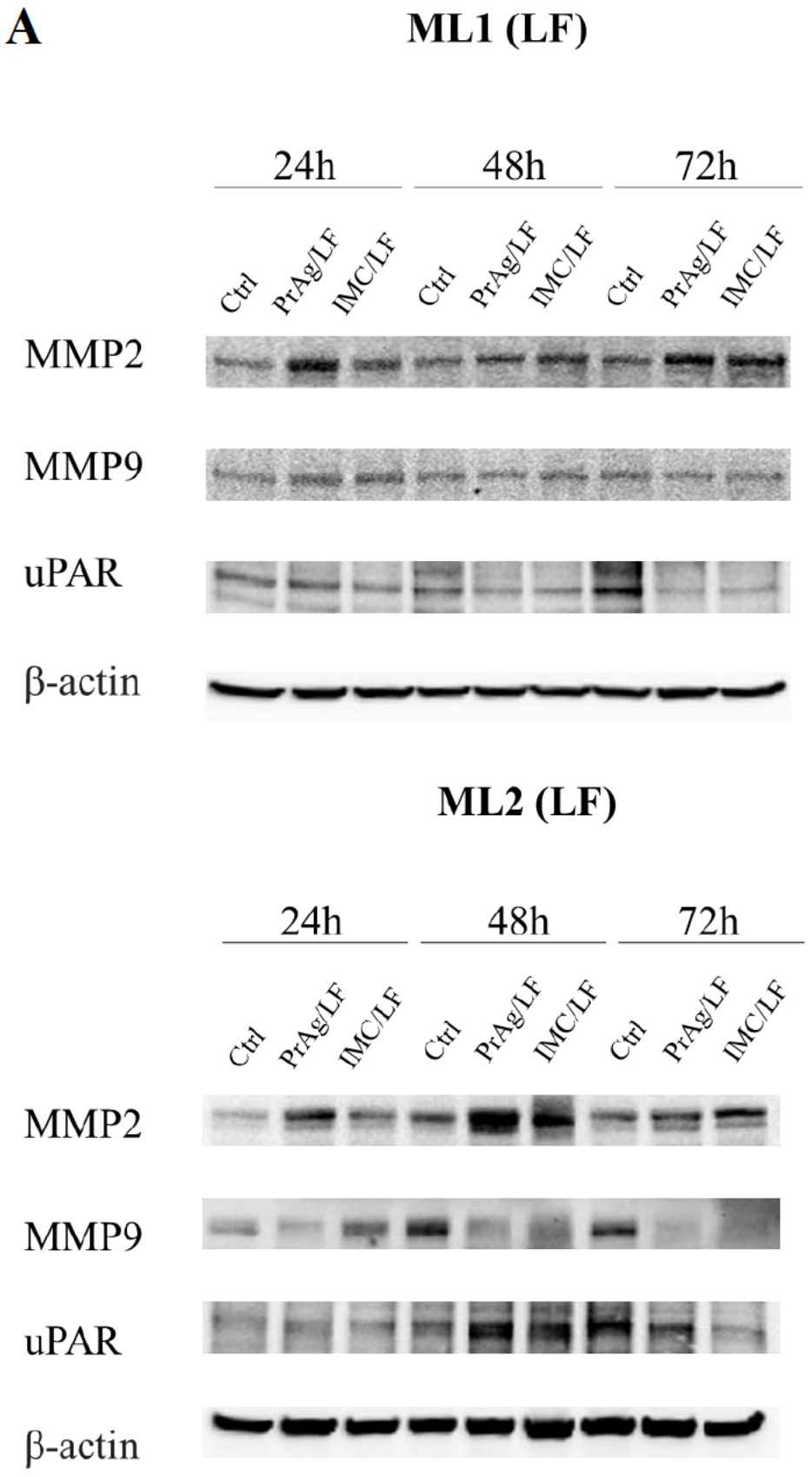
Previously, IMC/LF showed no cytotoxic effect on the AML cell lines tested, however MMP2, MMP9, and uPAR were basally expressed in almost all of them. As such we wanted to explore whether treatment with IMC/LF is leading to a downregulation of MMP2, MMP9 and uPAR, that might explain their response to the cytotoxicity of IMC/LF.

To investigate the impact of PrAg/LF and IMC/LF, we detected the expression of MMP2, MMP9 and uPAR before and after 24 h, 48 h, and 72 h of treatment in ML1, ML2, U937, TF1-vSrc, and TF1-HaRas AML cell lines.

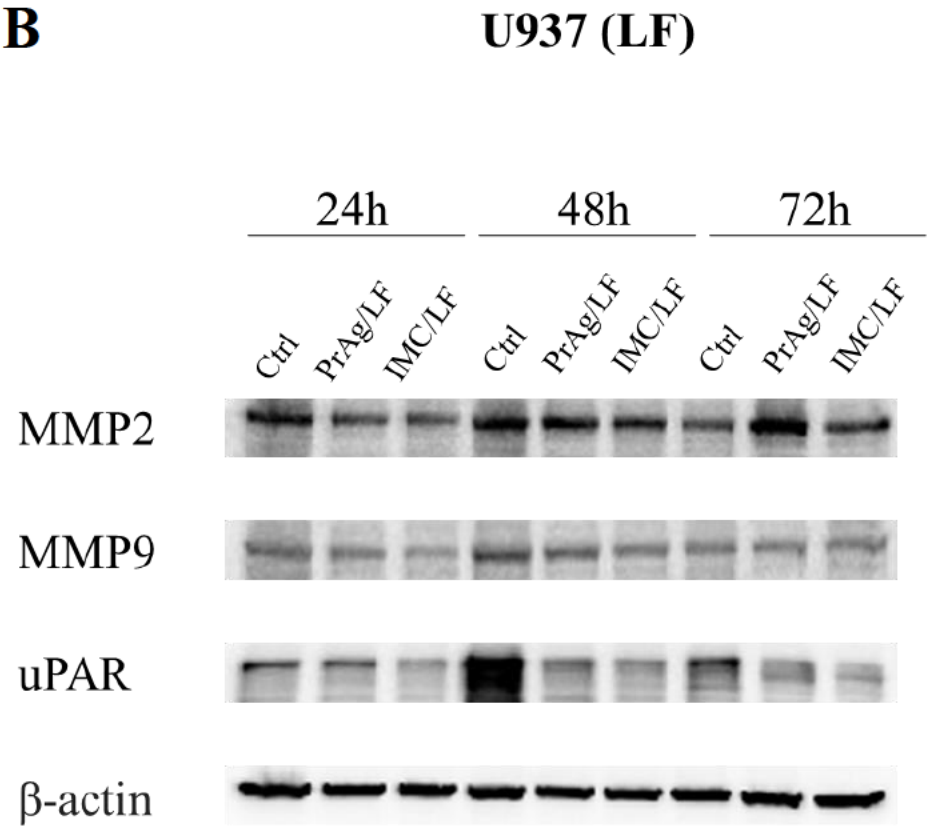
We demonstrated that MMP2 expression, in most cell lines, increases with PrAg/LF and IMC/LF treatments. However, investigating the levels of MMP9 and uPAR

showed the absence of a unified pattern of impact on their expression, where they were either upregulated, downregulated, or unaffected at different points (Figure 14 A, B and C).

In this investigation, PrAg/LF and IMC/LF both showed evidence of affecting the expression levels of MMP2, MMP9 and uPAR, even in cell lines that previously showed no sensitive response to IMC/LF treatments. However, these changes were not major with a unified pattern, hence the resistance towards IMC/LF is not due to the treatment causing a downregulation of the crucial proteases. The absence of a significant decrease in the expression to justify the lack of sensitivity towards IMC/LF treatment, further suggests that the activity of the proteases, not their expression, is insufficient to activate IMC/LF to get enough LF into the cells to induce cytotoxicity.


A
ML1 (LF)
24h
48h
72h
Ctrl
PrAg/LF
IMC/LF
MMP2
MMP9
uPAR
β-actin
ML2 (LF)

B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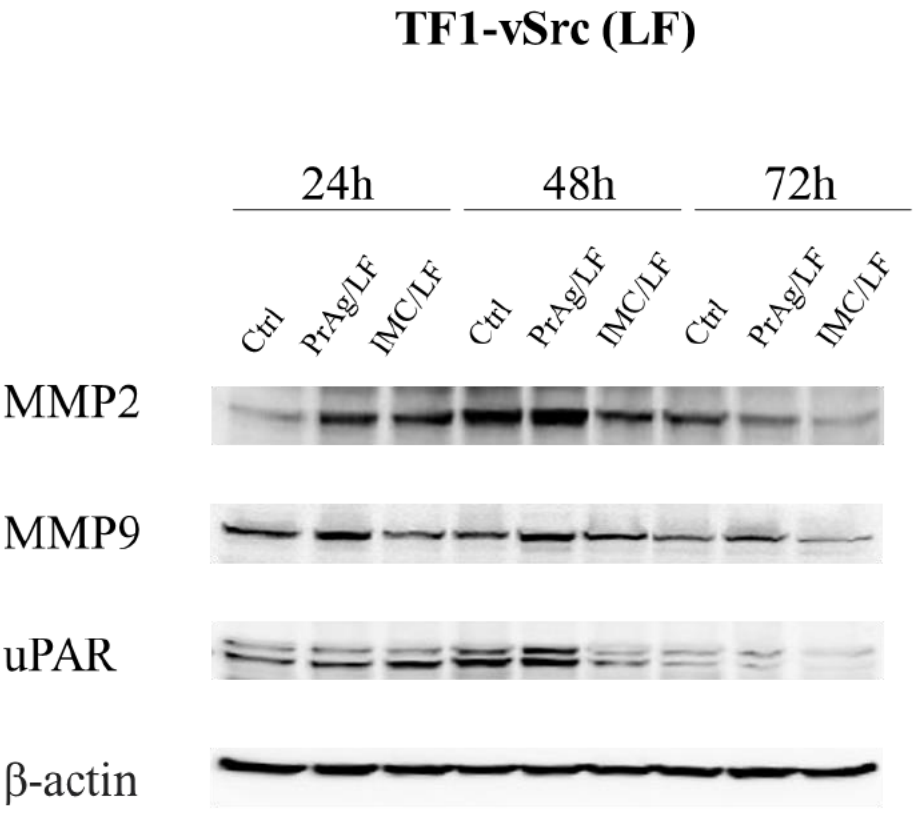
48h
72h
Ctrl
PrAg/LF
IMC/LF
MMP2
MMP9
uPAR
β-actin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72h
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PrAg/LF
IMC/LF
MMP2
MMP9
uPAR
β-actin

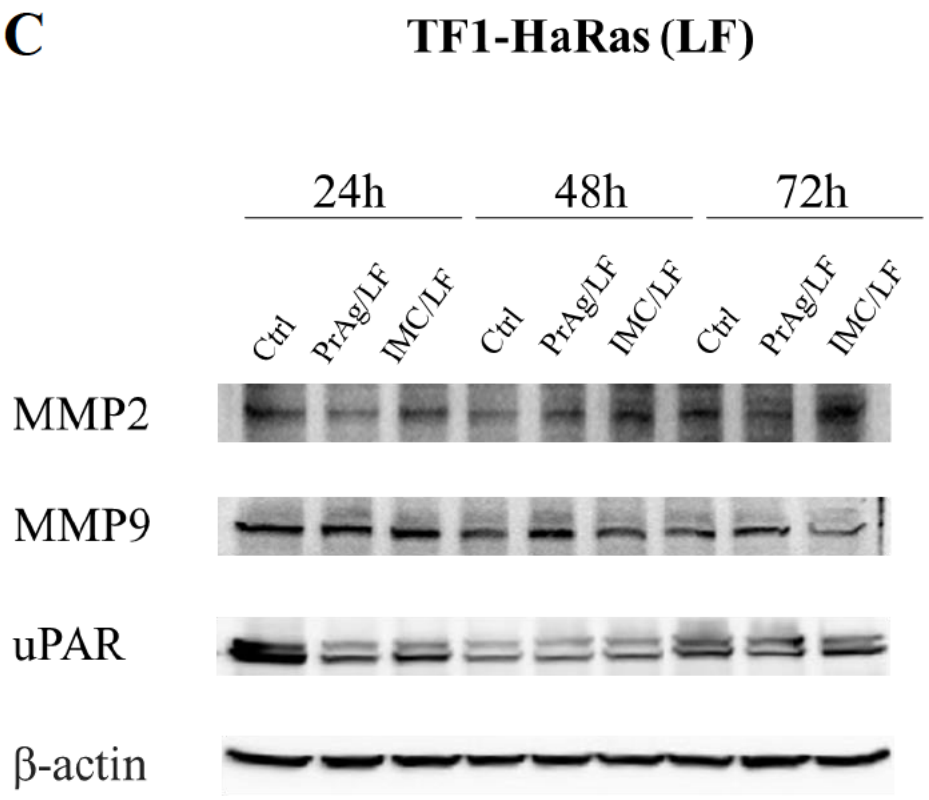


Figure 14:Effect of PrAg/LF and IMC/LF on the expression of MMP2, MMP9 and uPAR in AML cell lines. *(A, B, and C) Western blots showing the effect of PrAg/LF and IMC/LF on the expression of MMP2, MMP9 and uPAR after 24 h, 48 h, and 72 h of treatment in ML1, ML2, U937, TF1-vSrc, and TF1-HaRas. β-actin was used as a loading control (lower gel).*

3.4.3. The effect of PrAg/FP59 and IMC/FP59 on the level of expression

Furthermore, we wanted to investigate whether PrAg/FP59 and IMC/FP59 influence the expression of MMP2, MMP9 and uPAR, and if that correlates with the different levels of sensitivities seen towards IMC/FP59. As such, we tested a panel of 4 AML cell lines (ML2, U937, TF1-vSrc, and TF1-HaRas) for their expression of these proteins before and after treatment with PrAg/FP59 and IMC/FP59 at 24 h, 48 h, and 72 h. Notably, we couldn't examine ML1 at these conditions due to its extreme sensitivity to such treatments. We tried decreasing the concentration FP59 from 20 pM to 1 pM but still no significant surviving population was remaining to be analyzed.

Treatment of the moderately sensitive cell line ML2 with PrAg/FP59 caused a major decrease in the expression of MMP2 and MMP9, with a slight decrease in uPAR expression (Figure 15 A). This might be due to the fact that FP59 is a potent inhibitor of protein synthesis, leading even to a decrease in the expression of the loading control β-actin.

IMC/FP59 treatment of both ML2 and U937 either increased or didn't affect the expression of MMP2, MMP9 and uPAR when compared to the control untreated cells. As such, this demonstrates and explains why the sensitivity of these cell lines towards IMC/FP59 remains even at later time points (Figure 15 A and B).

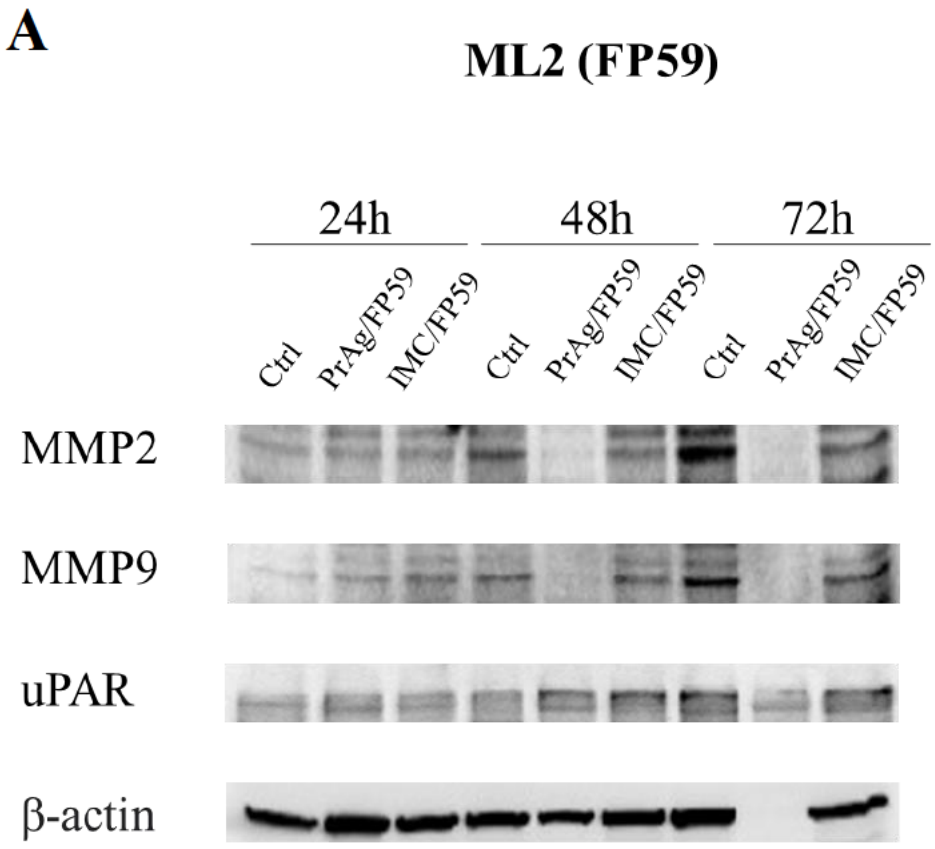
In addition, TF1-vSrc, the IMC/FP59 mildly sensitive but only at higher time points cell line, upregulated uPAR expression starting at 48h after treatment with IMC/FP59 while still expressing MMP2 and MMP9, which might indicate why this cell line is only responsive at high time points (Figure 15 B).

TF1-HaRas, had its uPAR downregulated after 72 h of IMC/FP59 treatment, which correlates with the insensitivity of this cell line towards IMC/FP59 treatment (Figure 15 C).

Notably, in some cases, the β-actin band in the lane treated with PrAg/FP59 is reduced. This might be due to the potent effect of this version, with FP59 inhibiting protein synthesis, and as such affecting the β-actin bands.

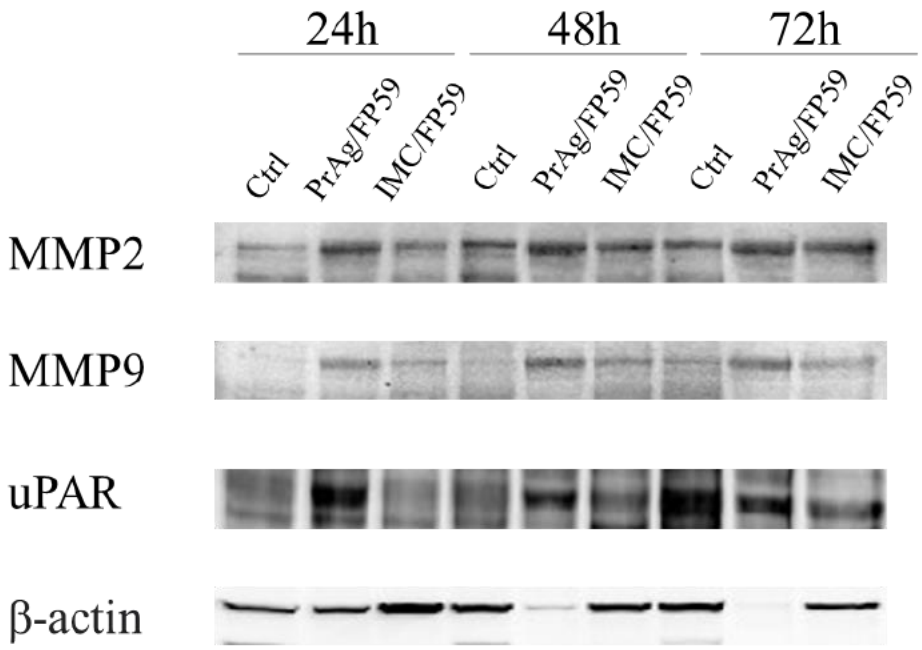
As a result, PrAg/FP59 and IMC/FP59 showed proof of affecting the expression levels of MMP2, MMP9 and uPAR in all the AML cell lines tested, both IMC/FP59 sensitive and resistant. This impact can either be a downregulation, upregulation, or no change in expression.

These changes did correlate to a certain extent with the sensitivity of the cell lines towards IMC/FP59. However, we still need to analyze the activity of the proteases, especially the uPA/uPAR system, to better understand the relationship between these proteases and the demonstrated sensitivity of the different cell lines.

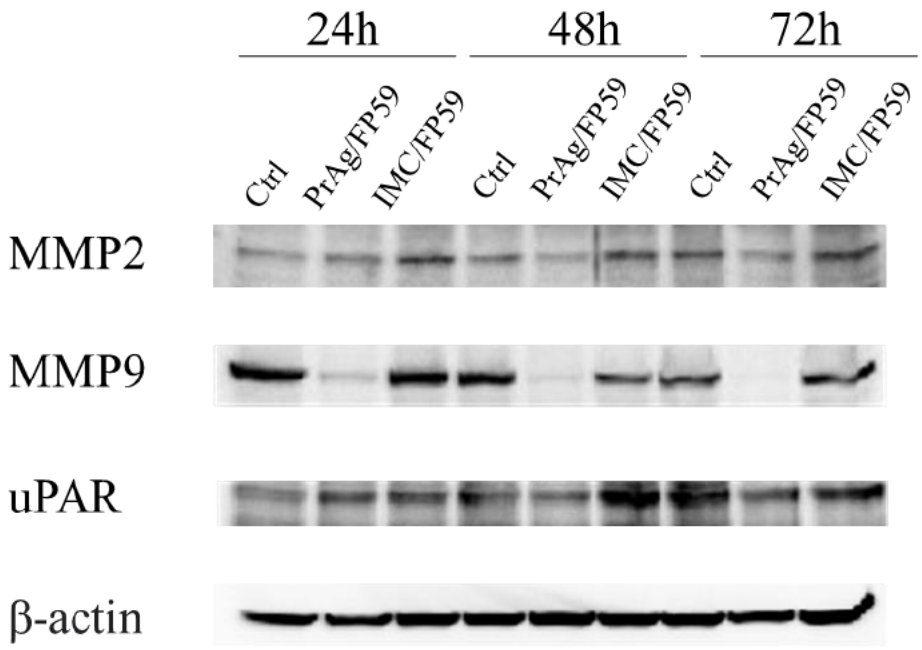


B

U937 (FP59)



TF1-vSrc (FP59)



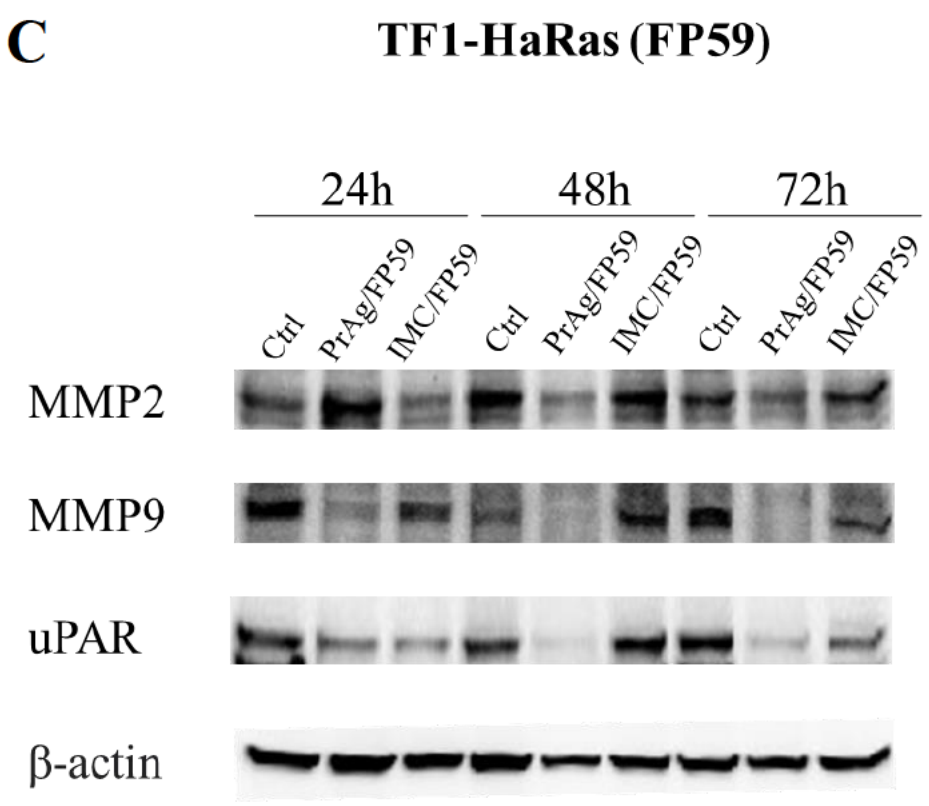


Figure 15:Effect of PrAg/FP59 and IMC/FP59 on the expression of MMP2, MMP9 and uPAR in AML cell lines. *(A and B) Western blots showing the effect of PrAg/FP59 and IMC/FP59 on the expression of MMP2, MMP9 and uPAR proteases after 24 h, 48 h, and 72 h of treatment in ML2, U937, TF1-vSrc, and TF1-HaRas, along with the Non-Linear regression curves that demonstrate the response of every cell line to PrAg/FP59, IMC/FP59, PAU2R200A/FP59 alone, and PAL1I207R/FP59 alone. β-actin was used as a loading control (lower gel).*

CHAPTER FOUR

DISCUSSION

AML is one of the most prevalent types of leukemias in adults. It continues to have a poor long-term survival rate with a large percentage of patients failing to achieve complete remission with the current treatment that is aggressive and doesn't suit all patients, especially the elderly. As such, this implies the necessity of novel targeted therapeutics for AML, to achieve a new era of treatment with enhanced responses, better survival rates and minimized toxicity from off-target effects (De Kouchkovsky et al., 2016).

Our laboratory and others have previously proved Anthrax Lethal Toxin (PrAg/LF) to be an effective targeted therapeutic in many human cancers, including melanoma and AML, demonstrating its toxicity on cells that depend on the MAPK pathway for survival, (Kassab et al., 2013) (Liu et al., 2009). However due to its off-target effects and *in vivo* toxicity, its tumor selectivity required further refining. Therefore, by taking advantage of the fact that the activation of PrAg requires cleavage by cell surface furin-like proteases, the first generation of more selective PrAg variants were generated termed PrAgU2 and PrAgL1. Their selectivity correlates with the fact that their activation mandates proteases that are abundant on the surface of tumor cells and not normal cells (Liu et al., 2005) (Liu et al., 2009), whereby PrAgU2/LF requires activation by the uPA/uPAR tumor specific protease system , while PrAgL1/LF mandates activation by the tumor expressed MMPs (Liu et al., 2003). Many studies,

have previously shown that both of these variants combined with LF did retain the potency observed with PrAg, while having an enhanced selectivity (Bekdash et al., 2015) (Liu et al., 2000). Moreover our laboratory tested both of these variants on AML cell lines and demonstrated their potency, as such showing that AML cells express both the uPA/uPAR system and MMPs (Bekdash et al., 2015)(data not published). This has paved the way to generate a modified intermolecularly complementing PAU2R200A-PAL1I207R version of PrAg that mandates two distinct proteolytic activities overexpressed by tumor tissues for its activation, both the uPA/uPAR system and MMPs simultaneously. As such, this version is thought to restrict its activation to cancerous cells, requiring no less than two criteria for its activation , thereby improving its selectivity (Liu et al., 2005).

In this study, we are the first to conduct testing of PAU2R200A-PAL1I207R intermolecularly complemented version (IMC) on AML cell lines. We started by conjugating the IMC version to LF and testing its potency on AML cells. IMC/LF demonstrated no cytotoxicity as opposed to PrAg/LF which suggests that AML cells may not have sufficient simultaneous activity levels of the uPA/uPAR protease system and the MMPs to sufficiently activate IMC/LF and translocate enough LF into the cytosol to inhibit the MAPK and cause cytotoxicity.

As a result, we then shifted to combining the IMC version to FP59, a more potent protein synthesis inhibitor. Out of the seven tested AML cell lines, two showed high sensitivity to the treatment of IMC/FP59 with IC_{50} values in the pM range, two others demonstrated moderate sensitivity, one cell line was moderately sensitive but only at later time point, and two cell lines were resistant. IMC/FP59 induced toxicity in AML aligns with previous findings that demonstrated its cytotoxicity to mouse melanoma B16-BL6 cells (Wein et al., 2015). Limited information exists on the expression and

activity level of uPA/uPAR and MMPs proteases on cancer cell lines, such studies are needed to further determine the source behind the different levels of toxicity demonstrated.

Importantly, a pattern was identified where the sensitivity of cells towards IMC/FP59 matched that of PAU2R200A/FP59 alone, while PAL1I207R alone wasn't cytotoxic at any point. This finding highlights the uPA/uPAR system, that is responsible for the activation of PAU2R200A, to be the rate limiting factor in the activation of IMC/FP59 in AML cells.

Even though IMC/LF did not show any signs of cytotoxicity on AML cell lines, we have shown that it significantly induces G0/G1 cell cycle arrest in a subset of cell lines. Moreover, we have further illustrated the cytotoxicity of IMC/FP59 by an increase in the percent of cells in the pre-G0/G1 phase in the subset of cells that are IMC/FP59 sensitive, displaying its cytotoxic effect. The cell cycle arrest analysis did correlate with the proliferation inhibition assay data, whereby the IMC/FP59 sensitive cell lines had their cell cycles arrested with IMC/LF, while those that are mildly sensitive or resistant had their cell cycle unaffected. The cell cycle effect exerted by IMC/LF demonstrates that LF is being translocated into the cytosol, however it is the activation level of IMC/LF by the proteases that is not high enough to translocate sufficient LF to cause cytotoxicity, but is enough to cause cell cycle arrest, rendering its effect as cytostatic.

Analysis of the mechanism of cell death in AML cell lines following IMC/FP59 treatment, revealed a significant double positivity staining with both annexin V and PI in the IMC/FP59 sensitive cell lines which indicates phosphoserine exposure and loss of membrane integrity, respectively. As such the mechanism of cell death appears to be non-apoptotic, however further studies should be done to eliminate apoptosis as a

contributing factor. Along with the double positivity staining, only one cell line (ML2) demonstrated another population that stained positively with annexin V only and not PI, following treatment IMC/FP59. Demonstrating that multiple mechanisms of cell death, apoptotic and non-apoptotic, might be involved for this cell line. Notably, IMC/FP59 showed a higher percentage of living population, and a lower but significant double positivity staining than PrAg/FP59. These findings strongly correlate with its higher IC_{50} values identified with the proliferation inhibition assay.

Furthermore, since the proliferation inhibition assay data showed that PAU2R200A/FP59 was cytotoxic to IMC/FP59 sensitive cells in a comparable manner to IMC/FP59, we hypothesized that the sensitivity to IMC/FP59 is dependent on uPAR expression. We first detected the basal levels of expression of MMP2, MMP9, and uPAR in a panel of 6 AML cells. The western blot analysis from two different trials along with their quantification demonstrated that MMP2 and uPAR have basal levels of expression in all tested cell lines with even TF1-HaRas, a resistant cell line, having significant levels of both. However, IMC/FP59 sensitive cell lines ML1, ML2, and U937 demonstrated to lack the basal expression of MMP9. These findings did not correlate with the sensitivity of cells towards IMC/FP59, and as such they indicate that the expression of uPAR might not depict the sensitivity towards IMC/FP59, since even resistant cells have a high significant expression. Subsequently, the uPA/uPAR activity rather than expression should be examined. In addition, these findings negate MMP9 from being a critical factor for the sensitivity of cells towards IMC/FP59, since sensitive cells lacked MMP9 expression.

We next explored whether treatment with IMC/LF is leading to a downregulation of MMP2, MMP9 and uPAR, that might explain their resistance towards IMC/LF. In this study, both PrAg/LF and IMC/LF demonstrated evidence of affecting MMP2, MMP9

and uPAR expression levels. However, over time such changes were not significant and lacked a unified pattern that justifies and correlates with the resistance towards IMC/LF. These observations indicate that the resistance of AML cells towards IMC/LF is not attributed to a downregulation of the crucial proteases after treatment. And as such, further suggest that the activity of the proteases, not their expression, is insufficient to activate IMC/LF to get enough LF into the cells to induce cytotoxicity.

Moreover, the effects of PrAg/FP59 and IMC/FP59 on MMP2, MMP9 and uPAR expression were displayed. PrAg/FP59 induced a major decrease of MMP2 and MMP9 expression in a subset of cells. These observations might be the result of FP59 inhibiting protein expression, whereby it even reduced the expression of β-actin, the loading control. A different loading control that is independent of FP59 activity should be used for further analysis.

This study also demonstrated that IMC/FP59 affects MMP2, MMP9 and most importantly uPAR expression in a way that correlates with their sensitivity status. Whereby, the sensitive ML2 and U937 express uPAR at all time points after treatment, while the mildly sensitive but only at later time points (TF1-vSrc) had its uPAR expression upregulated starting at 48 h post treatment with IMC/FP59, and the resistant cell line (TF1-HaRas) had its uPAR downregulated after 72 h of IMC/FP59 treatment. All these findings show that uPAR expression aligns with the response of cell lines to IMC/FP59. However, it is not sufficient to look at their expression, we will have to look at their activity levels to confirm such correlation.

Moving forward, future studies should focus on the advantage of this IMC PrAg version by investigating its effect on normal cells *in vitro* and *in vivo* to demonstrate its selectivity and lower non-specific toxicity compared to the previous versions. We also need to dive deeper into the mechanism of cell death induced by the IMC version,

while we have shown that IMC/FP59 induced non-apoptotic cell death, we still need to test for caspase activity to confirm whether or not apoptotic cell death is taking place.

Moreover, recent studies have shown that, although autophagy is considered to be a survival mechanism, it can be deleterious in some cases causing cell death (Kimmelman, 2011). Investigation of the role of autophagy in the IMC/FP59-mediated cell death is a must, which can be accomplished by detecting the autophagosomes or autophagy specific markers via flow cytometry, western blot, or immunostaining.

In this study we highlighted the uPA/uPAR system to be the rate limiting factor in the activation of the IMC/FP59, we also demonstrated that the protease activity is not sufficient to translocate enough LF to cause cytotoxicity with the IMC/LF, we as well showed indication that MMP9 expression might not be critical for the sensitivity of cells. However, to further confirm such findings, the activity of the protease systems should be extensively studied, not just their expression. This investigation will happen next, whereby we will detect the activity of the uPA/uPAR system via flow cytometry and we will try to correlate that to the levels of sensitivity we demonstrated. In addition, since MMP9 might not be crucial for the sensitivity of cells to be demonstrated, looking at the activity and role of other MMPs might reveal important involved factors. Furthermore, such findings can also be supported by implicating protease inhibitors such as uPA/uPAR inhibitors and determining whether such inhibitors will reverse the cytotoxicity seen by the IMC/FP59 on the AML cells.

CHAPTER FIVE

CONCLUSION

In conclusion, our study revealed that AML cells have different levels of sensitivity towards IMC/FP59, that can be divided into four groups of highly sensitive, moderately sensitive, only sensitive at later time points, or resistant. Importantly, the uPA/uPAR system indicated to be the rate limiting factor in the activation of IMC/FP59. In addition, although IMC/LF did not induce cytotoxicity in the tested cell lines, it demonstrated to cause cell cycle arrest in the subset of cells that is IMC/FP59 sensitive. Which indicated that LF is being translocated into the cytosol of cells, however the activation level of IMC/LF is not high enough to allow sufficient LF translocation to inhibit the MAPK pathway and induce cytotoxicity, but is high enough to cause cell cycle arrest, rendering its affect as cytostatic. Moreover, we demonstrated that the IMC/FP59-mediated cell death is non-apoptotic showing evidence of double positivity for annexin V/PI staining. Its double positivity staining was significant but lower when compared to PrAg/FP59 which correlates with its higher IC_{50} values. Furthermore, the basal level of expression of MMP2, MMP9 and uPAR did not correlate with the sensitivity of cells towards IMC/FP59, demonstrating that the activity of uPAR, rather than its expression, might be depicting the sensitivity. Moreover, expression of MMP9 showed not be crucial for IMC/FP59 sensitivity to manifest. In addition, we showed that the resistance of AML cells to IMC/LF is not attributed to a downregulation of the crucial proteases after treatment. We also demonstrated that over treatment with IMC/FP59, the uPAR expression does align with the response of cell lines to IMC/FP59, whereby IMC/FP59 sensitive cells

maintained uPAR expression over time. However, we still need to look at the activation level of the cell surface proteases and correlate them to the different levels of sensitivity seen.

The intermolecularly complementing PrAg version, PAU2R200A- PAL1I207R, demonstrated for the first time its potency and mechanism of action on AML, showcasing it to be a highly promising, more selective, targeted therapeutic.

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