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Dual Targeting of Matrix Metalloproteases and the MAPK Pathway in Acute Myeloid Leukemia Cells Using an MMP-activated anthrax lethal toxin (PrAgL1/LF)

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Dual Targeting of Matrix Metalloproteases and the MAPK Pathway in Acute Myeloid Leukemia Cells Using an MMP-activated anthrax lethal toxin (PrAgL1/LF)

Ahmad Al Saneh

ABSTRACT

Anthrax lethal toxin is a two-component toxin consisting of protective antigen (PrAg), the cell binding and translocation domain, and lethal factor (LF), the catalytic domain, which cleaves all mitogen-activated protein/extracellular regulated kinase kinases (MEKs), leading to the inhibition of the MAPK pathway. In order to increase selectivity of this toxin, we have replaced the furin cleavage site with a matrix metalloprotease cleavage site generating an MMP-activated anthrax lethal toxin; PrAgL1/LF. In this study, we attempt to target both the mitogen-activated protein kinase pathway and matrix metalloproteases in acute myeloid leukemia (AML) cell lines. Additionally, we also determine the level of autophagy activation, through quantification of autophagosomes on flow cytometry, and the impact of its inhibition, using the autophagy inhibitor chloroquine (CQ), on cell death secondary to MAPK inhibition. Moreover, we attempt to vertically inhibit the MAPK pathway in AML cell lines in order to overcome acquired resistance of AML cell lines to PrAg/LF by targeting the MAPKK protein MEK-1/2 as well as the MAPKKK protein ERK-1/2 via SCH772984, showing that coupling the two inhibitors may offer a novel targeting method in order to overcome acquired resistance to MEK-1/2 inhibitors. Our results demonstrate that vertical inhibition does offer increased potency in comparison to single targeting of the MAPK pathway when working with sensitive cell lines that are dependent on the cascade. Several cell lines tested were sensitive to PrAgL1/LF indicating that they express MMPs and are sensitive to the inhibition of the MAPK pathway. The expression of MMPs by AML cell lines was further confirmed by their sensitivity to PrAgL1/FP59, an MMP activated version of anthrax toxin whose cytotoxicity is not related to their dependence on the MAPK pathway. Inhibition of the MAPK pathway through PrAgL1/LF led to sustained activation of autophagy, starting at 24 hours and lasting up to 120 hours following treatment with PrAgL1/LF in the AML cell lines. Addition of CQ led to an increase in cell cytotoxicity following treatment with PrAgL1/LF, at all time points, indicating that the inhibition of the MAPK pathway in AML cells leads to the activation of autophagy, which in turn mediates cell death following treatment with PrAgL1/LF. Our study shows that AML cells express MMPs and can be targeted using an MMP-activated anthrax lethal toxin and that the LF-mediated inhibition of the MAPK pathway activates autophagy which is protective at later time points.

Key words: Anthrax Lethal Toxin, Autophagy, PrAg/LF, ERK inhibitor, Acute Myeloid Leukemia, Mitogen Activated Protein Kinase Pathway (MAPK).

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PrAg/LF	Furin-activated Anthrax Lethal Toxin
PrAgL1/LF	Matrix metalloprotease-activated Anthrax Lethal Toxin
PAU2/LF	Urokinase-activated Anthrax Lethal Toxin
AML	Acute Myeloid Leukemia
MAPK	Mitogen-activated protein kinase
MEK	Mitogen-activated protein kinase kinase
ERK	Extracellular signal-regulated kinase
FP59	Pseudomonas aeruginosa exotoxin A
CQ	Chloroquine
PrAg	Protective antigen
LF	Lethal Factor
EF	Edema Factor
MM-1	Mono-Mac-1
MM-6	Mono-Mac-6
mTOR	Mammalian target of rapamycin

Chapter One

Introduction

1.1 Acute Myeloid Leukemia

The cancer that takes place in blood/blood forming organs is referred to as leukemia. It can be classified into different types, primarily depending on whether it is acute (fast and aggressive growth) or chronic (slow growth and may be dormant for several years), and whether it begins in myeloid progenitors or lymphoid cells. Acute lymphocytic leukemia (ALL), Acute myeloid leukemia (AML), Chronic lymphocytic leukemia (CLL) and Chronic myeloid leukemia (CML) are considered to be some of the most severe types of leukemia (Redaelli et al., 2003). Around 8% of human malignancies are caused by leukemias and lymphomas (Cooper, 2000). "Acute lymphoblastic leukemia (ALL) is the second most common acute leukemia in adults, with an incidence of over 6500 cases per year in the United States alone. The hallmark of ALL is chromosomal abnormalities and genetic alterations involved in differentiation and proliferation of lymphoid precursor cells" (Terwilliger & Abdul-Hay, 2017). Chronic lymphocytic leukemia (CLL) is characterized by the clonal development and aggregation of neoplastic B lymphocytes in the blood, bone marrow, lymph nodes, and spleen (Zhang & Kipps, 2014). Chronic myelogenous leukemia (CML) is a long-standing myeloproliferative disease with prolonged course lasting for three to five years (Cortes et al., 1996). Eventually CML alters into an accelerated blast phase, which is generally lethal. Chronic myeloid leukemia is actually one of the first diseases with a chromosomal aberration identified to its name: Philadelphia chromosome, t(9;22)(q34;q11) (Cortes et al.,

1996). The prevalence of AML cases in men is higher than that in women, while the average lifetime risk of AML in both sexes is about half of 1% (Sami et al., 2020). Based on the American Cancer Society's evaluations of AML cases in the USA for 2019, there will be 21,450 new cases and 10,920 deaths from Acute Myeloid Leukemia (Sami et al., 2020). Of all cases of leukemia, AML accounts for 32% of cases in adults. The 5-year survival rate is just about 24% for adults (Sami et al., 2020).

1.1.1 Diagnosis of AML

Acute myeloid leukemia is considered to be a clinically and biologically heterogeneous disease which is characterized by the uncontrolled production of hematopoietic precursor cells (referred to as blasts), which would ultimately lead to the impairment of the production of normal blood cells and eventually cause symptoms such anemia and thrombocytopenia (Tallman et al., 2005). Bone marrow failure and impaired hematopoiesis are consequences of such clonal proliferation of undifferentiated myeloid precursors (Papaemmanuil et al., 2016). AML is diagnosed with 20 percent or more blasts in the bone marrow or peripheral blood (Döhner et al., 2010).

1.1.2 AML Classification

"The potential of the transformed (leukemic) multipotential hematopoietic cell to differentiate and mature along any myeloid lineage forms the basis for the phenotypic classification of acute and chronic myelogenous leukemia" (Lichtman & Segel, 2005). However in several cases, the genotype is heterogeneous, thus making phenotypic classification by the dominant lineage expressed difficult (Lichtman & Segel, 2005). Cytogenetics allow AML to be distinguished on the basis of their DNA mutations, thereby allowing classification according to the likelihood

that treatment would be efficient against the AML subtype (Lichtman & Segel, 2005).

There are two widely adapted classification systems that allow the proper distinction of the different subtypes of acute myeloid leukemia (De Kouchkovsky & Abdul-Hay, 2016). The French-American-British (FAB) classification is the first established system that represents eight subtypes (M0 through M7) of AML based on morphology of cells, as well as their cytochemical characteristics (De Kouchkovsky & Abdul-Hay, 2016). Aiming to better define acute myeloid leukemia subtypes in a more specific manner, the World Health Organization introduces a classification system that incorporates genetic information with morphology, immunophenotype and clinical presentation to define six disease entities: "AML with recurrent genetic abnormalities; AML with myelodysplasia-related features; therapy-related AML; AML not otherwise specified; myeloid sarcoma; and myeloid proliferation related to Down syndrome" (De Kouchkovsky & Abdul-Hay, 2016). The WHO system has been updated over the years (2001, 2008, 2016) which establishes it as a widely adapted classification system for AML (De Kouchkovsky & Abdul-Hay, 2016). These six major disease entities are also further categorized to incorporate more specific genetic information. One example is the eleven delineated subtypes of "AML with recurrent genetic abnormalities" based on different chromosomal translocations (De Kouchkovsky & Abdul-Hay, 2016).

1.1.3 Standard AML Therapy

While many AML patients respond to induction chemotherapy, refractory disease is common and relapse is the leading cause of failure in treatment (Papaemmanuil et al., 2016). That being said, different types of leukemia have different outlooks and approaches for therapy.

The first step for eligible acute myeloid leukemia patients is to undergo induction therapy in order to achieve CR (complete remission). Unfortunately, relapse would still have a high chance of occurrence if treatment is discontinued considering minimal residual disease often persists in CR (De Kouchkovsky & Abdul-Hay, 2016). Therefore, in order to eradicate any residual disease, induction therapy should be followed by consolidation therapy and achieve lasting remission (De Kouchkovsky & Abdul-Hay, 2016). The foundation of induction therapy consists of the '7+3' regimen, which combines 7 days of continuous infusion cytarabine with 3 days of anthracycline. It is usually applicable to patients with an intermediary to favorable prognosis and a low risk of treatment related mortality (De Kouchkovsky & Abdul-Hay, 2016). While studies have shown greater efficiency at higher doses, this added benefit is limited and generally increased at the cost of increased toxicity (De Kouchkovsky & Abdul-Hay, 2016). Thus, induction therapy with high-dose cytarabine is usually reserved for refractory disease (De Kouchkovsky & Abdul-Hay, 2016).

Bone marrow aspiration or biopsy are efficient clinical techniques that allow the assessment of an AML patient's response to induction therapy after two weeks of treatment (Döhner et al., 2010). Almost up to 50% of AML patients require additional courses of induction therapy after the first cycle, due to the importunate cytological evidence of the disease (Büchner et al., 2012). Eventually, most of the patients with newly diagnosed AML achieve CR with standard induction therapy (Büchner et al., 2012). As for consolidation therapy, it should be assigned to patients in remission to in turn exterminate remaining disease and avoid reversion. Chemotherapy and allogeneic hematopoietic stem cell transplant (allo-HSCT) are considered available options for consolidation (De Kouchkovsky & Abdul-Hay,

2016). Post induction therapy encompasses additional chemotherapy courses carried out with the same intensities as before; yet, the exact number of courses is uncertain (Burnett et al., 2011). For that matter, a study carried out by the United Kingdom Medical Research Council have tackled this issue and currently agree that a number of four courses, including induction, is considered to be adequate (Burnett et al., 2011).

Another possible approach is stem cell transplant (SCT). One such technique is autologous SCT which displays efficient anti-leukemic activity in comparison to other treatments such as chemotherapy (Burnett et al., 2011). However, autologous SCT does not evidently demonstrate increased overall survival (OS) (Burnett et al., 2011). Similarly, a well matched sibling can also act as an SCT donor, and such a technique has been clinically used for years (Burnett et al., 2011). However, considering the aggressive nature of such a treatment and the absence of an added benefit in terms of OS, this technique is usually reserved for second CR therapy (Burnett et al., 2011). This approach is also practical because it allows patients with low relapse risks to respond again in case they relapse, thus salvaging SCT for the second CR is efficient (Burnett et al., 2011). However, the potency of SCT is mostly demonstrated in patients with high-risk disease since their relapsed patients have low chances of getting a second response (Burnett et al., 2011). The risk score of SCT can actually be weighed and quantified through taking into account several factors such as age, degree of matching and female donor parity (Burnett et al., 2011). Unfortunately, relapse can still occur in around 40% of AML patients after stem cell transplant, as well as in two-thirds of patients with *de novo* AML within the first 18 months after CR (Yilmaz et al., 2019) (Tsirigotis et al., 2016).

1.1.4 Targeted Therapeutics

The fact that acute myeloid leukemia is considered to be a biologically and clinically heterogeneous disease renders progresses in supportive treatment and prognostic risk stratification that have streamlined proven therapies poor (De Kouchkovsky & Abdul-Hay, 2016). Innovative targeted therapies deliver the promise of operative anti-leukemic activity with decreased non-target toxicity (De Kouchkovsky & Abdul-Hay, 2016).

"Targeted therapy involves developing drugs that block cancer cell proliferation, promote cell cycle regulation or induce apoptosis or autophagy and targeted delivery of toxic substances specifically to cancer cells to destroy them. Targeted therapy involves the use of monoclonal antibodies or oral small drugs" (Padma, 2015). Combining antibody engineering with display and screening advances aided in monoclonal antibody production, such as phage display which facilitated the binding of antibody to a wide range of targeted antigens with unique specificity (Padma, 2015). Taking into account the relatively large nature of antibodies, they are generally administered intravenously (Padma, 2015). Monoclonal antibodies can also be used as targeting agents instead of just therapeutic agents. They can facilitate the delivery of active therapeutics in targeted therapy, in addition to prodrug activation enzymes chemotherapeutic drugs and radioactive substances, straight into tumor cells (Padma, 2015). Their mode of action involves blocking a particular target surrounding cancer cells (Padma, 2015). The small molecular inhibitors on the other hand, target molecular components that generally aid in specific cell functions such as proliferation, metastasis, or angiogenesis, which are imperative for cancer cell survival. Advances in cancer drug discovery and development revolve around small molecule inhibitors that function against new molecular targets which govern

therapeutic outcomes (Padma, 2015).

Another example of targeted therapeutics are fusion proteins such as “DAB389IL-2 (denileukin diftitox; ONTAK)” which is one of the first synthesized and utilized fusion proteins in its domain (Foss, 2000). Fusion proteins are characterized by combining a targeting mechanism (such as interleukin-2) to a cytocidal moiety (such as the enzymatic activity of the diphtheria toxin) (Foss, 2000). The human IL-2 sequence is engineered into the toxin to allow its targeting to cells displaying sufficient affinity IL-2 receptor on their surface (Kumar et al., 2019). This would permit further internalization of the toxin into the acidic cytosol upon binding, and the release of the catalytic domain through the pore formed by the translocation domain of the protein and exhibit protein synthesis inhibition (Kumar et al., 2019). This toxin’s potency has been widely displayed in an array of cancer and auto-immune diseases including HIV, psoriasis and B-cell non-Hodgkin’s lymphoma (Foss, 2000). This drug has also been proven to diminish human regulatory T cells which allows its successful usage in several human tumors (Kumar et al., 2019).

1.2 Anthrax Lethal Toxin

More novel and specific therapies are being developed to selectively target AML cells, without harming normal cells (Darwich, 2015). One such example of targeted therapy is the Anthrax Lethal Toxin (PrAg/LF) (Darwich, 2015).

Bacillus anthracis is a gram positive bacterium that discharges an exotoxin called the anthrax lethal toxin (Darwich, 2015). These virulent strains of the bacterium utilize the toxin to allow evading the immune system of their carrier and allow subsequent propagation and survival (Yilmaz et al., 2019). The bacterial spores can make their way into the organism through inhalation, permeation of the skin, or ingestion. Virulent strains of *Bacillus anthracis* acquire high levels of resistance and virulence

that allow them to be lethal to the host and thus result in the host's death shortly after infection. Although antimicrobials are efficient against the bacterium in early stages of the infection where the it is in its logarithmic phase and advancing in numbers, once cumulative levels of the toxins are secreted into the organism's bloodstream, the antibiotics lose their efficiency and the diseased host yields to the fatal consequences of the toxins (Yilmaz et al., 2019).

"Anthrax lethal toxin (PrAg/LF) is a binary toxin consisting of two proteins: protective antigen (PrAg) and lethal factor (LF). PrAg binds cells through its ubiquitously expressed receptors tumor endothelial marker–8 and capillary morphogenesis gene–2 and is cleaved by furin-like proteases leading to the generation of an active 63-kDa fragment (PrAg63). PrAg63 then forms oligomers, binds three to four molecules of LF, and undergoes endocytosis. Upon acidification of the endosome, PrAg63 oligomers undergo a conformational change leading to pore formation and translocation of LF into the cytosol. LF is a zinc metalloprotease that cleaves mitogen-activated protein/extracellular regulated kinase kinases (MEKs), leading to the inhibition of the MAPK pathway" (Bekdash et al., 2015).

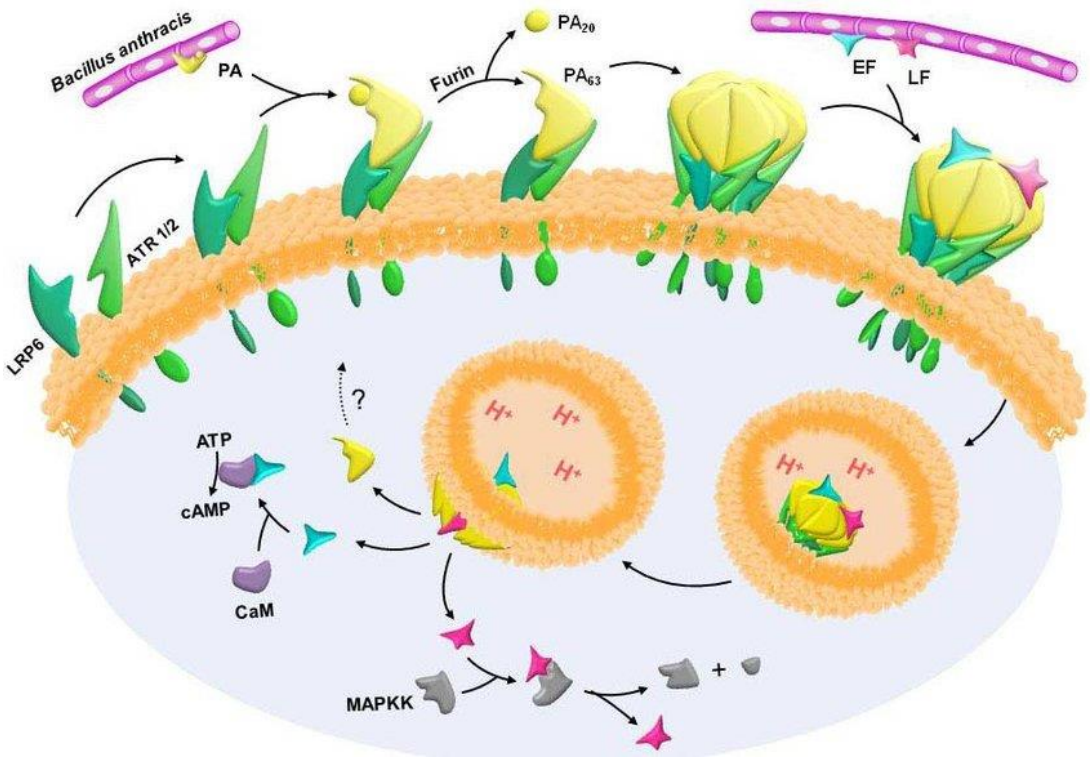


Figure 1: The mechanism of binding and activation of PA of the anthrax lethal toxin and the internalization of LF into the cell. (Adapted and modified from (Golden et al., 2009)).

1.2.1 Previous Work on Anthrax Lethal Toxin (PrAg/LF)

Anthrax lethal toxin has been widely used before and has proven efficacy as a potential targeted therapeutic in several human malignancies including Acute Myeloid Leukemia (Kasbo, 2015). Given the significance of MEK signaling in tumorigenesis, Duesbery et al., were among the first to assess the effects of anthrax lethal toxin (PrAg/LF) on tumor cells and found PrAg/LF to be efficient in impeding the activation of the mitogen-activated protein kinase activation in V12 H-ras-transformed NIH 3T3 cells (Duesbery et al., 2001). In vitro transformed cell treatment with the anthrax lethal toxin allowed the cells to return to their non-transformed morphology and repressed their abilities to form colonies in soft agar and to invade Matrigel without evidently affecting cell proliferation (Duesbery et al., 2001). As for in vivo studies, treatment with PrAg/LF inhibited growth of Ras-transformed cells implanted in athymic nude mice (in some cases causing tumor

regression) at concentrations that led to no evident animal toxicity (Duesbery et al., 2001). Unexpectedly, PrAg/LF also greatly reduced neovascularization in tumors (Duesbery et al., 2001). These results exhibit that PrAg/LF potently impedes tumor growth mediated by Ras and is a potential antitumor therapeutic (Duesbery et al., 2001).

Further studies have also established PrAg/LF's ability to reduce the levels of several proteins involved in triggering the MAPK signaling cascade. These proteins also play crucial roles in angiogenesis, tumorigenesis and metastasis of multiple human tumors, including renal cell carcinoma (RCC). PrAg/LF exposure decreased cell proliferation with no major effects on cell cycle progression and apoptosis. PrAg/LF was also proven to dramatically inhibit anchorage-independent growth of renal cell carcinoma cells. In addition, further in vivo studies demonstrated that tumor growth of renal cell carcinoma xenograft was greatly reduced upon treatment with PrAg/LF. The study also proved PrAg/LF's ability to induce extensive necrosis and a reduction of tumor neovascularization. PrAg/LF also exhibited direct obstruction of proliferation of endothelial cells in vitro (Kassab, 2013).

The ability of the anthrax lethal toxin to inhibit the highly activated MAPK pathway in melanoma shaped the use of PrAg/LF as an attractive method for melanoma therapy (Kassab, 2013). In a study carried out by Abi Habib et al., it was proven that anthrax lethal toxin is highly cytotoxic to a majority of melanoma cell lines and is efficiently selective, showing no cytotoxicity towards normal human melanocytes (Ralph J. Abi-Habib et al., 2005).

The anthrax lethal toxin's anti-melanoma efficacy was also exhibited *in vivo* (R. J. Abi-Habib et al., 2006). "When given systemically, PrAg/LF produced complete tumor regressions in a highly significant number of mice at all the doses and

schedules tested and up to 45 days after the end of treatment. Histologic examination of tumor injection sites at that point showed either a total absence of tumor cells or a persistence of a few degenerated cells, thus showing the ability of PrAg/LF to produce significant, long-term melanoma regression" (R. J. Abi-Habib et al., 2006).

1.2.2 Modifications of anthrax lethal toxin

Cytotoxicity of PrAg/LF can be enhanced by redirecting the internalization of LF, or any related toxin, by effectively altering the cleavage sequence required for proteolytic activation of PrAg, thus it will occur only in the presence of a stipulated proteolytic activity. (Peters et al., 2014). Matrix metalloproteinases (MMPs) are widely studied in cancer therapeutics as their overexpression in cancer tumors represents a novel target due to their role in extra-cellular matrix remodeling that allows tumor invasion and metastasis (S. Liu et al., 2000). Hence, a matrix metalloprotease-activated version of the anthrax lethal toxin (PrAgL1/LF) was engineered. Cell lines expressing MMPs should be capable of cleaving and activating the protective antigen, thus enabling the internalization of LF, leading to MEK protein inhibition in the mitogen activated protein kinase pathway (Arbuthnot et al., 2000). PrAgL1/LF also displayed a potent anti-tumor activity not only against human melanomas with BRAF V600E mutations, but also against other forms of human tumors irrespective of their BRAF status; for instance: colon and lung carcinomas and mouse tumors (S. Liu et al., 2008). Further experiments lead to the observation that PrAgL1/LF has the ability to obstruct the ability of primary endothelial cells to migrate toward a gradient of angiogenic factors, which is a vital step for tumor angiogenesis (S. Liu et al., 2008).

Several studies have demonstrated the potency of the matrix metalloprotease-activated anthrax lethal toxin (PrAgL1/LF) in antitumor activity against xenografts,

which previously displayed resistance towards the wild type, furin-activated, anthrax PrAg/LF (Alfano et al., 2010). PrAgL1/LF also seemed to inhibit the proliferation of anaplastic thyroid carcinoma xenograft, by reducing tumor vascularization and endothelial cell recruitment (Alfano et al., 2010). This effect was demonstrated in both: PrAg/LF-resistant and sensitive cell lines. Such results pave way for MMP-activated anthrax lethal toxin therapy against advanced tumors, thus exhibiting promising potential for clinical therapy of solid tumors with varying mutations (Alfano et al., 2010).

Another protease system that is overexpressed on tumor cells and virtually lacking from non-cancer cells is the urokinase plasminogen activator (uPA) (Bekdash et al., 2015). Thus by replacing the furin cleavage site of PrAg/LF with a corresponding urokinase-specific cleavage site (U2), a uPA-activated version of the anthrax lethal toxin was generated (Bekdash et al., 2015). PrAgU2/LF is a dual-selective toxin that targets two distinct tumor-specific markers: expression of the uPA/ uPAR system and dependence on the MAPK pathway for survival (Bekdash et al., 2015). PrAgU2/LF was proven to be cytotoxic to several AML cell lines as well as to primary blasts (Bekdash et al., 2015). Moreover, PrAgU2/LF appeared to induce non-apoptotic cell death, in AML cells. This sensitivity can be explained by the association of AML cells with the activity of both: MAPK and urokinase systems (Bekdash et al., 2015).

Anthrax lethal toxin name	PrAg/LF (Wild Type)	PrAgU2/LF	PrAgL1/LF	PrAgL1/FP59
Cell binding and translocation domain	Protective antigen activated by the presence of furin	Protective antigen activated by the presence of urokinase plasminogen activated system (uPA/uPAR)	Protective antigen activated by the presence of matrix metalloproteases	Protective antigen activated by the presence of matrix metalloproteases
Cytotoxic domain	*Bacillus anthracis* Lethal Factor	*Bacillus anthracis* Lethal Factor	*Bacillus anthracis* Lethal Factor	*Pseudomonas aeruginosa* exotoxin A

Figure 2: Different derivatives of the anthrax lethal toxin and PrAgL1/FP59 defining the cell binding and translocation domain as well as the cytotoxic domains of each.

Mitogen Activated Protein Kinase Pathway (MAPK)

There are several pathways that aid in a cell's survival and proliferation. However, the most vital of which for differentiation, apoptosis, metastasis and angiogenesis is the mitogen activated protein kinase pathway (MAPK) (Guo et al., 2020). The MAPK pathway comprises four cascades: "ERK, JNK/stress-activated protein kinase, p38 MAPK and ERK5 signal transduction pathways" (Guo et al., 2020). While some cascades play major roles in stress-induced apoptosis, the ERK/MAPK signaling pathway plays a more crucial role in signal transduction as well as proliferation of cells (Guo et al., 2020).

1.3.1 RAS-RAF-MEK-ERK Pathway

The ERK/MAPK signaling cascade comprises four major proteins involved in the regulation of the pathway: RAS, RAF, MEK and ERK (Guo et al., 2020). The upstream protein RAS is encoded by the *ras* oncogene (Guo et al., 2020). Upon the activation of a cell by an extracellular ligand such as the epidermal growth factor (EGF), a connector molecule called Grb2 (growth factor receptor-binding protein 2) binds to the receptor (Guo et al., 2020). Grb2 would then recruit and interact with

SOS (son of sevenless) to form a three-way complex with the receptor. Recruited SOS would thus allow switching the inactive conformation of RAS-GDP to an active conformation of RAS-GTP, thereby initiating the activation of the pathway (Guo et al., 2020). Upon its activation, RAS would then activate the downstream RAF protein kinase encoded by *raf.* RAF activation can be elicited in various ways, including but not limited to its dimerization, phosphorylation and dephosphorylation, or binding to the RAS kinase inhibitor (Guo et al., 2020). Activated isoforms of RAF such as Raf-1 would then proceed to phosphorylate and activate downstream MEK of the MAPK pathway (Guo et al., 2020). Consequently, MEK would exert its Tyr and Thr kinase activity by phosphorylating and activating downstream ERK, that would be anchored by MEK in the cytoplasm during its inactive state (Guo et al., 2020). Activated ERK would subsequently dimerize with itself upon activation and proceed to enter the nucleus in order to regulate the activity of various transcriptional and translational outputs. Considering the crucial physiological roles the ERK/MAPK pathway plays in a cell, it profoundly influences the cellular survival (Guo et al., 2020). Avruch *et al* also demonstrated the necessity of ERK dimerization by proving that phosphorylated ERKs dimerize with unphosphorylated ERKs before translocating into the nucleus to exert their transcriptional and translational modifications for gene expression (Avruch et al., 2001).

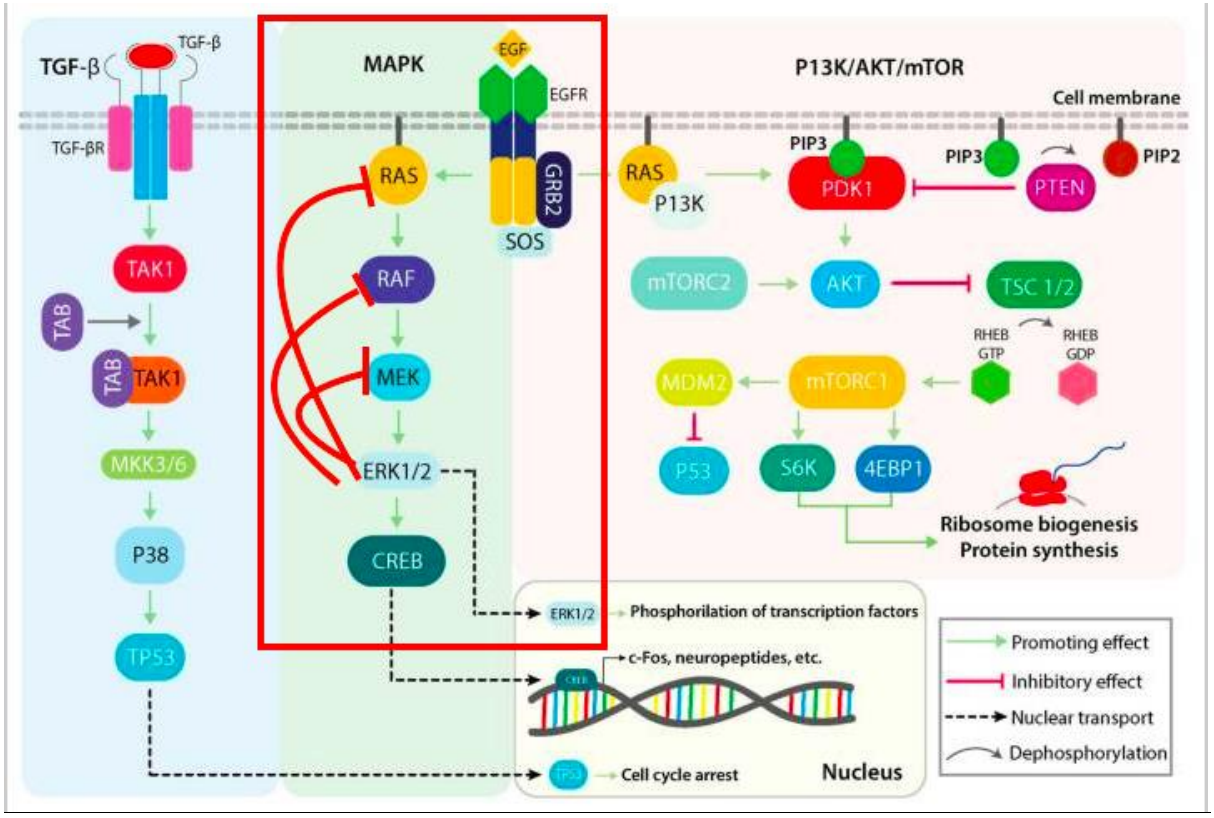


Figure 3: TGFβ/p38, Mitogen Activated Protein Kinase (outlined), and P13k/AKT/mTOR pathways. Under normal physiological conditions, the MAPK pathway is activated in a pulsatory manner where ERK-1/2 can halt its activation through inhibiting the activation of RAS/RAF/MEK. (Adapted and modified from (Braicu et al., 2019))

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

"The RAS/ RAF/ MEK/ ERK (MAPK) signaling cascade is essential for cell inter- and intra-cellular communication, which regulates fundamental cell functions such as growth, survival and differentiation. The MAPK pathway also integrates signals from complex intracellular networks in performing cellular functions" (Degirmenci et al., 2020). The presence of both positive and negative feedback loops plays a vital role in maintaining cellular homeostasis through the establishment of the pulsatile nature of the MAPK pathway (Amit et al., 2007). In contrast, mutations in the proteins of the MAPK cascade would lead to a constitutive activation of the pathway (Sever & Brugge, 2015). This higher basal enzymatic activity would eventually

allow an increase in proliferation and survival of the tumor cells harboring these mutations through an increase in transcriptional outputs (Sever & Brugge, 2015). Since the termination of the ERK signal plays a crucial role in regulating cell proliferation, the elicited MAPK pathway has to attain its initial basal status (Degirmenci et al., 2020). By the phosphorylation of multiple Serine/Threonine sites on RAF, ERK could employ negative feedback on RAF, breaking its interactions and inhibiting further dimerization of ERK (Degirmenci et al., 2020). As for MEK, N-terminus phosphorylation would allow the interception of its activity. Soon after inhibitory phosphorylation, the proteins of the signaling pathway would require de-phosphorylation in order to return to their basal/inactive state (Degirmenci et al., 2020).

Several mutations copious in Acute Myeloid Leukemia patients are associated with the Mitogen Activated Protein Kinase Pathway. Hence, shaping the pathway as a more attractive target for inhibition. One such mutation is the aberrant regulation of FLT-3. Under normal physiological conditions, FLT-3 is expressed by hematopoietic stem cells and progenitors (Thiele et al., 2012). FLT-3 is a transmembrane RTK activated by signaling ligands (Thiele et al., 2012). This RTK aids in promoting cell proliferation, survival and differentiation (Daver et al., 2019). While they confer poor prognosis, FLT-3 mutations such as FLT-3-ITD (internal tandem duplication) and FLT-3-TKD (tyrosine kinase domain) often result in significant consequences on patients with AML as the mutations account for approximately 30% of newly diagnosed patients (Daver et al., 2019). In addition, around 75% of newly diagnosed patients with a FLT-3 mutation tend to present the mutation during relapse (Daver et al., 2019). This suggests that aberrant regulation of FLT-3 expression is considered to be a driver mutation in AML (Daver et al., 2019). Such presence of FLT-3

mutations, whether in newly diagnosed or relapsed patients, leads to a decrease in overall survival, increased risk of relapse and a shorter duration of remission (Daver et al., 2019). As for RAS mutations, the different isoforms stand out in being cancer type dependent (Degirmenci et al., 2020). This means that different cancer types harbor different RAS mutant isoforms. For example, NRAS mutants are usually more prominent in melanoma, while other mutations such as HRAS are more distributed in adrenal glands. KRAS, which is the most common RAS mutation, is most prominent in pancreatic cancer (Degirmenci et al., 2020). One of the RAS protein mutational hotspots is the G12 or G13, which intercept GAP's Arg finger loop accession to the RAS GTPase site and obscure it from promoting hydrolysis (Degirmenci et al., 2020). This mutational hotspot is found in 90% of KRAS mutations. Other mutational hotspots also exist. For example Q61 mutations are found in 90% of NRAS mutations (Degirmenci et al., 2020). This shows that the site of mutational hotspots also comes into play in the cancer-type distribution (Degirmenci et al., 2020). The role of RAF as a cancer driver has been established after the discovery of the BRAF (V600E) mutation. While other mutations in RAF exist, such as CRAF, and ARAF, they are much less represented than BRAF mutations which comprise a major target of oncogenic mutations as they represent over 90% of events (Degirmenci et al., 2020). RAF mutations can be classified into three major classes based on how they trigger the pathway. Class I for example, mimic the phosphorylation of the activation loop of RAF, thus paving way for its activation (Degirmenci et al., 2020). Class II mutations on the other hand, rely on alleviating RAF's auto-inhibitory status (Degirmenci et al., 2020). Class III RAF mutations lack proper kinase activity which allows them to activate their wild type counterparts (Degirmenci et al., 2020). The fact that MEK mutations do not coexist

with Ras or RAF mutations, implies that they function as cancer drivers (Degirmenci et al., 2020). “MEK mutations are classified into two groups according to their activation mechanism. The first group of MEK mutations turns on the kinase activity of MEK by disrupting the inhibitory intramolecular interaction mediated by the regulatory helix A, while the second group does so through enhancing MEK homo-dimerization. These two types of MEK mutants also exhibit differential sensitivities to MEK inhibitors in clinic or under clinic trials. Like RAF, the elevated dimer affinity may also result in the resistance to inhibitors” (Degirmenci et al., 2020). As for ERK mutations, they play a minor role as cancer drivers for they are relatively rare mutations in cancer genomes (Degirmenci et al., 2020). Therefore, the MAPK pathway represents an attractive target for inhibition and thus essentially leading to the dysfunction of the cell and possibly cell death.

1.3 Vertical Targeting

The aberrant ERK/MAPK pathway activation has been spotted in multiple cancer types (around 30% of cancers) mostly in the form of K-Ras and BRAF mutations (Hatzivassiliou et al., 2012). Mitogen-activated protein kinase cascades are essential signaling pathways that play a crucial role in controlling normal cell proliferation, survival and differentiation (Shapira & Benhar, 2010). Any loss in control of the Mitogen Activated Protein Kinase pathway regulation could lead to cancer and other malignancies. Particularly, ERK has been the center of intense current research as it represents a new approach for clinical inhibitors in cancer therapy (Roberts & Der, 2007).

The downstream protein ERK regulates the activation/inactivation of the MAPK, under normal physiological conditions pathway, via negative feedback signals on several regulatory proteins. “In melanoma, instead, the aberrant hyper-activation of

MAPK pathway leads to an enhancement of proliferative stimuli, without an efficient negative feedback caused by the atypical phosphorylation of ERK. With the aim of blocking the hyper-activation of this pathway and the related uncontrolled neoplastic cell growth and dissemination, several small inhibitors have been synthesized and many strategies have been investigated to target the RAS/RAF/MEK/ERK pathway by vertical inhibition" (Grazia et al., 2014). Previous work has proved that combining selumetinib, which is a MEK1/2 inhibitor better known as AZD6244, with PLX4720 (a $BRAF^{V600E}$ inhibitor) effectively suppressed the ability of selumetinib-resistant cells to form colonies and inhibited their growth (Grazia et al., 2014). This was correlated with a synergistic decrease in phosphorylated ERK levels (Grazia et al., 2014). Nevertheless, combining Trametinib, which is also an inhibitor of MEK, with dabrafenib (a $BRAF^{V600E}$ inhibitor), lead to a cytostatic effect as well as caused decreased skin lesions development and cessation of tumor growth (Grazia et al., 2014). Furthermore, this co-treatment caused the expression of genes involved in apoptosis and the downregulation of transcripts associated with cell survival and proliferation in cells that have been proven to be resistant to dabrafenib (Andreucci et al., 2014). Similarly, the dual use of PLX4720, which is also a $BRAF^{V600E}$ inhibitor, and trametinib resulted in a decrease of growth of tumors *in vivo* and alleviated the skin toxicity correlated with the inhibition of MEK. Furthermore, upon combining vemurafenib ($BRAF^{V600E}$ inhibitor) and the MEK inhibitor AS703026, Grazia et al., were able to prove an increase in apoptosis along with a suppression of cell survival (Grazia et al., 2014). Such favorable outcomes are considered to be encouraging considering the fact that they reinforced clinical studies that have displayed promising results about vertically targeting the MAPK cascade (Grazia et al., 2014).

Alongside targeting MEK-1/2, targeting ERK is another utilized approach for inhibiting the MAPK pathway. Importantly, studies have shown that co-targeting of MEK and ERK by small molecule inhibitors presented synergistic inhibition and resulted in overcoming MEK inhibitor-related acquired resistance as well as prevented the development of other resistance (Hatzivassiliou et al., 2012). Furthermore, Hatzivassiliou et al., were able to prove for the first time that utilizing ERK inhibitors would allow overcoming the resistance related to MEK-1/2 inhibitors. This further proves that selective ERK inhibition may found a therapeutic opportunity for treating patients who have been facing relapses due to the reactivation of the MAPK pathway upon proceeding on MEK inhibitor therapy (Hatzivassiliou et al., 2012). Co-targeting MEK and ERK in K-Ras mutant cells with their respective inhibitors prevented the outgrowth of resistant cells (Hatzivassiliou et al., 2012). However, targeting such cells with acquired MEK inhibitor resistance via ERK inhibition efficiently obstructed the cells' proliferation (Hatzivassiliou et al., 2012). Such findings encourage the development of ERK inhibitors to be used in combination with MEK inhibitors as a therapeutic approach for overcoming MEK-related resistance (Hatzivassiliou et al., 2012).

SCH772984 is a potent and selective inhibitor of ERK1/2 that exhibits actions of both type I and type II kinase inhibitors (Lim et al., 2016). The selectivity of SCH772984 resides in the fact that with only 7 out of 300 kinases experimented, it successfully presents more than fifty percent inhibition at a concentration of 1 μM. The ERK inhibitor displays a nano-molar cytotoxic effect in cancer cells carrying several types of mutations including ones in: BRAF, KRAS and/or NRAS (Morris et al., 2013). SCH772984 interferes with the MAPK pathway activity through the competitive inhibition of ERK1/2 kinase activity as well as by initiating allosteric

inhibition of ERK1/2 phosphorylation through inhibition of MEK1/2 binding to ERK1/2 (Chaikuad et al., 2014).

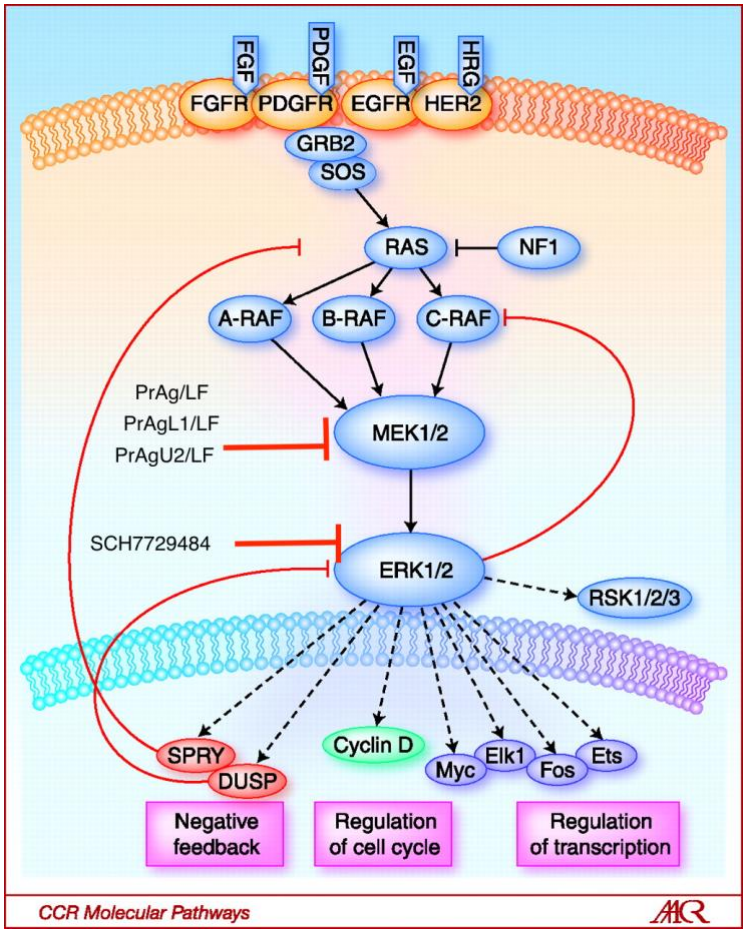


Figure 4: Vertical targeting of the MAPK pathway using the MEK inhibitor (anthrax lethal toxin) and the ERK inhibitor (SCH7729484) (adapted and modified from (Pratilas & Solit, 2010)).

1.5 Autophagy

Autophagy is an intracellular system of degradation which supplies the lysosome with cytoplasmic components. Despite its plainness, recent research has confirmed that autophagy plays a broad variety of, and often complex, physiological and pathophysiological roles (Kalmar et al., 2014) (Mizushima, 2007). Autophagy is a multistep process, during which sequestration, transport to lysosomes, degradation and utilization of degradation products take place (Mizushima, 2007). Autophagy is a different process than sequestering and degrading extracellular and plasma

membrane proteins via lysosomal endocytosis (Mizushima, 2007). While multiple types of autophagy exist, namely "macroautophagy, microautophagy and chaperone mediated autophagy", the term autophagy generally represents macroautophagy (Mizushima, 2007). Recent research has evidently established that autophagy varies more in a physiological and pathophysiological manner than anticipated; such as adapting to starvation, clearing of intracellular proteins and organelles, anti-aging, development, antigen presentation, cell death, elimination of microorganisms and tumor suppression (Mizushima 2005) (Adhauliya et al., 2016).

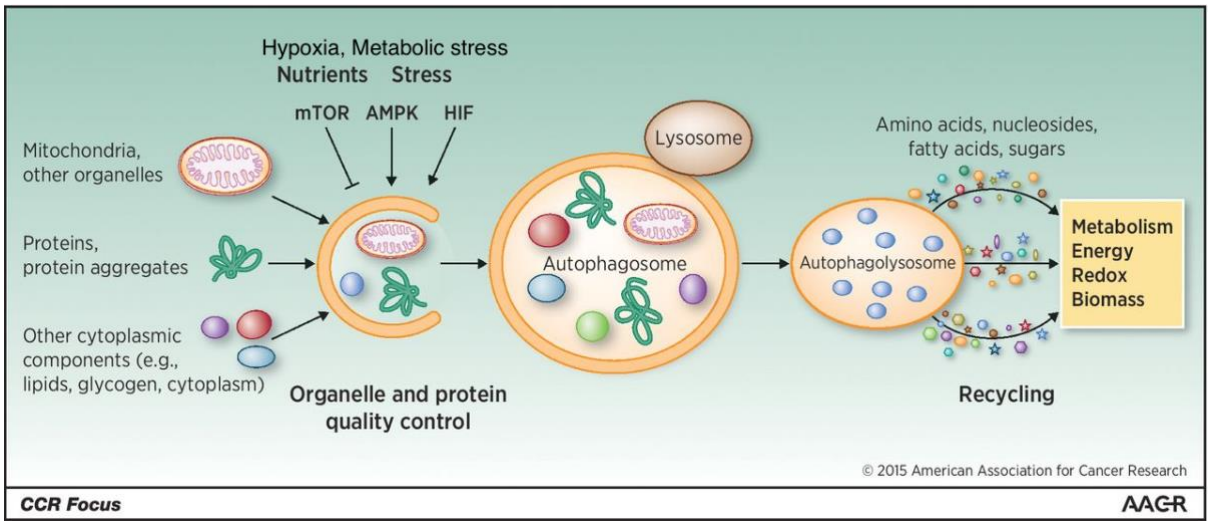


Figure 5: Cytoplasmic components, organelles and protein in the process of degradation during Autophagy. (Adapted and modified from (White et al., 2015))

Although better known as a pro-survival pathway, autophagy plays multiple roles in cell death. Autophagy's roles in cell death can be listed into three categories. One of the roles is autophagy-associated cell death, which indicates that cell death concurs with autophagy. Another role is autophagy-mediated cell death which proposes that cell death is triggered by autophagy. Finally, in the case where cell death is independent of apoptosis or necrosis, it is referred to as autophagy-dependent cell death (P. Bhat et al., 2018) (Galluzzi et al., 2017) (Yan et al., 2019). Death by autophagy has been reported in many studies especially in cancer cells. For instance,

the deprivation of HeLa cancer cells from aminoacids such as arginine is correlated to death by autophagy (Y. Liu et al., 2013). In addition, exposing glioblastoma cells, cervical cancer cells and human embryonic kidney cells to beta-mercaptoethanol and hydrogen peroxide led to death by autophagy (Chen et al., 2008). Nevertheless, depriving pancreatic cancer cell of arginine via [HuArgI(co)-PEG500] also demonstrated autophagy dependent cell death (Khalil & Abi-Habib, 2019). This increasing evidence proves that autophagy might have a fundamental role in cell death.

One of the main triggers of autophagy is nutrient deprivation/starvation; meaning the lack of any essential nutrient would activate the pro-survival process of autophagy as a way of compensation, hence facilitating the survival of the cell. Autophagy can also be activated by other factors such as reactive oxygen species (ROS), hypoxia and metabolic stress (Onorati et al., 2018). The process of autophagy is highly regulated and requires two key kinases: ULK and VPS34 as well as other regulatory factors such as Beclin-1 and BIF-1. Autophagy is greatly controlled by mTOR (mammalian target of rapamycin) as it plays a vital role in cell survival (F. A. Bhat et al., 2014). Upon nutrient starvation, mTOR would be inhibited. This would free ULK-1 from repression which would consequently phosphorylate and activate Beclin-1, further leading to the activation of the whole VPS34 complex thus forming a phagophore (Cicchini et al., 2015) (Jung et al., 2010). Under stressful conditions, several pathways, such as PI3K/Akt would be inhibited while pathways such as AMPK would be activated. This would lead to the inhibition of mTOR thus activating ULK and Beclin-1 to trigger autophagy (Cicchini et al., 2015) (Jung et al., 2010).

Several findings have demonstrated that the activation of ERK by active Raf or IGF-I receptor provoked a form of cell death that is associated with the formation of macro-vacuoles in the cytoplasm as well as extensive cell rounding, which further moved the cytoplasm and the nucleus to the side of the dying cell. While cell death has been correlated with the activation of caspase-8, this major vacuolization is not related to the classical apoptosis traits. This morphology may be a sign of programmed cell death mediated by autophagy (Cagnol & Chambard, 2011). Besides, under stress conditions, the MAPK cascade can regulate autophagy, eventually modulating the cellular response (Kim & Kim, 2019).

Chloroquine (an inhibitor of autophagy) is a drug that blocks the fusion of autophagosome with the lysosome (Mauthe et al., 2018) (Redmann et al., 2016). Along with CQ's role in the inhibition of autophagy, this drug also has an effect on p53, CXCR4-CXCL12, and the Toll-like receptor 9 in cancer cells (Verbaanderd et al., 2017). In our study, Chloroquine is to be used as a positive control in order to assess the cytotoxic effects of autophagy on the corresponding Acute Myeloid Leukemia cell lines.

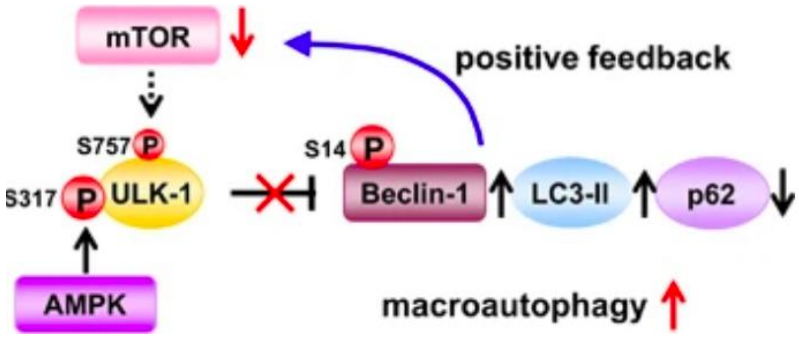


Figure 6: mTOR's decrease of inhibitory phosphorylation and AMPK's increase in phosphorylation on ULK-1 frees it from inhibition thus activating Beclin-1 and other regulatory proteins to promote autophagy induction. (Adapted and modified from (Hwang et al., 2017))

1.6 Aims and previous work done on AML

The effects of the wild type furin-activated anthrax lethal toxin, and its derivative urokinase-activated anthrax lethal toxin have been previously demonstrated on panels of acute myeloid leukemia cell lines as well as primary blasts. However, aiming to increase selectivity of the drug, we focus on exploiting the matrix metalloproteases present on the surface of AML cells and test the potency of the MMP-activated anthrax lethal toxin PrAgL1/LF. If targeting the matrix metalloproteases in acute myeloid leukemia proves to be a novel strategy, this would pave way for the construction of an inter-molecularly complementing version of the anthrax lethal toxin that targets both the uPA/uPAR system as well as MMPs. In a study carried out by Kassab et al., 64% of the cell lines tested demonstrated sensitivity to the inhibition of the MAPK pathway led by the Lethal Factor of the anthrax lethal toxin (Kassab et al., 2013). Flow cytometry analysis demonstrated that cell lines sensitive to the inhibition seemed to have high levels of phospho-ERK-1/2 levels, whereas resistant cell lines displayed low levels of pERK-1/2 (Kassab et al., 2013). This proves the dependence of such cell lines on MEK-1/2 and the activation of the MAPK pathway to regulate cell survival (Kassab, 2013). Upon the use of U0126, a specific MEK1/2 inhibitor, they noticed that its effect mimicked that of PrAg/LF; whereby the cell lines sensitive to the anthrax lethal toxin displayed a decrease in cell viability, whereas the cell lines resistant to the anthrax lethal toxin were also resistant to U0126 which further corroborates the necessity of the dependence of cells on the MAPK pathway in order to be sensitive to PrAg/LF (Kassab et al., 2013) (Ralph J. Abi-Habib et al., 2005). Aiming to overcome AML resistance to PrAg/LF, Kassab et al. utilized LYS294002, a phosphatidylinositol 3-kinase inhibitor. The use of the PI3K inhibitor would allow inhibition of the

PI3K/Akt pathway (Stoll et al., 2005). LYS294002 demonstrated cytotoxicity in cell lines that were resistant to the MEK-1/2 inhibition. However, coupling the two inhibitors did not show any synergistic effect. This indicates that the acute myeloid leukemia cell lines tested can be categorized into two distinct populations: either sensitive to MAPK pathway inhibition, or PI3K/Akt pathway inhibition (Kassab et al., 2013). The fact that no added benefits resulted from simultaneously targeting MEK-1/2 and PI3K in AML cells displays the inefficiency of instantaneously targeting two parallel pathways. This makes room for testing vertical inhibition instead of horizontal inhibition by co-targeting MEK-1/2 and ERK-1/2 of the MAPK cascade. In this study, we attempt to target both the mitogen-activated protein kinase pathway and matrix metalloproteases in acute myeloid leukemia (AML) cell lines. We also attempt to determine the level of autophagy activation, through quantification of autophagosomes on flow cytometry, and the impact of its inhibition, using the autophagy inhibitor chloroquine (CQ), on cell death secondary to MAPK inhibition. We also attempt vertical inhibition of the MAPK pathway in AML cell lines by targeting the MAPKK protein MEK-1/2 as well as the MAPKKK protein ERK-1/2 via SCH772984. The inhibition of both MEK-1/2 and ERK-1/2 simultaneously would allow us to compare and assess the cytotoxicity of the MEK-1/2 inhibitor (Anthrax Lethal Toxin) and the ERK-1/2 inhibitor (SCH 772984).

Chapter Two

Materials and Methods

2.1 Cell Lines

Human AML cell lines Mono-Mac-6, Mono-Mac-1, U-937, TF-1 HaRAS, HL-60, ML-2, THP-1 and TF1-vRaf were grown in RPMI 1640 (Lonza, Basel, Switzerland) culture media supplemented with 10% Fetal Bovine Serum (FBS) (Sigma-Aldrich) and 100U/ml penicillin/sterptomycin (Biowest). Cell lines were incubated at $37^{o}C$ / 5% CO_2.

2.2 Proliferation Inhibition Assay of Anthrax Lethal Toxin and its modifications (Cytotoxicity)

Cytotoxicity of PrAgL1/LF was determined using a proliferation inhibition assay. LF was added to yield a concentration of 10^{-9} M, and aliquots of 10^{4} cells/well in 100 μL cell culture medium were plated to the wells of 6 of the 8 rows in a flat-bottom 96-well plate (Corning Inc. Corning, NY). The last two rows included $6x10^{-5}$ mg/mL of FP59 instead of LF. This was followed by the addition of 50 μL of serially diluted PrAg in media to each well from a round-bottom 96-well plate (Corning Inc. Corning, NY) to yield concentrations ranging from 10^{-8} to 10^{-14} M in the first 3 rows. Same process was done for the wells of the next five rows where 50 μL of serially diluted PrAgL1 was added to yield concentrations ranging from 10^{-8} to 10^{-14} M. Absorbance was plotted against the log of concentration and on non-linear regression with a variable slop on GraphPad Prism 5 software (GraphPad Software, Sandiego CA).

2.3 Autophagy Assay

Cells were plated in a flat-bottom 6-well plate ($1x10^6$ cells/well) (Corning Inc. Corning, NY). Conditions included control, PrAg/LF, Rapamycin, and PrAg/LF with Chloroquine. LF and PA were added when needed to yield concentrations of 10^{-9} M and 10^{-8} M respectively. Chloroquine was set up to be added at a concentration of 50 μM. 0.5μM of Rapamycin was added as a control condition. Plates were incubated at $37^oC/5\%$ CO_2. Protocol was followed according to the company's manual (ENZO product manual - CYTO-ID® Autophagy Detection Kit, 2016). Results were read on a C6 flow cytometer.

2.4 Determining the Effect of Chloroquine on Cytotoxicity of PrAg/LF

LF was added to yield a concentration of 10^{-9} M, and aliquots of 10^4 cells/well in 100 μL cell culture medium were plated to the wells a flat-bottom 96-well plate (Corning Inc. Corning, NY). This was followed by the addition of 50 μL of serially diluted PrAg in media to each well from a round-bottom 96-well plate (Corning Inc. Corning, NY) to yield concentrations ranging from 10^{-8} to 10^{-14} M. 50 μM of chloroquine was the added to half the wells of the plate thus generating two conditions: PrAg/LF and PrAg/LF with CQ. Absorbance was plotted against the log of concentration and on non-linear regression with a variable slop on GraphPad Prism 5 software (GraphPad Software, Sandiego CA).

2.5 Cytotoxicity of the Combination of PrAg/LF and SCH772984

Cytotoxicity of PrAg/LF was determined using a proliferation inhibition assay. LF was added to yield a concentration of 10^{-9} M, and aliquots of 10^{4} cells/well in 100 μL cell culture medium were plated to the wells 96-well plate (Corning Inc. Corning, NY). This was followed by the addition of 50 μL of serially diluted PrAg in media from a round-bottom 96-well plate (Corning Inc. Corning, NY) to yield concentrations ranging from 10^{-8} to 10^{-14} M in the wells of the first 6 rows of the cell plate. 50 μL of diluted SCH772984 was added to the rest of the free wells as well as to some wells containing PrAg/LF with concentrations ranging from 200 μM to 10^{-4} μM. Thus producing three conditions: PrAg/LF, SCH772984, and PrAg/LF with SCH772984. Absorbance was plotted against the log of concentration and on non-linear regression with a variable slop on GraphPad Prism 5 software (GraphPad Software, Sandiego CA).

Chapter Three

Results

3.1 Sensitivity of AML Cells to PrAgL1/LF

Our first objective was to target both the mitogen-activated protein kinase pathway and matrix metalloproteases in acute myeloid leukemia (AML) cell lines. Hence, we first tested the cytotoxicity of the matrix metalloprotease-activated anthrax lethal toxin, PrAgL1/LF, on a panel of 8 human Acute Myeloid Leukemia cell lines (HL60, THP-1, ML-2, TF1-vRaf, Mono-Mac-6, TF1-HaRas, U937 and Mono-Mac-1). Since activity of PrAgL1/LF depended on two independent factors, namely expression of MMPs on the cell surface and dependence on the MAPK pathway for survival, we included two other toxins to test each of these parameters separately on AML cells. PrAg/LF was added to control for the dependence of AML cell lines on the MAPK pathway for survival, and PAL1/FP59 was added to control for expression of MMPs, independently of MAPK dependence. FP59 is a fusion of the PrAg binding domain of LF and the catalytic domain of Pseudomonas aeruginosa exotoxin A (Kassab et al., 2013). The mechanism of FP59 Binding to PrAg and translocating into the cytosol is identical to that of LF; however, FP59 does not target the MAPK pathway but ADP- ribosylates EF-2 leading to the inhibition of protein synthesis and subsequent cell death (Kassab et al., 2013). The combination of PrAgL1 and FP59, would, therefore, be cytotoxic to all cells that express the express MMPs (Kassab et al., 2013).

Out of the 8 AML cell lines tested, four were sensitive to the MMP-activated toxin, PrAgL1/LF (HL-60, THP-1, ML-2 and TF1-vRaf), indicating that these cell lines express MMPs and are sensitive to the inhibition of the MAPK pathway (Figure 4A).

As expected, these cell lines were also sensitive to wild-type PrAg/LF (Figure 4A). The remaining four cell lines (Mono-Mac-6, TF1-HaRas, U937 and Mono-Mac-1) HL-60, were not sensitive to neither PrAgL1/LF nor to PrAg/LF indicating that these cell lines are not dependent on the MAPK pathway for survival, hence are not sensitive to its inhibition by LF (Figure 4B). However, all cell lines, including these four LF-resistant cell lines were sensitive to the MMP-activated, MAPK-independent, PrAgL1/FP59, indicating that all the cell lines tested express MMPs (Figure 4). This is significant since it demonstrates that AML cells express both MMPs and the urokinase plasminogen activator (uPA) and can, therefore, be targeted using an intermolecularly complementing version of anthrax lethal toxin.

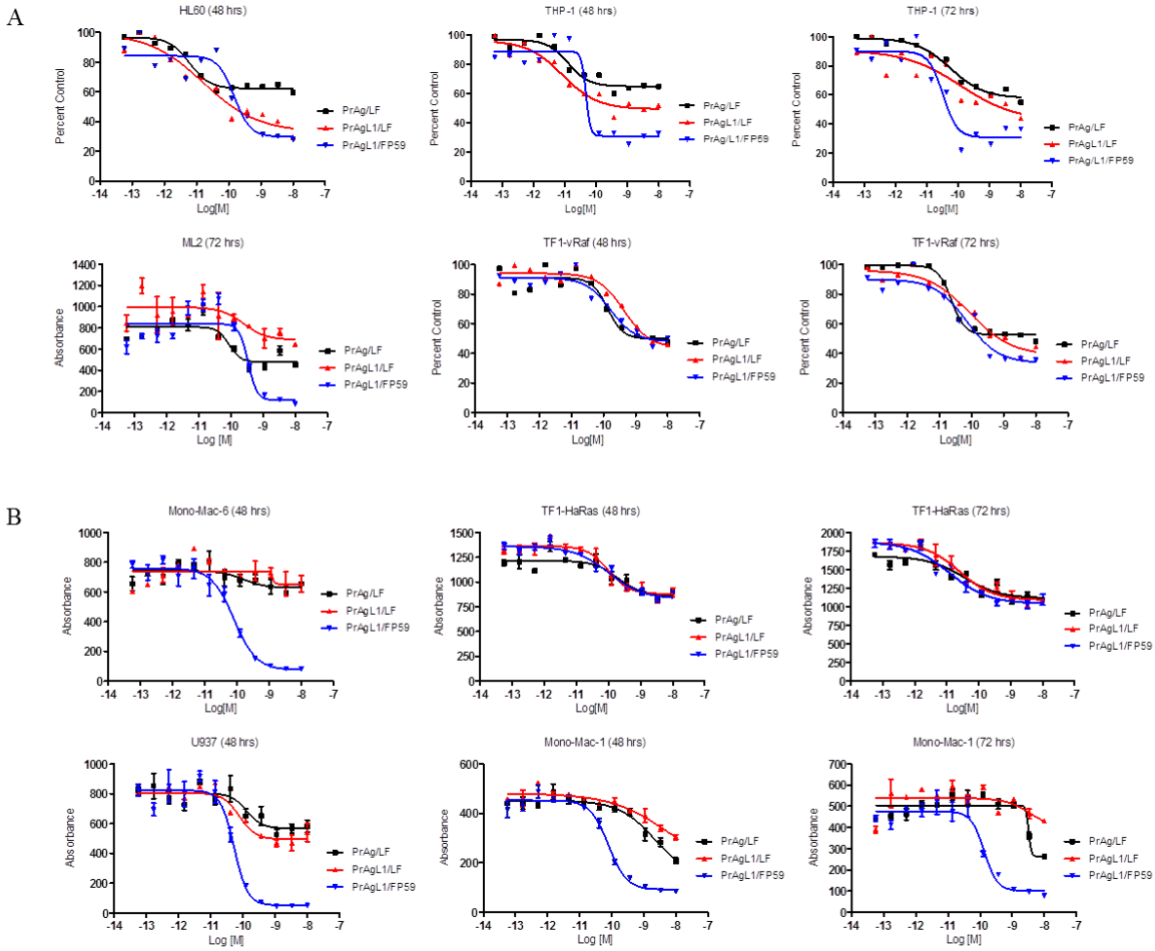
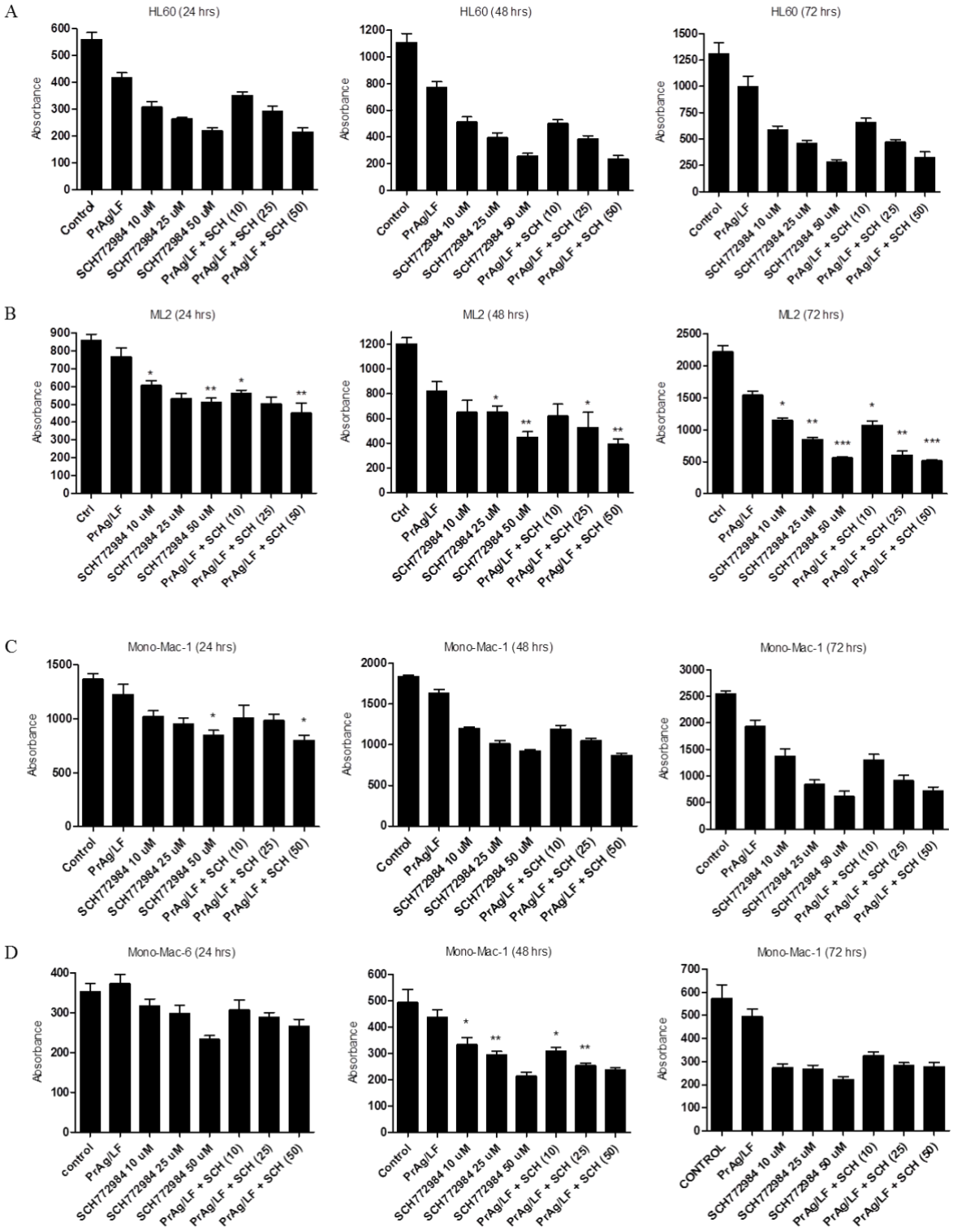


Figure 7: Cytotoxicity of PrAg/LF, PrAgL1/LF and PrAgL1/FP59 to HL-60 (48 hours), THP-1 (48 and 72 hours), ML-2 (72 hours), TF1-vRaf (48 and 72 hours), Mono-Mac-6 (48 hours), TF-1HaRas (48 and 72 hours), U937 (48 hours) and Mono-Mac-1 (48 and 72 hours). A) Cell lines sensitive to PrAgL1/LF and B) Cell lines resistant to PrAgL1/LF.

3.2 Effect of the Vertical Inhibition of the MAPK Pathway on AML Cells

Aiming to overcome the resistance displayed by some of the AML cell lines towards the inhibition of MEK1/2 by LF, we sought to apply vertical inhibition of the RAS/RAF/MEK pathway by simultaneously inhibiting MEK-1/2 (using PrAg/LF) and ERK1/2 (using the specific ERK-1/2 inhibitor SCH772984). First, we treated 5 AML cell lines (2 sensitive and 3 resistant to LF) with fixed concentrations of PrAg/LF (10^{-8}M PrAg/10^{-9}M LF) and SCH772984 (10μM, 25μM, and 50μM) for three time points: 24 hours, 48 hours and 72 hours. In all 5 cell lines, SCH772984 was significantly more potent than PrAg/LF, at all the concentrations used and at

time points tested ($p < 0.0001$ for all). Similarly, the combination of PrAg/LF and SCH772984 was significantly more potent than PrAg/LF alone in all cell lines, at all concentrations and time points ($p < 0.0001$ for all) (Figure 5). However, the combination of PrAg/LF and SCH7729484 was significantly more potent than SCH772984 alone mostly in ML-2 cells (Figure 5B) with the other cell lines either not showing any significance (HL60) (Figure 5A) or showing sporadic significance at some time points/concentration points (Mono-Mac-1, Mono-Mac-6 and U937) (Figure 5 C, D and E). In ML-2 cells, the combination of PrAg/LF and two of the three concentrations of SCH772894 is significantly more potent than the corresponding concentrations of SCH772984 alone at the 24 and 48-hour time points, while at the 72-hour time point, the combination of PrAg/LF and all three concentrations of SCH772984 is significantly more potent that the corresponding concentrations of SCH772984 alone ($p < 0.0001$) (Figure 5B). These results show that the vertical inhibition of the MAPK pathway through the inhibition of both MEK1/2 and ERK1/2 has an increased potency compared to PrAg/LF alone and may have an increased potency in some cell lines compared to SCH772984 alone. However, the limitation of this set of data is that it examines concentration end points by testing the highest concentration of PrAg/LF used in cytotoxicity assays and relatively high concentrations of SCH772894. This might obscure some beneficial effects of the combination at lower concentrations; hence testing a complete range of concentrations is warranted.


A
HL60 (24 hrs)
HL60 (48 hrs)
HL60 (72 hrs)
B
ML2 (24 hrs)
ML2 (48 hrs)
ML2 (72 hrs)
C
Mono-Mac-1 (24 hrs)
Mono-Mac-1 (48 hrs)
Mono-Mac-1 (72 hrs)
D
Mono-Mac-6 (24 hrs)
Mono-Mac-1 (48 hrs)
Mono-Mac-1 (72 hrs)
Absorbance
Control
Ctrl
CONTROL
PrAg/LF
SCH772984 10 uM
SCH772984 25 uM
SCH772984 50 uM
PrAg/LF + SCH (10)
PrAg/LF + SCH (25)
PrAg/LF + SCH (50)

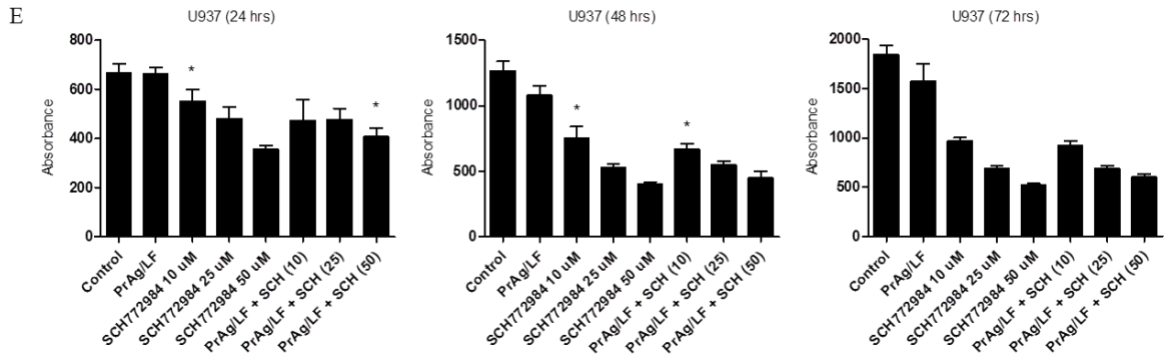
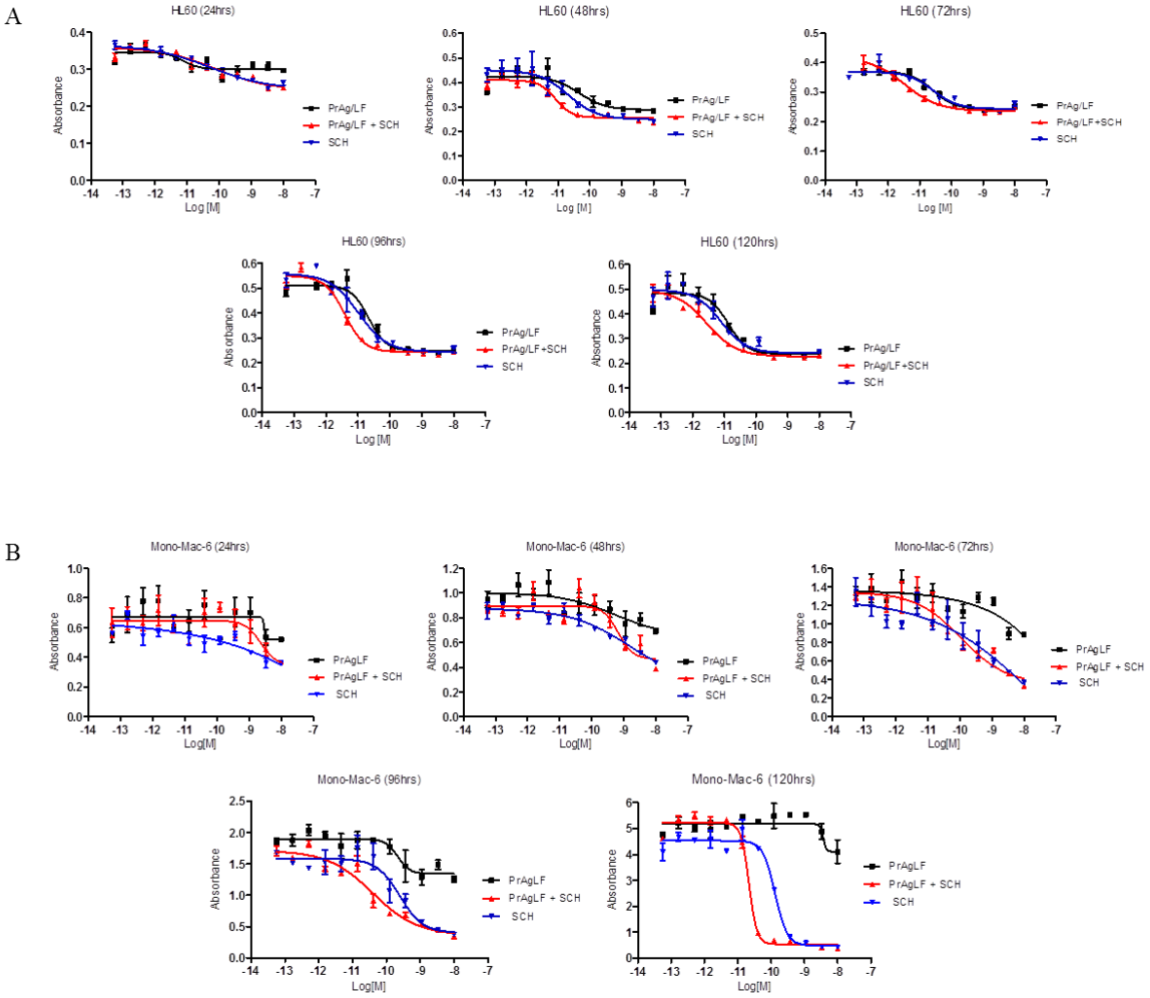


Figure 8: Cytotoxicity of PrAg/LF and SCH772984 (10, 25 and 50μM), alone or in combination on HL-60 (A), ML-2 (B), MonoMac-1 (C), MonoMac-6 (D) and U-937 (E) cell lines at three different time points. Symbols indicate a statistically significant difference.

To clearly compare and assess the effect of the dual inhibition of MEK-1/2 and ERK-1/2 on cell cytotoxicity, we tested a range of 11 different concentrations of both PrAg/LF and SCH772984 on 3 AML cell lines, one sensitive (HL60) and two resistant to PrAg/LF (Mono-Mac-6 and U937) thus increasing the range of concentrations and generating non-linear regression curves. Out of the three cell lines, only HL60 showed a clear increase in sensitivity with the combination of PrAg/LF and SCH772984 compared to either of them alone starting at 48 hours post-treatment. The IC_{50} for the combination was approximately 4-fold lower at 48 hours, 8-fold lower at 72 hours, 7-fold lower at 96 hours and 5 fold-lower at 120 hours, compared to either treatment alone (Figure 6A). In U937 cells, on the other hand, the combination had no added potency compared to either PrAg/LF alone and SCH772984 alone, indicating that this cell line is resistant to the inhibition of the MAPK pathway through the inhibition of either MEK1/2 alone, ERK1/2 alone or a combination of both Figure 6C). Mono-Mac-6 cells did not show any increase in potency of the combination, at the 24, 48 and 72-hour time points, compared to either treatment alone, but showed a significant increase in potency of the combination at 96 and 120 hours of treatment compared to each treatment alone. In addition, SCH772984 showed a significant increase in potency compared to PrAg/LF

to which cells remained resistant even at this late time point (Figure 6B). The IC_{50} of the combination was approximately 6-fold lower than that of SCH772984 alone at both 96 and 120 hours.

These results seem to indicate that vertical inhibition of the MAPK pathway, through targeting of both MEK1/2 and ERK1/2, does show an increased potency compared to single targeting in cell lines already sensitive to the inhibition of this pathway. However, this approach does not seem to have any added benefit in AML cells that are resistant to the inhibition of the MAPK pathway.



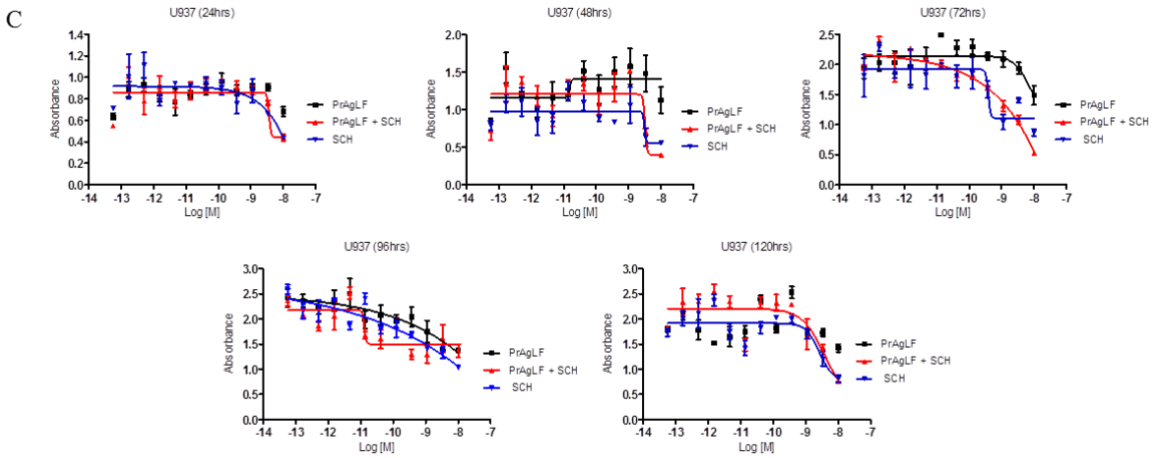


Figure 9: Non-linear regression curves of the cytotoxicity of varying concentrations of PrAg/LF, SCH772984 or the combination of both on HL-60 (A), Mono-Mac-6 (B) and U-937 (C) at 24, 48, 72, 96 and 120 hours.

3.3 Activation of Autophagy

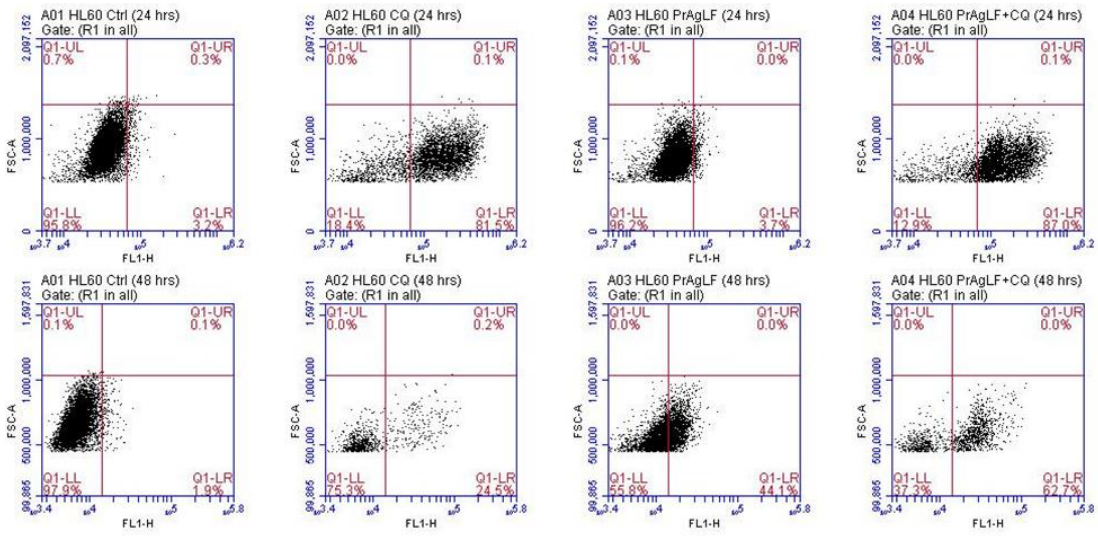
To determine whether the LF mediated inhibition of the MAPK pathway leads to the activation of autophagy in AML cells, we determined the level of autophagy activation in 4 AML cell lines (HL60, ML-2, Mono-Mac-1 and U937) following treatment with PrAg/LF for up to 120 hours.

Two of the cell lines tested (HL60 and U937) showed a clear activation of autophagy following treatment with PrAg/LF. In HL60 cells, the percentage of cells positive for autophagosomes increased from approximately 2% in control HL60 cells to 44% in cells treated with PrAg/Lf for 48 hours. In U937 cells, the percentage of cells positive for autophagosomes increased from 4.4%, 4.8%, 3.7% and 2.6% in control cells to 6.1%, 13.5%, 39.1% and 42.5% in cells treated with PrAg/LF, at 24, 48, 72 and 96 hours, respectively (Figure A and C). A third cell line, Mono-Mac-1, though not showing a clear accumulation of autophagosomes upon treatment with PrAg/LF, showed a clear increase in the accumulation of autophagosomes in cells treated with PrAg/LF and chloroquine (CQ), a downstream autophagy inhibitor, compared to with PrAg/LF alone or CQ alone at the later time points of 72 and 96 hours (Figure 7C). Autophagy being a flux, its activation can often be associated with an increase

in the rate of processing of autophagosomes which prevents their detectable accumulation. This is the case with these cells and this is why treatment with both PrAg/LF and CQ (which blocks the processing of autophagosomes) leads to an accumulation of autophagosomes, beyond each alone, indicating the activation of autophagy. One cell line, ML-2, did not show any sign of activation of autophagy at any time point (Figure 7B).

This indicates that autophagy is activated in a majority of AML cells following inhibition of anthrax lethal toxin-mediated inhibition of MEK1/2.

A



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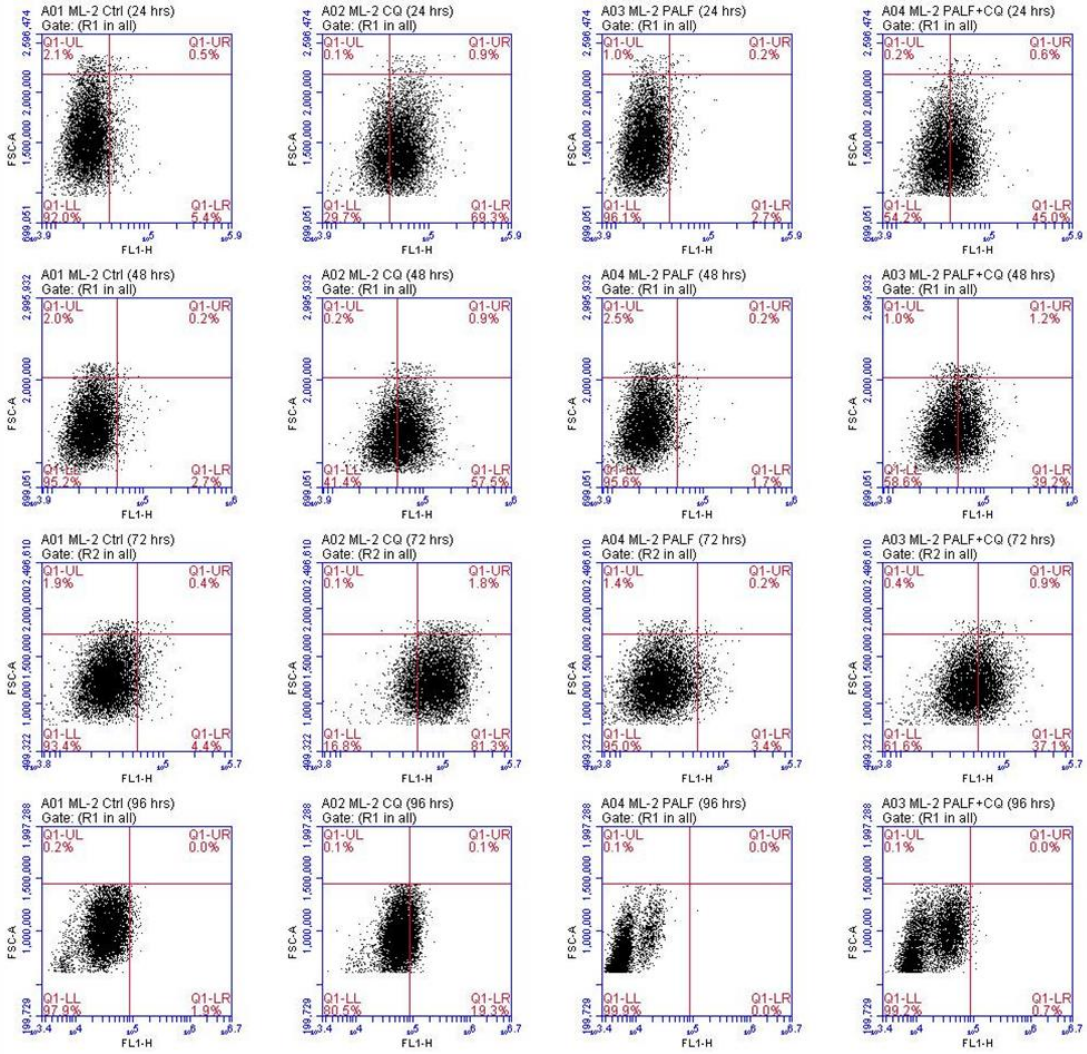
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Q1-LL 92.0%
Q1-LR 5.4%
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Gate: (R1 in all)
Q1-UL 0.1%
Q1-UR 0.9%
Q1-LL 29.7%
Q1-LR 69.3%
A03 ML-2 PALF (24 hrs)
Gate: (R1 in all)
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Q1-UR 0.2%
Q1-LL 96.1%
Q1-LR 2.7%
A04 ML-2 PALF+CQ (24 hrs)
Gate: (R1 in all)
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Q1-UR 0.6%
Q1-LL 54.2%
Q1-LR 45.0%
A01 ML-2 Ctrl (48 hrs)
Gate: (R1 in all)
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Q1-UR 0.2%
Q1-LR 2.7%
A02 ML-2 CQ (48 hrs)
Gate: (R1 in all)
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Q1-UR 0.9%
Q1-LL 41.4%
Q1-LR 57.6%
A04 ML-2 PALF (48 hrs)
Gate: (R1 in all)
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Q1-UR 0.2%
Q1-LR 1.7%
A03 ML-2 PALF+CQ (48 hrs)
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Q1-UR 1.2%
Q1-LL 58.6%
Q1-LR 39.2%
A01 ML-2 Ctrl (72 hrs)
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Q1-LR 4.4%
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Q1-LL 95.0%
Q1-LR 3.4%
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Q1-UR 0.9%
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Q1-LR 37.1%
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Gate: (R1 in all)
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Q1-LL 97.9%
Q1-LR 1.9%
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Q1-LL 80.5%
Q1-LR 19.3%
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Q1-UR 0.0%
Q1-LL 99.9%
Q1-LR 0.0%
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Gate: (R1 in all)
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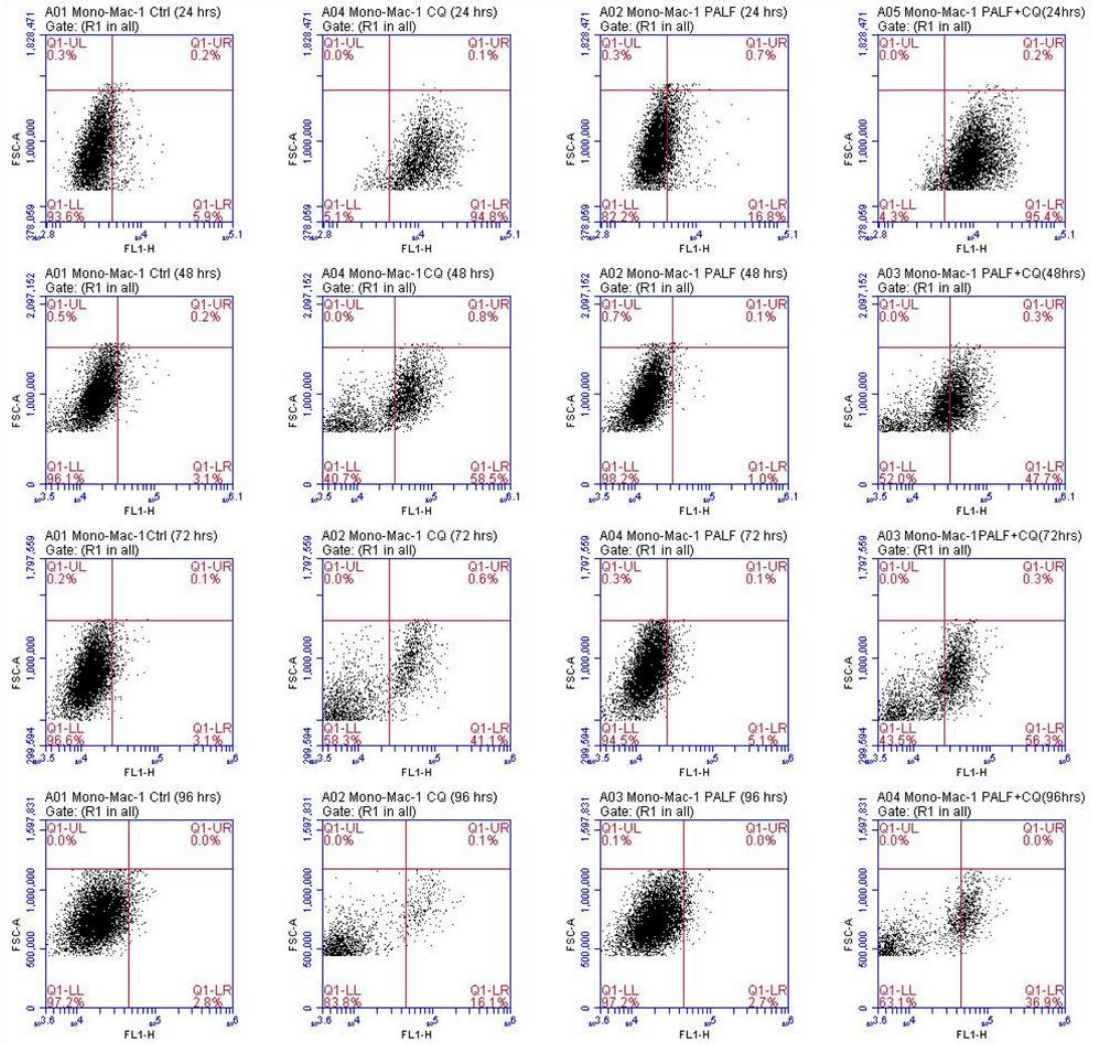
Q1-LL 99.2%
Q1-LR 0.7%
FSC-A
FL1-H

C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Q1-LR 5.8%
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Gate: (R1 in all)
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Q1-LR 94.8%
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Q1-LR 16.8%
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Q1-LR 3.1%
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Q1-LL 98.2%
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Q1-LR 47.7%
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Gate: (R1 in all)
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Q1-UR 0.1%
Q1-LL 96.6%
Q1-LR 3.1%
A02 Mono-Mac-1 CQ (72 hrs)
Gate: (R1 in all)
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Q1-UR 0.6%
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Q1-LR 41.1%
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Gate: (R1 in all)
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Q1-UR 0.1%
Q1-LL 94.5%
Q1-LR 5.1%
A03 Mono-Mac-1PALF+CQ(72hrs)
Gate: (R1 in all)
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Q1-UR 0.3%
Q1-LL 43.6%
Q1-LR 56.3%
A01 Mono-Mac-1 Ctrl (96 hrs)
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Q1-UR 0.0%
Q1-LL 97.2%
Q1-LR 2.8%
A02 Mono-Mac-1 CQ (96 hrs)
Gate: (R1 in all)
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Q1-UR 0.1%
Q1-LL 83.8%
Q1-LR 16.1%
A03 Mono-Mac-1 PALF (96 hrs)
Gate: (R1 in all)
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Q1-UR 0.0%
Q1-LL 97.2%
Q1-LR 2.7%
A04 Mono-Mac-1 PALF+CQ(96hrs)
Gate: (R1 in all)
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Q1-UR 0.0%
Q1-LL 63.1%
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FSC-A
FL1-H

D

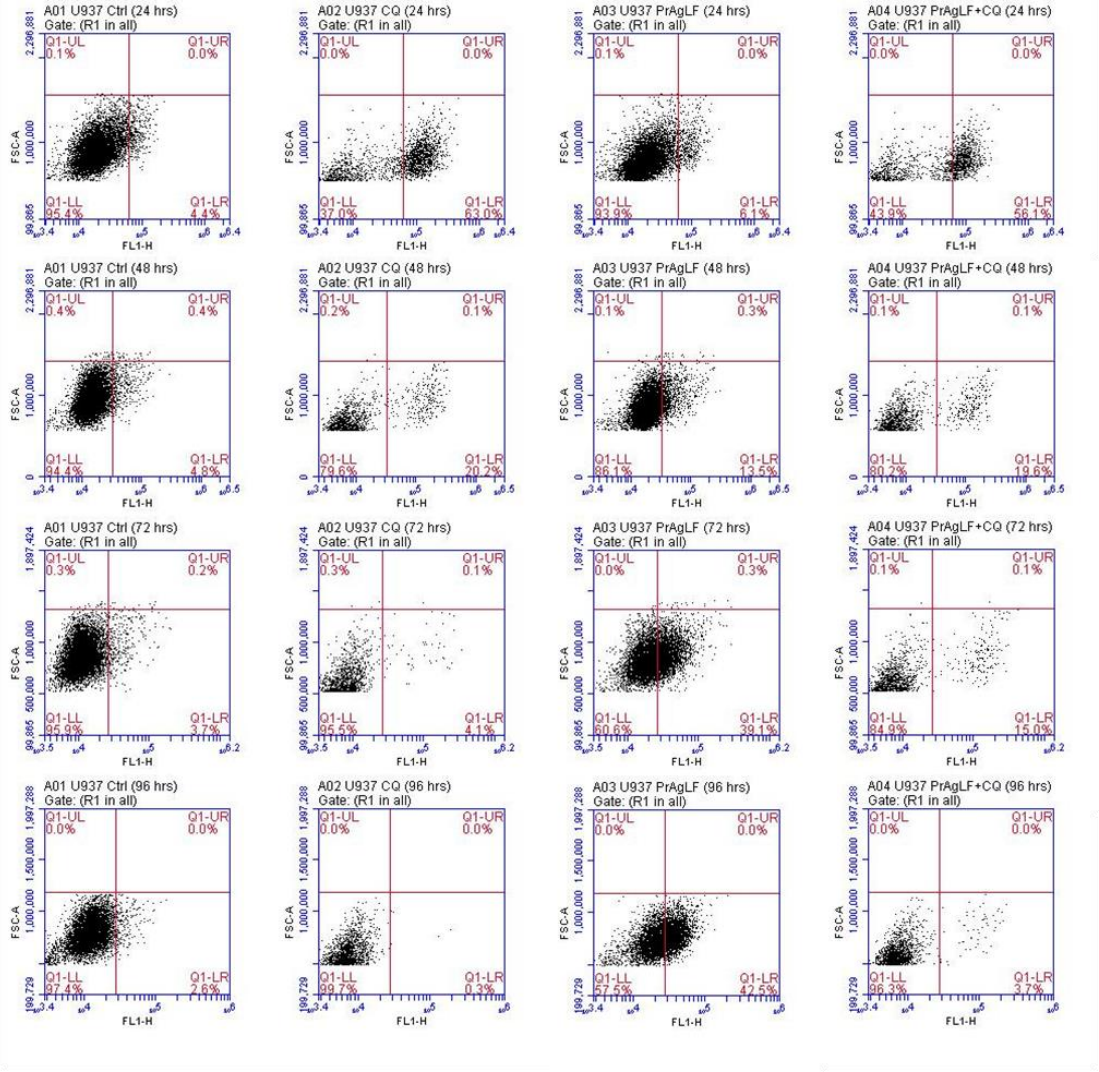

Figure 10: Autophagosome staining using the CytoID stain of HL60 (A), ML-2 (B), Mono-Mac-1 (C) and U937 (D) cells treated with PrAgL1/LF with or without chloroquine for 24, 48, 72 and 96 hours. Cells were gated on width versus forward scatter (R1)

3.4 Effect of the Activation of Autophagy on Cytotoxicity of PrAg/LF

To further investigate whether autophagosome formation secondary to LF treatment demonstrates autophagy-related cell death, we ran cytotoxicity assays on two cell lines (Mono-Mac-6 and TF-1HaRas) treating them with 50μM CQ and serially diluted PrAg/LF with CQ. MonoMac-6 from 48 hours up to 120 hours showed less cell viability upon coupling PrAg/LF with CQ than treating with PrAg/LF alone (Figure 8A). This was also demonstrated in TF-1HaRAS at all five time points (24,

48, 96 and 120 hours) (Figure 8B). This proves that Chloroquine-mediated inhibition of autophagy after LF treatment of the AML cell lines is more cytotoxic than allowing the regular flux autophagy to take place. This further proves the protective role autophagy plays in AML cell lines after treatment with the anthrax lethal toxin.

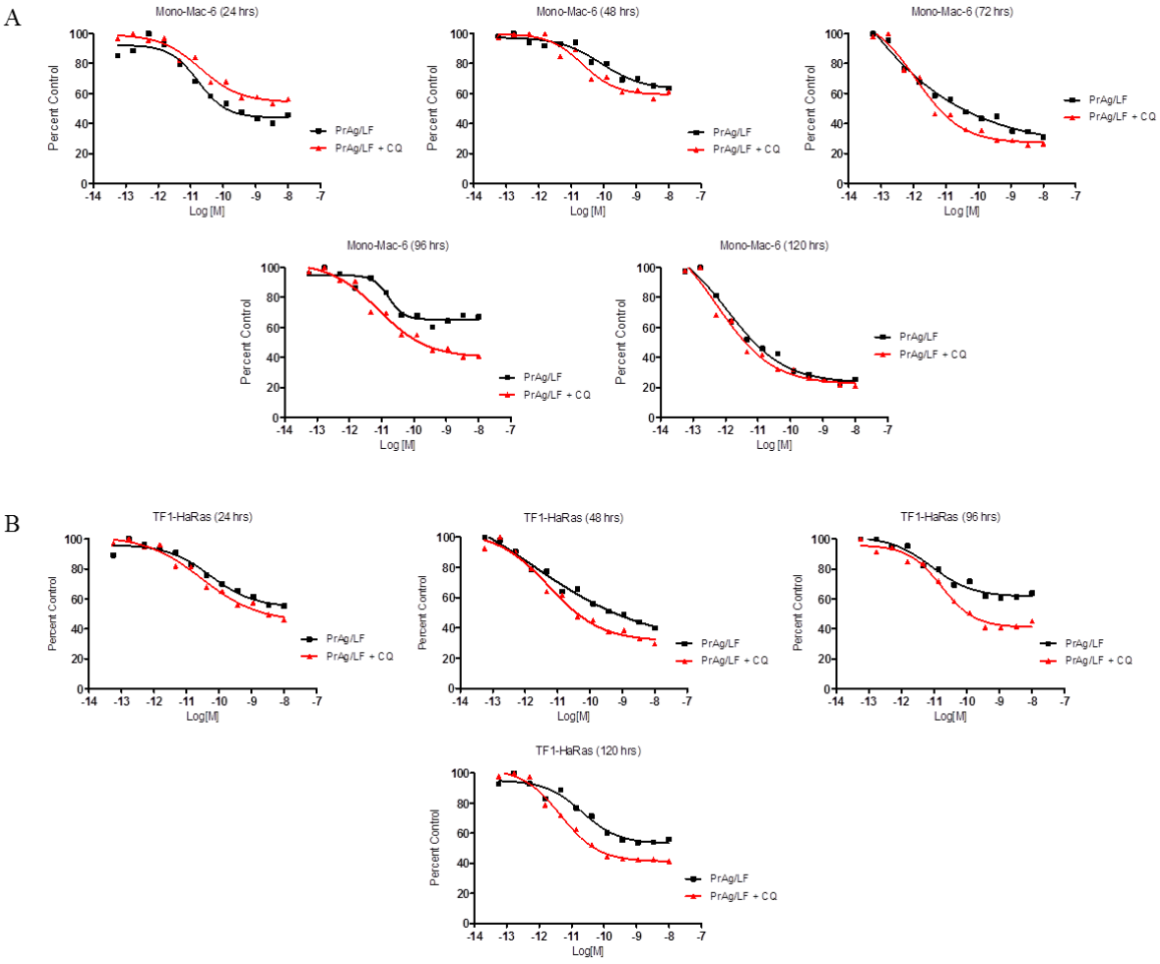

Figure 11: A Cytotoxicity of MonoMac-6 (A) and TF-1HaRas (B) cell lines treated with PrAgL1/LF with or without chloroquine.

Chapter Four

Discussion

Among the eight acute myeloid leukemia cell lines tested, four were shown to be sensitive to the furin-activated inhibition of PrAg/LF (HL-60, TF1-vRaF, ML-2 and THP-1). This indicates that these cell lines utilize the Mitogen Activated Protein Kinase pathway for survival. Treatment of these sensitive cell lines with PrAgL1/LF demonstrated a cytotoxic effect and thus a decrease in cell viability. Since PrAgL1/LF is a matrix metalloprotease activated-anthrax lethal toxin, this indicates that HL-60, TF1-vRaF, ML-2 and THP-1express matrix metalloproteases, allowing the LF moiety enter the cell and thus inhibit MEK-1/2 of the MAPK pathway. The remaining four cell lines (Mono-Mac-6, TF1-HaRas, U937 and Mono-Mac-1), were not sensitive to neither PrAgL1/LF nor to PrAg/LF indicating that these cell lines are not dependent on the MAPK pathway for survival, hence are not sensitive to its inhibition by LF. The eight AML cell lines were also treated with PrAgL1/FP59. FP59 does not target the MAPK pathway but ADP- ribosylates EF-2 thus leading to protein synthesis inhibition. Thus its mechanism of action is independent of the MAPK pathway. Since FP59 is coupled with PrAgL1 this means its activation is matrix-metalloprotease-dependent. Each of the eight cell lines (including the resistant ones) were sensitive to PrAgL1/FP59 indicating that FP59 was successfully translocated to the cytosol, thus all eight cell lines express matrix metalloproteases. In addition, cell lines such as HL-60 were more sensitive towards PrAgL1/LF than PrAg/LF, indicating a more selective method for targeting acute myeloid leukemia. This shows that AML cells express both MMPs and the urokinase plasminogen

activator (uPA) and can, therefore, be targeted using an intermolecularly complementing version of anthrax lethal toxin.

The fact that vertical targeting has been proven to be effective in recent studies especially in the course of the MAPK pathway, offered the possibility of utilizing the ERK-1/2 inhibitor SCH772984, along with the MEK-1/2 inhibitor anthrax lethal toxin PrAg/LF. By combining the two inhibitors, we seek to determine whether vertical inhibition would allow overcoming acquired resistance of AML cells to the anthrax lethal toxin. In all 5 cell lines (ML-2, Mono-Mac-1, HL-60, Mono-Mac-6 and U937) the combination of SCH772984 and PrAg/LF was significantly more cytotoxic than PrAg/LF alone, at all the concentrations of SCH772984 used (10μM, 25μM, and 50μM) and at time points tested (24, 48 and 72 hours). However, co-targeting MEK-1/2 and ERK-1/2 appeared to be more efficient than solely targeting ERK-1/2 only in the ML-2 cell line. As for the rest of the cell lines (Mono-Mac-1, Mono-Mac-6 and U937), only sporadic significance for that matter was displayed at certain time points and concentrations. This proves that vertical inhibition of MEK-1/2 and ERK-1/2 demonstrates more potency than only targeting MEK-1/2 via PrAg/LF, and in some cases more potent than only targeting ERK-1/2 via SCH772984.

To better display the effect of vertical inhibition, instead of using fixed concentrations of the inhibitors, we serially diluted PrAg/LF and SCH772984 and tested them on three AML cell lines one sensitive (HL60) and two resistant to PrAg/LF (Mono-Mac-6 and U937) in order to increase the range of concentrations of the two inhibitors. Interestingly, HL-60 revealed more sensitivity to vertical inhibition than individual inhibition of the MAPK pathway, especially starting from 48 hours. Furthermore, MonoMac-6 also displayed potency of co-targeting MEK-1/2

and ERK-1/2 of the MAPK pathway, however only at later time points (96 and 120 hours) when compared to the effect of each inhibitor alone. U-937 on the other hand, did not reveal any added benefit of vertical inhibition in comparison to targeting MEK alone or ERK alone. By that, we prove that coupling the two inhibitors may offer a novel targeting method in order to overcome acquired resistance to MEK-1/2 inhibitors. These results demonstrate that vertical inhibition does offer increased potency in comparison to single targeting of the MAPK pathway when working with sensitive cell lines that are dependent on the cascade. However, co-targeting resistant cell lines, such as U-937, do not display any added advantage.

To determine whether LF mediated inhibition of the MAPK pathway in acute myeloid leukemia cell lines activates autophagy, we used flow cytometry to quantify autophagosome formation. By that we can further investigate the impact of autophagy on cell death secondary to MAPK inhibition. Among the four AML cell lines tested (HL60, ML-2, Mono-Mac-1 and U937), HL-60 and U-937 displayed an increase in autophagosome formation upon treatment with PrAg/LF as time progressed. This indicates an activation of autophagy following treatment with the anthrax lethal toxin. Although MonoMac-1 did not show an increase in autophagosome formation after treatment with PrAg/LF in comparison to the control, it did display a clear increase in the accumulation of autophagosomes in cells treated with PrAg/LF and chloroquine (CQ), compared to with PrAg/LF alone or CQ alone at 72 and 96 hours. This can be explained by the fact that during autophagic flux, there is an increase in the rate of processing of autophagosomes, which prevents their detectable accumulation. This indicates that autophagy is activated in a majority of AML cells following inhibition of anthrax lethal toxin-mediated inhibition of MEK1/2.

To further investigate whether the autophagosome formation (thus autophagy activation) demonstrate autophagy-mediated cell death in acute myeloid leukemia cell lines, we performed cytotoxicity assays treating the cells with the anthrax lethal toxin, with or without the autophagy inhibitor chloroquine (CQ). MonoMac-6 displayed more sensitivity to the cytotoxic effects of PrAg/LF and CQ than PrAg/LF alone, starting from 48 hours up to 120 hours. Similarly, co-treatment of TF-1HaRas with PrAg/LF and the autophagy inhibitor chloroquine also displayed less cell viability than treatment with PrAg/LF alone at all five time points (24, 48, 72, 96 and 120 hours). This indicates that the inhibition of autophagy by chloroquine is more detrimental to AML cells when treated with PrAg/LF than when allowing autophagy activation to take place. Therefore, autophagy plays a protective role for AML cells secondary to MAPK pathway inhibition via anthrax lethal toxin.

Chapter Five

Conclusion

Our study shows that the AML cell lines tested express matrix-metalloproteases as they were sensitive to the treatment of PrAgL1/FP59, a matrix-metalloprotease-activated protein synthesis inhibitor. In addition, cell lines sensitive to PrAg/LF and PrAgL1/LF display dependence on the MAPK cascade for survival and proliferation, as cell viability decreased post inhibition of the pathway. Furthermore, seeking to overcome the resistance to the anthrax lethal toxin, vertical inhibition using the MEK-1/2 inhibitor (PrAg/LF) and the ERK-1/2 inhibitor (SCH772984) demonstrates more potency than only targeting MEK-1/2, and in some cases more potency than targeting ERK-1/2 alone. Moreover, results demonstrate that vertical inhibition does exhibit increased potency in comparison to single targeting of the MAPK pathway when working with sensitive cell lines that are dependent on the cascade for survival. However, dealing with resistant cell lines that do not depend on the MAPK pathway shows that co-targeting does not display an added benefit. Finally, while autophagy activation does seem to be present in a majority of the tested cell lines secondary to treatment with the anthrax lethal toxin, the process is proven to be protective of the AML cell lines. These findings take us a step closer in targeting resistant acute myeloid leukemia cell lines in a more direct and efficacious approach.

Cell line	HL-60	TF1-vRaF	ML-2	THP-1	Mono-Mac-6	TF1-HaRas	U937	Mono-Mac-1
Sensitivity to PrAg/LF	+	+	+	+	-	-	-	-
Sensitivity to PrAgL1/LF	+	+	+	+	-	-	-	-
Sensitivity to PrAgL1/FP59	+	+	+	+	+	+	+	+

Figure 12: Response of the different tested cell lines after treatment with the wild type and the matrix metalloprotease-activated anthrax lethal toxin as well as the MMP-activated Pseudomonas aeruginosa exotoxin A. (+ indicates sensitivity, - indicates resistance)

Cell line	HL-60	Mono-Mac-6	U937
Sensitivity to PrAg/LF	+	-	-
Sensitivity to SCH772984	+	-	-
Sensitivity to SCH772984 and PrAg/LF	+	-	-

Figure 13: Response of the different tested cell lines after treatment with the wild type anthrax lethal toxin as well as the ERK inhibitor SCH772984 each alone and in combination. (+ indicates sensitivity, - indicates resistance)

Cell line	HL-60	Mono-Mac-1	U937	ML-2
Autophagy Activation	+	+	+	-

Figure 14: Effect of the treatment of different tested cell lines with the wild type anthrax lethal toxin on the activation of autophagy. (+ indicates activation, - indicates inactivation)

Cell line	TF1-HaRas	Mono-Mac-6
Effect of Autophagy	Protective	Protective

Figure 15: Effect of autophagy activation on the tested cell lines after treatment with the wild type anthrax lethal toxin with and without chloroquine.

BIBLIOGRAPHY

Abi-Habib, R. J., Singh, R., Leppla, S. H., Greene, J. J., Ding, Y., Berghuis, B., Duesbery, N. S., & Frankel, A. E. (2006). Systemic Anthrax Lethal Toxin Therapy Produces Regressions of Subcutaneous Human Melanoma Tumors in Athymic Nude Mice. *Clinical Cancer Research*, *12*(24), 7437–7443. https://doi.org/10.1158/1078-0432.CCR-06-2019

Abi-Habib, Ralph J., Urieto, J. O., Liu, S., Leppla, S. H., Duesbery, N. S., & Frankel, A. E. (2005). BRAF status and mitogen-activated protein/extracellular signal-regulated kinase kinase 1/2 activity indicate sensitivity of melanoma cells to anthrax lethal toxin. *Molecular Cancer Therapeutics*, *4*(9), 1303–1310. https://doi.org/10.1158/1535-7163.MCT-05-0145

Adhauliya, N., Kalappanavar, A. N., Ali, I. M., & Annigeri, R. G. (2016). Autophagy: A boon or bane in oral cancer. *Oral Oncology*, *61*, 120–126. https://doi.org/10.1016/j.oraloncology.2016.09.001

Alfano, R. W., Leppla, S. H., Liu, S., Bugge, T. H., Ortiz, J. M., Lairmore, T. C., Duesbery, N. S., Mitchell, I. C., Nwariaku, F., & Frankel, A. E. (2010). Inhibition of Tumor Angiogenesis by the Matrix Metalloproteinase–Activated Anthrax Lethal Toxin in an Orthotopic Model of Anaplastic Thyroid Carcinoma. *Molecular Cancer Therapeutics*, *9*(1), 190–201. https://doi org/10 1158/1535-7163 MCT-09-0694

Amit, I., Citri, A., Shay, T., Lu, Y., Katz, M., Zhang, F., Tarcic, G., Siwak, D., Lahad, J., Jacob-Hirsch, J., Amariglio, N., Vaisman, N., Segal, E., Rechavi, G., Alon, U., Mills, G. B., Domany, E., & Yarden, Y. (2007). A module of negative feedback regulators defines growth factor signaling. *Nature Genetics*, *39*(4), 503–512. https://doi org/10 1038/ng1987

Andreucci, M., Faga, T., Russo, D., Bertucci, B., Tamburrini, O., Pisani, A., Sabbatini, M., Fuiano, G., & Michael, A. (2014). Differential Activation of Signaling Pathways by Low-Osmolar and Iso-Osmolar Radiocontrast Agents in Human Renal Tubular Cells. *Journal of Cellular Biochemistry*, *115*(2), 281–289. https://doi org/10 1002/jcb 24662

Arbuthnot, P., Capovilla, A., & Kew, M. (2000). Putative role of hepatitis B virus X protein in hepatocarcinogenesis: Effects on apoptosis, DNA repair, mitogen-activated protein kinase and JAK/STAT pathways. *Journal of Gastroenterology and Hepatology*, *15*(4), 357–368. https://doi.org/10.1046/j.1440-1746.2000.02069.x

Avruch, J., Khokhlatchev, A., Kyriakis, J. M., Luo, Z., Tzivion, G., Vavvas, D., & Zhang, X. F. (2001). Ras activation of the Raf kinase: Tyrosine kinase recruitment of the MAP kinase cascade. *Recent Progress in Hormone Research*, *56*, 127–155. https://doi.org/10.1210/rp.56.1.127

Bekdash, A., Darwish, M., Timsah, Z., Kassab, E., Ghanem, H., Najjar, V., Ghosn, M., Nasser, S., El-Hajj, H., Bazerbachi, A., Liu, S., Leppla, S. H., Frankel, A. E., & Abi-Habib, R. J. (2015). Phospho-MEK1/2 and uPAR Expression Determine Sensitivity of AML Blasts to a Urokinase-Activated Anthrax Lethal Toxin (PrAgU2/LF). *Translational Oncology*, *8*(5), 347–357. https://doi.org/10.1016/j.tranon.2015.07.001

Bhat, F. A., Sharmila, G., Balakrishnan, S., Singh, P. R., Srinivasan, N., & Arunakaran, J. (2014). Epidermal growth factor-induced prostate cancer (PC3) cell survival and proliferation is inhibited by quercetin, a plant flavonoid through apoptotic machinery. *Biomedicine & Preventive Nutrition*, *4*(4), 459–468. https://doi.org/10.1016/j.bionut.2014.07.003

Bhat, P., Kriel, J., Shubha Priya, B., Basappa, Shivananju, N. S., & Loos, B. (2018). Modulating autophagy in cancer therapy: Advancements and challenges for cancer cell death sensitization. *Biochemical Pharmacology*, *147*, 170–182. https://doi.org/10.1016/j.bcp.2017.11.021

Braicu, C., Buse, M., Busuioc, C., Drula, R., Gulei, D., Raduly, L., Rusu, A., Irimie, A., Atanasov, A. G., Slaby, O., Ionescu, C., & Berindan-Neagoe, I. (2019). A Comprehensive Review on MAPK: A Promising Therapeutic Target in Cancer. *Cancers*, *11*(10). https://doi.org/10.3390/cancers11101618

Büchner, T., Schlenk, R. F., Schaich, M., Döhner, K., Krahl, R., Krauter, J., Heil, G., Krug, U., Sauerland, M. C., Heinecke, A., Späth, D., Kramer, M., Scholl, S., Berdel, W. E., Hiddemann, W., Hoelzer, D., Hehlmann, R., Hasford, J., Hoffmann, V. S., … Pfirrmann, M. (2012). Acute Myeloid Leukemia (AML): Different treatment strategies versus a common standard arm--combined prospective analysis by the German AML Intergroup. *Journal of Clinical Oncology: Official Journal of the American Society of Clinical Oncology*, *30*(29), 3604–3610.https://doi.org/10.1200/JCO.2012.42.2907

Burnett, A., Wetzler, M., & Löwenberg, B. (2011). Therapeutic Advances in Acute Myeloid Leukemia. *Journal of Clinical Oncology*, *29*(5), 487–494. https://doi.org/10.1200/JCO.2010.30.1820

Cagnol, S., & Chambard, J.-C. (2011). ERK and cell death: Mechanisms of ERK-induced cell death – apoptosis, autophagy and senescence. *The FEBS Journal*, 2–21. https://doi.org/10.1111/j.1742-4658.2009.07366.x@10.1002/(ISSN)1742-4658(CAT)FreeReviewContent(VI)Reviews1011

Chaikuad, A., Tacconi, E. M. C., Zimmer, J., Liang, Y., Gray, N. S., Tarsounas, M., & Knapp, S. (2014). A unique inhibitor binding site in ERK1/2 is associated with slow binding kinetics. *Nature Chemical Biology*, *10*(10), 853–860. https://doi.org/10.1038/nchembio.1629

Chen, Y., McMillan-Ward, E., Kong, J., Israels, S. J., & Gibson, S. B. (2008). Oxidative stress induces autophagic cell death independent of apoptosis in

transformed and cancer cells. *Cell Death and Differentiation*, *15*(1), 171–182. https://doi.org/10.1038/sj.cdd.4402233

Cicchini, M., Karantza, V., & Xia, B. (2015). Molecular Pathways: Autophagy in Cancer—A Matter of Timing and Context. *Clinical Cancer Research : An Official Journal of the American Association for Cancer Research*, *21*(3), 498–504.https://doi.org/10.1158/1078-0432.CCR-13-2438

Cooper, G. M. (2000). The Development and Causes of Cancer. *The Cell: A Molecular Approach. 2nd Edition*. https://www.ncbi.nlm.nih.gov/books/NBK9963/

Cortes, J. E., Talpaz, M., & Kantarjian, H. (1996). Chronic myelogenous leukemia: A review. *The American Journal of Medicine*, *100*(5), 555–570. https://doi.org/10.1016/s0002-9343(96)00061-7

Darwich, M. E. (2015). *Potency and selectivity of a urokinase activated recombinant anthrax toxin to acute myeloid leukemia (AML) cells. (C2015)* [Thesis]. https://doi org/10 26756/th 2015 53

Daver, N., Schlenk, R. F., Russell, N. H., & Levis, M. J. (2019). Targeting FLT3 mutations in AML: Review of current knowledge and evidence. *Leukemia*, *33*(2), 299–312. https://doi org/10 1038/s41375-018-0357-9

De Kouchkovsky, I., & Abdul-Hay, M. (2016). 'Acute myeloid leukemia: A comprehensive review and 2016 update.' *Blood Cancer Journal*, *6*(7), e441–e441. https://doi.org/10.1038/bcj.2016.50

Degirmenci, U., Wang, M., & Hu, J. (2020). Targeting Aberrant RAS/RAF/MEK/ERK Signaling for Cancer Therapy. *Cells*, *9*(1), 198. https://doi.org/10.3390/cells9010198

Döhner, H., Estey, E. H., Amadori, S., Appelbaum, F. R., Büchner, T., Burnett, A. K., Dombret, H., Fenaux, P., Grimwade, D., Larson, R. A., Lo-Coco, F., Naoe, T., Niederwieser, D., Ossenkoppele, G. J., Sanz, M. A., Sierra, J., Tallman, M. S., Löwenberg, B., Bloomfield, C. D., & European LeukemiaNet. (2010). Diagnosis and management of acute myeloid leukemia in adults: Recommendations from an international expert panel, on behalf of the European LeukemiaNet. *Blood*, *115*(3), 453–474. https://doi.org/10.1182/blood-2009-07-235358

Duesbery, N. S., Resau, J., Webb, C. P., Koochekpour, S., Koo, H.-M., Leppla, S. H., & Woude, G. F. V. (2001). Suppression of ras-mediated transformation and inhibition of tumor growth and angiogenesis by anthrax lethal factor, a proteolytic inhibitor of multiple MEK pathways. *Proceedings of the National Academy of Sciences*, *98*(7), 4089–4094. https://doi.org/10.1073/pnas.061031898

Foss, F. M. (2000). DAB389IL-2 (ONTAK): A Novel Fusion Toxin Therapy for Lymphoma. *Clinical Lymphoma*, *1*(2), 110–116. https://doi org/10 3816/CLM 2000 n 009

Galluzzi, L., Baehrecke, E. H., Ballabio, A., Boya, P., Bravo-San Pedro, J. M., Cecconi, F., Choi, A. M., Chu, C. T., Codogno, P., Colombo, M. I., Cuervo, A. M., Debnath, J., Deretic, V., Dikic, I., Eskelinen, E.-L., Fimia, G. M., Fulda, S., Gewirtz, D. A., Green, D. R., … Kroemer, G. (2017). Molecular definitions of autophagy and related processes. *The EMBO Journal*, *36*(13), 1811–1836. https://doi.org/10.15252/embj.201796697

Golden, H., Watson, L., Lal, H., Verma, S., Foster, D., Kuo, S.-R., Sharma, A., Frankel, A., & Dostal, D. (2009). Anthrax toxin: Pathologic effects on the cardiovascular system. *Frontiers in Bioscience : A Journal and Virtual Library*, *14*, 2335–2357.

Grazia, G., Penna, I., Perotti, V., Anichini, A., & Tassi, E. (2014). Towards combinatorial targeted therapy in melanoma: From pre-clinical evidence to clinical application (Review). *International Journal of Oncology*, *45*(3), 929. https://doi.org/10.3892/ijo.2014.2491

Guo, Y.-J., Pan, W.-W., Liu, S.-B., Shen, Z.-F., Xu, Y., & Hu, L.-L. (2020). ERK/MAPK signalling pathway and tumorigenesis. *Experimental and Therapeutic Medicine*, *19*(3), 1997–2007. https://doi.org/10.3892/etm.2020.8454

Hatzivassiliou, G., Liu, B., O'Brien, C., Spoerke, J. M., Hoeflich, K. P., Haverty, P. M., Soriano, R., Forrest, W. F., Heldens, S., Chen, H., Toy, K., Ha, C., Zhou, W., Song, K., Friedman, L. S., Amler, L. C., Hampton, G. M., Moffat, J., Belvin, M., & Lackner, M. R. (2012). ERK Inhibition Overcomes Acquired Resistance to MEK Inhibitors. *Molecular Cancer Therapeutics*, *11*(5), 1143–1154.https://doi.org/10.1158/1535-7163.MCT-11-1010

Huang, D., Ding, Y., Luo, W.-M., Bender, S., Qian, C.-N., Kort, E., Zhang, Z.-F., VandenBeldt, K., Duesbery, N. S., Resau, J. H., & Teh, B. T. (2008). Inhibition of MAPK Kinase Signaling Pathways Suppressed Renal Cell Carcinoma Growth and Angiogenesis In vivo. *Cancer Research*, *68*(1), 81–88. https://doi org/10 1158/0008-5472 CAN-07-5311

Hwang, J.-Y., Gertner, M., Pontarelli, F., Court-Vazquez, B., Bennett, M. V. L., Ofengeim, D., & Zukin, R. S. (2017). Global ischemia induces lysosomal-mediated degradation of mTOR and activation of autophagy in hippocampal neurons destined to die. *Cell Death & Differentiation*, *24*(2), 317–329. https://doi org/10 1038/cdd 2016 140

Jung, C. H., Ro, S.-H., Cao, J., Otto, N. M., & Kim, D.-H. (2010). MTOR regulation of autophagy. *FEBS Letters*, *584*(7), 1287–1295. https://doi org/10 1016/j febslet 2010 01 017

Kalmar, B., Lu, C.-H., & Greensmith, L. (2014). The role of heat shock proteins in Amyotrophic Lateral Sclerosis: The therapeutic potential of Arimoclomol. *Pharmacology & Therapeutics*, *141*(1), 40–54. https://doi org/10 1016/j pharmthera 2013 08 003

Kasbo, Z. J. (2015). *Sensitivity of colorectal cancer cells to the recombinant Anthrax lethal toxin and the Urokinase-activated Anthrax lethal toxin. (C2014)* [Thesis]. https://doi org/10 26756/th 2014 40

Kassab, E. (2013). *Targeting the MAP Kinase pathway in human acute myeloid leukemia cells using a recombinant anthrax lethal toxin. (C2013)* [Thesis]. https://doi.org/10.26756/th.2013.13

Kassab, E., Darwish, M., Timsah, Z., Liu, S., Leppla, S. H., Frankel, A. E., & Abi-Habib, R. J. (2013). Cytotoxicity of Anthrax Lethal Toxin to Human Acute Myeloid Leukemia Cells Is Nonapoptotic and Dependent on Extracellular Signal-Regulated Kinase 1/2 Activity. *Translational Oncology*, *6*(1), 25–32.

Khalil, N., & Abi-Habib, R. J. (2019). [HuArgI (co)-PEG5000]-induced arginine deprivation leads to autophagy dependent cell death in pancreatic cancer cells. *Investigational New Drugs*.https://doi.org/10.1007/s10637-019-00883-4

Kim, S. H., & Kim, H. (2019). Astaxanthin Modulation of Signaling Pathways That Regulate Autophagy. *Marine Drugs*, *17*(10). https://doi org/10 3390/md17100546

Kumar, P., Kumar, A., Parveen, S., Murphy, J. R., & Bishai, W. (2019). Recent advances with Treg depleting fusion protein toxins for cancer immunotherapy. *Immunotherapy*, *11*(13), 1117–1128. https://doi org/10 2217/imt-2019-0060

Lichtman, M. A., & Segel, G. B. (2005). Uncommon phenotypes of acute myelogenous leukemia: Basophilic, mast cell, eosinophilic, and myeloid dendritic cell subtypes: A review. *Blood Cells, Molecules, and Diseases*, *35*(3), 370–383. https://doi org/10 1016/j bcmd 2005 08 006

Lim, J., Kelley, E. H., Methot, J. L., Zhou, H., Petrocchi, A., Chen, H., Hill, S. E., Hinton, M. C., Hruza, A., Jung, J. O., Maclean, J. K. F., Mansueto, M., Naumov, G. N., Philippar, U., Raut, S., Spacciapoli, P., Sun, D., & Siliphaivanh, P. (2016). Discovery of 1-(1H-Pyrazolo[4,3-c]pyridin-6-yl)urea Inhibitors of Extracellular Signal-Regulated Kinase (ERK) for the Treatment of Cancers. *Journal of Medicinal Chemistry*, *59*(13), 6501–6511. https://doi.org/10.1021/acs.jmedchem.6b00708

Liu, S., Netzel-Arnett, S., Birkedal-Hansen, H., & Leppla, S. H. (2000). Tumor Cell-selective Cytotoxicity of Matrix Metalloproteinase-activated Anthrax Toxin. *Cancer Research*, *60*(21), 6061.

Liu, S., Wang, H., Currie, B. M., Molinolo, A., Leung, H. J., Moayeri, M., Basile, J. R., Alfano, R. W., Gutkind, J. S., Frankel, A. E., Bugge, T. H., & Leppla, S. H. (2008). Matrix Metalloproteinase-activated Anthrax Lethal Toxin

Demonstrates High Potency in Targeting Tumor Vasculature. *Journal of Biological Chemistry*, *283*(1), 529–540. https://doi org/10 1074/jbc M707419200

Liu, Y., Shoji-Kawata, S., Sumpter, R. M., Wei, Y., Ginet, V., Zhang, L., Posner, B., Tran, K. A., Green, D. R., Xavier, R. J., Shaw, S. Y., Clarke, P. G. H., Puyal, J., & Levine, B. (2013). Autosis is a Na+,K+-ATPase-regulated form of cell death triggered by autophagy-inducing peptides, starvation, and hypoxia-ischemia. *Proceedings of the National Academy of Sciences of the United States of America*, *110*(51), 20364–20371. https://doi.org/10.1073/pnas.1319661110

Mauthe, M., Orhon, I., Rocchi, C., Zhou, X., Luhr, M., Hijlkema, K.-J., Coppes, R. P., Engedal, N., Mari, M., & Reggiori, F. (2018). Chloroquine inhibits autophagic flux by decreasing autophagosome-lysosome fusion. *Autophagy*, *14*(8), 1435–1455. https://doi.org/10.1080/15548627.2018.1474314

Mizushima, N. (2007). Autophagy: Process and function. *Genes & Development*, *21*(22), 2861–2873. https://doi org/10 1101/gad 1599207

Morris, E. J., Jha, S., Restaino, C. R., Dayananth, P., Zhu, H., Cooper, A., Carr, D., Deng, Y., Jin, W., Black, S., Long, B., Liu, J., Dinunzio, E., Windsor, W., Zhang, R., Zhao, S., Angagaw, M. H., Pinheiro, E. M., Desai, J., … Samatar, A. A. (2013). Discovery of a novel ERK inhibitor with activity in models of acquired resistance to BRAF and MEK inhibitors. *Cancer Discovery*, *3*(7), 742–750. https://doi.org/10.1158/2159-8290.CD-13-0070

Onorati, A., Dyczynski, M., Ojha, R., & Amaravadi, R. K. (2018). Targeting autophagy in cancer. *Cancer*, *124*(16), 3307–3318. https://doi.org/10.1002/cncr.31335

Padma, V. V. (2015). An overview of targeted cancer therapy. *BioMedicine*, *5*(4). https://doi org/10 7603/s40681-015-0019-4

Papaemmanuil, E., Gerstung, M., Bullinger, L., Gaidzik, V. I., Paschka, P., Roberts, N. D., Potter, N. E., Heuser, M., Thol, F., Bolli, N., Gundem, G., Van Loo, P., Martincorena, I., Ganly, P., Mudie, L., McLaren, S., O'Meara, S., Raine, K., Jones, D. R., … Campbell, P. J. (2016). Genomic Classification and Prognosis in Acute Myeloid Leukemia. *New England Journal of Medicine*, *374*(23), 2209–2221.https://doi.org/10.1056/NEJMoa1516192

Peters, D. E., Hoover, B., Cloud, L. G., Liu, S., Molinolo, A. A., Leppla, S. H., & Bugge, T. H. (2014). Comparative toxicity and efficacy of engineered anthrax lethal toxin variants with broad anti-tumor activities. *Toxicology and Applied Pharmacology*, *279*(2), 220–229. https://doi.org/10.1016/j.taap.2014.06.010

Petty, A. J., Heyman, B., & Yang, Y. (2020). Chimeric Antigen Receptor Cell Therapy: Overcoming Obstacles to Battle Cancer. *Cancers*, *12*(4). https://doi org/10 3390/cancers12040842

Pratilas, C. A., & Solit, D. B. (2010). Targeting the Mitogen-Activated Protein Kinase Pathway: Physiological Feedback and Drug Response. *Clinical Cancer Research*, *16*(13), 3329–3334. https://doi org/10 1158/1078-0432 CCR-09-3064

Redaelli, A., Stephens, J. M., Laskin, B. L., Pashos, C. L., & Botteman, M. F. (2003). The burden and outcomes associated with four leukemias: AML, ALL, CLL and CML. *Expert Review of Anticancer Therapy*, *3*(3), 311–329. https://doi org/10 1586/14737140 3 3 311

Redmann, M., Benavides, G. A., Berryhill, T. F., Wani, W. Y., Ouyang, X., Johnson, M. S., Ravi, S., Barnes, S., Darley-Usmar, V. M., & Zhang, J. (2016). Inhibition of autophagy with bafilomycin and chloroquine decreases mitochondrial quality and bioenergetic function in primary neurons. *Redox Biology*, *11*, 73–81. https://doi.org/10.1016/j.redox.2016.11.004

Roberts, P. J., & Der, C. J. (2007). Targeting the Raf-MEK-ERK mitogen-activated protein kinase cascade for the treatment of cancer. *Oncogene*, *26*(22), 3291–3310. https://doi org/10 1038/sj onc 1210422

Sami, S. A., Darwish, N. H. E., Barile, A. N. M., & Mousa, S. A. (2020). Current and Future Molecular Targets for Acute Myeloid Leukemia Therapy. *Current Treatment Options in Oncology*, *21*(1), 3. https://doi org/10 1007/s11864-019-0694-6

Sever, R., & Brugge, J. S. (2015). Signal transduction in cancer. *Cold Spring Harbor Perspectives in Medicine*, *5*(4). https://doi.org/10.1101/cshperspect.a006098

Shapira, A., & Benhar, I. (2010). Toxin-Based Therapeutic Approaches. *Toxins*, *2*(11), 2519–2583. https://doi org/10 3390/toxins2112519

Stoll, V., Calleja, V., Vassaux, G., Downward, J., & Lemoine, N. R. (2005). Dominant negative inhibitors of signalling through the phosphoinositol 3-kinase pathway for gene therapy of pancreatic cancer. *Gut*, *54*(1), 109–116. https://doi org/10 1136/gut 2004 046706

Tallman, M. S., Gilliland, D. G., & Rowe, J. M. (2005). Drug therapy for acute myeloid leukemia. *Blood*, *106*(4), 1154–1163. https://doi.org/10.1182/blood-2005-01-0178

Terwilliger, T., & Abdul-Hay, M. (2017). Acute lymphoblastic leukemia: A comprehensive review and 2017 update. *Blood Cancer Journal*, *7*(6), e577–e577. https://doi.org/10.1038/bcj.2017.53

Thiele, W., Krishnan, J., Rothley, M., Weih, D., Plaumann, D., Kuch, V., Quagliata, L., Weich, H. A., & Sleeman, J. P. (2012). VEGFR-3 is expressed on megakaryocyte precursors in the murine bone marrow and plays a regulatory role in megakaryopoiesis. *Blood*, *120*(9), 1899–1907. https://doi org/10 1182/blood-2011-09-376657

Tsirigotis, P., Byrne, M., Schmid, C., Baron, F., Ciceri, F., Esteve, J., Gorin, N. C., Giebel, S., Mohty, M., Savani, B. N., & Nagler, A. (2016). Relapse of AML after hematopoietic stem cell transplantation: Methods of monitoring and preventive strategies. A review from the ALWP of the EBMT. *Bone Marrow Transplantation*, *51*(11), 1431–1438. https://doi.org/10.1038/bmt.2016.167

Verbaanderd, C., Maes, H., Schaaf, M. B., Sukhatme, V. P., Pantziarka, P., Sukhatme, V., Agostinis, P., & Bouche, G. (2017). Repurposing Drugs in Oncology (ReDO)—Chloroquine and hydroxychloroquine as anti-cancer agents. *Ecancermedicalscience*, *11*. https://doi.org/10.3332/ecancer.2017.781

White, E., Mehnert, J. M., & Chan, C. S. (2015). Autophagy, Metabolism, and Cancer. *Clinical Cancer Research*, *21*(22), 5037–5046. https://doi org/10 1158/1078-0432 CCR-15-0490

Yan, X., Zhou, R., & Ma, Z. (2019). Autophagy—Cell Survival and Death. In Z.-H. Qin (Ed.), *Autophagy: Biology and Diseases: Basic Science* (pp. 667–696). Springer https://doi org/10 1007/978-981-15-0602-4_29

Yilmaz, M., Wang, F., Loghavi, S., Bueso-Ramos, C., Gumbs, C., Little, L., Song, X., Zhang, J., Kadia, T., Borthakur, G., Jabbour, E., Pemmaraju, N., Short, N., Garcia-Manero, G., Estrov, Z., Kantarjian, H., Futreal, A., Takahashi, K., & Ravandi, F. (2019). Late relapse in acute myeloid leukemia (AML): Clonal evolution or therapy-related leukemia? *Blood Cancer Journal*, *9*(2), 7. https://doi.org/10.1038/s41408-019-0170-3

Zhang, S., & Kipps, T. J. (2014). The Pathogenesis of Chronic Lymphocytic Leukemia. *Annual Review of Pathology*, *9*, 103–118. https://doi org/10 1146/annurev-pathol-020712-163955