Cross-Platform Screening for Novel Fluvastatin and Kinase Inhibitor Co-therapies in Cancer Cell Lines

by

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A thesis submitted in conformity with the requirements for the degree of Master of Science
Department of Medical Biophysics
University of Toronto

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2014

Abstract

Statins are widely used to manage hypercholesterolemia, and clinical trials have demonstrated that statins as monotherapies are effective anticancer agents in some patients. We aim to identify combination treatments that will expand the anticancer benefit of statins. We hypothesize that screening cancer cell lines with a genomic shRNA library and a small-molecule library will identify novel compounds to use in co-therapies with fluvastatin. A pooled shRNA dropout screen was performed to find genomic targets to inhibit alongside fluvastatin, which revealed several kinase targets as dropouts. This suggested that knocking down specific kinases can potentiate fluvastatin-induced cell death. To identify known kinase inhibitors that interact with fluvastatin to elicit cancer cell kill, we screened with a library of 320 kinase inhibitors, which revealed several putative hits. We anticipate that continued validation of candidates from both screens will lead to the identification of novel anticancer statin-based drug cocktails.
Acknowledgments

I am very fortunate to have had so many wonderful people supporting me through this chapter of my life. First, thank you to my supervisor, Dr. Linda Penn, for providing me with excellent mentorship. I am sincerely grateful for her encouragement and motivation, and for creating an inspiring environment in which to learn and grow. I would also like to thank my committee members, Dr. Aaron Schimmer and Dr. Jane McGlade, for their continued guidance and advice in all aspects of my research projects.

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To my lab mates, thank you for all of your help preparing me for seminars and conferences, and for late night chats and unforgettable memories. My search history has never been so interesting. I will certainly miss working with, and learning from, such a fun and inspirational team of people. To Aleks, you are the best mentor and friend I could have asked for. Thank you for always being willing to teach and support me, and for always going the extra mile, even all the way from Germany!

To my UC don family, you made more of an impact then you’ll know. If I listed you all, I’d need an extra page. Thank you for always being there for me when I resurfaced from the lab.

Last, but of course not least, thank you to my family. Mom, dad, Brian, thanks for always keeping me grounded and optimistic. I’m excited to start the next chapter!
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<th>Description</th>
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<tbody>
<tr>
<td>µM</td>
<td>Micromolar</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>ATF4</td>
<td>Activating transcription factor 4</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>AUY992</td>
<td>5-(2,4-Dihydroxy-5-isopropyl-phenyl)-N-ethyl-4-[4- (morpholinomethyl)phenyl]isoxazole-3-carboxamide</td>
</tr>
<tr>
<td>BCR-ABL</td>
<td>Breakpoint cluster region - Abelson</td>
</tr>
<tr>
<td>BiP/GRP78</td>
<td>Heat shock 70kDa protein 5 (glucose-regulated protein, 78kD)</td>
</tr>
<tr>
<td>CHOP/DDIT3</td>
<td>DNA-damage-inducible transcript 3</td>
</tr>
<tr>
<td>CML</td>
<td>Chronic myelogenous leukemia</td>
</tr>
<tr>
<td>CNA</td>
<td>Copy number alteration</td>
</tr>
<tr>
<td>CSNK2B</td>
<td>Casein kinase 2, beta, polypeptide</td>
</tr>
<tr>
<td>CTLA4</td>
<td>Cytotoxic T lymphocyte-associated antigen 4</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>eEF1A2</td>
<td>Eukaryotic protein translation elongation factor 1 alpha 2</td>
</tr>
<tr>
<td>EGFR</td>
<td>Epidermal growth factor receptor</td>
</tr>
<tr>
<td>EML4-ALK</td>
<td>Echinoderm microtubule-associated protein-like 4, anaplastic lymphoma kinase</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
</tr>
<tr>
<td>ERdj4/DNAJB9</td>
<td>DnaJ (Hsp40) homolog, subfamily B, member 9</td>
</tr>
<tr>
<td>ERN1/IRE1</td>
<td>Endoplasmic reticulum to nucleus signalling 1</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>FDA</td>
<td>Food and Drug Administration</td>
</tr>
<tr>
<td>FPP</td>
<td>Farnesylpyrophosphate</td>
</tr>
<tr>
<td>FTI</td>
<td>Farnesyltransferase inhibitors</td>
</tr>
<tr>
<td>GADD34/PPP1R15A</td>
<td>Protein phosphatase 1, regulatory subunit 15A</td>
</tr>
<tr>
<td>GARP</td>
<td>Gene activity ranking profile</td>
</tr>
<tr>
<td>GFP</td>
<td>Green fluorescent protein</td>
</tr>
<tr>
<td>GGPS</td>
<td>Geranylgeranyl diphosphate synthase 1</td>
</tr>
<tr>
<td>GGPP</td>
<td>Geranylgeranylpyrophosphate</td>
</tr>
<tr>
<td>GGTI</td>
<td>Geranylgeranyltransferase I inhibitor</td>
</tr>
<tr>
<td>GUI</td>
<td>Graphical user interface</td>
</tr>
<tr>
<td>HCA</td>
<td>High-content analysis</td>
</tr>
<tr>
<td>HCC</td>
<td>Hepatocellular carcinoma</td>
</tr>
<tr>
<td>HCS</td>
<td>High-content screening</td>
</tr>
<tr>
<td>HER2</td>
<td>Human epidermal growth factor receptor 2</td>
</tr>
<tr>
<td>HMEC</td>
<td>Human mammary epithelial cells</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>------------</td>
</tr>
<tr>
<td>HMGCR</td>
<td>3-hydroxyl-3-methylglutaryl coenzyme A reductase</td>
</tr>
<tr>
<td>HMGCS1</td>
<td>3-hydroxy-3-methylglutaryl coenzyme A synthase 1</td>
</tr>
<tr>
<td>HRAS</td>
<td>Harvey rat sarcoma viral oncogene homolog</td>
</tr>
<tr>
<td>HSP90</td>
<td>Heat shock protein 90</td>
</tr>
<tr>
<td>HTS</td>
<td>High-throughput screening</td>
</tr>
<tr>
<td>ICₕ₀</td>
<td>Half maximal inhibitory concentration</td>
</tr>
<tr>
<td>ICGC</td>
<td>International Cancer Genome Consortium</td>
</tr>
<tr>
<td>IKBKB</td>
<td>Inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase beta</td>
</tr>
<tr>
<td>JNK</td>
<td>c-Jun N-terminal kinase</td>
</tr>
<tr>
<td>KNN</td>
<td>K nearest neighbours</td>
</tr>
<tr>
<td>KRAS</td>
<td>Kirsten rat sarcoma viral oncogene homolog</td>
</tr>
<tr>
<td>KS</td>
<td>Kolmogorov-Smirnov</td>
</tr>
<tr>
<td>LDL</td>
<td>Low-density lipoprotein</td>
</tr>
<tr>
<td>LDLr</td>
<td>Low-density lipoprotein receptor</td>
</tr>
<tr>
<td>LPDS</td>
<td>Lipoprotein deficient serum</td>
</tr>
<tr>
<td>MAP2K4</td>
<td>Mitogen-activated protein kinase kinase 4</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MET</td>
<td>Met proto-oncogene</td>
</tr>
<tr>
<td>MK1</td>
<td>Mutachrome 1</td>
</tr>
<tr>
<td>MM</td>
<td>Multiple myeloma</td>
</tr>
<tr>
<td>MOI</td>
<td>Multiplicity-of-infection</td>
</tr>
<tr>
<td>mTOR</td>
<td>Mechanistic 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>NCBI</td>
<td>National Center for Biotechnology Information</td>
</tr>
<tr>
<td>NSCLC</td>
<td>Non-small cell lung cancer</td>
</tr>
<tr>
<td>OICR</td>
<td>Ontario Institute for Cancer Research</td>
</tr>
<tr>
<td>PCA</td>
<td>Principle component analysis</td>
</tr>
<tr>
<td>PDGFR</td>
<td>Platelet-derived growth factor receptor</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphoinositide 3-kinase</td>
</tr>
<tr>
<td>PI4KB</td>
<td>Phosphatidylinositol 4-kinase, catalytic, beta</td>
</tr>
<tr>
<td>PIM1</td>
<td>Pim-1 oncogene</td>
</tr>
<tr>
<td>PIM2</td>
<td>Pim-2 oncogene</td>
</tr>
<tr>
<td>PTEN</td>
<td>Phosphatase and tensin homolog</td>
</tr>
<tr>
<td>qRT-PCR</td>
<td>Quantitative real-time polymerase chain reaction</td>
</tr>
<tr>
<td>RNAi</td>
<td>Ribonucleic acid interference</td>
</tr>
<tr>
<td>SCLC</td>
<td>Small-cell lung cancer</td>
</tr>
<tr>
<td>SDA</td>
<td>Stepwise discriminant analysis</td>
</tr>
<tr>
<td>SERM</td>
<td>Selective estrogen-receptor modulators</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</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 ribonucleic acid</td>
</tr>
<tr>
<td>SNP</td>
<td>Single nucleotide polymorphism</td>
</tr>
<tr>
<td>SRE</td>
<td>Sterol regulatory element</td>
</tr>
<tr>
<td>SREBP2</td>
<td>Sterol regulatory element binding protein 2</td>
</tr>
<tr>
<td>SYK</td>
<td>Spleen tyrosine kinase</td>
</tr>
<tr>
<td>TBB</td>
<td>4,5,6,7-tetrabromo-1H-benzotriazole</td>
</tr>
<tr>
<td>TBK1</td>
<td>Tank-binding kinase 1</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumor necrosis factor alpha</td>
</tr>
<tr>
<td>TRC</td>
<td>The RNAi Consortium</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
</tr>
<tr>
<td>XBP1</td>
<td>X-box binding protein 1</td>
</tr>
</tbody>
</table>
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Chapter 1
Introduction

1 Introduction

1.1 Lung cancer

Cancer is the leading cause of death in Canada\(^1\), and lung cancer is the leading cause of cancer deaths worldwide\(^2\). While the main cause of lung cancer is smoking, incidences in non-smokers can be accounted for by environmental factors such as air pollution and asbestos\(^3\), and by genetic factors\(^4,5\). Lung cancer can be histologically divided into two subtypes: small-cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC)\(^6\). The latter often has poor prognosis and can be further histologically subdivided into adenocarcinoma, squamous-cell carcinoma and large-cell carcinoma\(^6\).

1.2 Conventional cancer therapies

Surgery, radiotherapy and chemotherapy are the pillars of traditional cancer therapy. These treatment strategies have well-documented, context-specific successes, but are limited as individual treatment modalities, and when used in combination. Surgery and radiotherapy are predominately used to manage localized malignancies, and chemotherapy is the foremost systemic cancer treatment. Surgical oncology encompasses preventative, diagnostic, staging, debulking, and curative purposes, amongst others\(^7\). Although vast in utility, surgery is ultimately limited as a curative treatment of cancer metastasis. Radiotherapy involves focusing electromagnetic radiation on malignant cells, which causes DNA damage and eventual cell death. This therapeutic option is a component of 40% of cancer management plans\(^8\), and purportedly, would be beneficial in 52% of cancer incidences\(^9\). As a localized treatment like surgery, radiotherapy is similarly limited as a curative therapy for most cancer types, including lung cancer.

In the 1940s, chemotherapy was introduced as an approach to combat both localized and metastatic cancer\(^10\). Many chemotherapeutic agents, including antifolates, purine analogues, alkylating agents, fluorouracil and platinum compounds have shown remarkable success in cancer treatment\(^10\). Unfortunately, these cytotoxic agents target not only cancer cells, but also normal cells. As such, cancer cell death elicited by these agents is accompanied by side effects
related to the damage of normal cells, especially highly proliferative cells in the skin, stomach and intestinal lining. In one report, over 80 percent of 238 breast cancer or malignant lymphoma patients reported nausea, fatigue and hair loss during chemotherapy in addition to emotional distress\textsuperscript{11}.

In addition to a narrow therapeutic index, acquired resistance is a major obstacle for chemotherapeutics. Acquired resistance was quickly observed by the pioneers of chemotherapy, and led to the development of combination chemotherapy\textsuperscript{12}. However, many malignancies, including lung cancer, have proven resistant even to combination chemotherapy. This necessitates treatments beyond non-specific chemotherapeutics. Improved understanding of molecular and signaling biology precipitated the inception of targeted cancer therapy. The complex, highly heterogeneous nature of cancer came to light, and cancer is now known not as a single disease, but as an umbrella term for over 100 diseases each with an independent set of underlying somatic mutations\textsuperscript{13}. The discovery of the \textit{HRAS} G12V point mutation as the genetic basis of bladder cancer\textsuperscript{14} steered research away from histology and towards cancer genetics. Research efforts were directed towards the identification of somatic mutations, understanding how they contribute to cancer evolution, and how they can be precisely targeted.

\subsection*{1.3 The era of personalized cancer medicine}

Understanding cancer genetics is crucial to combating the heterogeneity and adaptability of cancer cells. Comprehensively described by Hanahan and Weinberg, tumours form by acquiring multiple tumorigenic characteristics\textsuperscript{15}. These traits include sustaining proliferative signaling, evading growth suppressors, activating invasion and metastasis, enabling replicative immortality, inducing angiogenesis and resisting cell death\textsuperscript{15}. More recently recognized traits include the ability to deregulate cellular energetics and avoid immune destruction\textsuperscript{16}. Understanding the molecular underpinnings that give rise to these aberrant capabilities is needed if we are to develop appropriate, effective and precise drugs against a disease that can vary even within an individual tumour\textsuperscript{17-19}.

Personalized medicine involves precisely targeting somatic mutations within cancer cells. Targeted molecular therapies are rationally designed towards specific mutations that drive aberrant cell survival, growth and metastasis. Normal cells are ideally spared in this treatment context, creating a tumour-normal index lacking from cytotoxic systemic treatments. The goal of
Personalized medicine is to select therapies for patients with maximal potential efficacy, and minimal potential toxicity.

Dramatic improvements in DNA sequencing over the last decade have made the concept of individualized care realistic. Massively parallel sequencing technologies allow for quick and accurate identification of somatic mutations including single nucleotide polymorphisms (SNPs)\(^{20}\) and copy number alterations (CNAs)\(^{21,22}\). Indeed, entire human genomes can be sequenced\(^{22}\) and the cost continues to lower towards $1000 per genome\(^{13,23,24}\). Increasingly fast and inexpensive sequencing is propelling cancer therapeutics towards individualized care, where therapeutic vulnerabilities can be identified and exploited. A global, concerted effort to characterize oncogenic mutations found in 50 distinct cancer types has been undertaken by the International Cancer Genome Consortium (ICGC), which will ultimately lead to the development of more precise cancer therapies and guide individualized therapeutic management\(^{25}\).

The concept of oncogene addiction, whereby a single gene or pathway drives aberrant proliferation, provides strong rationale to pursue targeted therapies\(^{26-28}\). By identifying the ‘Achilles’ heel’ of cancers, targeted therapies can theoretically be designed or selected for\(^{28}\). For example, molecular determinants have been identified in NSCLC, designating which drugs to use in which patients. In NSCLC patients with EML4-ALK translocations, crizotinib yields stable disease or tumour shrinkage in 90% of patients\(^{29}\), and activating mutations in the tyrosine kinase domain of epidermal growth factor receptor (EGFR) strongly correlate with response to gefitinib, especially in female, Asian non-smokers\(^ {30,31}\).

Targeted cancer therapies can be divided into two major classes. One class is monoclonal antibodies, which target specific cell surface antigens. In brief, by targeting specific cell surface antigens, monoclonal antibodies can block proliferative signals, trigger the immune system to recognize and kill cancer cells and overcome immunosuppression in tumour microenvironments, or deliver cytotoxic chemicals to cancer cells. Trastuzumab was the first monoclonal antibody to reach the market and is primarily used to treat breast cancers with amplified or overexpressed human epidermal growth factor receptor 2 (HER2)\(^ {32}\). Other FDA approved monoclonal antibodies include: cetuximab, which targets EGFR for the treatment of metastatic colorectal cancer\(^ {33}\), and head and neck squamous cell carcinoma\(^ {34}\); bevacizumab, which targets vascular endothelial growth factor (VEGF) for the treatment of metastatic colorectal, lung, renal and other
cancers; and ipilimumab, targeting cytotoxic T lymphocyte-associated antigen 4 (CTLA4) for the treatment of advanced melanoma.

A second class of targeted molecular therapy is small-molecule inhibitors. This class includes selective estrogen-receptor modulators (SERMs) such as tamoxifen, chaperon protein inhibitors such as AUY922 targeted to heat shock protein 90 (HSP90), cancer stem cell inhibitors such as salinomycin, and tyrosine kinase inhibitors, which encompass the majority of small molecule inhibitors.

1.4 Kinase inhibitors as targeted cancer therapeutics

There are over 500 predicted protein kinases with diverse roles in regulating cell function, particularly signal transduction and cell cycle progression. Further, owing to their ability to be specifically inhibited, kinases are a class of proteins heavily pursued for personalized cancer medicine. To date, there are nearly 20 clinically approved kinase inhibitors for cancer therapy, and nearly 3000 clinical trials involving kinase inhibitors in the cancer context are registered with clinicaltrials.gov. Genomic determinants of sensitivity have been identified for many kinase inhibitors, which will continue to direct cancer treatment and management.

Most kinase inhibitors discovered and developed to date are ATP-competitive, and function by blocking the ATP-binding pocket to prevent phosphorylation reactions and cascades. Other classes include allosteric or non-ATP-competitive inhibitors, and covalent inhibitors, which bind irreversibly to a cysteine residue in the active site. The potential for dramatic clinical efficacy was realized with imatinib, which is marketed as Gleevec. Imatinib was first used for the treatment of chronic myelogenous leukemia (CML), and overall survival is more than 80 percent for chronic phase CML patients. The promise of kinase inhibitors continues to fuel the discovery of new inhibitors with improved specificity. Such discoveries are guided by structure-informed design and analogue synthesis.

Arguably, the largest hurdle to the utility of kinase inhibitors as anticancer agents is resistance. Resistance can occur through secondary mutation or amplification of the gene target, such as BCR-ABL amplification or mutation in imatinib resistance, and the T790M EGFR mutation that confers resistance to gefitinib. Resistance can also be conferred by loss of a chromosome, such as loss of chromosome 7 in EGFR-activated NSCLC. Activation of downstream signaling...
is yet another mechanism of resistance. For example, activation of the phosphoinositide 3-kinase (PI3K) pathway through the loss of phosphatase and tensin homolog (PTEN)\textsuperscript{48,49}, or mutations in the HER2 kinase domain\textsuperscript{50}, result in gefitinib resistance. Resistance can also be acquired by activation of compensatory pathways, such as met proto-oncogene (MET) amplification in NSCLC\textsuperscript{51}.

The cost of kinase inhibitors in the cancer treatment setting may also be prohibitive for clinical use. Cetuximab for the treatment of colorectal cancer, erlotinib primarily for the treatment of NSCLC and sunitinib for the treatment of renal cell carcinoma, cost about $80,000, $16,000 and $34,000 per patient, respectively\textsuperscript{52}. Meanwhile, the benefits of these drugs are being denounced as marginal, with increased overall survival ranging from ten days to less than three months\textsuperscript{52}. Toxicities of kinase inhibitors and other targeted therapies are also of concern. While targeted therapies were originally thought to have few toxicities, especially compared to systemic chemotherapies, cardiovascular\textsuperscript{53} and dermatologic\textsuperscript{54,55} toxicities and hyperthyroidism\textsuperscript{56} have all been documented and identified as limitations of kinase inhibitors.

Although there is still much to discover and heed caution to in regards to kinase inhibitors as anticancer agents, this type of targeted therapy remains promising and alluring. Second- and third-generation kinase inhibitors continue to be developed in attempts to overcome mechanisms of acquired resistance, and toxicities continue to be studied and clinically managed. Further, the costs of approved drugs will lower as more inhibitors go off-patent, and new insights to combining these targeted agents will lead to the discovery of efficacious kinase inhibitor combinations and multi-targeted inhibitors such as PI3K-mTOR inhibitors.

### 1.5 Drug repositioning, statins and cancer

Owing to financial burden, toxicity concerns, and other limitations of novel drug discovery, drug repurposing for the cancer context is has been highly pursued.\textsuperscript{57,58} Off-patent, approved drugs provide assurance in terms of low cost and safety. One class of approved drugs being pursued as anticancer agents is the statins. Statins are widely prescribed for managing hypercholesterolemia, and have undoubtedly earned blockbuster drug status. As small molecule inhibitors, statins target 3-hydroxyl-3-methylglutaryl coenzyme A reductase (HMGCR), the rate-limiting enzyme of the mevalonate pathway\textsuperscript{59} (\textbf{Figure 1}). By binding to HMGCR with 10,000 times greater affinity than its natural substrate\textsuperscript{60,61}, statins deplete products of the mevalonate pathway including
cholesterol, and trigger a homeostatic feedback response that ultimately decreases plasma low-density lipoprotein (LDL) cholesterol\textsuperscript{59}. This homeostatic feedback response is dependent on sterol regulatory element binding protein 2 (SREBP2), a transcription factor that is activated and translocated to the nucleus under low sterol conditions, where it binds to sterol regulatory elements (SRE) on sterol responsive genes including HMGCR and 3-hydroxy-3-methylglutaryl-CoA synthase 1 (HMGCS1) to restore end products of the mevalonate pathway\textsuperscript{62} (Figure 1).

**Figure 1. Simplified schematic of the mevalonate pathway**

Through a series of steps, acetyl-CoA is converted into mevalonate, which is subsequently converted into downstream products (blue) with varied functions (green). Statins competitively bind to HMGCR, the rate-limiting enzyme of the pathway, thereby preventing the production of mevalonate and depleting mevalonate pathway end products. Depletion of intracellular cholesterol triggers a homeostatic feedback response whereby SREBP2 translocates to the nucleus, binds to sterol regulatory elements, and upregulates sterol responsive genes including HMGCR, HMGCS1 and low-density lipoprotein receptor (LDLr) (not shown). This feedback mechanism restores mevalonate pathway end products, including cholesterol. Additionally, homeostatic uptake of extracellular cholesterol via LDLr decreases plasma cholesterol levels, which accounts for the clinical use of statins as cholesterol-lowering agents.
The antiproliferative properties of statins came to light in the early 1990s\textsuperscript{63,64}. Since then, many studies have tried to characterize these properties to determine if statins should be added to the catalogue of anticancer treatments. The wealth of epidemiological data shows discordant results, but of promise, some studies cite up to 50 percent reduction in cancer incidence in statin users\textsuperscript{65-67}. Particular to lung cancer, statin use was associated with 45% decreased risk in a population of Veterans patients\textsuperscript{68}. Disparities between studies have been attributed to the failure to account for inconsistencies within patient populations. Studies have disregarded differences in duration of statin use, failed to stratify patients by tumour subtypes, and considered the class of statins as a singular drug. There are currently seven statins marketed in North America, and their distinct pharmacological properties need to be considered for epidemiological, preclinical and clinical studies alike. While all statins share a common pharmacophore that is responsible for competitively binding to HMCGR, the ring moiety attached to this pharmacophore differs between statins, and dictates pharmacological properties including lipophilicity, drug half life, peak plasma concentration and drug metabolism\textsuperscript{69}.

Preclinical studies have suggested that a deregulated mevalonate pathway contributes to tumorigenesis, providing strong rationale to use statins to target this aberrant pathway regulation, and to repurpose statins as anticancer agents. For example, exogenous mevalonate has been shown to promote subcutaneous xenograft tumour growth\textsuperscript{70}, and the mevalonate pathway has been implicated in mediating the disruption of mammary acinar morphogenesis triggered by mutant p53\textsuperscript{71}. Associative epidemiological evidence for the role of statins in reducing cancer incidences and preclinical evidence supporting the putative role of the deregulated mevalonate pathway in promoting oncogenesis have led to clinical trials for the evaluation of statins as anticancer agents. There are over 20 clinical trials evaluating statins in the cancer context, most of which involve statins in a combination therapy setting. While statins are being paired with standard therapies including cisplatin for SCLC (NCT00433498, NCT01441349), erlotinib for squamous cell carcinoma and NSCLC (NCT00966472) and gefitinib for NSCLC (NCT00452244), combinations with non-standard therapies are emerging. Lovastatin plus standard of care treatment in hepatocellular carcinoma (HCC) increases median survival\textsuperscript{72}, and lovastatin, thalidomide and dexamethasone increases overall and progression-free survival in
multiple myeloma (MM)\textsuperscript{73}. Such evidence suggests that identifying novel statin co-therapies is warranted.

### 1.6 Combination therapy

The dismal productivity in pharmaceutical Research & Development for cancer therapeutics\textsuperscript{74} has led to a re-evaluation of the monotherapy paradigm for cancer treatment. The “magic bullet” concept, whereby a single drug is efficacious against malignancies rarely upholds, and even monotherapies deemed highly successful, such as imatinib, are eventually trodden by drug resistance. Since cancers arise from combinations of perhaps up to ten somatic mutations\textsuperscript{13}, and cancer cells are equipped with several mechanisms of resistance, combination treatments are clearly necessary. Network pharmacology and synthetic lethality have garnered enormous interest as new paradigms for cancer drug development\textsuperscript{75-77}. Various approaches have been taken to discover and design rational drug combinations and multi-targeted agents, including next-generation sequencing\textsuperscript{78} and high-throughput screening methods including systemic RNAi screening\textsuperscript{79,80} to identify co-occurring and interacting genomic alterations to be targeted.

High-throughput screening (HTS) is a common platform for drug discovery, and is heavily used by the pharmaceutical industry. HTS can involve chemical compound libraries or genetic RNAi libraries, and is typically automated and carried out in microtiter plates. Molecules from the chemical or genetic libraries are evaluated for a desired outcome, usually a reduction in cell viability in the cancer context, which is commonly determined using a colorimetric assay. Molecules demonstrating the desired outcome will progress to the validation stage of screening. In recent years, HTS has also been used to identify combination therapies. However, high-throughput screening is limited by the exponential number of potential drug combinations\textsuperscript{76}. Of course, not all compounds or combinations will validate, and there is a heavy time-cost to the validation phase of screening, which usually is not automated. Refining hit selection to increase validation rate would be highly beneficial. To focus resources on hits most likely to validate and be clinically safe and efficacious, \textit{in silico} screening methods that are run independent of, or in parallel with, experimental screening have been developed\textsuperscript{81,82}. High-content screening (HCS), whereby live cells are imaged to generate information-rich phenotypic data, can also direct hit selection towards compounds and molecules most likely to validate.
1.7 High-content screening

High-content screening has come to the forefront of drug discovery in recent years. The inaugural high-content screening system was introduced in 1997\textsuperscript{83}, and drastic improvements have led to the development of increasingly automated and user-friendly systems. This platform is based on automated microscopy, which allows for the visualization of cells and subcellular compartments. Multiple phenotypic measures or features including, but not limited to, changes in morphology, protein expression, protein-protein interactions, and intracellular and intercellular protein trafficking can be measured simultaneously\textsuperscript{84,85}. Not only does HCS capture multiparametric data, it also captures spatiotemporal information. As such, large quantities of drug response information can be generated with HCS as compared to traditional drug screening endpoint assays, such as colorimetric assays like MTT, MTS, alamarBlu\textsuperscript{®}, and CellTiterGlo\textsuperscript{®}, which have a single read-out. These traditional HTS assays rely on quantifying a singular cellular process, such as reduction of a compound by a mitochondrial enzyme, as a proxy for cell viability. Further, HCS can be used to study heterogeneous response to treatments by identifying and analyzing distinct cell subpopulations in a single treatment well\textsuperscript{84}. By contrast, HTS assays usually reduce cells in one well into one homogenous group. Accounting for heterogeneous response would be useful for studying drug sensitivity, as resistant and sensitive cells to any given drug could be distinguished and compared.

While HCS generally refers to large-scale screening with thousands of both compounds and microtiter plates, high-content analysis (HCA) refers to lower throughput screening involving fewer compounds and less than 10,000 data points\textsuperscript{84}. The high-content platform discussed in this thesis is low-throughput, but will be referred to as a ‘high-content screen’ to maintain consistent terminology across different screening platforms. Many fluorescence dyes are used for high-content imaging applications. Organelle-specific dyes exist to visualize particular parts of cells, such as nuclear-targeting or plasma-membrane targeting dyes. New dyes continue to be developed to overcome limitations such as specificity and toxicity, of which the latter prevents cell monitoring over time.

1.8 Research objectives and hypothesis

The broad research objective of this thesis project is to contribute to the translation of statins to the clinic for use as anticancer agents. The focused research objective is to identify small
molecules that potentiate the anticancer effects of fluvastatin in the A549 lung adenocarcinoma cell line, through the use of genomic and pharmacological screening platforms. We hypothesized that screening A549 cells with genomic and small-molecule libraries would uncover kinase inhibitors to use in co-therapies with fluvastatin. This project combines systemic gene knockdown, small-molecule screening and high-content imaging for anticancer drug discovery.

1.9 Approach

To address the research hypothesis, two screening libraries and three screening platforms were utilized to discover kinase inhibitor and statin co-therapies.

Aim 1: Validate candidate kinase-targeted small hairpin RNAs (shRNAs) as sensitizers to the antiproliferative effects of fluvastatin from a genome-wide shRNA screen. The first aim of this thesis project is a validation strategy based on a shRNA dropout screen performed by previous members of the Penn laboratory. From this screen, several shRNAs targeting kinases were found to putatively sensitize A549 cells to the antiproliferative effects of fluvastatin. This class of proteins is targetable and has wide-ranging roles in cell growth, proliferation and survival, prompting our interest in validating if their knockdown interacts favorably with fluvastatin to cause cancer cell death. Three kinases were selected for validation.

Aim 2: Design and conduct a high-content screen with fluvastatin and a kinase inhibitor library. The second aim of this project involves screening the A549 cell line with a library of 320 kinase inhibitors with and without fluvastatin to identify kinase inhibitors that potentiate the antiproliferative effects of fluvastatin. Two screening platforms, a classic antiproliferative colourimetric assay, and a novel high-content imaging strategy, were used.
Contributions and Acknowledgements:

The majority of the work in this chapter was completed by the author of this thesis. Additional contributions are indicated below.

Figure 2: The shRNA screen was carried out by Dr. Aleksandra Pandyra, Dr. Carolyn Goard and Dr. Elke Erikson.

Figure 3: Kevin Brown from the laboratory of Dr. Jason Moffat performed bioinformatic analysis of the shRNA screen data, and generated the representation of the screen results shown.

Figure 4: Immunoblot replicates were completed through a team effort between Dr. Aleksandra Pandyra, Dr. Carolyn Goard and the author.

Figure 5 and 6: Experiments for Figure 5 were carried out by Dr. Aleksandra Pandyra. Assays involving PIK-93 in MDA-MB-231s in Figure 6 were completed by Dr. Aleksandra Pandyra.

Figures 11-13: Jarkko Ylanko from the laboratory of Dr. David Andrews completed the technical screening of the MCF10A cell line. Data analysis was completed under the guidance Jarkko Ylanko.

Figure 24B: Schematic created by Ashley Hickman

Figure 34: Dr. Peter Mullen and Rosemary Yu provided technical assistance for MTT assays
2 Results

2.1 Validating candidate sensitizers to the antiproliferative effects of fluvastatin from a genome-wide shRNA dropout screen

Statins have been combined with several chemotherapeutics and small molecule inhibitors in both preclinical and clinical settings\textsuperscript{86}. Some combinations show promising additive and even synergistic anticancer effects, providing justification to explore rationally combined statin-based therapies. Previous members of the Penn laboratory undertook a shRNA dropout screen to impartially discover statin co-therapies. The TRC1 library assembled by The RNAi Consortium (TRC) at the Broad Institute was used, which contains short hairpins targeting approximately 16,000 genes\textsuperscript{87}. The pooled lentiviral library was transduced into A549 lung adenocarcinoma cells with a multiplicity-of-infection (MOI) that was expected to yield one stably expressing shRNA in each cell. Pools of stably transduced cells were subsequently subjected to either low-dose fluvastatin, optimized to yield approximately 80% viability, or to ethanol vehicle control. Three independent replicates were repeated for both the fluvastatin and ethanol conditions. The shRNA populations in both treatment conditions were assessed on Day 0, and every three days over 15 days by hybridizing harvested genomic DNA to custom microarrays. Underrepresented, or “dropout” shRNAs putatively sensitize cells to the antiproliferative effects of fluvastatin. shRNA abundance was determined for Days 0, 3, 6 and 12 only. Unexpectedly high cell counts on day 9 and 15 led to their exclusion. Figure 2 shows the workflow for the screen.

Relative shRNA abundances over time were collapsed into Z-score normalized shRNA Activity Ranking Profile Scores (shARP scores) for each shRNA in both treatment conditions, as previously described in Marcotte et al.\textsuperscript{88}. More negative shARP values are indicative of faster, and greater overall loss of a hairpin from the population over time. These values are normalized to baseline abundances on Day 0. For each gene targeted, there is an average of five shRNAs in the lentiviral library, which allows for sufficient redundancy. As such, shARP scores for shRNAs targeting the same gene were further reduced into a Gene Activity Ranking Profile score (GARP score). The GARP score is calculated by averaging the top two most negative shARP scores\textsuperscript{88}. The normalized GARP score for each gene under the ethanol treatment (GARP\textsubscript{ethanol}) was subtracted from the normalized GARP score under the fluvastatin treatment condition.
A549 cells stably transduced with the 80K shRNA pooled library

A549 were transduced with a pooled library of shRNAs (TRC1, Broad Institute), and these pools of cells were independently exposed to ethanol vehicle control or to a low, sublethal dose of fluvastatin. Three replicates of each treatment condition were performed. Every three days, the pools of cells were reseeded, and some cells were retained for harvesting genomic DNA. Genomic DNA from select time points shown above was hybridized to custom microarrays to determine the shRNA populations over time for each treatment condition. shRNAs that had low representation in the fluvastatin treatment condition compared to the ethanol condition were identified as candidate sensitizers to the antiproliferative effects of fluvastatin.

\[ \text{GARP}_{\text{fluvastatin}} \]

Genes with negative \( \left( \text{GARP}_{\text{fluvastatin}} - \text{GARP}_{\text{ethanol}} \right) \) values at least three standard deviations from the mean were defined as hits. In other words, knocking down these genes putatively increases the antiproliferative effects seen with fluvastatin treatment alone. In total, 151 hits were identified (Figure 3).

Notable classes of hits include mevalonate pathway genes, metabolic genes, and kinases. Kinases are targetable, and have wide-ranging roles in cell growth, proliferation and survival, which prompted our interest in validating their corresponding shRNAs. This class of hits was the focus.

Figure 2. shRNA dropout screen workflow
Figure 3. 151 candidate hits identified from the shRNA dropout screen

Each gene targeted in the shRNA library was assigned a Gene Activity Profiling Score (GARP score) for both the ethanol and fluvastatin treatment conditions. The difference between the Z normalized GARP<sub>fluvastatin</sub> and GARP<sub>ethanol</sub> for each gene represents how much and how quickly shRNAs targeted towards a particular gene were lost from the shRNA population. The more negative the score, the more likely knockdown of the gene interacts favorably with fluvastatin to induce antiproliferative effects and perhaps cell death. Genes with negative ΔZ at least three standard deviations from the mean were defined as hits. There are 151 hits according to this definition.

zFluva: Normalized Z-GARP score for fluvastatin treatment
zEtOH: Normalized Z-GARP score for the ethanol treatment
ΔZ: ZFluva-ZEtOH
of my validation efforts, and I also contributed to the validation of mevalonate pathway gene candidate hits, discussed in Appendix 1. Three kinases were selected for validation based on availability of specific small molecule inhibitors for each target, or its downstream products. This allowed for validation of genetic hairpins with pharmacological inhibitors, and the pharmacological inhibitors could theoretically serve as tool compounds for the development of clinically useful small molecule inhibitors if preclinical data supported such future work. The selected kinase targets were phosphatidylinositol 4-kinase, catalytic, beta (PI4KB), casein kinase 2, beta, polypeptide (CSNK2B) and mitogen-activated protein kinase kinase 4 (MAP2K4). PI4KB is a lipid kinase involved in lipid trafficking and signaling, which converts phosphatidylinositol (PI) to phosphatidylinositol-4-phosphate. CSNK2B is a serine/threonine kinase predominately involved in the regulation of cellular metabolism, but has many other cellular functions. MAP2K4 lies upstream of a stress activated protein kinase. The three selected kinases were not the top three hits, nor were they the top three kinases within the ranked list of 151 hits (Table 1). Rankings of the selected kinase were purposely varied, enabling us to preliminarily test the biological validity of our statistical definition of a hit.

2.1.1 Confirming target gene expression knockdown

Endogenous gene expression knockdown was verified for the top two performing shRNAs targeted to each of the three selected kinases. The shRNAs used are identical to those in the TRC1 library used in the dropout screen. First, A549 cells were stably transduced with each shRNA to create A549 sublines, which are denoted with the convention sh-target-#, where the number distinguishes between individual shRNAs (e.g. shPI4KB-1). Two control sublines were generated using shRNAs targeted to LacZ, a gene outside the human genome. Protein levels of the relevant gene target were assessed for all six validation sublines by immunoblot, and compared against the two control sublines. For PI4KB and CSNK2B, one of two shRNAs led to robust gene expression knockdown at the protein level, and both shRNAs targeted to MAP2K4 decreased gene expression (Figure 4). Additional approaches should be taken to validate the results to date. This may include validating additional shRNAs, or knocking down our targets with small interfering RNAs (siRNAs) or inducible shRNA systems. We have selected and begun a siRNA approach to complement the body of shRNA work.
Table 1. Rankings of candidate kinase genes from the shRNA dropout screen based on Z-normalized (GARP_{fluvastatin} – GARP_{ethanol}) scores

Rankings for the three selected kinases among the 151 hits from the shRNA dropout screen are shown. A more negative ZEtOH or ZFluva score reflects faster and a greater degree of shRNA dropout. More negative ΔZ scores are reflective of higher hit rankings, and indicate a greater differential in cell viability between the fluvastatin and ethanol treatments. The selected kinases are well distributed throughout the 151 ranked hits, allowing for validation of our defined threshold of a hit.

<table>
<thead>
<tr>
<th>Gene Symbol</th>
<th>Gene Name</th>
<th>Rank (of 151 hits)</th>
<th>ZEtOH</th>
<th>ZFluva</th>
<th>ΔZ</th>
</tr>
</thead>
<tbody>
<tr>
<td>CSNK2B</td>
<td>Casein kinase 2, beta polypeptide</td>
<td>73</td>
<td>-0.358</td>
<td>-2.097</td>
<td>-1.739</td>
</tr>
<tr>
<td>MAP2K4</td>
<td>Mitogen-activated protein kinase kinase 4</td>
<td>132</td>
<td>-0.220</td>
<td>-1.680</td>
<td>-1.460</td>
</tr>
<tr>
<td>PI4KB</td>
<td>Phosphatidylinositol 4-kinase, catalytic, beta</td>
<td>114</td>
<td>-1.723</td>
<td>-3.265</td>
<td>-1.542</td>
</tr>
</tbody>
</table>

Figure 4. Validating gene expression knockdown in A549 cells transduced with shRNAs from the TRC1 library

A549 sublines transduced with one individual shRNA each were grown to subconfluence and harvested for immunoblotting. Two control sublines, shLacZ-1 and shLacZ-2, were generated with shRNAs targeting LacZ, a bacterial gene. Expression of PI4KB, CSNK2B and MAP2K4 (A, B and C, respectively) in the validation and control sublines was determined by probing with appropriate antibodies. At least one shRNA targeted to each kinase robustly decreases protein expression as compared to expression levels in the control sublines.
2.1.2 Potentiating the anti-proliferative activity of fluvastatin

All six validation A549 sublines were treated with two doses of fluvastatin (5μM and 10μM), or with ethanol control for 72 hours. At this endpoint, the sublines were harvested and assessed for cell death by staining fixed cells with propidium iodide and determining the percent of cells in the pre-G1 population by flow cytometry. Results were compared to control sublines exposed to the same treatment, which were generated using shRNAs targeted to either GFP or LacZ. As seen in Figure 5, the control sublines (shGFP-1, shLacZ-1, shLacZ-2,) show minimal cell death in both the ethanol and fluvastatin treatment groups, confirming that shRNAs targeting GFP and LacZ induce low levels of cell death independently and in conjunction with fluvastatin, reflective of baseline cell death in cell culture conditions. By contrast, for each of the selected kinases, at least one shRNA was able to interact with fluvastatin to significantly increase cell death.

2.1.3 Using pharmacological inhibitors to complement genomic knockdown of targets

To further explore the validity of our putative shRNAs as sensitizers to the antiproliferative effects of fluvastatin, we used pharmacological inhibitors to complement genomic knockdown. PIK-93 was chosen as a potent inhibitor of PI4KB, and 4,5,6,7-tetrabromo-1H-benzotriazole (TBB) was selected as a specific inhibitor of CSNK2B. Complementary pharmacological data has not yet been generated for MAP2K4, however, SP600125, a specific and potent inhibitor of c-Jun N-terminal kinase (JNK), which is downstream of MAP2K4, would likely be selected for such studies. First, we determined if each inhibitor could lower the IC_{50} concentration of fluvastatin. Cells were treated with a dose-range of fluvastatin with and without low doses of each inhibitor. The low doses of each inhibitor were determined by treating cells with a range of concentrations to determine the highest possible dose that yielded negligible effects on cell viability in an MTT assay. The low-doses were determined to be 1μM for PIK-93 and 60μM for TBB. Dose-response curves were generated for both the fluvastatin-only and fluvastatin plus inhibitor conditions. As shown in Figure 6A, the MTT_{50} dose for fluvastatin in A549 cells was 24μM for the fluvastatin-only treatment, and was significantly decreased to less than 10μM when treated with 1μM PIK-93. For TBB, a dose of 60μM reduced the MTT_{50} dose of fluvastatin by 9.5μM (Figure 6C). This experiment was repeated in MDA-MB-231 cells, a relatively
A549 sublines stably expressing one shRNA each were subjected to either ethanol vehicle control or two doses of fluvastatin for 72 hours, upon which cells were harvested, fixed, stained with propidium iodide and analyzed by flow cytometry. Cells in the pre-G1 population are defined as dead. One of two validation sublines for each kinase demonstrates that shRNA knockdown of the indicated kinase potentiates cell death induced by fluvastatin. The mean percent pre-G1 population is displayed plus or minus standard deviation for three independent replicates. A one-way ANOVA with a Tukey post-test was performed for each subline to compare the pre-G1 populations in the ethanol versus fluvastatin treated groups. * = p < 0.05.
A549 and MDA-MB-231 cells were treated with low doses of fluvastatin or inhibitor or both combined for 72 hours. PIK-93, which potently inhibits PI4KB, is able to reduce the MTT<sub>50</sub> value of fluvastatin in both cell lines, but to significance in A549s only (A-B). TBB, which inhibits CSNK2B, reduces the MTT<sub>50</sub> value of fluvastatin in both cell lines, but to significance in MDA-MB-231 cells only (C-D). For all MTT assays, the mean ± SD MTT<sub>50</sub> value is shown for three independent replicates, *= p<0.05, unpaired, two-tailed t-test. PIK-93 and fluvastatin in combination increases cell death as assayed using the fixed propidium iodide assay in both A549 and MDA-MB-231 cells (E-F). Averages of three independent replications ± SD are shown. *= p< 0.05, unpaired, two-tailed t-test between solvent and each treatment condition.
fluvastatin-sensitive breast cancer cell line, to begin to determine if the effects of each of these two inhibitors in combination with fluvastatin can be generalized to other cancer types. Low-doses of PIK-93 and TBB determined for MDA-MB-231 cells were 1µM and 80µM, respectively. Reduction in the MTT₅₀ dose of fluvastatin produced by PIK-93 trended towards significance, while the reduction produced by TBB was significant in MDA-MB-231 cells (Figure 6B,D).

Further, we treated A549 cells with either low doses of fluvastatin, a low dose of PIK-93, or a combination of fluvastatin and inhibitor, and assessed these treatment groups for cell death using the fixed propidium iodide assay. The low PIK-93 and fluvastatin concentrations cause slight increases in cell death alone relative to solvent control, but in combination are able to significantly increase the percent of cells in the pre-G1 population in both A549 and MDA-MB-231 cells (Figure 6E-F).

2.2 High-content screening with fluvastatin and a kinase inhibitor library

The fluvastatin and kinase inhibitor combinations explored in section 2.1 are promising putative apoptosis-promoting and proliferation-suppressing co-therapies. As such, we were interested in pursuing additional kinase inhibitors. The kinases selected for validation from the shRNA dropout screen were handpicked based, in part, on the availability of selective inhibitors at the time. We resolved to take an unbiased approach to identify fluvastatin and kinase inhibitor combinations and set out to screen 320 kinase inhibitors (OICR Kinase Collection) in the presence and absence of fluvastatin. We were especially hopeful for FDA-approved kinase inhibitors, as the pharmacological inhibitors used in section 2.1 have not been optimized for clinical use. Identifying FDA-approved kinase inhibitors to use with FDA-approved statins may fast-track these statin and kinase inhibitor combinations to the clinic.

To create an opportunity to compare results from two screening platforms, namely the shRNA screen described in section 2.1, and the kinase inhibitor screen described here, we again screened with the A549 cell line for consistency. In addition to identifying new fluvastatin and kinase inhibitor co-therapies, we also aimed to preliminarily assess the utility of the OPERA® High-content Screening System in conjunction with a proprietary fluorescent membrane-targeted dye, mutachrome (MK1), for small-molecule inhibitor library screening. A MTS assay was performed
in parallel to provide a standard drug screen assay for comparison. Additional cell lines screened with the kinase inhibitor screen in combination with fluvastatin are discussed in Appendix 2.

2.2.1 Hit prediction

2.2.1.1 Library annotation and characterization

The kinase inhibitor library used was assembled by the Drug Discovery Program team at the Ontario Institute for Cancer Research and contains 320 inhibitors. The accompanying file for the kinase inhibitor library documents the names, compound structures, molecular weights, and gene targets for each inhibitor. To account for evolving gene names and newly discovered targets for inhibitors, the provided list of gene targets was updated. Gene targets for each inhibitor were determined by conferring with the NCBI PubChem Compound portal, the SuperTarget Database\(^9\), the PROMISCUOUS database\(^9\) and by surveying the literature to find drug-target interactions. All target genes were updated to the current and standard NCBI official gene symbols by conferring with the NCBI Gene portal. Library annotation enabled classification of the 320 kinase inhibitors into broad categories. Manning et al.,\(^4\) and the complementary website, www.kinase.com/human/kinome were consulted for this categorization. The library largely consists of inhibitors targeting cell cycle kinases, receptor tyrosine kinases, and kinases in the MAPK or PI3K pathways (Figure 7). Each inhibitor was categorized into a single group for these purposes. While this does not account for cross-talk between signalling pathways, it provides a representation of the inhibitor library.

2.2.1.2 Predicting hits for the kinase inhibitor library screen with the A549 cell line

The first goal was to anticipate which kinase inhibitors would potentiate the antiproliferative activity of fluvastatin in our impending kinase inhibitor screen with the A549 cell line. In order to gain preliminary insight to which inhibitors to expect as hits, the list of genes targeted by the 320 inhibitors was compared to the list of 151 candidate fluvastatin potentiators from the shRNA screen using the R Statistical Environment. The list of gene candidates from the shRNA screen was updated to standard NCBI official gene symbols prior to comparison. Of the 151 shRNA gene hits, 22 are kinases (Table 2). Of these kinases, ten are targeted by a total of 22 inhibitors in the kinase inhibitor library (Table 3). In other words, there are 22 inhibitors that share targets with shRNAs that putatively sensitized A549 cells to the antiproliferative effects of fluvastatin in
our shRNA screen. As such, we predicted that these 22 inhibitors may be candidates in the kinase inhibitor library screen. Any overlapping hits between the shRNA dropout screen and the kinase inhibitor library screen would be prioritized for validation. Of note, PIK-93 and TBB, inhibitors used in section 2.1 to validate whether knockdown of PI4KB and CSNK2B potentiates fluvastatin-induced antiproliferation, are compounds included in the library.

Figure 7. Classification of the library of 320 kinase inhibitors based on major signalling pathways or cellular processes inhibited

The 320 kinase inhibitors were classified based on which major signalling pathway or cellular process each targets. Each inhibitor was classified into one group based on the function of the predominant gene it is characterized to inhibit.
Table 2. Kinase hits from the shRNA dropout screen

Of the 151 hits from the shRNA dropout screen, 22 are kinases as determined by using the search term ‘kinase’ for all gene names. Any genes included in the list of hits that were withdrawn from NCBI gene were excluded. These 22 hits have varied rankings from the shRNA dropout screen results, and the lowest ranked gene preliminarily validated to date is MAP2K4, ranking 132.
Table 3. Predicted hits from the kinase inhibitor library screen in the A549 cell line based on shRNA screen results

By comparing hits that putatively potentiate fluvastatin-induced antiproliferative effects from the shRNA dropout screen against the gene targets of the 320 inhibitors in the kinase inhibitor library, 10 genes were found to overlap. Twenty-two independent kinase inhibitors from the library inhibit these 10 genes. Many of these inhibitors are promiscuous and target genes in addition to those listed, and some inhibit multiple genes in this list.

<table>
<thead>
<tr>
<th>shRNA Screen Hit</th>
<th>Kinase Inhibitor from Library</th>
</tr>
</thead>
<tbody>
<tr>
<td>PRKAA1 (AMPK)</td>
<td>Dorsomorphin dihydrochloride</td>
</tr>
<tr>
<td></td>
<td>STO-569 acetic acid</td>
</tr>
<tr>
<td>TBK1</td>
<td>BX-795</td>
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<td></td>
<td>SU-5668</td>
</tr>
<tr>
<td>CAMK2G</td>
<td>Staurosporine</td>
</tr>
<tr>
<td></td>
<td>STO-569 acetic acid</td>
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<td></td>
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<td>BI-2536</td>
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<td>Cyclopalatin</td>
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<td>GSK-461364, GSK-1364</td>
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<td></td>
<td>BI-6727, volasertib</td>
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<tr>
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<td>PI-93</td>
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<tr>
<td>CNK2B</td>
<td>TBB: NSC 231634</td>
</tr>
<tr>
<td></td>
<td>2-Dimethylamine-4,5,6,7-tetrahydro-1H-benimidazole, DMAT</td>
</tr>
<tr>
<td></td>
<td>Ellagic acid</td>
</tr>
<tr>
<td>PDPK1 (PK1)</td>
<td>OSU-03012 hydrochloride</td>
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<td>FKN2 (FAK2)</td>
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<td>DDF2</td>
<td>Dasatinib</td>
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<td>Imatinib</td>
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<td>Midatinib</td>
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<tr>
<td>MAP2K4</td>
<td>Staurosporine</td>
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<td></td>
<td>Berenstain</td>
</tr>
</tbody>
</table>
2.2.2 Pilot screen in MCF10A breast cells

We performed a proof-of-concept screen with the kinase inhibitor library in the presence and absence of fluvastatin with the MCF10A breast cell line. The purpose of this screen was to optimize and test the feasibility of our screening parameters for subsequent cancer cell lines. The cells were treated with the kinase inhibitor library plus fluvastatin or ethanol vehicle control, and imaged at our defined time points using the OPERA® High-content Screening System. Our collaborators in the laboratory of Dr. David Andrews had previously screened the MCF10A cell line with the OPERA®, and as such, the seeding density for 384-well plates, and the uptake of imaging dyes involved in our high-content imaging endpoint by these cells were already well established. This was desirable given our appeal for a prompt proof-of-concept screen. See Figure 8A for a schematic representation of the screen.

**Figure 8A.** Workflow and screening parameters for the high-content screen with MCF10A cells
MCF10A cells were seeded in 384-well plates, treated with 1µM of the kinase inhibitor library with or without fluvastatin for 24 and 48 hours, upon which images were taken with the OPERA® High Content Screening System and subsequently analyzed. An ‘alive-phenotype negative control’ and a ‘death-phenotype positive control’ were defined for data classification.

*HCS = high-content screening
MCF10A cells were treated for 24 and 48 hours with 1µM of the kinase inhibitor library (dissolved in DMSO) and either the MTT$_{20}$ dose of fluvastatin 5µM (Figure 9), or ethanol vehicle control. Low dose 5µM fluvastatin plus DMSO vehicle control was our ‘alive-phenotype negative control’ treatment and 5µM fluvastatin plus 1µM PIK-93 was our ‘death-phenotype positive control’ treatment. A summary of the screening parameters can be found in Figure 8B. Each treatment condition was assayed in two independent wells.

Figure 9. Determining the MTT$_{20}$ dose of fluvastatin in MCF10A cells for the pilot kinase inhibitor screen
A series of dose-response curves were performed on MCF10A cells to find the MTT$_{20}$ dose of fluvastatin. A dose of 5µM was selected as it yields about 20% reduction in MTT activity over three replicates (A). Variability between replicates (B) made calculation of the average MTT$_{20}$ value using the hillslopes and MTT$_{50}$ values from dose-response curves impractical for this dataset.
2.2.2.1 Feature extraction and image classification

At the treatment endpoints of 24 and 48 hours, two dyes were added to the culture media in every well to visualize the cells. DRAQ5 is a cell permeable, DNA-specific fluorescent dye, and MK1, or mutachrome 1, is a proprietary membrane-targeted fluorescent dye developed by our collaborator, Dr. David Andrews. Cells were incubated for 30 minutes with the dyes to allow for dye incorporation.

Image acquisition was completed using the OPERA® High-content Screening System. Four images were captured from independent fields-of-view per well, yielding eight images per treatment condition. The Acapella® high-content imaging and analysis software was then used for image processing. The script used for image segmentation, which is the identification of objects of interest (i.e. cells) in each image, and for feature extraction, which is the reduction the image data into quantifiable units or vectors, was written by members of the laboratory of Dr. David Andrews. Texture, morphology, dye intensity, and other features were extracted for every segmented cell in each image. Subsequently, MATLAB software and a companion graphical user interface developed by the laboratory of Dr. David Andrews were used for supervised feature reduction and classification of cells.

Two training groups were used for supervised feature reduction and classification: the alive-phenotype negative control group (5µM fluvastatin with DMSO); and the death-phenotype positive control (5µM fluvastatin with PIK-93). The objects within the two training groups were randomly divided into a training set and a prediction set (Figure 10). The software learns how to distinguish the two classification groups using the training set. This produces a classifier model that uses a reduced number of relevant features to classify data. The performance of this classifier model can be tested using the prediction set of control objects, for which the expected classification is known.

Feature reduction identified three features important for object classification at the 24 hour time point and four features important for object classification at 48 hours (Figure 11). Classification of our controls was promising, as over 80 percent of cells in the solvent control group (ethanol
Figure 10. Supervised feature reduction and classification schematic

Feature reduction and classification involve randomly dividing images of cells within each control group into a training set and a prediction set (top). The training set is used to generate a classifier model, which labels each cell in the prediction set as more similar to either the negative control or the positive control, thereby classifying all cells into one of two groups. The prediction group is also called the testing group, since this data has known labels for each cell, allowing the performance of the classifier model to be determined. The classifier model can be applied to any new cell in the remaining dataset. Adapted from http://nltk.org/book/ch06.html.
Figure 11. Features used for object classification in the MCF10A screen
Feature reduction by stepwise discriminant analysis for the defined negative and positive control groups (5µM fluvastatin plus solvent control, and fluvastatin5µM plus 5µM PIK-93, respectively) results in three features used for object classification for the 24 hour time point (A), and four features for the 48 hour time point (B). Features used for object classification include both nuclear and membrane features for both time points, indicating that both dyes used to visualize the cells are crucial for distinguishing between the two control groups. The mean z-score ± standard deviation is plotted for each feature compared to all features extracted.
and DMSO), negative control group (5µM fluvastatin and DMSO; “alive-phenotype”) and low-dose PIK-93 treatment group were classified into the negative control bin, as expected, while less than 10 percent of cells in the positive control group (5µM fluvastatin with PIK-93; “death-phenotype”) classified to the negative control bin (Figure 12).

**Figure 12. Classification of cells exposed to control treatments for the kinase inhibitor screen in MCF10A cells**

Cells from each treatment were classified into either the alive-phenotype negative control group or the dead-phenotype positive control group based on features in Figure 11 for the 24 hour (A) and 48 hour (B) time points. For the solvent control, PIK-93 alone, and negative control (fluvastatin + DMSO) treatment groups, over 85% of cells classify as the alive-phenotype negative control at both time points, as expected. Cells exposed to the positive control treatment (fluvastatin + PIK-93) are classified into the dead-phenotype positive control group, as expected.
Cells exposed to each of the 320 query kinase inhibitors plus either fluvastatin or ethanol conditions were also classified into either the positive control or negative control group (Figure 13). We are interested in kinase inhibitors for which cells treated with inhibitor alone (with ethanol vehicle) classify into the death-phenotype positive control group minimally (i.e. cause minimal cell death alone), and for which cells treated with inhibitor in combination with fluvastatin classify into the death-phenotype positive control group maximally (i.e. robust cell death seen with combination treatment). Such compounds are represented in the bottom right corner of Figure 13. To identify kinase inhibitors of interest, we set inclusion criteria for candidate hits. The first criterion is that less than 20% of cells treated with kinase inhibitor alone classify into the positive control group. The second criterion is that cells treated with kinase inhibitor and fluvastatin have a “percent classified as positive control” value at least 3 standard deviations from the negative control. With these criteria, we have 13 putative kinase inhibitors from the 24 hour time point and 9 putative kinase inhibitors from the 48 hour time point that result in a death-like phenotype when used in combination with fluvastatin (Tables 4 and 5, respectively). These kinase inhibitors potentially increase antiproliferative effects of fluvastatin.

Additional controls likely to induce cell death in culture conditions were used, including cyclohexamide, thapsagargin, staurosporine, TNF-α, tunicamycin, tamoxifen and rapamycin. A few of these compounds are seen in the upper right corner of Figure 13 (orange dots), indicating that these compounds produce a death-like phenotype alone and in combination with fluvastatin.

2.2.2.2 mTOR inhibitors, statins and the endoplasmic reticulum (ER) stress response

Of the 13 putative kinase inhibitors from the 24 hour time point, three were mammalian target of rapamycin (mTOR) inhibitors. One of these mTOR inhibitors, everolimus, recently received FDA-approval for the treatment of advanced hormone receptor positive, HER2 negative breast cancers. This provided rationale to characterize a combination of everolimus and fluvastatin in breast cell lines given the preliminary results from our pilot kinase inhibitor screen.

We developed a model for this drug combination based on the well-documented role of statins in eliciting the cytoprotective endoplasmic reticulum (ER) stress response. We hypothesized that statin treatment alone may be ineffective as a result of the activated cytoprotective ER stress response, which helps to prevent cell death (Figure 14A). Since mTOR inhibitors counter the ER
Figure 13. Classification of compound treatments in the ethanol and fluvastatin conditions for the kinase inhibitor screen in MCF10As (Previous page)

All cells exposed to the kinase inhibitor compound treatments in combination with ethanol or fluvastatin were classified into either the alive-phenotype negative control group or the dead-phenotype positive control group based on the features used in Figure 11. Classification results for 24 hours are shown in (A) and results for 48 hours are shown in (B). The greater the number of cells classifying into the dead-phenotype positive control group for any given treatment reflects greater implied cytotoxicity. Kinase inhibitors that have a low percentage of cells classifying into the positive control group when used as a single-agent (i.e. close to 0% on the y-axis), and that have a high percentage of cells classifying into the positive control group when used in combination with fluvastatin (i.e. close to 100% on the x-axis) are putatively potentiating fluvastatin-induced cell death. Our negative control, fluvastatin plus DMSO, causes very few cells to classify into the positive control group (red dot, bottom left), and our positive control, fluvastatin plus PIK-93 causes over 90 percent of cells to classify into the positive control group (green dot, bottom right). Importantly, PIK-93 on its own causes less than 20% of cell death alone, indicating that only treatment in combination with fluvastatin triggers a dead-phenotype. PIK-93 from the compound library is shown in blue. Compounds likely to elicit cell death were added as additional controls (orange dots). Cycloheximide, staurosporine, TNF-α and thapsigargin are represented in the top right corner, indicating that these compounds elicit a death-phenotype alone or in combination with fluvastatin. The other three orange dots account for tunicamycin, tamoxifen and rapamycin.
Table 4. Hits from the kinase inhibitor library high-content imaging screen in the MCF10A cell line at 24 hours

<table>
<thead>
<tr>
<th>Compound</th>
<th>Targets</th>
<th>Plate, Well ID</th>
<th>Standard Deviations from DMSO</th>
</tr>
</thead>
<tbody>
<tr>
<td>PIK-93</td>
<td>PI4KB, PI3K</td>
<td>External</td>
<td>4.76</td>
</tr>
<tr>
<td>HDS 029</td>
<td>EGFR, ERBB2, ERBB4</td>
<td>3-D09</td>
<td>4.54</td>
</tr>
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<td>Bisindoloylmaleimide I hydrochloride</td>
<td>PRKCA, PRKCB, PRKCG, PRKCE</td>
<td>2-I05</td>
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<tr>
<td>Rapamycin (Sirolimus)</td>
<td>mTOR, FKBP1A, IL2RA</td>
<td>1-C08</td>
<td>4.16</td>
</tr>
<tr>
<td>RAD-001 (Everolimus)</td>
<td>mTOR, FKBP1A</td>
<td>1-C11</td>
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<tr>
<td>Arctigenin</td>
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<td>3-G09</td>
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<td>WEE1</td>
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<td>Mutinib</td>
<td>ERBB2</td>
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<td>mTOR</td>
<td>2-C11</td>
<td>3.33</td>
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<td>AV-412</td>
<td>EGFR, ERBB2</td>
<td>4-E02</td>
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<td>SD169</td>
<td>MAPK14</td>
<td>1-H11</td>
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Thirteen inhibitors were defined as hits from the kinase inhibitor library screen at 24 hours. MCF10A cells were treated with the kinase inhibitor library with or without fluvastatin, and imaged after 24 hours (±1 hour). DRAQ5 and MK1 dyes were used to visualize the cells for imaging using the OPERA® high content screening platform. Treatment with these inhibitors plus ethanol causes less than 20 percent of cells to classify into the death-phenotype positive control group, implying minimal cell death caused by each inhibitor alone. Treatment with these inhibitors plus a sublethal dose of fluvastatin leads to a percentage of cells classifying into the death-phenotype positive control group at least 3 standard deviations from the negative control treatment (sublethal fluvastatin + DMSO), implying a large degree of cell death caused by inhibitor plus fluvastatin treatments. PIK-93 external control is defined as a hit under these criteria.
Nine inhibitors were defined as hits from the kinase inhibitor library screen at 48 hours. MCF10A cells were treated with the kinase inhibitor library with or without fluvastatin, and imaged after 48 hours (±1 hour). DRAQ5 and MK1 dyes were used to visualize the cells for imaging using the OPERA® high content screening platform. Treatment with these inhibitors plus ethanol causes less than 20 percent of cells to classify into the death-phenotype positive control group, implying minimal cell death caused by each inhibitor alone. Treatment with these inhibitors plus a sublethal dose of fluvastatin leads to a percentage of cells classifying into the death-phenotype positive control group at least 3 standard deviations from the negative control treatment (sublethal fluvastatin + DMSO), implying a large degree of cell death caused by inhibitor plus fluvastatin treatments. PIK-93 external control is defined as a hit under these criteria, as is the internal library PIK-93 compound.

Table 5. Hits from the kinase inhibitor library high-content imaging screen in the MCF10A cell line at 48 hours

<table>
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<th>Targets</th>
<th>Plate, Well ID</th>
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<td>MAP2K1, MAP2K2</td>
<td>3-A09</td>
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<tr>
<td>ZSTK474</td>
<td>PIK3CG</td>
<td>2-H04</td>
<td>3.41</td>
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<td>PIK-93</td>
<td>PI4KB, PI3K</td>
<td>4-G05</td>
<td>3.41</td>
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<tr>
<td>MK-1775</td>
<td>WEEl</td>
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<td>PHA690509</td>
<td>CDK2</td>
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<td>BIBU 1361 dihydrochloride</td>
<td>EGFR</td>
<td>1-G08</td>
<td>3.06</td>
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<td>CCI-779 (Temsirolimus)</td>
<td>mTOR</td>
<td>2-C11</td>
<td>3.04</td>
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<tr>
<td>ZM 306416 hydrochloride</td>
<td>KDR, FLT1</td>
<td>3-G08</td>
<td>3.04</td>
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stress response, we predicted that everolimus may block the ER stress response elicited in response to statin treatment, thereby preventing cells from circumventing statin-induced apoptosis (Figure 14B).

Figure 14. Model for statin and mTOR inhibitor interaction
Statins have been documented to elicit the ER stress response (A). The cytoprotective effects of the ER stress response may allow cells to evade statin-induced cell death. mTOR inhibitors block the ER stress response, and may therefore prevent evasion of statin-induced cell death. Following this model, statins and mTOR inhibitors may be a valuable therapeutic combination.
To test this model, a two-pronged approach was conceived. First, we would verify a fluvastatin-induced ER stress response, and second, we would test whether fluvastatin and everolimus together lower this response. To determine if a stress response was induced by fluvastatin, MCF10A cells were treated with 1μM fluvastatin, 10μM fluvastatin, ethanol control, or 1μM thapsagargin as a positive control for 4, 16 or 24 hours. The mRNA expression of a panel of stress response genes including BiP/GRP78, CHOP, ATF4, ERdj4, GADD34, and XBP1 (spliced and unspliced) was assessed at each time point by qRT-PCR. We expect to see increased mRNA expression of each of the first five genes listed, and to see a lowered ratio of unspliced XBP1 to spliced XBP1 if an ER stress response is present. One replicate of this set of experiments suggests fluvastatin does not elicit an ER stress response at the concentrations and time points surveyed. mRNA levels of BiP/GRP78, CHOP, ATF4, ERdj4, GADD34 are not increased or are only marginally increased by fluvastatin treatment compared to the solvent control (Figure 15). A three-fold increase in CHOP expression upon 1μM fluvastatin treatment at four hours may warrant further investigation (Figure 15C). Further, the ratio of unspliced to spliced XBP1 does not show a strong decreasing trend upon fluvastatin treatment, although the 10μM treatment at 4 hours may be worth additional investigation (Figure 15F). Thapsagargin, our positive control for ER stress, increased mRNA expression of all the stress response genes surveyed, usually in a time-dependent manner, and robustly decreased the ratio of unspliced to spliced XBP1 as compared to solvent.

To determine if everolimus potentiates the antiproliferative effects of fluvastatin, MCF10A cells were treated with a range of fluvastatin concentrations with or without 0.1nM everolimus, which alone caused less than 5% decrease in cell viability. Everolimus was not able to decrease the MTT_{50} value of fluvastatin (Figure 16A). Further, the combination was not able to induce cell death in MCF10As as determined using the fixed propidium iodide assay (Figure 16A).

The two-pronged approach was also started for two breast cancer cell lines: MDA-MB-231, a relatively statin-sensitive cell line representing basal-like breast cancer, and MCF-7, a relatively statin-insensitive cell line representing luminal breast cancer. The expression of the aforementioned six stress response genes at the mRNA level were assessed for the MDA-MB-231 line. Overall, fluvastatin treatment increases expression of these stress response genes marginally at best, while the positive control drug thapsigargin increases expression of all genes.
Figure 15. mRNA expression of ER stress response genes after fluvastatin treatment in MCF10A cells (Previous page)
The mRNA expression of six genes was measured by q-RT PCR after treatment with two doses of fluvastatin or solvent. Thapsigargin (Tg) was used as an ER stress positive control. Fluvastatin treatment at 1µM and 10µM for 4, 16 and 24 hours did not consistently increase mRNA expression of ATF4, BiP, CHOP, ErdJ4 and GADD34. Fluvastatin treatment also does not consistently trigger XBP1 mRNA splicing, although 10µM treatment at 4 hours may be worth further investigation. mRNA levels are normalized to expression GAPDH, and also normalized to solvent control. By contrast, the ER stress positive control, Tg, increases mRNA expression across all genes, and promotes XBP1 mRNA splicing. All graphs show results from one replicate. No further replicates were carried out.

Figure 16. Fluvastatin and everolimus in combination do not potentiate the antiproliferative effects or cell death over fluvastatin-only treatment in MCF10A cells
(A) MCF10A cells were treated with a range of fluvastatin doses with and without a low dose of everolimus (0.1nM), which does not reduce cell viability alone. Dose-response curves were generated using the MTT assay. Curves for fluvastatin-only treatment and fluvastatin plus everolimus treatment are nearly indistinguishable, as are the MTT₅₀ concentrations. (B) MCF10A cells were treated with fluvastatin (fluva) or everolimus (evero) separately, or in combination (F+E). At the concentrations used in this cell line, the combination treatment does not trigger cell death as assayed by fixed propidium iodide staining.
except for ATF4 and BiP at the 24 hour time point (Figure 17). For the MCF-7 line, mRNA expression of BiP does not increase with fluvastatin treatment, and mRNA expression of CHOP is slightly elevated at 16 hours upon 50µM fluvastatin treatment only (Figure 18). Upon combination fluvastatin and everolimus treatment, the pre-G1 population is not significantly increased in either cell line, but may be trending towards significance in the MCF-7 line (Figure 19). Taken together, after preliminary studies with three cell lines, we did not find strong evidence for fluvastatin inducing a stress response in breast cell lines, nor did we find strong evidence for fluvastatin and everolimus inducing cell death as a combination treatment.

Despite the seemingly negative data described above, many studies using dual PI3K/mTOR inhibitors sparked a renewed interest in everolimus. As a first-pass assessment of inhibiting PI3K and mTOR in combination with fluvastatin treatment, a triple combination including PIK-93, everolimus and fluvastatin was used to treat MDA-MB-231 and MCF-7 cells. PIK-93 inhibits PI4KB, but has also been shown to inhibit subunits of PI3K, and was therefore used here as a potential PI3K inhibitor. The triple combination significantly induced cell death as assayed with the fixed propidium iodide assay in the MCF-7 cell line (Figure 19). In MDA-MB-231 cells, it appears as though the triple combination may not provide much more benefit than pairing only PIK-93 and fluvastatin.

In sum, our pilot screen in the MCF10A cell line affirms the feasibility of using a high-content screening system to identify fluvastatin and kinase inhibitor drug combinations that induce a death-like phenotype. The primary goals of this screen were to establish screening parameters and evaluate the utility of our selected positive and negative controls for scoring potential hits. Our screen results indicate these goals have been met, as external controls such as staurosporine and thapsigargin, typical apoptosis-inducing tools, were categorized into the death-phenotype group. The lack of validation of one hit, everolimus, suggests that additional screen replicates may be necessary to limit false negative hits that progress to validation studies.

2.2.3 Screening in parallel with an MTS assay in A549 cells

Following completion of the pilot screen, a screen aimed at identifying cytotoxic fluvastatin and kinase inhibitor combinations in A549 lung adenocarcinoma cells was developed and carried out. This screen made use of two concentrations of the drug library: 0.1µM and 1µM, and again included two time points: 48 hours and 72 hours. The later time point is consistent with previous
Figure 17. ER stress response is not evident in MDA-MB-231 cells upon fluvastatin treatment (Previous page)
The mRNA expression of six genes was measured by q-RT PCR after treatment with two doses of fluvastatin or solvent. Thapsigargin (Tg) was used as an ER stress positive control. Fluvastatin treatment at 0.5µM and 1µM for 16 and 24 hours did not consistently increase mRNA expression of ATF4, BiP, CHOP, ErD4 and GADD34. Fluvastatin treatment also does not consistently trigger XBP1 mRNA splicing. mRNA levels are normalized to expression of GAPDH, and also normalized to solvent control. By contrast, the ER stress positive control, Tg, increases mRNA expression across all genes except for ATF4 and BiP, and promotes XBP1 mRNA splicing. All graphs show results from one replicate. No further replicates were carried out.

Figure 18. ER stress response genes, BiP and CHOP are not elevated by fluvastatin in MCF-7 cells
The mRNA expression of BiP and CHOP was measured by q-RT PCR after treatment with two doses of fluvastatin or solvent. Thapsigargin (Tg) was used as an ER stress positive control. Fluvastatin treatment at 10µM and 50µM for 4, 16 and 24 hours did not consistently increase mRNA expression of these two genes. mRNA levels shown were normalized to expression of glyceraldehyde 3-phosphate dehydrogenase (GAPDH), and also normalized to solvent control. Tg, increases mRNA expression across both genes at both time points. All graphs show results from one replicate. No further replicates were carried out.
Figure 19. Treating breast cancer cell lines with fluvastatin, PIK-93 and everolimus in combination increases cell death over each treatment alone
MDA-MB-231 and MCF-7 cells were treated with fluvastatin, PIK-93 and everolimus alone or in combination as indicated for 72 hours. Each compound on its own induces low levels of cell death detected by the fixed propidium iodide assay. Fluvastatin and PIK-93, as well as all three compounds in combination have a trend towards increased cell death in MDA-MB-231 cells (A). All three compounds in combination significantly increase cell death compared to each treatment alone in MCF-7 cells (B). Bars represent the mean with standard deviation of three independent replicates. * = p <0.05, unpaired t-test, two-tailed for the combination treatments against each compound alone.

F = fluvastatin
P = PIK-93
Evero, E = everolimus
experiments in this relatively fluvastatin-insensitive cell line and prolonged drug exposure may increase our number of candidate hits. Importantly, in addition to our high-content imaging endpoint, the MTS assay was included as a second, parallel endpoint. This colourimetric assay is a conventional drug screening endpoint. Having a parallel endpoint allows for cross-platform comparison, which will assist in determining if high-content screening with a small-molecule inhibitor library in this context is at least as useful, or potentially more useful than standard drug screening approaches. A workflow schematic can be found in Figure 20A.

Figure 20. Workflow and parameters for the kinase inhibitor library screen with A549 cells
A549 cells were plated in 384-well format, and treated with 0.1μM and 1μM of the kinase inhibitor library with or without fluvastatin for 48 or 72 hours. At endpoints, confocal images were taken using the OPERA® High Content Screening System, and an MTS assay was performed. An ‘alive-phenotype negative control’ and a ‘death-phenotype positive control’ were defined for data classification in the high content screening platform.
2.2.3.1 Parameter optimization for parallel screens

To begin, the MTT<sub>20</sub> dose, or the dose at which cell viability is reduced by 20%, was determined for fluvastatin. A549 cells were treated with a range of fluvastatin concentrations for 72 hours, and the MTT<sub>20</sub> dose was calculated from resulting dose-response curves. Three replicate dose-response curves gave a MTT<sub>20</sub> value of 5μM for this cell line (Figure 21), and so this concentration was selected as the fluvastatin-only dose for both screening platforms. The negative control representing the ‘alive-phenotype’ for the high-content screen was therefore 5μM fluvastatin plus DMSO vehicle control.

![Graph](image)

**Figure 21. Optimizing a low dose of fluvastatin to use in the A549 screen**

A549 cells were treated with a range of fluvastatin concentrations to generate dose-response curves (A) from which the MTT<sub>50</sub> concentration and slope were used to calculate the MTT<sub>20</sub> dose of fluvastatin to use in the screen (B). Three independent replicates were completed and the mean for each concentration with standard deviation is plotted in (A). A conservative dose choice of 5μM was selected.

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<thead>
<tr>
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<th>Slope</th>
<th>MTT&lt;sub&gt;20&lt;/sub&gt; (μM)</th>
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</tr>
<tr>
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<tr>
<td><strong>Average MTT&lt;sub&gt;20&lt;/sub&gt;</strong></td>
<td></td>
<td><strong>6.52</strong></td>
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</tbody>
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Given that PIK-93 and fluvastatin together reduce cell viability and induce cell death in A549 cells after 72 hours (Figure 6A,E from 2.1), a combination of PIK-93 and fluvastatin was to be used as the positive control for this screen. In addition to serving as the ‘death-phenotype positive control’, it also serves as a ‘combination treatment control’, whereby each compound alone does not induce a death-phenotype, but the combination does. To determine an appropriate dose of PIK-93 to combine with the optimized 5μM dose of fluvastatin, A549 cells were treated with three concentrations of PIK-93 (1μM, 5μM and 10μM) with and without 5μM fluvastatin.
At 72 hours, cell death was assessed using the fixed propidium iodide assay for all treatment groups. All three concentrations of PIK-93 in combination with 5μM fluvastatin are able to significantly increase cell death compared to 5μM fluvastatin alone (Figure 22). All three combinations were included in the screen, with the intention of selecting whichever combination performed as the best classifier for image classification as the ‘death-phenotype positive control’ (see 2.2.3.3). Further, as A549 cells showed a dose-dependent response to the combination treatment, all three concentrations plus a lower dose of 0.1μM were included in the screen to allow for future exploration of dose-dependent phenotypic changes. The external 0.1μM and 1μM doses of PIK-93 will also be compared to the internal PIK-93 compound within the kinase inhibitor library, which will be dosed at 0.1μM and 1μM. Doses of PIK-93 and fluvastatin were optimized for the 72 hour time point, and were used for both the 48 and 72 hour time points.

![Figure 22](image)

**Figure 22. Fluvastatin and a range of PIK-93 concentrations in combination can cause cell death in A549 cells**

A549 cells were treated with PIK-93 with and without the MTT$_{20}$ dose of fluvastatin determined in Figure 21. PIK-93 at all three concentrations, 1μM, 5μM and 10μM in combination with fluvastatin can increase cell death significantly over fluvastatin treatment alone. Bars represent averages plotted with standard deviation for three independent replicates. *=p<0.05, unpaired, two-tailed t-test.
The four PIK-93 concentrations plus or minus 5μM fluvastatin were also assessed using the MTT assay to forecast whether this pair of compounds would be defined as a hit in the parallel MTS screening platform. A549 cells were treated with PIK-93 and fluvastatin alone and in combination, and cell viability was significantly reduced by the combinations involving PIK-93 at 1μM and 5μM compared to 5μM fluvastatin alone (Figure 23). Since we know that PIK-93 does interact with fluvastatin to cause death in this cancer cell line at 1μM, 5μM and 10μM (Figure 22), these results suggest that PIK-93 is likely to be masked as a false negative in the MTS screen results at least for 10μM PIK-93. The high-content platform may comparatively have fewer false negatives. Of note, the combination of PIK-93 and fluvastatin is not a positive control for the MTS platform and is not needed for data analysis. The MTS plates have media-only, solvent-only and cells-only controls necessary for data analysis. A summary of the parameters for the A549 screen can be found in Figure 20B.

![Figure 23. PIK-93 and fluvastatin in combination can decrease MTT activity in A549 cells](image)

A549 cells were treated with a range of PIK-93 concentrations with and without the low dose of fluvastatin determined in Figure 21. Combination treatments decrease MTT activity significantly more than PIK-93 alone for 1.25μM and 5μM, suggesting that at these concentrations, PIK-93 would be captured as a potentiator of fluvastatin-induced antiproliferative effects. Average MTT activity is plotted with standard deviation for three independent replicates. *=p<0.05, unpaired, two-tailed t-test.
2.2.3.2 Treatment plate set-up

To reiterate, A549 cells were treated with the kinase inhibitor library at 0.1μM and 1μM, in combination with either 5μM fluvastatin or ethanol vehicle control. Each inhibitor is therefore assessed under four different conditions. The inhibitor library consisting of 320 inhibitors is supplied in four separate 96-well plates, with 80 inhibitors per plate (Figure 24A). Each set of 80 compounds was assayed on a single 384-well treatment plate. The four subsets of the library are denoted as KI1, KI2, KI3 and KI4 (for Kinase Inhibitor subset 1, etc.). All four conditions per kinase were assessed on the same 384-well treatment plate in an effort to avoid inter-plate variability effects (Figure 24B). Each treatment condition was assayed in a single treatment well. Although additional replicates per treatment would be ideal, we were limited by technical time constraints with the use of the OPERA High-content Screening System. In total, there are four treatment plates per time point per endpoint, and 16 plates total for this screen (Figure 24C). Controls were arrayed on each plate, shown in Figure 24B.

2.2.3.3 High-content screen platform analysis: Feature extraction and image classification

At each treatment endpoint, similar to the pilot screen, DRAQ5 and MK1 (described above) were added to each well to visualize the cells. Cells were incubated with these dyes for 30 minutes prior to image acquisition. Collected images were segmented, and features were extracted from identified cells with the Acapella software as described above. Supervised feature reduction and data classification was again carried out using the MatLab software and a customised graphical user interface (GUI), using two classifiers.

The two classification bins were again the ‘alive-phenotype negative control’ (5μM fluvastatin plus DMSO vehicle control) and the ‘death-phenotype positive control’ (5μM fluvastatin plus PIK-93) (Figure 25). All four concentrations of PIK-93 in combination with 5μM fluvastatin were assessed for performance as a ‘death-phenotype positive control’. PIK-93 at 5μM plus 5μM fluvastatin was selected as the best-performing classifier. This was determined by computing a rough representation of how distinct each potential positive control was from the defined negative control using principle component analysis (PCA). These graphs were an integrative part of the MatLab GUI.
The kinase inhibitor library is supplied in four 96-well plates, each of which contains 80 compounds (A). Each set of 80 compounds is plated at 0.1µM and 1µM with ethanol or fluvastatin on a single 384-well plate to avoid inter-plate variability within the evaluation of a single compound. Each treatment condition is evaluated in a single well, and treatment distribution is illustrated simplistically in (A), but treatments are plated in a grid-like fashion to accommodate pipetting equipment (B). Controls were distributed along the sides of the plates, and query kinase inhibitor treatments were distributed throughout the middle of the plates. The 80 compounds are coded from A02 through to H11 (B). DMSO negative control, the four doses of PIK-93, no treatment control (NONE) and a high dose of fluvastatin at 30µM (FS30) are shown. Following this dosing scheme, four plates were plated per time point for two screening endpoints for a total of 16 plates (C).

Figure 25. Representative images of A549 cells under control treatment conditions
Fluvastatin 5µM plus DMSO was used as the alive-phenotype negative control (top right), and fluvastatin 5µM plus PIK-93 5µM was used as the dead-phenotype positive control (bottom right) for feature reduction and subsequent image classification for all treatments in the kinase inhibitor screen. Representative images of cells in the solvent control (top left) and PIK-93 with ethanol (bottom left) treatment groups show that cells under these treatments closely represent the negative control. DRAQ5, a fluorescent DNA-binding dye is used to visualize the nuclei, and is shown in pseudoblue. Mutachrome 1, a fluorescent membrane dye is shown in yellow. Cell membrane staining is comparable between the solvent control and the negative control, but is quite different for the positive control.
After defining our two training groups, supervised feature reduction and classification were performed for each treatment plate separately. Data for the 72 hour time point is described. The features used for classification of each of the four library subsets differed by plate (Figure 26). Classification of negative controls was consistent over all four plates, as about 80% of cells in the negative control group (5µM fluvastatin and DMSO) were classified into the negative control bin, as expected (Figure 27). By contrast, classification of our positive controls was less optimal. While approximately 90% of cells exposed to the positive control treatment classify into the positive control group for KI1 and KI3, this is true for only about 50% of cells on the KI2 and KI4 plates (Figure 27). Different classifiers should be explored for these latter plates. Classification of all cells exposed to the range of PIK-93 and fluvastatin combinations reveals a dose-dependent reduction of cells with an ‘alive-phenotype’ (Figure 28). In other words, this data suggests dose-dependent death from exposure to PIK-93 and fluvastatin.

Cells exposed to each of the 320 query compounds in both the fluvastatin and ethanol conditions were also classified into either the positive control or negative control groups. Classification results for the two library concentrations are shown separately for each of the four subsets in Figures 29). We are again interested in compounds in the bottom right corner of plots in Figure 29, which represent kinase inhibitors that kill minimally alone, but robustly in combination with fluvastatin. To define hits for this screen, we imposed two inclusion criteria. The first criterion is that less than 25% of cells treated with kinase inhibitor alone classify into the positive control group. The second criterion is that cells treated with kinase inhibitor and fluvastatin have a “percent classified as positive control” value at least 1.5 standard deviations from the negative control. With these criteria, there are 20 putative kinase inhibitors that interact with fluvastatin to induce a ‘death-like’ phenotype (Table 6, Figure 30).

Figure 26. Features used for object classification in the A549 screen at the 72 hour time point (next page)
Feature reduction for the defined negative and positive control groups (5µM fluvastatin plus solvent control, and 5µM fluvastatin plus 5µM PIK-93, respectively) was completed separately for each plate (subsets KI1-4). Stepwise discriminant analysis (SDA) was used for KI1 (A) and KI2 (B), and KS was used for KI3 (C) and KI4 (D). A mix of nuclear and membrane features is represented for all plates except KI2, which includes only two membrane features. The list of features used for KI1 and KI3 include more nuclear features. The mean z-score ± standard deviation is plotted for each feature compared to all features extracted.
Figure 27. Classification of cells in control treatments for the A549 kinase inhibitor screen at 72 hours

Cells from each treatment were classified into either the alive-phenotype negative control group or the dead-phenotype positive control group based on features in Figure 26. Cells treated with the negative control (fluvastatin and DMSO) classify into the negative control group at least 80% of the time across all four treatment plates. Cells treated with the positive control (fluvastatin and PIK-93) yields cells that classify into the positive control group over 90% of the time for KI1 (A) and KI3 (C), but only about 50% of the time for KI2 (B) and KI4 (D).
Figure 28. Classification of PIK-93 treatments for the A549 kinase inhibitor screen at 72 hours

A dose dependent reduction in cell viability is suggested by our classification results for both PIK-93 treatment alone and combination treatment with fluvastatin for all four treatment plates. KI1 and KI3 show greater reduction in cell viability for the positive control treatment (fluvastatin + PIK-93 at 5μM, third bar from the right on each graph).
Figure 29. Classification of kinase inhibitor treatments in the ethanol and fluvastatin conditions at 72 hours in the A549 screen (this page and next)

All cells exposed to the kinase inhibitor treatments in combination with ethanol or fluvastatin were classified into either the alive-phenotype negative control group or the dead-phenotype positive control group based on the features used in Figure 26. Classification results are shown for the 72 hour time point for 0.1µM (A) and 1µM (B, next page) of the inhibitor library. The greater the number of cells classifying into the death-phenotype positive control group for any given treatment reflects a more cytotoxic treatment. Kinase inhibitors that putatively potentiate fluvastatin-induced cell death will have two characteristics. First, they will have a low percentage of cells classifying into the positive control group when used as a single-agent (i.e. close to 0% on the y-axis). Second, they will have a percentage of cells classifying into the positive control group that is close to the positive classifier (PIK-93 5µM) when used in combination with fluvastatin (i.e. close to the x-value of the positive control [blue dots]). The negative and positive controls, DMSO and PIK-93 5µM, respectively, are highlighted for each plate.
Figure 30. Two criteria for first-pass analysis of high-content screen data to define kinase inhibitors as hits
Kinase inhibitors had to meet two criteria to be defined as a hit.
Table 6. Hits from the kinase inhibitor library high-content imaging screen in the A549 cell line

Twenty inhibitors were defined as hits from the high-content imaging screen in the A549 cell line. Cells were treated with the kinase inhibitor library (1 µM dose shown only) with or without fluvastatin, and imaged after 72 ± 4 hours. DRAQ5 and MK1 dyes were used to visualize the cells for imaging using the OPERA® high content screening platform. Treatment with these inhibitors plus ethanol causes less than 25 percent of cells to classify into the death-phenotype positive control group, implying minimal cell death caused by each inhibitor alone (column 4, “kinase inhibitor + ethanol”). Treatment with these inhibitors plus a sublethal dose of fluvastatin leads to a percentage of cells classifying into the death-phenotype positive control group at least 1.5 standard deviations from the negative control treatment (sublethal fluvastatin + DMSO), implying a large degree of cell death caused by inhibitor plus fluvastatin treatments (last column).
2.2.3.4 MTS platform analysis

To analyse the MTS screen data, a generalize two-way ANOVA was used to determine which kinase inhibitor and fluvastatin combinations reduced cell viability significantly more than each compound alone (Figure 31). In this way, each kinase inhibitor was assigned a p-value, where p less than 0.05 indicates a kinase inhibitor that putatively potentiates the antiproliferative effects of fluvastatin. Using this mathematical model, 25 hits were identified (Table 7). Two of the hits were defined as significant at both library concentrations.

![Viability \( \alpha \) fluvastatin * kinase inhibitor]

\[
y = a_0 + a_1x_1 + a_2x_2 + a_3x_1x_2
\]

DMSO effect  Fluvastatin effect  Inhibitor:fluvastatin interaction

\[X_1, X_2 = \text{indicator or “dummy” variables}
\]
\[\text{If no drug, } X_i = 0
\]
\[\text{If drug, } X_i = 1
\]

Figure 31. Two-way ANOVA for analysis of MTS data

A two-way ANOVA was used to determine whether any given kinase inhibitor in combination with fluvastatin decreased cell viability more than each compound alone. p<0.05 indicated kinase inhibitors that putatively interact with fluvastatin to trigger a dead-phenotype.
Table 7. Hits from the kinase inhibitor library MTS screen in the A549 cell line

Twenty-five inhibitors were defined as hits from the MTS screen in the A549 cell line. Cells were treated with the kinase inhibitor library (0.1µM or 1µM) with or without fluvastatin for 72 hours, at which time MTS reagent was added. Absorbance values were determined using a spectrophotometer after 1 hour incubation with the reagent. To determine if the kinase inhibitor plus fluvastatin combination treatment reduced MTS activity significantly more than kinase inhibitor treatment alone and fluvastatin treatment alone, two-way ANOVA calculations were conducted. Separate calculations were performed for each inhibitor at each concentration of the kinase inhibitor library. Inhibitors for which the combination treatment absorbance reading was significantly lower (p<0.05) than inhibitor alone and fluvastatin alone readings were defined as hits. There are 25 hits in total. Two of the 25 hits were significant at both concentrations of the kinase inhibitor library.
2.2.3.5 Comparing platforms

To compare the MTS assay and the high-content screening results, the candidate kinase inhibitors that interact with fluvastatin to reduce cell viability or induce a death-like phenotype in each respective screen, were compared. A total of 8 kinase inhibitors overlapped between the two screening platform, leaving 17 independent MTS-platform hits and 12 independent HCS-platform hits (Figure 32, Table 8). Classifying the MTS-platform and HCS-platform hits into broad categories reveals similarities between the two platforms (Figure 33). Although the two platforms yielded distinct hits, the percent composition of the pathways these hits target are very similar. We also determined that 2 of the 22 predicted hits based on the shRNA screen were hits in the kinase inhibitor library screen (Table 9).

![Venn diagram showing overlap between MTS Hits, HCS Hits, 17, 8, 12](image)

**Figure 32. Overlap between the MTS screen hits and high-content screen hits**

Between the 25 MTS screen hits and 20 high-content imaging hits, 8 overlap. These were prioritized for validation. Additional high-content screening hits may be revealed as parameters for data analysis are optimized.
Table 8. Overlapping hits from the kinase inhibitor screen in the A549 cell line between the high-content imaging platform and the MTS platform

Of the 20 high-content imaging hits and the 25 MTS hits, 8 overlap. These inhibitors predominately target cell cycle kinases and receptor tyrosine kinases.

![Figure 33. Composition of hits from the MTS screen and high-content screen](image-url)

Although each screen has independent hits, the categorization of hits is quite similar between screens. This indicates that the two screens are not biased towards a specific type of kinase inhibitor.
The MTT assay was selected as a first-pass platform to validate kinase inhibitors hits. The focus for validation began with the eight overlapping hits from both screening platforms. Of these eight hits, two were found to be significant at both concentrations of the inhibitor library in the MTS platform. These are AG1296, targeting platelet derived growth factor receptor (PDGFR) and BAY, targeting spleen tyrosine kinase (SYK). Efforts were focused on validating these compounds, followed by the validation of the remaining six overlapping hits, and will be expanded to the 17 MTS-platform only hits. For validation, a sublethal dose of each inhibitor was determined in A549 cells. Cells were then treated with a range of fluvastatin concentrations with and without the sublethal dose of each kinase inhibitor to determine if each inhibitor could decrease the $MTT_{50}$ concentration for fluvastatin. Preliminary results show that AG1296 significantly decreases the $MTT_{50}$ for fluvastatin, and BAY trends towards decreasing the $MTT_{50}$

Table 9. Overlapping hits between the shRNA dropout screen and the kinase inhibitor library screen.

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<tr>
<th>shRNA Screen Hit</th>
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<th>MTS Results</th>
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<td>Inhibitor + Fluvastatin</td>
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2.2.4 Candidate drug combination validation using the MTT assay

The MTT assay was selected as a first-pass platform to validate kinase inhibitors hits. The focus for validation began with the eight overlapping hits from both screening platforms. Of these eight hits, two were found to be significant at both concentrations of the inhibitor library in the MTS platform. These are AG1296, targeting platelet derived growth factor receptor (PDGFR) and BAY, targeting spleen tyrosine kinase (SYK). Efforts were focused on validating these compounds, followed by the validation of the remaining six overlapping hits, and will be expanded to the 17 MTS-platform only hits. For validation, a sublethal dose of each inhibitor was determined in A549 cells. Cells were then treated with a range of fluvastatin concentrations with and without the sublethal dose of each kinase inhibitor to determine if each inhibitor could decrease the $MTT_{50}$ concentration for fluvastatin. Preliminary results show that AG1296 significantly decreases the $MTT_{50}$ for fluvastatin, and BAY trends towards decreasing the $MTT_{50}$.
for fluvastatin (Figure 34). Representative images of the eight overlapping kinase inhibitors in the ethanol and fluvastatin conditions taken with the OPERA® are shown in Figure 35. There are striking differences between the ethanol and fluvastatin conditions for all eight inhibitors.

Figure 34. Validation of overlapping hits from the MTS and high-content screen
To begin to validate the 2 overlapping hits that were significant at both library concentrations in the MTS screen, the highest dose of each inhibitor that does not cause a reduction in cell viability in the MTT assay was found. Fluvastatin dose-response curves were then generated with and without the low dose of each kinase inhibitor. Kinase inhibitors that potentiate the antiproliferative effects of fluvastatin are expected to lower the MTT50 concentration compared to fluvastatin alone. AG1296 significantly lowers the MTT50, and BAY shows a trend towards lowering MTT50.
Figure 35. Representative images of the eight overlapping hits from the kinase inhibitor screen taken with the OPERA® high content screening system (this page and next)

Each kinase inhibitor was treated in combination with fluvastatin or with ethanol vehicle control. By eye, for all eight overlapping inhibitors, the kinase inhibitor plus fluvastatin treatment leads to lowered cell count and disruptions to the cell membrane, which are not seen in the kinase inhibitor with ethanol condition. DRAQ5, a fluorescent DNA-binding dye is used to visualize the nuclei, and is shown in pseudoblue. Mutachrome 1, a fluorescent membrane dye is shown in yellow.
Chapter 3
Discussion

3 Discussion

Statins are under investigation as single-agents and in combination therapy settings for their utility as anti-cancer therapies. As with any other treatment, statins are likely to have greater efficacy when combined with other agents. To determine which agents to combine statins with to maximize clinical benefit, we made use of two screening libraries, one genomic and one small-molecule, and three screening platforms.

3.1 Validating candidate shRNAs targeting kinases

A shRNA dropout screen was carried out with the 80K shRNA library from The RNAi Consortium in the A549 cell line. Cells transduced with the pooled lentiviral library were treated with fluvastatin, and shRNA dropout in response to fluvastatin treatment was assessed over time. Within our definition of a hit, there were 151 putative genes that, when knocked down, sensitize A549 cells to the antiproliferative effects of fluvastatin. Twenty-two of these hits are kinases (Table 2), a class of targets we selected to validate based on druggability. To begin, three kinase targets were selected for validation: PI4KB, CSNK2B and MAP2K4.

On-target gene knockdown was tested for the top two shRNAs from the screen for each of the three kinases. A549 cells were transduced with each shRNA independently to generate A549 sh-sublines. Protein was harvested from these sublines for immunoblotting. Both shRNAs targeted to MAP2K4 were able to knock down gene expression at the protein level, and one of two shRNAs targeted to PI4KB and CSNK2B led to robust protein expression knockdown. Lack of knockdown by the second of two shRNAs may indicate that non-specific binding caused potentiation of fluvastatin-induced antiproliferative effects during the screen, or that the one on-target shRNA was robust enough to render the gene target a ‘hit’ within our statistical definitions despite low on-target knockdown by the second shRNA. Further, some immunoblots were carried out with later passages of the sublines, which may have enabled the activation of adaptive responses to overcome gene knockdown, or for the selection of cells with low-level gene knockdown. As such, we have begun to use siRNAs targeted to our selected kinases to
complement this body of work. The transient nature of siRNAs may help to prevent adaptive responses that occur with shRNA knockdown. All subsequent validation work should be repeated with the siRNA systems to improve our data interpretation, which is being performed by another member of the lab.

In parallel with immunoblotting for protein expression knockdown, we also assessed if gene knockdown by shRNAs in the six validation sublines sensitizes cells to fluvastatin-induced cell death. Using the percent of cells in the pre-G1 subpopulation as a proxy for cell death, as determined by staining fixed cells with propidium iodide and assaying DNA content by flow cytometry, we demonstrated significant increase in cell death upon fluvastatin treatment for one subline for each of the three kinase targets (Figure 5). We expected that both shMAP2K4-1 and shMAP2K4-2 sublines would undergo cell death given robust knockdown of MAP2K4 in both sublines, but only one did. The subline that did not undergo cell death may have compensated for the knockdown, thereby reversing the predicted sensitization to fluvastatin-induced cell death. Further, we anticipated that shCSNK2B-2 and shPI4KB-1 would undergo cell death upon fluvastatin treatment since robust knockdown was seen in these two sublines. Results were as expected for shCSNK2B-2, but surprisingly, shPI4KB-1 did not undergo cell death, while shPI4KB-2, which showed low protein knockdown, did. We can hypothesize that because the fixed propidium iodide assays were performed before the immunoblots for this cell line, that cell death seen in shPI4KB-2 in Figure 5 represents cells that initially had robust PI4KB knockdown. Cells with low-level knockdown may have out-competed these dying cells, and the selective advantage would result in a pool of cells with low-level protein knockdown as observed for shPI4KB-2 in Figure 4. Due to disparate knockdown between sublines, additional experiments with different shRNAs, or with siRNAs as mentioned will be needed to confirm that knockdown of each of these kinases sensitizes to fluvastatin-induced cell death. To give more confidence to the evidence so far suggesting that knocking down each of these kinases can potentiate fluvastatin-induced cell death, we took a pharmacological approach, anticipating that these inhibitors would mimic their corresponding shRNA molecules from the shRNA screen.

PIK-93 was selected as an inhibitor of PI4KB. We found that the combination of fluvastatin and PIK-93 was able to lower the MTT50 concentration of fluvastatin in both A549s and MDA-MB-231s, which supports the notion that knocking down PI4KB potentiates the antiproliferative properties of fluvastatin (Figure 6A-B). Further, we demonstrated that this combination
increases the pre-G1 subpopulation in both cell lines, and significantly in MDA-MB-231 cells, showing that PIK-93 is able to potentiate fluvastatin-induced cell death (Figure 6E-F). Of note, while PIK-93 is a potent inhibitor of PI4KB, and is selective for this beta subunit of PI4K at low concentrations, PIK-93 is also able to inhibit PI3K α, β and γ at varying concentrations. As such, we cannot conclude that the potentiation of antiproliferation caused by PIK-93 is attributable to PI4KB knockdown. However, the shRNA and pharmacological inhibitor results together increase confidence that PI4KB knockdown sensitizes cells to fluvastatin. To determine which PI3K and PI4K subunits are inhibited by PIK-93 at the concentrations we are using, immunoblotting with a panel of both PI3K- and PI4K-subunit specific antibodies should be carried out.

Taken together, pursuing the knockdown of PI4KB looks promising. PI4KB has received increased attention over recent years, as discoveries of its potential roles in cancer begin to emerge. These implicated roles in cancer support PI4KB knockdown as a potential anticancer strategy. This gene has been found to lie in an amplification hotspot in breast cancers and has been documented to disrupt mammary acinar formation, which is characteristic of breast oncogenes. This is particularly relevant to our results for the MDA-MB-231 breast cancer cell line where knockdown of this potential oncogene induces cancer cell death in the presence of fluvastatin. Disrupted vesicular trafficking caused by overexpression of PI4KB may underlie the oncogenic potential of this gene. Further, PI4KB is directly activated by eukaryotic protein translation elongation factor 1 alpha 2 (eEF1A2), a putative oncogene that is overexpressed in lung, breast and ovarian cancers. These studies, combined with our results so far, warrant further interrogation of PI4KB as a target to knockdown in combination with fluvastatin treatment.

TBB was selected as a selective inhibitor for CSNK2B. Our results show that TBB is able to decrease the MTT concentration of fluvastatin in both A549 and MDA-MB-231 cells, although significance is only achieved in the latter cell line (Figure 6C-D). This suggests that knocking down CSNK2B sensitizes these cells to the antiproliferative effects of fluvastatin. While TBB is considered a highly selective inhibitor, it has also been shown to inhibit pim-1 oncogene (PIM1) and pim-2 oncogene (PIM2), so selectivity of TBB at the concentrations used in the cell lines studied should be tested. Cell death was not studied for this combination treatment. Further study of the knockdown of CSNK2B is also warranted given that is has been linked to the promotion of...
of cell proliferation in endometrial\textsuperscript{102} and esophageal\textsuperscript{103} cancers, to metastasis in colorectal cancer\textsuperscript{104}, and to poor prognosis in gastric cancers\textsuperscript{105}. TBB has previously been shown to reduce cell viability, increase cell death, and inhibit survivin expression in colon and breast cancer cell lines, which counteracts survival-promotion by increased casein kinase 2 (CK2) expression\textsuperscript{106}. A pharmacological approach to inhibit MAP2K4 has not yet been taken. While some studies have characterized MAP2K4 with putative tumour suppressive functions in lung adenocarcinoma\textsuperscript{107} and ovarian cancers\textsuperscript{108}, another study suggests that MAP2K4 has an oncogenic role in breast and pancreatic cancers\textsuperscript{109}. Additional studies are required to determine if knocking down MAP2K4 with or without fluvastatin is an effective anti-cancer strategy.

Overall, validation of our screen hits is promising. Given emerging roles for each of these three kinases in cancer cell growth, proliferation and metastasis, studying their knockdown is highly relevant. We hope that pairing their knockdown with fluvastatin treatment will lead to the identification of impactful co-therapies for cancer treatment.

3.2 Kinase inhibitor library screen

Given the intriguing validation results for the shRNA screen, we were interested in discovering additional kinases whose inhibition augments the anticancer effects of fluvastatin. The two pharmacological inhibitors used in Chapter 2, PIK-93 and TBB, are tool compounds that have not been tested for human use. Pharmacological information of these inhibitors, including their metabolism, absorption, excretion and toxicity profiles need to be well characterized and optimized before use in humans is feasible. With this knowledge, we were especially interested in finding FDA-approved kinase inhibitors for which safety profiles are already known. However, we also saw value in surveying non-approved kinase inhibitors, as this would help to create a global picture of which classes of kinase inhibitors are likely to synergize with fluvastatin to elicit cancer cell kill.

A library of 320 kinase inhibitors, including both approved and non-approved compounds, was screened against the A549 cell line to identify kinase inhibitors that interact with fluvastatin to elicit cancer cell death. Cells were plated in 384-well format and treated with two doses of the kinase inhibitor library with and without a low dose of fluvastatin, for a total of 1280 treatment conditions, referred to as query treatments. Fluorescence-based imaging with the OPERA®
High-content Screening System was selected as the screening endpoint assay. This screening system is known for its rapid, high-quality, multicolour confocal imaging. Cells were visualized with the use of two fluorescent dyes: a nuclear localizing dye called DRAQ5, and a proprietary membrane-localizing dye called MK1. Images were taken for six fields of view per well, and images were segmented using the Acapella® software to identify all recognizable cells, by creating a mask, or outline, of both the nucleus and cytoplasm. The features of each cell were then extracted and quantified, such that every feature of a cell is represented by a numerical value. Features include the morphology, texture and dye intensity of both the nucleus and cytoplasm, and up to 785 features are extracted per cell, which can be used to group cells based on similarities and differences. A single feature, a set of known features, or all features can be used for this categorization or ‘classification’ process.

For supervised classification, which was used for this screen, carefully selected control groups must be designated and are ideally phenotypically distinct. Classification forces all objects of interest, or cells in the context of this screen, to be categorized into one of the designated control groups. As such, the more distinct the controls are, the more meaningful the classification results will be. Two control groups were designated for the purposes of this screen. The ‘alive-phenotype negative control’ was defined as cells treated with a sublethal dose of fluvastatin with DMSO vehicle, and the ‘death-phenotype positive control’ was defined as cells treated with a sublethal dose of fluvastatin treatment with PIK-93, a drug combination studied in Chapter 2. Using these controls, every cell exposed to each of the 1280 query treatments is computationally decided as being more phenotypically similar to one control or the other. In other words, each cell is classified as being more like the negative control, or “alive”, or more like the positive control, or “dead”. Any one treatment condition can be simply summarized as the percentage of cells that classified as either the negative or positive control. Essentially, this conveys the percent of alive or dead cells after exposure to a particular treatment. We are interested in the kinase inhibitors for which cells classify as “alive” under inhibitor-only treatment, and in combination with fluvastatin, classify as “dead”.

Owing to the novelty of this screening platform, a proof-of-principle screen was completed in MCF10A cells to ensure that our screening parameters were practical, and to learn how to interpret the output from a phenotypic screen. The classification results from the MCF10A screen illustrated that the positive and negative controls selected are in fact phenotypically
distinct (Figure 12 and Figure 13), providing confidence to our control selection. Classification of the rest of the screening data under our definitions of a candidate compound yielded 13 inhibitors from the 24 hour time point and 9 inhibitors from the 48 hour time point. Most of the treatment conditions did not have the desired death phenotype, which is to be expected (Figure 13). A large number of conditions yielding the death phenotype may indicate that the concentration of the compound library was too high, which would cause large-scale cytotoxicity. Our results confirm that our screening parameters are practical and give rise to interpretable results.

Validating drug combinations from this proof-of-concept screen may reveal high-content screening as a suitable platform for identifying fluvastatin co-therapies. In addition to expanding this screening strategy to cancer cell lines, as we have done with the A549 lung cancer cell line, we could also expand this screening platform to isogenic cell lines established with MCF10A cells. These isogenic cell lines would have overexpressed oncogenes or knocked down tumour suppressors representative of cancer-causing genomic alterations. Screening isogenic MCF10A cell lines against a compound library and fluvastatin may reveal synthetic lethal interactions with the genetic alteration introduced. Further, drug compounds or combinations of compounds that trigger cell death in isogenic MCF10As but not in unaltered MCF10As may have a desirable tumour-normal index. Screening strategies that combine genetic alterations and chemical compounds have already been successfully exploited to reveal resistance mechanisms to PI3K inhibitors\textsuperscript{110}, and therapeutic targets for MYC-driven cancers\textsuperscript{111}.

If treated as a stand-alone dataset, our results should be interpreted with caution. Although the MCF10A cell line is considered a ‘normal-like’ breast cell line, it has been characterized as cytogenetically abnormal, with p16 and p14ATF loci deletions, MYC amplifications and ER negative status\textsuperscript{112,113}. Further, introducing oncogenes into this cell line leads to transformation\textsuperscript{113}. As such, our results should be validated in both breast cancer cell lines and more normal-like breast cells, such as human mammary epithelial cells (HMECs) to derive biological value. Our hit drug combinations should only be pursued if they are found to have a tumour-normal index as demonstrated, for example, by the elicitation of cell death in breast cancer cell lines but not normal breast cells.
To begin to validate our potential hit combinations, we turned to one exciting family of hits that emerged from this pilot screen: the mTOR inhibitors. Rapamycin and two of its derivatives, temsirolimus (CCI-779) and everolimus (RAD001) were all defined as hits. mTOR mediates both cell cycle transition and the inhibition of apoptosis, and is widely studied as a target for anticancer research. Interestingly, everolimus was recently approved for the treatment of receptor positive HER2 negative breast cancers with letrozole. Additionally, the coadministration of everolimus and statins do not incite an adverse drug reaction and has been evaluated as safe in kidney transplant patients. These patients are prescribed everolimus, an immunosuppressant, for the prevention of organ rejection, and statins to manage elevated blood cholesterol that is common in transplant patients. Previous evaluation of the pharmacokinetic and pharmacodynamic properties of everolimus and statins together, and the approval of everolimus as an anticancer agent was intriguing. Further, we speculated that this combination could induce cancer cell apoptosis through each drug’s function in the ER stress response. The role of statins in activating the ER stress response has been well documented, and we speculated that this response may account for the failure of statins to elicit apoptosis as a monotherapy in some patient populations. mTOR inhibitors may block this ER stress response and discount its cytoprotective effects, and may thereby render cells susceptible to statin treatment.

To test this hypothesis, we first set out to verify that statins elicit the ER stress response in MCF10A cells. Cells were treated with two doses of fluvastatin, and mRNA expression of a panel of ER stress response genes was assessed by qRT-PCR. Verification of a triggered ER stress response would allow for subsequent demonstration that expression of the panel of stress genes is comparatively lower in the combined fluvastatin and everolimus treatment. The panel of stress genes selected are classically assessed for stress response. Five genes were anticipated to have increased mRNA expression following fluvastatin treatment. These genes were activating transcription factor 4 (ATF4), Heat shock 70kDa protein 5 (BiP/GRP78), DNA-damage-inducible transcript 3 (DDIT3/CHOP), DnaJ (Hsp40) homolog, subfamily B, member 9 (ERdj4) and protein phosphatase 1, regulatory subunit 15A (GADD34). We also assessed XBP1 splicing, by determining the ratio of unspliced to spliced XBP1 mRNA. Lower ratios are indicative of an ER stress response, because XBP1 mRNA is spliced under ER stress by endoplasmic reticulum to nucleus signalling 1 (ERN1/IRE1). Contradictory to our expectation,
fluvastatin treatment in MCF10A cells did not induce an ER stress response (Figure 15), nor was a stress response detected in MDA-MB-231 or MCF-7 cells (Figure 16, 17). Counter to other studies, a paper cited that statins reduce the ER stress response, which is implicated to protect against atherosclerosis\textsuperscript{120}. This indicates that the statin and stress response relationship may be more complex than initially anticipated and further studies may reveal circumstances under which this relationship is positive.

The kinase inhibitor library screen in A549 cells followed the same general workflow as the pilot screen in MCF10A cells. The major difference was the addition of a second parallel endpoint. In addition to imaging with a high-content screening system, the MTS cell viability assay was performed. The MTS assay is one of several convenient, commonly used colorimetric assays for measuring cell viability in drug screens. In particular, the MTS assay relies on active mitochondrial enzymes to convert a yellow tetrazolium salt into a purple formazan product, which can be measured using a spectrophotometer. This standard assay provides a benchmark of performance for our kinase inhibitor screen. We also hoped that this second endpoint would aid in data interpretation. The MTS assay is known to have high false negatives\textsuperscript{121}, and so we wanted to also assess whether high-content screening could reveal a greater number screen hits. The kinase inhibitor library screen in A549 cells included two concentrations of the compound library.

Classification results show that our positive and negative controls were distinct for plates 1 (K11) and 3 (K13), and less distinct for (K12) and 4 (K14) (Figure 27). Lower volumes of media or greater doses of fluvastatin may account for differences in cell viability for control treatments across plates, and highlights the issue of inter-plate variability. For the query compounds, a greater distribution of kinase inhibitors across the x-axes of Figure 29B is apparent for K11 and K13, as compared to K12 and K14, again suggesting that concentrations of fluvastatin varied between plates. The limited distribution of hits across the x-axes for the lower concentration of the drug library (Figure 29A) suggests that 0.1µM of the compound library was not sufficient to reveal candidate combinations. Perhaps 1µM and a higher concentration, such as 5µM, of the compound library would be more useful.

Under our criteria for defining candidate hits, the high-content screen revealed 20 putative kinase inhibitors that interact with fluvastatin to induce a death-phenotype, and inferentially, to cause
cell death. The thresholds for both criteria (maximum 25% kill by the kinase inhibitor alone, and minimum 1.5 standard deviations away from the negative control for the combination treatment) were arbitrarily set, and secondary screening should be undertaken to determine appropriate thresholds for hit definitions for our screen. Data classification should be repeated at least twice more to increase confidence in hit results. Supervised classification of our data involved randomly dividing the data for the positive and negative controls into training and prediction sets. Unrepresentative training sets, from which classifier models are generated, can skew classification results. Consequently, replicate classifications will allow for the identification and aversion of spurious output.

We anticipated that the depth of information provided by phenotypic data from the high-content platform would supersede the classic colorimetric MTS assay, and we aimed to explore how this additional information can be exploited to increase screening efficiency. Ideally, high-content screening would yield less false negatives, or have a higher rate of hit validation than the MTS assay. Data so far suggests that both platforms yield a similar number of hits: 20 candidate kinase inhibitors were identified with the high-content imaging platform, compared to 25 identified with the MTS screening platform. These raw numbers do not support the notion that the high-content screening platform reveals more hits compared to the MTS screen, which is known for high false negative hit rates. However, the classifier choice seems to be suboptimal for data from the plates encoded as KI2 and KI4 in the high-content screening platform (Figure 27). This may have masked hits from these plates. Indeed, there are fewer hits from these plates as compared to KI1 and KI3 (Table 6, third column). Further, the criteria used to define a hit for the imaging results remain flexible and arbitrary. As such, we cannot make definitive conclusions as to whether or not high-content screening yields more hits. Optimizing data analysis, including the exploration of additional classifiers, is required. Validation studies are needed to determine if high-content screening has a higher rate of hit validation.

Interestingly, there are eight hits that overlap between the MTS platform and high-content imaging platform (Figure 31, Table 8), and these were prioritized for validation. It will be interesting to determine if these eight overlapping hits have a greater rate of validation as inhibitors that potentiate fluvastatin-induced antiproliferative effects. If this is the case, parallel screens such as the one we have undertaken, or using high-content imaging for secondary screening following an MTS screening may prove beneficial in streamlining the validation
process for drug discovery. In essence, a cross-platform screening strategy such as ours may help to identify treatments that are more likely to validate, thus decreasing time and resources spent on validating false positives.

Of the eight overlapping kinase inhibitors, two were significant hits in the MTS platform at both concentrations of the drug library (Table 7), suggesting that these drugs are able to elicit cell death at a nanomolar concentration in the presence of fluvastatin. These two inhibitors are tyrphostin AG1296 (AG1296) and BAY 61-3606 (BAY). Validation efforts were focused on these two inhibitors, and will be expanded to the remaining six overlapping hits, and then to the remaining hits. AG1296 selectively inhibits platelet-derived growth factor receptor (PDGFR)\textsuperscript{122}, which drives breast cancer metastasis as well as ovarian and colon cancer\textsuperscript{123,124}. AG1296 has also been shown to inhibit the growth of small-cell lung cancer cell lines\textsuperscript{125}. BAY selectively inhibits spleen tyrosine kinase (SYK), which has been characterized as a tumour suppressor in breast cancer\textsuperscript{126}, but is largely unexplored in lung cancer. Representative images of cells exposed to kinase inhibitor alone or with fluvastatin for the eight overlapping kinase inhibitors look extremely promising (Figure 35). There is definite intraocular impact between the inhibitor only treatments and combination treatments, and we are eager to validate these combinations using additional cell death assays.

3.3 Future work

Validation results from the shRNA screen are promising. We have preliminary evidence to show that knocking down PI4KB, CSNK2B and MAP2K4 individually elicit cell death upon fluvastatin treatment, suggesting that these targets should be further pursued. As mentioned, additional genomic knockdown strategies should be undertaken to confirm our results, since only one of two hairpins per kinase in our validation experiments induce cell death in the presence of fluvastatin. Experiments with siRNAs targeted to PI4KB have begun, and will follow for the other two targets. A limitation to our results thus far is that specificity of the shRNAs and pharmacological inhibitors used has not been evaluated. shRNA specificity can be determined with the introduction of overexpression constructs, which should reverse fluvastatin-induced cell death if the shRNA is on-target. Further, to extend pharmacological inhibitor experiments and to overcome the potential promiscuous activity of PIK-93 and TBB, we can explore more specific inhibitors for PI4KB\textsuperscript{127} and CSNK2B\textsuperscript{101} which have been discovered in recent years. The
hairpins and pharmacological inhibitors can also be evaluated with fluvastatin in xenograft mouse models to begin to explore potential in vivo effects.

Of the 151 hits from the shRNA screen, three kinase targets were selected for validation. Three targets from another class of hits were also validated, as discussed in Appendix 1. Clearly, there are many additional targets to consider, included unexplored kinase hits (Table 2). Perhaps pathway analysis of the full list of hits will give better insight as to which targets or groups of targets should be inhibited alongside fluvastatin treatment. Surveying the literature highlights tank-binding kinase 1 (TBK1) as another interesting kinase target to pursue from the shRNA screen. A systemic genomic screen demonstrated that TBK1 is synthetic lethal with KRAS-driven cancers\textsuperscript{79}, which is modelled by the A549 cell line we used in our screen. Adding fluvastatin treatment to TBK1 knockdown in KRAS-driven cancers may trigger even more robust cancer cell death.

The kinase inhibitor screen results are equally interesting. The overlapping hits between our two screening platforms suggest that we have a set of robust hits to follow up on. The validity of these hits is also suggested by representative images of cells exposed to these kinase inhibitor and fluvastatin drug combinations (Figure 35). Validation of these hits is ongoing. The technical aspects of the screen deserve greater attention, as we continue to learn how best to analyze the wealth of phenotypic data collected from the screen. Although we have chosen an unbiased approach to identify features from our controls with which to classify all query treatments, we continue to search for the best classification strategy that will generate the most informative list of hits. Refining these strategies will ultimately lead to the development of an efficient drug discovery process.

### 3.4 Concluding Remarks

Through the use of genomic, small-molecule and high-content screening techniques, we have identified several potential kinase inhibitor and fluvastatin co-therapies. Kinases and statins both target cellular processes that are crucial to cell proliferation and survival, and combining these two classes of drugs as anticancer agents has enormous potential to impact patient care. Validation of hits from both the shRNA dropout screen and kinase inhibitor library screen are still in early stages. However, results to date and emerging evidence of cancer relevance for our
candidate genomic targets from the shRNA screen, and our candidate kinase inhibitors from the kinase inhibitor screen, warrant greater investigation and more in-depth validation of our hits. Through continued validation, we anticipate that our screening efforts will identify novel and effective anticancer drug cocktails.
Materials and Methods

4 Materials and Methods

4.1 Materials

All cell culture media contained phenol red, except for RPMI media used for LNCaP cells (Appendix 2) and was supplemented with antibiotics, as prepared by the Ontario Cancer Institute Tissue Culture Media Facility (Toronto, Canada). Fetal bovine serum (FBS) and Trypsin-EDTA were both purchased from Gibco. Fluvastatin was purchased from United States Biological and dissolved in ethanol. Thiazolyl blue tetrazolium bromide was purchased from Sigma-Aldrich.

The OICR Kinase Inhibitor Collection of 320 kinase inhibitors and Toolkit Collection of 160 compounds were obtained from the Drug and Development Platform at OICR by the Andrews Lab. DRAQ5 was purchased from LifeTechnologies. Mutachrome (MK1), the proprietary membrane dye is developed and made in-house by the Andrews lab.

4.2 Cell Culture

Cell lines were maintained at 37°C with 5% carbon dioxide in humidified incubators, and grown in monolayer. A549, MDA-MB-231, and MCF-7 cells were grown in DMEM H21 media, DU145 cells were grown in alpha-MEM media and LNCaP cells were grown in RPMI media (no phenol red). Cell culture media was supplemented with 10% FBS. All shRNA-induced sub-lines generated using A549 cell lines were maintained in the same way. The A549s stably transduced with the pooled TRC1 library in the shRNA screen was generated by the laboratory of Dr. Jason Moffat (Donnelley Centre, University of Toronto, Toronto, Canada).

MCF10A cells were cultured in DMEM H21/HAM F12 media supplemented with 5% horse serum (Gibco), 10μg/mL insulin (Sigma-Aldrich, I9278), 0.5μg/mL Hydrocortisone (Sigma-Aldrich, H-0888), 20ng/mL EGF (R&D Systems, 236-EG) and 0.1μg/mL cholera toxin (Sigma-Aldrich, C8052).
4.3 Lentiviral and shRNA subline generation

DNA was prepared using the Qiagen Plasmid Maxiprep kit and TRC1 library shRNA bacterial stocks obtained from Dr. Jason Moffat. TRC1 shRNA clones are listed below:

Kinases (shRNA subline ID, TRC Clone ID):
- shCSNK2B-1: TRCN0000003794
- shCSNK2B-2: TRCN0000003796
- shMAP2K4-1: TRCN0000001393
- shMAP2K4-2: TRCN0000039916
- shPI4KB-1: TRCN0000005695
- shPI4KB-2: TRCN0000005696

Mevalonate pathway genes (shRNA subline ID, TRC Clone ID):
- shGGPS1-1: TRCN0000045788
- shGGPS1-2: TRCN0000045790
- shHMGCS1-1: TRCN0000045843
- shHMGCS1-2: TRCN0000045845
- shSREBF2-1: TRCN0000020664
- shSREBF2-2: TRCN0000020667

Control shRNAs (shRNA subline ID, TRC Clone ID):
- shGFP-1: TRCN0000072181
- shLacZ-1: TRCN0000072235
- shLacZ-2: TRCN0000072240

293TV cells underwent calcium phosphate transfection to generate lentiviral particles. Cell culture media was replaced with 8.5mL of 25μM chloroquine in cell culture media. A solution of 10μg TRC pLKO.1-puro shRNA construct, 5μg pMDG1.vsvg, 5μg pRSV-Rev and 5μg pMDLg/pRRE construct DNA was combined with 375μL of 0.5M calcium chloride prepared in sterile distilled water, to a final volume of 750 μL. 750mL of 2X HEPES-buffered saline (2X HBS, 140 mM sodium chloride, 1.5 mM Na2HPO4, 50 mM HEPES, pH 7.0) was added dropwise and mixed with air bubbling with a pipettor, followed by a ten minute room temperature incubation. The 1.5mL mixture was added to the chloroquine-containing media already on culture plates, which were then incubated at 37 °C overnight. Transfection media was replaced 16 hours later with 5mL cell culture media. Virus-containing media was harvested 24 and 48 hours later, after filtration with a 0.45 μm filter.
Subconfluent A549 cells were infected with 2 mL of the pLKO.1-puro-based virus stocks described above, combined with 1 mL media, 8 μg/mL polybrene. Three hours later, 2mL of media were added to the plates. Media was changed the next day. The following day, cells were split into selection media with 2 μg/mL puromycin. Selection occurred for nine days. Cells were used immediately for pre-G1 DNA content assays and immunoblotting described below.

4.4 MTT Assays

Thiazolyl blue tetrazolium bromide was purchased from Sigma-Aldrich, and dissolved in PBS to a 5mg/mL stock concentration of MTT reagent to be stored at -4°C in a light sensitive container. Cells were seeded in 96-well tissue culture plates in 75μL of media. Seeding density was adjusted such that final cell populations were subconfluent at the time of MTT reagent addition. On the next day, cells were treated as described. For fluvastatin dose-response curves, A549s were treated with 200µM, and decreasing doses in 1:1 dilutions in cell culture media to 0.78µM. MDA-MB-231s were treated similarly, with 10µM as the highest dose. Media only controls and solvent controls were included. All treatments were delivered in triplicate to a total well volume of 150μL. Upon 72 hours treatment, 40µL of 5mg/mL MTT reagent dissolved in PBS was added to each well, and the plate was incubated for 3 hours, enabling the formation of the purple formazan product in wells containing viable cells. At the 3 hour end point, 80µL of 10% SDS with 0.01N hydrochloric acid (HCl) was added to each well. Plates were incubated overnight at 37°C allowing the formazan product to dissolve fully before absorbance readings. Absorbance of each well was measured using a SpectraMax M5 microplate reader (Molecular Devices) at 570nM. Media only wells served as 0% MTT activity controls and cell only wells served as 100% MTT activity controls. All sample wells were normalized to these values. Dose response curves were plotted using Prism (version 5.0, GraphPad Software), and half-maximal inhibitory concentrations (IC₅₀) values were calculated.

4.5 Immunoblotting

Cells were seeded overnight on 10cm tissue culture plates and treated with fluvastatin or ethanol as described for 72 hours. Cells were harvested for immunoblotting about 3 passages later than for fixed PI experiments. At 72 hours, media as aspirated from the cells, and the cells were washed with PBS. 500μL of SDS lysis buffer (10% β-mercaptoethanol, 11% glycerol, 1% SDS, 0.1M Tris pH 6.8) boiled to 100 degrees Celcius was added to the plates, and lysates were
scraped into eppendorf tubes. 4x Laemmli’s loading dye was added to a final 1x concentration, and samples were stored at -20 degrees Celsius. Samples were boiled at 100 degrees Celsius for 15 minutes prior to SDS-PAGE. Samples were loaded into 10% resolving gels at 25µL volumes. Amersham ladder was added at 1.5µL. Proteins were resolved by SDS-PAGE according to standard protocol, and transferred onto nitrocellulose membranes. Membranes were blocked with 5% milk in 0.01% PBS-T, and probed with primary antibodies overnight at -4 degrees Celsius. Antibodies are listed below with the company and catalog number information.

Anti-MAP2K4 (Cell Signaling Technology, 91525)
Anti-PI4KB (BD Pharmingen, 611817)
Anti-CSNK2B (EMD Millipore, 04-1128)
Anti-Actin (Sigma-Aldrich, A2066)
Anti-Tubulin (EMD Millipore, CP06)

Membranes were washed 3 times in 5-10 minute intervals in 0.01% PBS-T, and subsequently incubated in IRDye-labeled secondary antibodies (LICOR Biosciences) for one hour at room temperature. Membranes were washed twice for 5 minutes in 0.01% PBS-T, and once for five minutes in PBS, and imaged using the Odyssey infrared imaging system (LICOR Biosciences).

For HMGCR detection (Appendix 4), cells were lysed on ice for 15 minutes using a Triton X-based buffer instead of with SDS lysis buffer. The Triton lysis buffer contains 1mM 2-β-glycerophosphate, 0.5µg/mL antipain, 1µg/mL aprotinin, 1mM EDTA, 1mM EGTA, 1µg/mL leupeptin, 150mM NaCl, 1mM phenylmethylsulfonyl fluoride, 1µg/mL prestatin A, 20mM Tris pH 7.5, 0.5% Triton X-100, 1mM sodium orthovanadate, 2.5mM sodium pyrophosphate. Lysates were transferred to eppendorf tubes and centrifuged at 14,000 rpm for 10 minutes at 4 degrees Celcius. The supernatant was collected, and an aliquot was reserved to determine protein concentration with the Bradford assay. Fresh DTT and Laemmli’s loading were added to the rest of the supernatant to final 1X concentrations. Primary anti-HMGCR A9 antibody was used to detect HMGCR (prepared in-house from hybridoma number CRL-1811 from ATCC). All other aspects of the immunoblotting procedure was the same as described above.

4.6 Fixed propidium iodide (PI)

Cells were seeded overnight in 10cm tissue culture plates, and treated as indicated for 72 hours. At the endpoint, media was collected from plates into falcon tubes. The remaining adherent cells were washed with PBS, trypinized with 1x Trypsin and collected into the corresponding falcon
tube. The falcon tubes were centrifuged at 1400 rpm for 5 minutes to pellet the cells. The supernatant was removed, and the pellet was washed with PBS. The tubes were centrifuged again at 1400 rpm for 5 minutes, and the supernatant was removed. Seventy percent ethanol was added drop-wise to the pellet to fix the cells, and incubated at -20 degrees Celsius at least overnight, and up to two weeks. Ethanol was removed and cells were washed with PBS prior to treatment with DNase free RNase for one hour at 37 degrees, and staining with 50 µg/mL propidium iodide (Sigma-Aldrich) for 15 minutes. A FACS Calibur cytometer (BD Biosciences) was used to determine the pre-G1 population by flow cytometry. Ten thousand events were acquired, and only single-cells were included for analysis. Data was analyzed using FlowJo (TreeStar, Inc.).

4.7 Quantitative real-time PCR

Cells were grown to sub-confluence, and RNA was harvested using 1mL TRIZOL (Invitrogen) according to manufacturer’s protocol. cDNA was synthesized using 500ng RNA using SuperScript III (Invitrogen) according to the manufacturer’s protocol, and diluted 1:5 with sterile water (Sigma). Quantitative real-time RT-PCR (qRT-PCR) of ER stress gene mRNA expression was performed using 1x SYBR Green Master Mix (Applied Biosystems). Primer sequences:

**GAPDH:**
- Forward: GAAGGTGAAGGTCGGAGTC
- Reverse: GAAGATGGTGATGGGGATTTC

**ATF4:**
- Forward: TGGCCAAGCACTTCAAACCT
- Reverse: GTGTGGAGGGACTGACCAA

**BiP:**
- Forward: TGACATTTGAAGACTTCAAAGCT
- Reverse: CTGCTGTATCCTCTTCAACCAGT

**CHOP:**
- Forward: GGAGCATCAGTCCCCCCTTT
- Reverse: TGTGGGATTGGGAGGTCACATC

**ERdj4:**
- Forward: AAAATAAGAGCCCGGATGCT
- Reverse: CGCTTTTGGATCCAGTGGTT

**GADD34:**
- Forward: CCCAGAAACCCCTACTCATGATC
- Reverse: GCCCAGACAGCCAGGAAAT

**XBP1 (spliced):**
- Forward: CGCTTGGGGATGGATGCCCCTG
- Reverse: CCTGCACCTGTGCGGACT

**XBP1 (unspliced):**
- Forward: AGTCCGCAGCAGCTAGACTACG
- Reverse: TGGCAGGCTCTGGGGAGGAGG

Quantitative RT-PCR reactions were carried out in 384-well plates. Each reaction was plated in triplicate, in 10µL volumes containing 2µL of diluted cDNA. Data was acquired and analyzed with the ABI Prism 7900 Sequence Detection System. Target gene transcript levels were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH).
For HMGCR regulation studies in Appendix 4, quantification of HMGCR mRNA also involved 1x SYBR Green Master Mix (Applied Biosystems) and transcript levels were normalized to GAPDH (primer sequences listed above). HMGCR primer sequences:

**HMGCR:**
- Forward: GTTCTGAACTGGAACATGGGC
- Reverse: TTCATCCTCCACAAGACAATGC

For HMGCR exon 1A and 1B quantification (Appendix 4), Taqman Master Mix (Applied Biosystems), and Taqman primer and probe sets were used. Primer and probe sequences:

**GAPDH:**
- Hs99999905_m1 (Applied Biosystems)

**HMGCR Exon 1A**
- Forward: GGATGTCGCACACAAGAGA
- Reverse: GGTTGTCGCCCTCTAGTG
- Probe: TCCTTGGATCTCCAGATCT (FAM reporter)

**HMGCR Exon 1B**
- Forward: AATGGATGTCGCACACAAGAGA
- Reverse: CCATGCATTGAAAAAGTCTTGACA
- Probe: CTTGGATCTCCTGCCTTCTCT (FAM reporter)

4.8 IC20 dose calculations

To calculate the IC20 dose, dose response curves were generated as described under ‘MTT Assay’. Dose response curves were plotted using Prism (version 5.0, GraphPad Software), and half-maximal inhibitory concentrations (IC50) values and the hillslope were calculated. Using the equation, $\text{IC20} = \left( \frac{80}{100-50} \right)^{\text{hillslope}} \times \text{IC50}$, the IC20 value was solved for.

4.9 Plating cells for kinase inhibitor screen

Cells were seeded at optimized cell densities into 384-well clear, flat-bottom tissue culture treated plates for MTS reactions (BD Falcon) and 384-well cell carriers (Perkin Elmer) using the Multidrop Combi Reagent Dispenser (Thermo Scientific) at 40uL per well with a standard tube dispensing cassette. Cells were incubated at 37 degrees Celcius overnight and treated the next day.

4.10 Treatments for kinase inhibitor screen

Plates were flick-aspirated immediately prior to treatment. *Fluvastatin and ethanol treatment:* Fluvastatin or ethanol was added to media to create 1.25x stocks of the optimized IC20
fluvastatin dose and corresponding ethanol vehicle control. The Liquidator 96 manual 96-well pipettor (Rainin) was used to dispense 40µL of the fluvastatin- or ethanol-containing media into desired wells. **Kinase library treatment:** Source plates of the inhibitor library were made in DMSO to a concentration of 200µM and stored at -20 degrees Celcius. Each one of four source plates contains 80 kinase inhibitors (See Figure 24). The four kinase inhibitor source plates were diluted to working stock concentrations of 5µM and 0.5µM in HEPES solution in 384-well plates separate from the treatment plates (working stock compound plates). Control compounds were arrayed into the first and last two columns of the working stock compound plates at 5x the final desired concentration. 10µL of the 5x concentration working stock compound plates were added to treatment plates containing 40µL ethanol or fluvastatin in each well.

**4.11 MTS**

The CellTiter 96® AQueous Non-Radioactive Cell Proliferation Assay (MTS) was purchased from Promega, and PMS was purchased from Sigma-Aldrich. Stocks of the MTS and PMS reagents were made according to manufacturer’s protocol (Promega). At treatment endpoint, 10µL of PMS supplemented MTS was added to the 50µL contents of each well, according to ratios supplied by the manufacturer. Plates were incubated at 37°C to allow the formazan product to develop, and the TECAN Infinite® M1000 PRO was used to measure absorbance of each well at 490nM at one hour, and again every 30 minutes up to four hours. Media only wells served as 0% MTS and readings from solvent only wells were subtracted as background from all readings. A two-way ANOVA was calculated for each kinase inhibitor using the solvent only, inhibitor only, fluvastatin only and inhibitor plus fluvastatin absorbance readings with the R Statistical Environment.

**4.12 OPERA® image capture**

The Perkin Elmer OPERA system was used to capture images of the cells at the 72 hour endpoint. A stock of dye solution containing DRAQ5 and mutachrome at 3x the final concentration was made in cell culture media. The final concentration of DRAQ5 and mutachrome in each treatment well was 5µM and 1µM, respectively. 25µL of dye solution was added to the 40µL base treatment of fluvastatin or ethanol treatment plus 10µL kinase library treatment, for a total well concentration of 75µL thirty minutes prior to plate imaging. Filter pass
and parameters were defined according to dye emission and absorbance spectra. Images were captured at 40X magnification using a water-immersion lens.

4.13 Image segmentation and feature extraction

Perkin Elmer Acapella® High-content Imaging and Analysis Software was used to segment images and extract feature data from images. The script used for both processes is written and maintained by the Andrews lab, and based on the Acapella® (Perkin Elmer) script.

4.14 Image classification

MATLAB® software was used to classify images into defined negative control and positive control bins. The corresponding graphical user interface for image classification was created by Jarkko Ylanko in the Andrews Lab. The interface allows for selection of treatment wells, features to include in feature reduction and subsequent classification, and for controls to be defined. K nearest neighbours plus the best of three algorithms (principle component analysis (PCA), stepwise discriminant analysis (SDA) or Kolmogorov–Smirnov (KS) are used for dimensional reduction.
Contributions and Acknowledgements:

Figures A1 and A2: These figures were completed through a team effort between Dr. Aleksandra Pandyra, Dr. Carolyn Goard and the author

Figures A6-8: The technical screen in MCF10A cells with the toolkit compound library was completed by Jarkko Ylanko in the laboratory of Dr. David Andrews. Data analysis was completed under the guidance of Jarkko Ylanko.

Figures A9-10: MTT assays for Figure A9 and qRT-PCR experiments were completed by Dr. Carolyn Goard

Figure A12: Dr. Peter Mullen, Katherine Hicks and the author contributed to qRT-PCR experiments for quantifying HMGCR expression

Figure A13: HMGCR exon 1a and 1b expression quantification was carried out by Dr. Peter Mullen and Katherine Hicks

Figure A14: Schematic created by Dr. Peter Mullen
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1 Appendix 1 – Validating hairpins targeting mevalonate pathway genes as sensitizers to the antiproliferative effects of fluvastatin from a genome wide shRNA dropout screen

1.1 Overview

The anti-tumour benefit of statins may increase if statins are administered with co-therapies. To identify genes to knockdown in combination with statins, a shRNA dropout screen with fluvastatin was designed (described and discussed in Chapter 2). A few classes of hits emerged from this screen, one of which was kinases, as discussed throughout the main text of this thesis. Genes within the mevalonate pathway comprise another class of hits.

Three mevalonate pathway genes were candidate hits from the screen: sterol regulatory element binding protein 2 (SREBP2); geranylgeranyl diphosphate synthase 1 (GGPS1); and 3-hydroxy-3-methylglutaryl-CoA synthase 1 (HMGCS1). SREBP2 is a transcription factor that upregulates key mevalonate pathway genes in response to low sterol levels to maintain mevalonate pathway homeostasis. GGPS1 is an enzyme involved in synthesizing geranylgeranyl pyrophosphate (GGPP), which is involved in protein prenylation for key signal transduction proteins including the Ras superfamily and other small GTP-ase binding proteins including the Rab and Rho families. HMGCS1 is an enzyme involved in the second step of the mevalonate pathway signalling cascade, directly upstream of the reaction inhibited by statins (Refer to Figure 1). These three hits allude to the benefit of concurrently targeting the mevalonate pathway at multiple nodes to enhance statin-induced anticancer effects. We endeavored to evaluate this added benefit.

1.2 Hypothesis and Aims

If genes within the mevalonate pathway are knocked down in combination with fluvastatin treatment, then greater antiproliferative effects and cell death will be induced compared to fluvastatin treatment alone.
To address this hypothesis we will 1) confirm on-target knockdown of the three mevalonate pathway genes by their corresponding hairpins obtained from the screening library and 2) determine if these hairpins in combination with fluvastatin can increase cell death as assayed using the fixed propidium iodide assay.

1.3 Results

Validation studies involved the top two hairpins from the screen for each of the three mevalonate pathway genes. Six A549 sublines were generated, each stably expressing one of the six hairpins of interest, obtained from the original TRC1 library used in the drop out screen. Sublines are denoted as sh-target-#, where the number distinguishes between the two hairpins per gene. In addition, three control sublines were generated, which stably expressed hairpins targeting either GFP or LacZ.

On-target gene knockdown was assessed for all six sublines by immunoblotting. Sublines were grown to subconfluence, protein was harvested and run on an acrylamide gel, and probed with respective antibodies. Protein levels within each query subline were assessed against the three control sublines. Knockdown of SREBP2 in both the sh-SREBP2 sublines and of HMGCS1 in both the sh-HMGCS1 sublines are evident, while clear knockdown of GGPS1 is not observed (Figure A-1).

To determine if each shRNA in combination with fluvastatin treatment induces increased cell death, the six sublines were treated with two doses of fluvastatin or ethanol vehicle control, and the cells were harvested for the fixed propidium iodide assay. Compared to ethanol treatment, the percent of cells in the pre-G1 population increased significantly in all sublines upon treatment with at least one of the doses of fluvastatin (Figure A-2). By contrast, for the control sublines (shGFP, shLacZ), fluvastatin treatment does not increase the pre-G1 population over ethanol treatment.
Figure A-1. Gene expression knockdown in A549 cells transduced with shRNAs targeting mevalonate pathway genes

A549 sublines stably expressing one individual shRNA targeting SREBP2, HMGCS1 or GGPS1 were grown asynchronously, and protein was harvested for immunoblotting. Two sublines were generated using independent shRNA molecules for each gene of interest, and are denoted as 1 and 2. Protein expression for each subline is compared to protein expression in three control sublines stably expressing shRNAs targeting GFP or LacZ. Both shSREBP2 sublines (A) and both shHMGCS1 sublines (B) show decreased protein expression, whereas shRNAs targeted to GGPS1 do not decrease protein levels as noticeably (C). Blots shown are representative of three replicates.
1.4 Discussion

Preliminary validation studies for our three screen hits within the mevalonate pathway support targeting the mevalonate pathway at nodes separate from, and concurrently with, statin treatment. Both shGGPS1 sublines underwent robust cell death upon fluvastatin treatment (Figure A-2), which is promising despite low protein expression knockdown observed (Figure A-1). Although these two results together may suggest off-target hairpin activity interacting with fluvastatin to trigger cell death, there are two potential explanations for these seemingly inconsistent results. First, the GGPS1 hairpins may have induced low level gene knockdown as observed in Figure A-1. The $Z_{\text{ethanol}}$ score for GGPS1 is much lower than for other hits evaluated (Table A-1), indicating that GGPS1 knockdown alone robustly elicits cell death and that GGPS1 is crucial for cell viability. Given this demonstrated importance of GGPS1 to cell viability, the low level GGPS1 knockdown by the hairpins may have been sufficient to render
cells sensitive to fluvastatin as observed in Figure A-2. Second, there may have been robust knockdown of

Table A-1. Rankings of candidate mevalonate pathway genes from the shRNA dropout screen based on Z-normalized (GARPfluvastatin – GARPethanol) scores

<table>
<thead>
<tr>
<th>Gene Symbol</th>
<th>Gene Name</th>
<th>Rank (of 151 hits)</th>
<th>ZEtOH</th>
<th>ZFluva</th>
<th>ΔZ (ZFluva - ZEtOH)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HMGCS1</td>
<td>3-hydroxyl-3-methylglutaryl-Coenzyme A synthase 1 (soluble)</td>
<td>6</td>
<td>-0.268</td>
<td>-3.016</td>
<td>-2.748</td>
</tr>
<tr>
<td>SREBF2</td>
<td>Sterol regulatory element binding transcription factor 2</td>
<td>12</td>
<td>-0.387</td>
<td>-3.048</td>
<td>-2.661</td>
</tr>
<tr>
<td>GGPS1</td>
<td>Geranylgeranyl diphosphate synthase 1</td>
<td>17</td>
<td>-3.952</td>
<td>-6.342</td>
<td>-2.389</td>
</tr>
</tbody>
</table>

Inhibition of GGPS1 has been studied previously, given its role in protein prenylation and thus membrane localization of key signal transduction molecules such as Ras. This localization has been demonstrated to be crucial for Ras-dependent cellular transformation\(^{128}\), which is relevant to our cell line under investigation, which harbours a Kras mutation. Further, isoprenoid end-products of the mevalonate pathway, including geranylgeranylpyrophosphate (GGPP) and farnesylpyrophosphate (FPP), can reverse statin-triggered apoptosis\(^{129}\), suggesting that inhibiting isoprenoid biosynthesis with farnesyltransferase inhibitors (FTIs) and geranylgeranyltransferase I inhibitors (GGTIs) in combination with statin treatment may elicit an even greater degree of apoptosis. Indeed, GGPS1 inhibition using specific bisphosphonate inhibitors combined with lovastatin treatment has been demonstrated to result in synergistic induction of apoptosis in leukemia cells\(^{130}\). Clinically, three farnesyltransferase inhibitors (FTIs)
and a dual FTI and geranylgeranyltransferase I inhibitor (GGTI) inhibitor have been evaluated. Unfortunately, clinical results to date have been disappointing\textsuperscript{131}. Our results encourage further development and optimization of combined statin and GGPS1 inhibitor treatment.

Both shSREBP2 sublines show decreased SREBP2 protein expression (Figure A-1) and robust induction of cell death upon fluvastatin treatment (Figure A-2). Inhibition of SREBP2, a transcription factor that can mediate restoration of low mevalonate pathway end products, and thus can potentially aid cells in evading statin-triggered apoptosis, is attractive. Targeting the protein-DNA interaction to inhibit transcription factor function is widely perceived as difficult, but latent cytoplasmic protein transcription factors such as SREBP2 offer an opportunity to disrupt the activation or maturation of the transcription factor instead\textsuperscript{132}. Compounds such as fatostatin and betulin have been demonstrated to inhibit SREBP2 activation\textsuperscript{133,134}, and our results support the exploration of combining statins with these agents, or other compounds with similar activity, as potential anti-cancer combination therapies.

Decreased HMGCS1 protein expression (Figure A-1) and increased cell death upon fluvastatin (Figure A-2) was also observed for both shHMGCS1 sublines. HMGCS1 expression is increased in response to potential anticancer therapies including androgen exposure\textsuperscript{135} and STAT inhibition\textsuperscript{136}, which allude to the potential benefit of inhibiting HMGCS1 in combination therapies. Further, with the crystal structure of HMGCS1 solved, potent and specific inhibitors are being discovered for use as cholesterol-lowering and antibiotic agents\textsuperscript{137}, which could, in light of our data, be used in combination with fluvastatin in the cancer context as well.

Overall, our preliminary validation of shRNAs targeting genes within the mevalonate pathway as sensitizers to fluvastatin-induced apoptosis supports multi-nodal targeting of the mevalonate pathway.
2 Appendix 2 – Screening a kinase inhibitor library in breast and prostate cancer cell lines to identify co-therapies to use with fluvastatin

2.1 Overview

In addition to the MCF10A and A549 cell lines screened with the 320 kinase inhibitor library and fluvastatin in Chapter 2, additional cell lines were evaluated. These include two prostate cancer cell lines, DU145 and LNCaP, two breast cancer cell lines, MDA-MB-231 and MCF-7.

2.2 Hypothesis and Aims

If prostate and breast cancer cell lines are screened against a library of 320 kinase inhibitors with fluvastatin, then co-therapies likely to induce cell death with fluvastatin will be identified.

2.3 Results

Each of the four cell lines was treated with the kinase inhibitor library plus either a sublethal dose of fluvastatin or ethanol vehicle control for 72 hours. At this time point, an MTS assay was performed, and high-content images were collected for each treatment condition. For high-content image analysis, the death-phenotype positive control for all cell lines was PIK-93 plus fluvastatin, and the alive-phenotype negative control was DMSO plus fluvastatin.

To begin, the IC<sub>20</sub> or IC<sub>25</sub> dose of fluvastatin was determined for each cell line. Each cell line was treated with a range of fluvastatin concentrations and dose response curves were generated using the MTT assay. From these curves, the IC<sub>50</sub> concentration and slope were determined and used to calculate the IC<sub>20</sub> or IC<sub>25</sub> dose of fluvastatin. Doses vary by cell line and are shown in Figure A-3. These doses were used to guide selection of the dose of fluvastatin to be used in combination with each of the 320 kinase inhibitors in the compound library.

Next, cells were treated with four doses of PIK-93 (0.125μM, 1.56μM, 5μM and 10μM) with and without the predetermined sublethal dose of fluvastatin to determine if the PIK-93 and fluvastatin combinations could increase cell death induced by each compound alone at each
Figure A-3. MTT$_{20}$ or MTT$_{25}$ values of fluvastatin for two breast cancer and two prostate cancer cell lines

Four cell lines were treated with a range of fluvastatin concentrations for 72 hours, upon which an MTT reaction was conducted. The MTT activity values over the range of concentrations were used to generate dose-response curves, as shown. The curves represent an average of 3-5 replicates, plotted with standard deviation. The IC$_{50}$ values and slope were used to calculate the IC$_{25}$ or IC$_{20}$ dose of fluvastatin, which results in approximately 75-80 percent cell viability. The final dose for use in the screen was selected based on these calculated values. The IC$_{50}$ values were higher for two replicates of five for the DU145 cell line, causing the average IC$_{20}$ value to be higher than anticipated given raw MTT activity values. The selected dose for this cell line was therefore lower than the calculated IC$_{20}$ value.
respective concentration. All PIK-93 doses were included in the screens for future study of dose-dependent phenotypic changes. Combinations of PIK-93 and fluvastatin significantly induce cell death over PIK-93 alone for the MCF-7 and DU145 cell lines (Figure A-4). These combinations may be appropriate to use as death-phenotype positive control treatments for the analysis of high-content screen data. Data analysis for the LNCaP and MDAMB231 cell lines may require a death-phenotype positive control distinct from fluvastatin with PIK-93, since the combination treatments do not significantly increase the percent of cells in the pre-G1 population. Large error bars suggest that additional replicates are needed for these two cell lines. Additionally, PIK-93 at 1µM in combination with 0.25µM or 0.5µM fluvastatin showed robust increase in percent of cells in the pre-G1 population in previous experiments (Figure 6F, main text), and may indicate that higher concentrations of fluvastatin are required for lethal interaction with PIK-93. Further, PIK-93 and fluvastatin combinations significantly decrease cell viability as determined with the MTT assay for all four cell lines, suggesting these combinations are likely to score as hits in the MTS assay (Figure A-5).

To date, DU145, LNCaP and MDA-MB-231 cells were screened and both MTS and image data has been collected. MCF-7 cells have been screened with only MTS data collected thus far. All screening data from these cell lines remains to be analyzed.

2.4 Discussion

Assessing multiple cell lines from different cancer types may reveal a kinase inhibitor and fluvastatin combination that may be commonly lethal among all cell lines, or unique combinations for each cell line may emerge. Comparing results across cell lines may reveal common pathways to inhibit to potentiate fluvastatin’s anticancer effects.
Figure A-4. Combined fluvastatin and PIK-93 treatment increases the pre-G1 population in MCF-7 and DU145 cell lines
Two breast cancer cell lines and two prostate cancer cell lines were treated with four concentrations of PIK-93 with or without a sublethal dose of fluvastatin specific to each cell line for 72 hours. Combination treatments significantly increase the percent of cells in the pre-G1 population in MCF-7 and DU145 cell lines, but not the MDA-MB-231 and LNCaP cell lines, as determined with the fixed propidium iodide assay. Variable replicates may be masking an effect in MDA-MB-231s and LNCaPs, and additional replicates may be required. Bars represent averages plotted with standard deviation for three to four independent replicates. * = p<0.05, unpaired, two-tailed t-test between each PIK-93 dose alone and in combination with fluvastatin.
Figure A-5. PIK-93 and fluvastatin in combination decreases MTT activity in breast cancer and prostate cancer cell lines

Two breast cancer cell lines and two prostate cancer cell lines were treated with four concentrations of PIK-93 with or without a sublethal dose of fluvastatin specific to each cell line for 72 hours. At least one concentration of PIK-93 in combination with fluvastatin decreases MTT activity significantly compared to the PIK-93 dose alone. Average MTT activity is plotted with standard deviation for three to four independent replicates. *=p<0.05, unpaired, two-tailed t-test.
3 Appendix 3 – Screening MCF10A cells with a 160-compound drug library to identify co-therapies to use with fluvastatin

3.1 Overview

A collection of 160 compounds was screened against MCF10A cells with and without fluvastatin. This compound library is diverse in composition, as compared to the 320 compound library consisting only of kinase inhibitors discussed in Chapter 2. This screen was also completed as a proof-of-concept screen to determine the feasibility of identifying fluvastatin co-therapies with a high-content screening platform. This screening technique may be expanded to MCF10A isogenic cell lines to determine drug combinations that are toxic in MCF10A lines transformed with oncogenes but not in MCF10A cell lines without oncogene addition.

3.2 Hypothesis and aims

If MCF10A cells are screened with fluvastatin and a library of 160 compounds, co-therapies to use in combination with fluvastatin will be revealed.

3.3 Results and discussion

A 160 compound library assembled by the OICR Drug Development Platform, referred to as the ‘toolkit library’, was screened in the presence and absence of fluvastatin in MCF10A cells. Cells plated in 384-well plates were treated for 24 hours with 1μM of the toolkit library dissolved in DMSO plus the IC\textsubscript{20} dose of fluvastatin or ethanol vehicle control. Cells were imaged using the OPERA® High-content Screening System, and two control groups were defined for feature reduction and object classification. One group was the alive-phenotype negative control, which was the IC\textsubscript{20} dose of fluvastatin plus DMSO vehicle (Refer to Figure 9). The second group was the death-phenotype positive control, which was the IC\textsubscript{20} dose of fluvastatin plus 1μM PIK-93, an inhibitor of PI4KB discussed in the main body of this thesis.
At 24 hours, DRAQ5, a DNA-specific fluorescent dye, and MK1, a membrane fluorescent dye, were added to each treatment well to visualize the cells, which were imaged 30 minutes later. Each treatment condition was assayed in two wells, and four field-of-view images were captured per well. In total, eight images were captured per treatment condition. Using the Acapella® high-content imaging and analysis software, feature reduction yielded 15 features required for object classification between the two defined controls (Figure A-6). The control groups classified as expected. The negative control group (5µM fluvastatin plus DMSO) as well as the solvent control and PIK-93 only treatment all classified into the negative control group over 80% of the time, and the positive control (5µM fluvastatin plus 1µM PIK-93) classified into the positive control group over 90% of the time (Figure A-7).

![Figure A-6](image-url)

**Figure A-6. Features used for object classification for the MCF10A toolkit library screen at 24 hours**

Feature reduction was completed using defined negative and positive control groups (5µM fluvastatin plus solvent control, and 5µM fluvastatin plus 5µM PIK-93, respectively). A mix of nuclear and membrane features was used for subsequent data classification. The mean z-score ± standard deviation is plotted for each feature compared to all features extracted.
The 160 compound treatments with ethanol control (single compound treatment) or with fluvastatin (combination treatment) were also classified into either the negative or positive control groups. We are interested in compounds that result in an alive-phenotype when used as a single compound treatment and result in a death-like phenotype when used in combination with fluvastatin. Of the 160 compounds, most are minimally toxic alone and in combination with fluvastatin, as expected for a small-molecule library screen (Figure A-8). Two inclusion criteria were used to define hits. First, compound alone treatments resulted in less than 20% of cells classifying in the death-like phenotype, and second, the compound treatment is at least three standard deviations away from the negative control, 5µM fluvastatin plus DMSO. The hits are visualized in the bottom right corner of Figure A-8, and are listed in Table A2. Additional known cytotoxic compounds were added as positive controls (Figure A-8, orange dots). Compounds that induced a death-like phenotype alone and in combination with fluvastatin include staurosporine, thapsigargin, TNFα and cycloheximide. Interestingly, two controls induced a death-like phenotype over 70% of the time in combination with fluvastatin, and were barely outside of the first criteria for a hit, as each compound alone caused about 21% of cells to

**Figure A-7. Classification of cells exposed to control treatments for the MCF10A toolkit library screen at 24 hours**

Cells from each treatment were classified into either the alive-phenotype negative control group or the dead-phenotype positive control group based on features in Figure A6. For solvent control, PIK-93 alone, and our defined negative control (fluvastatin + DMSO) treatment groups, over 80% of cells classify as the alive-phenotype negative control as expected. Over 90% of cells exposed to the positive control treatment (fluvastatin + PIK-93) are classified into the dead-phenotype positive control group.
Figure A-8. Classification of all compounds in the MCF10A toolkit library screen
All cells exposed to the 160 compounds in the toolkit library in combination with ethanol or fluvastatin for 24 hours were classified into either the alive-phenotype negative control group or the dead-phenotype positive control group based on the features used in Figure A6. The greater the number of cells classifying into the dead-phenotype positive control group for any given treatment suggests a more cytotoxic treatment. Kinase inhibitors that have a low percentage of cells classifying into the positive control group when used as a single-agent (i.e. close to 0% on the y-axis), and that have a high percentage of cells classifying into the positive control group when used in combination with fluvastatin (i.e. close to 100% on the y-axis) are putatively potentiating fluvastatin-induced cell death. Our negative control, fluvastatin plus DMSO, causes few cells to classify into the positive control group (red dot, bottom left), and our positive control, fluvastatin plus PIK-93 causes over 95 percent of cells to classify into the positive control group (green dot, bottom right). Importantly, PIK-93 on its own causes less than 20% of cell death alone, indicating that only treatment in combination with fluvastatin triggers a dead-phenotype. PIK-93 from the compound library is shown in blue. Additional compounds likely to elicit cell death (orange dots) were added as additional controls and include cycloheximide, staurosporine, TNF-α, thapsigargin, tunicamycin, tamoxifen and rapamycin (discussed in text).
classify into the death-like phenotype group. These include rapamycin, and tunicamycin. Tamoxifen did not induce a death-like phenotype alone or in combination, and acts similar to the negative control.

<table>
<thead>
<tr>
<th>Plate - well ID</th>
<th>Compound Name</th>
<th>Compound type</th>
<th>Standard deviations from negative control</th>
</tr>
</thead>
<tbody>
<tr>
<td>External</td>
<td>PIK-93</td>
<td>DNA Topoisomerase inhibitor</td>
<td>7.76</td>
</tr>
<tr>
<td>2-C07</td>
<td>Daunorubicin hydrochloride</td>
<td>HDAC inhibitor</td>
<td>7.09</td>
</tr>
<tr>
<td>2-H07</td>
<td>Trapoxin A</td>
<td>SHH antagonist</td>
<td>6.96</td>
</tr>
<tr>
<td>2-G04</td>
<td>JK 184</td>
<td>Estrogen-Related Receptor beta</td>
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</tr>
<tr>
<td>2-D02</td>
<td>GSK 4716</td>
<td>DNA Topoisomerase II inhibitor</td>
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<tr>
<td>2-C09</td>
<td>Epirubicin hydrochloride</td>
<td>Antifungal</td>
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<tr>
<td>1-H02</td>
<td>Sentaconazole nitrate</td>
<td></td>
<td>3.67</td>
</tr>
</tbody>
</table>

Table A-2. Putative hits from the toolkit library screen in the MCF10A cell line

Our inclusion criteria for screen hits yield six compounds as candidate potentiators of fluvastatin-induced cell death, defined by phenotypic response to fluvastatin and PIK-93 in MCF10A cells. Our external PIK-93 compound was used as a positive control and classifies as a hit under our hit definition.

3.4 Discussion

Screening the MCF10A cell line with a 160 compound library in the presence and absence of fluvastatin has identified putative compounds to use in co-therapies with fluvastatin. High-content imaging suggests that low doses of these compounds and the selected low dose of fluvastatin each cause minimal cell death alone, but the compound plus fluvastatin combinations elicit robust cell death in the MCF10A cell line. Validation of these compounds may show that this proof-of-concept screen involving a high-content screening platform is suitable for identifying compounds that elicit cell death in combination with fluvastatin.

As with the proof-of-concept screen with a kinase inhibitor library discussed in Chapter 2, we can again expand this screening platform to isogenic cell lines established with MCF10A cells, which are considered a normal-like breast line. Screening such isogenic cell lines against compound libraries can reveal therapeutic targets for specific genetic alteration driven cancers, and mechanisms of resistance to certain compounds as mentioned above

110,111.
Again, if we want to pursue the drug combinations from this screen, we should proceed with caution. Our results should be validated in both breast cancer cell lines and more normal-like breast cells to determine clinical utility.
4 Appendix 4 – Exploring expression of HMGCR and HMGCR exon 1 splice variants in response to fluvastatin treatment or lipid deprivation as biomarkers of statin sensitivity

4.1 Overview

Evidence from our group has suggested that statin sensitivity is correlated to mRNA expression of mevalonate pathway genes upon statin treatment. Through microarray analysis and subsequent validation studies with multiple myeloma (MM) cell lines, it was found that statin-sensitive and statin-insensitive cell lines respond differently to lovastatin treatment: while the statin-sensitive cell lines did not upregulate mevalonate (MVA) pathway genes such as HMGCR, the rate-limiting enzyme of the MVA pathway, statin-insensitive lines had robust upregulation of these genes\(^{138}\). This generated a hypothesis whereby deficient mevalonate pathway upregulation (i.e. lack of a mevalonate pathway feedback response) is indicative of tumour cell death\(^{138}\). A dysregulated mevalonate pathway may therefore serve as a potential biomarker of statin sensitivity, which would be clinically advantageous for predicting patient response to statins.

In a recent clinical trial for statin treatment in breast cancer, neo-adjuvant fluvastatin treatment induced significant increase in tumour cell apoptosis in 60% of grade-3 breast cancer patients\(^{139}\). As such, we hoped to explore HMGCR regulation in response to statin treatment as a biomarker of response in the breast cancer setting. A panel of breast cancer cell lines was studied, and heterogeneous statin sensitivity was again observed (Figure A-9, Goard et al., 2014, Breast Cancer Research Treatment, In Press). Selecting two relatively statin-sensitive and two relatively statin-insensitive cell lines from this panel, we explored HMGCR expression and HMGCR splice variant regulation in response to fluvastatin treatment as well as to lipid deprivation, a surrogate for statin-triggered sterol depletion. The second exon 1 variant, HMGCR exon 1b, was recently identified\(^{140}\).
4.2 Hypothesis

If breast cancer cell lines are treated with fluvastatin or subjected to lipid deprivation by being cultured in lipoprotein deficient serum (LPDS), changes in HMGCR expression or in the expression of HMGCR exon 1 variants may distinguish statin-sensitive cell lines, and serve as biomarkers for statin sensitivity.

To address this hypothesis we aim to 1) treat two statin-sensitive and two statin-insensitive cell lines with fluvastatin, and compare HMGCR expression between the two groups, 2) culture the four aforementioned breast cancer cell lines in LPDS supplemented media and compare HMGCR expression between the two groups, and 3) culture the cell lines in LPDS supplemented media and compare HMGCR exon 1A and 1B expression between the two groups.

![Figure A-9. Sensitivity to fluvastatin varies across a panel of 19 breast cell lines](image)

Nineteen breast cell lines were treated for 72 hours with a range of fluvastatin concentrations. The MTT assay was used to generate dose-response curves for each cell line. The average MTT$_{50}$ value is shown as a mean of at least three independent replicates. The MTT$_{50}$ values span a range of 200µM, demonstrating that sensitivity to fluvastatin varies by cell line. Figure in Goard et al., 2014, Breast Cancer Research Treatment (In Press).
4.3 Results

Two relatively statin-sensitive cell lines, MDA-MB-231 and HCC1954, and two relatively statin-insensitive cell lines, MCF-7 and CAMA1, were selected for our studies (refer to Figure A9 for comparative sensitivity across a panel of 19 breast cell lines). We first sought to determine if HMGCR expression is correlated with statin sensitivity. The four cell lines were treated with 10 mM fluvastatin for 8, 16 and 24 hours, and mRNA gene expression was quantified using q-RT-PCR. There were no striking differences in the upregulation of HMGCR between statin-sensitive and statin-insensitive breast cancer cell lines (Figure A-10). The effect of fluvastatin treatment on HMGCR expression was also assessed at the protein level in MDA-MB-231s and MCF-7s (Figure A-11). Cells were treated with 10 mM fluvastatin or ethanol solvent for 8, 12, 16 or 24 hours, and protein lysates were harvested for immunoblotting. Fluvastatin induces increased HMGCR expression similarly in both cell lines (Figure A-11, column 2 for each blot).

Figure A-10. Fluvastatin treatment in two sensitive and two insensitive breast cancer cell lines does not reveal marked differences in HMGCR regulation between the two sensitivity groups

Four breast cancer cell lines were treated with 10µM fluvastatin for 8, 16 or 24 hours. HMGCR mRNA was harvested and subsequently quantified using qRT-PCR. There is no strong trend towards differential HMGCR regulation upon statin treatment between the two relatively sensitive versus the two relatively insensitive cell lines.
A  MDA-MB-231 (sensitive)

B  MCF-7 (insensitive)

Figure A-11. HMGCR protein regulation upon fluvastatin treatment and sterol withdrawal in MDA-MB-231 and MCF-7 cells

MDA-MB-231 and MCF-7 cells were treated with fluvastatin or ethanol control, in either FBS or lipoprotein deficient serum (LPDS) supplemented media. Protein was harvested using a Triton-X based lysis buffer and run on an agarose gel. Sterol withdrawal via culturing in LPDS causes upregulation of HMGCR in the relatively sensitive MDA-MB-231 cell line (A), especially at 16 and 24 hours, but not noticeably in the MCF-7 cell line (B) (compare first and third lanes). Fluvastatin treatment plus culturing with LPDS supplemented media causes an upregulation of HMGCR in both cell lines, but to a greater degree in the statin sensitive MDA-MB-231 cell line (forth band). UT2 cells are Chinese hamster ovary cells. Lysates from UT2s ectopically expressing an empty control vector are used as the negative control (-ve cont.) and UT2s ectopically expression HMGCR are used as a positive control (+ve cont.).
We also sought to determine if culturing cell lines in LPDS-supplemented media could induce differential HMGCR expression at the protein level between statin-sensitive and -insensitive cell lines. MDA-MB-231 cells and MCF-7 cells were cultured in media supplemented with either 10% fetal bovine serum (FBS) or 10% LPDS. Only the statin-sensitive MDA-MB-231 cells show an increase in HMGCR expression when cultured in LPDS (Figure A-11, column 4 for each blot) and culturing in LPDS combined with fluvastatin treatment induces greater increases in HMGCR expression in the statin-sensitive MDA-MB-231 cells compared to MCF-7 cells.

To further explore the effect of lipid deprivation on MVA pathway gene expression, all four breast cancer cell lines were cultured in FBS or LPDS supplemented media for 16 hours, the time point at which HMGCR upregulation seemed to be most robust at the protein level as seen in Figure A-11. At 16 hours, HMGCR mRNA expression was surveyed by q-RT-PCR. Expression was highest in the most-sensitive cell line, MDA-MB-231 (Figure A-12A, leftmost), and is lower in the less sensitive cell line, MCF-7 (Figure A-12A, second from right). These results mirror the immunoblots from Figure A-11. However, expanded to other cell lines, the trend is less convincing, as the most insensitive cell-line shows HMGCR expression similar to the second most sensitive cell line. This trend is replicated when HMGCS1 expression is assessed (Figure A-12B).

![Figure A-12](https://example.com/image)

**Figure A-12.** HMGCR and HMGCS1 regulation in breast cancer cell lines cultured in FBS and LPDS supplemented media

Four breast cancer cell lines were cultured in either FBS or LPDS supplemented media. mRNA was harvested and HMGCR expression was assessed by qRT-PCR. HMGCR expression for each cell line is normalized to the FBS supplemented media condition. HMGCR (A) and HMGCS1 (B) expression tends to be higher in the sensitive cell lines and lower in the insensitive cell lines.
This unconvincing trend in HMGCR expression as a correlate of statin sensitivity led to the exploration of HMGCR splice variant regulation as potential correlates. Two HMGCR exons, exon 1A and exon 1B were studied. Cells were grown in either LPDS or FBS supplemented media as with previous experiments, and mRNA expression levels of each exon were quantified by q-RT-PCR. HMGCR exon 1A response to sterol deprivation in the four cell lines is similar to total HMGCR expression levels (Figure A-13). By contrast, HMGCR exon 1B expression trends to being inversely correlated with statin-sensitivity in either LPDS or FBS supplemented media (Figure A-13).

Figure A-13. HMGCR exon 1 variant regulation in breast cancer cell lines in FBS and LPDS supplemented media

HMGCR exon 1A expression (A) is regulated similarly to HMGCR expression seen in Figure A12. HMGCR exon 1B expression (B) increases as fluvastatin sensitivity decreases in both FBS and LPDS supplemented media. Low HMGCR exon 1B expression may be a biomarker of statin sensitivity in breast cancer cell lines.
4.4 Discussion

A deficient mevalonate pathway feedback response may be attributable to statin sensitivity in MM cell lines. By contrast, this model does not hold true for breast cancer cell lines, as there were no striking differences in the upregulation of HMGCR between statin-sensitive and statin-insensitive breast cancer cell lines at either the mRNA or protein levels after statin treatment (Figure A-10, Figure A-11). Consequently, HMGCR regulation in response to fluvastatin treatment is unlikely to be used as a marker of statin sensitivity in the breast cancer setting. Differences in feedback responses between these MM and breast cancer cells suggests that HMGCR regulation may be cell-system dependent. Perhaps MM cell lines are more dependent on the mevalonate pathway, and as such, require its upregulation to promote cell survival. These differences allude to a potential need for cancer-type specific biomarkers of statin response. HMGCR regulation and HMGCR exon 1A regulation in response to sterol deprivation also is not correlated to statin sensitivity across a panel of four breast cancer cell lines (Figure A-12, A-13). Interestingly, HMGCR exon 1B mRNA expression seems to be inversely correlated with statin sensitivity, and therefore has biomarker potential (Figure A-13). The trend is upheld in both FBS and LPDS supplemented cultures, suggesting that patient lipid levels would not affect this biomarker of statin sensitivity. A model for HMGCR exon 1b as a marker for statin sensitivity can be found in Figure A-14. Further exploration of HMGCR exon 1B as a biomarker of statin sensitivity in a larger panel of breast cancer cell lines is warranted. Defining a biomarker of statin response is crucial to proving the clinical utility of statins. HMGCR exon 1B regulation may help to enrich for patients who are likely to respond favorably to statin treatment.

Figure A-14. Model for HMGCR exon 1b as a marker for statin sensitivity

HMGCR exon 1B expression is higher in statin insensitive cells than statin sensitive cells. This suggests that low HMGCR exon 1B activity may be predictive of statin sensitivity.
References


