Preclinical potentiators of statin-induced anticancer effects

By

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A thesis submitted in conformity with the requirements
for the Degree of Doctor of Philosophy
Department of Medical Biophysics
University of Toronto

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2014

Abstract

Statins have been used for decades in the treatment of hypercholesterolemic patients to decrease the incidence of adverse cardiovascular events. Statins target the rate-limiting enzyme of the mevalonate (MVA) pathway, 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGCR). Statin-mediated HMGCR inhibition in hepatocytes leads to depletion of MVA derived sterols, including cholesterol, and a subsequent uptake of low-density lipoprotein (LDL) cholesterol from the plasma. Aside from the intended cholesterol-lowering properties, statins exert many pleiotropic effects including anti-tumour activity. The well characterized pharmacokinetic profiles, safety and the fact that many statins are off patent has ensured their prompt entry into clinic for evaluation of anti-cancer efficacy. We hypothesize that the therapeutic window of statins can be increased through (i) the use of a pharmacological screen to identify compound/s that synergize with statins to induce tumour-specific apoptosis and (ii) the use of a genome-wide small hairpin ribonucleic acid (shRNA) screen to identify novel pathways/targets whose knockdown enhances the vulnerability of tumour cells to statin-inhibition. Firstly we carried out a screen in a multiple myeloma (MM)
cell line where we combined atorvastatin with other off-patent FDA approved drugs. We identified dipyridamole, a commonly used anti-platelet agent as synergizing with and potentiating statin-induced apoptosis in a variety of MM and acute myelogenous leukemia (AML) cell lines and primary patient samples. The efficacy of the combination was demonstrated \textit{in vivo}. Secondly, through genome-wide shRNA screen, we identified several putative targets whose knockdown potentiated statin induced anti-cancer effects in the A549 lung cancer cell line. Amongst the most promising hits, sterol regulatory element binding transcription factor 2 (SREBF2) was validated in lung and breast cancer cell lines. The knockdown of SREBF2 was found to dramatically potentiate statin-induced apoptosis and block the upregulation sterol-responsive gene targets. Taken together, this research has uncovered a novel combination of clinically translatable drugs with strong preclinical efficacy in hematological malignancies. Furthermore we have identified several novel validated targets that sensitize the MVA pathway to statin-induced apoptosis.
Acknowledgements

First, I would like to thank my supervisor, Dr. Linda Penn, for the opportunity to work in her lab, in one of the best research centers in the world. I have learned so much from her and I will never forget my experience in this incredibly supportive lab. Along with Dr. Linda Penn’s unwavering support, I would like to thank the members of my supervisory committee, Drs Aaron Schimmer and Robert Kerbel for their scientific guidance. I felt like I could always go to them for advice and this has been instrumental in helping me through graduate school.

Furthermore, I would like to thank my family for their never-ending support and love, most important Mom, Tat, and Magda and certainly every other member of the Polish gang, Kate, Aga, all the Piotr’s, Ciocia, Wujek, Grandma, Grandpa and the little ones, and the new ones (Jon and Allison). I would like to thank Mark, my best friend, who has always pushed me to be the best I can be in every aspect of my life and go through life screaming and fighting. And of course my other friends, I can’t even imagine going through life without knowing you: Tracy, Amanda, Sara, Pete, Cory, Manpreet, Carolyn, Paul B., Janice, Laura, Cynthia, Ric, Lisa, Trudy, Larry, Jamie, Kevin, Stepho, Tracey, Valery. A special thanks to Maria K., I wish I could have been a better friend.

Moreover, I would like to thank all the collaborators, Drs. Jason Moffat, Corey Nislow, Mark Minden, Paul Boutros and Suzanne Trudel, who have all been instrumental in contributing to the work presented in this thesis.
Also, I would like to thank Dr. Karl Lang for giving me the great opportunity to work in his outstanding, excellent and awesome lab, his everlasting support and guidance on present and future research endeavours.

Finally I would like to thank Philipp Lang, for everything. There’s too much to say than a few sentences could cover.
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<th>Full name</th>
</tr>
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<tbody>
<tr>
<td>3D</td>
<td>3 dimensional</td>
</tr>
<tr>
<td>4-MD</td>
<td>4-{[3′,4′-(Methylenedioxy)benzyl]amino}-6-methoxyquinazoline</td>
</tr>
<tr>
<td>4S</td>
<td>Scandinavian Simvastatin Survival Study</td>
</tr>
<tr>
<td>5-FU</td>
<td>5-fluorouracil</td>
</tr>
<tr>
<td>AACR</td>
<td>American Association of Cancer Research</td>
</tr>
<tr>
<td>ALK</td>
<td>anaplastic lymphoma kinase</td>
</tr>
<tr>
<td>AML</td>
<td>acute myelogenous leukemia</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>AV</td>
<td>annexin V</td>
</tr>
<tr>
<td>b.i.d.</td>
<td>bis in die</td>
</tr>
<tr>
<td>BRAF</td>
<td>B Rapidly Accelerated Fibrosarcoma</td>
</tr>
<tr>
<td>C</td>
<td>concentration</td>
</tr>
<tr>
<td>cAMP</td>
<td>Cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>CD</td>
<td>cluster of differentiation</td>
</tr>
<tr>
<td>cGMP</td>
<td>cyclic guanosine monophosphate</td>
</tr>
<tr>
<td>CHD</td>
<td>coronary heart disease</td>
</tr>
<tr>
<td>CI</td>
<td>combination index</td>
</tr>
<tr>
<td>CML</td>
<td>chronic myelogenous leukemia</td>
</tr>
<tr>
<td>CoA</td>
<td>Coenzyme A</td>
</tr>
<tr>
<td>CTLA-4</td>
<td>cytotoxic T lymphocytes antigen 4</td>
</tr>
</tbody>
</table>
CYP3A4 cytochrome p450 A4
db-cAMP dibutyryl cAMP
DCIS ductal carcinoma in-situ
DMEM H21 Dulbecco's modified eagle's medium H21
DMSO dimethyl sulfoxide
DNA Deoxyribonucleic acid
dUTP deoxyuridine-triphosphat
EC effective concentration
eEF1A2 eukaryotic elongation factor 1 alpha 2
EGFR epidermal growth factor receptor
EML4 Echinoderm microtubule-associated protein-like 4
ENT1 equilibrative nucleoside transporter
EORTC European Organisation for Research and Treatment of Cancer
ER estrogen receptors
Erk Extracellular signal-regulated kinases
esiRNA endoribonuclease-prepared siRNA
FBS fetal bovine serum
FDA Food and Drug Administration
FDPS farnesyl pyrophosphate synthase
FITC fluorescein isothiocyanate
FTIs farnesyltransferase inhibitors
g gram
GARP gene activity ranking profile
GCSF  Granulocyte colony-stimulating factor
GGPP  geranylgeranyl pyrophosphate
GGPS1 geranylgeranyl diphosphate synthase 1
GGTase-I geranylgeranyltransferases I
GLUT  glucose transporters
HCC   hepatocellular carcinoma
HDL   high-density lipoprotein
Her2  Human Epidermal Growth Factor Receptor 2
HMGCR hydroxy-3-methylglutaryl coenzyme A reductase
HMGCS1 hydroxymethylglutaryl coenzyme A synthase 1
HMGCS1 3-hydroxy-3-methylglutaryl-Coenzyme A synthase 1
i.p.   intraperitoneal
IC     inhibitory concentration
IMDM  Iscove modified Dulbecco medium
JNK   c-Jun N-terminal kinases
kg    kilogramm
LDL   low-density lipoprotein
LDLr  low-density lipoprotein receptor
m     meter
MAPK  mitogen-activated protein kinase
MAP2K4 mitogen-activated protein kinase kinase 4
MEM   modified Eagle's medium
mg    milligramm
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>MM</td>
<td>multiple myeloma</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger ribonucleic acid</td>
</tr>
<tr>
<td>mTOR</td>
<td>mammalian target of rapamycin</td>
</tr>
<tr>
<td>MTS</td>
<td>3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium</td>
</tr>
<tr>
<td>MTT</td>
<td>3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide</td>
</tr>
<tr>
<td>MVA</td>
<td>mevalonate</td>
</tr>
<tr>
<td>NCI</td>
<td>National Cancer Institute</td>
</tr>
<tr>
<td>nM</td>
<td>nanomolar</td>
</tr>
<tr>
<td>NSCLC</td>
<td>non–small cell lung cancer</td>
</tr>
<tr>
<td>OATB</td>
<td>organic anion transporting polypeptide</td>
</tr>
<tr>
<td>P-gp</td>
<td>P-glycoprotein</td>
</tr>
<tr>
<td>p.o.</td>
<td>per os</td>
</tr>
<tr>
<td>p53</td>
<td>protein 53</td>
</tr>
<tr>
<td>PARP</td>
<td>Poly (adenosinediphosphate-ribose) polymerase</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PBSC</td>
<td>peripheral blood stem cells</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PD-1</td>
<td>Programmed cell death protein 1</td>
</tr>
<tr>
<td>PDEs</td>
<td>phosphodiesterases</td>
</tr>
<tr>
<td>PEGylated</td>
<td>polyethylene glycol modified</td>
</tr>
<tr>
<td>PI</td>
<td>propidium iodide</td>
</tr>
<tr>
<td>PIK4B</td>
<td>phosphatidylinositol 4-kinase, catalytic beta</td>
</tr>
<tr>
<td>PR</td>
<td>progesterone receptor</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>--------------</td>
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</tr>
<tr>
<td>RNAi</td>
<td>Ribonucleic acid interference</td>
</tr>
<tr>
<td>RPMI</td>
<td>Roswell Park Memorial Institute</td>
</tr>
<tr>
<td>SCAP</td>
<td>SREBP cleavage activating protein</td>
</tr>
<tr>
<td>SCID</td>
<td>Severe combined immunodeficiency</td>
</tr>
<tr>
<td>SD</td>
<td>standard deviation</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulfate polyacrylamide</td>
</tr>
<tr>
<td>SHARP</td>
<td>shRNA Activity Ranking Profile</td>
</tr>
<tr>
<td>shRNA</td>
<td>small hairpin ribonucleic acid</td>
</tr>
<tr>
<td>siRNA</td>
<td>small interfering RNA</td>
</tr>
<tr>
<td>SRE</td>
<td>sterol responsive elements</td>
</tr>
<tr>
<td>SREBF2</td>
<td>sterol regulatory element binding transcription factor 2</td>
</tr>
<tr>
<td>TACE</td>
<td>transarterial chemoembolization</td>
</tr>
<tr>
<td>TAE</td>
<td>transcatheter arterial embolization</td>
</tr>
<tr>
<td>TD</td>
<td>thalidomide and dexamethasone</td>
</tr>
<tr>
<td>TRC1</td>
<td>RNAi Consortium</td>
</tr>
<tr>
<td>TUNEL</td>
<td>Terminal deoxynucleotidyl transferase dUTP nick end labeling</td>
</tr>
<tr>
<td>VAD</td>
<td>vincristine, doxorubicin and dexamethasone</td>
</tr>
<tr>
<td>VEGF</td>
<td>vascular endothelial growth factor</td>
</tr>
<tr>
<td>zGARP</td>
<td>Z normalized GARP</td>
</tr>
<tr>
<td>µL</td>
<td>microliter</td>
</tr>
<tr>
<td>µM</td>
<td>micromolar</td>
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Chapter 1 : Introduction
1.1 Treatment of Cancer

Cancer poses a significant global health problem and accounts for 13% of deaths worldwide\(^1\). Advances in molecular biology have revealed cancer to be an extremely heterogenous and innately complex disease. High-throughput genomic and transcriptomic analysis has demonstrated that there exist multiple unique molecular subsets not only within a tumour type such as breast\(^2-4\) and lung\(^5\), but complexity can exist within the context of a patient’s individual tumour\(^6-9\). As summarized by Hanahan and Weinberg in their seminal review, tumour cells are characterized by replicative immortality, resistance to apoptosis and anti-growth signals, induction of angiogenesis, as well as the capacity to invade and metastasize\(^10\). Amongst these tumour-defining characteristics, there are the novel emergent hallmarks namely altered metabolism and immune evasion.

Several mechanisms of triggering cell death have been described. There is the classical non-inflammatory programmed apoptosis molecularly mediated by caspases and morphologically characterized by nucleus condensation, membrane blebbing and eventual phagocytosis of apoptotic cells\(^11\). Cells can also die through necrosis, a process mediated by the activation of RIP1 and RIP3 that causes a local inflammatory response\(^11\). Autophagic cell death entails a caspase-independent vacuolization of cellular organelles\(^11\). Although not strictly leading to cell death, aberrations during mitosis termed mitotic catastrophe, can also lead to oncosuppressive cell senescence\(^11\). Resistance to cell death is a pivotal hallmark of cancer that can contribute to tumorigenesis and many anti-cancer agents target this hallmark by triggering tumour cell apoptosis. Increasingly detailed elucidation of the cell death machinery as well as different forms of cell death has afforded novel and more specific ways to target cancer cells\(^12\).

The arsenal of current drug therapies available to treat cancer broadly falls into two general categories: cytotoxic chemotherapy and targeted molecular therapies.
1.1.1 Cytotoxic Chemotherapy

Ever since their inception in the early 1970s for the curative treatment of advanced Hodgkin’s disease\textsuperscript{13}, cytotoxic chemotherapeutic drugs have been used for decades in the treatment of solid tumours and hematological malignancies at various stages of therapeutic intervention. These drugs include: the alkylating agents\textsuperscript{14} such as temozolomide used in treating gliomas\textsuperscript{15}; carboplatin of the platinum compounds\textsuperscript{16} which is the mainstay of ovarian cancer chemotherapy\textsuperscript{17}; the antimetabolites\textsuperscript{18} of which 5-fluorouracil is used in first-line therapy of colorectal cancer; the topoisomerase inhibitors\textsuperscript{19} an example of which is idarubicin used in consolidation and maintenance therapy of acute myelogenous leukemia (AML) patients\textsuperscript{20}; and, the tubulin-binding drugs\textsuperscript{21} such as docetaxel recommended for adjuvant therapy in luminal B and triple negative breast cancer\textsuperscript{22}. These drugs reduce tumour burden by targeting rapidly proliferating cells, and as a consequence, normal tissues are also collaterally affected leading to general systemic side effects such as central and peripheral neurotoxicity, cardiotoxicity, gastrointestinal symptoms and immune suppression\textsuperscript{23-26}. Despite problems of wide-ranging toxicities, intrinsic and acquired drug resistance\textsuperscript{27}, and controversies over clinical benefits in many cancers\textsuperscript{28}, these cytotoxic drugs are still the mainstay of most treatment regimens.

1.1.2 Targeted Molecular Therapies

An increased understanding of the aberrant molecular pathways that drive tumorigenesis has subsequently fuelled the rational design of agents to inhibit them. A target required for aberrant tumour growth, survival and metastasis is usually mutated or over-expressed in the tumour but not normal cells thereby providing the rationale for a tumour-
normal index. The two primary types of targeted therapy are monoclonal antibodies or small molecule inhibitors.

The development of monoclonal antibodies to relieve immune suppression and enhance anti-tumour immunity in immunogenic tumours has begun to fill an important gap in the treatment of metastatic melanoma, a cancer with few treatment options and among the highest mortality rates. Ipilimumab, a monoclonal antibody targeting cytotoxic T lymphocytes antigen 4 (CTLA-4) was approved by the European Union in 2010 and the Food and Drug Administration (FDA) in March 2011 for the treatment of metastatic melanoma patients. CTLA-4 is an inhibitory molecule expressed on T cells during activation. There are currently eight monoclonal antibodies targeting other immune targets such as Programmed cell death protein 1 (PD-1) in clinical trials. Bevacizumab, another monoclonal antibody, blocks vascular endothelial growth factor (VEGF), and stops tumour growth by preventing the formation of new blood vessels. It has been approved for the treatment of metastatic colorectal, breast, kidney and ovarian cancer.

Over the last decade, many small molecule inhibitors have been approved for the treatment of various tumour types. Recent approvals include vemurafenib in the treatment of melanoma with the BRAF V600E mutation, and crizotinib an agent that targets the EML4–ALK (Echinoderm microtubule-associated protein-like 4 - anaplastic lymphoma kinase) fusion protein in non-small-cell lung cancer. The successful drug imatinib, which targets the BCR-ABL fusion protein in Philadelphia chromosome-positive chronic myelogenous leukemia (CML), has been used in the clinic for years and has improved the outcomes of many CML patients. Imatinib also targets c-KIT and has been approved for treated patients with gastrointestinal stromal tumors. The list of other targeted agents, currently approved and in development, is extensive. However, what was once considered the best new method of specifically targeting tumours is fraught with unexpected challenges.
Due to the specificity of the targeted agents they were thought to be less toxic than the traditional cytotoxic agents. However, it is now apparent that the safety of the targeted agents has been over-estimated. Targeted agents often cause organ-specific toxicities. The EGFR inhibitors can cause dermatologic toxicity, the angiogenesis inhibitors and tyrosine kinase inhibitors cardiovascular toxicity, the mTOR (mammalian target of rapamycin) inhibitors metabolic toxicities. These toxicities were not evident during clinical trials as the positive selection of responsive patients masked the effects apparent in a broader patient population characterized by other comorbidities not evaluated in clinical trials. A recent meta-analysis of 38 randomized clinical trials testing novel agents approved by the FDA for the treatment of solid tumours between 2000 and 2010 reported that despite modest improvements in outcomes, these new drugs, were generally more toxic than the old drugs. Despite initial claims of safety, targeted agents also have side effects that have a significant impact on a patient’s quality of life.

At the recent 24th EORTC-NCI-AACR (European Organisation for Research and Treatment of Cancer-National Cancer Institute-American Association of Cancer Research) Symposium on ‘Molecular Targets and Cancer Therapeutics’ in Ireland, the progress and effective incorporation of targeted agents into the clinic were discussed. Redundancies of signaling pathways, innate or acquired resistance, presence of feedback loops that dampen efficacy are all obstacles facing the clinical utility of the targeted agents. The use of targeted agents in anti-cancer therapeutic regimens incurs high costs. For example, cetuximab, a drug targeting the epidermal growth factor receptor (EGFR), approved for the treatment of non-small cell lung cancer (NSCLC) costs $80,000 for an 18-week treatment in the United States and has an extended survival benefit of 1.2 months. On average, bevacizumab costs $90,000 to treat a patient with an overall survival benefit of 1.5 months. Future implementation of cost-effectiveness analyses as well as companion studies to identify biomarkers of response
and consequently sub-populations of cancer patients most likely to benefit from these treatments is necessary for the realistic implementation of these therapies into the standard of care.

While the traditional cytotoxic agents and targeted therapies have made great impact in cancer patient care, their toxicities, lack of efficacies in treating certain tumours and prohibitive costs highlights an urgent need for the development of novel therapeutic strategies.

1.1.2 Drug Repositioning

Exploiting drugs already approved for non-cancerous diseases is an attractive alternative for development of novel anti-cancer therapies\(^{44, 45}\). Since the pharmacokinetic, pharmacodynamic and toxicity profiles of these drugs are already known, their translation into clinical trials testing for their anti-cancer efficacy has the potential to be rapid. As many of these compounds have been on the market for many years in the treatment of other diseases, they are often off-patent and readily accessible form the economic perspective.

Identification of drug candidates for repositioning can occur through chemical screening where vulnerabilities of cancer cell lines exposed to FDA-approved compounds is tested. Using this approach, several such candidates have been recently identified such as the antimicrobial tigecycline for the treatment of AML\(^ {46}\). Similarly, the antiparastistic agent ivermectin was found to have anti-leukemic activity\(^ {47}\). Tumour cell kill can occur through a mechanisms related to the compound’s known mode of action (“on-target repositioning) or a combination of on-target effects and several other pleotropic effects. The oral antiparastic agent, clioquinol, whose mechanism of action related to its antiparastistic efficacy is unknown, was found anti-leukemia activity through inhibition of the proteasome\(^ {48}\).
Some drugs have been used for decades in the treatment of conditions affecting large portions of the population. This can lead to epidemiological evidence suggesting that these drugs could affect the incidence of other conditions such as cancer. Two such examples include metformin and statins. The former has been used for the treatment of non-insulin required diabetes for over 50 years. Numerous case-control and cohort studies have linked metformin usage with reduced cancer incidence\textsuperscript{49,50}. Metformin, whose anticancer effects are attributable to both direct (insulin-independent) and indirect (insulin-dependent) actions of the drug, is currently being evaluated in prospective clinical trials where efficacy is being tested in breast cancer patients\textsuperscript{51,52}.

Statins are a family of drugs that have been used to lower cholesterol for decades in patients with cardiovascular and coronary heart disease, also have anti-cancer effects. Their application as anti-cancer therapeutics is the focal point of this body of work.

1.2 Repositioning Statins as anti-cancer agents

1.2.1 Statins in the cardiovascular setting

The observation that patients dying from occlusive vascular disease had thick and irregular artery walls eventually led to the development of the lipid hypothesis linking coronary heart disease (CHD) death with high levels of LDL-cholesterol. In 1973, the pioneering work of Goldstein and Brown demonstrated that 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGCR) activity, already known to be the rate-limiting enzyme of cholesterol biosynthesis, was correlated to the extracellular levels of lipoproteins in cultured fibroblasts\textsuperscript{53} It was correctly rationalized that by inhibiting HMGCR, plasma LDL-cholesterol could be lowered. Efforts to find such a compound culminated in the discovery of mevastatin, a microbial metabolite capable of potently inhibiting HMGCR\textsuperscript{54,55}. Mevastatin’s \textit{in vivo}
efficacy was subsequently demonstrated in humans\textsuperscript{56}. Despite demonstrated efficacy in lowering serum cholesterol levels, mevastatin was not marketed and the first commercially available statin was lovastatin, a natural product isolated from \textit{Aspergillus terreus}.

In the early 1990’s other synthetic lovastatin analogues soon followed with simvastatin and pravastatin becoming available. However, it wasn’t until the seminal Scandinavian Simvastatin Survival Study (4S) that established the role of statins in the prevention of adverse cardiovascular events\textsuperscript{57} including mortality. The study randomized 4444 patients with angina pectoris or previous myocardial infarctions into two-arms, with one arm receiving simvastatin and the other a placebo. After a five year follow up, it was found that patients in the simvastatin arm suffered coronary deaths, less major coronary events and a decreased risk of undergoing myocardial revascularisation procedures. These reductions were correlated to decreased in total plasma cholesterol, LDL-cholesterol and increases in high-density lipoprotein (HDL) cholesterol. Similar large studies\textsuperscript{58,59} demonstrating the efficacy in the treatment of hypercholesterolemia followed, cementing their role in the prevention of cardiovascular disease and treatment of hyperlipidemia.

1.2.2 The pharmacology of statins

There are currently seven statins approved in North America for cardiovascular indications. Structurally, synthetic and naturally occurring statins are composed of a core pharmacophore, common to all statins and an attached moiety, a ring (reduced naphthalene, indole, pyrrole or quinolone) with different substituents is the distinguishing feature between statins. The pharmacophore binds HMGCR in a competitive, reversible way and the substituents on the ring define a statin’s solubility and other pharmacological properties listed in Table 1-1\textsuperscript{60,61}. Typical cholesterol-lowering dose range from 20-80 mg and are orally
administered daily in tablet form. Statins undergo extensive first-pass uptake and metabolism by the liver. The lipophilic statins passively diffuse into hepatocytes and peripheral tissues whereas hydrophilic statins are transported into the liver by organic anion transporting polypeptide (OATB) family of drug transporters and as such their systemic penetration into extra-hepatic sites is limited. Potential adverse drug interactions between statins and other drugs occur with the statins metabolized by the cytochrome p450 A4 (CYP3A4) enzyme, mainly simvastatin, lovastatin and atorvastatin. Statins are generally well tolerated with mild side effects. Hepatotoxicity⁶² and myotoxicity which may progress to rhabdomyolysis⁶³ are amongst the most serious side-effects but do not occur in the majority of patients. High statin doses (Table 1-1), up to 25 times the normal cholesterol-lowering doses, have been administered to humans and were tolerated. Taken together, statins are safe, well tolerated and their pharmacology and toxicology has been well investigated.
<table>
<thead>
<tr>
<th>Statin</th>
<th>Lipophilic</th>
<th>Requires Metabolic Activation</th>
<th>Bioavailability (%)</th>
<th>Binding (mg/ml)</th>
<th>Plasma Protein (%)</th>
<th>Plasma Protein Binding Site</th>
<th>Plasma Protein Peak Concentration (mg/ml)</th>
<th>Plasma Protein (mg)</th>
<th>Metabolism CYP3A4</th>
<th>Highest Known Lipophilic Stain</th>
<th>Tolerated dose in cancer patients</th>
<th>Maximum allowed cholesterol-lowering dose (20-80 mg/day)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lovastatin</td>
<td>yes</td>
<td>no</td>
<td>yes</td>
<td>160-260</td>
<td>&gt;95</td>
<td>35</td>
<td>160-260</td>
<td>no</td>
<td>1680 mg/day (ref 104)</td>
<td>Pitavastatin</td>
<td>yes (CYP2C9)</td>
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</tr>
<tr>
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<td>yes</td>
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<td>yes</td>
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<td>&gt;95</td>
<td>11</td>
<td>47-55</td>
<td>no</td>
<td>1680 mg/day (ref 104)</td>
<td>Simvastatin</td>
<td>yes (CYP2C9)</td>
<td>yes (CYP2C9)</td>
</tr>
<tr>
<td>Atorvastatin</td>
<td>yes</td>
<td>no</td>
<td>yes</td>
<td>10-20</td>
<td>94-95</td>
<td>3</td>
<td>10-20</td>
<td>no</td>
<td>1680 mg/day (ref 104)</td>
<td>Fluvastatin</td>
<td>yes (CYP2C9)</td>
<td>yes (CYP2C9)</td>
</tr>
<tr>
<td>Simvastatin</td>
<td>no</td>
<td>yes</td>
<td>yes</td>
<td>7-10</td>
<td>80-98</td>
<td>2</td>
<td>448 (1050)</td>
<td>no</td>
<td>10-20</td>
<td>no (CYP2C9)</td>
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<tr>
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<td>no</td>
<td>yes</td>
<td>yes</td>
<td>20-30</td>
<td>80-98</td>
<td>12</td>
<td>445 (1100)</td>
<td>no</td>
<td>10-20</td>
<td>no (CYP2C9)</td>
<td>10-20</td>
<td></td>
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<tr>
<td>Pravastatin</td>
<td>no</td>
<td>yes</td>
<td>yes</td>
<td>20-30</td>
<td>80-98</td>
<td>30</td>
<td>445 (1100)</td>
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<td>yes</td>
<td>20</td>
<td>88</td>
<td>&gt;95</td>
<td>10-20</td>
<td>no</td>
<td>1680 mg/day (ref 104)</td>
<td>no (CYP2C9)</td>
<td>10-20</td>
<td></td>
</tr>
</tbody>
</table>

* Table was adapted and modified from Gazzerro et al. (57) and Shitara et al. (58)
** Following a typical cholesterol-lowering dose (20-80 mg/day)
1.2.3 The mevalonate pathway and cancer

The mevalonate (MVA) pathway is an essential metabolic pathway whose end products provide the cell with bioactive molecules (Figure 1-1). These include: sterols such as cholesterol involved in membrane integrity and steroid production; farnesyl and geranylgeranyl isoprenoids which are substrates for the post-translational modification of proteins such as those of the Ras family; dolichol; ubiquinone; and isopentenyladenine. In hepatocytes, inhibition of HMGCR, the rate-limiting enzyme of the MVA pathway, and subsequent depletion of sterols leads to a restorative feedback response mediated by the transcription factor sterol regulatory element binding transcription factor 2 (SREBP2). When deprived of sterols, the latent SREBF2 is activated, transported to the golgi, cleaved by golgi-resident site-1 and -2 proteases and translocated to the nucleus where it binds to sterol responsive elements (SRE) initiating transcription of sterol responsive genes such as HMGCR and low-density lipoprotein receptor (LDLr). Increased LDLr transcription leads to higher LDLr protein at the membrane surface and LDL-uptake leading to decreased plasma cholesterol levels\(^{53, 64-66}\). Drs Goldstein and Brown revealed this elegant regulation of cholesterol homeostasis for which they were awarded the Nobel prize\(^{67}\). Studies in other normal cells, limited mostly to circulating mononuclear cells have suggested that normal cells are subject to similar regulation. A recent report demonstrated that following treatment of healthy subjects with atorvastatin, LDLr mRNA levels were increased in circulating mononuclear\(^{68}\) and others have reported elevated HMGCR activity in mononuclear leukocytes of healthy subject following statin administration\(^{69}\).
Figure 1-1: The MVA pathway.

Statins block the mevalonate pathway by inhibiting the rate-limiting enzyme, HMG-CoA reductase (HMGCR). Statins compete with the natural substrate, HMG-CoA, for the active site of HMG-CoA reductase. Mevalonate (MVA), the product of the reaction, is the precursor to many critical cellular end-products essential for cell proliferation and survival.
Several lines of evidence suggest that the MVA pathway is dysregulated in cancer at several levels and not subject to normal feedback control. Acute leukemia patients are often hypocholesterolemic and their leukemic cells were found to have higher LDL-receptor (LDLr) activity inversely correlated with their plasma cholesterol levels\textsuperscript{70} and as a consequence higher LDL uptake\textsuperscript{71}. Furthermore, there was elevated HMGCR activity in mononuclear cells from leukemic patients relative to mononuclear cells from healthy subjects\textsuperscript{72} and leukemic cells were not as responsive to sterol changes as normal cells\textsuperscript{73}. Others groups have found that AML cells isolated from patients increase intracellular cholesterol in response to treatment with chemotherapeutic agents although this was not correlated with increased LDL accumulation indicating that de novo cholesterol synthesis was responsible for this increase\textsuperscript{74}. Taken together, aberrant cholesterol homeostasis and elevated HMGCR activity has been found in AML patient cells indicating that a window of therapeutic intervention by agents that target the MVA pathway such as statins exists.

Aberrant MVA pathway regulation has also been documented in other hematological malignancies. Our laboratory has surveyed 17 multiple myeloma (MM) cell lines and stratified them according to their susceptibility to undergo lovastatin-induced apoptosis\textsuperscript{75}. Yielding approximately equal sized cohorts defined by clear differences in statin-sensitivity, enabled the further characterization of the panel and find molecular markers of statin sensitivity. The MM cell lines had a wide range of genetic abnormalities and some abnormalities differed and were common to both cohorts of MM cell lines. However, no clear cohort-defining abnormality that could be correlated with statin-sensitivity was found although this lack of a genetic biomarker could be a consequence of the underpowered study. Interestingly, in subsequent studies, our group found that the MM cell lines that were sensitive to lovastatin-induced apoptosis were deficient in sterol-feedback control and unable to upregulate HMGCR and hydroxymethylglutaryl coenzyme A synthase 1 (HMGCS1)\textsuperscript{76}. The
conferral of sensitivity in MM cell lines with abnormal sterol-feedback responses further strengthens the concept that tumour cells with dysregulated MVA pathway are targetable by statins.

Our group found that over-expression of HMGCR mRNA, and other members of the MVA pathway including HMGCS1 and farnesyl pyrophosphate synthase (FDPS), was correlated with poor prognosis of and decreased survival of breast cancer patients. In the same study we found that ectopic introduction of the catalytic portion of HMGCR induced transformation in non-transformed cells and increased oncogenic potential in transformed cells cell culture and in vivo. A recent study investigating mutant protein 53 (p53)’s disruptive effect on mammary acinar morphogenesis showed that the mutant’s effects are mediated by the MVA pathway which was over-expressed in breast cancer cells harbouring the mutant and grown in 3D (3 dimensional) culture. Following analysis of five breast cancer patient data sets, it was determined that the samples harboring p53 mutations were also characterized by elevated expression of sterol biosynthesis genes compared to patient tumours bearing wild-type p53. Furthermore, clustering analysis demonstrated that the cluster with the highest MVA gene expression was associated with the poorest prognosis. Examined individually, over-expression of MVA pathway genes, including HMGCR and HMGCS1, was also correlated with worse prognosis. An earlier report noted that exogenous addition of MVA promoted in vivo tumour growth of subcutaneous xenografts. Although it is not clear whether the dysregulation of the MVA pathway plays a causal role in oncogenesis, MVA pathway activity is important in cancer. MVA pathway dysregulation has been demonstrated at multiple levels in cancer cell lines and primary patient samples. A dysregulated MVA pathway presents a tumour vulnerability with the potential of positive therapeutic consequences when targeted by agents, such as statins, that inhibit it.
1.2.4 Statins as preventative anti-cancer agents

Millions of patients are prescribed statins on a yearly basis enabling retrospective case-control and prospective cohort studies to uncover potential protective non-cardiovascular effects. Accumulating epidemiological evidence accrued over the last two decades suggests statin use may reduce cancer incidence but conclusive evidence recommending statin use for primary prevention is lacking. Ample epidemiological analysis exists for the most common cancers such as breast and lung cancer. In breast cancer, several large cohort studies amongst them the Cancer Prevention Study II Nutrition Cohort and the Kaiser Permanente and case-control studies have demonstrated no relationship between statin use and incidence of breast cancer. Some of these studies were criticized for lack of information on potential confounders such as physical activity and diet, short follow-up times, lack of consideration for duration and type of statins used. When considering key variables such as the nature of the statin used, duration of usage, and tumour sub-type positive associations were evident. A large cohort study found that there was an 18% reduction in breast cancer risk amongst lipophilic statin users. A case-control study found that long term statin use of greater than 5 years was associated with a decreased breast cancer risk. A retrospective cohort study analyzing the occurrence of ER/PR-negative (estrogen receptors/progesterone receptors) tumours amongst statin users found that breast cancer patients taking statins had fewer ER/PR-negative tumours, which were of lower grade and stage.

Although studies examining the use of statins for the secondary prevention of breast cancer have not been prevalent the few published studies have been extremely encouraging of statin use to prevent breast cancer recurrence. A prospective cohort study found that lipophilic statin use amongst early stage breast cancer patients, post-cancer diagnosis, was associated with decreased risk of breast cancer recurrence. The risk of recurrence in this study was furthermore inversely correlated with duration of statin use. A recently published, much larger
and better-powered Danish study found a significant decrease in breast cancer recurrence amongst lipophilic statin users\textsuperscript{87}. Of twenty-five epidemiologic studies composed of case-control, cohort and examining statin use and incidence or recurrence of breast cancer, seven found that there was a reduction in breast cancer incidence or recurrence associated with statin use.

Amongst lung cancer patients, many studies reported no association between statin use and lung cancer risk\textsuperscript{88,89}. However, these studies were characterized by relatively low numbers of statin users. A large cohort study of 2000 veteran lung cancer patients, on the other hand found that statin use was associated with a 45% decreased risk of lung cancer\textsuperscript{90}. Another retrospective cohort study composed of veterans reported a 30% reduction in the risk of lung cancer amongst statin users\textsuperscript{91}. Taken together, there is supporting epidemiological evidence to indicate that statins may play a role in the prevention of lung cancer amongst male users. Of fourteen epidemiologic studies composed of case-control, cohort and examining statin use and incidence of lung cancer, three found that there was a reduction in lung cancer incidence associated with statin use. However, unlike in breast cancer, few attempts have been made to focus on effects specific to lung cancer sub-types and types of statin used.

In hematological malignancies there has been some evaluation of preventive statin use but this has been largely limited by relatively low numbers of patients suffering from these cancers. The largest study examining lymphoid neoplasms, the European case-control EPI-LYMPH study, found that there was a significant reduction in lymphoma risk amongst statin-users. The same risk reduction were maintained when the 2,362 patients were stratified according to histological subtype including multiple myeloma (MM) although that group contained only 288 MM patients\textsuperscript{92}. Mention of the role of statins as preventative agents in other tumour types not presented in this thesis is beyond the scope of this introduction and
this topic comprehensively reviewed elsewhere\textsuperscript{93}. There is supportive evidence that statin use decreases the incidence and recurrence of breast cancer, lung cancer and hematological malignancies, especially when the type of statin used, tumour sub-type and duration of statin use is carefully considered. However, overall evidence supporting the chemopreventive effects of statins is inconsistent and reflects the limited nature of observational studies. Many of these observational studies did not properly evaluate links between prevention and statin dose and duration of statin use. Confounding by indication cannot be ruled out. Statin-users may differ from non-statin users in a way that might positively impact cancer risk factors independent of any specific effects of statins. Adherence to drug therapy especially for preventative purposes has been linked to individuals that are generally more health-conscious and have more education. It has been suggested that low levels of cholesterol are associated with increased cancer incidence\textsuperscript{94, 95}, and it may be that hypercholesterolemic patients are protected for years by their increased cholesterol levels before they start to use statins. Taken together, to ultimately address whether statin use is associated with a positive anti-cancer effect, prospective clinical trials directly assessing statin benefit in cancer patients need to carried out.

1.2.5 Statins in the clinic

Early cell culture studies in the nineties exploring the anti-cancer effects of statins primarily used lovastatin in a variety of solid and hematological tumour cell lines. Lovastatin was shown induce cell cycle arrest in a dose and time-dependent manner in the solid tumour cell lines\textsuperscript{96-98} and in hematological cells\textsuperscript{99-101} at concentrations ranging from 2-10 μM. The statin induced anti-cancer effects were determined to be a result of direct HMGCR inhibition as they were reversible with the addition of MVA. As the cell culture lovastatin
concentrations used to inhibit growth or induce apoptosis were clearly above what was clinically achievable with the typical 10-80 mg (~0.15-1 mg/kg/day) oral daily dose, initial prospective clinical trials involving the evaluation of statin use in cancer patients have sought to address key issues such elevated dosing and potential dose limiting toxicities. Early dose-finding studies established that lovastatin can be tolerated at high concentrations greatly exceeding cholesterol-lowering doses. In two separate studies, short-term oral lovastatin administration at doses ranging from 1 to 45 mg/kg/day to patients with advanced pre-treated malignancies was tolerated\textsuperscript{102,103}. In the earliest study, lovastatin was administered 88 patients with a variety of solid tumours for seven consecutive days in monthly cycles and was well tolerated at doses up to 25 mg/kg; above that dosing, side effects such as myopathy, fatigue and nausea occurred but could be partly controlled with the concomitant administration of ubiquinone\textsuperscript{102}. In a similarly dosed phase I study, 18 patients with glioma and glioblastoma multiforme were administered lovastatin of 30 mg/kg/day and did not experience adverse reactions during treatment\textsuperscript{103}. High doses of other statins such as simvastatin are also well tolerated. Simvastatin at doses of 15 mg/kg/day was administered in patients with relapsed or refractory myeloma or lymphoma\textsuperscript{104}. Taken together, statins are well tolerated at levels greatly exceeding cholesterol-lowering doses.

The pharmacodynamics of statins administered at higher doses have been also been addressed albeit in fewer studies. A trial where lovastatin was administered at a dose of 10 mg/m\textsuperscript{2} to 415 mg/m\textsuperscript{2} every 6 hours for 96 hours measured lovastatin peak plasma bioactivity levels of 0.06-12.3 µM\textsuperscript{105}. However, the measured concentrations were not dose-dependent and any dose-dependent relationship is likely complicated by patient variability to hydrolyze lovastatin to the active form of the drug. The above-mentioned early lovastatin trial also detected micromolar concentrations of lovastatin peaking at 3.9 µM\textsuperscript{105}. Although concentrations of lovastatin equivalent to doses needed in tissue culture studies to achieve an
anti-cancer effect were detected in some patients, very few objective responses were evident in those trials were lovastatin was tested in a monotherapy setting.

Statin efficacy, when combined with other agents has been demonstrated. A few studies have documented statin therapy success in hepatocellular carcinoma (HCC). HCC patients receiving pravastatin (20-40 mg/day) and with the standard of care transarterial chemoembolization (TACE) experienced a significantly longer median survival when compared to patients receiving TACE alone\textsuperscript{106}. Another group demonstrated pravastatin efficacy in HCC as an adjuvant treatment following transcatheater arterial embolization (TAE) and oral 5-FU treatment\textsuperscript{107}. Recently, a trial combining lovastatin with thalidomide and dexamethasone (TD) in patients with relapsed or refractory MM illustrated lovastatin prolonged overall survival and progression-free survival\textsuperscript{108}. Encouraging response rates in an AML phase I trial where pravastatin of doses of up to 1680mg/day (24mg/kg/day) was safely combined with idaraubicin and high dose cytarabine, in newly diagnosed and salvage AML patients\textsuperscript{109} has warranted an ongoing follow-up multi-site phase II trial (NCT00840177). In a neo-adjuvant trial, women with stage I breast cancer and ductal carcinoma in-situ (DCIS) were administered 80 mg or 20 mg of fluvastatin 3-6 weeks before surgery. Fluvastatin treatment reduced proliferation and increased apoptosis in the high-grade tumours\textsuperscript{110}.

According to ClinicalTrials.gov, there are 35 clinical trials involving statins being evaluated in the clinic as anti-cancer agents: 6 trials in breast cancer patients, 5 in lung cancer patients and 6 in patients with hematological malignancies. Out of the 35 studies, 4 are statin prevention studies involving women with either high-inherited risk for breast cancer or who have previously undergone surgery for ductal carcinoma. Monotherapeutic use of statins is a feature of 11 of the 35 clinical trials; 2 are early phase safety studies. Interestingly, another 2 of the clinical trials where statin is used as a monotherapy will attempt to elucidate a biomarker of statin response, assess intracellular statin levels (NCT00828282), and test the
hypothesis of statin-selective effects on basal subtype breast cancer (NCT00807950). The majority of the ongoing studies are testing statins in a combination setting. Twenty clinical studies are evaluating statins in combination with other agents at various points of therapeutic intervention. Most of these agents are the standard of care chemotherapies. Interestingly, there are two active studies also combining statins with non-standard agents. One single group assessment trial is combining simvastatin with metformin in treating men with recurrent prostate carcinoma (NCT01561482). Another study, also currently recruiting, will treat chemotherapy resistant, refractory MM patients with simvastatin and zoledronic acid in addition to the chemotherapy (NCT01772719). The results from the large number of ongoing studies are eagerly awaited and will be instructive in how best to apply statins in clinical use.

These prospective trials have been and will continue to be inexorably far more informative than the non-interventional retrospective analyses.

Statins are tolerated at high doses but it remains unclear whether they will need to be administered at high doses as clinical anti-cancer efficacy has been demonstrated at both cholesterol lowering doses\textsuperscript{106, 107, 110-113} and higher statin doses\textsuperscript{108, 109}. Statin-responsive tumours include hematological malignancies, AML\textsuperscript{109, 111} and MM\textsuperscript{108, 113} and breast\textsuperscript{110, 112}. Although there are clinical trials testing statins as single agents, many of these studies are not only evaluating patient response but are also seeking to establish the safety of statins in a particular clinical setting or attempting to establish a biomarker of statin response. Statins are unlikely to be used as a monotherapy with the possible exception in neo-adjuvant setting. This is not only a consequence of limited statin anti-cancer efficacy when used alone\textsuperscript{102, 103, 114, 115}, but a consequence of trying to eradicate an inherently complex and heterogeneous disease necessitating the need for multi-modal combinatorial approaches.
1.3 Combination therapy

There are many obstacles to effective drug therapy when treating cancer patients. Not only are tumour cells heterogeneous and dependent on many altered molecular pathways subject to complex intertwining feedback loops, but they can quickly develop resistance to single agents. Resistance, intrinsic or acquired is equally a problem for the classically used cytotoxics and novel targeted therapies. Single agents are rarely able to provide sustained long-term response and there are numerous benefits to combinatorial approaches. Drugs with different mechanisms of action share non-overlapping toxicities. Their co-administration minimizes individual resistance. For targeted agents, combination therapy can combat feedback loops and a redundancy in signaling, factors that often times limit the efficacy of a single agent.

The therapy-limiting presence of feedback loops was elegantly demonstrated in a recent screen that sought to understand the unresponsiveness of colon cancer harboring the BRAF (V600E) oncoprotein. BRAF (V600E) is targeted by vemurafenib, a small molecule inhibitor approved for the treatment of melanoma. Through an RNA interference screen in colon tumour lines, it was shown that knockdown of the epidermal growth factor receptor (EGFR) displayed strong synergy with vemurafenib. Colon cancer cells rapidly activate EGFR in response to vemurafenib treatment, in contrast to melanoma cells that express low EGFR levels, thereby negating any anti-tumoural vemurafenib effects. Therefore, using vemurafenib in combination with an EGFR targeted agent such as erlotinib in the treatment BRAF (V600E) bearing-oncoprotein colon tumours would circumvent this therapy-limiting feedback activation. These rationally crafted combinations of targeted agents are currently being evaluated in the clinic but require a thorough molecular understanding of each individual targeted agent and the signaling
pathways they perturb. Furthermore, the costs involved in combining two targeted agents in chemotherapeutic regimen are likely cost-prohibitive.

Recent efforts to identify novel combinations of anti-cancer agents, discussed at the 24th EORTC-NCI-AACR Symposium on ‘Molecular Targets and Cancer Therapeutics’\(^{42}\) have explored unbiased approaches. Led by S. Holbeck, 100 FDA-approved small-molecule anti-cancer agents are being screen in combination on the NCI’s panel of 60 cell lines and thousands of unexpected combinations are evaluated for their anti-cancer efficacy. Preclinically successful combinations, that include agents like statins, can be fast-tracked into clinical trials because their pharmacokinetic, dosing and toxicity have already been established. The quest for novel, efficacious combinations is paramount to the successful treatment of patients that do not respond to the standard of care treatments currently available.
1.4 Research Objectives and thesis outline

My global hypothesis is that targeting the mevalonate (MVA) pathway in tumour cells is an effective strategy to induce tumour cell apoptosis. My research objectives are to explore how to maximize statin induced apoptosis: (i) through the use of a pharmacological screen to identify compound/s that synergize with statins in inducing apoptosis in AML and MM, (ii) through the use of a genome-wide shRNA screen to identify novel pathways/targets whose knockdown enhances the vulnerability to statin-inhibition in lung and breast cancer.

Chapter 2 of this thesis uncovers a novel drug combination with strong antimyeloma and antileukemic activity. Our focus in hematological malignancies stems from preclinical and clinical evidence generated by our group and others, demonstrating dysregulation of the MVA pathway in both AML and MM. Furthermore, positive evidence hinting at statin efficacy AML and MM patients following statin treatment suggested to us that statins will be clinically useful in these tumour types. Through the use of a pharmacological screen composed of 100 off-patent, FDA-approved drugs, dipyridamole was found to potentiate the anti-cancer effects of atorvastatin in multiple myeloma. Subsequent validation in a panel of MM and AML cell lines demonstrated that the combination was synergistic and capable of inducing apoptosis. Primary MM and AML patient samples were also vulnerable to the combinations’ apoptosis-inducing effects and effectiveness in a leukemia xenograft model was demonstrated. The chapter is concluded by explorations into the mechanism of drug synergy.

Chapter 3 of this thesis details the execution of a genome-wide shRNA screen in the A549 lung cancer cell line stably transduced with the RNAi Consortium (TRC1) shRNA library. Sublethal fluvastatin exposure over several passages uncovered drop-out hits identified through microarray hybridization of amplified genomic DNA. Several hits were
validated in lung and breast cancer cells, the most promising of which, the transcription factor SREBF2, was also characterized in vivo. Other promising kinases were also identified which are subject to further exploration.

Chapter 4 of this thesis discusses the overall feasibility of effectively repurposing statins as anti-cancer agents and focuses on discussing the gaps between the cell culture molecular mechanisms postulated to be responsible for the anti-cancer effects and the in vivo translational relevance of these findings. Furthermore, a discussion on the importance of finding statin-responsive patients and a biomarker of statin response or intrinsic sensitivity will be discussed. This is paramount to extending the benefit of statins to as many patients without incurring unwarranted side effects. Overall, this thesis extends our understanding of how to maximize the anti-tumour effects of statins by successfully combining them with novel, unexpected agents and shRNAs targeting kinases and enzymes of the MVA pathway.
1.5 References


Chapter 2: Combining statins and dipyridamole effectively targets acute myelogenous leukemia and multiple myeloma

Contributions:

The majority of work presented in this chapter was completed by the author of this thesis. Additional contributions are as follow:

Zhihua Li carried out experiments for Figure 2.6 D

Dr. Peter J Mullen and Janice Pong carried out experiments for Figure 2.9
2.1. Abstract

Statins have been successfully utilized for decades to treat patients with hypercholesterolemia. Promising preclinical evidence indicates that statins may be useful as anti-cancer agents and that their anti-tumour efficacy is increased when combined with other agents. To identify novel therapeutic combinations with elevated anti-cancer activity, we screened atorvastatin in combination with FDA approved drugs and identified dipyridamole, a drug used for the prevention of cerebral ischemic events, as a potentiator of the anti-myeloma activity of statins. The statin-dipyridamole combination was synergistic and induced apoptosis in cell lines and primary cells from myeloma and acute myelogenous leukemia patients. Additionally, this novel drug combination decreased tumour growth of leukemia xenografts. To decipher which activities of dipyridamole contribute to the synergy, we combined statins with drugs mimicking each of the many pharmacological actions of dipyridamole. We determined that modulators of cAMP (Cyclic adenosine monophosphate), in combination with statins, also have anti-cancer activity. This suggests that the mechanism of synergy between statins and dipyridamole may be attributable to the inhibitory effect of dipyridamole on phosphodiesterases (PDEs). This work provides a strong rationale to evaluate the therapeutic efficacy of a novel combination of the FDA approved drugs, statins and dipyridamole, in further clinical investigations.
2.2. Introduction

There is an urgent need for novel therapeutic strategies in treating both acute myelogenous leukemia (AML) and multiple myeloma (MM) especially in heavily pre-treated and relapsed patients. Despite recent advances in MM treatment with the introduction of thalidomide, landolidomide and bortezomib, it is difficult to achieve progression free survival beyond 36 months\(^1\). In AML, survival is poor following relapse and 40-50% of older AML patients and 20-30% of younger AML patients will experience primary inductive failure\(^2\).

Statins, potent inhibitors of the rate-limiting enzyme in the mevalonate (MVA) pathway, 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGCR)\(^3\) have been used for decades in the treatment of patients with hypercholesterolemia. Their frequent use in the prevention of adverse cardiovascular events has led to accumulating epidemiological evidence that suggests statin use may reduce cancer incidence\(^4\)-\(^6\). In hematological malignancies, we and others have shown that statins can effectively trigger tumour-specific apoptosis\(^7\)-\(^12\),\(^13\) The apoptotic effects of statins have been attributed to direct inhibition of HMGCR in tumour cells followed by depletion of fundamental MVA-derived end-products such as isoprenoids and cholesterol\(^10\),\(^14\),\(^15\). In tumour cells, dysregulation of the MVA pathway at several levels has been postulated to be responsible for the observed therapeutic index. For example, higher tumour levels of HMGCR and other MVA pathway enzyme mRNA are associated with poor prognosis and reduced survival in cancer patients\(^16\),\(^17\). Dysregulation of the MVA pathway at the level of the restorative sterol-feedback coordination is present in both MM\(^9\) and AML\(^18\),\(^19\). In AML, dysregulation of the MVA pathway can also cause abnormal cholesterol homeostasis leading to overactive production and import of cholesterol contributing to decreased therapeutic efficacy\(^11\),\(^20\). Taken together, dysregulation of the MVA pathway in hematological malignancies provides a strong rationale for statin therapy.
Initial prospective clinical trials involving the evaluation of statin use in cancer patients have sought to address key issues such as dose limiting toxicities. Early dose-finding studies established that statins can be tolerated at high concentrations greatly exceeding cholesterol-lowering doses which range from 20 to 80 mg/day (~0.3-1 mg/kg/day). In two separate studies, short-term oral lovastatin administration at doses ranging from 1 to 45 mg/kg/day to patients with advanced pre-treated malignancies was well tolerated\textsuperscript{21,22}. Simvastatin was tolerated at doses of 15 mg/kg/day in patients with relapsed or refractory myeloma or lymphoma\textsuperscript{23}. In a phase I trial combining pravastatin with idaraubicin and high dose cytarabine, in newly diagnosed and salvage AML patients, pravastatin doses of up to 1680 mg/day (24 mg/kg/day) were safely administered\textsuperscript{24}. Therefore, high doses of statins can be safely administered in the clinical cancer care setting but the ideal dosing regimen remains unclear as anti-cancer efficacy has been observed with both high\textsuperscript{24} and low\textsuperscript{25,26} cholesterol-lowering doses.

Statins have also been safely combined with the standard of care therapy regimens in MM and AML. Recently, a trial combining lovastatin with thalidomide and dexamethasone (TD) in patients with relapsed or refractory MM illustrated lovastatin prolonged overall survival and progression-free survival\textsuperscript{27}. Encouraging response rates as well as the well-tolerated addition of pravastatin in the abovementioned AML phase I trial\textsuperscript{24} has warranted an ongoing follow-up phase II trial (NCT00840177). However, in a trial of twelve refractory or relapsed MM patients simvastatin was administered with vincristine, doxorubicin and dexamethasone (VAD chemotherapy) and only one partial response was observed\textsuperscript{28}. Statins can be combined with the standard of care in AML and MM patients without serious side effects in inductive, consolidation and maintenance therapy settings. While this approach has shown some promise, there remain many non-responsive patients, highlighting an urgent need to develop new additional synergistic combinatorial approaches utilizing statin chemotherapy.
Building on promising results of statins as anti-cancer agents in AML and MM, we conducted a pharmacological screen of FDA approved drugs in combination with statins to identify novel combinations with anti-cancer efficacy in hematological malignancies that could be immediately used for patient care. The screen identified dipyridamole, a commonly used anti-platelet agent for the prevention of secondary stroke and ischemic heart attack, as a potentiator of the anti-proliferative effects of statins in AML and MM cell lines. The combination, synergistic and capable of inducing apoptosis at low micromolar doses in AML and MM cells, effectively slowed tumour growth in a leukemia xenograft model and induced apoptosis in primary AML and MM patient samples. Dipyridamole is known to elicit numerous effects including phosphodiesterase (PDE) inhibition. Modulators of cAMP signaling including other PDE inhibitors, also induced apoptosis in combination with statins. Taken together, these findings not only postulate a role of the cAMP pathway in MVA pathway regulation, but provide a strong rationale for developing statin-dipyridamole therapy for AML and MM patients.
2.3. Results

2.3.1 A screen of pharmacologically active drugs identifies dipyridamole as a potentiator of the anticancer effects of atorvastatin

To identify a therapeutically effective combination of drugs with novel anticancer activities using an unbiased approach, we screened atorvastatin in combination with a library of 100 on-and off-patent drugs composed of antimicrobials and metabolic regulators. Well known pharmacokinetic profiles as well as high achievable plasma concentrations were amongst the characteristics of the drugs in the library. The KMS11 MM cell line was treated for 72 hours with either a singularly sublethal dose of atorvastatin (0-20% effect on viability), each of the 100 drugs alone or in combination with atorvastatin. The combination of dipyridamole, a well-known anti-platelet agent and atorvastatin was found to decrease cell viability as assessed by MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) activity (Figure 2-1A). Validation in a panel of malignant AML and MM cell lines showed that dipyridamole was capable of significantly potentiating the antiproliferative effects of atorvastatin at multiple doses (Figure 2-1B) following 48 hours of incubation and as measured by cellular metabolic activity, an indicator of cell viability, assessed by the MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. Dipyridamole alone, used at a concentration of 5 µM, did not have significant effects on cell viability. A significant increase in the anti-cancer effect of atorvastatin was also observed when combined with lower micromolar doses (2.5 µM) of dipyridamole in the AML and MM cell lines (data not shown). Statins, of which there are six approved in North America, are often used interchangeably. However, structural differences of each statin governing key facets such as metabolism, drug interactions and lipophilicity, not only impact their cholesterol-lowering efficacies but also pleiotropic effects. We therefore extended our
investigations to include fluvastatin, another lipophilic statin and found that dipyridamole was also able to potentiate its anti-cancer effects (Supplementary Figure 2-1A).
Figure 2-1: A drug screen reveals dipyridamole as a potentiator of the anti-proliferative effects of atorvastatin.

(A) The KMS11 MM cell line was exposed to 100 drugs (at concentrations of 5-50 µM) alone and in combination with a sublethal dose of atorvastatin (3.5 µM) for 72 hours. Dipyridamole (open black square) was identified as a compound decreasing MTS activity in combination with atorvastatin. Results are representative of two independent screens. (B) Dipyridamole potentiated the cytotoxic effects of atorvastatin at various doses, in AML (OCI-AML2, OCI-AML3, HL-60) and MM (LP1, KMS11, 8226) cell lines as assessed by MTT assay following 48 hours of compound exposure, either atorvastatin (closed circle) or atorvastatin and 5 µM dipyridamole (closed square). *p < 0.05 (t-test, unpaired, two-tailed). Data represent the mean ± SD of three independent experiments.
2.3.2. The combination of statins and dipyridamole is synergistically antiproliferative and induces apoptosis in AML and MM cell lines

We next evaluated whether the statin-dipyridamole combination was synergistic in the panel of AML and MM cell lines. We treated cells with increasing concentrations of statin and dipyridamole alone and in combination (using a constant ratio at all doses) for 48 hours and used the MTT assay to assess effects on viability. Synergy at multiple effect levels was evaluated using the combination index (CI), where CI values of less than 1 indicate synergy\textsuperscript{30}. The combination of atorvastatin and dipyridamole synergistically decreased cell viability at multiple effective concentrations as indicated by the CI values at the EC\textsubscript{50} (50% effective concentration), EC\textsubscript{25}, EC\textsubscript{75} and EC\textsubscript{90}, which were significantly lower than 1 in all AML and MM cell lines (Figure 2-2).
Figure 2-2: The statin-dipyridamole combination is synergistic in AML and MM cell lines.

AML and MM cell lines were exposed to increasing concentrations of atorvastatin, dipyridamole, or atorvastatin-dipyridamole combination in a fixed ratio and dose-response curves were generated. Results were analyzed to evaluate synergy using the combination index (CI). CI < 1 indicates synergy, CI = 1 indicates additivity and CI > 1 indicates antagonism. Representative CI-fractions effect ($f_a$) plots show the atorvastatin-dipyridamole combination is synergistic at multiple effective concentrations in (C) AML and (D) MM cell lines. The EC50 (50% effective concentration) EC25, EC75 and EC90 from at least three independent experiments are shown for (C) AML (D) MM cell lines. *p < 0.05 (one sample t-test, comparing EC values to 1.0) Data are the mean ± SD.
Similar remarkable effects were observed with the fluvastatin-dipyridamole combination (Figure 2-3).
A

Viability (%)

OCI-AML2

OCI-AML3

HL-60

LP1

KMS11

8226

Fluvastatin (uM)

B

Fluvastatin + Dipyridamole

Combination Index

AML-2

AML-3

HL-60

AML Cell lines

C

Fluvastatin + Dipyridamole

Combination Index

LP1

KMS11

8226

MM Cell lines
Figure 2-3: Dipyridamole potentiates the anti-proliferative effects of fluvastatin and the combination is synergistic.

(A) Dipyridamole potentiated the anti-proliferative effects of fluvastatin at various doses, in AML (OCI-AML2, OCI-AML3, HL-60) and MM (LP1, KMS11, 8226) cell lines as assessed by MTT assay following 48 hours of compound exposure. *p < 0.05 (t-test, unpaired, two-tailed). (B) AML and (C) MM cell lines were exposed to increasing concentrations of fluvastatin, dipyridamole, or fluvastatin-dipyridamole combination in a fixed ratio and dose-response curves were generated. Results were analyzed to evaluate synergy using the combination index (CI). CI < 1 indicates synergy, CI = 1 indicates additivity and CI > 1 indicates antagonism. The EC50 (50% effective concentration) EC25, EC75 and EC90 from at least three independent experiments are shown. *p < 0.05 (one sample t-test, comparing EC values to 1.0) Data represent the mean ± SD.
The limitation of ectrophotometric cell viability assays such as the MTT and MTS assays is reliance on mitochondrial enzymes whose rates of conversion of the MTT and MTS substrates do not directly assess apoptosis. We therefore chose representative cell lines from the AML and MM panel, OCI-AML3 and KMS11, respectively, and assessed for apoptosis using TUNEL (Terminal deoxynucleotidyl transferase dUTP nick end labeling) and PARP (Poly (adenosinediphosphate-ribose) polymerase) cleavage. We treated OCI-AML3 (Figure 2-4A, left panel) and KMS11 (Figure 2-4B, left panel) cells with atorvastatin and dipyridamole and found that there is a dramatic induction of apoptosis when atorvastatin is combined with dipyridamole with no effect of either drug alone. The apoptotic effect was abrogated with the co-administration of mevalonate (MVA) and therefore was deemed to result specifically from the inhibition of HMGCR, the target of statins. The degree of apoptosis was proportional to the concentration of dipyridamole with a 1 µM dose of dipyridamole resulting in significant induction of apoptosis when combined with statins in OCI-AML3 cells. Cleavage of PARP also occurred in OCI-AML3 and KMS11 cells (Figure 2-4A-B, right panels respectively) following exposure to the atorvastatin-dipyridamole combination for 48 hours. Thus, the statin-dipyridamole combination is synergistic and induces apoptosis in AML and MM cells.
**Figure 2-4: The statin-dipyridamole combination induces apoptosis in AML and MM cell lines.**

Treatment of the OCI-AML3 (A) or the KMS11 (B) cell lines with sublethal low micromolar doses of atorvastatin (Ator.) and dipyridamole (DP) induces apoptosis following 48 hours of compound exposure as assessed by TUNEL (left panels). The apoptosis was abrogated in the presence of exogenous mevalonate (MVA). The atorvastatin-dipyridamole combination also caused PARP cleavage in both cell lines (right panels). *p < 0.05 (one-way ANOVA with a Tukey post test, the statin-dipyridamole group being significantly different than all other groups). TUNEL data represent the mean ± SD of at least three independent experiments. All immunoblots are representative of a minimum of three independent experiments.
2.3. 3. The combination of statins and dipyridamole induces apoptosis in primary AML and MM cells

To determine whether primary AML and MM cells are sensitive to the statin-dipyridamole combination, we exposed patient samples to statins and dipyridamole for 48 hours. In AML samples, cell death was measured using annexin V/propidium iodide (AV/PI) staining and then summing the AV+/PI- and AV+/PI+ cell populations. Alternative to TUNEL and PARP cleavage, the Annexin V/PI stain requires fewer cells and is suitable for primary samples. The assays are comparable and interchangeable as similar levels of death were measured using TUNEL (Figure 2-4A) and AV/PI (Figure 2-5) in OCI-AML3 cells.
Figure 2-5: The statin-dipyridamole combination induces apoptosis in the OCI-AML3 cell line.

OCI-AML3 cells were treated for 48 hrs with vehicle, 2 μM atorvastatin, 5 μM dipyridamole or the atorvastatin-dipyridamole combination and cells were assessed for Annexin V (AV)-propidium iodide (PI) stating flow cytometry. Cell death following atorvastatin-dipyridamole treatment was comparable to TUNEL (Figure 3A, left panel). *p < 0.05 (one-way ANOVA with a Tukey post test, the statin- dipyridamole group being significantly different than all other groups). Data represent mean ± SD of four independent experiments.
Primary cells from AML patients with intermediate-risk cytogenetics (n = 2) and adverse cytogenetics (n = 3) were treated with ranges of doses of statins and dipyridamole (Table 1).
Table 2-1: The statin dipyridamole combination induces apoptosis in primary AML cells

<table>
<thead>
<tr>
<th></th>
<th>Patient 1</th>
<th>Patient 2</th>
<th>Patient 3</th>
<th>Patient 4</th>
<th>Patient 5</th>
</tr>
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<td>ND</td>
<td>21</td>
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<tr>
<td>Dipyridamole 2.5 μM</td>
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<td>ND</td>
<td>16</td>
<td>18</td>
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</tr>
<tr>
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<td>23</td>
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<td>16</td>
<td>10</td>
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<tr>
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<tr>
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<td>ND</td>
<td>23</td>
<td>ND</td>
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<td>34</td>
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<td>46</td>
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<tr>
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<td>ND</td>
<td>ND</td>
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<tr>
<td>Atorvastatin 5 μM + Dipyridamole 10 μM</td>
<td>38</td>
<td>ND</td>
<td>ND</td>
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* Following treatment for 48 hours, primary cells were assessed for Annexin V (AV)–propidium iodide (PI) staining by flow cytometry, and percent apoptosis was evaluated by summing the AV+PI− and AV−PI+ quadrants. Patients 1 and 3 were classified into the intermediate prognosis groups based on cytogenetics and patients 2, 4 and 5 into adverse prognosis groups. ND indicates not determined.
The statin-dipyridamole combination significantly induced apoptosis in primary AML cells (Figure 2-6B), and a representative dot blot is shown in Figure 2-6A. Death was dipyridamole and statin dose-dependent and observed at similar doses used in cell lines. The effects of the combination were minimal in PBSCs (peripheral blood stem cells) obtained from GCSF (Granulocyte colony-stimulating factor)-mobilized peripheral blood to be utilized for allotransplantation (Figure 2-6C). The PBSCs were treated at two-times higher statin micromolar doses than the primary AML cells to thoroughly evaluate potential cytotoxicity to non-transformed cells. Even at these higher doses, cytotoxicity was not significant. Primary MM samples were similarly stained with AV to determine apoptosis induced by the statin-dipyridamole combination and CD138 (cluster of differentiation 138) to identify MM cells. As shown in Figure 2-6D, patient 1 was sensitive to statin-dipyridamole induced apoptosis as assessed by the decrease in the viable upper left quadrant CD138+/AV- cell population without a concomitant decrease in the CD138-/AV- normal population. Upon undergoing apoptosis, MM cells rapidly lose CD138 (syndecan-1), and therefore, apoptotic MM cells can possibly be found in both CD138+/AV+ and CD138-/AV+ quadrants. Therefore, when examining effects of drugs on the viability of MM cells, changes in the CD138+/AV- population were considered. MM patient 2 was not sensitive to the statin-dipyridamole combination and this is consistent with our previous results showing that not all MM patients were responsive to statins. Taken together, these data underscore the therapeutic utility of the statin-dipyridamole combination in AML and MM patient samples.
Figure 2-6: The statin-dipyridamole combination induces apoptosis in primary AML and MM patient cells.

Primary AML patient cells (B, n = 5) and primary MM patient cells (D, n = 2) were treated for 48 hrs with vehicle, 5 µM fluvastatin, 10 µM dipyridamole and the fluvastatin-dipyridamole combination. Primary normal hematopoietic cells (PBSCs) (C, n = 4) were treated for 48 hrs with vehicle, 10 µM atorvastatin, 10 µM dipyridamole or the atorvastatin-dipyridamole combination. Primary AML and PBSCs were assessed for Annexin V (AV)-propidium iodide (PI) staining by flow cytometry. MM cells were assessed for AV and CD138 staining by flow cytometry. A representative primary AML patient sample is shown in (A). For PBSCs and AML cells, percent apoptosis was evaluated by summing the AV+/PI- and AV+/PI+ quadrants. For MM primary samples, a decrease in the viable CD138 positive cells marks apoptosis. *p < 0.05 (one-way ANOVA with a Tukey post test, the statin-dipyridamole group being significantly different than all other groups).
2.3.4. The combination of statins and dipyridamole delays tumour growth in leukemia xenografts

To evaluate the statin-dipyridamole combination in vivo, we conducted a treatment trial in SCID (Severe combined immunodeficiency) mice harboring established xenografts of OCI-AML2 cells. We chose to orally administer atorvastatin because this is the route of delivery for humans, a longer serum half-life is evident orally compared to other routes of delivery and previously demonstrated in vivo efficacy. Although oral administration of dipyridamole, like of atorvastatin is the conventional route of delivery in patients, oral gavage of dipyridamole resulted in significantly lower dipyridamole serum concentrations when compared to intraperitoneal (i.p.) administration (Figure 2-7).
Figure 2-7: Dipyridamole plasma concentrations are higher following i.p. administration than p.o.

Higher dipyridamole plasma concentrations were achieved with i.p. administration as compared to oral dosing three hours post administration. *p < 0.05 (t-test, unpaired, two-tailed).
As differences in gastric pH levels between mice and humans have been reported to impair the oral bioavailability of dipyridamole in mice\textsuperscript{34}, we reasoned that i.p. administration would be suitable for evaluating the \textit{in vivo} efficacy of combining statins and dipyridamole. The dipyridamole concentration in serum of mice treated with dipyridamole reached the micromolar concentration range (Figure 2-8A) three hours post administration and was comparable to the doses used in our cell culture studies. Mice were injected with OCI-AML2 cells subcutaneously and when xenografts were palpable, mice were orally treated daily with vehicle alone, 50 mg/kg atorvastatin, 120 mg/kg dipyridamole, or the combination of atorvastatin and dipyridamole. The combination of atorvastatin and dipyridamole significantly decreased tumour volume (Figure 2-8B) and the tumour weight at the time of sacrifice (Figure 2-8C).
Figure 2-8: The statin-dipyridamole combination delays tumour growth in leukemia xenografts.

(A) Plasma concentrations of dipyridamole 3 hours post-administration of 120 mg/kg dipyridamole and vehicle intraperitoneally (i.p.) (B) SCID mice were injected subcutaneously with $10^6$ OCI-AML2 cells. After tumours became palpable, mice were randomized into groups and treated daily with 50 mg/kg atorvastatin orally (p.o.), 120 mg/kg dipyridamole (i.p.), a combination of dipyridamole and atorvastatin or PBS (phosphate buffered saline) (p.o. = per os) and vehicle (i.p.) (Con.). Tumour volume was measured every two days. After 14 days of treatment, mice were sacrificed and tumours were resected and weighed (B). *p < 0.05 (one-way ANOVA (Analysis of variance) with a Tukey post test, the statin-dipyridamole group being significantly different than all other groups. For tumour weights the statin-dipyridamole group was significantly different than the PBS and the atorvastatin groups. (C) Plasma concentrations of dipyridamole 3 hours post-administration as administered in (A). Data represent the mean ± SD, and are representative of two independent in vivo experiments, both showing similar results.
2.3.5. Mediators of cAMP signaling in combination with statins induce apoptosis and raise intracellular cAMP levels in leukemia cells

The observed anti-cancer effects of statins occur through on-target inhibition of HMGCR as concomitant addition of MVA abrogated apoptosis (Figure 2-4). Identifying the mechanism of how dipyridamole contributes to the observed synergy is more difficult as dipyridamole is known to elicit many molecular effects. Our strategy to uncouple which of these effects contributed to the observed synergy was to systematically evaluate pharmacological inhibitors of each known activity of dipyridamole that could plausibly contribute to the anti-tumour effects for their ability to phenocopy dipyridamole in potentiating statin induced anti-cancer effects in AML and MM cells.

Dipyridamole is a reported P-glycoprotein (P-gp) inhibitor. P-gp is an ATP (adenosine triphosphate) -binding cassette transporter; its overexpression in cancer cells can contribute to efflux of chemotherapeutic drugs leading to treatment resistance. P-gp modulatory interactions of statins either as a substrate or inhibitor have consistently been reported for lovastatin, and the majority of evidence shows atorvastatin and fluvastatin are not significantly transported by transported by P-gp. We first determined whether dipyridamole modulating P-gp could be contributing to the observed statin-dipyridamole synergistic effect. We used a system of MM 8226 paired cells lines, one parental and one over-expressing P-gp (Figure 2-9A). The P-gp over-expression was induced by long-term exposure to doxorubicin a common P-gp substrate, leading to these 8226 cells (8226DOX) being resistant to doxorubicin. Dose-response curves were obtained by treating the parental 8226 and 8226DOX cells to increasing concentrations of doxorubicin with and without 5 µM dipyridamole. Dipyridamole did not reverse doxorubicin resistance in 8226DOX cells (Figure 2-9B). The doxorubicin IC50
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(inhibitory concentration 50) values in the 8226
DOX cells were still in the high micromolar range upon addition of dipyridamole compared to the parental 8226 cells in which the doxorubicin IC50 values were in the nanomolar range. There was a slight decrease in IC50 values upon addition of dipyridamole, however this was evident in both parental and 8226
DOX cells. Thus, evidence shows that dipyridamole inhibition of P-gp is unlikely involved in the synergistic efficacy of the statin-dipyridamole combination.
Figure 2-9: The relative sensitivity to doxorubicin in 8226<sub>DOX</sub> and 8226 parental cells is not significantly altered in response to dipyridamole exposure.

(A) P-gp expression histogram of parental 8226 cells and P-gp overexpressing doxorubicin resistant 8226 cells (8226<sub>DOX</sub>). Histogram is representative of three independent experiments.

(B) Parental 8226 and 8226<sub>DOX</sub> were treated with increasing concentrations of doxorubicin for 48 hours generating dose-response curves from which IC<sub>50</sub> values were computed as described in methods. The addition of dipyridamole (5 µM) to the 8226<sub>DOX</sub> cells did not reverse doxorubicin resistance. *p < 0.05 (t-test, unpaired, two-tailed). Data represent the mean ± SD of three independent experiments.
We further investigated dipyridamole’s other pharmacological activities to determine whether they could contribute to the anti-tumour effects observed in combination with statins (Figure 2-10A). Dipyridamole inhibits the equilibrative nucleoside transporter (ENT1) which transports nucleosides such as adenosine into cells\(^{37}\). In addition, inhibition of glucose uptake through glucose transporters (GLUT1, GLUT4) is another reported dipyridamole activity\(^{38}\). Also, dipyridamole is a multi-isoform phosphodiesterase (PDE) inhibitor with varying degrees of inhibition reported for PDE 5, 6, 7, 8, 10, 11\(^{39}\) and an ability to increase both cAMP and cGMP (cyclic guanosine monophosphate) levels in cell culture and \textit{in vivo}\(^{40}\). To investigate which, if any, of the above reported effects of dipyridamole were responsible for the potentiation of the anti-tumour effects of statins, we chose representative compounds of each of these pathways, and then evaluated response of AML and MM cells upon exposure to sublethal doses of these compounds alone and in combination with statins.

There were no significant effects when statins were combined with the glucose transport inhibitor fasentin\(^{41}\) (Figure 2-10B) or the equilibrative nucleoside transport inhibitor NMBPR (Figure 2-10C). Similarly, the cGMP substrate-specific PDE inhibitor 4-\{[3’,4’- (Methylenedioxy)benzyl]amino\}-6-methoxyquinazoline (4-MD)\(^{42}\) also failed to potentiate statin induced anti-proliferative effects (Figure 2-10D). Interestingly, the cAMP substrate-specific PDE3 inhibitor cilostazol\(^{43}\), another anti-platelet drug currently in clinical use for the treatment of intermittent claudication did promote statin-induced anti-proliferative effects in AML and MM cells (Figure 2-10E).
A

P-gp inhibitor (Doxorubicin) → DP → PDEs inhibitor (4-MD, Cilostazol)

Glucose transport inhibitor (Fasentin) → DP

ENT transport inhibitor (NMBPR) → DP

B

C

D

E

Graphs showing the effects of different compounds on cell viability for various cell lines (OCI-AML2, OCI-AML3, KMS11).

Graph B: Effect of Fasentin and NMBPR on cell viability.

Graph C: Effect of 4-MD on cell viability.

Graph D: Effect of Cilostazol on cell viability.
Figure 2-10: Cilostazol, a PDE3 inhibitor, potentiates the anti-proliferative activity of statins.

(A) A schematic displaying some of dipyridamole’s known pharmacological effects (black) and representative drugs (red) of each class. The glucose transport inhibitor fasentin (at concentrations of 12.5 µM, 6.3 µM and 12.5 µM in OCI-AML-2, OCI-AML-3 and KMS11 cells respectively) (B), equilibrative nucleoside transport inhibitor NMBPR (at concentrations of 20 µM for all cell lines) (C) and the PDE5-specific inhibitor 4-MD (at concentrations of 10 µM for all cell lines) (D), did not potentiate the anti-proliferative effects of atorvastatin (at concentrations of 4 µM, 2 µM and 4 µM in OCI-AML2, OCI-AML3 and KMS11 cells respectively) following 48 hrs of treatment while cilostazol (at concentrations of 25 µM, 12.5 µM and 25 µM in OCI-AML2, OCI-AML3 and KMS11 respectively) (E), a cAMP elevating PDE3 inhibitor did potentiate atorvastatin-mediated anti-proliferative effects. *p < 0.05 (one-way ANOVA with a Tukey post test, the statin-dipyridamole group being significantly different than all other groups) Data represent the mean ± SD of at least three independent experiments.
To evaluate dipyridamole’s functionality as a PDE inhibitor, we tested whether dipyridamole could raise intracellular cAMP levels. We measured intracellular cAMP levels and detected a three-fold induction in AML cells following exposure to dipyridamole (Figure 2-11A). Statin and dipyridamole co-treatment also resulted in raised cAMP levels in AML cells similar to that of singular dipyridamole treatment (Figure 2-11A). To ensure that the observations extended to other statins, we assayed fluvastatin in subsequent experiments. We next tested whether this observation was limited to the single agent cilostazol or could be broadened to include other modulators of the cAMP signaling arm. We therefore combined sublethal doses of forskolin, an adenylate cyclase activator which is known to raise intracellular cAMP levels, as well as a cell permeable analog of cAMP, dibutyryl cAMP (db-cAMP), with fluvastatin and assessed apoptosis using AV/PI staining after treatment for 48 hours. When combined with fluvastatin, all three modulators of the cAMP signaling arm, cilostazol, forskolin and db-cAMP, resulted in a dramatic increase in apoptosis in AML cells similar to that observed with the statin-dipyridamole combination (Figure 2-11B). Indeed, this greater apoptotic effect of statins in combination with cilostazol, forskolin and db-cAMP was also observed in primary AML patient cells (Figure 2-11C). These data suggest that the potentiation of statin-induced apoptosis by dipyridamole can be mediated through the modulation of cAMP signaling as three different agents used at concentrations known to raise cAMP levels, when combined with statins, also induced apoptosis in AML cells and primary AML patient cells. This observation is further strengthened by the ability of dipyridamole to raise cAMP levels in AML cells.
Figure 2-11: Modulators of cAMP induce apoptosis and increase intracellular cAMP levels in AML cells.

(A) Intracellular cAMP levels were raised in OCI-AML3 cells following 15 min treatment with 5 μM dipyridamole, and the fluvastatin (2 μM)-dipyridamole combination relative to control. The PDE3 inhibitor cilostazol (20 μM), the adenylate cyclase activator forskolin (10 μM), and a cell permeable analog of cAMP, dibutyryl cAMP (0.1 mM, db-cAMP) in combination with fluvastatin (2 μM, 4 μM and 5 μM for OCI-AML3, OCI-AML2 and primary cells respectively) induced apoptosis in AML cells (B) and primary patient samples (C). *p < 0.05 (one-way ANOVA with a Tukey post test, the statin-dipyridamole group being significantly different than all other groups) Data represent the mean ± SD of at least three independent experiments.
2.4. Materials and Methods

Cell Culture and Compounds

Human MM cell lines (LP1, KMS11, 8226) were maintained in RPMI (Roswell Park Memorial Institute) 1640 medium. Human AML cell lines (OCI-AML3, HL-60 and OCI-AML2) were maintained in alpha modified Eagle's medium (αMEM) and Iscove modified Dulbecco medium (IMDM) respectively. 8226DOX cells were additionally cultured in 0.3 μM doxorubicin. Media was supplemented with 10% fetal bovine serum (FBS, GIBCO) and penicillin-streptomycin. Cells were incubated at 37°C in 5% CO₂ and all cell lines were routinely confirmed to be mycoplasma-free (MycoAlert mycoplasma detection kit, Lonza). Atorvastatin calcium (21 CEC Pharmaceuticals LTD) and fluvastatin (US Biologicals) were dissolved in ethanol. Dipyridamole (Sigma), Cilostazol (Tocris Bioscience), 6-S-heacep₂₂-6-thioinosine (NBMPR, Tocris Bioscience), 4-\{3',4'-(Methylenedioxy)benzyl\}amino}-6-methoxyquinazoline (4-MD) (Calbiochem), Fasentin (Sigma) were dissolved in dimethyl sulfoxide (DMSO).

Primary Cells

Primary AML and MM patient samples were obtained from consenting AML and MM patients respectively. Peripheral blood mononuclear cells (PBSCs) were obtained from healthy volunteers donating cells for allotransplantation. The PBSCs were granulocyte colony-stimulating factor (GCSF)-mobilized. Mononuclear cells were fractioned by Ficoll-Hypaque gradient sedimentation. AML and PBSC primary cells were cultured in IMDM medium supplemented with 20% FBS and 5% 5367-conditioned medium. MM primary cells were cultured in IMDM medium supplemented with 10% fetal bovine serum, 1% glutamine, and penicillin-streptomycin. Primary AML cells were obtained from frozen stocks stored in
liquid nitrogen, thawed, and within 2-10 hours, treated for 48 hours. PBSC and primary MM cells were obtained fresh and treated as indicated and as previously reported<sup>9</sup>. Use and collection of human tissue for this study was approved by the University Health Network Institutional Review Board (Toronto, ON).

**Chemical Screen for Cytotoxic Drugs**

96 well non-tissue culture treated plates (BD Falcon) of KMS11 cells (20000 cells/well) were treated with aliquots of a chemical library<sup>29</sup> of 100 drugs dissolved in DMSO (3-50 µM) using a Biomek FX Laboratory Automated Workstation (Beckman Coulter). One plate had been pre-treated with 3.5 uM of atorvastatin. Following 72 hours of incubation, cell viability was assessed using the (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) (MTS) reduction assay (Promega) as per the manufacturer’s protocol and as previously described<sup>29</sup>.

**MTT, TUNEL and Annexin V apoptosis assays**

(3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma-Aldrich) assays were carried out as previously described<sup>44</sup>. Briefly, 2 x 10<sup>5</sup> to 3 x 10<sup>5</sup> cells/ml (MM and AML cell lines) were plated in 96 well plates and after 24 hours, treated with the indicated compounds and concentration ranges for 48 hours. Half-maximal inhibitory concentrations (IC<sub>50</sub>) values were computed from dose-response curves using Prism (v5.0, GraphPad Software). For TUNEL assays, 2.5 x 10<sup>5</sup> cells/ml were seeded in 6 well plates and treated for 48 hours as indicated. Cells were fixed in ethanol and staining was performed using terminal deoxynucleotide transferase dUTP nick end labeling (TUNEL) according to the manufacturer’s instructions (APO-BRDU Apoptosis Kit, Phoenix Flow Systems). Annexin V (AV) apoptosis assays (Biovision) were carried out as per the manufacturer’s protocol.
Briefly, $2.5 \times 10^5$ cells/ml (cell lines) and $5 \times 10^5$ cells/ml (primary cells) were treated for 48 hours and stained with AV-fluorescein isothiocyanate–conjugated annexin (FITC) and propidium iodide (PI). Primary MM cells were labeled with anti-CD138-phycoerythrin (Immunotech) and AV-FITC (R&D Systems). Cells were analyzed for apoptosis by flow cytometry; ten thousand events were acquired using a FACSCalibur cytometer (BD Biosciences). CD138-positive and AV negative cells constitute viable myeloma cells and apoptotic myeloma cells fall within the CD138-negative AV-positive population.

**Drug Combination Studies**

Synergy between statins and dipyridamole was evaluated using the combination index (CI)$^{30}$. Dose response curves were generated for statins and dipyridamole alone and in combination at a constant ratio following compound exposure for 48 hours. Viability was assessed by MTT assay. CalcuSyn software (biosoft) was used to evaluate synergy using the median effect model.

**Immunoblotting**

$2.5 \times 10^5$ cells/ml were seeded in 6 well tissue-culture plates and treated as indicated for 48 hours. For PARP detection, cells were washed with PBS and lysed using boiling hot SDS (sodium dodecyl sulfate polyacrylamide) lysis buffer (1.1% SDS, 11% glycerol, 0.1M Tris pH 6.8) with 10% β-mercaptoethanol. Blots were probed with anti-tubulin (Santa Cruz Biotechnology) and anti-PARP (Cell Signaling).

**Intracellular cAMP assays**

Intracellular levels of cAMP were measured using the Cyclic AMP Chemiluminescent Immunoassay Kit (Cell Technology, INC) as per the manufacturer’s protocol. Briefly, 1.5 x
10^6 cells per well of a 6 well plate were incubated with compounds as indicated, washed with PBS and lysed using 150 µL (microliter) of the provided lysis buffer. Samples were stored at -80 °C if not immediately used.

**Leukemia xenograft models**

Severe Combined Immunodeficiency (SCID) male mice (7-9 week old) were subcutaneously injected with 10^6 OCI-AML2 cells. When tumours became palpable (15 mm³), mice were randomized and treated daily with 120 mg/kg dipyridamole administered intraperitoneally (i.p.) (from a solution of 5 mg/ml dipyridamole, 50mg/ml polyethylene glycol 600, and 2 mg/ml tartaric acid), 50 mg/kg atorvastatin administered by oral gavage (p.o.), a combination of dipyridamole and atorvastatin, or vehicle (PBS orally and vehicle i.p.). Tumours were measured every two days using digital calipers and tumour volume was calculated using the following formula: (tumour length x width^2)/2. Animal work was carried out with the approval of the Princess Margaret Hospital ethics review board in accordance to the regulations of the Canadian Council on Animal Care.

**Assessment of dipyridamole levels in serum**

Levels of dipyridamole in the serum of mice were determined as previously described by spectrofluorometry using differences in the fluorescence of dipyridamole between acidic and basic conditions. Briefly 52 µL of serum from one mouse was equally divided into two solutions containing glycine buffer (186 µL, 67.8 mM) and ethanol (37.5 µL) pH 2.6 and pH 9.8 respectively. For standard curves, accompanying dipyridamole control solutions dissolved in ethanol were prepared using serum from untreated mice. Fluorescence spectra (450-600 nm, maximum fluorescence occurring at 490 nM, excitation at 420 nM) were measured using a SpectraMax M5 plate reader (Molecular Devices).
P-glycoprotein (P-gp) cell surface expression

Briefly, 1.0 x 10^6 cells/ml were stained with 10 μL of FITC-tagged anti-P-gp antibody (BD Biosciences) and expression was detected by flow cytometry using a FACSCalibur cytometer (BD Biosciences).
2.5. Discussion

From a screen of FDA approved drugs, we identified an effective statin-dipyridamole combination as having previously unrecognized anti-cancer activity against AML and MM cells. The combination was synergistic and significantly induced apoptosis not only in cell lines but also in primary myeloma and leukemia patient samples. A leukemia xenograft model confirmed the \textit{in vivo} efficacy of the combination in its ability to slow tumour growth following treatment of pre-established tumours. The mechanism by which dipyridamole potentiated statin-induced apoptosis was explored by combining pharmacological inhibitors representative of each of the previously reported molecular effects of dipyridamole with statins. Using this approach, we eliminated the possibility that the synergy was mediated through dipyridamole’s inhibitory effects on P-glycoprotein, equilibrative nucleoside transporters and glucose transporters. Cilostazol, a cAMP specific PDE inhibitor was able to potentiate statin-induced apoptosis suggesting that dipyridamole’s PDE inhibitory activities could be responsible for the observed synergy. Indeed, dipyridamole was able to raise intracellular levels of cAMP in AML cells and other modulators of cAMP signaling were also able to induce apoptosis when combined with statins in AML cells and primary patient cells. Taken together, we identify a novel combination of two FDA-approved agents for the treatment of hematological cancers and identify the critical mechanism of action.

Statins are well tolerated in the clinical cancer setting including hematological malignancies. In dose-escalating studies where cancer patients were administered lovastatin doses of up to 45 mg/kg/day, average low micromolar peak plasma bioactivity statin levels were achieved \textsuperscript{21}. Atorvastatin and fluvastatin, used in the current study, have pharmacokinetic properties that are systemically more favorable than lovastatin, including a longer half-life and higher peak concentration ($C_{\text{max}}$), respectively. The cholesterol lowering 40 mg oral dose of fluvastatin
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has a $C_{\text{max}}$ 200-440 ng/ml$^{33}$, which is equivalent to 0.49-1.0 µM. Although the administration of higher than cholesterol-lowering fluvastatin or atorvastatin doses have not yet been evaluated in cancer patients, the tolerability observed with other statins suggests that elevated doses of fluvastatin and atorvastatin will be similarly tolerated and that low micromolar range (2–5 µM) doses used in our cell culture studies are likely readily achievable in humans. Importantly, the anti-proliferative activity of statins is dose and time dependent, and evidence shows that even cholesterol lowering-doses can decrease tumor burden in cancer patients$^{36,25}$. Thus, the optimal dose of statins to use for cancer patient treatment remains unclear, yet evidence strongly suggests effective dosing can be achieved in vivo.

Like all anti-cancer agents, the optimal treatment strategy is to administer in multimodal and combinatorial treatment strategies to increase tumor-specific anti-proliferative activities. Statins can be successfully combined preclinically with number of diverse agents$^{46}$. The addition of statins to the standard of care regimen in the clinical cancer setting has also been used to successfully treat some AML$^{24}$ and MM$^{27}$ patients. Here, we provide a complimentary approach of combining statins with an already FDA-approved agent in the treatment of hematological malignancies.

Dipyridamole has been used as part of antithrombotic therapy for decades and its pharmacology has been thoroughly investigated. Recently, the extended-release formulation of dipyridamole in combination with aspirin has been widely accepted for use in the prevention of cerebral ischemic events in patients with stroke. Dipyridamole is constantly being re-formulated to maximize systemic exposure and extended release formulations of dipyridamole have a reported half-life of 13.6 hours following typical 200 mg twice daily (b.i.d.) dosing with steady state peak plasma concentrations of 1.0-4.0 µg/ml (2.0 – 7.9 µM)$^{47}$. Dipyridamole is tightly protein bound to $\alpha_1$-acid-glycoprotein (AGP) in serum$^{48}$, however this does not preclude dipyridamole activity as an antithrombotic, suggesting that the steady
state micromolar-range concentrations of dipyridamole achieved with antithrombotic doses of 200 mg b.i.d. (bis in die) will be sufficient for effective anti-cancer therapy when combined with statins. Interestingly, much higher dipyridamole doses have been tolerated in humans as reported from overdose case reports\(^49\), suggesting dosing could potentially be elevated. Taken together, evidence suggests the combination of statins and dipyridamole will be a well-tolerated and efficacious strategy for the clinical treatment of leukemia and myeloma.

Our apoptosis assays in primary cells demonstrated that the combination of statin and dipyridamole was capable of inducing apoptosis in primary AML and MM patient samples. Moreover, even when used at elevated concentrations, primary normal PBSCs did not demonstrate significant toxicity in response to dipyridamole and statin exposure. This further attests to the potential therapeutic window of the statin-dipyridamole combination. Indeed, the statin-dipyridamole combination was shown to be safely combined and well-tolerated in humans in studies of cardiovascular protection\(^40\) and renal function\(^51\). Therefore given that we have shown that the statin-dipyridamole combination minimally affects normal hematopoietic cells and this combination has been safely administered to humans, we predict that a substantial therapeutic window exists in the treatment of AML and MM patients.

Dipyridamole has been associated with multiple pharmacological actions including the inhibition of P-gp, nucleoside transport, glucose uptake and PDEs. These attributes have led to explorations of the potential benefits of dipyridamole in diseases other than cardiovascular conditions, including cancer. Dipyridamole has been combined with antimetabolite agents such as 5-fluorouracil (5-FU) in an effort to prevent salvage metabolism through dipyridamole’s nucleoside transport inhibitory activities. Although addition of dipyridamole at 300 mg daily to the regimen of 5-FU, leucovirin and mitomycin-C to patients with advanced pancreatic cancer was well tolerated, these studies lacked encouraging therapeutic responses when compared to the standard of care\(^52\). This is consistent with our data showing
the combination of statins with other nucleoside transport inhibitors, or inhibitors of glucose transport, was ineffective at triggering cell death of AML and MM cells. Interestingly, the combination of statins and the PDE3 inhibitor cilostazol did have anti-leukemia and anti-myeloma activity. Cilostazol is a selective PDE3 inhibitor whose reported effects include cAMP elevation and like dipyridamole, is also currently in clinical use. As the specific cGMP elevating agent, 4-MD, showed no anti-leukemia and myeloma activity in combination with statins, we further explored other modulators of cAMP signaling and tested their potential to induce apoptosis in combination with statins. The well-characterized cAMP elevating agent forskolin, cilostazol, and the cell permeable cAMP analog db-CAMP all induced apoptosis in leukemia cells and higher levels of cAMP were observed in cells treated with the statin-DP combination. There is some evidence to indicate that pro-apoptotic responses to cAMP signaling occur. In one report, albeit limited to one cell line, IPC-81, treatment with a cAMP analog not only induced apoptosis as a single agent but, when combined at sublethal doses with a chemotherapeutic drug daunorubicin, the two agents synergized in promoting apoptosis. Another report identified cAMP selective PDE 3, 4 and 7 inhibitors, including cilostazol, in combination with adenosine receptor agonists as having strongly synergistic anti-proliferative effects in MM cells and increases in intracellular cAMP levels were also observed with the combination though in vivo preclinical efficacy was not demonstrated. Here we show that the inhibition of the MVA pathway using statins and dipyridamole’s effects on intracellular cAMP levels may contribute to the observed synergy in inducing tumour cell kill.

Interestingly, the statin-dipyridamole combination effectively reduced proliferation in MM cell lines previously characterized as being statin-insensitive. The division of MM cells into statin-sensitive and statin-insensitive cohorts, has been linked to differences in the sterol-feedback loop. Whereas in the statin-sensitive KMS11 MM cells the upregulation of sterol
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responsive genes such as HMGCR in response to statin exposure is limited, the statin-insensitive LP1 have a robust feedback loop. The anti-myeloma effects of the statin-dipyridamole combination observed in difficult to treat MM cells further demonstrates its therapeutic potential.

In summary, we have identified a synergistic combination of statins and dipyridamole that is preclinically effective in treating AML and MM. Our mechanistic investigations have uncovered other FDA-approved drugs, such as cilostazol that, in combination with statins, could also have applications in the hematological cancer therapeutic setting. These studies may serve as a foundation for developing a phase I clinical trial involving the combination of statins and dipyridamole for the effective treatment of AML and MM.
2.6. References


23. van der Spek E, Bloem AC, van de Donk NW, Bogers LH, van der Giessen R, Kramer MH *et al.* Dose-finding study of high-dose simvastatin combined with standard


Chapter 3: Genome wide shRNA screen reveals novel sensitizers of statin-induced apoptosis

Contributions:

The majority of work presented in this chapter was completed by the author of this thesis. Additional contributions are as follow:

Screen design, execution and interpretation: Aleksandra Pandyra, Dr. Carolyn A. Goard and Dr. Elke Ericson.

Genomic DNA extraction, hairpin amplification and array hybridization: Dr. Elke Ericson and Marinella Gebbia.

All bioinformatics analysis was performed by Dr. Kevin Brown

Figure 3.3. Dr. Carolyn A. Goard and Janice Pong contributed to harvesting and immunoblotting replicates.

Figure 3.4 A and B. Dr. Carolyn A. Goard and Janice Pong contributed to the generation of the cells and some replicates of the apoptosis assays.

Figure 3.8 C. Dr. Peter J. Mullen carried out the proliferation assays
3.1 Abstract

Statins have been used for decades to treat hypercholesterolemic patients and their widespread use in the clinic has uncovered some pleiotropic effects including anti-tumour efficacy. Statins target the rate-limiting enzyme of the mevalonate (MVA) pathway, 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGCR), a pathway that supplies the cell with essential sterol end products such as cholesterol. The MVA pathway is dysregulated in many cancers and statins are promising novel agents currently being evaluated in the clinic for efficacy in treating cancer. To uncover novel targets that potentiate statin induced apoptosis, we carried out an unbiased genome wide shRNA screen in the A549 lung cancer cell line stably transduced with the RNAi Consortium (TRC1) shRNA library. A549 cells were exposed to sublethal doses of fluvastatin over 12 days and significant shRNAs under represented in the fluvastatin treated group relative to control yielded a list of putative potentiators of statin induced anti cancer-effects. Among 151 hits, the sterol regulatory element binding transcription factor 2 (SREBF2) was validated and found to dramatically sensitize A549, MDAMB231 and MCF7 breast cancer cells to statin-induced apoptosis. Stable SREBF2 knockdown in lung and breast cancer cells completely abrogated the statin-induced upregulation of sterol responsive genes HMGCR and 3-hydroxy-3-methylglutaryl-CoA synthase 1 (HMGCS1). Subcutaneous xenografts of stably expressing SREBF2 sublines in NOD SCID mice were more sensitive to oral fluvastatin treatment than the control sublines. Taken together, we have identified and validated an important novel potentiator of statin induced apoptosis in lung and breast cancer cells.
3.2 Introduction

Statins, potent inhibitors of the rate-limiting enzyme in the mevalonate (MVA) pathway, 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGCR)\(^1\) have been used for decades in the treatment of patients with hypercholesterolemia. Their widespread use for the prevention of adverse cardiovascular events has led to the accumulation of retrospective epidemiological evidence that indicates statin use can reduce cancer incidence\(^2\)–\(^5\). Beyond lowering cholesterol, statins exert pleiotropic effects including direct anti-proliferative and pro-apoptotic effects on tumor cells.

The anti-cancer effects of statins have been attributed to direct target HMGCR in tumor cells leading to depletion of fundamental MVA-derived end-products such as isoprenoids and cholesterol\(^6\)–\(^8\). The consequential downstream effects of this depletion affect oncogenic pathways such as the MAPK/Erk (Mitogen-activated protein kinases/Extracellular signal-regulated kinases)\(^9\),\(^10\), JNK (c-Jun N-terminal kinases)\(^11\),\(^12\) pathways and the epidermal growth factor receptor (EGFR)\(^13\). Statin induced anti-proliferative effects and apoptosis have been shown to be mainly p53 independent\(^11\),\(^14\),\(^15\) and abrogated by the extopic expression of BLC\(^2\)\(^16\),\(^17\). Statins are currently being evaluated as anti-cancer agents in patients with a broad range of tumor types. Data from completed clinical trials data indicates that statins are well tolerated in the clinical cancer care setting at cholesterol lowering\(^18\),\(^19\) and higher doses\(^20\)–\(^22\), alone or when co-administered with the standard of care\(^23\)–\(^26\). Taken together, preclinical, epidemiological and clinical evidence has demonstrated that repurposing statins for the treatment of cancer has potential clinical utility.

To further extend the benefit of statins and maximize anti-tumour efficacy, we sought to identify novel targets capable of potentiating statin-induced anti-cancer effects through a genome wide shRNA screen. The advent of RNA interference (RNAi) technology\(^27\) has
revolutionized the experimental landscape and consequently augmented our understanding of cancer genetics. Knockdown approaches, encompass small interfering RNAs (siRNAs)\textsuperscript{28}, endoribonuclease-prepared siRNAs (esiRNA)\textsuperscript{29} and short hairpin RNAs (shRNA)\textsuperscript{30}, coupled with completely sequenced human and mouse genomes, have enabled the creation of multiple RNAi libraries which are of great utility in functional screens. The advantages of using a functional screen to identify products of genes which regulate a particular biological activity following a perturbation lie in its unbiased and genome-wide global nature\textsuperscript{31}. Such screens have been successfully used to identify essential genes in tumour cell lines\textsuperscript{32}, oncogene-associated synthetic lethal interactions\textsuperscript{33}, determinants of response to therapies\textsuperscript{34, 35} and sensitizers to anti-cancer drugs\textsuperscript{36}. Although all RNAi methods can be used for transient short-term knockdown approaches, shRNA libraries when combined with viral mediated transduction integrate into the genome of a target cell and are advantageous for long-term experiments particularly when employing nontransfectable cells.

We conducted a shRNA screen using the A549 non-small-cell lung cancer (NSCLC) stably transduced with the RNAi Consortium (TRC1) shRNA library (shA549) and treated the cells with sublethal doses of fluvastatin for twelve days. Significantly under-represented shRNAs in the fluvastatin-treated group were identified as potential gene targets whose knockdown enhanced the anti-proliferative effects of fluvastatin. Subsequent validation of four gene hits demonstrated that knockdown of geranylgeranyl diphosphate synthase 1 (GGPS1), sterol regulatory element-binding protein 2 (SREBF2), mitogen-activated protein kinase kinase 4 (MAP2K4) and phosphatidylinositol 4-kinase, catalytic beta (PIK4B) in combination with fluvastatin possessed pro-apoptotic and anti-proliferative effects. Extending the validation to breast cancer, revealed that, SREBF2 and PI4KB dramatically enhanced fluvastatin’s pro-apoptotic and anti-proliferative effects in MCF7 and MDA MB 231 breast cancer cells, effects which were corroborated with a PI4KB pharmacological inhibitor.
Expression analysis in the lung and breast cells stably expressing shRNAs targeting SREBF2 revealed that these sublines lacked the ability to upregulate HMGCR in response to fluvastatin exposure, a phenomenon likely responsible for their remarkable fluvastatin-sensitivity. Therefore concomitant inhibition of the MVA pathway through targeting HMGCR using statins and the transcription factor SREBF2 is an effective therapeutic strategy that induces apoptosis in lung and breast cancer cells.

Using a genome wide shRNA screen we have identified novel targets whose inhibition, combined with fluvastatin, possesses anti-tumour therapeutic efficacy in lung and breast cancer cells. The development of novel therapeutic cocktails in the treatment of these tumours is imperative especially in patients unsuccessfully treated with several front-line treatments.
3.3 Results

To identify novel sensitizers of statin-induced apoptosis, we designed a genome-wide shRNA screen in which some shRNA-stably expressing cells when combined with sublethal doses of fluvastatin, were outcompeted over time. The genes targeted by these dropout shRNAs were identified as potentiators of statin-induced anti-cancer effects and further validated in attempts to identify novel targets and or pathways which will increase statin anti-tumour efficacy.

3.2.1 A genome-wide screen identifies shRNA drop-outs after fluvastatin exposure

The A549 adenocarcinoma lung cancer cell line, stably transduced with the RNAi Consortium (TRC1) shRNA library (shA549), was utilized for screen execution. The TRC1 library is composed of lentiviral shRNAs targeting nearly 16,000 human genes. Five or four hairpins exist per gene and nearly 7,000 genes have been validated. The A549 cells are characterized by elevated HMGCR activity. Elevated HMGCR activity relative to normal cells not only establishes an active target but is indicative of a potential therapeutic index making the A549 cells a suitable choice for the screen. Furthermore, the A549 cells are relatively statin insensitive and uncovering potentiators of statin anti-cancer activity will broaden the applicability of statins to tumours that would otherwise remain unresponsive to statins. shA549 cells were exposed to sublethal doses of fluvastatin over twelve days, with cell-splitting, harvesting cells for genomic DNA and re-seeding of remaining cells occurring every 3 days (Figure 3-1 A). Cell viability, relative to ethanol, was maintained constant throughout the duration of the experiment at 60-70% (Figure 3-1 B). Genomic DNA from shA549 cells on days 0, 3, 6 and 12 was, PCR amplified and hybridized onto custom
Affymetrix Gene Modulation Array Platform (GMAP) arrays. Following extraction and normalization, each shRNA was assigned an shRNA Activity Ranking Profile score (SHARP) which, within each condition, incorporates not only the abundance of the shRNA relative to time 0 but shA549 doubling time, enabling shRNA drop-out assessment at multiple time points. On average, 5 hairpins form the TRC1 library target one gene. SHARP scores were converted to the gene activity ranking profile (GARP) scores by averaging the two lowest SHARP scores for each gene in order to quantify the gene’s shRNA dropout rate. A scatterplot of Z normalized GARP scores (zGARP) in the fluvastatin-treated shA549’s plotted against the ethanol-treated shA549’s (Figure 3-1 C) shows the gene drop-out distribution. A relatively negative zGARP score attests to the general essentiality of a gene as related to cellular proliferation.
Stably transduced with 80K shRNA pool

A549 cells

3 biological replicates

Day 0 (D0)

Vehicle

Day 3 (D3)

Day 6 (D6)

Day 12 (D12)

Amplification and hybridization of shRNA populations from gDNA

Fluvastatin

Control (EtOH) Treatment (zGARP)

Fluvastatin Treatment (zGARP)

% viability relative to ethanol control

Days

D

ZFluva - ZEthanol

D

151

D

C

Fluvastatin Treatment (zGARP)

Control (EtOH) Treatment (zGARP)

D

151

D

zFuva - zEtOH

x = 3x

zFuva - zEtOH

151

D
Figure 3-1: A genome wide dropout screen uncovers putative shRNAs that potentiate fluvastatin-induced cell death.

(A) A schematic representing the steps of the screen. A549 cells were treated with sublethal doses of fluvastatin (4-5 µM) every 3 days, over 12 days. Viability relative to ethanol in the fluvastatin-treated replicates was consistently in the 60-70% range on days 3, 6 and 12 (B). gDNA was amplified and shRNA populations hybridized onto custom Affymetrix Gene Modulation Array Platform (GMAP) arrays. Differences between shRNA abundances over the 12 days were incorporated into a SHARP score, collapsed into a GARP score and z transformed. A scatterplot of fluvastatin treated zGARP scores plotted against the ethanol (EtOH) treated ones show the comparative shRNA zGARP score distribution. Significant hits chosen for follow-up are indicated on the scatterplot (yellow diamonds) (C). Hits were defined as genes where the $\Delta Z_{\text{fluvastatin}}$ was more than three standard deviations lower than the mean $\Delta Z_{\text{fluvastatin}}$ of all genes (D).
In order to uncover fluvastatin-potentiating drop-outs, zGARP score differences between the fluvastatin and ethanol treated groups ($Z_{\text{fluvastatin}} - Z_{\text{ethanol}} = \Delta Z_{\text{fluvastatin}}$) were computed. Genes were the $\Delta Z_{\text{fluvastatin}}$ was more than three standard deviations lower than the mean $\Delta Z_{\text{fluvastatin}}$ of all genes were defined as hits (Figure 3-1 D, Table 3.1). Taken together, we have identified 151 potential putative genes whose knockdown could potentiate the anti-cancer effects of fluvastatin.
<table>
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**Table 3-1:** Putative gene hits targeted by shRNAs that potentiate the anti-cancer effects of fluvastatin.
3.2.2 Validation of shRNA hits in the A549 cells uncovers potentiators of statin-induced apoptosis and anti-proliferative effects.

The genome-wide shRNA screen yielded a list of 151 potential hits (Table 3-1). Amongst the hits, ranked based on negativity of the zGARP scores, there were several MVA pathway related genes namely 3-hydroxy-3-methylglutaryl-Coenzyme A synthase 1 (HMGCS1), geranylgeranyl diphosphate synthase 1 (GGPS1), sterol regulatory element-binding protein 2 (SREBF2). Immediately preceding the conversion of 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) to MVA catalyzed by HMGCR, HMGCS1 is responsible for the production of HMG-CoA. Downstream of HMGCR, GGPS1 catalyzes the synthesis of geranylgeranyl pyrophosphate (GGPP) from farnesyl diphosphate and isopentenyl diphosphate. GGPP is integral to the post-translation modification of many proteins and GGPP incorporation to their C-terminus results in protein prenylation. SREBF2 is a key transcription factor involved in the maintenance of cholesterol homeostasis through a sterol-mediated feedback loop. Upon sterol-depletion, the latent SREBF2 is activated and induces transcription of sterol-responsive genes including HMGCR, HMGCS1 and low-density lipoprotein receptor (LDLr) (Figure 3-2)\textsuperscript{38,39}. 
Figure 3-2: HMGCR inhibition by statins triggers a restorative feedback loop in hepatocytes

(A) HMGCR maintains normal levels of MVA derived end-products in hepatocytes. (B) Upon statin-mediated HMGCR inhibition, the cell is depleted of sterols such as cholesterol (1). In sterol-depleted cells, the latent transcription factor, SREBF2, is translocated from the endoplasmic reticulum to the Golgi apparatus where it is cleaved by proteases (2) and its mature, active form translocates to the nucleus (3). (C) Once in the nucleus, SREBF2 binds to promoter regions containing sterol response elements (SREs) inducing the transcription of HMGCR, HMGCS1 and LDLr. (D) Resulting increased LDLr at the surface of the membrane internalizes and thereby lowers serum LDL-cholesterol levels.
Targeting the MVA through inhibition of HMGCR using statins has been suggested for decades as being an effective anti-cancer therapeutic strategy. Dysregulation of the MVA pathway at the level of increased activity and expression of HMGCR has been implicated in a variety of cancers. Targeting the MVA pathway at more than one nodal point has the potential to be an effective therapeutic strategy and we therefore chose two MVA pathway related hits, GGPS1 and SREBF2 for further validation. Subsequent putative hits chosen for validation were mitogen-activated protein kinase kinase 4 (MAP2K4) and phosphatidylinositol 4-kinase, catalytic beta (PIK4B). Targeting these genes as an anti-cancer therapeutic strategy, either alone or in combination with statins has not been previously explored but is attractive since pharmacological compounds targeting the MAP2K4 and PIK4B proteins directly or indirectly are available.

Distribution of gene hits chosen for validation are highlighted in the scatterplot of fluvastatin and ethanol zGARP scores (Figure 3-1C). The individual drop-out profiles of the two best shRNAs for each gene over time are shown Figure 3-3.
Figure 3-3: Line plots of the two best hairpins for each hit chosen for validation show the dropout over time.

Line plots of the two best hairpins for each gene (GGPS1, MAP2K4, PI4KB, and SREBF2) from ethanol (solid line) and fluvastatin (dashed line) treated shA549 cells. Matching hairpins in each condition are color coded in red and blue.
Both hairpins targeting the MVA pathway related GGPS1 and SREBF2 show a robust decrease in the fluvastatin treated group relative to ethanol. In order to validate the four hits, we chose two independent shRNAs from the original TRC1 library targeting each gene and generated A549 sublines stably expressing each shRNA (Table 3-2). Two control sublines, transduced with shRNAs targeting a non-human gene, LacZ, were concurrently generated.
Table 3-2: TRC Clone ID's of shRNA hits chosen for validation.

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Protein expression of each target was assessed for knockdown following selection of cell lines for the stably expressing shRNA construct (Figure 3-4).
Figure 3-4: Generation of A549 sublines stably expressing shRNA constructs demonstrates knockdown of protein or mRNA target.

Following selection, protein lysates harvested from asynchronously growing A549 cells were immunoblotted with the appropriate primary and secondary antibodies. Knockdown of GGPS1 was also validated using real-time PCR (polymerase chain reaction) relative to GAPDH and expression was normalized to the shLacZ #2 control. All immunoblots are representative of a minimum of three independent experiments. Real-time data represent the mean ± SD of three independent experiments *p < 0.05 (one-way ANOVA with a Dunnet post test, the shGGPS1 groups being significantly different from the control shLacZ.)
Stable knockdown of SREBF2 was evident in both sublines when compared to the LacZ A549 controls and growth immediately following selection of each subline was comparable to the LacZ subline controls. Following exposure to sublethal concentrations of fluvastatin, both shSREBF2 A549 sublines showed a remarkable increase in apoptosis as measured by assessing cellular pre-G1 DNA content (Figure 3-5 A). The fluvastatin-induced apoptosis was dose dependent and minimal in the shLacZ A549 sublines, as expected. Furthermore, the percent MTT activity and the half-maximal fluvastatin inhibitory concentrations (IC$_{50}$) values generated by treating A549 sublines with a range fluvastatin doses were significantly decreased in both shSREBF2 A549 sublines, respectively (Figure 3-5 C and D). The IC$_{50}$ values for both LacZ control A549 sublines, ranging from 30-45 µM, were reduced to 5 µM in the shSREBF2 A549 sublines.

GGPS1 knockdown at the protein level (Figure 3-4) was not strong and GGPS1 mRNA levels were reduced by only 50% in both GGPS1 A549 sublines. However, there was sufficient knockdown to observe a dramatic induction fluvastatin-mediated apoptosis (Figure 3-5A). Again, as with the SREBF2 A549 sublines, the apoptosis was dose-dependent. The concomitant addition of GGPP with fluvastatin, the immediate downstream product formed by GGPS1 mediated catalysis, abrogated apoptosis (Figure 3-5 B) indicating that the knockdown of GGPS1 was on-target.

A dramatic reduction in fluvastatin IC$_{50}$ in the GGPS1 A549 sublines when compared to LacZ controls was also apparent (Figure 3-4 C). Variations in cellular growth between the different sublines could impact fluvastatin sensitivity irrespective of any potentiating effects caused by the specific knockdown the target. All cell lines were equally seeded at the commencement of an experiment and but there were no significant differences between the final net absorbance in the control cells (Figure 3-4 E). Although the MTT assay measures the activity of mitochondrial succinate dehydrogenase and is therefore only a proxy for cellular
proliferation, the lack of observable differences in the final absorbance between the untreated controls in the assessed A549 sublines suggests that growth between the cells was similar.

At least one of the A549 sublines expressing shRNA targeting MAP2K4 and PIK4B, shMAP2K4 #2 and shPI4KB #2 respectively, showed increased sensitivity to fluvastatin exposure when compared to the A549 control sublines (Figure 3-5A). Although a small degree of potentiation upon treatment with fluvastatin was observed in the shMAP2K4 #2 subline, the drop-out over time was minimal (Figure 3.3). Furthermore, as both shMAP2K4 sublines were characterized by similar levels of knockdown at the protein level (Figure 3.4) and only one demonstrated an appreciable effect, it is possible that this is an off target effect. Further validation using different shRNA constructs or siRNA’s would need to be conducted before concluding that knockdown of MAP2K4 potenitates statin-induced apoptosis.

In the case of PI4KB, degree of shRNA knockdown was not correlated to levels of protein expression. Although the shPI4KB #1 A549 subline consistently expressed lower amounts of PI4KB protein following multiple passages, this subline was not sensitive to fluvastatin-induced apoptosis (Figure 3-5 A). The generation of A549 sublines stably expressing shRNAs targeted against GGPS1, PI4KB and MAP2K4 were difficult to grow following selection and sublines needed several passages before cells could be utilized for experiments. Adaptation of a subline to stable knockdown of a target essential for growth is a possibility, potentially confounding assessments of sensitivity due to compensatory upregulation of other proteins or pathways. A subline such as A549 shPI4K #1, expressing lower amounts of PI4KB protein relative to the A549 shPI4K #2 cells took longer to adapt following selection and did not show increased fluvastatin-induced apoptosis. In both of the MAP2K4 sublines showed that the knockdown of the kinase targets was decreased over time (data not shown) indicating selection of subpopulations with higher protein levels of MAP2K4. Furthermore, the shMAP2K4 #2 A549 subline had higher levels of basal apoptosis
when compared to all other sublines (Figure 3-5A). In the shGGPS1 A549 sublines, a 50% mRNA target reduction was sufficient to potentiate the apoptosis-inducing effects of fluvastatin. Taken together, validation of hits using the approach of generating sublines stably expressing shRNA constructs is not appropriate for every target and highly dependent on whether the target is essential for growth.

Whereas stable knockdown of SREBF2 was well tolerated and differences between the shSREBF2 A549 sublines and control cells were apparent only when challenged with statins, probable adaptation upon knockdown of MAP2K4 and PI4KB in the A549 cells led to a masking of their ability to behave as potenitators of fluvastatin-induced apoptosis and anti-proliferative effects. Therefore, for further validation of non-MVA related targets we chose the approach of transiently knocking down the target using small interfering RNAs (siRNA). The availability of a direct pharmacological inhibitor of PI4KB led us to further validate this candidate hit.
Figure 3-5: shRNAs targeting gene hits identified in the screen potentiate fluvastatin-induced apoptosis and anti-proliferative effects.

(A) Exposure of A549 sublines stably expressing the indicated shRNA construct to sub-lethal doses of fluvastatin for 72 hours significantly induced apoptosis in at least one of the two cell lines expressing an shRNA targeting a single gene hit. Apoptosis was assessed by fixed propidium iodide staining and dead cells were characterized by pre-G1 DNA content. Bars represent the mean ± SD of at least three independent experiments *p < 0.05 (one-way ANOVA with Tukey post test within each A549 subline comparing all groups, the fluvastatin treated groups being significantly different from the ethanol control). (B) Fluvastatin-induced apoptosis in the shGGPS1 #1 subline was reversed by the concomitant addition of GGPP. Bars represent the mean ± SD of at least 3 independent experiments *p < 0.05 (one-way ANOVA with Tukey post test within each A549 subline comparing all groups, the fluvastatin treated group being significantly different from the others). The A549 sublines were independently generated a second time and similar results were obtained. (C) Knockdown of SREBF2 and GGPS1 significantly decreased the fluvastatin IC₅₀ values computed as described in methods. Sublines were exposed to a range of fluvastatin doses for 72 hours and viability was assessed using the MTT assay. *p < 0.05 (one-way ANOVA with Tukey post test comparing all groups, all SREBF2 and GGPS1 sublines being significantly different from the LacZ controls). Representative dose-response curves for the SREBF2 sublines are shown in (D) and corresponding final net absorbances (following subtraction of blank) at 590 nm for the untreated controls 96 hours post-growth for each subline are shown in (E) Bars represent the mean ± SD of at least 3 independent experiments.
3.2.3 Concomitant targeting of PI4KB and HMGCR showed anti-proliferative effects in lung and breast cell lines.

The reduction of target mRNA using siRNA’s is short-term and therefore advantageous if adaptive responses to knockdown of a specific target occur. In order to clarify whether the inconclusive results obtained with the use of the shRNAs targeting PI4KB were off-target or a result of adaptive responses, we employed transient knockdown approaches. We therefore used siRNA duplexes at low nanomolar concentrations and transiently transfected parental A549 cells. Reduction of PI4KB protein was evident 72 hours post transfection as compared to A549 cells transfected with a negative scrambled siRNA control and an untransfected control (Figure 3-6 A). As cells were treated with fluvastatin 48 hours post transfection, reductions in PI4KB protein levels coincided with fluvastatin exposure allowing for potentiation of anti-proliferative activity to be assessed. In A549 cells transfected with siRNA #1, which resulted in the greatest decrease of PI4KB protein, there was a significant reduction in fluvastatin IC50 values compared to cells transfected with the siRNA negative control (Figure 3-6 B).

To exclude the possibility that the identified hit shRNA hits that potentiated fluvastatin-mediated anti-cancer effects are limited to one cell line, we extended our experiments to a different cell line of another tumour type, the triple negative (ERα-, PR-, and HER2-negative) basal-like MDAMB231 breast cancer cells. Breast cancers characterized by triple negative tumours have fewer effective therapeutic options upon recurrence. As lipophilic statins such as fluvastatin have already shown clinical promise in the treatment of breast cancer, and additional clinical trials are underway, uncovering potentiators of fluvastatin’s anti-cancer effects in this tumour type has high potential for immediate clinical utility.
Similar to the A549 cells, reduction of PI4KB protein also occurred 72 hours post transfection relative to the negative scrambled siRNA and untransfected controls (Figure 3-6C). Consequently, transfection with all three siRNA duplexes targeting PI4KB significantly decreased fluvastatin IC50 values relative to negative siRNA control (Figure 3-6D). In the breast cancer cells, all three siRNA duplexes consistently knocked down the PI4KB target and this was accompanied with concomitant decreases in the fluvastatin IC50 values whereas in the A549 cells, only one duplex, which demonstrated the greatest knockdown resulted in sensitization to fluvastatin’s anti-cancer effects. Higher concentrations of the other two duplexes could be used to achieve greater knockdown and further verify PI4KB as a potenitator of statin induced anti-cancer effects.
1 = Control
2 = siRNA Control
3 = PI4KB siRNA #1
4 = PI4KB siRNA #2
5 = PI4KB siRNA #3

A549

MDAMB231

IC50 Fluvastatin (uM)
Figure 3-6: Transient knockdown of PI4KB sensitizes lung and breast cancer cells to the anti-proliferative effects of fluvastatin.

PI4KB protein expression in A549 lung and MDA MB231 breast cancer cells was decreased 72 hours following transient transfection with 10 nM of three different siRNAs targeting PI4KB mRNA relative to a negative scrambled siRNA control and an untransfected control (A and C respectively). Fluvastatin IC$_{50}$ values in A549 and MDA MB231 cells were significantly lower following siRNA transfection targeting PI4KB. 24 hours post-transfection, cells were re-seeded in 96 well plates, allowed to adhere overnight and treated with a range of fluvastatin doses for 72 hours (B and D respectively). Bars represent the mean ± SD of least 3 independent experiments *p < 0.05 (one-way ANOVA with Tukey post test comparing all groups, the indicated groups being significantly different from the siRNA control).

Immunoblots are representative of a minimum of three independent experiments for the MDA MB231 cells and two independent experiments for the A549 cells at the 72 hour time-point.
To corroborate the observations using a pharmacological approach we used a PI4KB inhibitor, PIK-93\textsuperscript{41,42} and co-treated the A549 and MDAMB 231 cells. PIK-93 when used at a concentration of 1 µM is reported to specifically inhibit PI4KB and not the type III counterpart phosphatidylinositol 4-kinase, catalytic alpha (PI4K-alpha) which requires PIK-93 concentrations greater than 10 µM to achieve inhibition\textsuperscript{42}. Furthermore, this dose was sublethal in our cell systems allowing for the assessment of potentiation. Remarkably, the fluvastatin-PIK-93 combination not only significantly lowered fluvastatin IC\textsubscript{50} values in A549 (Figure 3-7A and B) and MDAMB231 cells (Figure 3-7 C) but also induced apoptosis in the MDAMB231 cells (Figure 3-7 D and E). In combination with PIK-93, the fluvastatin concentrations needed to induce apoptosis were in the sub micromolar range (0.25 µM). Using genetic and pharmacological approaches we have validated a novel target that sensitizes breast and lung cancer cells to fluvastatin induced apoptosis.
**A549**

**MDAMB231**

**Fluvastatin + PIK93 (1 uM)**

**Fluvastatin (0.25 μM)**

**Fluvastatin (0.5 μM)**

**PIK93 (1 μM)**

**Control**

**Fluvastatin (0.5 μM)**

**PIK93 (1 μM)**

**Fluvastatin + PIK93**

**Normalized MTT Activity**

**Pre-G1 Population**

**IC50 Fluvastatin (uM)**

**% Normalized MTT Activity**

**% Pre-G1 Population**

**Fluvastatin (0.5 μM)**

**PIK93 (1 μM)**

**Fluvastatin + PIK93 (1 uM)**

**Fluvastatin (0.25 μM)**

**Fluvastatin + PIK93 (1 uM)**

**Fluvastatin**

**Fluvastatin + PIK93 (1 uM)**

**Fluvastatin**

**Fluvastatin + PIK93 (1 uM)**
Figure 3-7: The combination of fluvastatin and a pharmacological inhibitor of PI4KB is anti-proliferative and induces apoptosis in lung and breast cancer cells.

Fluvastatin IC$_{50}$ values in A549 and MDAMB231 cells were decreased following concomitant treatment with 1 µM of a PI4KB inhibitor PIK93 for 72 hours (A and C respectively). Representative fluvastatin dose-response curves in the presence and absence of PIK93 for A549 cells are shown in B, viability was assessed using the MTT assay. Bars represent the mean ± SD of least 3 independent experiments. *p < 0.05 (t-test, unpaired, two-tailed). The combination of sublethal doses of PIK93 and fluvastatin induce apoptosis in MDAMB231 cells following 96 hours of treatment (D), representative histograms are shown in (E). Apoptosis was assessed by fixed propidium iodide staining and dead cells were characterized by pre-G1 DNA content. Bars represent the mean ± SD of 3 independent experiments. *p < 0.05 (one-way ANOVA with Tukey post test comparing all groups, the indicated groups being significantly different from control, fluvastatin 0.25 µM, fluvastatin 0.5 µM and PIK93 1 µM groups).
3.2.4 Down-regulating the SREBF2 mediated sterol-feedback loop in combination with statins is an effective anti-cancer strategy to induce lung and breast tumour cell kill.

The remarkable sensitization to the anti-cancer effects of fluvastatin upon stable SREBF2 knockdown in A549 cells (Figure 3-5) prompted a more thorough investigation into the generalizability of this phenomenon in another solid tumour type. Due to recent positive clinical results in the treatment of breast cancer patients with fluvastatin\textsuperscript{18}, we chose two breast cancer cell lines, the triple negative basal-like MDAMB231 and the luminal MCF7 (ER\textsubscript{α}+, PR+, and HER2- (Human Epidermal Growth Factor Receptor 2), both previously characterized to have some molecular fidelity to the primary tumours whose subtypes they represent\textsuperscript{43}. The MDAMB231 and MCF7 cells display a differential range of statin sensitivities; while the MDAMB231 are characterized by fluvastatin IC\textsubscript{50} values in the low micromolar range, those of the MCF7 cells are ten fold higher. As there is no universal biomarker of statin-sensitivity, identifying a target that sensitizes cells displaying a wide range of statin sensitivities to statin-mediated anti-cancer effects is therapeutically attractive. SREBF2 knockdown in the A549 shSREBF2 sublines was well tolerated; we therefore generated MDAMB231 and MCF7 sublines stably expressing shRNAs targeting SREBF2. SREBF2 protein levels in the MDAMB231 sublines were decreased relative to shLacZ sublines (Figure 3-8A) and consequently this resulted in a significant reduction of fluvastatin IC\textsubscript{50} values (Figure 3-8B). Although in both MDAMB231 and MCF7 shSREBF2 sublines there was a reduction of fluvastatin IC\textsubscript{50} relative to controls, the magnitude of difference was greater in the MCF7 cells (Figure 3-8D). Control fluvastatin IC\textsubscript{50} values in the MCF7 control sublines ranged from 30 to 50 µM and were in the lower micromolar range (5 to 10 µM) in the shSREBF2 sublines.
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Exposure of representative shSREBF2 and control shLacZ MDAMB231 and MCF7 sublines to sublethal doses of fluvastatin resulted in a dramatic increase in apoptosis in the shSREBF2 sublines (Figure 3-8 C and E respectively).
**A** MDAMB231

1. Uncleaved SREBF2
2. IB: SREBF2
3. Cleaved SREBF2
4. IB: Tubulin

1 = shLacZ #1
2 = shLacZ #2
3 = shSREBF2 #1
4 = shSREBF2 #2

**B** MDAMB231

IC50 Fluvasatin (µM)

1 2 3 4

% Pre-G1 Population

2 3

**C** MDAMB231

**D** MCF7

1. Uncleaved SREBF2
2. IB: SREBF2
3. Cleaved SREBF2
4. IB: Tubulin

**E** MCF7

IC50 Fluvasatin (µM)

1 2 3 4

% Pre-G1 Population

2 3 4

**F** MCF7

ethanol
10 uM fluvasatin

% Pre-G1 Population

2 4
Figure 3-8: Stable knockdown of SREBF2 sensitizes breast cancer MCF7 and MDAMB231 cells to the pro-apoptotic and anti-proliferative effects of fluvastatin.

Parental MDAMD231 and MCF7 cells were infected with lentiviral particles and puromycin-selected for 48 hours. Following puromycin-selection, protein lysates harvested from asynchronously growing MDAMB231 and MCF7 cells were immunoblotted and showed decreased SREBF2 protein expression relative to cells transduced with the LacZ controls (A and D respectively). Fluvastatin IC$_{50}$ values in the MDAMB231 and MCF7 sublines expressing the SREBF2 shRNAs were significantly lower when compared to the control sublines. Dose response curves were generated following exposure to fluvastatin for 72 hours and viability was assessed using the MTT assay (B and E respectively). Exposure of representative MDAMB231 and MCF7 sublines to sublethal doses of fluvastatin caused a significant increase in apoptosis relative to a control LacZ subline (C and F respectively). Apoptosis was assessed by fixed propidium iodide staining and dead cells were characterized by pre-G1 DNA content. Bars represent the mean ± SD of at least 3 independent experiments *p < 0.05 (one-way ANOVA with Tukey post test comparing all groups, the indicated groups being significantly different from control sublines in C and F and from all other groups in B and E). Immunoblots are representative of a minimum of three independent experiments.
SREBF2 is a key transcriptional regulator of cholesterol homeostasis. SREBF2’s role in inducing the expression of sterol responsive genes upon sterol-depletion for instance following statin treatment prompted us to assess the levels of HMGCR and other sterol-responsive genes in the shSREBF2 sublines.

Remarkably, the knockdown of SREBF2 by two independent shRNAs nearly completely abolished the upregulation of sterol-responsive genes HMGCR and HMGCS1 following treatment with fluvastatin. While in the shLacZ A549 and MCF7 sublines there was a robust upregulation of HMGCR and HMGCS1 following 24 hours of fluvastatin treatment, this was largely abrogated in the shSREBF2 sublines (Figure 3-9 A and B respectively). Levels of SREBF2 itself did not increase upon fluvastatin treatment and robust knockdown was confirmed in both cell lines. As sensitivity to drugs may be affected by proliferation, we conducted proliferation assays using all shSREBF2 and shLACZ sublines. There were no significant differences in doubling times in all four sublines in MCF7, MDAMB231 and A549 cells (Figure 3-9 C).

The sensitization to fluvastatin-induced apoptosis upon knockdown of SREBF2 was not only evident in A549 lung cancer cells but observable in breast cancer cells characterized by a wide range of statin sensitivities. Next we investigated whether targeting HMGCR using statins in combination with SREBF2 knockdown is an effective therapeutic strategy in vivo.
A

**Relative HMGCR levels**

- Ethanol
- Fluvasatin

<table>
<thead>
<tr>
<th>shSREBF2 #1</th>
<th>shSREBF2 #2</th>
<th>shLacZ #1</th>
<th>shLacZ #2</th>
</tr>
</thead>
<tbody>
<tr>
<td>*</td>
<td>*</td>
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</tr>
</tbody>
</table>

B

**Relative HMGCS1 levels**

- Ethanol
- Fluvasatin

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<th>shSREBF2 #2</th>
<th>shLacZ #1</th>
<th>shLacZ #2</th>
</tr>
</thead>
<tbody>
<tr>
<td>*</td>
<td>*</td>
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</tbody>
</table>

C

**Relative SREBF2 levels**

- Ethanol
- Fluvasatin

<table>
<thead>
<tr>
<th>shSREBF2 #1</th>
<th>shSREBF2 #2</th>
<th>shLacZ #1</th>
<th>shLacZ #2</th>
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</table>

**A549**

**MCF7**

**Doubling time (hours)**

- MDAMB231
- MCF7
- A549

<table>
<thead>
<tr>
<th>shLacZ #1</th>
<th>shLacZ #2</th>
<th>shSREBF2 #1</th>
<th>shSREBF2 #2</th>
</tr>
</thead>
</table>
Figure 3-9: Knockdown of SREBF2 abrogates the upregulation of HMGCR and HMGCS1 upon fluvastatin exposure.

Parental A549 and MCF7 cells were infected with lentiviral particles and puromycin-selected for 48 hours. Four sublines were generated from each cell line. 2 sublines stably expressing shRNAs targeting SREBF2 and 2 control sublines stably expressing shRNAs targeting LacZ. Following 48 hours of puromycin-selection of infected cells, RNA was harvested from subconfluent cells grown over several passages and treated for 24 hours with 10 μM of fluvastatin or ethanol control and assessed for expression of HMGCR, HMGCS1 and SREBF2 relative to GAPDH in shA549 (A) and shMCF7 (B) sublines (shSREBF #1, shSREBF #2, shLacZ #1, shLacZ #2). Data represent the mean ± SD of at least three independent experiments. *p < 0.05 (t-test, unpaired, two-tailed, comparison made within each subline). For basal comparisons across four sublines, *p < 0.05 (one-way ANOVA with a Tukey post test, the LacZ sublines being significantly different than both SREBF2 sublines). There were no significant differences in the growth rates between the shSREBF2 and control shLacZ sublines in MDAMB231, MCF7 and A549 cells as assessed using the CyQuant cell proliferation assay kit (C).
3.2.5 Fluvastatin delays tumour growth in MDAMB231 xenografts.

To evaluate whether SREBF2 potentiated the anti-cancer effects of fluvastatin \textit{in vivo}, we treated NOD-SCID mice harboring established xenografts of MDAMB231 shSREBF2 #1 and shLacZ #2 sublines. We chose to orally administer fluvastatin because this is the route of delivery for humans. Mice were subcutaneously injected with MDAMB231 sublines and when xenografts were palpable, mice were orally treated 3 times a week with PBS, or 25 mg/kg fluvastatin for ten days. Fluvastatin significantly decreased final tumour weight (Figure 3-10 A) and final tumour volume (Figure 3-10 B) in the MDAMB231 shSREBF2 xenograft group. Although not reaching statistical significance likely due to limited number of mice in a group, fluvastatin also had an effect on final tumour volume and weight within the MDAMB231 shLacZ group.

A similar experiment was conducted using the shSREBF2 #2 and shLacZ #2 sublines. Mice were treated with lower oral doses of fluvastatin (10 mg/kg), and tumours were allowed to reach a larger volume. Treatment of the mice harboring the shSREBF2 xenografts with fluvastatin resulted in a small but significant reduction in tumour volume following 21 days of treatment. There were no differences between the PBS and fluvastatin treated groups in the mice harboring the shLaCZ #2 controls. Experiments are underway with the remaining the cell lines.
NOD-SCID mice were injected subcutaneously with 4 million MDAMB231 shSREBF2 or shLACZ cells. After tumours became palpable (2-3 weeks), mice within each subline xenograft were randomized into groups and treated three times a week with 25 mg/kg fluvastatin orally (p.o.) or PBS (p.o.) and vehicle (i.p.) (Con.). Tumour volume was measured every two-three days. After 10 days of treatment, mice were sacrificed and tumours were resected and weighed (B). n= 3-5 mice per group. *p < 0.05 (t-test, unpaired, two-tailed, comparison made within each subline).

Figure 3-10: Fluvastatin delays tumour growth in MDAMB231 xenografts.
3.4 Methods

Compounds.
Fluvastatin, dissolved in ethanol was purchased from US Biologicals. PIK-93, dissolved in DMSO was purchased from SelleckBio and geranylgeranyl pyrophosphate (GGPP) was obtained as a solution from Sigma. DNAse free RNAse was purchased from Roche.

Cell Culture.
All cell lines were cultured in Dulbecco's modified eagle's medium H21 (DMEM H21). Media was supplemented with 10% fetal bovine serum and penicillin-streptomycin. Cells were grown asynchronously as monolayers at 37°C in 5% CO₂ and cell lines were routinely confirmed to be mycoplasma-free (MycopAlert mycoplasma detection kit, Lonza).

shRNA dropout screen.
A549 adenocarcinoma lung cancer cell line stably transduced with a TRC1 shRNA library (sh-A549) containing over 80,000 shRNAs encoded in the pLKO.1 vector targeting 16,000 human genes, approximately 7,000 of which have been validated, were seeded in factory tissue culture vessels (Nunc, 16.5 million sh-A549 cells/vessel). 2 hours post-seeding and upon adherence, cells were treated with sublethal doses fluvastatin (4-5 µM) or ethanol control. Fluvastatin dosing was chosen and accordingly adjusted throughout the duration of the experiment such that treatment led to decrease cell viability of 20-40% relative to ethanol control. Every 3 days cells were re-seeded and excess cells were pelleted and stored at -80 °C for subsequent genomic DNA extraction (QIAmp Blod Maxi Kit, Qiagen). PCR amplified shRNA populations were hybridized to the custom Affymetrix Gene Modulation Array Platform (GMAP) arrays as detailed elsewhere.
shRNA screen analysis and scoring.

Data was analyzed as previously described\textsuperscript{32}. Briefly, data was extracted and normalized using the CCBR-OICR Lentiviral Technology (COLT) database\textsuperscript{48}. Affymetrix Power Tools v1.12.0 were used for extraction and the Bioconductor affy package (v1.26.1) for normalization. Within each treatment condition and time point, the fold changes relative to time zero (t\textsubscript{0}), with cell doublings being accounted for, were calculated and an shRNA Activity Ranking Profile score (SHARP) was assigned. For each gene, two hairpins with the most negative SHARP scores were averaged and collapsed to the gene activity ranking profile (GARP)\textsuperscript{32} and Z-score normalized. Z score differences between the fluvastatin and ethanol treated groups (Z\textsubscript{fluvastatin} – Z\textsubscript{ethanol} = ΔZ\textsubscript{fluvastatin}) were computed. A negative Z score is indicative of an shRNA that was depleted over time. A lower ΔZ\textsubscript{fluvastatin}, is indicative of an shRNA that was more rapidly depleted in the fluvastatin treatment group and therefore the absence of the gene targeted by the shRNA, could be a potentiator of statin-induced anti-cancer effects. Genes were the ΔZ\textsubscript{fluvastatin} was more than three standard deviations lower than the mean ΔZ\textsubscript{fluvastatin} of all genes were defined as hits.

MTT, fixed propidium iodide (PI) assays.

(3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma-Aldrich) assays were done as previously described\textsuperscript{49}. In brief 1.5 x 10\textsuperscript{4} to 3 x 10\textsuperscript{5} cells/ml were plated in 96 well plates and after 24 hours, treated with the indicated compounds and concentration ranges for 72 hours. Half-maximal inhibitory concentrations (IC\textsubscript{50}) values were computed from dose-response curves using Prism (v5.0, GraphPad Software). For fixed PI assays, 3-3.5 x 10\textsuperscript{5} cells/dish were seeded in 10 cm dishes, after 24 hours, treated for 72 hours as indicated. Cells were fixed in 70% ethanol, treated with DNase free RNase and stained with 50 µg/mL PI
(Sigma-Aldrich). Cells were analyzed for the dying pre-G1 population by flow cytometry; ten thousand events were acquired using a FACSCalibur cytometer (BD Biosciences).

**Immunoblotting.**

Sub-confluent growing cells were washed with PBS and lysed using boiling hot SDS lysis buffer (1.1% SDS, 11% glycerol, 0.1M Tris pH 6.8) with 10% β-mercaptoethanol. Blots were probed with anti-SREBF2 (BD Pharmingen), anti-MAP2K4 (Cell Signaling Technology), anti-PI4KB (BD Pharmingen), anti-tubulin (Santa Cruz Biotechnology), anti-actin (Sigma). Primary antibodies were detected using the Odyssey infrared imaging system with IRDye-labeled secondary antibodies (LI-COR Biosciences).

**Real-Time PCR.**

RNA was harvested from sub-confluent growing cells using TRIzol Reagent (Invitrogen). cDNA was synthesized from 0.5 µg using SuperScript III (Invitrogen). HMGCR mRNA levels relative to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were assayed as previously described using SYBR Green master mix (Applied Biosystems)\(^5\). LDLR mRNA levels were assayed with TaqMan Gene Expression Assays, SREBF2 (Hs01081784_m1), GGPS1 (Hs00191442_m1), GAPDH (Hs99999905_m1) using TaqMan master mix (Applied Biosystems). ABI Prism 7900 Sequence Detection System was used for the acquisition and analysis of relative transcript levels.

**Generation of shRNA validation cell lines.**

DNA from bacterial glycerol stocks of shRNAs from the TRC1 library (Table 3.) was generated (Qiagen Plasmid Maxiprep kit). Lentiviral particles were generated by calcium phosphate transfection of sub-confluent (50-60%) 293TV cells with 10 µg of TRC pLKO.1
puro shRNA construct, 5 µg each of pMDG1.vsvg, pRSV-Rev and pMDLg/pRRE constructs. Virus was collected 24 and 48 hours later, filtered though a 0.45 µm filter and stored at -80 °C. Stably expressing shRNA A549, MCF7 and MDAMB231 cell lines were generated following infection of cells (60-70% confluent) with 2 ml of the lentiviral particles combined with 1 ml of medium containing 8 µg/ml of polybrene. After 48 hours cells were split and selected using 2 µg/ml of puromycin and maintained as pooled populations.

**siRNA transient transfections.**

Cells were seeded in (1.2-1.5 x 10^5 cells/well) and 24 hours later transfected with individual siRNA duplexes (Origene). Briefly, duplexes at a of concentration of 250 nM were incubated in 100 µL of serum and antibiotic free DMEM medium with 20 µL of HiPerfect (Qiagen) for 10 minutes following which they were added to the cells. Cells were either harvested for protein expression analysis or re-seeded in 96 wells for MTT assays.

**Xenografts models.**

Logarithmically growing MDAMB231 cells stably transduced with shRNA SREBF2 and control constructs (4 million) were subcutaneously injected into the flanks of NOD SCID mice. When tumours became palpable, 2-3 weeks post-injection, treatment commenced. Mice were orally treated with 25 mg/kg of fluvastatin suspended in PBS 5 times a week. Following ten days of treatment, tumour weight and volume were measured. Final tumour volume was calculated using the following formula: (tumour length x width^2)/2. Animal work was carried out with the approval of the Princess Margaret Hospital ethics review board in accordance to the regulations of the Canadian Council on Animal Care.
3.5 Discussion

Statins are currently being evaluated in the clinic as anti-cancer agents. To increase their utility in a combination setting, we used a genome-wide shRNA screen to identify potentiators of statin-induced anti-tumour effects. In this screen we treated the adenocarcinoma A549 cell line stably transduced with the TRC1 shRNA library with sublethal doses of fluvastatin and identified significant shRNAs that dropped out in response to fluvastatin exposure over time. The A549 cell line is an adenocarcinoma NSCLC line and represents a tumour type faced with considerable treatment challenges. Lung cancer is a principal cause of cancer-related deaths and the histologically complex NSCLC comprises the majority of lung cancer. Patients with the best prognosis are those with surgically resectable early stage NSCLC but even those patients have a 70% 5-year survival and a high recurrence rate\textsuperscript{51,52}. Although chemotherapy is used at various stages of intervention in NSCLC patients, there are few effective treatment options and novel anti-cancer agents are urgently needed.

From the 151 putative genes whose knockdown could potentiate the anti-cancer effects of statins in A549 cells, we validated four, SREBF2, GGPS1, MAP2K4 and PI4KB. Indicative of the robustness of our screen was the identification of GGPS1 as a potentiator of fluvastatin’s anti-proliferative effects. Targeting isoprenoid biosynthesis with agents specifically developed to inhibit the isoprenoid transferase enzymes downstream of HMGCR, has been investigated for its ability to perturb essential pathways in cancer cells such as Ras. The most clinically advanced class of the isoprenoid transferase-inhibiting compounds, are the farnesyltransferase inhibitors (FTIs), but they have yet to demonstrate real clinical efficacy\textsuperscript{53}. It has been suggested that the limited clinical anti-tumour activity of the FTIs results from alternative prenylation stemming from the activity gernaylgeranyltransferases I (GGTase-I). This has launched the development of dual FTI and
GGTase-I inhibitor, however these compounds have dose-limited toxicities precluding clinical use\textsuperscript{54}. To date, four drugs have been tested in more than seventy clinical trials: tipifarnib, lonafarnib, BMS-214662, and L-778123. The first three drugs are FTIs and L-778123 is the abovementioned dual FTI and GGTase-I inhibitor which is no longer in development. The anti-cancer efficacy of these drugs was tested in solid and haematological malignancies, alone or in combination with other agents and the results have been largely negative\textsuperscript{55}. Only one specific GGTase-I inhibitor has been tested in clinical trials and the GGTase-II inhibitors have not yet reached clinical testing. Given the overall disappointing clinical results of targeting the FTIs and GGTase’s, alternative therapeutic strategies of disrupting protein prenylation in cancer cells, are needed. Here we demonstrate that targeting the MVA pathway using statins and by knockdown of GGPS1, resulted in the dramatic induction of apoptosis in A549 cells, reversible by the concomitant addition of GGPP (Figure 3-4 A and B). Our finding is supported another group who have found that inhibiting GGPS1 with newly synthesized GGPS1 inhibitors and statins has synergistic anti-cancer activity\textsuperscript{56}. This strategy of targeting GGPS1 and HMGCR is advantageous as it precludes the use of clinically limited high doses of a single compound to induce tumour kill. The difficulty in generating A549 sublines with highly sustained GGPS1 knockdown (Figure 3-3) is supported by high negative Z\textsubscript{ethanol} values (Table 3-1) indicting that GGPS1 is essential for cancer cell growth and potentially a singularly useful target. Taken together, we have shown that inhibition of the MVA pathway using statins and knockdown of another enzyme in the MVA pathway, GGPS1 results in a robust induction of apoptosis reversible by concomitant addition of GGPP and therefore likely caused by isoprenoid depletion and disruption of protein prenylation.

HMGCR expression is in part controlled by SREBF2 mediated transcriptional regulation. Sensing sterol depletion as for instance upon treatment with a statin, latent
SREBF2 is activated and induces transcription of sterol-responsive genes including HMGCR. SREBF2 was identified as a drop-out hit in our genome-wide shRNA screen. The generation shSREBF2 A549 sublines with stable suppression of SREBF2 protein and mRNA levels was remarkably well tolerated and shSREBF2 A549 sublines were characterized by the same growth rates as control sublines. The dramatic induction of apoptosis and remarkable reduction in IC\textsubscript{50} values upon fluvastatin exposure in the shSREBF2 sublines prompted further validation in breast cancer where statins have shown tremendous therapeutic promise\textsuperscript{18}. SREBF2 knockdown resulted in a dramatic sensitization to the pro-apoptotic and anti-proliferative effects of fluvastatin in MCF7 and MDAMB231 cells (Figure 3-7) without any changes to the proliferation of the shSREBF2 sublines. Remarkably, despite residual levels of SREBF2 mRNA (~10 %) in all shSREBF2 sublines, there was no upregulation of HMGCR upon fluvastatin exposure.

Recently, an independent group published a second window-of-opportunity trial in which patients with primary invasive breast cancer were treated with the lipophilic atorvastatin 2 weeks prior to surgery\textsuperscript{57}. In addition to evaluating tumour proliferation (Ki67 index), HMGCR protein expression prior to and following atorvastatin treatment in tumour tissue was assessed. HMGCR-positive patient tumours not only experienced a significant decrease in Ki67 following atorvastatin treatment but a concomitant increase in HMGCR protein levels. These findings are of importance because they suggest that statins directly target the tumour cell and that the tumour cell then experiences a feedback loop mediating HMGCR upregulation following statin exposure. The anti-proliferative response in HMGCR-positive tumours after statin treatment suggests a potential predictive biomarker for statin response. We have previously shown that elevated HMGCR mRNA was correlated with poor prognosis and reduced survival of breast cancer patients\textsuperscript{58}. Taken together, HMGCR is an important anti-cancer target whose over-expression has been linked to poor patient prognosis.
and direct HMGCR inhibition in a patient tumour cell has demonstrated anti-proliferative efficacy. The consequential implications of concomitant HMGCR upregulation within a tumour following statin exposure in a clinical setting have not been addressed but as demonstrated by our results, blocking the upregulation via SREBF2 knockdown could have profound anti-tumour consequences. In cell culture, the implications of HMGCR upregulation following statin exposure as it relates to statin-sensitivity, has been explored in the context of multiple myeloma (MM)\(^50\). In MM cell lines, statin-sensitive MM cell lines did not demonstrate a robust feedback loop and the expected upregulation of sterol-responsive genes such as HMGCR. In contrast, statin-insensitive MM cell lines exhibited a robust feedback loop. In the current study, we have demonstrated the functional consequences of blocking the feedback loop in breast and lung cancer cells in the context of the remarkable sensitization to statin-induced apoptosis (Figure 3-11).

Targeting SREBF2 with an shRNA was an effective cell culture strategy to demonstrate anti-cancer efficacy in combination with statins. \textit{In vivo} however, the clinical utility of RNAi technology has yet to be demonstrated. The chief obstacle to systemic RNAi non-viral delivery has been the appropriate delivery vehicle. While advances have been made particularly by Alnylam Pharmaceuticals and Tekmira Pharmaceuticals in their use of polyethylene glycol modified (PEGylated) lipid nanoparticle technology coupled with stabilizing nucleic acid modifications, their lead compounds are still only in early phase clinical trials\(^59\). Although there are no specific direct SREBF2 inhibitors, recently, compounds such as betulin that indirectly inhibit SREBP activity by preventing its maturation through inducing interaction of SREBP cleavage activating protein (SCAP) and Insig have been identified\(^60\). Our work supports future evaluation of statins in combination with inhibitors of SREBP activity for their anti-cancer efficacy.
A. Normal cell

Serum LDL-cholesterol

Restoration of cholesterol and other MVA derived end-products

B. Tumour cell

C.}

1) Sterol-depletion

2) SREBF2

3) HMGCR

4) FTIs, GGTPs

Protein prenylation
Figure 3-11: Targeting the MVA pathway in tumour cells.

(A, upper left panel) In normal hepatocytes, HMGCR maintains levels of MVA derived end-products. (upper right panel) Upon statin-mediated HMGCR inhibition, the cell is depleted of sterols such as cholesterol (1). In sterol-depleted cells, the latent transcription factor, SREBF2, is translocated from the endoplasmic reticulum to the nucleus (3). (lower right panel) Once in the nucleus, SREBF2 binds to promoter regions containing sterol response elements (SREs) inducing the transcription of HMGCR, HMGCS1 and LDLr. (lower left panel) Resulting increased LDLr at the surface of the membrane internalizes LDL-cholesterol from the serum thereby lowering LDL-cholesterol levels.

(B) In tumour cells, the MVA pathway can be dysregulated in several ways. (1) LDLr activity and expression can be elevated in cancer cells as can HMGCR expression and activity (2). Tumour cells are vulnerable to the depletion of sterol derived MVA endproducts that are used for protein prenylation of several key oncogenic proteins such as Ras (3). Dysregulation at the level of the restorative sterol feedback loop mediated by the SREBF2 can also occur. Some cancer cells are not able to upregulate sterol-responsive genes following statin inhibition while others are characterized by abnormally high cholesterol import despite already high endogenous cholesterol levels (4). Points of potential therapeutic intervention of the MVA pathway in cancer cells are illustrated in (C). Statins target HMGCR, the rate-limiting enzyme of the MVA pathway. FTIs and GGTIs prevent protein prenylation of key oncogenic molecules such as Ras. Newly defined mechanisms by which the MVA pathway can be targeted include the knockdown of GGPS1, an enzyme responsible for the production of GGPP and one level upstream of the germaylgeranyl transferase enzymes, and knockdown of the transcription SREBF2 which prevents the upregulation of sterol-responsive genes in cancer cells where the feedback loop is intact.
In addition to SREBF2 and GGPS1, we have identified non-MVA related targets PI4KB and MAPK4, whose knockdown potentiated the anti-cancer effects of statins. PI4KB regulates transport of ceramide, required for the synthesis of sphingomyelin, between the endoplasmic reticulum (ER) and Golgi, and phosphorylates phosphatidylinositol (PI) to phosphatidylinositol 4-phosphate (PI(4)P). Recent reports implicate PI4KB’s oncogenic involvement in mammary neoplasia and ability to cooperate with eukaryotic elongation factor 1 alpha 2 (eEF1A2) in disrupting acinar morphogenesis. Recently, a genomic and transcriptomic analysis of 2,000 breast tumours identified the PI4KB gene as being located in a genomic region prone to amplification and characterized by putative driver aberrations. This may support a role for targeting breast tumours characterized by both this genomic abnormality and HMGCR-positivity using PI4KB inhibitors and statins. Indeed, we have already shown in our cell culture studies that combining fluvastatin with the PI4KB inhibitor PIK-93 induces apoptosis in the MDAMB231 breast cancer cell line. The same study identified MAP2K4 as being located in a genomic region of recurrent deletions in breast tumours. As suggested by our results where knockdown of MAP2K4 potentiated the anti-cancer effects of fluvastatin, targeting tumours with deletions encompassing MAP2K4 could potentially widen the therapeutic index of statins.

Through a genome-wide shRNA screen we have identified and validated several potentially clinically relevant sensitizers of statin-mediated anti-cancer effects. Recent clinical evidence showing statin efficacy in breast cancer patients treated with the same doses used to treat hypercholesterolemic patients underscores their importance in the clinical cancer care setting. We have shown that targeting the MVA pathway at multiple points maximizes tumour cell kill. Targeting SREBF2 effectively suppresses the restorative feedback loop that causes upregulation of HMGCR in response to statin treatment, effectively widening statins’
therapeutic window. Statins are safe, FDA-approved and, many are off patent making them cost-effective anti-cancer agents. Our study has identified targets whose inhibition has the potential to extend the benefit of statins and maximize tumour cell kill.
3.6. References:


Chapter 4 : Discussion
4.1 Statins as anti-cancer agents – How translational is the cell culture work?

Studies exploring the anti-cancer properties of statins in cell culture studies of cell lines or primary patient samples have been numerous and span a wide variety of solid and liquid tumours. Establishment of a statin-induced anti-proliferative or apoptotic effect is usually followed by an assessment of statin-induced perturbation of an activated oncogenic pathway or effects on an over-expressed protein known to contribute to oncogenic progression in that particular tumour model. Work in our laboratory has shown that lovastatin suppresses the Raf/MEK/ERK pathways in primary AML patient samples responsive to statin-induced apoptosis\(^1\) and others have reported similar effects in breast cancer concomitant with decreases in NF-κB activity and adapter protein 1 DNA binding activities\(^2\).

Anti-proliferative simvastatin effects on breast cancer cell lines have also been attributed to activation of JNK and c-Jun phosphorylation\(^3\),\(^4\). In MM, Rho proteins, whose isoprenylation by the addition of GGPP is critical for proper membrane localization, have been shown to mediate statin-induced apoptosis and reversal of cell-adhesion mediated drug resistance through statin mediated inhibitory effects on Rho kinase\(^5\). Suppressive effects on the AKT pathway have been reported in lung cancer\(^6\),\(^7\) and breast cancer cells\(^8\).

Nearly all the cell culture studies showing statin anti-cancer efficacy were accompanied by effect reversal following the addition of MVA. Most, though not all of the effects have been attributed to depletion of the isoprenylation arm of the MVA pathway as most commonly the addition of GGPP blocked statin-mediated effects. Amidst this plethora of statin reported molecular effects in cell lines and \textit{ex vivo} cultured primary patient samples, it is difficult to identify a specific biomarker of statin response since many of these effects following HMGCR inhibition appear to be indirect consequences of perturbations on commonly active pathways. While it is clear that inhibition of geranylgeranylation of Rho...
GTPases is a critical mediator of statins’ anti-cancer activity, the identification of a specific proteins responsible for these effects has been elusive although some potential candidates such as Ras-related C3 botulinum toxin substrate 1 (Rac1) and cell division cycle 42 (Cdc42)\textsuperscript{9} have been identified.

So far there has been a general lack of a translational link between cell culture studies and clinical investigations. Most of these cell culture studies address effects of statins directly on a tumour cell but there is little clinical evidence to indicate that a statins directly target a tumour cell bringing up the issue of how much statin actually reaches the extra-hepatic sites. Usually, the concentrations used in cell culture are not clinically achievable. Although the early dose-finding studies did show that lovastatin and simvastatin can be tolerated at very high doses, these studies did not actually demonstrate any real efficacy and micromolar doses of around 10 were detected in only a few patients. Furthermore, statins are not formulated for administration at such high doses as the maximum dose of a single pill is 80 mg, and this limits long-term administration. Oftentimes, the rationale of the statin used in a clinical trial does not reflect the cell culture hypothesis upon which efficacy was based. Recently, lovastatin was found to inhibit EGFR dimerization and synergize with the EGFR inhibitor gefitinib in inducing cytotoxicity in various cell lines\textsuperscript{10}. Based on this preclinical data, a clinical trial, currently ongoing, testing the efficacy of a statin combined with an EGFR inhibitor, erlotinib, in patients with squamous cell carcinomas and NSCLC was initiated (NCT00966472). The statin being used in this clinical trial is rosuvastatin, which is a hydrophilic statins that does not passively diffuse through a plasma membrane but requires carrier-mediated active transport for entry into hepatocytes. Hepatocytes express organic anion transporting polypeptides (OATP) which facilitates the entry of hydrophilic statins such as rosuvastatin and pravastatin into hepatocytes from the portal blood\textsuperscript{11}. Rosuvastatin is therefore unlikely to reach cells of the extra-hepatic tissues including the solid tumour and
rarely used in cell culture studies to demonstrate anti-cancer efficacy. Although this fact does not preclude anti-cancer efficacy of the rosuvastatin-erlotinib combination, as other clinical pleiotropic effects of statins might contribute to anti-cancer efficacy, it might complicate interpretation of results and confound subsequent biomarker discovery. Clinically, in addition to their cholesterol lowering abilities, statins are known to exert anti-inflammatory and immunomodulatory effects. Taken together, there are still many obstacles in unifying cell culture anti-cancer statin activity with clinical relevance.

On the other hand, a few clinical trials have sought to unify the preclinical studies with relevant clinical correlates. The phase I study in AML patients where pravastatin was administered with idaraubicin and cytarabine at pravastatin doses 40-1680mg/day was based on the cell culture findings showing that inhibition of cholesterol synthesis sensitized AML cells to chemotherapeutic treatment. AML cells upon chemotherapeutic treatment mounted adaptive responses and increased their intracellular cholesterol. The newly diagnosed patients with unfavorable cytogenetics fared better than historical controls when evaluating complete remission. Amongst the responders, LDL/cholesterol levels were lower than baseline throughout therapy. In the cell culture study that found simvastatin reversed cell-adhesion mediated drug resistance, simvastatin was used at clinically achievable low micromolar doses to treat 6 refractory multiple myeloma patients saw encouraging initial responses as ascertained by decreases in paraprotein levels compared to historical controls. A recent study found evidence of inhibition of geranylgeranylation in the bone marrow mononuclear cells of one AML patient out of 4 following seven days of treatment with simvastatin. The above trials are encouraging in their demonstration, albeit limited, of clinical correlates with efficacy, future efforts should be directed at uncovering molecular indicators of statin activity within a tumour.
A recent window-of-opportunity trial in patients with primary invasive breast cancer compared HMGCR expression in paired tumour samples before and after treatment with standard doses of atorvastatin (80 mg/day)\textsuperscript{18}. This pivotal study presented some key findings, chiefly that post statin-treatment, increases in HMGCR levels in 68\% of the samples were observed. Interestingly, significant decreases in Ki67 expression following atorvastatin treatment in the tumours with initial detectable HMGCR levels were observed. HMGCR upregulation in response to atorvastatin treatment is quite suggestive of atorvastatin exerting a direct anti-tumour effect in response to depletion of intracellular sterol pools as occurs upon statin-mediated HMGCR inhibition\textsuperscript{19,20}. The clinical study did not indicate whether cholesterol levels in patients were lowered following administration but presumably this would have been the case. It is therefore also possible that HMGCR upregulation within the tumour occurred due to decreased cholesterol levels in the plasma. However, the tumours with the significant decreases in the Ki67 index were the ones with targetable HMGCR prior to statin-treatment. Taken together, this study suggests that HMGCR-positive breast tumours can be successfully targeted by statins. Other recent clinical trials have specifically started to hone in on patient subpopulations and specific tumour types predicted to respond to statin therapy. An ongoing trial (NCT00807950) where simvastatin will be pre-operatively administered to patients with primary breast cancer is testing the hypothesis that simvastatin will selectively affect tumours of the basal subtype\textsuperscript{21}. The clinical trial intends to examine genomic changes in the tumour following simvastatin treatment and correlate them with biological effects. These clinical exploration into the availability of biomarkers to predict statin response and assess statin-directed tumour effects are key to selecting statin responsive patients in future clinical trials and increasing the chances of demonstrating statin clinical efficacy.
Despite the rapid translation of statins into the clinic, there are gaps between cell culture anti-cancer activity and clinical relevance. Many studies employ high statin concentrations not clinically attainable or assume that statins are interchangeable despite their clearly different pharmacological profiles. Furthermore studies using statins with more favorable extra-hepatic and capable of reaching the tumour pharmacokinetic properties such as fluvastatin have been extremely encouraging in demonstrating the benefits of using statins in cancer patient care.

4.2 Potentiating statin-induced apoptosis – combinatorial approaches

In Chapter 2, we carried out a pharmacological screen intended to identify compounds that potentiate the anti-cancer effects of statins. Challenges to successful chemotherapeutic and targeted agent drug treatments, include innate and acquired resistance, redundancy of signaling pathways, tumour heterogeneity, presence of feedback loops that activate other pathways, and toxicity.

As elegantly summarized by Dr. Chou, a founder of multiple synergy algorithms extensively used in pre-clinical biology, combining drugs has multiple potential benefits. Combining drugs might increase therapeutic efficacy, decrease toxicity by being able to decrease the dosage of one or two drugs while maintain the same efficacy and potentially retard drug resistance.

4.2.1 Statins and rationally crafted combinations

Statins have been combined with a multitude of currently available chemotherapeutics as well as targeted agents. The combination of statins with reagents that are currently already
in the clinic and comprise the standard of care is a logical progression to broadening the benefits of statins. In addition to potentiating the activity of already available anti-cancer agents it has been important to evaluate the interaction of statins with many drugs as many elderly cancer patients receive statins for their cardiovascular problems. Although statins have well-established safety profiles, the potential adverse reactions with other drugs administered to already compromised patients necessitates their combinatorial evaluation.

Statins have been combined with anthracyclines, mainly doxorubicin, and synergism and potentiation have been demonstrated in tissue culture and murine cancer xenograft models. The addition of lovastatin attenuated doxorubicin’s cardiotoxicity and protected endothelial cells from doxorubicin’s cytotoxic effects. The exact mechanisms of synergy have been elusive and often confounded by contrasting actions of the individual drugs on the same mediator. For example, it has been suggested that inhibition of the activation of nuclear factors κβ (NF-κβ) by lovastatin contributed to lovastatin’s sensitization to the cytotoxic effects of doxorubicin. However, it has also been reported that NF-κβ activation by doxorubicin is essential for its cytotoxic effects. Lovastatin’s inhibitory effects on P-glycoprotein have also been implicated as being responsible for the synergy observed when combining lovastatin with doxorubicin, a substrate for P-glycoprotein. Statins have been similarly combined with an arsenal of other classical chemotherapeutic such as platinum compounds, antimetabolites particularly 5-fluorouracil and tubulin-binding drugs such as paclitaxel, with synergy and potentiation being cell-dependent and mechanism of action not fully elucidated.

Statins have also been combined with other drugs that target the MVA pathway, namely the yet to be approved farnesyltransferase inhibitors (FTIs) and geranygeranyltransferase inhibitors (GGTIs). Synergy between lovastatin and multiple FTIs and GGTIs in inducing anti-cancer effects against multiple myeloma was previously
demonstrated\textsuperscript{34}. Synergy with inhibitors of geranylgeranyl diphosphate synthase, the enzyme upstream of geranylgeranyltransferase which synthesizes geranylgeranylpyrophosphate (GGPP), and lovastatin were similarly observed in leukemia cells\textsuperscript{35}. Statins have also been, combined with some targeted agents, and have shown to enhance the activity EGFR inhibitors, gefitinib and erlotinib in glioblastoma\textsuperscript{36}, vascular endothelial growth factor receptor (VEGFR) inhibitors\textsuperscript{37}, sorafenib in melanoma\textsuperscript{38}, trastuzumab in breast cancer\textsuperscript{39}, the m-TOR inhibitors in leukemia\textsuperscript{40}, imatinib in chronic myelogenous leukemia\textsuperscript{41}.

Statins can successfully be combined with many agents that are currently approved for clinical use in the treatment of cancer patients and some of them are even being evaluated in the clinic. However, many patients will and have not responded to the classic chemotherapeutic agents, alone or in combination with statins and the targeted agents are still cost prohibitive. We therefore pursued an alternative approach of discovering novel unexpected combinations. In chapter 2 we used of a pharmacological screen composed of FDA-approved drugs for non-cancer indications and combined it with statins to uncover novel combinations with anti-cancer activity. In Chapter 3, we undertook a genomic approach and carried out an shRNA screen to discover novel targets/pathways whose knockdown combined with inhibition of the MVA pathway maximized anti-tumour effects.

\textbf{4.2.2 Statins and dipyridamole}

The screen in chapter 2 carried out the in the KMS11 MM cell line uncovered dipyridamole, a drug that has been used for decades in the clinic as an antithrombotic agent. The MM KMS11 cell line was chosen for the screen because it was a relatively statin sensitive cell line\textsuperscript{42,43}. The rationale in choosing this cell line was that enhancing tumour kill in a cell line that already responds to statins will further maximize tumour kill.
Strong preclinical activity was demonstrated using multiple MM and AML cell lines. Interestingly, in the LP1 MM cell line, the statin-dipyridamole combination demonstrated robust synergy and dipyridamole-mediated potentiation of the statins’ anti-cancer effects indicating that the combination has the potential to target tumour cell intrinsically insensitive to statin-induced apoptosis.

Primary AML and MM patients were also susceptible to the combination and in vivo tumour delaying activity was demonstrated in OCI-AML2 xenografts. In attempts to phenocopy dipyridamole’s many molecular effects we utilized pharmacological inhibitors representative of each class and also combined them with statins. With the exception of cilostazol, the other compounds representing nucleoside and glucose transport inhibitors, and the cyclic guanosine monophosphate (cGMP) elevating phosphodiesterase (PDE) inhibitors did not potentiate statin induced anti-cancer effects. As other PKA activating, cyclic adenosine monophosphate (cAMP) elevating agents such forskolin also significantly potentiated statin-induced apoptosis in AML cells and primary patient samples, that activation of that signaling arm is implicated in contributing to the synergistic effect observed. We thus identified another class of drugs, the cAMP elevating PDE inhibitors such as cilostazol also capable of inducing tumour apoptosis when combined with statins. Cilostazol is also an antiplatelet agent currently in clinical use.

As discussed in Chapter 2, dipyridamole’s inhibitory activities exerted upon P-glycoprotein and the nucleoside transporter inhibitors have been of interest when combined with standard chemotherapeutic drugs whose actions are dampened by the expression of P-glycoprotein and nucleoside salvage metabolism. The early clinical trials based on these principles of dipyridamole activity in combination chemotherapy in solid tumours were not successful in demonstrating efficacy44-47 and some of the failure was attributed to dipyridamole’s binding to α1-acid-glycoprotein (AGP) in serum48. However, as novel and
better dipyridamole formulations have since entered the market\textsuperscript{49,50} there is likely to be a resurgence of this drug for use other than those in the cardiovascular setting.

Indeed in recent years there has been a resurgence of dipyridamole being used as an anti-cancer agent particularly in breast cancer. One study treated MMTV-PyMT transgenic mice, which develop a highly aggressive breast cancer, with 10 mg/kg of dipyridamole for almost three months and found that dipyridamole exhibited chemopreventive activities against tumorigenesis and metastasis\textsuperscript{51}. Another group which administered dipyridamole to breast cancer tumour bearing xenografted mice found that the drug decreased primary tumour growth, metastasis, macrophage tumour infiltration and resulted in decreased activation of the Wnt, ERK1/2-MAPK and NF-\kappa B pathways\textsuperscript{52}. The mechanism by how dipyridamole perturbed those pathways was not elucidated.

4.2.3 Limitations and future directions of this work

Although we attributed the involvement of cAMP elevation and PKA activation as being partly responsible for the mechanism by which dipyridamole potentiates statin-induced apoptosis, we did not demonstrate the functional consequences of the reverse. Over-expression of PDE’s, knockdown of PKA or abrogation of its activity would have strengthened the significance of our findings and this work is underway. We have also demonstrated that the statin-dipyridamole combination abrogates the statin-induced upregulation of HMGCR and this is also being further explored especially since we have demonstrated in Chapter 3 the functional consequences of this abrogation via knockdown of SREBF2.

The frequent utilization of the MTS and MTT assays to assess cell viability limits interpretation and does not measure apoptosis. The calorimetric assays, such as the MTS and
MTT, although cost-effective and relatively quick, should only represent a first pass attempt at discovering potential anti-cancer effects. Although in the current work, validation of hits was accompanied by apoptosis assays, utilizing these assays for cancer drug screening purposes should be approached with caution as it can potentially result in the pursuit of hits that are anti-proliferative and do not cause tumour cell apoptosis.

The use of pharmacological modulators of cAMP signaling to mimic the statin-dipyridamole combination and prove its significance in modulating the synergistic interaction is also problematic. Although the pharmacological inhibitors were used at concentration shown in the literature to elicit the intended effects, they are likely not specific and like dipyridamole have other modes of action. It is very difficult to accurately ascertain by which mechanism two drugs interact to yield the observed net effect especially when drugs have multiple mechanisms of action. As cautioned by Dr. Chou, it is rather improbable to ever fully ascertain the mechanism of synergy and especially to demonstrate it clinically\textsuperscript{53}, especially since rarely do we ever fully know the mechanism of a single drug at the biochemical, molecular, cellular and pharmacological level\textsuperscript{22,54}. When two drugs are combined, there may be several hundred steps occurring from time of treatment to death or cycle arrest of a cell. However, the manner in which well-characterized pathways are perturbed by either drug or their concomitant addition is a good method to exclude certain possibilities and narrow down through which signaling cascades effects might be occurring.

There are limitations with the \textit{in vivo} model used to assess drug efficacy. The statin-dipyridamole combination merely delayed tumour growth and did not regress or keep the tumour volume constant. In a clinical situation, it might therefore be difficult to translate this result into therapeutic efficacy. On the other hand, the dosing regimen could be further optimized. Animals were treated once daily, and as the half-life of dipyridamole is less than six hours, more frequent administration might result in improved \textit{in vivo} efficacy. The \textit{in vivo}
studies involved the use of xenografts implanted in immune-deficient animals. There are limitations to using this murine model to predict \textit{in vivo} efficacy. Firstly, statins exert many immunomodulatory effects, most of them immunosuppressive and the NOD SCID mice have impaired T and B lymphocyte development, a feature which prevents them from rejecting human cell line xenografts but therefore does not take into account key statin-mediated clinical responses. Furthermore, it is difficult to modulate cholesterol levels in these mice and special transgenic models are required\textsuperscript{55} to effectively lower cholesterol levels using statins. Again, as this is a primary mode of statin activity, anti-tumour contribution resulting from lowering cholesterol may be missed when using immunodeficient mice. Furthermore, AML is not a disease where solid tumours are formed and therefore more sophisticated models are required to recapitulate the disease. Primarily future work will focus on evaluating whether the statin-dipyridamole combination can effectively target the leukemic cancer stem (LCS) cell. Others\textsuperscript{56,57} and we\textsuperscript{58} have demonstrated that statins can effectively target the LCS. Engraftment potential of CD34 purified primary AML samples injected into the femur of sublethally irradiated NOD SCID mice, followed by weeks of treatment of the mice with statin, dipyridamole or the combination will be evaluated.

More broadly, another general concern in utilizing xenografts and even some of the more sophisticated transgenic spontaneous tumour models is that drug efficacy is being tested against primary tumours. However, many cancer patients, especially in clinical trials, present advanced metastatic disease that has already failed primary therapy. Many of the experimental drugs that have fared well preclinically, fail in clinical trials because of inadequate preclinical models that do not recapitulate metastatic disease. Researchers have sought to address this question by resecting the primary tumours in mice to enable the progression of metastatic disease and have successfully tested drugs in this setting\textsuperscript{59,60}. 

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4.3 Targeting the MVA pathway – unbiased knockdown approaches

In Chapter 3, pan genome-wide shRNA screen identified several hits that were further pursued as putative potentiators of statin induced anti-cancer effects. Although the screen was carried out over several passages and doubling times, putative drop-out shRNA hits were successfully verified using shorter term assays.

4.3.1 The MVA pathway’s other targets

The presence of several hits from the MVA pathway emphasized its importance in being targeted as an anti-cancer therapeutic strategy. HMGCR is the rate-limiting enzyme of the MVA pathway and its inhibition will lead to a depletion of all downstream end products. Inhibition of other nodal points of the MVA pathway will lead to a complex interplay of accumulation and inhibition of its various intermediate products and potentiation is not always obvious. For instance treating with a statin will lead to depletion of farnesyl pyrophosphate (FPP) but this might be negated if GGPS1 is simultaneously blocked because it will lead to an accumulation of FPP. Therefore if the statin induced anti-cancer effect is mediated by blocking ras farnesylation for instance then synergy is unlikely. We demonstrated that knockdown of GGPS1 dramatically potentiated statin-induced apoptosis. However, the cells lines stably expressing the shRNA targeting GGPS1 were difficult to generate, highlighting the overall importance of this target for maintaining cancer cell growth. Inhibiting GGPS1 will affect a largest amount of proteins when targeting just one of the downstream geranylgernayltransferases. An independent group that has synthesized pharmacological inhibitors to GGPS1 has also noted dramatic synergy of the GGPS1 inhibitors when combined with statins in inducing tumour cell kill in leukemia cells.
The identification and validation of the transcription factor sterol regulatory element-binding protein 2 (SREBF2) as potentiating stain-induced apoptosis in several tumour types provides strong evidence for the functional consequences of the restorative sterol feedback loop. The stably expressing shSREBF2 lung and breast cancer sublines were characterized by similar growth rates but underwent a dramatic induction of apoptosis upon treatment with sublethal doses of fluvastatin when compared to the control sublines. Despite residual SREBF2 levels (mRNA knockdown was approximately 85% effective), the shSREBF2 sublines lacked the ability to upregulate HMGCR and HMGCS1 levels in response to fluvastatin exposure within 24 hours, an effect which likely blunts the effects of the statins in the control sublines.

4.3.2 Limitations and future directions of this work

We have identified numerous other novel hits such as phosphatidylinositol 4-kinase, catalytic beta (PIK4B) that were validated in both breast and lung cancer cells using genomic and pharmacological approaches. The sensitizing effects upon targeting PI4KB to statin induced apoptosis should be further mechanistically explored. Net effects of the combination of pathways known to be perturbed by inhibiting either targets such as the Raf/MEK/ERK and Akt pathways will be examined. Furthermore, as pharmacological inhibitors to PIK4B are available in vivo efficacy and safety of the combination can be evaluated.

Although we have shown that SREBF2 knockdown strongly potentiates statin induced apoptosis, the effects and potential toxicity to normal cells has not been explored. There are no known specific inhibitors of SREBF2 and as such, this question cannot be readily
answered using murine \textit{in vivo} models. The question can perhaps be addressed by cell culture studies using normal primary cells.

HMGCR is the rate-limiting enzymes of the MVA pathway and its inhibition, by definition, should render inhibition of enzymes downstream of it such as GGPS1 inconsequential. However, as we clearly observe strong potentiation upon knockdown of GGPS1 and inhibition of HMGCR using statins, this suggests that the degree of HMGCR inhibition was sub-optimal and further inhibition at the level of GGPS1 was able to cooperate with statins to further block this arm of the pathway. This should be directly investigated. For example, the level of mevalonate and/or other end products such as GGPP before and after statin inhibition could be measured using high-performance liquid chromatography. By assessing the mevalonate flux, a clearer idea about the kinetics and degree of HMGCR inhibition will emerge and answer key questions pertaining to whether knockdown of the GGPS1, HMGCS1 and SREBF2 in combination with statins is due to MVA pathway inhibition and/or potential involvement of other molecular pathways perturbed in response to GGPS1, HMGCS1 and SREBF2 knockdown.
4.4 Concluding remarks and anticipated impact

In this body of work we have identified a translatable combination of safe and readily available FDA approved drugs, statins and dipyridamole which have the potential to directly impact patient care. We have demonstrated strong preclinical antmyeloma and antileukemia efficacy of the combination. Through our investigations into uncovering the mechanism by which dipyridamole potentiates statin-induced apoptosis, we have identified an additional class of drugs, the cAMP elevating PDE inhibitors, some of which are also clinically approved, with antileukemia activity. The targets uncovered through the shRNA screen have not only increased our understanding of the key branches of the MVA pathway that maximize statin induced anti-tumour effects but have also uncovered a plethora of novel target kinases such as PIK4B and MAP2K4 that can be further assessed in the future for preclinical efficacy.
4.5 References:


Publications (2008-2013):


