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# HIGH DOSE SIMVASTATIN AS A POTENTIAL ANTICANCER THERAPY IN LEUKEMIA PATIENTS

Tamer Ahmed

University of Kentucky, tamer.kafafy@gmail.com

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Tamer Ahmed, Student

Dr. Markos Leggas, Major Professor

Dr. Jim Pauly, Director of Graduate Studies

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HIGH DOSE SIMVASTATIN AS A POTENTIAL ANTICANCER THERAPY IN  
LEUKEMIA PATIENTS

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DISSERTATION

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A dissertation submitted in partial fulfillment of the  
requirements for the degree of Doctor of Philosophy in the  
College of Pharmacy at the University of Kentucky

By

Tamer Ahmed

Lexington, Kentucky

Director: Dr. Markos Leggas, Associate Professor of Pharmaceutical Sciences  
Co-Director: Dr. Val Adams, Associate Professor of Pharmacy Practice

Lexington, Kentucky

2013

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## ABSTRACT OF DISSERTATION

### HIGH DOSE SIMVASTATIN AS A POTENTIAL ANTICANCER THERAPY IN LEUKEMIA PATIENTS

Simvastatin is a 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitor that is used for the treatment of hyperlipidemia. Simvastatin has recently been studied for its potential use in cancer therapy. In-vitro studies have shown that simvastatin displays anticancer activity, but at concentrations unlikely to be achieved in patients being receiving typical antihyperlipidemic treatment doses. Thus, several clinical trials were conducted to study the tolerability of high dose statins in cancer patients. The maximum tolerated dose of simvastatin was determined to be 15 mg/kg/day, 25-fold higher than a typical dose. However, it is not known if simvastatin plasma concentrations can reach those found to be effective in-vitro. In this context, we initiated a clinical study to determine the pharmacokinetics of high dose simvastatin in patients with chronic lymphocytic leukemia. For this purpose, an LC-MS/MS method was developed and validated for the quantitation of simvastatin and its acid form in plasma and peripheral blood mononuclear cells obtained from CLL patients. Results show that simvastatin concentrations were dose proportional relative to the antihyperlipidemic doses, but lower than those required for in-vitro cytotoxicity against cancer cells. These findings demonstrate that the in-vitro effective concentrations of simvastatin are not achievable clinically, which might explain the limited effectiveness of high dose simvastatin in this study and in previous clinical trials. In view of these data, the use of simvastatin as a sole therapy in cancer treatment was not encouraging and led us to examine the use in combination with other anticancer drugs.

After screening several chemotherapeutic agents in combination with simvastatin, we showed that tipifarnib (a farnesyltransferase inhibitor) interacts synergistically in several leukemia cell lines. Mechanistically we showed that simvastatin augments the cytotoxicity of tipifarnib by disrupting the localization of RAS in the cell membrane and by subsequent deactivation of the ERK pathway. Consistent with this observation, drug treatment led to the induction of apoptosis through the caspase cascade activation and the cleaved PARP upregulation. Notably, this synergistic effect

was observed at clinically achievable concentrations of simvastatin and tipifarnib. Thus, the effectiveness of this combination should be explored further in future clinical studies.

KEYWORDS: Simvastatin, Leukemia, LC-MS/MS, Pharmacokinetics, Tipifarnib

Tamer Ahmed  
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Student's Signature

01/07/13  
\_\_\_\_\_  
Date

HIGH DOSE SIMVASTATIN AS A POTENTIAL ANTICANCER THERAPY IN  
LEUKEMIA PATIENTS

By  
Tamer Ahmed

Markos Leggas  
\_\_\_\_\_  
Director of Dissertation

Val Adams  
\_\_\_\_\_  
Co-Director of Dissertation

Jim Pauly  
\_\_\_\_\_  
Director of Graduate Studies

01/07/13  
\_\_\_\_\_  
Date

## DEDICATION

To my parents, who have provided me with love, endless support and encouragement throughout my entire life. To my wife Rehame and kids Youssef, Abdelrahman and little

Omar, you are the joy of my life.

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## **Chapter 1 : Introduction**

### **A. Background**

Statins have become well established as safe and effective drugs in the treatment of hypercholesterolemia. The beneficial effects of statins in primary and secondary prevention of cardiovascular diseases were demonstrated in several clinical trials, such as Scandinavian Simvastatin Survival Study (4S) [1], Long-term Intervention with Pravastatin in Ischemia Disease (LIPID) [2], Cholesterol and Recurrent Events (CARE) [3] , West of Scotland Coronary Prevention Study (WOSCOPS) [4], Air Force/Texas Coronary Atherosclerosis Prevention Study (AFCAPS/TexCAPS) [5] and the Heart Protection Study (HPS) [6]. Statins mediate their effect through the inhibition of the 3-hydroxy-3-methylglutaryl-coenzyme A (HMG CoA) reductase, an enzyme of the mevalonate pathway. In a rate limiting process this enzyme catalyzes the conversion of HMG CoA to mevalonate, the precursor of cholesterol [7, 8]. Thus, statins exert their therapeutic effect primarily by decreasing the intracellular hepatic cholesterol levels and by upregulating the hepatic LDL receptor expression, which results in an increase of LDL cholesterol hepatic uptake and substantial decline in plasma LDL cholesterol levels [7, 8].

The statin family is composed of eight members that are naturally derived or chemically synthesized (Figure 1.1). Lovastatin, simvastatin, and pravastatin are naturally derived from fungal fermentation, whereas fluvastatin, atorvastatin, cerivastatin, rosuvastatin and pitavastatin are synthetically derived. Cerivastatin was withdrawn from the market in August 2001 due to risk of serious rhabdomyolysis. All statins possess a common structural characteristic which is an HMG-CoA like moiety that enables statins to compete with HMG-CoA on the enzyme active site. In all of the statins, this side chain moiety exists in an open ring (active, acid) form which is responsible for binding the

HMG CoA reductase active site. However, lovastatin and simvastatin are present in a lactone prodrug form which undergoes hydrolysis in vivo to the active open ring form by carboxyesterases in the liver and plasma.

## **B. Statins and Pleiotropic effects**

The strong correlation between serum cholesterol levels and coronary artery disease [9, 10] supported the assumption that the protective effect of statins in cardiovascular disease is mainly attributed to the ability of statins to lower the serum cholesterol levels. However, this notion seemed to be imprecise when subgroup analyses of large clinical trials have suggested possible beneficial effects of statins that may not be entirely dependent on cholesterol reduction. For instance, the risk of myocardial infarction was found to be significantly lower in individuals treated with statins than those treated with other cholesterol lowering agents with both groups showing comparable reduction in serum cholesterol levels [11, 12]. Likewise, administration of statins was associated with a substantial lower risk of developing dementia relative to those treated with other lipid lowering agents. This effect was independent of the presence or absence of untreated hyperlipidemia suggesting no central role of LDL cholesterol levels in the effect of statins [13]. Moreover, the vascular protective effects of statins were demonstrated in a clinical study where four weeks of simvastatin treatment improved the endothelial functions in patients with heart failure compared to those treated with ezetimibe, despite the comparable levels of LDL cholesterol in both groups [14]. Several reports have also demonstrated the association between the use of statins and the reduced risk of osteoporosis and multiple sclerosis [15, 16]. In a similar context, several studies have reported the association of statins use with decreased risk of cancer [17-19]. In a retrospective study, statins significantly reduced the risk of renal cell carcinoma by 48% in almost half a million patients, irrespective of age, sex, smoking,

and obesity [19]. Conversely, a recent systemic review with 42 studies failed to show any evidence on the protective effect of statins in cancer [20]. Conclusions driven from these studies were inconsistent which may be attributed to the nature of the studies as being observational and retrospective [21]. Long follow up studies might be needed to detect any potential long-latency effects of statins in cancer. Overall, these findings strongly suggest the possible beneficial effects of statins beyond cholesterol reduction. Thus, investigators began to unravel the molecular mechanism and the clinical implications of these pleiotropic effects.

### **C. Statins and mevalonate pathway**

As mentioned above, statins mediate their anti-hypercholesterolemic effect through blocking the mevalonate pathway and subsequent decrease in cholesterol production (Figure 1.2). Cholesterol is a vital cell membrane component and its production is necessary for cellular membrane structure and integrity. It also acts as a precursor for steroid hormones and bile acids synthesis [22]. However, there are several other downstream products of the mevalonate pathway such as ubiquinone (coenzyme Q10), dolichol and isoprenoids that were found to play a critical role in the different cell functions. For instance, Dolichol, in the form of dolichol phosphate, plays an important role in glycoprotein synthesis. It works as a carrier molecule of oligosaccharide in N-linked protein glycosylation. Ubiquinone (coenzyme Q10) is involved in the electron transport chain in mitochondrial respiration and functions as an antioxidant in the inhibition of lipid peroxidation [23]. Isoprenoids, including geranylgeranyl pyrophosphate (GGPP) and farnesyl pyrophosphate (FPP), are used to modify small G proteins such as RAS and RHO GTPases that play a crucial role in cell motility, proliferation and survival [24]. In fact, several studies demonstrated that most of the pleiotropic effects induced by

statins including their antitumor activity are mediated through the depletion of isoprenoids and the subsequent impairment of the small G proteins functions [25].

#### **D. G-proteins**

Guanine nucleotide-binding proteins (G proteins) are a large family of GTP binding proteins that act as a molecular switch regulating wide variety of cell functions. There are two main classes of G Proteins, the heterotrimeric G proteins (large G proteins) that are composed of  $\alpha$ ,  $\beta$ , and  $\gamma$  subunits and are activated by membrane G proteins coupled receptors. The second class is monomeric G proteins that are also known as small G proteins or small GTPases because of its low molecular weight of 20 - 40 KDa. Small G proteins are classified into five major families including RAS, RHO, RAB, ARF and RAN families [26, 27].

G proteins are known to alternate between inactive GDP bound and active GTP-bound states (Figure 1.3) [28, 29] . Switching between GDP and GTP binding conformations allow these proteins to function as a molecular switch regulating several cellular functions (Figure 1.3) [30] . Several protein classes regulate the activity of the G proteins including; Guanine nucleotide exchange factors (GEFs) which stimulate the dissociation of GDP from the G proteins and allow GTP binding in a passive rebinding process; GTPase activating proteins (GAPs) which acts as negative regulators of G proteins activity through accelerating the rate of hydrolysis of GTP to GDP [31, 32].

#### **E. Small G proteins and Cancer**

Small G proteins comprise a large class of membrane proteins with broadly diverse functions. Recent study has indicated approximately 600 genes in the human genome that encode proteins with C-terminal CXXX motif, a conserved recognition motif in most of the prenylated proteins [33]. However, only more than 100 proteins have been

identified to undergo prenylation [34]. The members of the small G protein families were shown to play critical role in regulating several cellular responses including signal transduction, cytoskeletal organization, and intracellular vesicle trafficking [26] . For example, members of the RAS family are essential element in transducing signals mediated by the extracellular microenvironment that regulate several fundamental processes such as cell growth, proliferation and apoptosis [35-37]; the RHO family proteins regulate signaling networks involved in cytoskeletal organization, cell cycle progression, gene expression and cell proliferation and survival [38-41]. Both RAB and ARF family members are involved in regulating intracellular vesicular transport and protein trafficking between different organelles [42, 43], whereas RAN proteins are responsible for the transport of RNA and proteins across the nuclear membrane [44, 45].

Both RAS and RHO GTPases are activated in response to signals, initiated either extracellularly or intracellularly, that generate the active GTP-bound form and propagate further downstream signaling events. The role of RAS and RHO GTPases in carcinogenesis is well established [35]. Constitutive activation of RAS and RHO GTPases, either by point mutation or over expression, will trigger downstream signaling which are involved in cell growth and proliferation leading to uncontrolled cell growth and proliferation and will result in tumor development [35, 38]. In 20 - 30 % of human tumors, RAS proteins are constitutively activated by a point mutation that reduces the GTPase activity of RAS and prevent the hydrolysis of GTP to GDP [36, 46]. The incidence of the mutation of the RAS genes varies greatly among the different tumors, the highest rate of mutation was found in pancreatic cancer (90%) [47], colon cancer (50%) [48, 49] and lung cancer (30%) [50]. In addition to activation through mutation, RAS was also found to be hyperactivated as a result of deregulated expression or by an activating mutation of upstream signaling molecules such as growth factor receptor tyrosine kinases. The

most common examples are EGFR and HERs that are hyperactivated in many types of tumor such as lung, breast and ovarian cancers [51, 52]. Unlike RAS, no work has reported activating mutations in RHO proteins; however, accumulating evidence has shown that RHO proteins are hyperactivated in human tumors relative to normal tissue [39].

Given the crucial role of RAS and RHO GTPases in regulating several downstream pathways that mediate cancer growth and progression, substantial efforts were made to target these signaling pathways in cancer therapy. Several therapeutic strategies have been developed to inhibit these pathways through blocking the upstream signaling molecules or inhibiting the activities of the downstream effectors [53]. One attractive approach is to target the RAS and RHO themselves through interrupting their posttranslational modification process, which is crucial for the proteins in order to get anchored into the membrane and attain full activity.

#### **F. Post translational modifications of small G proteins**

It was first recognized over twenty years ago that the function of small G proteins is dependent on a post-translational modification process that enables small G proteins from attaching to the cellular membranes and subsequently being activated [46]. Small G proteins are synthesized in the cytosol as hydrophilic soluble proteins that undergo a series of modifications in order to add a lipidated hydrophobic moiety that facilitate the anchoring of small G proteins into the lipophilic cellular membranes. These modifications take place at the CAAX (C, cysteine; A, aliphatic amino acid; X, any amino acid) motif in the protein carboxyl terminus at several steps including prenylation, proteolysis and carboxymethylation (Figure 1.4). Prenylation is the first and the rate limiting step in the modification and it includes the covalent attachment of a lipid isoprenoids moiety into the cysteine residue of the CAAX motif through the interaction with either

farnesylpyrophosphate (FPP, 15-carbon isoprenoids) or geranylgeranyl pyrophosphate (GGPP, 20-carbon isoprenoids), which are intermediate products of the mevalonate pathway [54]. In general, proteins carrying leucine or phenylalanine as the X residue in the CAAX motif get geranylgeranylated and is catalyzed by geranylgeranyl transferase I (GGTase I) enzyme, otherwise the protein gets farnesylated in the presence of farnesyl transferase (FTase) enzyme [55]. After prenylation, Rce1 endopeptidase catalyzes the cleavage of the three terminal amino acids (AAX) of the proteins and then the isoprenylated cysteine will get methylated by the isoprenylcysteine carboxyl methyltransferase (ICMT) [56]. N- RAS, H- RAS and K- RAS (4A) isoforms undergo additional palmitoylation modification (addition of palmitoyl moiety) at the C-terminus besides farnesylation, whereas K- RAS (4B) isoform attaches to the membrane through farnesyl moiety and a polybasic, lysine rich, sequence near the terminal cysteine. These additional modifications are also critical for the association and stability of the small G proteins at cellular membranes. In general, these posttranslational modifications are critical for RAS and RHO GTPases to associate with cellular membrane in order to execute their biological functions. Given the fact that RAS and RHO GTPases play an essential role in carcinogenesis, deactivation of these proteins through targeting their posttranslational process is thought to be a promising strategy to fight cancer. Several approaches were postulated to target this process either through inhibiting the rate limiting enzymes such as FTase and GGTase (using FTase and GGTase inhibitors), or by interfering with the mevalonate pathway (using statins), in order to inhibit the biosynthesis of FFP and GGPP, which are critical substrates for the prenylation process.

### **G. Statins and antitumor activity**

Although most of the epidemiological and meta-analyses reports suggest no helpful or harmful effect of statins on cancer risk [20, 21], accumulating evidence from in-

vitro and in-vivo studies have demonstrated the anti-tumor effects of statins in several tumor types [57, 58]. The ability of mevalonate to abrogate the antitumor activity of statins indicated the importance of the mevalonate pathway in mediating the antitumor activity of statins [59, 60]. In fact, depletion of the intracellular pools of both FPP and GGPP, as a result of the upstream inhibition of mevalonate synthesis, with consequent dysfunction of small G proteins is suggested to be the underlying mechanism of the antitumor activity of statins. This finding was supported by several add back studies that demonstrated the ability of GGPP to abrogate the statin induced apoptosis in cancer cells, whereas addition of FPP only showed partial reversal [59, 61-64]. Other products of the mevalonate pathway including cholesterol, squalene, lanosterol, desmosterol, dolichol, dolichol phosphate, ubiquinone and isopentenyladenine were not able to reverse the apoptotic effect of statins in cancer cells [59, 61, 65].

Antitumor effects exhibited by statins include growth arrest, induction of apoptosis, inhibition of angiogenesis, and repression of tumor metastases [58, 66]. Statins have been shown to induce growth arrest at the G1/S phase in both solid tumors and hematologic malignancies [67-72]. Many studies have shown that statins antiproliferative effect is mediated through the induction of cyclin dependent kinase inhibitors (CKIs), p21<sup>Waf1/Cip1</sup> and/or p27<sup>Kip1</sup> that downregulate the kinase activity of cyclin-dependent kinase 2 (CDK-2), essential for G1/S transition [67, 71, 73]. Statin induced growth arrest was shown to be rescued by the addition of mevalonate and GGPP, indicating the role of geranylgeranylation inhibition in mediating the cytostatic effect of statins [74, 75].

Similarly, statin induced apoptosis in different tumor types has been found to be abrogated by the addition of mevalonate and geranylgeranyl pyrophosphate (GGPP) and was partially reversed by farnesyl pyrophosphate (FPP) [59]. The apoptotic activity of statins is thought to be mediated through the disruption of the balance between

proapoptotic and antiapoptotic members of the Bcl2 family, which are important regulators of cell survival. Downregulation of antiapoptotic proteins (e.g. Bcl2 and Mcl1) as well as increasing the expression of proapoptotic protein (e.g. Bax and Bim) was associated with lovastatin induced apoptosis in different tumor cells [62, 76]. Furthermore, statins were found to induce apoptosis through the activation of caspase proteases involved in programmed cell death [77, 78]. However, the molecular mechanism by which statins generate apoptosis in tumor cells is not well defined. The wide intracellular pool of small G proteins as well as their complicated downstream network of signaling pathways makes it difficult to define a specific mechanism of action. The downregulation of the Raf/MEK/ERK pathway was suggested to contribute to lovastatin mediated apoptosis in AML cells [79]. Conversely, the apoptotic effect of statins in both ovarian and breast cancer cells was associated with the activation of JNK pathway and the phosphorylation of the transcriptional factor c-Jun [64, 80]. In lung carcinoma cells, lovastatin was shown to inhibit the EGF induced EGFR autophosphorylation and inhibits the AKT activation by EGF in combination with gefitinib [81]. In addition to the in vitro antitumor activity, statins have been shown to have in vivo antitumor activity in different animal models where simvastatin was found to have an inhibitory effect on the proliferation of human AML cells in SCID mice [82]. Furthermore, statins were shown to inhibit the growth of colon tumors in rats and mice [83-85].

Building upon the evidential results obtained from both in vitro and in vivo studies that indicate the diverse antitumor effects of statins on the different types of tumors, clinical investigators were tempted to assess whether this antitumor activity will translate into significant clinical benefits.

## H. Clinical trials of statins

Several clinical studies investigated the value of statins as an adjuvant treatment, at typical doses used in the treatment of hypercholesterolemia, in improving the efficacy of standard treatment in cancer. A randomized controlled trial in patients with advanced hepatocellular carcinoma suggested that the addition of pravastatin, at a daily dose of 40 mg, to standard treatment prolonged the median survival to 18 months versus 9 months in patients receiving standard treatment only [86]. Other clinical trials have shown that the administration of simvastatin improved the efficacy of standard therapies in patients with multiple myeloma and non-small cell lung cancer [87, 88]. Similarly, addition of simvastatin to irinotecan, 5-fluorouracil and leucovorin (FLOFIRI) modestly prolonged the time to progression from 6.7-8.5 months with FLOFIRI alone to 9.9 months in patients with metastatic colorectal cancer [89]. In contradiction with previous findings, addition of pravastatin to chemotherapy in advanced gastric carcinoma in a phase II trial did not improve the outcome in those patients [90]. Likewise, simvastatin, at 40mg/daily, could not improve the clinical status in chronic lymphocytic leukemia patients [91]. This discrepancy in clinical data of statins at typical doses could be attributed to several factors including the tumor type being treated, the limited number of patients in the study, or the advanced stage of the disease.

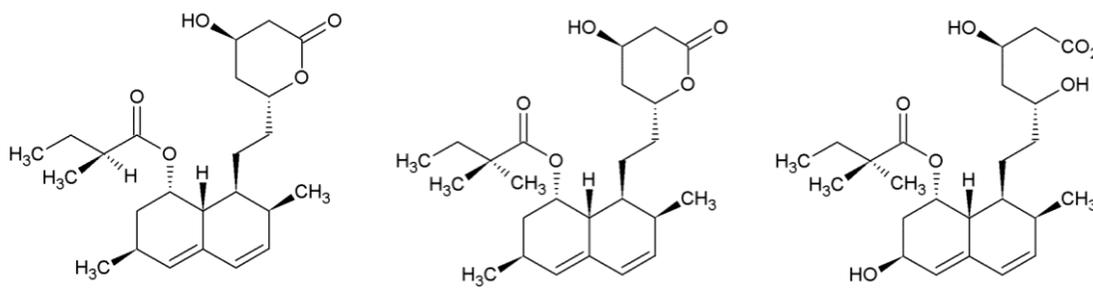
Furthermore, the safety and tolerability of statins at high doses were assessed in limited clinical trials. Lovastatin was found to be safe and well tolerated at maximum tolerated doses 25 mg/kg/day and 35 mg/kg/day (with concomitant administration of ubiquinone to prevent rhabdomyolysis) in cancer patients [92, 93]. In a phase I trial, patients with myeloma or lymphoma were able to tolerate simvastatin at 7.5 mg/kg given orally, twice daily for seven consecutive days [94]. These high doses of lovastatin and simvastatin are more than 40 and 25 fold higher than the regular dose (40mg/day) used

for hypercholesterolemia therapy, respectively. However, further evaluation of statins at high doses in phase II trials showed limited efficacy in cancer patients [92, 95-97]. Based on those negative results, it becomes important to know whether statins at high doses are able to achieve therapeutically effective plasma concentrations. Currently, none of the conducted clinical trials looked at the pharmacokinetics of statins at high doses. Thus, the work contained in this dissertation characterizes the pharmacokinetics of simvastatin given at high doses in cancer patients.

### **I. Statins combined with other anticancer drugs**

Given the ability of statins to inhibit the biological function of small GTPases and its impact on several important cellular functions, numerous preclinical studies were in favor of exploring the potential benefits of statins in combination with other anticancer treatments. In addition, using statins in a synergistic or additive combination will give the opportunity to utilize reduced concentrations of statins that could be achieved in the clinic. Recently, Jakobisiak and Golab have published a review article that includes numerous combination studies of statins with other anticancer drugs and discussed their clinical relevance [98]. Briefly, statins have been shown to potentiate the effects of anticancer drugs from different classes such as 5-fluorouracil, etoposide, anthracycline, doxorubicin, cisplatin and paclitaxel [99-104]. It is interesting to note that cerivastatin augmented the cytotoxic effect of 5- fluorouracil to a degree that only 10 -500 fold lesser concentrations of 5- fluorouracil were required to yield similar effect of the drug alone [101]. Likewise, synergistic interactions of statins with anticancer agents were seen at much lower concentrations of statins relative to what have been used with statins alone [99-104]. Moreover, statins were also shown to potentiate the antitumor activity of several molecular targeted therapies such as celecoxib, cetuximab, sorafenib and gefitinib [105]. In fact, fluvastatin at clinically achievable concentrations was able to

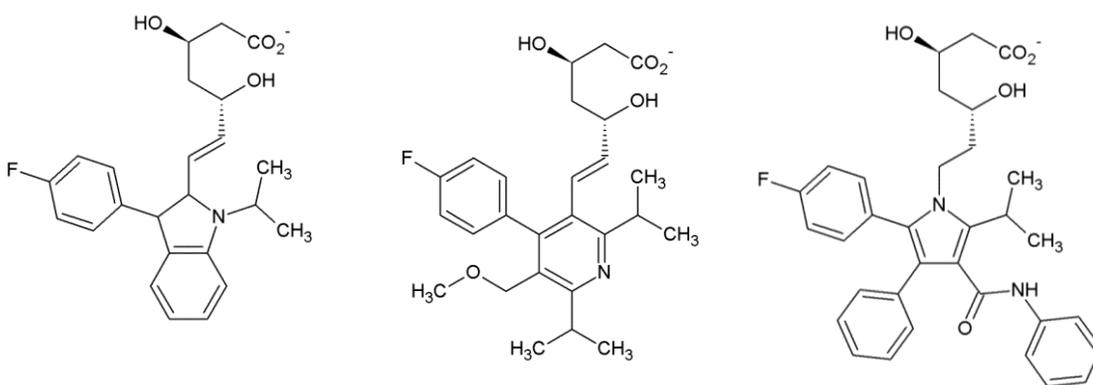
induce synergistic interaction in combination with trastuzumab in breast cancer cells [106]. In general, synergistic combination of statins with anticancer drugs may warrant dose reduction of statins to clinically achievable concentrations that facilitate a smooth transition from the in-vitro settings into clinical application. In this dissertation, a combination of simvastatin and tipifarnib at clinically achievable concentrations was shown to induce cytotoxic effect in leukemia cells in a synergistic fashion.



**Lovastatin**

**Simvastatin**

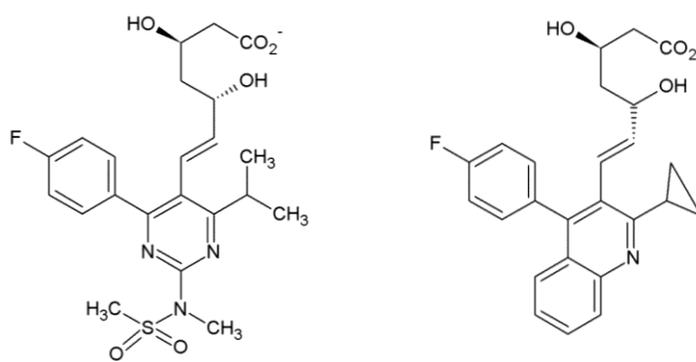
**Pravastatin**



**Fluvastatin**

**Cerivastatin**

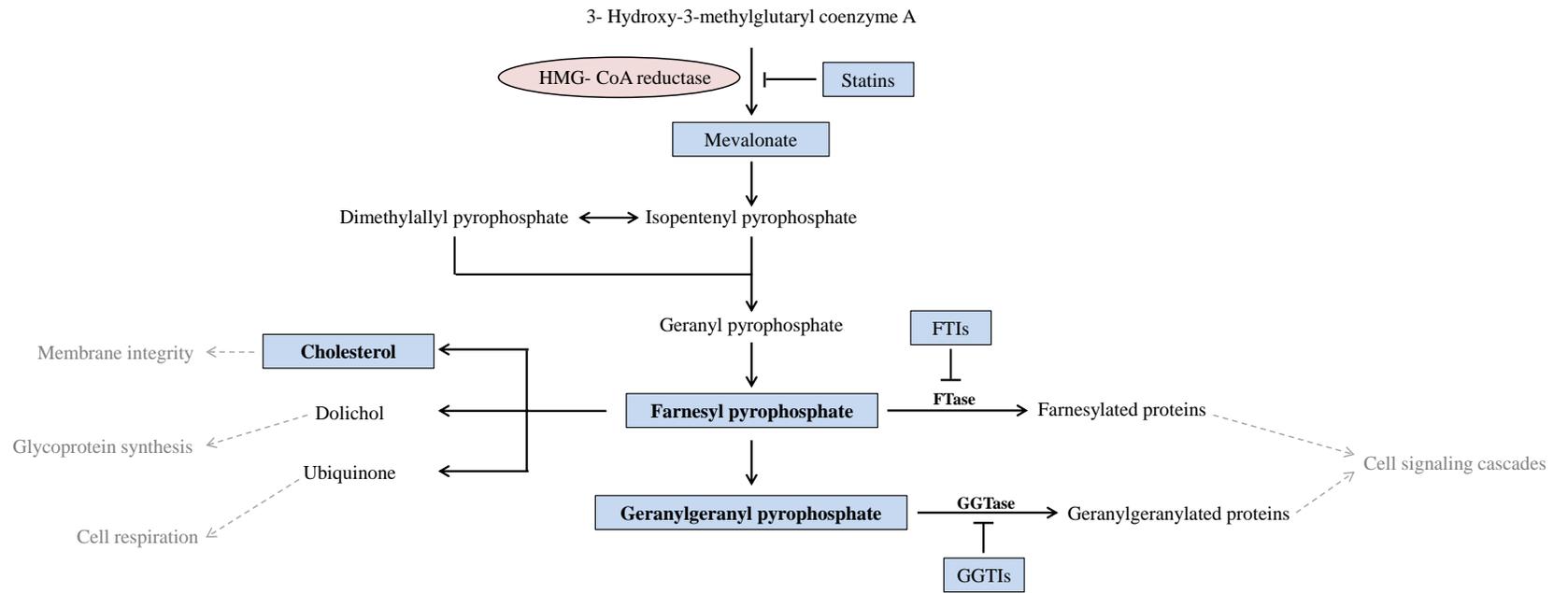
**Atorvastatin**



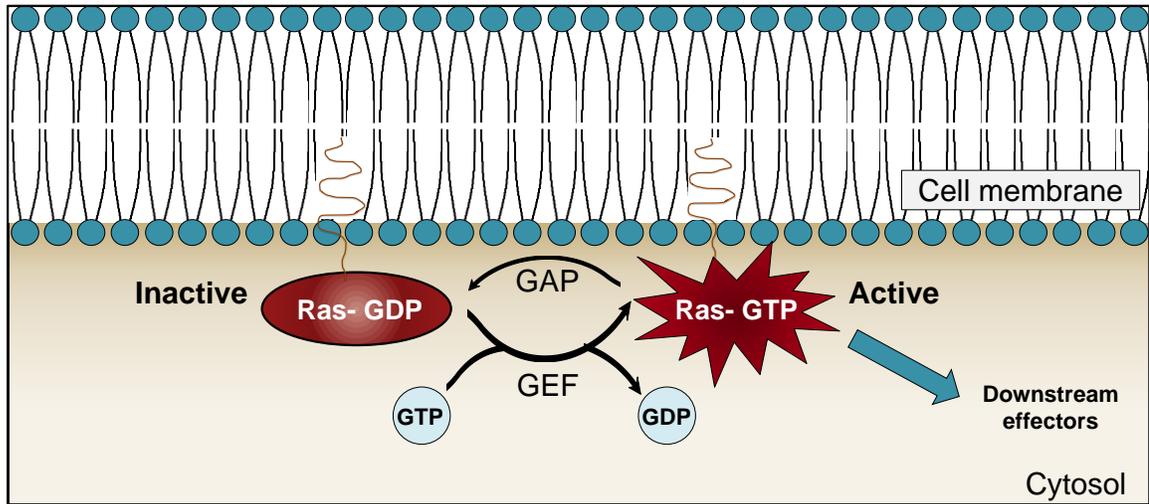
**Rosuvastatin**

**Pitavastatin**

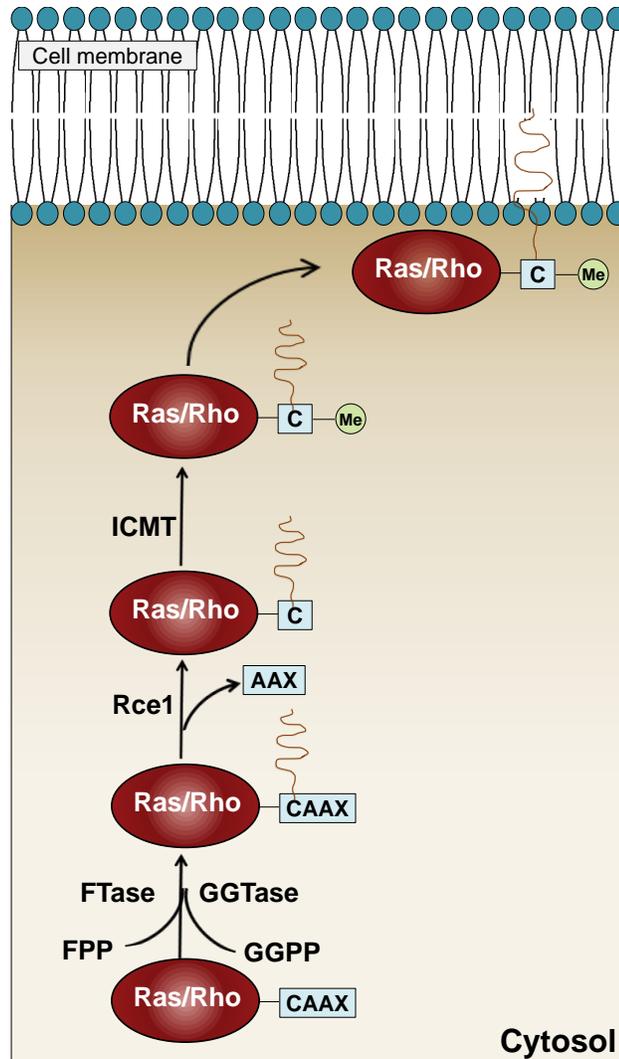
**Figure 1.1.** Chemical structures of the statins.



**Figure 1.2.** Overview of the mevalonate pathway, its downstream products and targets for inhibition by statins, farnesyltransferase inhibitors and geranylgeranyltransferase inhibitors.



**Figure 1.3.** A schematic of small G protein activation. Small G proteins switch between GDP (inactive) and GTP (active) conformations which is controlled by GEF, guanine nucleotide exchange factors and GAP, GTPase activating proteins.



**Figure 1.4.** Posttranslational modification of RAS and RHO GTPases. Farnesyltransferase (FTase) and geranylgeranyltransferase (GGTase) catalyze the addition of farnesyl pyrophosphate (FPP) and geranylgeranyl pyrophosphate (GGPP) to the C-terminus of the small G proteins, respectively. Rce1 endopeptidase removes the last three amino acids from the carboxyl terminus. Following the removal of the AAX amino acids, the carboxyl terminus is then methylated by isoprenylcysteine carboxyl methyltransferase (ICMT). Me, Methyl.

## Chapter 2 : Hypothesis and Specific Aims

The long term goal of our research is to evaluate the clinical utility and prospects of simvastatin in cancer therapy. An initial objective for this dissertation work included a phase-II clinical trial using high dose simvastatin as a therapy in patients with chronic lymphocytic leukemia (CLL). Therefore, our studies used simvastatin as a model drug. In efforts to develop statins into the clinic for cancer therapy, limited Phase I and II studies have assessed the safety and efficacy of high dose statins in cancer patients. Lovastatin has been previously studied in phase-I and phase-II studies, in solid tumors, and simvastatin was studied in myeloma and lymphoma patients. However, the pharmacokinetics of high dose simvastatin, although vital for its clinical development, were not previously defined. Statins are 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase inhibitors and exert their anticancer effect by inhibiting prenylation (lipidation) of low molecular weight GTPases, such as RAS and RHO oncoproteins, which play a key role in intracellular cancer cell signaling. In tumor cells, these signaling pathways are deregulated and contribute to cell proliferation and inhibit apoptosis. Our initial hypothesis was that *high dose simvastatin administered for one week every 21 days for 6 cycles is safe and efficacious in adults with recurrent or refractory CLL*. In addition, we hypothesized that *high dose simvastatin treatment will disrupt the cellular localization of proteins that depend on prenylation for their trafficking and will induce apoptosis in CLL cells*.

To facilitate the planned clinical study, we first sought to determine the pharmacokinetics of simvastatin, as well as its apoptotic effects, in a limited number (n=3) of patients that were administered high dose at its MTD (7.5 mg/kg, twice daily, for seven days). Therefore we conducted the studies outlined in Specific Aim 1.

**Specific aim 1:** Evaluate the pharmacokinetics of high dose simvastatin in a pilot clinical trial in (CLL) patients.

**Aim 1.1:** Develop, implement and validate a bioanalytical method for accurate and precise quantitation of simvastatin lactone and simvastatin acid in plasma and peripheral blood mononuclear cells. This work is outlined in **Chapter 3**.

**Aim 1.2:** Determine the pharmacokinetics and peripheral blood mononuclear cells (PBMCs) biodistribution in patients treated with high dose simvastatin. This work is outlined in **Chapter 4**.

**Aim 1.3:** Determine whether high dose simvastatin treatment induces apoptosis in CLL cells from treated patients. This work is outlined in **Chapter 4**.

Based primarily on the findings from Aim 1.2, we reconsidered the use of simvastatin as a single agent and sought to determine combination treatments that could potentially be synergistic. In these studies we focused on combining statin with a farnesyl transferase inhibitor (i.e., tipifarnib) which was designed to prevent lipidation (farnesylation) of RAS. Our rationale for this combination was based on the capacity of RAS to become lipidated by geranylgeranylation, which is a process that can be inhibited by simvastatin. Although each drug alone can induce apoptosis, we reasoned that the combination may allow for synergistic effects that can be achieved at lower concentrations. Our hypothesis is that *the combination of simvastatin and tipifarnib is synergistic and this synergy is conferred by the capacity of simvastatin to block the alternative lipidation pathway of RAS, which induces apoptosis*. Therefore we conducted the studies outlined in Specific Aim 2.

**Specific aim 2:** Assess the interaction between simvastatin and tipifarnib in inducing cytotoxicity in leukemia cell lines. This work is outlined in **Chapter 5**.

**Aim 2.1:** Determine whether simvastatin synergistically induces apoptosis in combination with tipifarnib in leukemia cells.

**Aim 2.2:** Determine the underlying molecular mechanisms that induce apoptosis in simvastatin/tipifarnib treated cells including RAS membrane localization, downstream signaling, and apoptosis.

## **Chapter 3 : Validated LC-MS/MS method for simultaneous determination of simvastatin and its acid form in human plasma and cell lysate: Pharmacokinetic Application**

### **A. Introduction**

Simvastatin is a well-established drug for the treatment of hyperlipidemia. Simvastatin is a prodrug administered in the lactone form, which is converted in the liver into the active acid form (Figure 3.1). It is this active carboxylate form that reduces cholesterol biosynthesis by competitively inhibiting the 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, the rate limiting enzyme in the mevalonate pathway [8]. Additionally, statins inhibit the synthesis of other downstream products in the mevalonate pathway, such as the isoprenoids [8]. Isoprenoids, including farnesyl pyrophosphate (FPP) and geranylgeranyl pyrophosphate (GGPP), are known to be involved in important cellular processes such as proliferation and apoptosis [24]. Thus, statins have recently been tested for their potential use as anticancer agents. As with all agents in this class, in vitro studies have shown that simvastatin displays anticancer activity, but only at concentrations that are higher than those observed in plasma of patients being administered typical doses associated with hyperlipidemia therapy [107].

Several clinical trials were subsequently conducted to study the safety and tolerability of high dose statin analogues, including simvastatin, in cancer patients [92-94]. Oral statins were found to be well tolerated at high doses with minor side effects. In a phase I study, lovastatin given orally at a dose of 25 mg/kg daily was well tolerated and safe in patients with solid tumor [93]. In the case of simvastatin, a phase I study in patients with myeloma or lymphoma has shown that the maximal tolerated dose (MTD) of simvastatin, given orally, is 7.5 mg/kg twice a day, which is 25-fold higher than typical dose. The most common side effects of high dose simvastatin were nausea, diarrhea,

muscle weakness and myalgia [94]. However, pharmacokinetics (PK) was not defined and it is not known if simvastatin plasma concentrations can reach the levels necessary for the antitumor activity observed in vitro. In this context, we initiated a clinical study to characterize the pharmacokinetics of simvastatin lactone and its acid form in plasma and peripheral blood mononuclear cells (PBMCs) after oral administration of simvastatin at 7.5 mg/kg twice daily in patients with recurrent and refractory chronic lymphocytic leukemia (CLL).

Simvastatin has low systemic bioavailability which is attributed to the high extraction by the liver, the main site of action for treating hyperlipidemia. Therefore, sensitive analytical methods have previously been developed to assay both simvastatin (SIM) and its acid form (SIMA) in plasma [109-112]. The first analytical method developed was an LC coupled with UV detection (238 nm); nonetheless, low sensitivity for quantitation of SIM and SIMA in biological fluids was reported [113]. Better sensitivity using UV detection was achieved later with an LOQ of 0.5 ng/mL but with run time > 28.7 min [114]. A more sensitive HPLC-FD method using 1-bromoacetylpyrene for derivatization has been reported with an LOQ of 0.1 ng/mL for both analytes [115]. Although this LC-FD method is highly sensitive, sample preparation using solid phase extraction and analyte derivatization is inconvenient and time consuming. On the other hand, several LC-MS/MS methods have been developed for the determination of SIM and SIMA in biological fluids which are more sensitive and specific [109-112]. These methods are coupled with either solid phase extraction (SPE) or liquid-liquid extraction (LLE) procedures. Solid phase extraction has yielded good recoveries for SIM but SIMA recovery was low [116]. LLE showed better recoveries for both SIM and SIMA compared to SPE [109, 111, 112]. Current analytical methods have not been validated for the analyses of SIM and SIMA in cell lysates. Moreover, few assays have been validated to

measure plasma concentration of SIM and SIMA at higher levels [117-119]. Here we report the development and validation of an LC-MS/MS method for the analysis of simvastatin and its acid form in human plasma and PBMCs.

## **B. Methods**

### **1. Chemicals and reagents**

Simvastatin was purchased from Toronto Research Chemicals Inc. (North York, Canada). Ammonium acetate (Mallinckrodt Baker, Philipsburg, NJ, USA) and sodium hydroxide (EM Science, Gibbstown, NJ, USA) were purchased from VWR (West Chester, PA, USA). HPLC grade acetonitrile and diethyl ether were obtained from Sigma-Aldrich (St Louis, MO, USA). Lovastatin (Alexis Biochemicals, San Diego, CA, USA), hydrochloric acid and glacial acetic acid were from Fisher Scientific (Fair Lawn, NJ, USA). Anhydrous ethanol was obtained from IBI Scientific (Peosta, IA, USA). K562, a chronic myelogenous leukemia (CML) cell line, was purchased from ATCC (Manassas, VA, USA).

### **2. LC-MS/MS instrumentation and conditions**

All analyses were performed using an HPLC system consisting of a Shimadzu LC-20AD pump and a Shimadzu SIL-20AC VP auto sampler (Shimadzu, Columbia, MD, USA). The LC system was interfaced to an API 2000 ESI-MS/MS (Applied Biosystems, Foster City, CA, USA). The analytical column used was a Phenomenex Luna C<sub>18</sub> (2.0 mm x 100 mm i.d.; 2.5 µm particle size), connected to a C<sub>18</sub> guard column (Phenomenex C<sub>18</sub>, 2.0 mm x 4 mm; 5 µm particle size). An isocratic mobile phase was used consisting of 75:25 (% v/v) acetonitrile : ammonium acetate (0.1M, pH 5.0 adjusted with acetic acid). The flow rate was 0.15 mL/min under ambient temperature. The auto sampler temperature was maintained at 4°C and the injection volume was 20 µL. The run time

was 10 min. All analytes and internal standard were detected on a triple quadrupole mass spectrometer (API 2000), equipped with a turbo ion spray source (MDS SCIEX, Toronto, Canada) and operating in the positive ion mode. Lovastatin (LOV) was used as an internal standard (IS). Quantitation was performed using multiple reaction monitoring (MRM) of precursor/product ion transitions at  $m/z$  419.3/199.3 for SIM; 437.3/303.3 for SIMA; and 405.2/199.3 for LOV.

The optimized source parameters for SIM, SIMA and LOV were as follows: the nebulizer gas pressure was set at 30 psi, the heater gas at 90 psi, the ion spray voltage was 5500 V and the turbo heater temperature was 500°C. The curtain gas pressure was set at 40 psi and the collision activation dissociation (CAD) gas at 10 psi. Lastly the entrance potential, declustering potential, collision energy and cell exit potential applied were set at 8.27, 14, 17 and 5.25 V for SIM, 7, 3.8, 14 and 8.5 V for SIMA and 8.7, 12.5, 21.2 and 5.4 V for LOV, respectively. All the parameters were controlled by Analyst software version 1.4.2 (Applied Biosystems, Foster City, CA, USA).

### **3. Preparation of standard and quality control samples**

Stock solutions of SIM, SIMA and LOV (1 mg/mL) were prepared in ethanol. Simvastatin acid was prepared by alkaline hydrolysis of simvastatin [120]. Standard working solutions of SIM and SIMA were prepared by serial dilution of the appropriate stock solutions with mobile phase. Standards were prepared fresh for each run by spiking 25  $\mu$ L of the appropriate working solutions of both analytes and internal standard into 425  $\mu$ L of drug free human plasma to obtain calibration concentrations of 2.5, 5, 10, 50, 100, 250, 500 ng/mL SIM, 5, 10, 50, 100, 250, 500 ng/mL SIMA and 50 ng/mL LOV. Similar to plasma calibration standards, cell lysate calibration standards were prepared at calibration concentrations of 2.5, 5, 10, 50, 80, 100, 250 ng/mL SIM, 5, 10, 50, 80,

100, 250 ng/mL SIMA and 50 ng/mL LOV. Cell lysate matrix was prepared by lysing K562 cells in deionized water ( $3 \times 10^7$  cells/mL) via sonication.

Plasma quality control (QC) sample concentrations were 7.5, 150 and 400 ng/mL for SIM and 15, 150 and 400 ng/mL for SIMA. Cell lysate QC sample concentrations were 7.5, 90 and 200 ng/mL for SIM and 15, 90 and 200 ng/mL for SIMA. QC samples were prepared using stock solutions other than those used for calibration standards preparation. Both calibration standards and QC samples were prepared at 4°C in an ice bath.

#### **4. Processing of plasma and cell lysate samples**

All plasma and cell lysate samples were stored at -80°C and thawed at room temperature. A 25 µL aliquot of LOV was added to 475 µL of plasma or cell lysate sample in 16 mm x 100 mm glass test tube. The tubes then were vortexed for 10 s. After the addition of 500 µL of ammonium acetate buffer (0.1 M, pH 5.0), tubes were vortexed again for 1 min. Diethyl ether (3 mL) was then added to each tube and samples were placed on a shaker at 200 rpm for 15 min at 4°C. Extracted samples were centrifuged at 3000 rpm for 10 min at 4°C. After centrifugation, the aqueous layer was frozen by placing the tubes in dry ice for 1 min. The organic layer was decanted into a new 16 mm x 100 mm test tube and was evaporated till dryness at room temperature using a gentle stream of nitrogen. The residue was reconstituted in 50 µL of mobile phase and 20 µL was injected onto the HPLC column.

#### **5. Method validation**

The method validation of SIM and SIMA in human plasma and cell lysate was performed according to the FDA guidelines [121]. The assay was validated for specificity

and sensitivity, linearity, precision and accuracy, extraction recovery, matrix effect, and stability.

5.1. Specificity and sensitivity: Assay specificity and sensitivity were conducted in eight different lots of blank plasma that was either left blank or spiked with both analytes and IS. Analytes were extracted using the previously described extraction procedure and analyzed to determine the extent of interference by endogenous plasma components at the retention time of both analytes and IS. The lowest limit of quantitation (LLOQ) was assessed in the same plasma lots used for specificity. The determination of LLOQ was based on the criteria that the deviation of the measured concentrations should not be more than 20% from the nominal concentration and that the signal to noise ratio be  $\geq 5$ .

5.2. Linearity: Linearity was evaluated using plasma samples spiked with both SIM and SIMA at concentration ranges of 2.5-500 ng/mL and 5-500 ng/mL, respectively. The internal standard, LOV, concentration was 50 ng/mL in all calibration standards. Three calibration curves were prepared and analyzed by plotting area ratios of analyte to internal standard against the concentration of each calibration standard. The results were fitted into a linear regression model using  $(1/y)$  as a weighting factor for both SIM and SIMA. A cell lysate calibration curve was prepared similar to plasma calibration curve, but at concentration ranges of 2.5-250 ng/mL and 5-250 ng/mL for SIM and SIMA, respectively.

5.3. Precision and accuracy: The intra-day precision and accuracy was evaluated at three different QC levels (low, medium and high) in eight replicates on the same day and in five replicates on three different days for inter-day precision and accuracy determination. Acceptable deviation was set within 15% of the nominal concentration for accuracy and within 15% relative standard deviation for precision.

5.4. Extraction recovery and matrix effect: The recovery efficiency of the extraction procedure was performed at low and high QCs using the extraction procedure described in section 2.4. Recovery was evaluated as a percentage of the peak area of analytes that were spiked into a matrix before extraction to the peak area of analytes that had been spiked after extraction of a blank matrix. Endogenous substances present in biological matrix can possibly enhance or suppress analyte ionization to affect the sensitivity, precision or accuracy of the described method. Matrix effect was assessed as a percentage of the peak areas of control plasma extracted and then spiked with analyte, to neat standards injected directly in the same reconstitution solvent. Matrix effect was carried out on five different lots of blank plasma and at low and high QC levels.

5.5. Stability: The short term and long term stability of SIM and SIMA in plasma and cell lysate samples was evaluated under different storage conditions. All stability experiments were performed at low and high QC levels. Both analytes were spiked individually in order to assess the potential for interconversion between the lactone and acid forms.

Short term stability of SIM and SIMA was evaluated in plasma and cell lysate samples at 4°C (ice-bath) for 6 h. The autosampler storage stability was determined by storing the reconstituted QC samples for 6 h under autosampler conditions (i.e., 4°C). Samples were stored for a month at -80°C to evaluate long term stability of SIM and SIMA. Lastly, the stability of SIM and SIMA in plasma and cell lysate samples was assessed after repeated cycles of freeze and thaw (2 cycles). In each cycle the samples were removed from -80°C storage and allowed to thaw at room temperature.

## **6. Pharmacokinetic study**

In a pilot clinical trial, patients received an oral dose of 7.5 mg/kg simvastatin twice daily for one week. Only patients who signed a written consent form were enrolled in this study. Blood samples (8 mL) were collected after the first oral dose of simvastatin at pre-dose, 0.25, 1, 2, 3, 6, 8, and 12 hours. All samples were collected in heparinized BD Vacutainer Cell Preparation Tubes (CPT) and immediately centrifuged (1800 x g for 30 min at room temperature) to separate plasma and PBMCs. Collected plasma and PBMCs were stored at -80°C until analysis. At time of analysis, PBMC pellets were thawed and lysed in 1 mL deionized water via sonication then processed as described in section 4.

## **C. Results and Discussions**

### **1. Performance of LC and MS/MS**

The Phenomenex Luna C<sub>18</sub> column, used in this study, gave a symmetric peak shape for all analytes with an acceptable run time (10 min). Mobile phase components were selected based on previous works where ammonium acetate was used to enhance ionic strength of the analytes [110]. Also, different volumetric ratios of acetonitrile and ammonium acetate buffer were tested to obtain the best peak shape for both analytes with reasonable retention time (<10 min). In previously developed methods, simvastatin and lovastatin (Figure 3.1) were detected in positive ion mode whereas negative ion mode was typically favored for simvastatin acid detection [109, 110, 112]. Few studies have utilized the positive ion mode for detecting simvastatin acid [117, 118]. However, in our studies simvastatin acid gave better fragmentation in positive ion mode with higher product ion signal intensities. Thus, both analytes (SIM and SIMA) and IS (LOV) were detected in positive ion mode without the need to switch polarity during the sample run.

MS source parameters, as well as analytes parameters, were optimized to achieve the highest signal intensity.

## 2. Selectivity and sensitivity (LLOQ)

Plasma samples from eight different sources were tested for the presence of endogenous substances that might interfere at the retention times of peaks of interest as evaluated by chromatograms of blank plasma and cell lysate, plasma and cell lysate spiked with SIM and SIMA at QC1 level or LOV at 50 ng/mL, plasma and PBMCs collected from patients at predose and 12 h after receiving simvastatin at 7.5 mg/kg twice daily (Figure 3.2). Both SIMA and SIM were well separated with retention times of 2.65 and 7.1 min, respectively. LOV was detected at 5.6 min. The chromatograms show no interfering peaks at the retention times of both analytes and IS in the blank plasma. However, in-source lactonization of SIMA into SIM was recognized as shown in Figure 3.2 B, where a small peak (**Peak 1**) can be seen on the simvastatin MRM channel ( $m/z$  419.3/199.3) at the retention time of SIMA. A similar peak (**Peak 2**) occurs on the simvastatin acid MRM channel ( $m/z$  437.3/303.3) at the retention time of SIM, this peak was explained as the interference of A+1 isotope from  $[M+NH_4]^+$  of the simvastatin lactone form but not by in-source hydrolysis [122]. Therefore, chromatographic separation between SIM and SIMA is needed to eliminate the contribution of the post column in-source lactonization and the interference of  $[M+NH_4]^+$  isotope of simvastatin lactone.

The LLOQ was tested at different levels ranging from 1-10 ng/mL and it was found to be 2.5 ng/mL for SIM with an accuracy of 97% and 8% precision while SIMA showed an LLOQ of 5 ng/mL with 105% accuracy and 7% precision. Previous analytical methods have proven to be highly sensitive with a limit of quantitation ranging from 0.05-0.1 ng/mL [109, 110, 112, 117]. These methods developed for the determination of low

SIM and SIMA plasma levels achieved by typical doses (40 mg). However, our method is developed for PK study of high dose simvastatin that is 25 fold higher than typical doses, thus LLOQ achieved was sufficient for the purpose of this study.

### 3. Linearity, precision and accuracy

The calibration curves of SIM (2.5-500 ng/mL) and SIMA (5-500 ng/mL) in human plasma and SIM (2.5-250 ng/mL) and SIMA (5-250 ng/mL) in cell lysate showed acceptable linearity. These ranges encompassed the concentrations observed in human plasma and PBMCs collected in a pharmacokinetic study following the oral administration of high dose simvastatin. Calibration curves (n= 3) prepared in human plasma yielded the following regression equations  $y= 0.005 (\pm 0.001) + 0.61(\pm 0.03) x$  with  $R^2=0.997$  and  $y= 0.002 (\pm 0.002) + 0.23 (\pm 0.02) x$  with  $R^2=0.997$  for SIM and SIMA, respectively. Similarly, calibration curves (n= 3) prepared in cell lysate yielded the following regression equations  $y= 0.003 (\pm 0.002) + 0.65 (\pm 0.11) x$  with  $R^2=0.997$  and  $y= 0.001 (\pm 0.002) + 0.31 (\pm 0.09) x$  with  $R^2=0.992$  for SIM and SIMA in cell lysate, respectively.

Inter- and intra-day precision and accuracy were determined at three concentration levels (7.5, 200 and 400 ng/mL for SIM and 15, 90 and 150 ng/mL for SIMA). As shown in Table 3.1, inter- and intra-day precision values of SIM and SIMA, expressed as % relative standard deviation (RSD), ranged from 1.1 to 5.3%, whereas accuracy values ranged between 88.6 - 110.2%. The results from intra and inter-day precision and accuracy indicate that the method reproducibility is acceptable within the same day and on different days.

#### **4. Recovery and matrix effect**

Analytes were extracted from biological samples using a liquid-liquid extraction procedure; several organic solvents were tested for their extraction efficiencies such as methyl tertiary butyl ether (MTBE), diethyl ether, ethyl acetate, acetonitrile and methanol. Ethyl acetate showed fair recovery for SIM, but extraction efficiency for SIMA was poor. MTBE and diethyl ether were found to have comparable extraction efficiencies for both SIM and SIMA and were higher than those obtained by the other organic solvents used. Although MTBE was commonly used in previous methods, diethyl ether was chosen for LLE procedure in this study. Mean recovery values of SIM and SIMA in human plasma were found to be 75.3% and 73.2% at QC1 level whereas at QC3 level they were 68.6% and 58.9%, respectively. In cell lysate, mean recovery of SIM and SIMA were higher at QC1 levels compared to plasma recovery with 95.7% and 98.1%, respectively. Recovery values of both SIM and SIMA in cell lysate at QC3 level were similar to those in human plasma. Furthermore, mean matrix effect values are within the acceptable range for both SIM and SIMA, indicating that the matrix effect has no impact on the analytes quantification. The results of the recovery and matrix effect are summarized in Table 3.2.

#### **5. Stability**

The interconversion between simvastatin and simvastatin acid is a result of hydrolysis of SIM and lactonization of SIMA. It has been found that the interconversion can be reduced either at low temperature or when pH is adjusted between pH 4 and pH 5 [116]. Acidified samples stored under low temperature conditions display very low interconversion ( <1% at 4°C and 0.05% at -20°C for 4 weeks) [116]. Thus, during method validation, the plasma and cell lysate samples were kept at 4°C at all stages of analysis and the reconstitution solution was buffered at pH 5. As shown in Table 3.3, simvastatin and simvastatin acid were found to be stable in human plasma, cell lysate

and the buffered reconstitution solution for at least 6 h at 4°C. For long term stability, both analytes were stable in human plasma and cell lysate for at least one month at -80°C (Table 3.4). Over two freeze-thaw cycles of human plasma and cell lysate, SIM and SIMA were also found to be stable (Table 3.5).

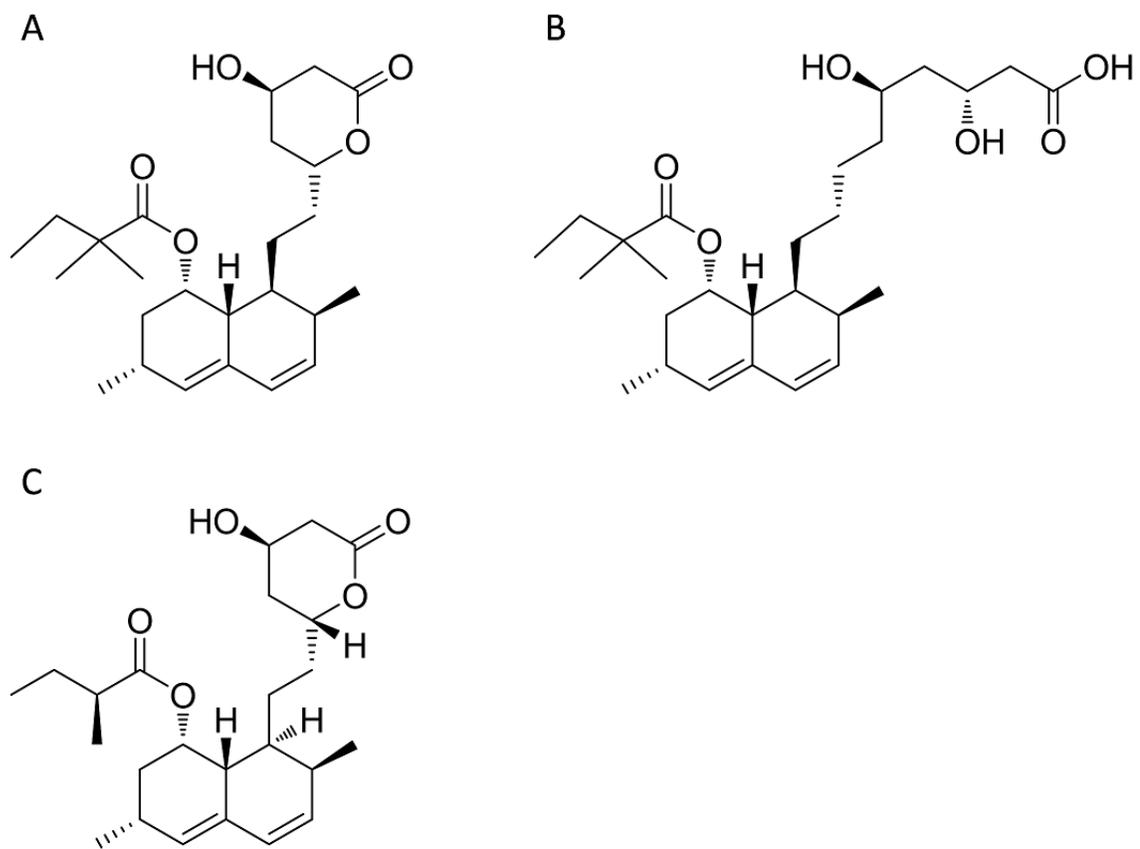
Stability of SIM and SIMA in stock and working solutions has been tested in several previous works. Over different solutions compositions both SIM and SIMA were found to be stable for at least one month [109, 112, 116, 117]. However, we have tested the stability of both SIM and SIMA in working solution kept at -80°C, and they were found to be stable for at least one year. Lastly, no stability studies were carried out for lovastatin as it has previously been shown to be stable under similar storage conditions [123].

## **6. Pharmacokinetic study**

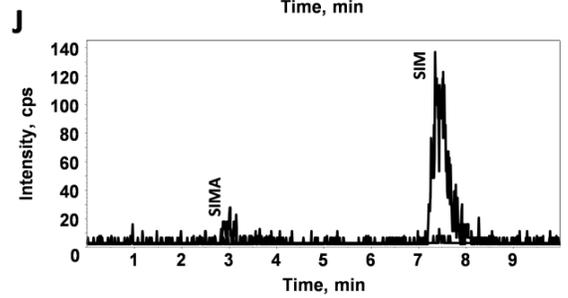
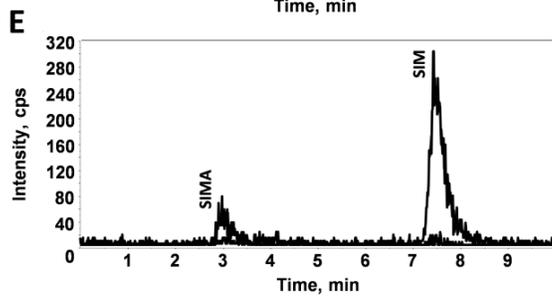
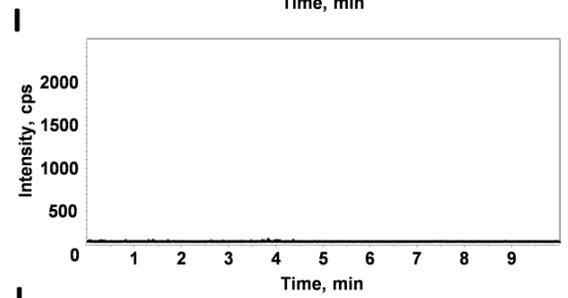
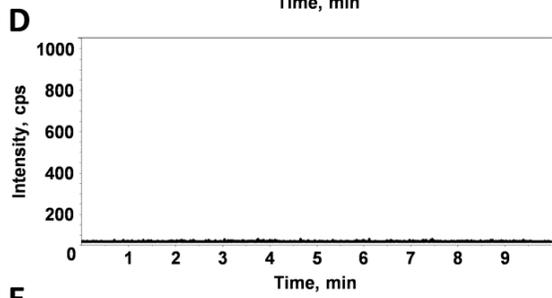
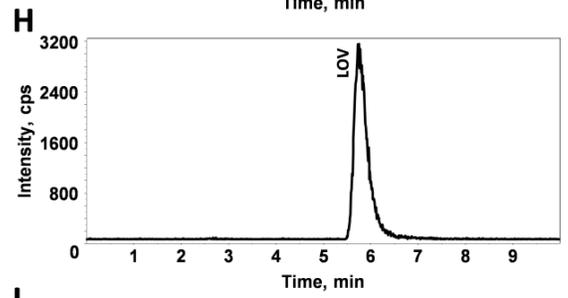
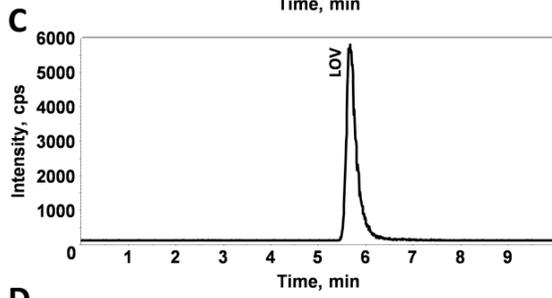
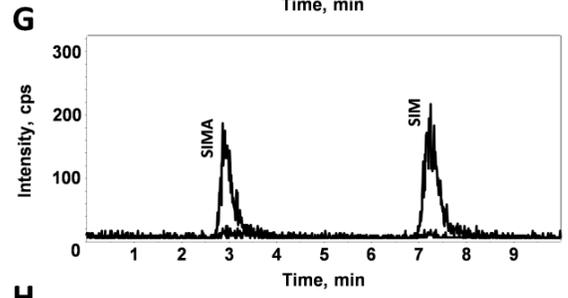
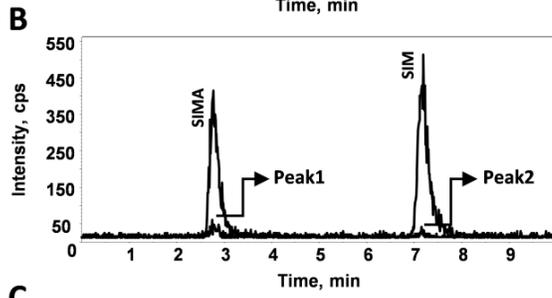
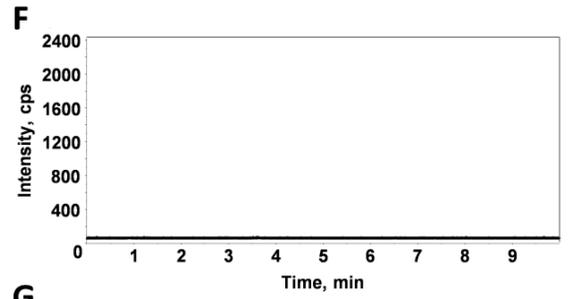
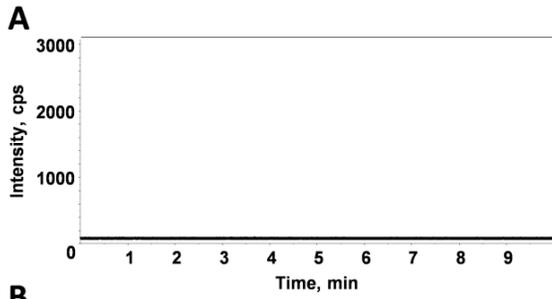
This method was successfully applied for the determination of simvastatin and its acid form in human plasma and PBMCs samples collected from leukemia patients following the oral administration of high dose simvastatin. Figure 3.2 shows the MRM chromatograms of both plasma and PBMCs samples collected from a patient 12 h after receiving simvastatin at 7.5 mg/kg twice daily. Figure 3.3 depicts a typical pharmacokinetic profile of SIM and SIMA in plasma and SIM in PBMCs from a CLL patient who received high dose simvastatin. Unlike SIM, SIMA concentrations in PBMCs were below the detection limit of the assay at all the time points of the PK study. This could be attributed to the hydrophilicity of the carboxylate form which may limit its accessibility to the PBMCs. Alternatively, the carboxylate may be subject to efflux by an ATP-binding cassette transporter.

## **D. Conclusions**

In conclusion, an LC-MS/MS was developed and validated for the determination of simvastatin and its acid form in human plasma and cell lysate. This assay is the first method developed for the analysis of SIM and SIMA in cell lysate. Moreover, this assay spans the concentration range of quantification of both SIM and SIMA that is applied for high dose simvastatin administration. Overall, this analytical method has proved to be successful for the analysis of SIM and SIMA in plasma and PBMCs samples collected from a high dose simvastatin pharmacokinetic study.



**Figure 3.1.** Chemical structures of (A) simvastatin, (B) simvastatin acid and (C) lovastatin.



**Figure 3.2.** Representative chromatograms of: blank plasma (A) and cell lysate (F), plasma (B) and cell lysate (G) spiked with both SIM and SIMA at QC1 level, plasma (C) and cell lysate (H) spiked with LOV at 50 ng/mL, patient plasma (D) and PBMCs (I) samples collected at predose and patient plasma (E) and PBMCs (J) samples collected 12 h after oral administration of simvastatin (7.5 mg/kg).

**Table 3.1.** Intra- and Inter-day precision and accuracy

Analyte	Nominal concentration (ng/mL)	Intra-day (n=8)		Inter-day (n=5)	
		Accuracy (mean $\pm$ SD, %)	Precision (%RSD)	Accuracy (mean $\pm$ SD, %)	Precision (%RSD)
Simvastatin					
QC1	7.5	110.2 ( $\pm$ 5.7)	5.2	96.5 ( $\pm$ 2.5)	2.6
QC2	150	105.2 ( $\pm$ 1.4)	1.4	97.1 ( $\pm$ 2.4)	2.4
QC3	400	99.6 ( $\pm$ 2.1)	2.1	94.7 ( $\pm$ 2.3)	2.4
Simvastatin acid					
QC1	15	95.3 ( $\pm$ 3.0)	3.2	92.9 ( $\pm$ 4.9)	5.3
QC2	150	89.4 ( $\pm$ 1.0)	1.1	90.3 ( $\pm$ 2.3)	2.5
QC3	400	86.8 ( $\pm$ 2.1)	2.4	91.1 ( $\pm$ 1.4)	1.5

SD, standard deviation. RSD, Relative standard deviation.

**Table 3.2.** Recovery and matrix effect

Analyte	Recovery (mean $\pm$ SD, %) (n=3)		Absolute matrix effect (mean $\pm$ SD, %) (n=5)
	Human plasma	Cell lysate	
Simvastatin			
QC1	75.3 ( $\pm$ 5.8)	95.7 ( $\pm$ 4.1)	98.9 ( $\pm$ 3.3)
QC3	68.6 ( $\pm$ 5.4)	67.5 ( $\pm$ 7.6)	99.0 ( $\pm$ 5.6)
Simvastatin acid			
QC1	73.2 ( $\pm$ 4.4)	98.1 ( $\pm$ 5.8)	96.4 ( $\pm$ 3.9)
QC3	58.9 ( $\pm$ 4.1)	63.8 ( $\pm$ 10.1)	98.7 ( $\pm$ 1.0)

SD, standard deviation.

**Table 3.3.** Short term stability of the analytes in mobile phase extract, human plasma and cell lysate stored at 4°C (n=3)

Analyte	Analyte concentrations at different time points (mean $\pm$ SD) <sup>a</sup>								
	Mobile phase extract			Human plasma			Cell lysate		
	1h	3h	6h	1h	3h	6h	1h	3h	6h
Simvastatin									
QC1	99.7 ( $\pm 6.7$ )	106.7 ( $\pm 11.8$ )	102.7 ( $\pm 10.2$ )	96.6 ( $\pm 4.0$ )	99.8 ( $\pm 14.4$ )	101.9 ( $\pm 8.8$ )	118.9 ( $\pm 11.7$ )	121.9 ( $\pm 21.6$ )	109.9 ( $\pm 10.7$ )
QC3	100.3 ( $\pm 2.4$ )	101.0 ( $\pm 2.9$ )	97.9 ( $\pm 2.8$ )	94.0 ( $\pm 4.7$ )	113.5 ( $\pm 3.0$ )	115.7 ( $\pm 0.6$ )	97.1 ( $\pm 12.0$ )	110.4 ( $\pm 11.8$ )	97.3 ( $\pm 8.6$ )
Simvastatin acid									
QC1	101.2 ( $\pm 4.6$ )	89.6 ( $\pm 3.2$ )	92.8 ( $\pm 3.7$ )	85.5 ( $\pm 24.6$ )	94.5 ( $\pm 8.5$ )	102.8 ( $\pm 13.5$ )	121.0 ( $\pm 16.8$ )	111.9 ( $\pm 8.6$ )	101.1 ( $\pm 9.2$ )
QC3	100.0 ( $\pm 3.9$ )	98.3 ( $\pm 1.8$ )	96.8 ( $\pm 3.1$ )	95.0 ( $\pm 10.4$ )	97.4 ( $\pm 10.1$ )	97.5 ( $\pm 2.9$ )	89.9 ( $\pm 1.2$ )	81.4 ( $\pm 3.4$ )	90.3 ( $\pm 3.0$ )

<sup>a</sup> Analyte concentrations are expressed as the mean percentage of time zero concentrations ( $\pm$  standard deviation).

**Table 3.4.** Long term stability in human plasma and cell lysate (n=3)

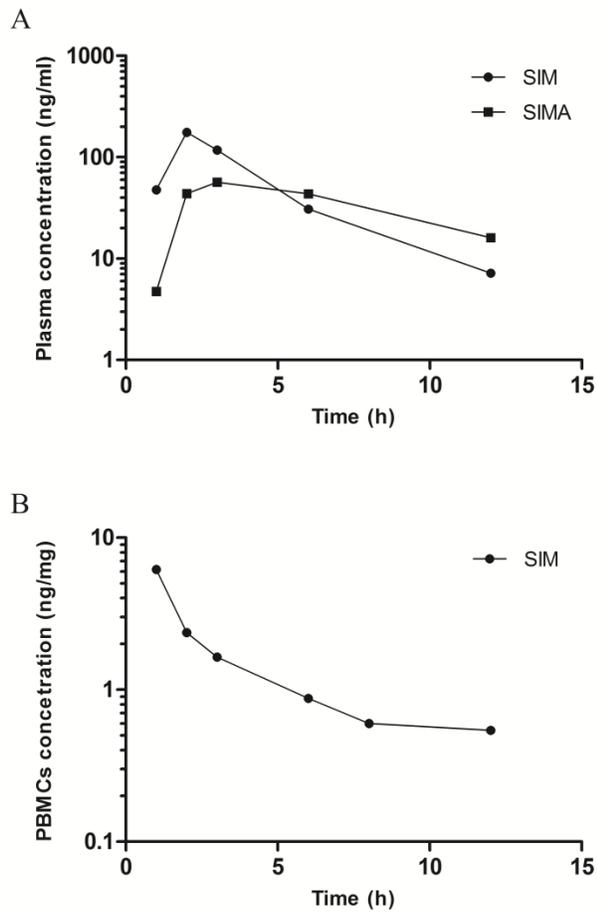
Analyte	Analyte concentrations at different time points (mean $\pm$ SD) <sup>a</sup>								
	Human plasma					Cell lysate			
	1 Day	3 Days	1 Week	2 Weeks	4 Weeks	1 Day	1 Week	2 Weeks	4 Weeks
Simvastatin									
QC1	105.5 ( $\pm$ 7.6)	95.6 ( $\pm$ 9.4)	102.5 ( $\pm$ 10.9)	99.6 ( $\pm$ 5.2)	97.0 ( $\pm$ 6.5)	99.7 ( $\pm$ 7.8)	90.2 ( $\pm$ 9.2)	98.3 ( $\pm$ 4.8)	90.5 ( $\pm$ 5.7)
QC3	112.1 ( $\pm$ 9.4)	97.3 ( $\pm$ 3.6)	102.3 ( $\pm$ 2.6)	101.4 ( $\pm$ 18.3)	95.4 ( $\pm$ 11.9)	87.4 ( $\pm$ 4.4)	106.5 ( $\pm$ 8.4)	99.9 ( $\pm$ 5.2)	101.6 ( $\pm$ 9.2)
Simvastatin acid									
QC1	91.3 ( $\pm$ 7.7)	106.1 ( $\pm$ 13.4)	105.9 ( $\pm$ 11.0)	106.8 ( $\pm$ 6.5)	91.3 ( $\pm$ 16.4)	97.1 ( $\pm$ 5.6)	104.3 ( $\pm$ 8.7)	104.5 ( $\pm$ 3.2)	114.7 ( $\pm$ 7.1)
QC3	99.1 ( $\pm$ 7.3)	106.5 ( $\pm$ 3.6)	96.7 ( $\pm$ 2.2)	106.9 ( $\pm$ 17.3)	108.3 ( $\pm$ 7.2)	90.4 ( $\pm$ 14.0)	96.2 ( $\pm$ 9.4)	83.0 ( $\pm$ 13.2)	96.2 ( $\pm$ 12.5)

<sup>a</sup>Analyte concentrations are expressed as the mean percentage of time zero concentrations ( $\pm$  standard deviation).

**Table 3.5.** Freeze and thaw stability in human plasma and cell lysate (n=3)

Analyte	Analyte concentrations at given cycle (mean $\pm$ SD) <sup>a</sup>			
	Human plasma		Cell lysate	
	1st cycle	2nd cycle	1st cycle	2nd cycle
Simvastatin				
QC1	113.9 ( $\pm$ 11.8)	103.7 ( $\pm$ 4.7)	101.5 ( $\pm$ 10.0)	95.6 ( $\pm$ 5.1)
QC3	102.9 ( $\pm$ 5.1)	104.5 ( $\pm$ 5.1)	88.7 ( $\pm$ 6.5)	100.3 ( $\pm$ 10.1)
Simvastatin acid				
QC1	85.4 ( $\pm$ 4.9)	95.1 ( $\pm$ 16.5)	91.4 ( $\pm$ 5.3)	109.9 ( $\pm$ 6.8)
QC3	96.5 ( $\pm$ 11.7)	90.3 ( $\pm$ 3.3)	86.1 ( $\pm$ 6.9)	105.3 ( $\pm$ 3.3)

<sup>a</sup>Analyte concentrations are expressed as the mean percentage of time zero concentrations ( $\pm$  standard deviation).



**Figure 3.3.** Pharmacokinetic profiles of (A) simvastatin lactone and carboxylate in plasma and (B) simvastatin lactone in PBMCs after oral administration of high dose simvastatin in a CLL patient. SIM concentration in PBMCs is normalized to the protein concentration of each PBMCs sample.

## **Chapter 4 : Pharmacokinetics of high dose simvastatin in refractory and relapsed chronic lymphocytic leukemia patients**

### **A. Introduction**

Over the past two decades, statins have been used safely and effectively for the treatment of hypercholesterolemia and for lowering the incidence of cardiovascular disease. Statins inhibit 3-hydroxy-3-methylglutaryl coenzyme A reductase, the rate limiting enzyme at the top of the mevalonate pathway, which is responsible for cholesterol synthesis [7, 8]. In addition to cholesterol, the mevalonate pathway yields other downstream products such as isoprenoids, dolichol and ubiquinone [7, 8], which are critical components for a wide range of cellular metabolic and signaling processes. In particular, isoprenoids (farnesyl pyrophosphate and geranylgeranyl pyrophosphate) are crucial for the anchoring of small GTPases, such as RAS and RHO family proteins, to the cell membrane. Membrane attachment allows the subsequent activation of these proteins, which mediate intracellular signaling for several downstream survival and proliferation processes [24].

In this context, statins have been tested for their potential use as anticancer agents in several tumor types. Several reports have shown that statin mediated inhibition of isoprenoid synthesis disrupts small GTPases localization to the membrane and is likely the underlying mechanism for the in vitro observed antitumor activity [59, 75, 79]. Notably, these reports have demonstrated that statins display anticancer activity only at concentrations higher than those observed in plasma of patients being administered typical doses associated with hyperlipidemia therapy [107].

Therefore, clinical investigators were prompted to study the safety and tolerability of high dose statins in cancer patients. In a lovastatin phase-I study in patients with solid tumors, the maximum tolerated dose of lovastatin was 25 mg/kg daily [93]. One minor

response was also reported in a patient with recurrent high grade of glioma. In this study, the peak plasma concentrations of lovastatin were in the range of 0.1 to 3.9  $\mu\text{M}$ , which are comparable to its  $\text{IC}_{50}$  values in glioma cells (0.2 - 2  $\mu\text{M}$ ) [124]. However, in a subsequent phase I/II study of high dose lovastatin in patients with malignant glioma only one partial response and one minor response were observed out of nine patients [95]. A later phase II study, in patients with advanced gastric adenocarcinoma, tested the effect of an even higher lovastatin dose (35 mg/kg/day), by using concomitant administration of ubiquinone to prevent rhabdomyolysis, but the results were negative[92]. In the case of simvastatin, a phase I study was conducted in patients with myeloma or lymphoma and the maximum tolerated dose (MTD) of oral simvastatin was determined to be 7.5 mg/kg twice a day, for seven days. The most common side effects of high dose simvastatin were nausea, diarrhea, muscle weakness and myalgia [94]. However, the study design did not include pharmacokinetics and it remains unknown whether simvastatin at high doses can reach the concentrations required for the antitumor activity observed in vitro. In a subsequent phase II study, simvastatin at MTD was given for 7 days followed by rapid intravenous diffusion of vincristine (0.4 mg), adriamycin (9 mg/m<sup>2</sup>), and dexamethasone 40 mg orally (VAD) on days 7- 10. High dose simvastatin failed to reverse clinical resistance to VAD chemotherapy in myeloma patients [97]. Authors of this study attributed the limited efficacy of simvastatin to the short period of treatment as well as the treatment strategy. However, failure to reach therapeutically effective concentrations might be a possible explanation of these unsuccessful clinical results. Here we report the pharmacokinetics of simvastatin given at MTD in patient with recurrent and refractory chronic lymphocytic leukemia (CLL).

## **B. Methods**

### **1. Materials**

Simvastatin for in vitro studies was purchased from Toronto Research Chemicals Inc. (North York, Canada). Ammonium acetate (Mallinckrodt Baker, Philipsburg, NJ, USA) was purchased from VWR (West Chester, PA, USA). HPLC grade acetonitrile and diethyl ether were obtained from Sigma-Aldrich (St Louis, MO, USA). Lovastatin (Alexis Biochemicals, San Diego, CA, USA) and glacial acetic acid were from Fisher Scientific (Fair Lawn, NJ, USA). Heparinized BD Vacutainer Cell Preparation Tubes CPT tubes were purchased from Becton-Dickinson (Franklin Lakes, NJ). RPMI 1640 medium, penicillin/streptomycin, MEM vitamins and MEM non-essential amino acids were from Life Technologies (Grand Island, NY) whereas fetal bovine serum was obtained from Atlanta Biologicals (Lawrenceville, GA).

### **2. Study Design and subjects**

Eligible subjects were at least 18 years old and diagnosed with CLL utilizing WHO classification criteria [125]. All patients had previously received treatment and either had refractory or relapsed CLL. At the time of treatment subjects had either disease-related symptoms or progressive disease with deterioration of blood counts, discomfort from lymphadenopathy or hepatosplenomegaly, recurrent infections, or associated autoimmune disorders that necessitated further therapy. Patients were required to have a normal serum bilirubin level and serum transaminase levels of no more than 50% above the upper limit of institutional normal limits. All patients provided written informed consent for this study, which was approved by the institutional review board of the University of Kentucky (Lexington, KY).

Simvastatin was administered orally twice daily for seven consecutive days at a dose of 7.5 mg/kg per dose. All doses were administered using 80 mg tablets and were rounded to the nearest 80 mg increment. A 14-day washout followed the seven days of treatment comprising a 21-day treatment cycle. Patients were evaluated for progression and unexpected toxicities prior to commencing with each treatment cycle and were to be treated for 6 cycles. All grade 3 or 4 adverse events, except for nausea or diarrhea that resolved to less than grade 1 with appropriate anti-emetics or anti-diarrhea medications required a treatment reduction. Additionally, any patient experiencing grade II muscle weakness or grade II creatine phosphokinase (CPK) elevation required a dose reduction.

During study treatment, patients underwent weekly or bi-weekly evaluations that included history, physical examination, complete blood counts and comprehensive chemistry profiles. Toxicities were graded according to the National Cancer Institute Common Toxicity Criteria (version 3.0). The NCI CLL revised guidelines for diagnosis and treatment were utilized to determine the level of clinical response [126]. The clinical trial was registered with [clinicaltrials.gov](http://clinicaltrials.gov), NCT00828282, prior to enrolling patients and was conducted in compliance with the principles of the Declaration of Helsinki.

### **3. Pharmacokinetic study design**

Serial blood samples (8 mL) were collected in heparinized BD Vacutainer Cell Preparation Tubes (CPT) tubes during cycle 1 at predose, 15 min and 1, 2, 3, 6, 8, 12, and 24 hours and at predose on day 7. Upon collection, samples were immediately centrifuged (1800 x g for 30 min at room temperature) to separate plasma and peripheral blood mononuclear cells (PBMCs) from whole blood. Top layer (plasma and PBMCs) was collected and centrifuged at 1500 rpm for 5 min to separate plasma from PBMCs and samples were stored at -80°C until analysis.

#### 4. LC-MS/MS Analysis

An LC-MS/MS method was developed and validated to measure simvastatin and its acid form in plasma and PBMCs obtained from CLL patients enrolled in this pilot trial [127]. Briefly, all analyses were performed using an HPLC system consisting of a Shimadzu LC-20AD pump and a Shimadzu SIL-20AC VP auto sampler (Shimadzu, Columbia, MD, USA). The LC system was interfaced to an API 2000 ESI-MS/MS (Applied Biosystems, Foster City, CA, USA). Chromatographic analyte separation was carried out on a reverse-phase Phenomenex Luna C<sub>18</sub> column (2.0 x 100 mm i.d.; 2.5 µm particle size), connected to a C<sub>18</sub> guard column (Phenomenex C<sub>18</sub>, 2.0 x 4 mm). An isocratic mobile phase was used consisting of 75:25 (% v/v) acetonitrile: ammonium acetate (0.1M, pH 5.0 adjusted with acetic acid). The flow rate was 0.15 mL/min under ambient temperature. The autosampler temperature was maintained at 4°C and the injection volume was 20 µL. The run time was 10 min. All analytes and internal standard were detected on a triple quadrupole mass spectrometer, equipped with a turbo ion spray source and operating in the positive ion mode. Lovastatin was used as an internal standard. Quantitation was performed using multiple reaction monitoring (MRM) of precursor/product ion transitions at m/z 419.3/199.3 for simvastatin lactone; 437.3/303.3 for simvastatin carboxylate; and 405.2/199.3 for lovastatin. All the parameters were controlled by Analyst software version 1.4.2 (Applied Biosystems, Foster City, CA, USA).

For analysis, plasma and PBMC pellets were thawed and PBMC pellets were lysed in 1 mL deionized water via sonication. A 25 µL aliquot of lovastatin (2.5 µM) was added to 475 µL of plasma or cell lysate sample in 16 x 100 mm glass test tube. The tubes then were vortexed for 10 s. After the addition of 500 µL of ammonium acetate buffer (0.1 M, pH 5.0), tubes were vortexed again for 1 min. Diethyl ether (3 mL) was then added to each tube and samples were placed on a shaker at 200 rpm for 15 min at

4°C. Extracted samples were centrifuged at 3000 rpm for 10 min at 4°C. After centrifugation, the aqueous layer was frozen by placing the tubes in dry ice for a minute. The organic layer was decanted into a new 16 x 100 mm test tube and was evaporated till dryness at room temperature using a gentle stream of nitrogen. The residue was reconstituted in 50 µL of mobile phase and 20 µL was injected onto the HPLC column.

## **5. Pharmacokinetic data analysis**

Plasma concentrations versus time data were evaluated by compartmental modeling using Phoenix WinNonlin 6.2 (Pharsight Corporation, Mountain View, CA). Various compartment models were tested to determine the most appropriate model. The plasma pharmacokinetic parameters of simvastatin lactone and carboxylate, including the maximum observed plasma concentration ( $C_{max}$ ) and time to  $C_{max}$  ( $T_{max}$ ), terminal phase elimination half-life ( $t_{1/2}$ ) and the area under the plasma concentration-time curve (AUC) from time 0 to time of the last measurable concentration ( $AUC_t$ ) were also calculated by non-compartmental analysis. PBMCs concentration of simvastatin was calculated based on the cellular volume of the collected PBMCs sample with considering the volume of CLL cell = 200 fL [128]. CLL cell count in each sample was determined through measuring the protein concentration of the sample relative to those obtained from standard CLL samples with known cell count.

## **6. Specimen collection and CLL cell isolation from PBMCs**

All samples were processed as described above. A portion of the separated PBMC pellets was resuspended in FACS buffer (phosphate-buffered saline (PBS), pH 7.2, 0.5% bovine serum albumin (BSA), and 2 mM EDTA) at concentration of 2 million cells/mL. Cells were stained with anti-CD5-PE (2.5 µL) and anti-CD19-FITC (2.5 µL) antibodies (Becton-Dickinson, Franklin Lakes, NJ) per 250 µL of suspended cells (500,000 cells) in polystyrene tubes. Samples were mixed and incubated for 30 minutes

in the dark at 4°C. After incubation cells were washed twice with 1 mL FACS buffer, resuspended in 300 µL of buffer and then analyzed using FACScan flow cytometer (Becton Dickinson, Franklin Lakes, NJ). CLL cells in PBMC samples were both CD5 and CD19 positive. If staining was  $\geq 85\%$  in the PBMC (i.e., CLL cells represent  $\geq 85\%$  of PBMCs), the sample was used without any further separation. Otherwise, CLL cells were isolated using CD19 microbeads (Miltenyi Biotec, Auburn, CA).

Briefly, a 20 µL aliquot of CD19 magnetic microbeads was added to 80 µL FACS buffer containing  $1 \times 10^7$  cells. Samples were mixed and incubated for 15 min at 4°C. Cells were washed using 1 mL FACS buffer, centrifuged at 300 x g for 10 min, and resuspended in 500 µL of buffer. A MACS LS column was used to separate CD19 labeled cells. After applying the cell suspension, the column was washed with buffer to elute unlabeled cells. CD19 magnetic microbead labeled cells were then flushed out from the column by firmly pushing the plunger into the column. The collected CLL cell sample was again stained with CD5/CD19 to ensure that the CLL cells were enriched to 85% or more.

## **7. Western blotting**

A portion of the isolated cells collected from CLL patients at pre-dose on day 1 and day 7 of cycle 1 were washed twice with ice cold PBS buffer. Cell pellets were lysed in RIPA buffer (50 mM Tris, pH8.0, 150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholate, 1 mM EDTA, 0.1% SDS) supplemented with Complete® protease inhibitor cocktail (Roche Diagnostics, Indianapolis, IN) and incubated for 30 min (4°C) on a rotating shaker. Cell lysates were then centrifuged at 1500 rpm for 5 min to remove any particulates. Protein concentrations were measured using the BCA protein assay (Thermo Scientific, Rockford, IL). Samples were boiled at 95°C for 10 min with NuPage LDS sample buffer (Invitrogen, Eugene, OR) and 0.1 M DTT (Sigma, St. Louis, MO).

Protein electrophoresis was performed on 10% SDS-polyacrylamide gel (SDS-PAGE) at room temperature and proteins were transferred to a polyvinylidene fluoride (PVDF) membrane (Millipore, Billerica, MA) at 4°C. Membranes were blocked for 2 hr at room temperature with tris-buffered saline (TBST) or phosphate-buffered saline (PBST) containing 0.05% (v/v) Tween-20 and supplemented with 5% (w/v) nonfat milk or BSA. Membranes were incubated overnight at 4°C with the following primary antibodies at 1:1000 dilution: anti-cleaved PARP, Bcl2, phospho ERK, ERK, phospho p38, p38, phospho JNK, JNK and GAPDH rabbit polyclonal antibodies (Cell Signaling Technology Inc.). After washing with TBST or PBST, the membranes were probed with HRP-conjugated secondary antibody for 1 hr at room temperature. Following washes with TBST or PBST protein bands were visualized by enhanced ECL (Thermo Fisher Scientific, Rockford, IL) using the Kodak Image Station 2000 MM (Eastman Kodak, Rochester, NY).

#### **8. Cell Culture of immortalized cell lines and primary CLL cells**

Primary cultures were derived from the peripheral blood of the CLL patients (other than those involved in the clinical trial) with informed consent before therapy. CLL cells were isolated from PBMCs as described above. CLL cells were maintained in RPMI 1640 medium (supplemented with 20% FBS, 1% MEM non-essential amino acids, 1% MEM vitamins and penicillin/streptomycin) [129] and were allowed to recover for 24 hr before use in the designed experiments. All cultures were maintained at 37°C in a humidified atmosphere of 5% CO<sub>2</sub> and 95% air.

#### **9. Cell viability assay**

Primary CLL cells were plated in 96 well plates at a density of  $5 \times 10^5$  cells per well in 100  $\mu$ L of medium. Cells were incubated with increasing concentrations of simvastatin (0 - 200  $\mu$ M) for 48 hr at 37°C. At the end of the incubation period, 20  $\mu$ L of

MTS reagent (Promega Corporation, Madison, WI) was added to each well and further incubated for 4 hr at 37°C. Cell viability was determined by measuring the absorbance at 490 nm wavelength using Molecular Devices Spectramax M5 plate reader.

### **10. Apoptotic assay**

Primary CLL cells ( $5 \times 10^6$  cells/mL) were incubated in a 6 well plate with different concentrations of simvastatin (0, 10, 50 and 100  $\mu$ M) for 48 hr at 37°C. After incubation, cells were harvested and washed twice with ice cold PBS buffer. The cell pellet was resuspended in Annexin binding buffer (50 mM HEPES, 700 mM NaCl, 12.5 mM CaCl<sub>2</sub>, pH 7.4) at a concentration of  $1 \times 10^6$  cells/mL. To identify dead and apoptotic cells, 1  $\mu$ L propidium iodide (PI) and 5  $\mu$ L Annexin V-FITC (Invitrogen, Eugene, OR) were added to each 100  $\mu$ L of cell suspension and samples were incubated for 15 min at room temperature. Samples were diluted to 500  $\mu$ L using annexin binding buffer before analysis using FACScan flow cytometer (Becton Dickinson, Franklin Lakes, NJ). Cells that were positive for Annexin-V but negative for PI were those in early stage apoptosis while cells positive for both annexin-V and PI were in late stage apoptosis.

### **11. Statistical analysis**

Data are reported as mean values  $\pm$  SD and analyzed statistically with one-way ANOVA followed by Turkey's post-hoc test. P values  $\leq$  0.05 were considered significant.

## **C. Results**

### **1. Plasma and PBMCs pharmacokinetics**

Three patients were accrued between July 2009 – January 2011. The first participant accrued, remained on treatment for three cycles of therapy before experiencing disease progression. Of note, this participant reported an initial decrease in constitutional symptoms including fatigue and the clinical investigators noted a

substantial decrease in the patient's palpable adenopathy. Due to the waxing and waning nature of CLL, it is unknown if the change in symptoms and adenopathy is attributable to the effect of simvastatin. The subsequent two participants experienced progression of leukemia during their first cycle of therapy and were subsequently removed from therapy. One participant experienced grade 1 limb pain as the only toxicity attributed to the treatment.

Simvastatin lactone and carboxylate analyses in plasma and PBMC samples were performed using a validated LC/MS/MS assay. Figure 4.1 illustrates the chromatograms of simvastatin lactone and carboxylate in plasma and PBMC collected from one patient, 1 hr after oral administration of high dose simvastatin.

As shown in Figure 4.2, simvastatin lactone was more predominant than carboxylate in plasma. Non-compartmental analysis using the concentrations from Figure 4.2 was initially used to determine the AUC,  $C_{max}$ ,  $T_{max}$ , and terminal half-life of each simvastatin form in these patients and values are summarized in Table 4.1. As expected by the low number of patients and the magnitude of the oral dose, we observed high interpatient variability in all parameters for both simvastatin lactone and carboxylate. Conversely, several structural models were fitted to the plasma data obtained from the three CLL patients. In contrast to a previous population study [130], two compartment structural model representing only the central compartments of lactone and carboxylate did not adequately fit the data of both the second and third patients. However, a four compartment model was found to best describe the data and the distribution phase of both forms to the peripheral compartment, as depicted in Figure 4.2. To minimize the number of parameters to be estimated by the model some assumptions were made. Based on the previous simvastatin pharmacokinetic studies [130, 131], we assumed the interconversion clearance ( $CL_{12}$ ) of simvastatin lactone to

carboxylate to be 40% of the elimination clearance ( $CL_{10}/F$ ) of the lactone form. Furthermore, we considered the reconversion of carboxylate to the lactone form as being negligible [131]. Also, both clearances from and to peripheral compartments of simvastatin lactone and carboxylate were assumed to be equal ( $CL_{13}=CL_{31} \neq CL_{24}=CL_{42}$ , respectively) and were fixed. Lastly, fixing the absorption rate constant ( $K_a$ ) at 2.76 1/hr [130] as well as the peripheral volume of distribution of simvastatin lactone ( $V_3$ ) or carboxylate ( $V_4$ ) was found to improve the fit and the accuracy of the estimated parameters. Figure 4.2a and b show the plasma concentration versus time profile of both simvastatin lactone and carboxylate after oral administration of simvastatin at 7.5 mg/kg to the three CLL patients.

As shown in Figure 4.2, patient 2 had higher plasma concentrations of both forms of simvastatin relative to the other two patients. Simulation of multiple dosing of simvastatin based upon the final PK model for 6 days revealed no accumulation of either simvastatin lactone or carboxylate after the second dose or at day 6 in the three patients (Figure 4.2). The model predicted clearance also showed that there was a 5-fold variation in the estimated lactone clearance (i.e.,  $CL/F$ ) (Table 4.1).

Similarly, simvastatin lactone and carboxylate was measured in PBMCs and as shown in Figure 4.3, patient 2 had the highest concentrations, as compared to the other patients, which correlated with their plasma concentrations ( $R^2= 0.9715$ , Figure 4.4). Notably, it was only the simvastatin lactone that was detectable in the PBMC of these three patients.

## **2. In vivo antitumor activity of simvastatin in CLL patients**

Although simvastatin at high dose showed limited efficacy in all three patients, molecular analyses showed that simvastatin had an effect. Upon treatment with high dose simvastatin for 7 consecutive days, CLL cells collected from patient 1 and 2

showed elevated level of cleaved PARP (apoptotic marker) expression compared to day-1 predose sample (Figure 4.5). In fact, the Bcl2 protein is often highly expressed in CLL cells and is thought to slow the apoptosis process which leads to their accumulation in peripheral blood [132, 133]. Moreover, the MAPK pathways were shown to be important regulators of CLL survival [134-137]. Therefore, we examined the effect of simvastatin on the expression of Bcl2 as well as MAPKs (p38, JNK and ERK) in CLL cells collected from the three treated patients. As shown in Figure 4.5, simvastatin treatment does not seem to affect the expression of Bcl2 protein or any of the MAPK family members in the second and third CLL patients. Of note, the first CLL patient showed elevated expression of both basal and phosphorylated MAPKs after treatment with simvastatin.

### **3. In vitro antitumor activity of simvastatin in primary CLL cells**

The anti-proliferative activity of simvastatin was also assessed in primary cells collected from CLL patients using the MTS colorimetric assay. This assay relies on the ability of viable cells to actively metabolize the MTS tetrazolium salt into its formazan product that has an absorbance measured at 490 nm wavelength. A dose dependent decrease in cell viability of primary CLL cells was observed upon continuous simvastatin (0-200 $\mu$ M) treatment for 48 hours (Figure 4.6a). The IC<sub>50</sub> values ranged from 47.98-112.6  $\mu$ M (Mean  $\pm$  SD, 94.4  $\pm$  26.6  $\mu$ M). We next assessed the ability of simvastatin to induce apoptosis in primary CLL cells collected from three patients. Similarly, primary CLL cells were exposed to increasing doses of simvastatin (0-200  $\mu$ M) for 48 hours and cells were stained with Annexin V and PI. Staining demonstrated a statistically significant increase in the percentage of cell apoptosis when treated at 100  $\mu$ M simvastatin for 48 hr relative to control (Figure 4.6b).

## **D. Discussion**

Beyond their cholesterol lowering effect, several reports have shown that statins have anticancer properties in different tumor types [57, 66]. This effect is believed to be mediated through the inhibition of isoprenoid synthesis and the subsequent deactivation of small GTPases, which are involved in regulating multiple cellular functions including proliferation and survival [75, 79]. However, these in vitro studies have shown that statins display their anticancer activity at micro molar concentrations that cannot be achieved with typical anti cholesterolemia doses [107]. This provided the rationale for testing the safety and tolerability of statins at high doses in cancer patients. Simvastatin was well tolerated and its MTD was 7.5 mg/kg twice daily for 7 consecutive days in a 21-day cycle. This pilot clinical study demonstrated that simvastatin administered at its MTD achieved low micro-molar concentrations ( $C_{max}$ ), which based on in vitro evidence, are unlikely to be effective.

Initial attempts to fit the pharmacokinetic data to a two-compartment model, as previously reported, were not successful in two of three patients. A four compartment model was found to better characterize the data obtained from these patients. However, in order for the model to fit the data, several assumptions, based on previous pharmacokinetic publications, had to be made. Furthermore, although the model was adequately fit to data from day 1, it did not predict the modest accumulation of either form of simvastatin, which was observed on day 7. This observed accumulation maybe due to slight saturation of metabolic and/or transport processes following the repetitive administration of high dose simvastatin.

Our results are in accord with previous studies of high dose lovastatin. In that study, patients with solid tumors were administered lovastatin and the MTD was 25 mg/kg [93]. As a part of the study pharmacokinetics were conducted and peak plasma

concentrations ranged from 0.1-3.9  $\mu\text{M}$  with an average concentration 2.32  $\mu\text{M}$ . These in vivo concentrations were found to be comparable to those effective in glioma cells in vitro. Nonetheless, this approach did not show success in the clinic where high dose lovastatin exhibited limited efficacy in glioma patients in a subsequent phase II trial [95]. Although lovastatin is known to cross blood brain barrier [138], it is not known whether it can reach the brain at similar concentrations as those achieved in plasma. Similarly, simvastatin at its MTD (7.5 mg/kg, given orally, twice a day) failed to reverse clinical resistance to VAD chemotherapy (vincristine 0.4 mg, adriamycin 9 mg/m<sup>2</sup>, and dexamethasone 40 mg) in myeloma patients [97]. The short period of treatment (7 days) as well as the treatment strategy was denoted as potential factors that contributed to the unsuccessful clinical results. However, a longer period of treatment (21 days) with lovastatin at 7.5 mg/kg/day did not show any objective responses in patients with head and neck squamous cell carcinoma or cervical cancer [139]. Recently, a phase II study found no evidence of beneficial effect of high dose simvastatin on disease markers in multiple myeloma patients [96]. The investigators of those two clinical studies of high dose simvastatin assumed that simvastatin reaches similar concentrations in plasma to those achieved with lovastatin. This was a reasonable assumption, since the pharmacokinetics of these two statins is similar at lower doses [140]. Although few patients were accrued in our study, results from the plasma analysis of simvastatin after high dose have proven this assumption. The simvastatin plasma concentrations in our patients showed similar but relatively lower  $C_{\text{max}}$  concentrations (0.08 - 2.2  $\mu\text{M}$ ) compared to lovastatin ( $C_{\text{max}}$ : 0.1 – 3.9  $\mu\text{M}$ ). This higher  $C_{\text{max}}$  range of lovastatin is likely within the interpatient variability range and may also be attributed to the difference in dosage regimen. Lovastatin dosing was more frequent (6.25 mg/kg four times daily) relative to simvastatin (7.5 mg/kg twice a day). Overall, our study was in agreement with previous lovastatin studies that reported low micro molar concentrations in plasma after

administering high doses [93, 141]. Moreover, the high interpatient variability seen in these studies was also observed among patients enrolled in our study which may be attributed to several factors including, differences in metabolism, as well as differences in oral absorption, due to efflux or incomplete dissolution of the high doses administered [142].

The few aforementioned Phase II trials of high dose statins were initiated considering the fact that plasma peak concentrations achieved by these doses have been shown to be effective in vitro. However, the limited activity of statin seen in these clinical trials addresses some concerns about whether statins at high doses are really achieving therapeutically effective concentrations at the relevant tissues. Several in vitro studies have reported that statins were effective against glioma and myeloma cells at low micro-molar concentration ranges 1–10  $\mu\text{M}$  [143-146] and 0.8–13.3  $\mu\text{M}$  [62, 147, 148], respectively. Noteworthy is the fact that the primary cells collected from glioma and myeloma patients were found to be more resistant to statins compared to established cell lines. For example, lovastatin were found to inhibit the proliferation of primary cells obtained from myeloma patients at 10- 100  $\mu\text{M}$  [149]. Similarly, lovastatin inhibited cell proliferation of primary glioma cells at  $\text{IC}_{50}$  values ranging from 6 – 63  $\mu\text{M}$  [148], while it was shown to induce 10 – 30 % apoptosis in primary cells at 10  $\mu\text{M}$  [150]. Together, these observations indicate that the maximum plasma concentrations achieved with high dose statins are only approaching the lower range of effective concentrations required for anticancer activity in primary myeloma and glioma cells. Therefore, comparing effective in-vitro concentrations of statins in established cancer cells with those seen in patients may not be a valid approach in these cases. In agreement with this observation, our in vitro data indicate that simvastatin induces apoptosis in primary CLL cells only at suprapharmacologic concentrations (~100  $\mu\text{M}$ ), which are not attainable in vivo. This

may in part explain the progression of leukemia in the CLL patients treated with high dose simvastatin in this study.

From another perspective, simvastatin carboxylate is known to be the active form that mediates the antitumor activity of simvastatin through the inhibition of the HMG-CoA reductase enzyme. In our study, simvastatin carboxylate was found to be present in plasma at lower concentrations compared to simvastatin lactone. Moreover, it was not observed (or below the detection limit 5 ng/mL (0.01  $\mu$ M)) in CLL cells isolated from these patients, even at high level of exposure as in second CLL patient. The hydrophilic nature of the carboxylate form may have hindered its accessibility into CLL cells. In general, limited accessibility of the simvastatin active form to the tumor site may be considered a critical factor added to other factors that contribute to the poor response seen in all the previous clinical trials. Despite the limited efficacy shown in all the CLL patients, CLL cells collected from two patients after treatment with simvastatin were shown to undergo apoptosis. However, this apoptotic effect was independent of the survival pathways of CLL cells, such as MAPK pathways or Bcl2 protein, which were not affected by treatment. Interestingly, there was no correlation between level of exposure to simvastatin in CLL patients and the molecular apoptotic effect of simvastatin on CLL cells isolated from these patients.

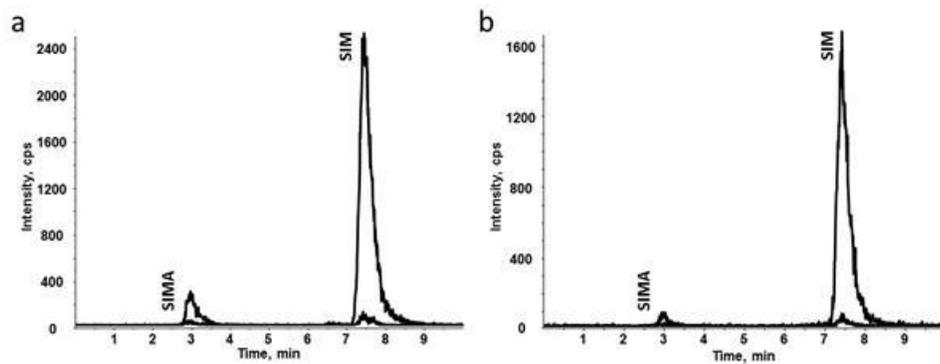
## **E. Conclusion**

In conclusion, pharmacokinetic data in CLL patients showed that simvastatin administered at its MTD achieves plasma concentrations that are far lower than those shown to be effective *ex vivo* in primary CLL cells. In view of these data, the use of simvastatin as a sole therapy for treatment of CLL, and perhaps other cancer types, is unlikely to be successful. However, rational combination therapy that includes statins may still provide clinical benefit.

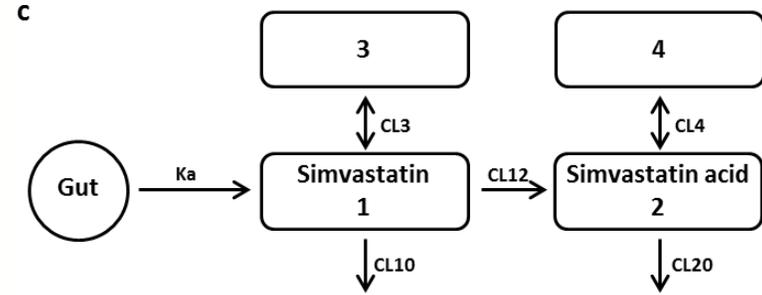
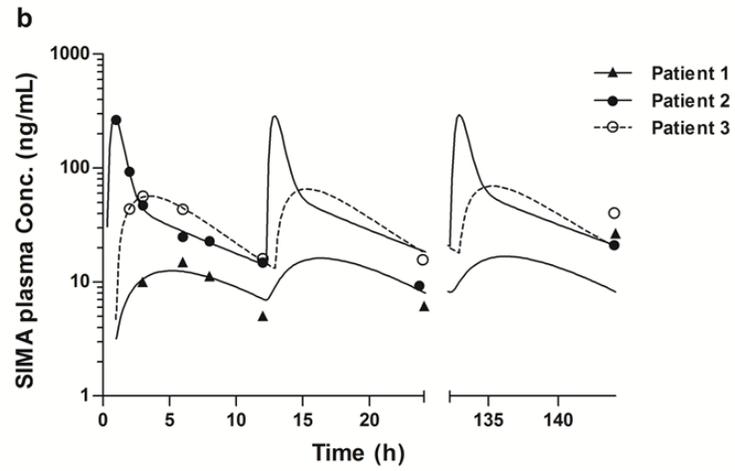
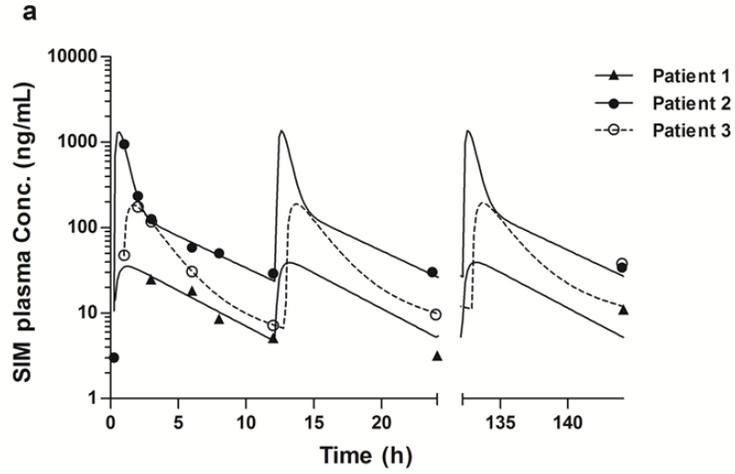
**Table 4.1.** Pharmacokinetic parameters in plasma for simvastatin lactone and carboxylate after oral administration of MTD of simvastatin to CLL patients ( $n=3$ ).

PK Parameters	Simvastatin lactone*				Simvastatin carboxylate			
	Patient #1	Patient #2	Patient #3	Average (SD)	Patient #1	Patient #2	Patient #3	Average (SD)
$t_{1/2}$ (hr)	3.7	3.5	2.1	3.1 (0.8)	3.7	4.1	4.8	4.2 (0.5)
$C_{max}$ ( $\mu\text{M}$ )	0.08	2.2	0.42	0.9 (1.1)	0.03	0.6	0.13	0.25 (0.3)
$AUC_{12}$ ( $\mu\text{M}\cdot\text{hr}$ )	0.42	4	1.46	1.9 (1.8)	0.25	1.3	0.93	0.8 (0.5)
$T_{max}$ (hr)	1	1	2	1.3 (0.5)	6	1	3	3.3 (2.5)
CL/F (L/hr)	1811	375	606	927 (774)	963	440	281	567 (354)
V/F (L)	12968	292	2046	5102 (6868)	3919	145	15	1363 (2214)

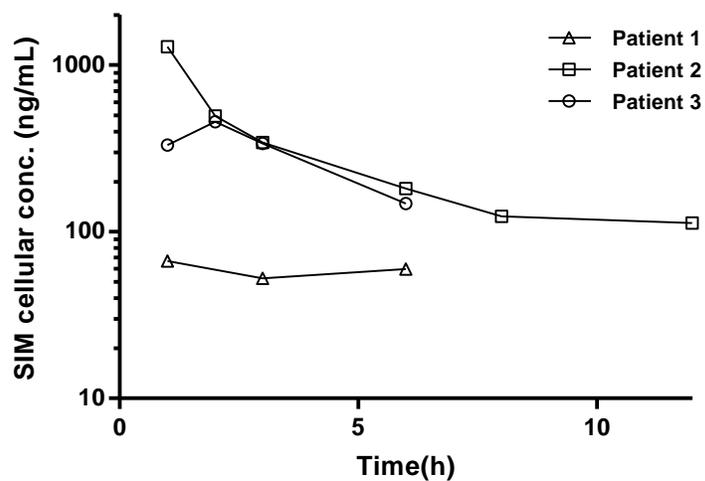
$t_{1/2}$ : terminal half-life;  $C_{max}$ : maximum concentration;  $AUC_{12}$ : area under the concentration versus time curve for 12 hours; CL: clearance and V: volume of distribution; F: bioavailability.



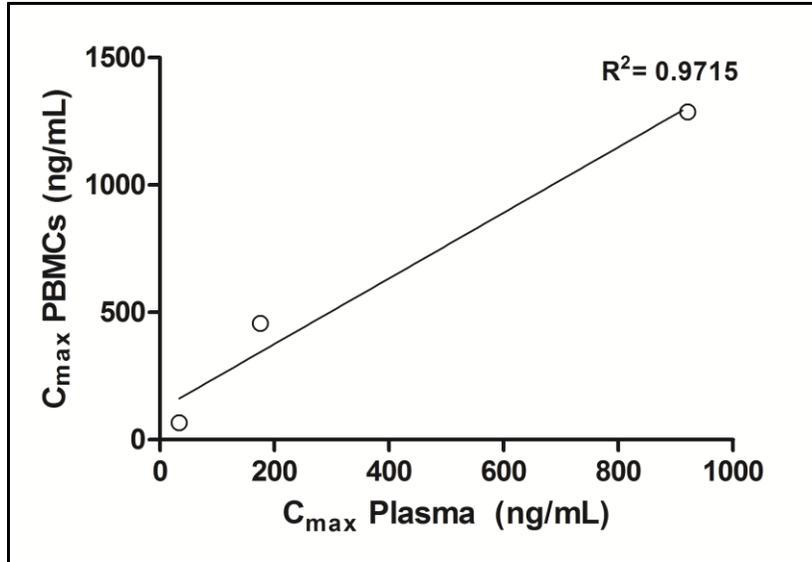
**Figure 4.1.** Representative chromatograms of patient plasma (a) and PBMCs (b) samples collected 1 hr after oral administration of simvastatin (7.5 mg/kg). SIM, simvastatin lactone and SIMA, simvastatin carboxylate.



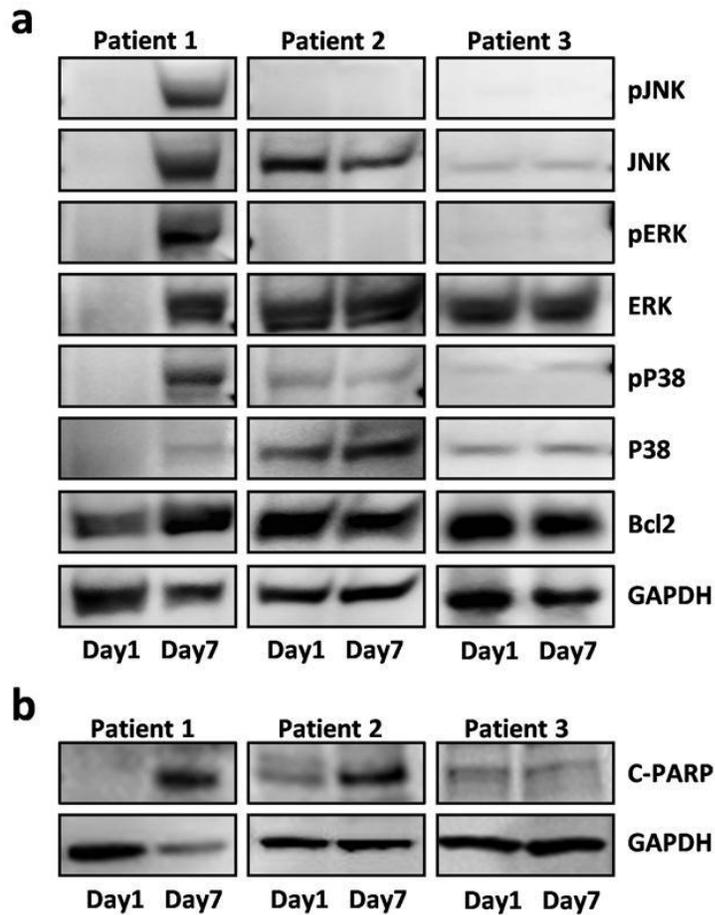
**Figure 4.2.** Simulated plasma concentration versus time profiles in CLL patients (n=3) after oral administration of simvastatin at MTD regimen (7.5 mg/kg/twice a day), (a) simvastatin lactone, SIM (b) simvastatin carboxylate, SIMA. The solid lines represent simulated estimated concentrations which were generated using Phoenix WinNonlin 6.2, (c) Schematic representation of the final four compartment PK model with first order oral absorption.



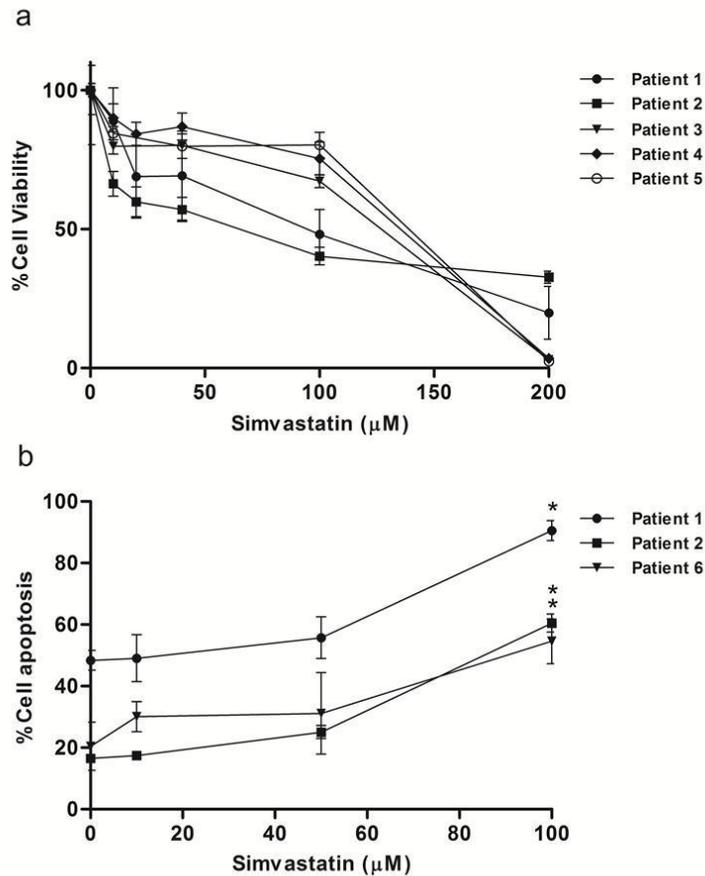
**Figure 4.3.** PBMCs concentration-time profile of simvastatin lactone following oral administration of MTD simvastatin in CLL patients. Simvastatin concentrations were normalized to the cellular volume of CLL cells in each PBMCs sample. SIM, simvastatin lactone. CLL cell volume = 200 fL [128].



**Figure 4.4.** Association of  $C_{max}$  concentrations of simvastatin lactone in plasma and PBMCs of CLL patients.



**Figure 4.5.** Effect of high dose simvastatin on the expression level of MAPK and Bcl-2 proteins (a) and cleaved PARP protein (b) in CLL cells isolated from patients before and after therapy with 15 mg/kg/day simvastatin for 7 consecutive days. CLL cells were isolated from treated patients and sorted by FACS (CD5<sup>+</sup>/CD19<sup>+</sup>). Cells were processed for western blot analysis to assess the expression of JNK, phospho JNK, ERK, phospho ERK, p38, phospho p38, Bcl-2 and c-PARP proteins. GAPDH, a cytosol protein, was used as a loading control.



**Figure 4.6.** Treatment of CLL patient cells with simvastatin reduces cell viability (a) and induces apoptosis (b). CLL cells were freshly isolated from patients and sorted by FACS (CD5<sup>+</sup>/CD19<sup>+</sup>). CLL cells were treated for 48 hr with increasing concentrations of simvastatin (0-200μM). The percentage of viable cells was measured using MTS assay. Apoptosis was measured using Annexin V/PROPIDIUM IODIDE assay and results represent percentage of apoptosis (early and late). Data are presented as mean ± SD. \* P ≤ 0.05.

## **Chapter 5: Simvastatin interacts synergistically with tipifarnib to induce apoptosis in human leukemia cells through the disruption of RAS membrane localization and interruption of ERK pathway**

### **A. Introduction**

The RAS/MEK/ERK signaling pathway encompasses several proteins that play key roles in cell proliferation as well as in the prevention of apoptosis of leukemic cells [151]. Aberrant regulation of this pathway is observed in leukemia because of RAS mutations, which lead to its constitutive activation, as well as genetic alteration of upstream signaling molecules of the RAS [152, 153]. Pharmacologic intervention to attenuate this pathway is thus a potential therapeutic strategy for leukemia treatment. However, it has been difficult to identify molecules that directly inhibit the function of RAS, and alternative approaches to prevent or block the membrane localization of RAS have been tried as a way to pharmacologically limit the activation of this pathway [24, 154].

RAS is a small GTP-binding protein that functions as a molecular switch regulating several signaling pathways that play a crucial role in controlling the activity of cell proliferation, differentiation and malignant transformation [30, 35-37]. RAS activation requires a series of posttranslational modifications to allow its association with the inner face of the cell membrane, where it can interact with membrane receptors and activate downstream signaling cascades [24]. The first and most crucial step in RAS posttranslational modification is the covalent attachment of the farnesyl moiety into RAS carboxyl terminal in a process called farnesylation and is catalyzed by the farnesyltransferase (FTase) enzyme [24].

FTase inhibition was proposed as a strategy to impede RAS localization to the membrane and its subsequent activation. Therefore, several farnesyltransferase

inhibitors (FTIs) were developed and evaluated in preclinical and clinical settings against a variety of human cancers. The preclinical evaluation of FTIs in cell culture and animal models has shown promising results as potential therapeutic agents and several FTIs progressed into clinical trials [155]. However, the efficacy of FTIs as a single agent in patients with solid tumors was limited, but some modest efficacy was observed in hematologic malignancies [156, 157]. Resistance to FTIs has been attributed to posttranslational modification of RAS by an alternative lipidation pathway, whereby RAS can be geranylgeranylated by geranylgeranyltransferase I (GGTase I) in the presence of FTIs [158]. This alternative isoprenylation mechanism enables RAS to associate with the cell membrane and retain full biologic activity despite of the blockage of the farnesylation pathway.

Understanding the mechanism by which RAS escapes the effect of FTIs tempted the investigators to change their strategy by targeting both prenylation pathways in order to avoid the cross-geranylgeranylation of RAS and knockdown its activity. Therefore, considerable effort has been made to evaluate the FTIs with geranylgeranyltransferase inhibitors (GGTI) in combination. Although several studies have demonstrated synergistic cytotoxicity and apoptotic activity of FTI/GGTI combinations in different tumor types, significant toxicity was reported in preclinical models, which is mostly related to GGTI, thereby limiting the therapeutic potential of this combination [159, 160]. Recently, GGTI-2418, a novel geranylgeranyltransferase inhibitor, was found to be well tolerated with minimal side-effects in a phase I trial in patients with refractory solid tumors [161].

Simvastatin , an anti-hyperlipidemic drug that inhibits 3-hydroxy 3-methylglutaryl coenzyme A (HMG-CoA) reductase, has been shown in several studies to induce apoptosis in cancer cells through blockade of the geranylgeranylation pathway of small GTPases [59, 75, 79]. Unlike GGTIs, statins are known to be well tolerated and have a

wide margin of safety. Given the ability of simvastatin to inhibit the alternative pathway of RAS prenylation as well as its good safety profile, we postulated that simvastatin could overcome tipifarnib resistance and augment its antitumor activity in leukemia cells.

## **B. Methods**

### **1. Chemicals**

Simvastatin was purchased from Toronto Research Chemicals Inc. (North York, Canada). Tipifarnib was purchased from Selleck Chemicals LLC, Houston, TX, USA. Absolute ethanol was obtained from Sigma-Aldrich (St Louis, MO, USA). RPMI-1640 medium and penicillin/streptomycin were from Life Technologies (Grand Island, NY, USA), whereas fetal bovine serum (FBS) was obtained from Atlanta Biologicals (Lawrenceville, GA, USA). Alexa Fluor® 488 annexin V/Dead Cell Apoptosis Kit and NuPage LDS sample buffer were from Invitrogen (Eugene, OR). Antibodies were purchased from Cell Signaling (Danvers, MA, USA). Mevalonate, farnesyl pyrophosphate and geranylgeranyl pyrophosphate were from Sigma-Aldrich (St Louis, MO, USA). Propidium iodide was obtained from (Invitrogen Corporation, Carlsbad, CA). Riponuclease A from bovine pancreas, resazurin and dithiothreitol (DTT) were purchased from Sigma-Aldrich (St Louis, MO, USA). BCA protein assay was from Thermo Scientific, Rockford, IL, whereas Complete protease inhibitor cocktail was obtained from Roche Diagnostics, Indianapolis, IN.

### **2. Cell culture and treatment**

All cell lines (KG1 and HL60 acute myelogenous leukemia; K562, chronic myelogenous leukemia; Molt4, Jurkat and HSB2, acute T cell leukemia) were purchased from ATCC (Manassas, VA, USA). Cells were cultured in RPMI-1640 medium supplemented with FBS, penicillin (100 IU/mL) and streptomycin (100 µg/mL) at 37°C in

a humidified incubator with 5% CO<sub>2</sub>. FBS was added to medium as follows: 20 % for KG1 and HL60 cells, 10 % for Jurkat, Molt4 and HSB2 cells or 5% for K562 cells.

Cells were suspended in growth medium at 5 x 10<sup>5</sup> cells/mL for KG1, HL60 and HSB2 and 2.5 x 10<sup>5</sup> cells/mL for K562, Molt4 and Jurkat and placed in 6- well plates and treated with simvastatin (1 or 4 μM), tipifarnib (0.1 or 1 μM) or their combinations for 72 hr. Cells incubated with 10 μL/mL DMSO were used as a control.

### 3. Cell viability assay and combination index calculation

A panel of six cell lines of varied leukemic origin including KG1, HL60, K562, Jurkat, Molt4 and HSB2 was used to determine the cytotoxicity of simvastatin and tipifarnib following single drug or combination treatment. Cells were placed in 96 well plates at a density of 50 x 10<sup>3</sup> cells per well for KG1, HL60 and HSB2 or 25 x 10<sup>3</sup> cells per well for K562, Molt4 and Jurkat in 100 μL of the appropriate growth medium. Cells were incubated with increasing concentrations of simvastatin (0, 0.4 - 400 μM), tipifarnib (0, 0.01 – 10 μM) or their combination at different concentrations for 72 hr at 37°C. At the end of the incubation period, 10 μL of resazurin was added to each well and further incubated for 3 hr at 37°C. Cell viability was determined by measuring the fluorescence at 560 nm excitation wavelength and 590 nm emission wavelength using Molecular Devices Spectramax M5 plate reader. Synergism between simvastatin and tipifarnib was assessed using the combination index (CI) method of Chou and Talalay [162-164].

$$CI = d_1/D_1 + d_2/D_2$$

In this equation,  $D_1$  and  $D_2$  represent the doses of drug 1 and drug 2 alone, required to produce x% effect, and  $d_1$  and  $d_2$  are the doses of drugs 1 and 2 in combination required to produce the same effect. CI value < 1 indicates synergy while

values = 1 or > 1 indicate additivity and antagonism, respectively. Experiments were performed in triplicates.

#### **4. Apoptosis Assay**

Cells were harvested and washed with ice cold phosphate-buffered saline (PBS) buffer, pH 7.2. Cell pellets were resuspended ( $1 \times 10^6$  cells/mL) in annexin binding buffer (50 mM HEPES, 700 mM NaCl, 12.5 mM  $\text{CaCl}_2$ , pH 7.4). To identify dead and apoptotic cells, 1  $\mu\text{L}$  propidium iodide (PI) and 5  $\mu\text{L}$  Annexin V-FITC were added to each 100  $\mu\text{L}$  of cell suspension and samples were incubated for 15 min at room temperature. Samples were diluted to 500  $\mu\text{L}$  using annexin binding buffer before analysis using FACScan flow cytometer (Becton Dickinson, Franklin Lakes, NJ). Cells that were positive for Annexin-V but negative for PI were those in early stage apoptosis while cells positive for both annexin-V and PI were in late stage apoptosis.

#### **5. Cell cycle analysis**

Cells were harvested and washed twice with ice cold PBS buffer. Cells were then fixed in 3 mL of absolute ethanol overnight at 4°C. Cells were washed twice with ice cold PBS buffer and incubated with propidium iodide (100  $\mu\text{g}/\text{mL}$ ) and Ribonuclease A from bovine pancreas (200  $\mu\text{g}/\text{mL}$ ) in the dark for 30 min at 37°C. Processed samples were kept at 4°C and protected from light until analysis using a FACScan flow cytometer (Becton Dickinson, Franklin Lakes, NJ).

#### **6. Total and fractionated protein isolation**

For total lysate preparation, cells were harvested after treatment and washed twice with ice cold PBS. Cell pellets were lysed in RIPA buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholate, 1 mM EDTA, 0.1% SDS) supplemented with Complete protease inhibitor cocktail and incubated for 30 min (4°C)

on a rotating shaker. Cell lysates were then centrifuged at 1500 rpm for 2 min to remove any particulates. Protein concentrations of membrane, cytosolic fractions and total cell lysate were measured using the BCA protein assay.

For cytosolic and membrane protein fractions, cells were collected after treatment and washed with ice cold PBS buffer. Cells ( $1 \times 10^7$ ) were lysed via sonication for 15s in 200  $\mu$ L lysis buffer I (1 mM EDTA, 20 mM Tris-HCl (pH 7.4)) supplemented with Complete protease inhibitor cocktail. Lysates were centrifuged at  $100,000 \times g$  at  $4^\circ\text{C}$  for 1 hr using an ultracentrifuge (OptimaMax, TLA55 rotor; Beckman Coulter). The supernatant containing the soluble fraction (cytosolic fraction) was collected and the pellet (membrane fraction) was then washed with 1 mL lysis buffer I twice, to remove any remnant of the cytosolic fraction. The membrane pellet was solubilized in 50  $\mu$ L lysis buffer II (150 mM NaCl, 10 mM Tris-HCl, 5 mM EDTA, 1% Triton X-100) supplemented with Complete protease inhibitor cocktail and then sonicated for 5s to solubilize any particulate left in the buffer. Protein samples were stored at  $-20^\circ\text{C}$  until analysis or were processed immediately for immunoblotting.

## **7. Western blot analysis**

The expression of total-PARP, cleaved PARP, cleaved caspases 3, 7 and 9 and procaspases 3, 7 and 9, Bcl2, Mcl1, Bcl-xL, Bax, phospho ERK, total ERK, phospho AKT, total AKT, RAS, calnexin and GAPDH was evaluated in protein lysates or subcellular fractions, as indicated. Protein samples were boiled at  $95^\circ\text{C}$  for 10 min with NuPage LDS sample buffer and 0.1 M DTT. Protein electrophoresis was performed on 10% SDS-polyacrylamide gels (SDS-PAGE) at room temperature and proteins were transferred to a polyvinylidene fluoride (PVDF) membrane (Millipore, Billerica, MA) at  $4^\circ\text{C}$ . Membranes were blocked for 2 hr at room temperature with tris-buffered saline (TBST) containing 0.05% (v/v) Tween-20 and supplemented with 5% (w/v) nonfat milk or

bovine serum albumin. Membranes were incubated with primary antibodies overnight at 4°C at 1:1000 dilution. After washing with TBST, the membranes were probed with HRP- conjugated secondary antibody at 1:2000 dilution (Cell Signaling Technology Inc.) for 1 hr at room temperature. Following washes with TBST, protein bands were visualized by enhanced ECL (Thermo Fisher Scientific, Rockford, IL) using the Kodak Image Station 2000 MM (Eastman Kodak, Rochester, NY).

## **8. Statistical analysis**

Data are reported as mean values  $\pm$  SD and analyzed statistically with one-way ANOVA followed by Turkey's post-hoc test. P values  $\leq$  0.05 were considered significant.

## **C. Results**

### **1. Simvastatin and tipifarnib combination has a synergistic antiproliferative effect in leukemia cell lines**

To evaluate the potential for synergy between simvastatin and tipifarnib we treated KG1, HL60, K562, Jurkat, Molt4 and HSB2 cell lines with either drug alone, to determine the respective IC<sub>50</sub> (Table A.1 and A.2, Figure A.1 and A.2), and then with different concentrations of simvastatin (1 and 4  $\mu$ M) and tipifarnib (0.05, 0.1, 0.5 and 1  $\mu$ M) in combination. Cell viability was measured after 72 hr of treatment using a fluorometric cell proliferation assay as described under methods. Synergism was assessed by calculating CI values, which is based on the mathematical model described by the Chou and Talalay [162-164]. As shown in Figure 5.1, with the exception of the KG1 cells the combination of simvastatin and tipifarnib was synergistic at all concentrations. Overall, simvastatin at high dose yielded higher fractional effect (FE) in combination with tipifarnib, relative to its lower dose. This effect was more substantial in HL60 cells for both CI and FE values. These results indicate that the combination of

simvastatin and tipifarnib synergistically enhanced the cytotoxic effects in various leukemia cell lines.

## **2. The synergistic effects of simvastatin/tipifarnib are mediated by apoptosis**

To determine if the combination of simvastatin/tipifarnib was cytotoxic, we investigated whether the reduced cell viability was attributed to apoptosis. Leukemia cells were treated with simvastatin (1 or 4  $\mu\text{M}$ ) and tipifarnib (0.1 or 1  $\mu\text{M}$ ) concentrations alone or in combination for 72 hr. Subsequently, western blot analysis was performed to analyze the activation of the caspase cascade. As shown in Figure 5.2A, proteolytic cleavage of caspase 3 and 7 to their active forms triggered the apoptotic process through the cleavage of other important intracellular substrates such as poly (ADPribose) polymerase (PARP), which is involved in DNA repair. These results were consistent in all leukemia cell lines tested. However, caspase 7 was only activated in Jurkat, Molt4 and HSB2 cells. Moreover, we examined caspase 9 activation (cleavage of caspase 9), which acts upstream of caspase 3 and 7, using western blot analysis. Elevated expression of cleaved caspase 9 was observed in K562, Jurkat, Molt4 and HSB2 cells. HL 60 cells showed no expression of both the full length and cleaved forms of caspase 7 and 9 and PARP. Conversely, KG1 cells were more resistant to simvastatin/tipifarnib with no signs of caspase cascade activation or PARP cleavage.

In fact, several reports suggest the importance of the antiapoptotic and proapoptotic Bcl-2 family proteins in regulating cell survival and apoptosis [165-169]. To better understand the apoptotic effect of simvastatin/tipifarnib, we also examined the expression of Bcl2 family proteins in leukemia cells treated with simvastatin and tipifarnib alone or in combination for 72hr using western blot analysis. As shown in Figure 5.2B, combined treatment of simvastatin and tipifarnib did not substantially alter the expression of either antiapoptotic (e.g., Bcl2 and Bcl-xL) or proapoptotic (e.g., Bax)

proteins. However, simvastatin/tipifarnib resulted in a significant reduction on the expression of the antiapoptotic Mcl1 protein in all leukemia cells except KG1.

To further confirm the apoptosis inducing effect of simvastatin/tipifarnib, Annexin V-FITC and PI analysis was performed on a subset of cells. The HL60 and Jurkat cells were treated with simvastatin (1 or 4  $\mu\text{M}$ ) and tipifarnib (0.1 or 1  $\mu\text{M}$ ) alone or in combination. As shown in Figure 5.2C and Figure 5.2D, combined treatment of simvastatin and tipifarnib showed a significant increase in early (AnnexinV+/PI-) and late (Annexin V+/PI+) apoptosis in both cell lines, compared to control and single treatments. Together these findings indicate that the synergistic interaction between simvastatin and tipifarnib in human leukemia cells is mediated by apoptosis. Also, KG1 was shown to be more resistant to this combination than other leukemia types.

### **3. Synergistic cytotoxicity of simvastatin/tipifarnib in leukemia cells is not associated with cell cycle arrest**

Since the cell proliferation assay cannot distinguish between apoptotic and arrested cells, we examined whether cell cycle arrest is contributing to the decrease in cell viability following simvastatin/tipifarnib exposure. Leukemia cells including KG1, HL60, K562 and HSB2 were treated with simvastatin (4  $\mu\text{M}$ ) and tipifarnib (1  $\mu\text{M}$ ) alone and in combination for 72hr. After treatment, cell cycle distribution was assessed using flow cytometry. The distribution of the cell cycle phases (G1, S and G2/M) showed no significant changes after treatment relative to control in all leukemia cells tested (Figure 5.3). These results suggest that the synergetic interaction of simvastatin/tipifarnib is cytotoxic and not cytostatic.

#### **4. Co-Treatment of simvastatin and tipifarnib disrupts the localization of RAS in the cell membrane**

The RAS GTPases are important mediators of cell signaling pathways involved in the survival and proliferation of cancer cells. FTI drugs were developed to prevent the farnesylation of RAS and thus inhibit its membrane incorporation and ultimately the RAS mediated signaling. As reported previously, the limited efficacy of tipifarnib, as well as that of other FTIs, may be attributed to the continued signaling of RAS by alternative isoprenylation (geranylgeranylation). Mounting evidence suggests that the anticancer activity of simvastatin is mediated by its capacity to disrupt the geranylgeranylation of small G-protein, primarily RHO proteins [59, 75, 79]. We therefore reasoned that coadministration of simvastatin and tipifarnib could disrupt the RAS prenylation process and its membrane association through blocking both the farnesylation and the alternative geranylgeranylation pathways. Leukemia cells were treated with simvastatin (4 $\mu$ M) and tipifarnib (1 $\mu$ M) alone or in combination for 72hr and cells subjected to a fractionation procedure to isolate the membrane and the cytosolic protein fractions. Western blot analysis was performed to determine RAS location in both fractions. Interestingly, simvastatin/tipifarnib robustly inhibited the membrane association of RAS with its subsequent sequestration into the cytosol, Figure 5.4. This effect was not observed in KG1 cells where RAS localization in the cell membrane did not change upon treatment with the combination compared to the control and single treatments. Overall, these findings indicate that simvastatin in the presence of tipifarnib disrupts membrane association of RAS and most likely results in loss of RAS function because of its inability to associate with membrane bound effectors.

## **5. Simvastatin/tipifarnib downregulates the ERK downstream signaling in human leukemia cell lines.**

It is well known that RAS activation is a crucial step for several cytoprotective and stress related signaling pathways. Therefore, we examined the effect of simvastatin/tipifarnib on the phosphorylation status (activity) of two main RAS downstream signaling pathways; ERK and AKT pathways. As shown in Figure 5.5, a 24 hr treatment of simvastatin/tipifarnib significantly decreased the phosphorylation of ERK in three of the tested leukemia cell lines including HL60, K562 and HSB2. Both KG1 and Jurkat cells showed no expression of the phosphorylated form of ERK. In addition, only Jurkat cells showed high levels of phosphorylated AKT. Interestingly, upon treatment with simvastatin alone or in combination the AKT phosphorylation was abolished. This result indicates that downregulation of the ERK signaling is most likely because of the effect of simvastatin/tipifarnib in disrupting the upstream RAS membrane localization and its subsequent deactivation.

## **6. Addition of mevalonate and isoprenoids prevents simvastatin/tipifarnib induced apoptosis and reverses the disrupted RAS isoprenylation**

To further investigate whether blocking the isoprenylation routes is responsible for the apoptotic effect of simvastatin/tipifarnib, leukemia cells were treated with mevalonate and isoprenoids (FPP and GGPP) in the presence of simvastatin/tipifarnib. Annexin V apoptosis assay and western blot analysis of caspase 3 and Mcl1 were employed to assess apoptosis. The Annexin V assay revealed that the apoptotic effect of simvastatin/tipifarnib was reversed by the addition of mevalonate, FPP or GGPP in HL60 cells (Figure 5.6A and B). Similarly, western blot analysis, in Figure 5.6C, indicated that caspase 3 activation (cleaved caspase 3) and Mcl1 downregulation induced by simvastatin/tipifarnib, in both K562 and HSB2 cells, were reversed by the addition of

mevalonate, FPP or GGPP. On the other hand, the effect of this combination on RAS disruption from the membrane was reversed by the addition of mevalonate and GGPP and partially by FPP (Figure 5.6D). These findings indicate that simvastatin/tipifarnib is mediating its apoptotic effect and RAS membrane disruption through the blocking of both the farnesylation and the geranylgeranylation pathways.

#### **D. Discussion**

Aberrant activation of the oncogenic RAS signal transduction is commonly observed in hematological malignancies. RAS mutations have been reported in 30% of leukemia, most frequently acute leukemia. FTIs were initially developed to inhibit RAS activation through blocking its farnesylation process. Despite the encouraging preclinical results, FTIs showed limited activity in clinical trials. This is thought to be due to the ability of RAS to get activated through the geranylgeranylation pathway, which acts alternatively to the farnesylation pathway once FTIs are administered. This escape mechanism was only noticed in K-RAS and N-RAS isoforms, which are commonly mutated in solid tumors and leukemia, respectively [158]. Blocking this alternative prenylation pathway is an attractive strategy to evade the resistance to FTIs. Here we report that simvastatin was able to augment the cytotoxicity of tipifarnib in a panel of leukemia cells through blocking the alternative geranylgeranylation pathway of RAS.

Our data show that simvastatin significantly increased the cytotoxic effect of tipifarnib in the different leukemia cells tested. Results from both cell viability and apoptotic assays indicate that leukemia cells were differentially affected by the simvastatin/tipifarnib combination. The increased sensitivity to this combination was more significant in HL60 compared to other leukemia cells tested whereas KG1 cells were more resistant with no signs of apoptosis. Our finding was in agreement with previous work, which demonstrated that KG1 is one of the insensitive cell lines that

required higher doses of simvastatin or tipifarnib relative to other leukemia cell lines [170]. Moreover, heterogeneity in response to statins and FTIs in primary CD34<sup>+</sup> AML cells has been reported recently [171].

In our studies, we employed AML, ALL and CML cells. With the exception of one AML cell line (KG1) the simvastatin/tipifarnib combination was synergistic in all other cell lines. However, the observed synergy appears to be independent of RAS mutations. Specifically, HL60 (N-RAS mutation, c.182A>T), Molt4 and HSB2 cells (N-RAS mutation, c.34G>T) and K562 and Jurkat cells (N-RAS wild type) were all sensitive, while KG1 (N-RAS mutation, c.35G>A) was resistant. An alternative explanation for the antagonism observed in the KG1 cells could be the presence of efflux transporters, such as P-gp and BCRP, which could be potentially effluxing the lactone and carboxylate species, respectively. However, those proteins were not found in these cells by Western blot analyses (data not shown). This does not preclude the presence of other transporters that may limit the accessibility of the simvastatin carboxylate form to the cancer cell. For example, MRP1 is an efflux transporter that is ubiquitously expressed and primarily transports anionic compounds. Additionally, further studies will be required to assess if the KG1 resistance is attributed to deregulated function of the mevalonate pathway.

Here we determined that the observed synergy following combination treatment was due to increased apoptosis. The balance between antiapoptotic (e.g. Bcl-2, Mcl-1 and Bcl-xL) and proapoptotic (e.g. Bax and Bad) proteins regulates the release of cytochrome c from the mitochondria into the cytosol, which will lead to the activation of the caspase cascade and the induction of apoptosis. In this study, we demonstrate that the simvastatin/tipifarnib combination initiates apoptosis through the downregulation of Mcl1 protein. Mcl-1 is an anti-apoptotic member of the Bcl-2 protein family that prevents apoptosis by forming heterodimers with proapoptotic Bcl-2 family members.

Downregulation of Mcl1 protein allows the proapoptotic Bcl2 proteins to initiate mitochondrial collapse and subsequent release of cytochrome c into the cytosol where it activates the apoptotic caspase cascade. Conversely, our results show that Bax expression was unchanged by treatment in all cell lines that expressed it.

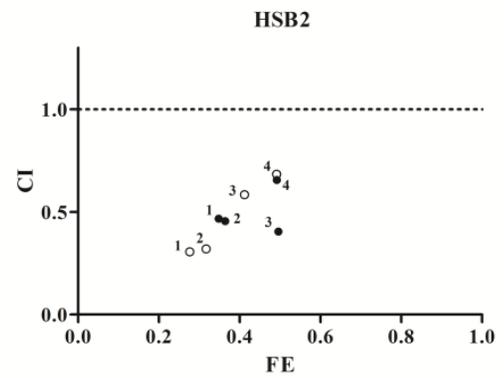
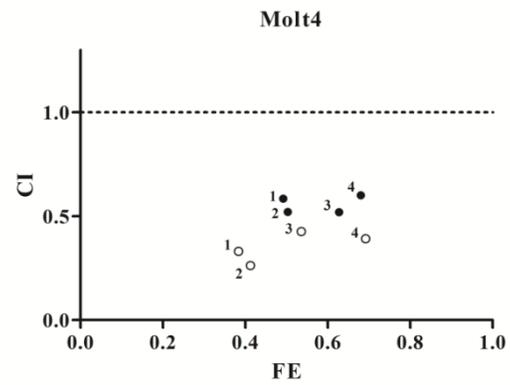
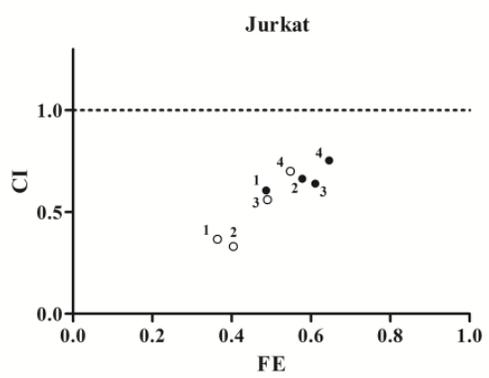
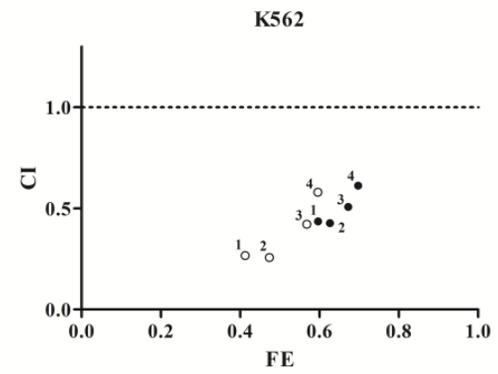
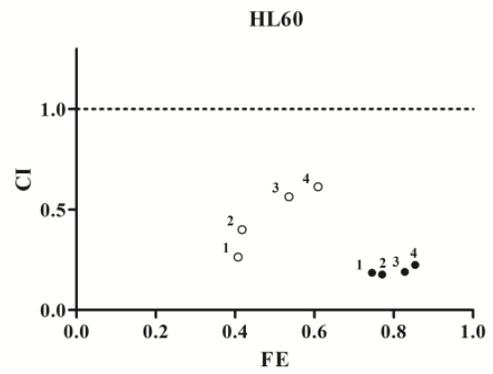
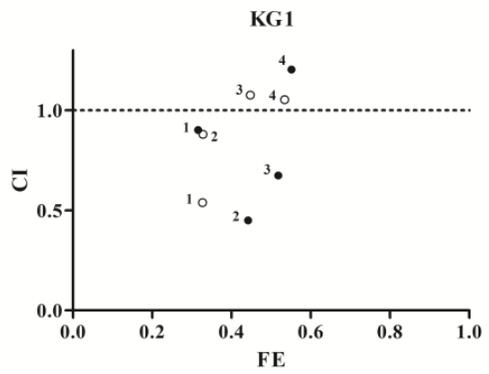
In fact, RAS plays a central role in activating several downstream effectors that are known to regulate different cell functions including cell growth, survival and differentiation. Therefore, therapeutic targeting of RAS signaling pathways has become a major endeavor in cancer therapy. Our results demonstrated that disrupting RAS membrane localization, by simvastatin/tipifarnib cotreatment, significantly decreased ERK phosphorylation in the cell lines tested. This finding is in line with previous work, which demonstrated the disruption of RAS/ERK signaling in multiple myeloma cells treated with FTI/lovastatin [172]. In general, our results indicate that RAS/MEK/ERK pathway might be involved in simvastatin/tipifarnib induced cytotoxicity. However, the lack of the basal level of ERK activity in Jurkat cells, which are sensitive to this combination, may indicate the involvement of other RAS downstream pathways. Conversely, the insensitive cell line KG1 showed no inhibition of RAS isoprenylation when treated with simvastatin/tipifarnib combination. Previous report has shown that higher concentrations of simvastatin (100  $\mu$ M) were required to block the isoprenylation of RAS in resistant AML cell lines [170].

On the other hand, reversal of simvastatin/tipifarnib combination induced apoptosis by mevalonate, FPP and GGPP was notably consistent in the sensitive leukemia cells. This observation confirms that prenylation pathways are the cellular targets of this combination. In line with this finding, RAS membrane disruption was also abrogated by the addition of mevalonate and GGPP and partially by FPP. This partial effect of FPP could be attributed to the higher binding affinity of tipifarnib to FTase,

therefore higher FPP concentrations might be required to completely reverse the effect of tipifarnib in this combination. Although, the disruption of RAS prenylation was clearly seen upon treatment with simvastatin/tipifarnib combination and was strongly correlated with cellular response, RAS as an exclusive target for this combination is still questionable. Several reports suggested that RAS may not be the only target for FTI treatment and other elusive targets may be involved [173]. The wide pool of proteins that undergo prenylation makes it difficult to identify a true therapeutic target for simvastatin/tipifarnib. Nonetheless, the ability of GGPP and FPP to rescue the effect of simvastatin/tipifarnib combination on the membrane localization of RAS demonstrates the role of the alternative prenylation as a mechanism of resistance to tipifarnib monotherapy.

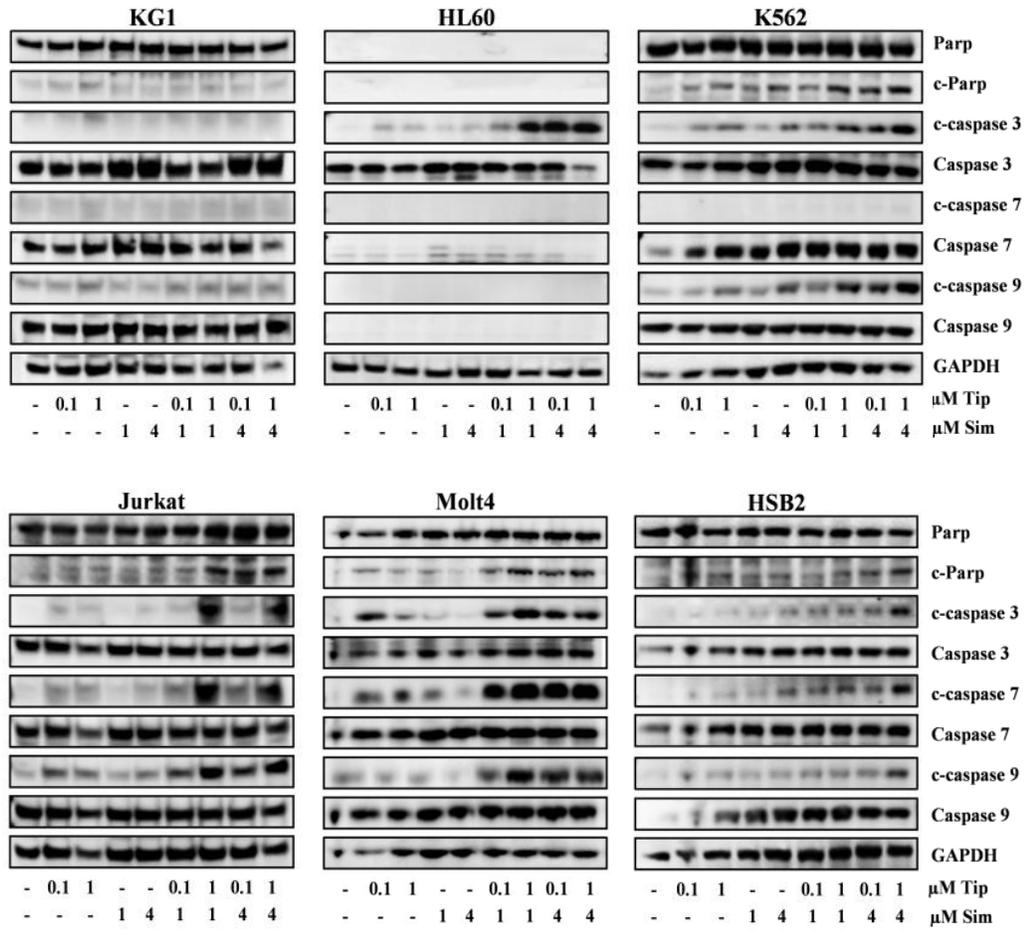
Furthermore, the use of simvastatin as anticancer agent was limited by the high doses required to mediate its antitumor activity. However, in our study, simvastatin was shown to induce apoptosis in combination with tipifarnib at lower concentrations, as low as 1 $\mu$ M. It is interesting to note that simvastatin, given at maximum tolerated dose (7.5mg/kg, twice daily) to leukemia patients, was found to achieve plasma levels comparable to those used in our study (**Chapter 4**).

In summary, the results obtained in this study suggest that synergistic cytotoxic effect of simvastatin/tipifarnib combination is, at least in part, due to the disruption of RAS membrane localization. Reversal of such effect by the addition of GGPP and FPP indicates the role of alternative geranylgeranylation as an escape mechanism for RAS activation in the presence of tipifarnib. However, such preliminary evidence of in vitro data needs further in vivo investigation.

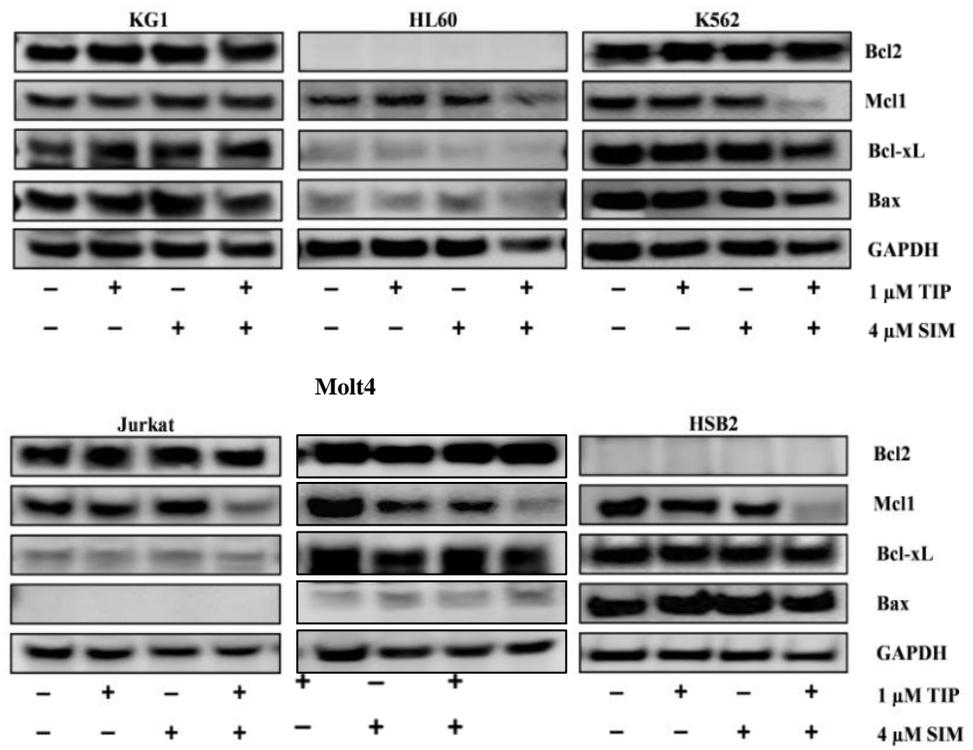


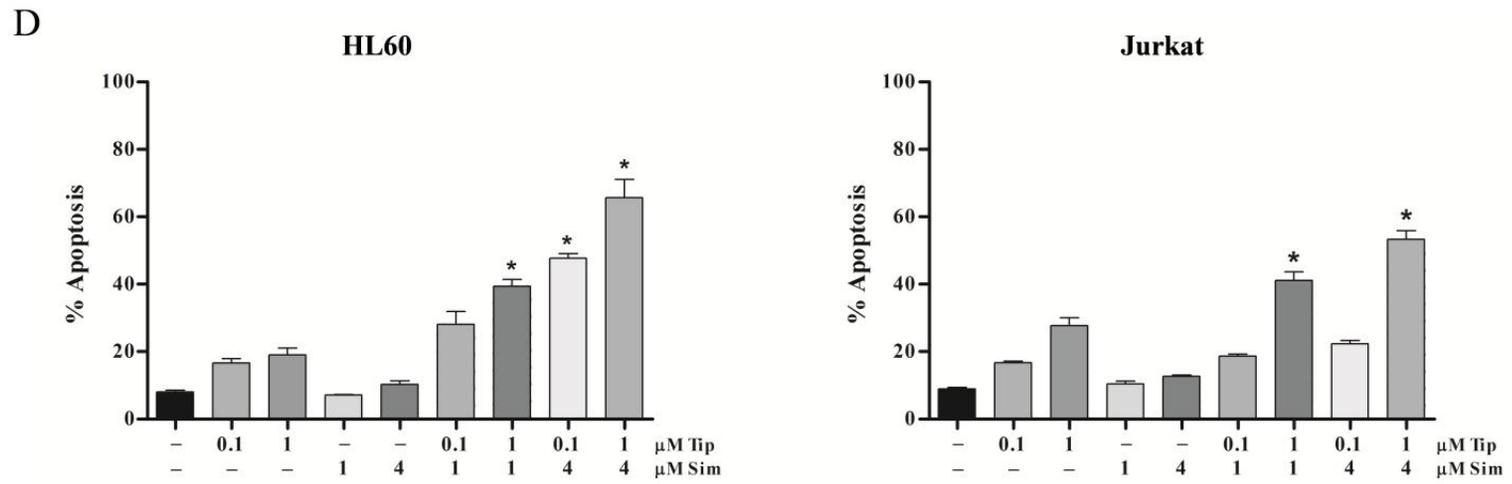
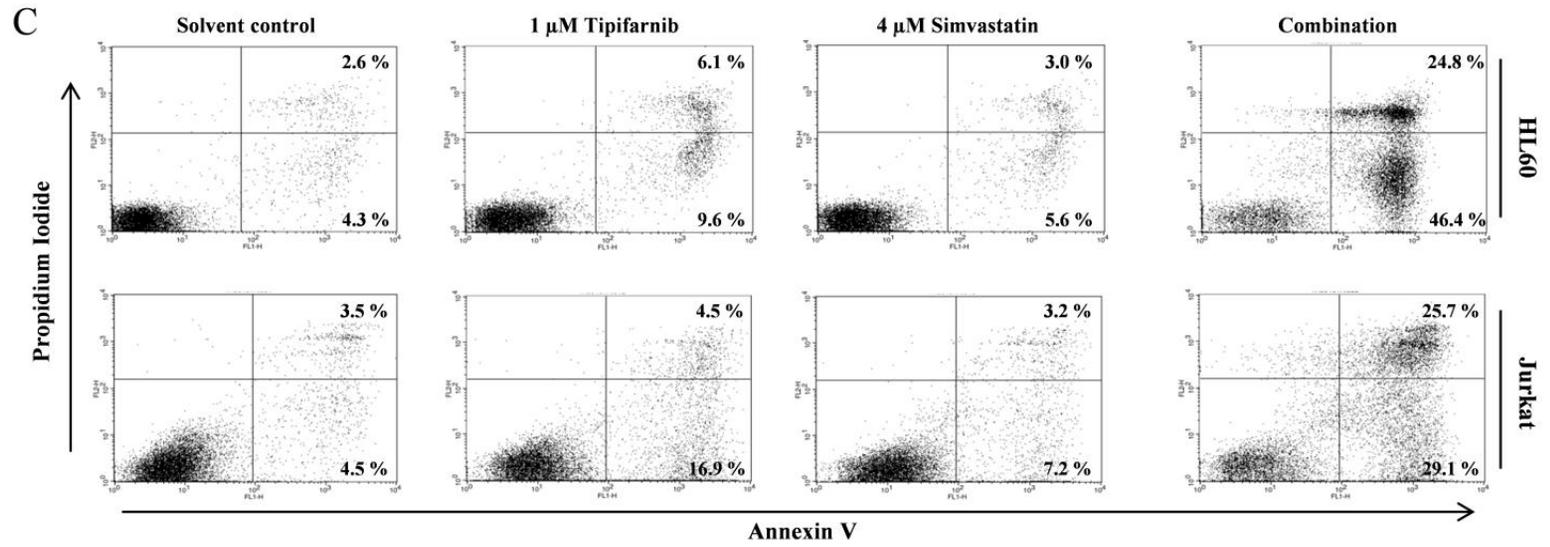
**Figure 5.1.** Simvastatin synergistically potentiates tipifarnib mediated lethality in human leukemia cells. Leukemia cell viability was determined following combination treatment with simvastatin (1 or 4  $\mu\text{M}$ ) and tipifarnib (0.05, 0.1, 0.5 or 1  $\mu\text{M}$ ) for 72 hours. Fractional effect (FE) values were determined by comparing results with those of untreated controls. Open and closed circles represent 1 and 4  $\mu\text{M}$  simvastatin treated sets, respectively. Numbers from 1 to 4 denote tipifarnib concentrations in ascending order. Results are the mean of 3 independent experiments.

A

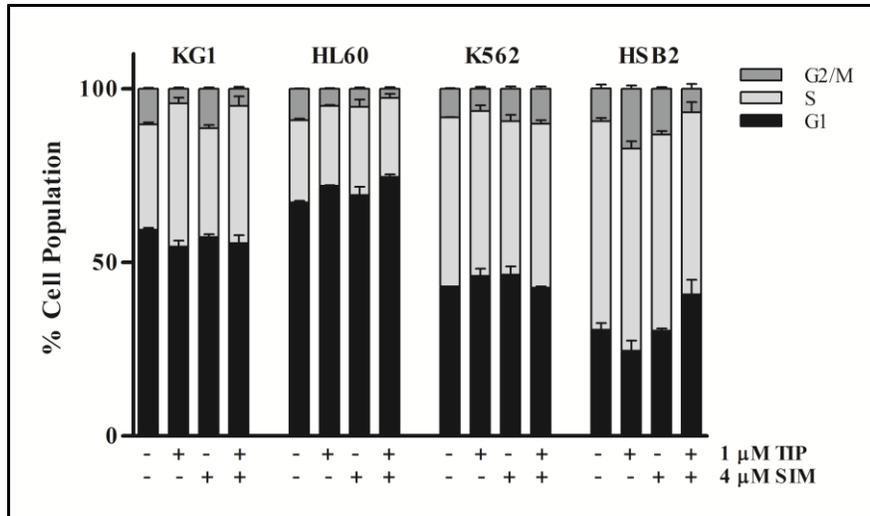


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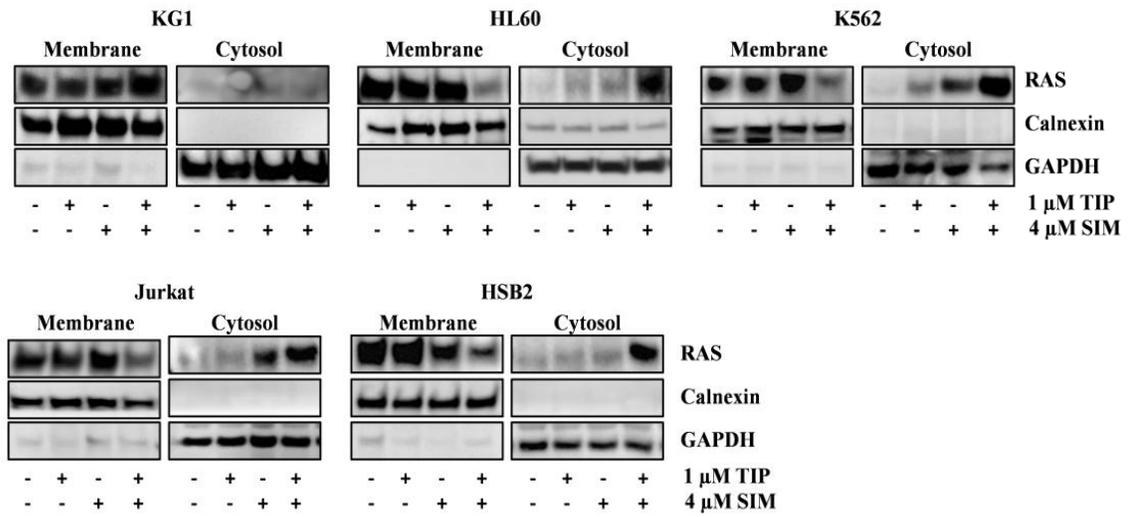




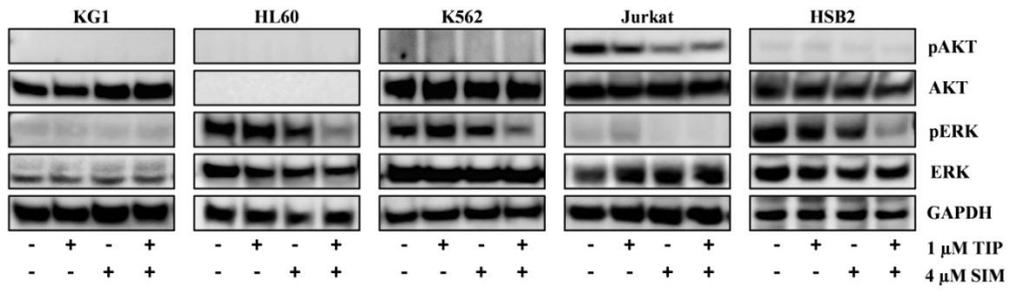
**Figure 5.2.** Combined exposure of leukemia cells to simvastatin and tipifarnib induces apoptosis through caspase activation and downregulation of Mcl1. Leukemia cells were treated for 72 hours with simvastatin and tipifarnib at concentrations indicated, either alone or in combination. At the end of the incubation period, cells were lysed and western blot analysis was performed to monitor the cleavage of caspases and PARP (A) and the expression of BCL2 family proteins (B). GAPDH was used as a loading control to ensure equivalent loading. Alternatively, HL60 and Jurkat cells were treated with simvastatin (4  $\mu$ M) and tipifarnib (1  $\mu$ M) alone and in combination for 72hr. Cells were then costained with Annexin and PI with subsequent flow cytometric analysis. A representative dot-plot is shown for each condition (C). AnnexinV+/PI- stained cells in the bottom right quadrant represent early apoptotic cells whereas late apoptotic or necrotic cells are located in the upper right quadrant with Annexin+/PI+ staining. In panel (D), representative figures of AnnexinV/PI staining of HL60 and Jurkat cells showing the sum of the percentages of early and late apoptotic cells. Results represent means of 3 independent experiments. Data are presented as mean  $\pm$  SD. \*,  $P > 0.05$ , significantly greater than values for cells exposed to simvastatin or tipifarnib alone. SIM, simvastatin. TIP, tipifarnib.



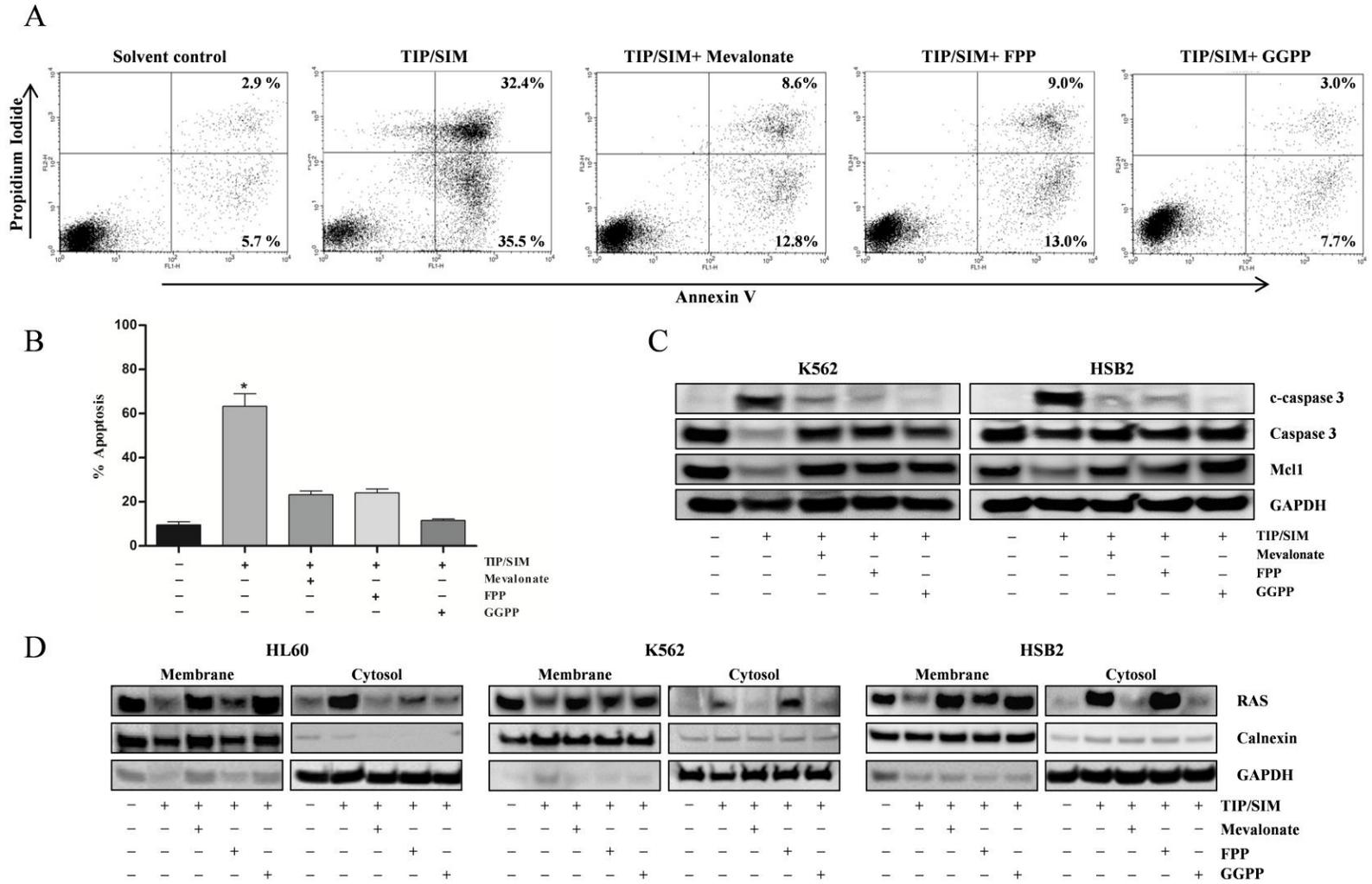
**Figure 5.3.** Simvastatin/tipifarnib does not induce cell cycle arrest in leukemia cells. Leukemia cells were treated for 72 hours with simvastatin (4 μM) and tipifarnib (1 μM), alone and in combination, before being stained with PI and analyzed by flow cytometry. SIM, simvastatin. TIP, tipifarnib



**Figure 5.4.** Simvastatin/tipifarnib alters subcellular localization of RAS in human leukemia cells. Leukemia cells were treated with simvastatin (4 μM) and tipifarnib (1 μM), alone and in combination for 72hrs. Cytosolic and membrane fractions were prepared and western blot analysis was performed as described in the method section using the indicated antibodies. Calnexin was used as a membrane marker, whereas GAPDH is a marker of the cytosolic fraction. SIM, simvastatin. TIP, tipifarnib.



**Figure 5.5.** Co-treatment of simvastatin and tipifarnib blocks ERK phosphorylation in human leukemia cells. Upon treatment with simvastatin (4 μM) and tipifarnib (1 μM), alone and in combination for 24hr, leukemia cells were processed for western blot analysis using the indicated antibodies. GAPDH was used as a loading control. SIM, simvastatin. TIP, tipifarnib.



**Figure 5.6.** Depletion of prenyl substrates by simvastatin/tipifarnib is associated with apoptosis induction, caspase activation, Mcl1 downregulation and RAS membrane disruption. HL60 cells were cotreated with simvastatin (4  $\mu$ M) and tipifarnib (1  $\mu$ M) in the presence of mevalonate (200  $\mu$ M), farnesyl pyrophosphate (10  $\mu$ M) and geranylgeranyl pyrophosphate (10  $\mu$ M). After 72 hour incubation, cells were harvested and costained with Annexin V/PI with subsequent flow cytometric analysis. A representative dot-plot and bar-figure were shown for each condition (A) and (B), respectively. Alternatively, K562 and HSB2 cells were treated similarly and then processed for western blot analysis to assess the expression of caspase 3 and Mcl1 (C). GAPDH was used as a loading control. Under the same conditions, HL60, K562 and HSB2 cells were processed for western blot analysis to monitor the expression of RAS in both the cytosolic and membrane fractions (D). Calnexin was used as a membrane marker, whereas GAPDH is a marker of the cytosolic fraction. Results represent means of 3 independent experiments. Data are presented as mean  $\pm$  SD. \*,  $P > 0.05$ , significantly lower than values for cells exposed to simvastatin-tipifarnib combination. SIM, simvastatin. TIP, tipifarnib.

## Chapter 6 : General Discussion

Statins are inhibitors of 3-hydroxy-3methylglutaryl (HMG) Co-A reductase that prevent cardiovascular diseases and lower LDL cholesterol. In recent years, increasing evidences from in vitro and in vivo studies have established the antitumor activity of statins, independent of cholesterol reduction. Besides their preclinical activity, statins have favorable safety profile and are available orally at a relatively inexpensive cost. Therefore, investigators were tempted to bring statins into the clinic for cancer therapy. However, clinical experience with high dose statins in cancer patients has reported unsuccessful outcomes [92, 95-97]. This thesis discusses the clinical utility and prospects of statins in cancer therapy.

As a part of the clinical development of high dose simvastatin in cancer patients, understanding the pharmacokinetics of this drug at high doses was important. Our pharmacokinetic study is the first to examine simvastatin concentrations in both plasma and tumor cells after high dose administration in leukemia patients. In fact, simvastatin at maximum tolerated dose (7.5 mg/kg, twice daily) achieved higher plasma concentrations (0.08-2.2  $\mu\text{M}$ ) relative to a typical dose (40mg) which has a peak plasma concentration of 0.02-0.08  $\mu\text{M}$  (10-34 ng/mL). However, the high plasma concentrations achieved are still lower than those found to be effective in vitro. These insufficient levels of simvastatin are the most likely explanation of the limited efficacy of high dose simvastatin observed in previous clinical studies [96, 97]. These low levels of simvastatin are mainly attributed to the extensive first pass extraction of simvastatin that hinders the drug reaching the systemic circulation at sufficient concentrations. Overall, this poor delivery process of statins to the circulation urges further exploration of different strategies to improve bioavailability and consequent clinical activity.

Most of the clinical studies assessing the antitumor activity of high dose statins, have no rationale for the choice of statins. The statin member that is most effective and shows favorable clinical profile have yet to be determined. Currently, there are seven FDA approved statins in the market that possess the same mechanism of action; however, they differ in terms of their chemical structures, pharmacokinetic profiles and potencies. In terms of efficacy, preclinical studies have demonstrated that lipophilic statins (e.g. lovastatin, simvastatin, atorvastatin, fluvastatin and pitavastatin) have better antitumor activity relative to hydrophobic statins (e.g. pravastatin and rosuvastatin) [174-177], which is logical since lipophilic statins are more accessible to the tumor cells. On the other hand, the low systemic bioavailability of statins is considered a major barrier that may impede their clinical activity as anticancer agents. In this regard, both simvastatin and lovastatin show the lowest bioavailability (below 5%) relative to other statin members; yet, they were the most studied statins in clinical trials. Another disadvantage of simvastatin and lovastatin is their fast elimination with half-life less than three hours. Moreover, simvastatin and lovastatin are substrates for CYP3A4 metabolizing enzyme [178] that may limit their use in combination with other anticancer agents, if a CYP3A4 interaction exists. In view of the poor pharmacokinetic profile of both simvastatin and lovastatin, they are unlikely to be considered as an optimal statin model for cancer therapy. Therefore, achieving higher bioavailability for prolonged period of time is vital for statins in order to score better distribution in the tumor and subsequently to attain better efficacy. Fluvastatin, has shown improved bioavailability (10-35%) and limited CYP3A4 metabolism; however, the very short half-life of fluvastatin (0.5 – 2.3 hr) is a major pitfall that may hold back its clinical development as an anticancer therapy. Conversely, pitavastatin, a lipophilic statin recently approved by the FDA, shows superior systemic bioavailability (80%) relative to other statins as well as longer half-life (11 hours) and limited CYP450 metabolism [178]. Despite of the very few

studies that have evaluated the antitumor activity of pitavastatin, its favorable pharmacokinetic profile makes it a promising candidate that warrants further evaluation in cancer therapy.

With respect to statin dose, there has been a debate in the last decade about the optimal dosage regimen of statins in cancer therapy. In view of the clinical experience of statins in cancer treatment, most of clinical studies have favored continuous administration of low dose statins over intermittent high dose regimens in terms of safety. Moreover, continuous low dose statin was thought to achieve better efficacy through a sustainable blockage of the mevalonate pathway. In fact, most of these studies have evaluated the significance of using statins at typical doses as an adjuvant treatment in cancer; yet, outcomes turned to be controversial. Of note, high dose statins were evaluated in clinical studies as a sole therapy not in combination with standard therapy which is the case in low dose statins; thus, it is not clear whether low-dose statins is better than high doses in terms of efficacy.

The lack of clinical benefits of high dose statins in previous studies does not preclude that statins at high dose could be useful in combination with other anticancer agents. Several preclinical studies have shown the ability of statins to interact synergistically with various antitumor treatments [100-104]. Recently, a phase II study has evaluated high dose simvastatin in combination with vincristine (0.4 mg), adriamycin (9 mg/m<sup>2</sup>), and dexamethasone 40mg orally (VAD) in a sequential administration [97]. Addition of high dose simvastatin showed no response that could be attributed to the treatment strategy (sequential versus simultaneous) as well as the short half-life of simvastatin. However, in vivo treatment with high dose simvastatin for 7 consecutive days in leukemia patients displayed an increase in the in vitro chemosensitivity of their AML cells [179].

In recent years, the development of molecular targeted therapeutics is dramatically evolving over conventional cytotoxic drugs. Many targeted agents that modulate specific oncogenic proteins have been approved or still under development with the hope to achieve better anticancer activity and fewer side effects. However, the ability of the tumor to confer resistance (intrinsic or acquired) against these molecular targeted agents is common. Thus, combination therapies become a well-established principle in cancer therapy to circumvent cancer resistance. In this thesis, simvastatin was used in combination with tipifarnib to evade cancer resistance developed against tipifarnib when used alone. Tipifarnib is a farnesyl transferase inhibitor that was initially developed to target RAS farnesylation. However, when cells were treated with tipifarnib, K-RAS and N-RAS become geranylgeranylated and remain fully functional. Our study showed that simvastatin in combination with tipifarnib, at clinically achievable concentrations, displayed a synergistic interaction in leukemia. This synergistic combination was based on a mechanistic rationale that targets farnesylation pathway of RAS as well as its alternative geranylgeranylation pathway. Here we demonstrate that inhibition of both prenylation pathways, by combining simvastatin with tipifarnib, induces synergistic lethality that was not attained by the inhibition of each prenylation pathway separately.

Although our study was limited to the in vitro setting and to established cancer cell lines, recent study have demonstrated that simvastatin was able to inhibit geranylgeranylation pathway in primary AML cells at concentrations similar to those used in our study [179]. Generally, these in vitro findings warrant further investigation of high dose statins in combination with tipifarnib in leukemia patients. In addition, given the fact that this combination was able to knockdown RAS from the cellular membrane, further exploration of this combination in other cancer models that harbor K- RAS mutation such as pancreatic, colorectal and non-small cell lung cancers is encouraged.

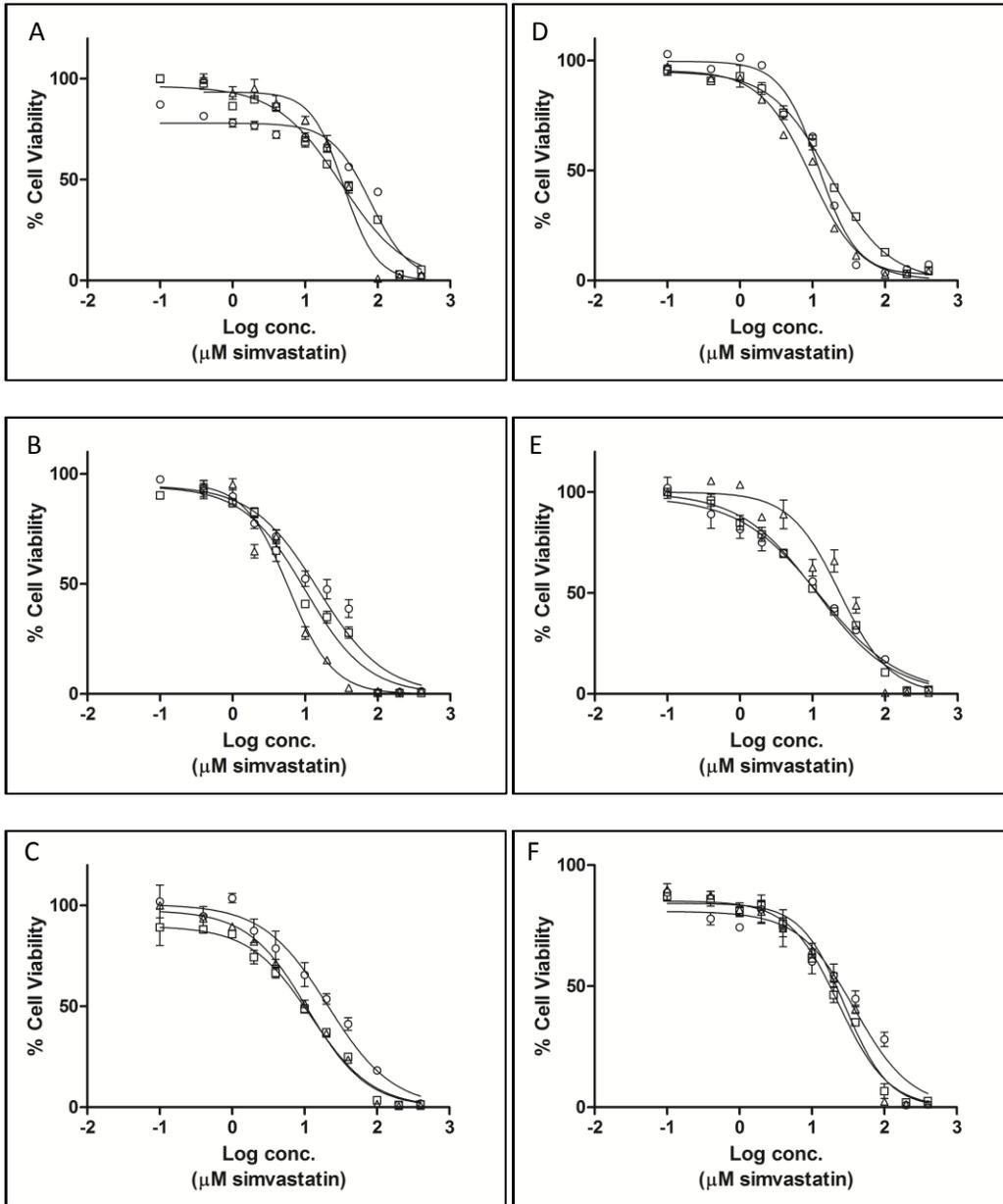
It is also worth noting that this combination showed variable response among the different leukemia cell lines where AML cell line (HL-60) was the most sensitive one toward this combination. This finding is supported by previous work that has reported similar heterogeneity in response among group of AML cell lines toward simvastatin, tipifarnib or GGTI-298 when they were used separately [170]. The differential sensitivity of AML cells was attributed to the difference in interference with prenylation pathways. This was in line with our finding that simvastatin/tipifarnib combination was shown to disrupt RAS isoprenylation in the sensitive HL-60 cells relative to KG1 cells which were more resistant. Interestingly, this heterogeneity in response was observed in primary AML cells when treated with simvastatin or FTI/lovastatin. However, further investigation is needed to unravel the molecular basis of this differential behavior among AML cells and to find whether it can be exploited in the identification of leukemia patient population who are most likely to respond for this given combination.

In conclusion, this thesis provides insights into the clinical feasibility of simvastatin and the new approaches of its use in cancer therapy. Using LC-MS/MS analytical tool, we have measured simvastatin lactone and carboxylate levels in both plasma and PBMCs collected from chronic lymphocytic leukemia patients after high dose administration. Despite the limited number of patients enrolled in this pilot trial, our data indicate that simvastatin at high doses showed insufficient plasma and tumor concentrations which are below those found effective in-vitro. This finding discourages the use of high dose statins as a sole therapy in cancer patients, and that, further exploration of strategies to improve its clinical activity is required. One promising approach is considered in this thesis which is the combination of simvastatin with other anticancer agents that may show synergy. Our studies demonstrated a synergistic interaction of simvastatin and tipifarnib combination which might be mediated by the

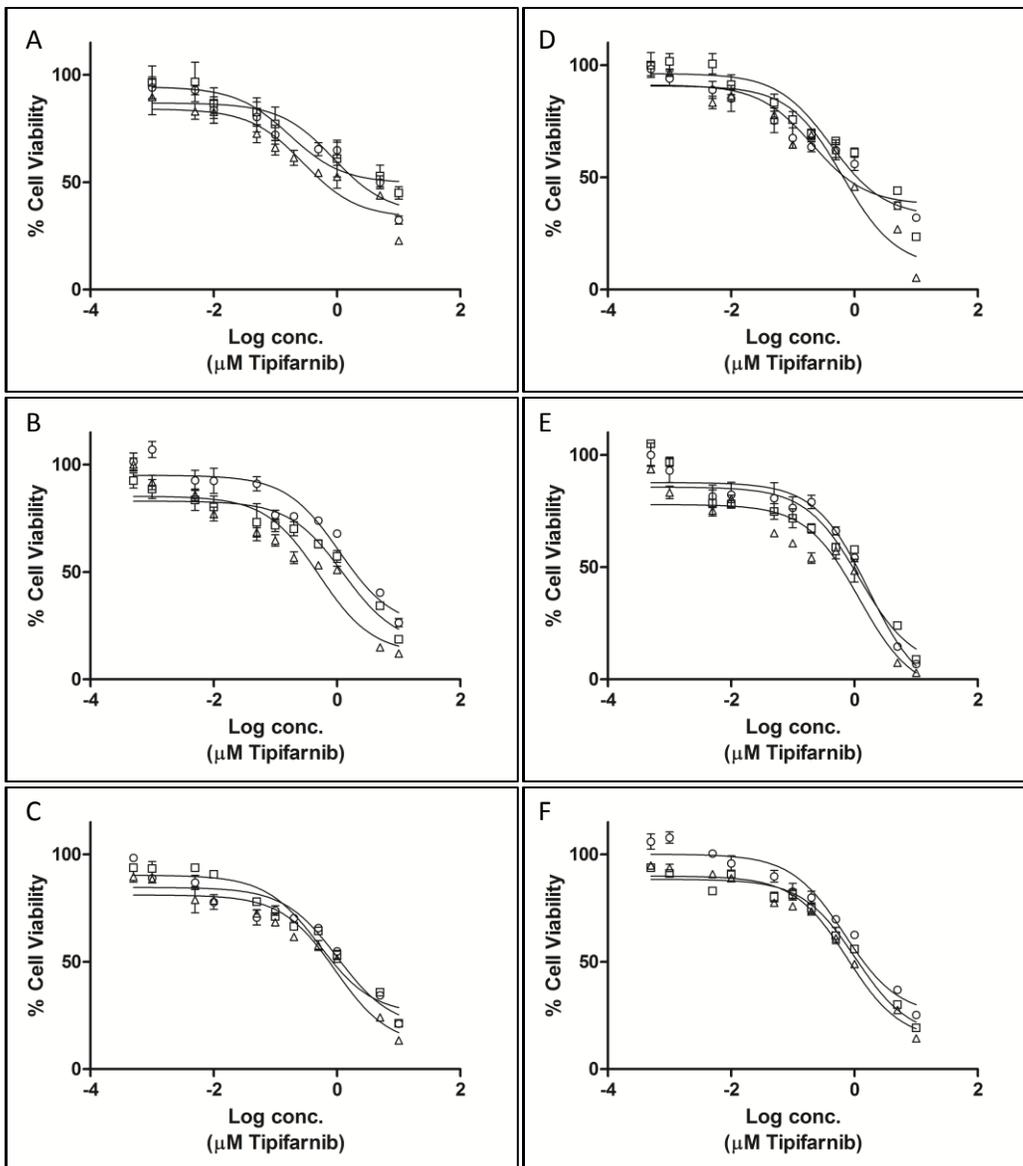
RAS/MEK/ERK pathway disruption. These promising preclinical results warrant further investigation in other cancer models and in animal models as a step toward future clinical application.

## APPENDICES

**Appendix A:** Evaluation of the antiproliferative effect of simvastatin and tipifarnib in leukemia cell lines.



**Figure A.1.** Dose response curve of simvastatin in human leukemia cell lines. Cell lines, KG1 (A), HL-60 (B), K562 (C), Molt4 (D), Jurkat (E) and HSB2 (F) were treated for 72 hrs with different concentrations of simvastatin (0-400 $\mu$ M). At the end of the incubation period, 10  $\mu$ L of resazurin was added to each well and further incubated for 3 hr at 37°C. Cell viability was determined by measuring the fluorescence at 560 nm excitation wavelength and 590 nm emission wavelength using Molecular Devices Spectramax M5 plate reader. Experiments were performed in triplicates.



**Figure A.2.** Dose response curve of tipifarnib in human leukemia cell lines. Cell lines, KG1 (A), HL-60 (B), K562 (C), Molt4 (D), Jurkat (E) and HSB2 (F) were treated for 72 hrs with different concentrations of simvastatin (0-10 $\mu$ M). At the end of the incubation period, 10  $\mu$ L of resazurin was added to each well and further incubated for 3 hr at 37°C. Cell viability was determined by measuring the fluorescence at 560 nm excitation wavelength and 590 nm emission wavelength using Molecular Devices Spectramax M5 plate reader. Experiments were performed in triplicates.

**Table A.1.** IC<sub>50</sub> values (μM) of simvastatin in leukemia cell lines (n=3).

Cell line	IC <sub>50</sub> Values (95%CI)			Mean (SD)
	Sample# 1	Sample# 2	Sample# 3	
KG1	79.29 (61.49 to 102.2)	31.03 (22.52 to 42.75)	34.01 (29.79 to 38.84)	48.1 (27.0)
HL60	17.33 (14.83 to 20.25)	9.419 (8.202 to 10.82)	12.17 (10.57 to 14.02)	12.9 (4.0)
K562	15.14 (10.40 to 22.04)	10.02 (7.911 to 12.70)	5.938 (4.692 to 7.515)	10.3 (4.6)
Molt4	12.59 (8.204 to 19.33)	11.50 (8.793 to 15.04)	23.89 (17.26 to 33.05)	15.9 (6.8)
Jurkat	20.98 (15.58 to 28.26)	12.31 (10.32 to 14.69)	10.96 (9.695 to 12.40)	14.7 (5.4)
HSB2	39.08 (26.54 to 57.56)	23.47 (20.49 to 26.88)	28.34 (23.22 to 34.59)	30.2 (7.9)

**Table A.2.** IC<sub>50</sub> values (μM) of tipifarnib in leukemia cell lines (n=3).

Cell line	IC <sub>50</sub> Values (95%CI)			Mean (SD)
	Sample# 1	Sample# 2	Sample# 3	
KG1	0.878 (0.558 to 1.383)	0.287 (0.169 to 0.487)	0.169 (0.085 to 0.337)	0.44 (0.37)
HL60	0.208 (0.135 to 0.322)	0.414 (0.266 to 0.645)	0.650 (0.400 to 1.057)	0.42 (0.22)
K562	1.094 (0.593 to 2.015)	1.254 (0.798 to 1.970)	0.511 (0.311 to 0.842)	0.95 (0.38)
Molt4	1.688 (1.012 to 2.814)	1.187 (0.659 to 2.136)	1.201 (0.720 to 2.004)	1.35 (0.28)
Jurkat	1.019 (0.619 to 1.676)	0.513 (0.358 to 0.736)	0.936 (0.660 to 1.329)	0.81 (0.26)
HSB2	0.696 (0.483 to 1.003)	1.041 (0.774 to 1.399)	0.765 (0.604 to 0.970)	0.83 (0.18)

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geranylgeranylation in AML blast cells in a subset of AML patients, *Experimental hematology*. 2012. **40**(3): p. 177-86 e6.

## VITA

**Tamer Ahmed**, Doctoral candidate

### Birth

January 17, 1979  
Lawrence, KS

### Education

May 2005. Master of Pharmaceutics, Faculty of Pharmacy, Assiut University, Egypt.

June 2000. Bachelor degree in Pharmaceutical sciences, Assiut University, Egypt.

### Work experience/ research experience

2006 - present. Assistant lecturer, Clinical Pharmacy Dept., Faculty of Pharmacy, Ain Shams University, Egypt.

2001-2006. Assistant lecturer, Pharmaceutics Dept., Faculty of Pharmacy, Assiut University, Egypt.

Summer. 1999. Summer Internship, Pharmaceutical Sciences Dept., University of Cincinnati, USA (Dr. Sakr lab).

### Awards

2007-2010. Kentucky Graduate Scholarship

2011. AACR- Minorities in Cancer Research award

### Publications

Shaaban KA, **Ahmed TA**, Leggas M and Rohr J. Saquayamycins G-K, Cytotoxic Angucyclines from *Streptomyces* sp.: Containing Two Angucycline Derivatives Bearing the Unusual Ami-nosugar Rednose. J Nat Prod. 2012; 75(7):1383-92.

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**Ahmed TA**, Hayslip J and Leggas M.. Pharmacokinetics of high dose simvastatin in patients with relapsed chronic lymphocytic leukemia. (*In preparation*)

**Ahmed TA**, Hayslip J and Leggas M. Simvastatin synergistically potentiates tipifarnib cytotoxic and apoptotic effects in human leukemia cells through the disruption of RAS/MEK/ERK pathway. (*In preparation*)

### **Presentations**

**Ahmed TA**, VanZant G, Hayslip J and Leggas M. Pharmacokinetic, safety and efficacy of high dose simvastatin in refractory and relapsed chronic lymphocytic leukemia (CLL) patients. American Association of Cancer Research (AACR) Annual Meeting. March, 2012, Chicago, IL.

**Ahmed TA**, Hayslip J and Leggas M. Simvastatin synergistically potentiates tipifarnib cytotoxic and apoptotic effects and disrupts Ras membrane localization in human leukemia cells. AACR-NCI-EORTC International Conference: Molecular Targets and Cancer Therapeutics. November, 2011, San Francisco, CA.

**Ahmed TA**, Hayslip J and Leggas M. Pharmacokinetics and pharmacodynamics of high dose Simvastatin in chronic lymphocytic leukemia patients. Markey Cancer Center Research Day, March 2011, Lexington, KY.

**Ahmed TA**, Hayslip J and Leggas M. High dose Simvastatin as a potential anticancer therapy in patients with relapsed chronic lymphocytic leukemia. Markey Cancer Center Research Day, April 2010, Lexington, KY.

**Ahmed TA**, El-Gendy G, Khaled K and Hafez E. Design and evaluation of biodegradable chitosan sponges as wound and burn dressings. Egyptian Pharmaceutical Society Meeting, December 2004, Cairo, Egypt.

**Ahmed TA**, El-Gendy G, Khaled K and Hafez E. Design and evaluation of chitosan membranes containing nitrofurazone. Assiut University Fourth Pharmaceutical Science conference, March 2004, Assiut, Egypt.

Tamer Ahmed  
\_\_\_\_\_  
Student's Signature

01/07/13  
\_\_\_\_\_  
Date