LEBANESE AMERICAN UNIVERSITY

The Mechanisms of Resistance of Anthrax Lethal Toxin (LeTx)-Induced Cytotoxicity in AML Cells

By

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ABSTRACT

Anthrax lethal toxin has recently been established to induce cytotoxicity in acute myeloid leukemia cell lines by the inhibition of the MAPK pathway. The aim of this study was to investigate the mechanisms by which some acute myeloid leukemia cell lines develop resistance to the LeTx-induced inhibition of the MAPK pathway. This was achieved by determining the differences in the Map Kinase pathway between LeTx-sensitive and resistant AML cell lines. In order to determine if autophagy could be affecting a cell lines capability of developing resistance, the autophagy inhibitor chloroquine was used against both sensitive and resistant cell lines. Our data showed that autophagy is a contributing mechanism to the resistance of Mono-Mac-1 and U937 to the LeTx-mediated inhibition of the MAPK pathway. Another potential mechanism of resistance to the inhibition of MEK1/2 is the negative feedback loop initiated by ERK1/2. In order to investigate this mechanism, we tested the effects of the vertical inhibition of the MAPK pathway using both the MEK1/2 inhibitor LeTx and specific ERK inhibitors (VX-11e and SCH772984). Our results indicate that vertical inhibition by SCH772984 rendered the resistant cell lines sensitive to the inhibition of the MAPK-pathway and showed an increased cytotoxic effect once used in combination with anthrax lethal toxin compared to the use of anthrax lethal toxin alone.

Key words: Anthrax Lethal Toxin, Autophagy, Chloroquine, ERK inhibitors, Acute Myeloid Leukemia.
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<tr>
<td>LeTx</td>
<td>Anthrax Lethal Toxin</td>
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<td>AML</td>
<td>Acute Myeloid Leukemia</td>
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<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
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<tr>
<td>MEK</td>
<td>Mitogen-activated protein kinase kinase</td>
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<tr>
<td>ERK</td>
<td>Extracellular signal-regulated kinase</td>
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<tr>
<td>FP59</td>
<td>Pseudomonas aeruginosa exotoxin A</td>
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<tr>
<td>CQ</td>
<td>Chloroquine</td>
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<tr>
<td>PrAg</td>
<td>Protective antigen</td>
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<tr>
<td>ANTXRs</td>
<td>Anthrax toxin receptors</td>
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<tr>
<td>LF</td>
<td>Lethal Factor</td>
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<tr>
<td>EF</td>
<td>Edema Factor</td>
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<td>MM1</td>
<td>Mono-Mac-1</td>
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<tr>
<td>MM6</td>
<td>Mono-Mac-6</td>
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<td>RTK</td>
<td>Receptor Tyrosine Kianse</td>
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Chapter 1
Introduction

1.1 Acute Myeloid Leukemia

Research and treatment of cancer is based on the fact that all normal cells have similar molecular networks that control cell death, proliferation, and growth (Hejmadi, 2010). These normal cells are converted into cancerous cells when a disruption or mutation occurs that results in changes in these networks such as genetic modifications of numerous proto-oncogenes and tumor-suppressor genes in addition to other genomic changes which ultimately lead to continuous cell proliferation and the formation of tumors (Karakosta et al., 2005). Cancer differentiates itself from all other diseases by developing at any point or stage in life in any organ within the body. No two cancer cases can behave precisely alike. Some cancers develop in an aggressive manner in which the cancer develops at a very rapid pace while other types grow slowly or they may remain dormant for years. The majority of cancers are categorized under three key groups: sarcomas, lymphomas or leukemias, and carcinomas. Sarcomas are the most rare type of cancer accounting for less than 1% of all adult solid malignancies and are characterized as solid tumors of connective tissues (Burningham, Hashibe, Spector, & Schiffman, 2012). 8% of human malignancies arise from Leukemias (blood forming cells) and Lymphomas (cells of the immune system) (Cooper, 2000). Lastly Carcinomas make up the majority of human cancers (90%) and are malignancies of epithelial cells (Cooper, 2000). The main focus of this study is dealing with leukemia specifically the
inner workings/mechanisms of resistance of acute myeloid leukemia cell lines to anthrax lethal toxin.

Acute myeloid leukemia (AML) is a disorder which is characterized by the clonal expansion of myeloid progenitors in the peripheral blood and bone marrow. Adult acute myeloid leukemia (AML) is not a single disease, it is actually a collection of associated diseases, and patients with dissimilar subtypes of AML can have diverse reactions and responses to treatment. AML can be present either as a leukemia without an apparent precursor illness or as a progression from evident marrow disorders such as aplastic anemia, myelodysplasia and Fanconi anemia, or after the having received therapy for other types of cancers or nonmalignant illnesses (Schiffer & Stone, 2003). AML is the dominant type of acute leukemia found in most adults, as it represents 80 to 85 percent of the cases in this group (Yamamoto & Goodman, 2008)

Latest studies have shown that the disorder is brought about by an accumulation of genetic alterations in bone marrow hematopoetic stem cells with increasing age (Saultz & Garzon, 2016). Recurring chromosomal structural variations have been established as prognostic and diagnostic markers for acute myeloid leukemia; this finding suggests that genetic abnormalities that are acquired play a vital role in leukemogenesis (Carretero, Remeseiro, & Losada, 2010). In order to establish a diagnosis for AML, myeloblasts must encompass a minimum of 20% nucleated cells in the blood or bone marrow (Döhner et al., 2010). While advances in the treatment of AML have resulted in a substantially better prognosis for younger patients, outcomes in the elderly who make up the majority of new cases remains inadequate (Shah, Andersson, Rachet, Björkhol, & Lambert, 2013). Even with existing therapies, as many as 70% of patients above the
age of 65 will succumb to their disease within one year of diagnosis (Meyers, Yu, Kaye, & Davis, 2013). Several types of treatment may be used for people with AML. The typical treatment for AML is induction chemotherapy with a combination of anthracycline and cytarabine. This is generally followed by either chemotherapy or stem cell transplantation, depending on the ability of the patient to undergo intensive treatment and the possibility of cure with chemotherapy alone (Roboz, 2012). In special circumstances surgery and radiation therapy may be used. AML is cured in 35–40% of people who are less than 60 years old and 5–15% who are more than 60 years old. This population showing an average survival of only 5 to 10 months (Medinger, Lengerke, & Passweg, 2016). The available therapies are not too effective and are quite intensive and damaging particularly to people over the age of 60 (in which the highest rate of AML occurs) that cannot withstand such treatments. Most of the patients with AML who achieve complete remission end up relapsing and having the leukemia recur within 3 years of diagnosis (Döhner et al., 2010). Overall, the prognosis of patients after relapse is poor and treatment possibilities insufficient. Therefore it is evident that novel therapeutic approaches are essential especially considering that acute myeloid leukemia incidence rates have increased since the late 1970s by 73% (Smith et al., 2010). One such approach is determining the role autophagy is playing with respect to acute myeloid leukemia cell lines.
1.2 Autophagy

Autophagy is an essential cell recycling program which is responsible for the removal of damaged or long-lived proteins and organelles (Redmann et al., 2016). Pharmacological modulators of this pathway have been comprehensively utilized in a broad range of research and clinical studies. Under ideal conditions, basal levels of autophagy are required to warrant cell fitness and to sustain quality control of crucial cellular components, by eliminating unfolded, unnecessary and/or aged proteins, as well as impaired or surplus organelles (Evangelisti et al., 2015). Autophagic flow can significantly increase in response to cellular stress, such as nutrient deficiency, hypoxia, starvation, DNA damage and pathogen infection, allowing the cell to survive with that specific “emergency” status. Disruption of the autophagic pathway might have severe consequences considering that the deregulation of autophagy triggers diverse pathological disorders such as autoimmunity, inflammatory disorders, cancer, and neurodegeneration (Winslow & Rubinsztein, 2008).

When cells are not receiving sufficient growth signals or when inhibition of oncogenic kinases occurs this leads to a decrease in glucose metabolism. This triggers the self-digestive role of autophagy in order to break down unnecessary organelles and bulk cytoplasm. On the other hand, autophagy can be a mechanism of survival for cells throughout periods of starvation where self-digestion offers a substitute of energy source and can assist in the discarding of unfolded proteins under stressful conditions (Komatsu et al., 2005). The majority of evidence is in correlation with the role of autophagy in sustaining tumour cell survival as a reaction to metabolic stress in vitro as well as in hypoxic tumour regions in vivo (Degenhardt et al., 2006). In preclinical models once the
pro-survival mechanism of autophagy was inhibited this lead to the death of tumor cells and instigated apoptotic cell death (Amaravadi et al., 2007). Moreover, recent studies have shown that autophagy inhibitors given in combination with chemotherapy repressed tumor development and activated cell death in a much larger magnitude compared to the sole use of chemotherapy alone (in vitro and in vivo) (Yang, Chee, Huang, & Sinicrope, 2011). These findings indicate that pro-survival autophagy may represent a major impediment to successful cancer therapy and thus represents a novel therapeutic target. Accordingly, the manipulation of pro-survival autophagy may be a major key to successful cancer therapy and may be a novel strategy for targeted disruption of oncogenic proteins in specific types of acute myeloid leukemia (Zhang et al., 2013).

Chloroquine (CQ) (an inhibitor of autophagy) is an anti-malarial drug that blocks the fusion of autophagosome with the lysosome and inhibits lysosomal acidification (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 vivo studies have shown that CQ administration in cancer leads to advantageous results. In one study after exposing mice (with overexpressed glioblastomaxenografts) to CQ the results indicated hindered tumor development (Jutten et al., 2013). Moreover after CQ was administered intracranially the amount of apoptotic cells increased and the number of mitotic cells was considerably reduced in a different glioblastoma xenograft study (Kim et al., 2010). Also, in three separate studies CQ was shown to decrease tumor size and frequency in mice with liver cancer stem cells (Song et al., 2013). This lead to a decline in tumor development and
mass in an orthotopic xenograft model of liver cancer (Redmann et al., 2016), and decreased tumor size and weight in a human melanoma xenograft model (Lakhter et al., 2013).

The specific mechanism by which CQ leads to the inhibition of autophagy is through its accumulation inside acidic organelles, subsequently resulting in the inhibition of lysosomal enzymes and the subsequent prevention of the fusion of endosomes (refer to Figure 3), including autophagosomes, and lysosomes, leading the inhibition of autophagy (Akache et al., 2016).

Figure 1. The factors that trigger autophagy and the role Chloroquine plays in inhibiting autophagy.

The two types of self-destruction at the cellular level: apoptosis (self-killing) and autophagy (self-eating) - are believed to be tumor suppressive. Conversely, recent studies, demonstrate that autophagy can lead to cell survival under conditions of nutrient restriction, therefore, inhibition of autophagy by chloroquine can inhibit tumorigenesis
(Dang, 2008). Given its possible function in metabolism and cell survival, autophagy may play a significant role in oncogenesis and in the response of several tumor types, including leukemias, to cancer therapeutics.
1.3 Anthrax Lethal Toxin

The antitumour potential of wild type LeTx was investigated in several tumor types particularly in mitogen-activated protein kinase (MAPK)-dependent tumours such as human melanomas. In one study it was experimentally proven that in vivo systemic LeTx treatment of subcutaneous human xenograft melanoma tumors in athymic nude mice actually led to significant tumor regression with only minor toxicity observed in mice, hence demonstrating the potency and selectivity of LeTx (Abi-Habib et al., 2006). In another study it was demonstrated that anthrax lethal toxin is potent against melanoma cell lines carrying the V599E BRAF mutation, hence it is potentially a useful therapeutic for melanoma patients especially those carrying the V599E BRAF mutation (80% of melanoma patients) (Abi-Habib et al., 2005). It has also previously been established that LeTx-sensitivity in melanoma cell lines is dependent on the levels of phosphorylated MEK1/2 with LeTx-induced cytotoxicity in melanoma cells being dependent on phospho-MEK1/2 levels (Abi-Habib et al., 2005). In 2013 a study proved that the majority of acute myeloid leukemia cell lines were in fact sensitive to inhibition of the MAPK pathway via anthrax lethal toxin (Kassab et al., 2013). It was essentially the first study that showed acute myeloid leukemia exhibiting cytotoxic responses to inhibition of the MAPK pathway. The present work is a direct continuation of that paper that demonstrated the importance of using anthrax lethal toxin in targeting AML cell lines.

Anthrax is caused by a Gram-positive spore-forming bacteria, Bacillus anthracis. Entry of the bacterial spores into the organism can occur either through the skin, or by inhalation or ingestion. This bacterium has evolved a very high degree of virulence
which leads to the killing of the host within a few days of infection. Antibiotics are capable of being effective against anthrax in the preliminary stages of the infection when the bacillus is still advancing in numbers but once increasing quantities of the toxins are secreted in the bloodstream the antibiotics lose their effectiveness and the infected organism submits to the lethal effects of the toxins (Agrawal & Pulendran, 2004). Systemic infection is therefore usually lethal since the initial symptoms are comparable to those of the flu and are very difficult to detect (Liu, Moayeri, & Leppla, 2014).

Patients usually display non-specific symptoms at the initial stages of infection (Holty et al., 2006). An ulcerated small erythematous lesion is a characteristic developed in cutaneous anthrax patients, the lesions which form end up resembling a spider bite. Abdominal uneasiness, vomiting, nausea, and diarrhea are symptoms which develop with patients who suffer from gastrointestinal anthrax. Pulmonary anthrax patients have chest pain, malaise, diaphoresis, fever, cough, dyspnea and may also have nausea and vomiting (Frankel et al., 2009).

The toxins expressed by *B. anthracis* consist of three separate exotoxin components: lethal factor (LF, 89 kDa), protective antigen (PrAg, 83 kDa), and edema factor (EF, 90 kDa). The two later components, LF and EF are enzymes whose activity is responsible for the lethality of anthrax. The route of spore entry into the organism characterizes the type of anthrax disease that will follow: gastrointestinal, cutaneous, and inhalational anthrax. Recent studies showed that spores are phagocytosed by local macrophages and dendritic cells, these cells may serve as a ‘Trojan horse’ to transport them from marginal sites to local lymph nodes where they propagate and germinate to become toxin-producing vegetative bacteria.
The exotoxins of *B. anthracis* act in a binary combination to form either the anthrax lethal toxin (LeTx), which is composed of two proteins, PrAg (protective antigen) and LF (lethal factor) or the anthrax edema toxin (EdTx), consisting of PrAg and EF (edema factor) (Langer et al., 2012). PrAg is the cellular binding and translocation moiety while lethal factor (LF) and edema factor (EF) constitute the catalytic moieties of the toxins. PrAg is an essential component of the anthrax toxins since it allows cell binding and translocation of the catalytic moieties (LF or EF) into the host cell (Friebe, van der Goot, & Bürgi, 2016). PrAg binds to the ubiquitously expressed anthrax toxin receptors (ATRs), the tumor endothelial marker 8 (TEM8) and the capillary morphogenesis protein 2 (CMG2). TEM8 is mostly expressed in blood vessel of tumours and in vasculature since it plays a role in angiogenesis and the regulation of neovasculature. CMG2 is expressed in capillary cells and its expression is correlated with capillary morphogenesis.

The mechanism of action of anthrax toxins (refer to Figure 2) starts with the binding of PrAg83 (83 kDa protein) to anthrax toxin receptors (TEM8 or CMG2) through interactions between domains 2 and 4 of PrAg with Mg$^{2+}$ and Ca$^{2+}$ ions at the metal ion adhesion domain of the receptors (Santelli, Bankston, Leppla, & Liddington, 2004). PrAg83 is then proteolytically cleaved by furin or furin-like proteases at the RKKR cleavage sequence of the protein. This cleavage leads to the release of a 20 kDa fragment from the N terminus, resulting in the formation of the active, receptor-bound 63 kDa fragment (PrAg63) (Liu & Leppla, 2003). The active PrAg63 then oligomerizes (forming either heptamers or octomers) and binds 3 to 4 LF or EF molecules (Abrami, Liu, Cosson, Leppla, & Goot, 2003). The number of LF and EF molecules bound to the
PrAgoliogomer is determined by steric hindrance between the molecules at adjacent binding sites. The complex then internalizes through receptor-mediated endocytosis into a clathrin-coated endosome (Young & Collier, 2007).

The clathrin-coated endosome then fuses with a lysosome leading to the acidification of the vesicle and a subsequent conformational change in the structure of PrAg63 that transforms the pre-pore complex into a β-barrel pore structure that allows the translocation of the catalytic moiety (LF or EF) into the cytosol (Abrami, Leplla, & Goot, 2006). Once in the cytosol these catalytic moieties enzymatically alter their corresponding targets leading to the observed effect of the anthrax toxins (van der Goot & Young, 2009). Edema factor (EF) binds to its target calmodulin (a calcium sensor), and then in turn mimics the effects of adenylatecyclase raising the intracellular concentration of cAMP. Lethal factor (LF) is a Zinc-metalloprotease that catalytically cleaves all MEKs (MEK1, 2, 3, 4, 6 and 7) at their N-terminal region, leading to the complete inhibition of all three branches of the MAPK pathway (Naik et al., 2013). These enzymatic moieties (LF or EF) are thought to unfold partially or completely as they pass through the constricted PrAg63 oligomeric pores then refold in the cytosol back into their native, enzymatically-active conformation (Bradley, Mogridge, Mourez, Collier, & Young, 2001). Since LF inactivates MEKs, particularlry MEK1/2, and inhibits the MAPK pathway, it is of particular importance in targeting cancers carrying oncogenic mutations in this pathway. Hence, anthrax lethal toxin (LeTx) is a potential targeted therapeutics for the treatment of several tumors (Abi-Habib et al., 2006, Tanioset al., 2013).
The detailed mechanism of entry of anthrax lethal toxin and how it releases its cytotoxic effects within the cell.

This inhibition of the MAPK pathway, which is essential for several functions such as proliferation, inflammation and survival in all cell types, leads to multisystem dysfunction in the host. The inhibition of MEK1/2 (MAPKK) is an attractive strategy for selective targeting of tumors since it can impede inappropriate, oncogenic, signal transduction independently of the upstream position of the oncogenic mutation (refer to Figure 3) (Huang et al., 2008). Modification of the PrAg proteolytic activation site, allowing activation by different proteases, changes the activation profile of PrAg and is a potential approach for increasing the selectivity of LeTx to cancer cells. Others have modified the furin-activation site (RXXR) on PrAg to a urokinase plasminogen activator (uPA)-activation site or to a matrix metalloprotease (MMP)-activation site, both
proteases overexpressed on cancer cells and showing limited expression on normal cells, hence the increase in selectivity (Liu, Schubert, Bugge, & Leppla, 2003).

Figure 3. Multisystem dysfunction in the host due to the interference of the MAPK pathway by Lethal Factor.

Cytotoxicity of anthrax lethal toxin will solely take place when a specific proteolytic activity is in existence and this can be manipulated by changing the cleavage sequence which is necessary for the proteolytic triggering of PrAg (Peters et al., 2014). FP59 is a fusion of the PrAg binding domain of LF and the catalytic domain of Pseudomonas aeruginosa exotoxin A. FP59 is not targeting the MAPK pathway, it ADPribosylates elongation factor 2 resulting in the obstruction of protein synthesis and consequent cell death (Kassab et al., 2013). The binding to PrAg and translocation of FP59 into the cytosol is indistinguishable to those of LF; the resulting combination of PrAg and FP59 leads to MAPK-independent cytotoxicity to all the cells that express the anthrax toxin receptors (Kassab et al., 2013). This is a useful tool for evaluating the existence of toxin receptors and the reliability of the uptake process, since cell death is easily measured. By determining the mechanism of resistance of some cell lines to anthrax lethal toxin this
can help establish novel targeted therapeutics that will be able to bypass the mechanism of resistance. Another main form of targeted therapeutics in acute myeloid leukemia cell lines is developing new drugs that target the mitogen-activated protein kinase (MAPK) pathway.
1.4 MAPK Pathway

The MAPK signaling pathway is an extremely precise signal transduction pathway that attempts to guarantee that the signal input is firmly associated with the duration of ERK activation, hence under normal conditions, the activation of the MAPK pathway is of a pulsatile nature, in which the pathway (eventually the effector ERK/2) is being activated for short periods of time followed by long periods of inactivation. The activation of ERK begins with the phosphorylation of MEK which then phosphorylates threonine and tyrosine residues on ERK leading to its activation. ERK then translocates from the cytosol to the nucleus to phosphorylate and activate several transcription factors, which are responsible for cell growth, regulation of differentiation and mitosis (refer to Figure 4)(Seger & Krebs, 1995).

![Figure 4. The MAPK signaling pathway.](image)

A lot of evidence suggests that both negative and positive feedback loops play significant roles in regulating the baseline sensitivity to input of the MAPK pathway.
thus preserving cellular homeostasis (Amit et al., 2007). In contrast, MAPK- deregulated tumors are characterized by a constitutively activated MAPK pathway that has lost its normal pulsatile activation scheme, resulting in a continuous activation of ERK and a higher basal enzymatic activity, subsequently leading to continuous stimulation of transcriptional and translational outputs, and abnormal cell growth (Sever & Brugge, 2015). The fact so many characteristics of cells are controlled by the MAPK signaling pathway and that, components of this pathway are some of the most frequently mutated oncogenes in human cancers (particularly Ras, followed by Raf and RTK deregulations) gives reason to be optimistic that approaches based on targeting this pathway will be constitute successful targeted cancer therapeutic approaches.

FLT3 is a class III family receptor tyrosine kinase which is strongly expressed in hematopoietic stem cells and this RTK has pivotal functions when it comes to cell proliferation and continued survival (Maroc et al., 1993). Mutations in FLT3 are the most abundant mutations which are seen in AML (Levis et al., 2011). The majority of these mutations are positioned in the juxtamembrane domain and they are located within the tyrosine kinase domain in 28% of circumstances in AML. Internal tandem duplications in FLT3 lead to continuous activation of the tyrosine kinase which ultimately leads to heightened activation of MAPK pathway and signal transduction (Gale et al., 2008). These findings indicate the importance of inhibiting the MAPK pathway with regards to acute myeloid leukemia since the majority of cases result in the constitutive activation of this pathway.
### 1.5 ERK Inhibitors

Constitutive triggering of the MEK1/2-ERK1/2 pathway has been detected in a large number of human cancers, following the occurrence of oncogenic mutations in Ras or Raf (Bos, 1989). This is why inhibitors of the MAPK pathway are very important considering the significant role this pathway plays in the majority of cancer cell lines. The primary MAPK pathway inhibitors that were developed consisted of inhibiting Ras, Raf and MEK1/2. Even though signaling through the ERK pathway have been shown to be blocked by inhibitors of Ras, Raf and MEK1/2, the overall effectiveness of such inhibitors in the clinical setting has been limited. This is mostly due to the fact that the proteins of the ERK pathway are tangled in numerous cellular functions, hence, it may be more valuable to inhibit the ERK protein directly given its distinctive role in the Ras→Raf→MEK/1/2→ERK1/2 pathway, regulating the pathway of upstream signals to its cytosolic and nuclear effectors (Yap, Worlikar, MacKerell, Shapiro, & Fletcher, 2011). The RAS-RAF-MEK-ERK pathway is far more complex than initially conceived to be. The pathway’s overall structure encompasses a small G protein (RAS) and three protein kinases (RAF, MEK and ERK). The beginning point for this pathway is the binding of ligand to a receptor tyrosine kinase (RTK) transmembrane protein. This activates a signaling cascade that results in the phosphorylation of ERK and its translocation to the nucleus, where it activates transcription factors leading to changes in gene expression (McCain, 2013). During growth factor signaling, under normal conditions, distinctive pulses of ERK activity lead to transcription and translational events that affect proliferation and cellular behavior in general (Kholodenko, 2000).
Until recently, it was presumed that RAF and/or MEK inhibitors would be enough to inhibit ERK1/2 activity and that there would be no added advantage of directly inhibiting ERK. Therefore, development of ERK inhibitors trailed behind RAF and MEK drugs. However, because most of the resistance mechanisms to RAF and MEK drugs leads to the reactivation of ERK1/2, inhibiting ERK1/2 directly may defeat the existing limitations of RAF or MEK inhibitors (Ryan, Der, Wang-Gillam, & Cox, 2015). Therefore, the mechanisms of resistance to ERK inhibitors will possibly be diverse and distinct from those of resistance to MEK inhibitors.

Two potent and selective ERK inhibitors: VX-11e and SCH772984 were used in this study in order to determine the efficacy of these ERK inhibitors and the effect of the vertical inhibition of the MAPK pathway (both MEK1/2 and ERK1/2) in both sensitive and resistant acute myeloid leukemia cell lines (refer to Figure 5). SCH772984 is an innovative pyridine-indazole inhibitor with an uncommon extended piperazine-phenyl-pyrimidine (Morris et al., 2013). It is extremely selective, with only 7 out of 300 kinases tested displaying more than 50% inhibition at a concentration of 1 µM. SCH 772984 has a nanomolar cytotoxicity in tumor cells carrying NRAS, KRAS or BRAF mutations (Morris et al., 2013). It is efficacious in vivo, inducing xenograft tumor regression in mice. SCH772984 has the capability of effectively inhibiting the downstream effector of the MAPK pathway (ERK), hence it has the potential of overcoming resistance to MEK or BRAF inhibitors. SCH772984 has a dual mechanism of action, leading to the allosteric inhibition of ERK1/2 phosphorylation through inhibition of MEK1/2 binding to ERK1/2 (inhibition of ERK1/2 activation) and through the competitive inhibition of ERK1/2 kinase activity (inhibition substrate activation by ERK1/2) (Chaikuad et al.,
SCH772984 binding adjacent to the ATP binding pocket prompts the formation of a new allosteric pocket that then optimally accommodates the inhibitor (Chaikuad et al., 2014). Regarding VX-11e it is a type-I kinase inhibitor with distinctive kinetic properties defined by slow dissociation rates (Rudolph, Xiao, Pardi, & Ahn, 2015). This ERK inhibitor results in potent inhibitor activity against both recombinant ERK1 and ERK2. Though VTX-11e and SCH772984 display distinctive mechanisms of ERK inhibition, both exhibit a slow off-rate, a characteristic that extends their inhibitory activities (Rudolph et al., 2015).

**Figure 5.** The specific inhibition of ERK by VX-11e and SCH772984.

One of the primary objectives of this study is to determine the mechanisms of resistance of some AML cell lines to anthrax lethal toxin, and determining the link between the inhibition of MAPK signaling pathway and LeTx-mediated cytotoxicity. Discovering the mechanism of resistance in turn opens up a large amount of possibilities in creating new ways of targeting these cells using anthrax lethal toxin. One such way consists of vertical targeting of the MAPK pathway using both LeTx and an ERK1/2 inhibitor such
as VX-11e and SCH772984. We will test the combination of the novel ERK inhibitors and LeTx in order to determine the effect of vertical inhibition of the MAPK pathway on both and resistant and sensitive AML cell lines. Another important objective of this study is determining the contribution of autophagy to Anthrax lethal toxin-induced cytotoxicity. Autophagy could be playing a vital role in allowing some cell lines to survive the LeTx-mediated inhibition of the MAPK pathway.
Chapter 2
Materials and Methods

2.1 Expression and Purification of LeTx

Recombinant LeTx proteins PrAg and LF, as well as FP59 (fusion of the PrAg binding domain of LF and the catalytic domain of Pseudomonas aeruginosa exotoxin A), were expressed and purified as described previously (Ramirez, Leppla, Schneerson, & Shiloach, 2002).

2.2 Cells and Cell Lines

Human AML cell lines HL60, U937, Mono-Mac-1, Mono-Mac-6, were grown as described previously (Ladokhin, Vargas-Uribe, Rodnin, Ghatak, & Sharma, 2017).

2.3 ERK inhibitor and Chloroquine method of preparation

For the preparation of Chloroquine 0.5 grams of powdered chloroquine were measured and 10 mL of deionized water were added to the powder using a sterilized syringe. The prepared stock solution was then stored at 4 °C. There have not been a lot of published reports on the preparation of the two novel ERK inhibitors therefore in order to prepare a 0.1M stock solution of VX-11e 50 mg of this inhibitor was added to 1 mL of DMSO and then vortexed and stored at 4 °C. In the preparation of SCH772984 5 mg was added to 1 mL of DMSO to make a 0.014 M stock solution which was also stored at 4 °C. Concentrations of 1 and 10 Micro Molar were used for both VX-11e and SCH772984.
2.4 Proliferation Inhibition Assay (Cytotoxicity)

Sensitivity of AML cell lines to LeTx was determined using a proliferation inhibition assay as described previously (Abi-Habib et al., 2005). We have also used a recombinant protein, termed FP59, consisting of the PrAg binding domain of LF fused to the catalytic domain of P. aeruginosa exotoxin A. PrAg/FP59 was used as a control for catalytic domain entry into the cytosol of AML cells. Briefly, aliquots of 104 cells/well, in 100 μl of cell culture medium, containing a fixed concentration of 10−9 M LF or FP59, were plated onto a flat-bottom 96-well plate (Corning Inc, Corning, NY). Then, 50 μl of PrAg in media were added to each well to yield concentrations ranging from 10−8 to 10−13 M. Following a 48-72-hour incubation at 37°C/5% CO2, 50 μl of XTT cell proliferation reagent (Roche, Basel, Switzerland) was added to each well and the plates incubated for another 4 hours. Absorbance was then read at 450 nm using a microplate reader (Thermo Fisher Scientific, Waltham, MA). Nominal absorbance and percent maximal absorbance were plotted against the log of concentration.

2.5 Statistical Analysis

Nonlinear regression with a variable slope sigmoidal dose-response curve was generated along with inhibitory concentration 50 (IC50) using GraphPad Prism 5 software (GraphPad Software, San Diego, CA). All assays were performed at least twice with an interassay range of 30% or less for IC50. The GraphPad Prism 5 software was also used for T-test analysis in order to compare groups with different variables.
Chapter 3

Results

3.1 Cytotoxicity of LeTx

First we tested the cytotoxicity of LeTx on a panel of 4 human AML cell lines using an XTT proliferation inhibition assay. One of the AML cell lines (HL60) was sensitive to the LF-mediated inhibition of the MAPK pathway, another cell line displayed medium sensitivity (Mono-Mac-6) and two cell lines (Mono-Mac-1 and U937) were not sensitive to the LF-mediated inhibition of the MAPK pathway. In order to further investigate the mechanism of resistance of these cell lines, proliferation inhibition assays were performed testing the different responses of these cell lines against both PrAg/LF and PrAg/FP59 in the same inhibition assay. In Figure 5 section 5.1 the resistant cell line Mono-Mac-1 is capable of taking up FP59 since this cell line is exhibiting a strong cytotoxic response to PrAg/FP59. In figure 5 section 5.2 U937 responds to PrAg/FP59 with increased cell death also indicating that FP59 is being translocated properly into the cytosol. In Figure 5 section 5.3 the intermediate sensitivity cell line Mono-Mac-6 is not completely inhibited by anthrax lethal toxin but is completely sensitive to inhibition by PrAg/FP59. And in the sensitive cell line in Figure 5 section 5.4 HL60 is clearly able to uptake FP59 leading to MAPK independent cytotoxicity to the cells.
Figure 6. Nonlinear regression curves of LeTx (PrAg/LF) (red) and PrAg/FP59 (blue) on human AML cell lines

**Figure 6.1 Mono-Mac-1**. Mono-Mac-1 is resistant to PrAg/LF, but it is sensitive to inhibition by PrAg/FP59 indicating that the resistance to anthrax lethal toxin inhibition is not due to aberrant translocation of LF. This cell line is resistant to LF-mediated inhibition by the MAPK pathway. Figure A is at 48 hours, Figure B at 72 hours, and Figure C at 96 hours.
Figure 6.2 U937. U937 is resistant to PrAg/LF, but it is sensitive to inhibition by PrAg/FP59 indicating that the resistance to anthrax lethal toxin inhibition is not due to aberrant translocation of LF. This cell line is resistant to LF-mediated inhibition by the MAPK pathway. Figure A is at 48 hours, Figure B at 72 hours, and Figure C at 96 hours.
**Figure 6.3** Mono-Mac-6. Mono-Mac-6 is sensitive to PrAg/LF, and it is sensitive to inhibition by PrAg/FP59. This cell line is therefore sensitive to LF-mediated inhibition by the MAPK pathway. Figure A is at 48 hours, Figure B at 72 hours, and Figure C at 96 hours.
Figure 6.4 HL60. HL60 is sensitive to PrAg/LF, and it is sensitive to inhibition by PrAg/FP59. This cell line is therefore sensitive to LF-mediated inhibition by the MAPK pathway. Figure A is at 48 hours, Figure B at 72 hours, and Figure C at 96 hours.
3.2 Effect of Chloroquine

In order to determine the role autophagy might be playing in response to the inhibition of the MAPK pathway in AML cell lines inhibition assays were performed against all four cell lines using PrAg/LF, PrAg/LF+CQ, and CQ alone. First cytotoxicity assays were performed in Figure 6.1 and 6.2 with the two resistant cell lines U937 and Mono-Mac-1. In the cell line Mono-Mac-1 it was evident that PrAg/LF had no effect on the cell line. CQ alone did result in increased cell death and cell cytotoxicity on the cell line, however the combination of both CQ and anthrax lethal toxin lead to an increased cytotoxic effect compared to that of CQ alone. In U937, which is also resistant to anthrax lethal toxin also had an increased cytotoxic effect once subjected to the combination of both CQ and anthrax lethal toxin (PrAg/LF) which was stronger than the cytotoxic effect of CQ alone. In the two cell lines HL60 (sensitive) and Mono-Mac-6 (intermediate sensitivity) it was clear that regardless of exposing the cells to PrAg/LF alone or in combination with CQ there was not a significant difference in cytotoxicity.
Figure 7. Nonlinear regression curves of LeTx (PrAg/LF) (red), PrAg/LF + CQ (black), and CQ (blue) on human AML cell lines.

Figure 7.1 Mono-Mac-1. According to the results above Mono-Mac-1 had cytotoxic responses once subjected to an autophagy inhibitor CQ as well as becoming more sensitive when CQ was added in combination with PrAg/LF. There was an increased cytotoxic effect with the (CQ+PALF) compared to PALF alone. Figure A is at 48 hours, Figure B at 72 hours, and Figure C at 96 hours.
Figure 7.2 U937. According to the results U937 is slightly sensitive to inhibition by CQ alone whereas the combination of PrAg/LF+CQ lead to a significant increase in cytotoxicity compared to that of CQ alone or PALF. Figure A is at 48 hours, Figure B at 72 hours, and Figure C at 96 hours.
According to the results above Mono-Mac-6 which has been previously established to be sensitive to PrAg/LF displayed no increase in cell death or cytotoxicity once exposed to either PALF or a combination of both PALF and CQ. Figure A is at 48 hours, Figure B at 72 hours, and Figure C at 96 hours.
According to the results above, HL60, which has been previously established to be sensitive to PrAg/LF, has shown to have no increased cytotoxic effects once being exposed to an autophagy inhibitor compared to the use of PALF alone. Figure A is at 48 hours, Figure B at 72 hours, and Figure C at 96 hours.
3.3 Effect of ERK inhibitors

The 100 Micro Molar concentration was leading to a significant amount of cell death in all the cell lines, and this was because the concentration that was used was too high. According to the results in (Figure 7.1 section A) it is clear that Mono-Mac-1 which is resistant to anthrax lethal toxin is not sensitive to inhibition by VX-11e at all varying concentrations. It showed no significant difference with using PALF alone or in combination with VX-11e at 1 or 10 Micro Molar. After being subjected to SCH772984 the cell line does show increased cytotoxicity in response to this ERK inhibitor. There is a significant difference between PALF and PALF+ SCH at 1 Micro Molar (P value =0.0041). In Figure 7.2 the resistant cell line U937 was also insensitive to inhibition by VX-11e. However the cell line was sensitive to inhibition by SCH and had significantly increased cytotoxicity once a combination of SCH at 10 Micro Molar + PALF was used in comparison to PALF alone (P value= 0.0083). In figure 7.3 Mono-Mac-6 is exhibiting cytotoxic responses to both inhibitors. There was a significant increase in cytotoxicity in comparison to PALF alone at PALF+VX-11e (10 Micro Molar) and PALF+SCH (1 MicroMolar) (P value= 0.0001). In (Figure 7.4 section A) HL60 was extremely sensitive to anthrax lethal toxin which is something that has already been established. However it is also sensitive to VX-11e + PALF at a concentration as low as 1 MicroMolar as well as being sensitive to SCH+ PALF at 1 Micro Molar compared to that of PALF alone (P value= 0.0003).
Figure 8. Column statistics of AML cell lines treated with varying concentrations of VX-11e and SCH772984 alone and in combination with Anthrax lethal toxin. (with SEM).

Figure 8.1 Mono-Mac-1. In figure A the results indicate that Mono-Mac-1 is not sensitive to inhibition by VX-11e at all the variable increasing concentrations with or without PrAg/LF. (except 100 Micro Molar) Figure B shows that Mono-Mac-1 is sensitive to inhibition by SCH772984 at 1 MicroM with PrAg/LF compared to PrAg/LF alone.
Figure 8.2 U937. In figure A the results indicate that U937 is not sensitive to inhibition by VX-11e at all varying concentrations (except 100 Micro Molar). Figure B shows that U937 is sensitive to inhibition by SCH772984 at 1 and 10 MicroMolar with PrAg/LF.
Figure 8.3 Mono-Mac-6. In figure A the results indicate that Mono-Mac-6 is sensitive to inhibition by VX-11e. The results show that PrAg/LF + VX-11e at 10MicroM had an increased cytotoxic effect compared PrAg/LF alone. Figure B shows that Mono-Mac-1 is sensitive to inhibition by SCH772984 at as low as 1 Micro Molar + PrAg/LF.
Figure 8.4 HL60. In figure A the results indicate that HL60 is sensitive to inhibition by VX-11e 1MicroM in combination with PrAg/LF. Figure B shows that HL60 is sensitive to inhibition by SCH772984 at a very low concentration of 1MicroM with PrAg/LF.
Chapter 4
Discussion

In this study, we have been able to rule out the mechanism of resistance of AML cell lines as being dependent on the lack of proper translocation of LF and we have shown increased cytotoxicity in the AML cell lines once subjected to CQ or the novel ERK inhibitors. We have demonstrated through the use of FP59 that both resistant and sensitive cell lines were capable of translocating the LF moiety into the cytosol. We used FP59 because FP59 binds to PrAg and is translocated into the cytosol in an identical matter to that of LF; however, it does not target the MAPK pathway but rather ADP-ribosylates elongation factor 2 leading to inhibition of protein synthesis and cell death (Kassab et al., 2013). It was clear that all four cell lines were sensitive to inhibition by PrAg/FP59 indicating that the translocation of LF into the cell membrane was not the causative factor leading to some cell line’s developing resistance to anthrax lethal toxin. This finding takes us a step closer in determining the exact mechanism of resistance of some AML cell lines. Therefore from the results of this experiment it is clear that LF is being translocated into the cells in a proper manner in both resistant and sensitive cell lines.

We were also able to determine the role autophagy is playing with respect to resistance in these AML cell lines. According to the cytotoxicity assays it was evident that the use of a combination of both CQ and anthrax lethal toxin lead to increased cytotoxicity in the resistant cell lines compared to the use of anthrax lethal toxin alone. This indicates that the administration of CQ and subsequently the inhibition of autophagy lead to the
resistant cells lines becoming more sensitive to inhibition by anthrax lethal toxin. In previous studies it has been established that autophagy-related stress tolerance can trigger a survival mechanism in cells by sustaining energy production that can ultimately result in tumor advancement and therapeutic resistance (Sui et al., 2013). Preclinical models have also shown that the inhibition of autophagy can reestablish chemosensitivity and boost tumor cell death (Amaravadi et al., 2007). These results along with the results from this paper establish autophagy as a potential therapeutic target. However, autophagy can be considered as a double-edge sword since continued or constitutive tumor cell autophagy can actually lead to increased cell death in tumor cells, specifically in apoptosis-defective cells (Kimura, Takabatake, Takahashi, & Isaka, 2013). Another limiting factor is whether or not CQ administration is possible considering its toxic effects. Conversely, recent studies show that short-term administration of CQ rarely causes severe side effects, long term exposure on the other hand will lead to severe side effects such as bone marrow suppression and hypoglycemia (Goyal & Bordia, 1995), (Nagaratnam, Chetiyawardana, & Rajiyah, 1978). Otherwise, daily small doses (up to 250mg of CQ) can be tolerated (Marmor et al., 2016). Therefore inhibiting autophagy and exposing the cells to anthrax lethal toxin was successful in causing increased cell death to resistant cell lines but did not have a significant increased cytotoxic effect on the already sensitive cell lines.

Lastly, we showed the potential use of the ERK inhibitors (SCH772984 and VX11e) with the AML cell lines. Mutations in the ras-mediated signal transduction pathway are existent in 30-50% of AML, and nearly 50% of AML cases at diagnosis have abnormal phosphorylation of ERK (Appelbaum, Rowe, Radich, & Dick, 2001). Therefore targeted
therapy specifically inhibiting ERK could result in increased cell death in these AML cell lines. The results indicated that the novel ERK inhibitors that were used (SCH772984 and VxX11e) had cytotoxic effects at on both the intermediate sensitive cell line (Mono-Mac-6) and sensitive cell line HL60. The resistant cell lines however were not sensitive to inhibition by VX-11e. What was very interesting from the results was that the two resistant cell lines were not sensitive to inhibition by VX-11e however the two sensitive cell lines were displaying significant cytotoxic responses once being exposed to VX-11e. This might have something to do with the fact that the resistant cell lines might have a different type of ERK mutation that is best inhibited by SCH772984. This indicates that the type of mutation at the level of the ERK within the resistant cell lines is best inhibited by the SCH772984 and not VX-11e, due to its allosteric inhibition of ERK phosphorylation, MEK1/2 binding and also the ATP-competitive inhibition of ERK phosphorylation of its substrates. For future research it would be advised to take a concentration between 10 and 50 MicroMolar in order to determine the optimal concentration to be used since 100MicroMolar was a very high concentration to be used with both ERK inhibitors and this concentration was as leading to complete cell death.
Chapter 5

Conclusion

Throughout this study the use of cytotoxicity assays was essential in allowing us to determine the cytotoxic responses that the cell lines may or may not have once being exposed to different variables. We were able to determine that lethal factor was in fact being translocated properly into the cell and this allows us to narrow our search for the exact mechanism of resistance of some AML cell lines to anthrax lethal toxin. Another vital component of this study is determining that autophagy does in fact play a crucial role in allowing resistant cell lines to survive anthrax lethal toxin cytotoxicity. Consequently we were able to discover that the use of a combination of CQ and anthrax lethal toxin has synergistic cytotoxic effects on the resistant cell lines. Targeting autophagy in cancer provides new opportunities for drug development in order to create an autophagy inhibitor for therapeutic advantage. And this study provides a potential use for chloroquine at small doses for a short period of time in combination with chemotherapy or targeted agents. We were also able to determine that both ERK inhibitors VX-11e and SCH772984 are very effective at having cytotoxic effects on the sensitive cell lines whereas the resistant cell lines were only sensitive to inhibition by SCH772984. Correspondingly, we were also able to conclude that the use of the ERK inhibitors in combination with anthrax lethal toxin had a significantly stronger cytotoxic effect on the AML cell lines compared to using anthrax lethal toxin alone. These findings take us a step closer in targeting resistant acute myeloid leukemia cell lines in a more direct and efficacious approach.
References


