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Multiplexed High-throughput Screenings for Immune Modulators of Cytotoxic T Lymphocyte

Ziyan Zhao

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Immune modulation is an important therapeutic approach. Down regulation using immune inhibitors can treat autoimmune diseases and prevent transplant rejection, whereas up regulation using immune augmentors can increase cancer cell killing and better clear viral and bacterial infections. Currently available immune inhibitors include mitotic blockers, monoclonal antibodies that reduce activated immune cell numbers or proinflammatory cytokine levels, and specific small molecule inhibitors that target signaling events involved in immune cell activation and proliferation; immune augmentors are mostly monoclonal antibodies that help immune cells overcome inhibitory immune checkpoints. Small molecule immune augmentors are available, but they are greatly limited by number and mechanisms. There is an unmet medical need to look for more immune modulators. A cell-based assay was devised using cytotoxic T lymphocytes as a model cell system in high-throughput screens to look for active compounds. Activation of cells results in the externalization of lysosome-associated membrane protein to the cell surface, which can be detected by fluorescent antibody present in extracellular solution in flow cytometry. By using different methods to activate cells in an increasingly multiplexed design, the assay gains more power to look for interesting compounds as immune modulators. The first version of the assay used small molecule stimulants to achieve maximal cell activation, revealing 75 inhibitory compounds from an NIH chemical library. Mechanism of action analysis subsequently led to 8 novel compounds being identified, and two are being investigated as molecular probes for target identification. The second version of the assay used antibody-coated beads to achieve a submaximal level of cell activation, allowing one augmenting compound to be identified from a natural product library. Mechanism of action analysis determined that it is a potent activator of protein kinase C, and structural deconvolution led to its identification as teleocidin A-1. The last
version of the assay incorporated multiple treatment time of cells with compounds using fluorescent cell barcoding technique. It was used to screen the NCI diversity library, revealing 5 augmenting compounds of lytic granule exocytosis. Therefore, multiplexed assays of cytotoxic T lymphocyte can be used in high-throughput screens to look for immune modulators.
Multiplexed High-throughput Screenings for Immune Modulators of Cytotoxic T Lymphocyte

Ziyan Zhao

B.E., Beihang University, 2010

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Submitted in Partial Fulfillment of the
Requirements for the Degree of
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at the
University of Connecticut

2018
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Ziyan Zhao

2018
Multiplexed High-throughput Screenings for Immune Modulators of Cytotoxic T Lymphocyte

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First and foremost, I would like to thank my major advisor, Dr. Adam Zweifach, who mentored me throughout my graduate program. Adam is extremely knowledgeable and meticulous when it comes to scientific research and experiment design. He is also true to his words and never shy about sharing his opinions. It is my great honor to have joined his lab and worked on many interesting projects under his guidance. Adam taught me to be suspicious of everything I read in the scientific journals, and he often said that results try to trick you especially when they seem too good to be true. Over the past several years, I have grown to become an independent thinker. I owe my scientific achievements to him, and I am more than grateful for having him as my mentor.

Adam is also very good at managing project progress. Without his help, I would not have achieved so many publications during the graduate program. He and his wife Elisabeth have been very hospitable to me, as they always invite me to their family gatherings during holidays, making me feel not lonely in a foreign country.

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## Abbreviations Used

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<th>initials</th>
<th>full names</th>
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<tbody>
<tr>
<td>ABHD</td>
<td>α/β-hydrolase domain-containing</td>
</tr>
<tr>
<td>AID</td>
<td>assay ID</td>
</tr>
<tr>
<td>APC</td>
<td>antigen-presenting cell</td>
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<tr>
<td>CaN</td>
<td>calcineurin</td>
</tr>
<tr>
<td>CANAR</td>
<td>CaN Activity Reporter</td>
</tr>
<tr>
<td>CD</td>
<td>cluster of differentiation</td>
</tr>
<tr>
<td>CLR</td>
<td>C-type lectin receptor</td>
</tr>
<tr>
<td>CTL</td>
<td>cytotoxic T lymphocyte</td>
</tr>
<tr>
<td>CTLA-4</td>
<td>cytotoxic T lymphocyte antigen-4</td>
</tr>
<tr>
<td>DAG</td>
<td>diacylglycerol</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>EC50</td>
<td>half of maximal level effective concentration</td>
</tr>
<tr>
<td>EKAR</td>
<td>ERK activity reporter</td>
</tr>
<tr>
<td>ELISA</td>
<td>enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>erk</td>
<td>extracellular signal-regulated kinase</td>
</tr>
<tr>
<td>FACS</td>
<td>fluorescence-activated cell sorting</td>
</tr>
<tr>
<td>FCB</td>
<td>fluorescent cell barcoding</td>
</tr>
<tr>
<td>FRET</td>
<td>forster resonance energy transfer</td>
</tr>
<tr>
<td>GPCR</td>
<td>G protein-coupled receptors</td>
</tr>
<tr>
<td>HSP</td>
<td>heat shock protein</td>
</tr>
<tr>
<td>HTS</td>
<td>high-throughput screening</td>
</tr>
<tr>
<td>IL</td>
<td>interleukin</td>
</tr>
<tr>
<td>IMiDs</td>
<td>immune modulatory drugs</td>
</tr>
<tr>
<td>INF</td>
<td>interferon</td>
</tr>
<tr>
<td>IP3</td>
<td>inositol triphosphate</td>
</tr>
<tr>
<td>irAes</td>
<td>immune related adverse events</td>
</tr>
<tr>
<td>ITAM</td>
<td>immunoreceptor tyrosine-based activation motif</td>
</tr>
<tr>
<td>Jak</td>
<td>Janus activated kinase</td>
</tr>
<tr>
<td>LAMP</td>
<td>lysosome-associated membrane protein</td>
</tr>
<tr>
<td>LP</td>
<td>lysophospholipid</td>
</tr>
<tr>
<td>MAPK</td>
<td>mitosis-activated protein kinase</td>
</tr>
<tr>
<td>MAPKK</td>
<td>MAPK kinase</td>
</tr>
<tr>
<td>MMOA</td>
<td>molecular mechanism of action</td>
</tr>
<tr>
<td>MS</td>
<td>mass spectrometry</td>
</tr>
<tr>
<td>mTOR</td>
<td>mammalian target of rapamycin</td>
</tr>
<tr>
<td>NCI</td>
<td>National Cancer Institute</td>
</tr>
<tr>
<td>NFAT</td>
<td>nuclear factor of activated T cells</td>
</tr>
<tr>
<td>NFκB</td>
<td>nuclear factor kappa-light-chain-enhancer of activated B cells</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>NIH</td>
<td>National Institute of Health</td>
</tr>
<tr>
<td>NK</td>
<td>natural killer</td>
</tr>
<tr>
<td>NLRP3</td>
<td>nucleotide-binding domain, leucine-rich-containing family, pyrin domain-containing-3</td>
</tr>
<tr>
<td>NMR</td>
<td>nuclear magnetic resonance</td>
</tr>
<tr>
<td>NP</td>
<td>natural product</td>
</tr>
<tr>
<td>NR</td>
<td>normal Ringer's</td>
</tr>
<tr>
<td>NSC</td>
<td>Cancer Chemotherapy National Service Center number</td>
</tr>
<tr>
<td>PD</td>
<td>programmed death</td>
</tr>
<tr>
<td>PFA</td>
<td>paraformaldehyde</td>
</tr>
<tr>
<td>PIP2</td>
<td>phosphatidylinositol-4,5-biphosphate</td>
</tr>
<tr>
<td>PKC</td>
<td>protein kinase C</td>
</tr>
<tr>
<td>PLC</td>
<td>phospholipase C</td>
</tr>
<tr>
<td>PMA</td>
<td>phorbol 12-myristate 13-acetate</td>
</tr>
<tr>
<td>RIG-1</td>
<td>retinoic acid-inducible gene I</td>
</tr>
<tr>
<td>RLR</td>
<td>RIG-1-like receptors</td>
</tr>
<tr>
<td>SAR</td>
<td>structure-activity relationship</td>
</tr>
<tr>
<td>SID</td>
<td>substance ID</td>
</tr>
<tr>
<td>SMILES</td>
<td>simplified molecular-input line-entry system</td>
</tr>
<tr>
<td>STAT</td>
<td>signal transducer and activator of transcription</td>
</tr>
<tr>
<td>STING</td>
<td>stimulator of interferon gene</td>
</tr>
<tr>
<td>TALL-104</td>
<td>T acute lymphoblastic leukemia-104</td>
</tr>
<tr>
<td>TCR</td>
<td>T cell receptor</td>
</tr>
<tr>
<td>TG</td>
<td>thapsigargin</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
</tr>
<tr>
<td>TNF</td>
<td>tumor necrosis factor</td>
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Chapter 1: Introduction and Goal of Study

1.1 Overview of immune system components

The immune system is the first-line defense mechanism composed of innate and acquired systems under strict regulation. Major components of the immune system include immune cells, soluble cytokines, complement and chemokines. The role of the immune system is to recognize non-self organs, tissues, cells and subcellular organisms and mount a cohort of immune response to clear out the potential threat.

The white blood cells in the immune system derive from pluripotent stem cells located in the bone marrow. Two major lineages are the lymphoid and myeloid. Typically, cells of lymphoid lineage regulate the adaptive immune response, and cells of myeloid lineage are the first responders to infections. The lymphoid progenitors differentiate into T or B lymphocytes that can become effector cells when activated: T lymphocytes become either CD8+ cytotoxic T lymphocytes to directly lyse the target cells or CD4+ helper T cells that secrete cytokines to activate other immune cells; B lymphocytes become plasma cells that produce antibodies which opsonize antigens and assist antibody-dependent cell-mediated cytotoxicity (Figure 1). The predominant maturation site for T cells is the thymus, and for B cells is the bone marrow in mammals (bursa of Fabricius in birds). A third class of lymphoid cells is natural killer (NK) cell that lacks antigen-specific receptors and therefore belongs to the innate immune system. Dendritic cells are derived from lymphoid progenitors as well. They function as antigen-presenting cells by engulfing and processing foreign organisms into recognizable peptides bound to their cell surface proteins.

The myeloid progenitors differentiate into granulocytic monocyctic lineage, megakaryocytes and erythrocytes. Granulocytic monocyctic lineage gives rise to granulocytes such as eosinophil, basophil, polymorphonuclears, involved in the initial immune response to a foreign attack; mast cells that play an important role in the development of allergies; and monocytes that could turn
into tissue-specific macrophages involved in phagocytosis and antigen presentation. Megakaryocytes are broken down into platelets that assist blood clotting. The oxygen carrier, red blood cells, are derived from erythroid progenitors.

Figure 1 An overview of the humoral and cell-mediated responses in the adaptive immune system.

When foreign pathogens enter the mammalian body, B cell-mediated humoral response can generate antibodies to eliminate the antigen present in the bloodstream, and T cell-mediated immune response can either recognize target cells hosting intracellular pathogens and directly kill infected cells or secrete cytokines to regulate the activity of other immune cells. Figure is adapted from Kuby Immunology¹.
Cytokines are the soluble mediators of immune response. They can bind to specific receptors with very high affinity on their target cell membranes, trigger signal transduction pathways and cause target cells to activate, proliferate or differentiate. The molecular weight is usually less than 30 kDa, making them diffusible to carry autocrine, paracrine or even endocrine effects. Some examples of cytokines are interferons, interleukins and tumor necrosis factors. Depending on the binding receptors, cytokines can fine tune a cell response. Chemokines are chemotactic cytokines that could attract immune cells to the secretion source, often the site of infection.

Complement is comprised of serum proteins that interact with one another in a catalytic cascade. They can form membrane attack complexes and cause direct lysis of bacteria. Some complement subunits enhance antibody responses and antigen presentation process. Others are involved in clearing immune complexes from tissues and apoptotic cells.

1.2 A typical immune response

Before an antigen is introduced to the body, naïve immune cells circulate between blood stream and lymphoid tissues such as thymus, lymph nodes, spleen and mucosa-associated lymphoid tissue. When the body first encounters an infectious agent, myeloid lineage cells are the first line defense as innate immune system. They can recognize both extracellular and intracellular pathogens through pathogen-recognition receptors such as Toll-like receptors (TLRs). The engagement of receptors initiates killing of pathogens directly or alerts the adaptive immune system to the presence of pathogen. Granulocytes can produce cytokines and chemokines that attract neutrophils and natural killer cells to the infection site. In turn, neutrophils can produce more chemokines and attract antigen presenting cells.

Once antigen presenting cells are attracted to sites phagocyte the pathogens, two types of antigen processing and presentation occur. Type I of major histocompatibility complex (MHC I) presents cytosolic bacterial or viral proteins and interact with CD8+ T cells. These endogenous antigen
proteins are processed by the proteasome and pumped into endoplasmic reticulum through Transmembrane-Associated Antigen Processing (TAP) proteins. Eventually these peptides are loaded onto MHC I molecules and presented at the cell surface for recognition. Type II (MHC II) presents processed extracellular pathogens for CD4+ T cells. Exogenous antigens are taken up, degraded and routed to endosomes. Eventually endosomes fuse with lysosomes, where antigenic peptides are switched for the invariant chain peptide residual that are initially bound by MHC II. MHC II loaded with the foreign peptide is then transported to cell surface for antigen presentation. Antigen presenting cells travel to lymph nodes and present the processed antigens to T cells. Unprocessed antigens also gain access to lymph nodes and become recognized by B cells. These antigens could be opsonized by complements and/or antibody complexes, trapped by specialized macrophages and transferred to B cells.

In the draining lymph nodes, naïve T cells encounter antigen presenting cells and arrest their movements. They cluster around APCs and start to proliferate once they detach. B cells also establish stable interactions with helper T cells through surface receptors such as T cell receptor-MHC II-peptide and adhesion molecules. These signals, especially CD40 engagement, are required for B cell proliferation and differentiation. CD8+ T cells are also activated in lymph nodes via interaction with APCs, CD4+ T cells at the same time. Once activated, they differentiate into cytotoxic T lymphocytes.

Activated lymphocytes leave the lymph node and circulate in the blood stream. Plasma cells travel to different sites, depending on the isotypes of antibodies. Effector and memory CD4+ and CD8+ T cells travel to the site of infection following chemokine and cytokine gradients. The proliferation of all lymphocytes declines within 10-14 days. Once the pathogen is cleared, there is a decrease in inflammatory signals. Activated T cells engage surface receptor for apoptosis, and regulatory T cells release anti-inflammatory cytokines to tune down the immune response.
In response to chemokines, circulating effector lymphocytes express a different profile of cell-adhesion molecules and chemokine receptors that allow them to interact with inflamed vascular endothelium at the infected site. They roll along the endothelial cells, become activated through chemokine and G-protein linked receptors, engage in cell adhesion to the luminal side of the vessel wall through integrin molecules, and eventually undergo transendothelial migration to the infected tissue.

**1.2.1 Cytotoxic T Lymphocytes (CTLs) Activation and Effector Function**

CTLs are CD8+ T cells with cytotoxic effector function, and they can recognize and kill target cells by releasing their lytic granules. The basic process of activation from the perspective of CTLs includes T cell receptor engagement, formation of signalosome, downstream signaling events and effector function (Figure 2). The surface T cell receptor can recognize and bind to MHC I loaded with foreign processed peptides on target cells, and CD8 molecule stabilizes the interaction by binding to nonpolymorphic regions on MHC I molecule. Another important function of CD8 engagement is the delivery of Lck to immune synapse. This is crucial as Lck has tyrosine kinase activity that is lacked in TCR cytoplasmic domain. Lck can phosphorylate the immunoreceptor tyrosine-based activation motifs (ITAMs) in intracellular domains of CD3 subunit chains. Phosphorylated ITAMs then recruit Zeta-chain associated protein (ZAP) kinase to the immune synapse and phosphorylates linker for activated T cells, which recruits multiple proteins to form a signalosome complex and activates downstream signaling events.

Among the many proteins in the signalosome complex, phospholipase C plays a key role in T cell activation, as it cleaves a signal molecule, phosphatidylinositol-4,5-biphosphate (PIP2) into two parts, inositol triphosphate (IP3) and diacylglycerol (DAG). Cytosolic inositol triphosphate binds to its receptor located on ER membrane and causes storage of calcium ion to be released. The fall in calcium level in ER causes the conformational change of stromal interaction molecules (STIM 1), which then bind to calcium release-activated calcium modulator 1 (ORAI 1) on the cell
surface. ORAI 1 is the essential subunit of calcium release-activated calcium (CRAC) channel which is highly selective for calcium ions and can cause calcium influx from extracellular solution when activated\(^\text{10,11}\). Increased intracellular calcium can modulate calmodulin and calcineurin activity, and it is involved in the translocation of nuclear factor of activated T (NFAT) cells from cytosol to nucleus\(^\text{12,13}\). DAG is a membrane-bound molecule that can recruit PKC to the cell membrane by binding to its C1 domain. Conventional PKC activation also requires calcium binding to C2 domain\(^\text{14}\). Upon activation, PKC can phosphorylate the downstream kinases and initiate a three-step kinase cascade: PKC-MAPKK-MAPK. These are important to promote cytoskeleton polarization in activated T cells\(^\text{15}\). Both kinase activation and calcium signaling are indispensable for T cell activation and lytic granule exocytosis.

![Figure 2 An overview of TCR signaling events.](image)

*T cells become activated through the engagement of TCR with antigen-MHC molecules. The recruitment of CD4 (or CD8) results in the phosphorylation of ITAMs on CD3 intracellular domains. This recruits ZAP kinase which activates the key adaptor molecule linker for activation of T cells (LAT) and initiates the signalosome complex. As a result, PIP2 is cleaved into DAG and IP3, activating downstream kinases and triggering calcium release respectively. Figure is adapted from Nature Reviews\(^8\).*
Effector functions of T cell include direct cytotoxicity via lytic granule exocytosis, engagement of FasL/FasR, and secretion of soluble mediators. Microtubule network is polarized towards the immune synapse and positions lytic granules to close proximity. Secretory lytic granules contain granzymes and perforins, and they are released when granule membrane fuses with CTL cell membrane. Perforins can perforate the target cell membrane, and granzymes can pass through those pores into target cell cytosol and initiate caspase-dependent or -independent signaling and cause target cell death. Fas ligand externalized onto CTL surface via lysosomal membrane fusion can engage Fas receptor on target cells and initiate caspase activation cascade. CTLs can also cause indirect killing of target cells by releasing INF-γ and TNF-α. When engaged with these cytokines, INF receptor can upregulate the expression level of Fas receptor and MHC molecules at the target cell surface, and TNF receptor can activate caspase cascades.

**Figure 3** Direct killing of target cells by CTLs via lytic granule exocytosis.

Once activated through TCR, CTLs undergo signal transduction and reorient the microtubule network to form an immunological synapse, releasing lytic granule contents to act on the target cell, resulting in target cell death.
cell membrane perforation and intracellular caspase activation. Figure is adapted from Nature Immunology\textsuperscript{16}.

CTLs are involved in both acute and chronic transplant rejection. Their cytotoxic effects can result in the lysis or apoptosis of non-self cells in transplant organs. The persistent infiltration of T cells in transplants can result in progressive graft function decline\textsuperscript{19}. This is to be distinguished from graft-versus-host-disease, which is a result of transplanted hematopoietic stem cells attacking the recipient tissues\textsuperscript{20}. Current immunosuppressants are targeting T cell biology, including its trafficking, activation, proliferation and effector functions. Non-biologics such as glucocorticoids and rapamycin have side-effects on immune cells other than T cells. Calcineurin is involved in CTL activation and proliferation, however calcineurin inhibitors such as cyclosporine A have severe toxicity and cannot be used to prevent long-term transplant rejection\textsuperscript{19}.

CTLs are also found to be involved in the pathogenesis of autoimmune diseases such as rheumatoid arthritis due to their autoreactive inflammatory behaviors\textsuperscript{21}. Patient samples showed the presence of CTLs in both synovial fluid and synovial membrane, and they can secrete pro-inflammatory cytokines that potentiate bone degradation by stimulating osteoclasts\textsuperscript{22}. CD8+T cells also play a role in the establishment of germinal centers in rheumatoid arthritis patients, which are follicular structures resembling secondary lymphoid follicles\textsuperscript{23,24}. Current therapies of autoimmune disease are mostly biologics, and they are focused on inflammation control in order to relieve the disease symptoms\textsuperscript{25}. However, biologics such as anti-TNF-\(\alpha\) and CTLA4 antagonist are limited by cell surface receptors or extracellular cytokines as targets. The complicated heterogeneity of autoimmune disease makes it hard to design specific immunosuppressant biologics. The administration dosage is also limited for biologics.

1.3 Immune modulation is important

The immune system is tightly regulated. On the one hand, results from normal immune activity may become detrimental sometimes, such as in allergy and transplantation. In the case of allergy,
T and B cells become activated and undergo clonal expansion in response to allergens such as food antigens, autoantigens, infectious agents and superantigens. They migrate to tissues and initiate inflamed reactions. This in turn results in prolonged survival of inflammatory cells, cytokine production, chemokine release, and tissue remodeling even function loss. In the case of transplantation, recipient's immune system can recognize new graft or transplant from the donor and attack them, leading to immediate or chronic transplant rejection; T cells from hematopoietic stem-cell transplant can recognize recipient's tissues as foreign and attack them, leading to graft-versus-host disease. On the other hand, dysregulated immune activity is often associated with disease. A dysregulated immune system can be either overactive or under-responsive. An overactive immune system often leads to self-attack, the onset of autoimmune diseases, where the immune system mistakenly mounts a response against self-tissues or organ. In comparison, an under-responsive immune system is unable to clear transformed cells, leading to tumor evasion and metastasis. Therefore, modulating the immune system activity to a proper level is critical to treat diseases, alleviate syndromes and prevent transplant rejection.

Immune modulation is the regulation of the immune system activity using pharmaceutics, and it has two directions of modulation. Down-regulatory modulations using immune inhibitors such as cyclosporine A and glucocorticoids have proven useful in treating autoimmune diseases and to prevent transplant rejection. Up-regulatory modulations using immune augmentors such as imiquimod and interleukin-2 have been shown to improve pathogen recognition and cancer immunotherapies.

1.3.1 Currently available immune modulators and their limitations

Currently available immune modulators can be categorized into immune inhibitors and immune augmentors. This section will give some specific examples of immune modulators, explain their mechanisms and list their limitations. It is worth noting that some drugs categorized into one mechanism could have effects on other mechanisms as well.
For immune inhibitors, some drugs work by blocking cell proliferation in general. When responding to an antigen, lymphocytes need to undergo activation and proliferation to generate enough effector cells. An anti-proliferative agent or antimetabolite can block activated lymphocyte proliferation and reduce the number of effector cells. Glucocorticoids bind to intracellular glucocorticoid receptors and block genes involved in cell proliferation\(^{31}\). Another mechanism in glucocorticoid-induced immune inhibition is glucocorticoid-induced leucin zipper that inhibits CD3-dependent T cell activation, which explains its broad innate immune suppressing effects\(^{32,33}\).

Methotrexate was first introduced as an antiproliferative agent that blocks purine biosynthesis and works as a mitotic inhibitor. Later it was found that methotrexate also increases extracellular adenosine level, which binds to adenosine receptors on the plasma membrane of many immune cell types. Adenosine is a potent endogenous mediator with anti-inflammatory effects. When adenosine receptor is activated, it interrupts superoxide anion formation in neutrophils and protects the vascular endothelial cells. Adenosine can also inhibit Fc-\(\gamma\) receptor in monocytes and inhibits their phagocytic activity\(^{34}\). Both glucocorticoids and methotrexate have been in use since the 1950s and are still the gold standard in treating rheumatoid arthritis even with long-term side effects, including osteoporosis, metabolic disease and increased risk of cardiovascular disease\(^{35,36}\).

Some immune inhibitors are monoclonal antibodies or decoy receptors, and they can reduce the number of activated immune cells or proinflammatory cytokines in circulation. For example, IL-2R antagonist can block the proliferation signal needed for activated T cells\(^{37}\). Antithymocyte globulin is synthesized by other animals in response to human thymocytes, and it can bind to human lymphocytes \textit{in vivo} and results in their phagocytosis\(^{38}\). They can both reduce activated lymphocyte number in circulation. A major proinflammatory cytokine is TNF with various effects on immune responses. There are five approved biologics that target the proinflammatory cytokine TNF, and they are among the best sellers on the drug market. Infliximab, adalimumab,
Certolizumab, golimumab and etanercept can bind to both the soluble and the transmembrane form of TNF\textsuperscript{39}. Although both forms of TNF are biologically active, the soluble form drives the inflammatory response, and the transmembrane form maintains the innate immune response to infections such as tuberculosis, listeriosis and leishmaniasis\textsuperscript{40,41}. Inhibiting both forms at the same time could explain the side effects associated with currently available TNF inhibitors, including active tuberculosis, invasive fungal infections, viral or bacterial infections due to opportunistic pathogens including \textit{Listeria} and \textit{Legionella}\textsuperscript{39}. IL-1 is a highly active proinflammatory cytokine involved in autoinflammatory diseases such as gout and type 2 diabetes. Rilonacept is a decoy receptor for soluble IL-1. It binds to IL-1, prevents it from binding to its endogenous receptor, and reduces its inflammatory effects\textsuperscript{42}.

There are small molecule immune inhibitors that act more specifically to target signaling events involved in immune cell activation. For example, cyclosporin A inhibits calcineurin activity, a key phosphatase that removes phosphate groups from NFAT protein, unmasking its nuclear localization sequence and allowing it to bind \textit{IL-2} promoter gene sequences\textsuperscript{43–45}. Rapamycin binds to mammalian target of rapamycin (mTOR) and blocks T cell activation, proliferation and differentiation, currently in use to prevent kidney transplant rejection\textsuperscript{46}. Jak-STAT inhibitor such as tasocitinib inhibits the important kinase involved in cell proliferation and survival\textsuperscript{47}. Fingolimod was first synthesized from an immunosuppressive natural product, myriocin, and it is found to interact with sphingosine 1-phosphate (S1P) receptors and can reduce peripheral lymphocyte count in multiple sclerosis\textsuperscript{48}. S1P is one member of the lysophospholipids (LP) family, which bind to specific cell surface G-protein coupled receptors to transduce signals. Other members of LPs family and their receptors are also explored for their involvement in autoimmune diseases, neurodegenerative diseases, fibrosis, pain, cancer, inflammation etc\textsuperscript{49}. Recently, lysophosphatidylserines (lyso-PS) is found to be involved in neurological disease polyneuropathy\textsuperscript{50}. ABHD12 was identified to be the mutant gene, and it functions as a major
brain lyso-PS lipase to generate more lyso-PS in cells\textsuperscript{51,52}. A small-molecule inhibitor of ABHD16A, KC01, can deplete lyso-PS from cells, including lymphoblasts derived from subjects with polyneuropathy, indicating the central role of ABHD12-ABHD16A in lyso-PS metabolism and potential targets for treating neuroimmunological disorders\textsuperscript{53}.

For immune augmentors, most pharmaceutical interventions are antibodies against cell surface receptors that serve as inhibitory immune checkpoints. For example, T cell surface molecule programmed death-1 (PD-1) binds to programmed death-1 ligand 1 (PD-L1) expressed by many tumor types and sends an inhibitory signal to T cells by antagonizing PI3K activity directly\textsuperscript{54}. Therefore, antibodies against either PD-1 or PD-L1 can interfere their binding and overcome the inhibitory effect of PD-1 engagement. Some examples of anti-PD-1 are nivolumab (by Bristol-Myers Squibb), MK3475 (by MERCK), pidilizumab (by Medivation). Some PD-L1 antibodies are BMS-936559 & MPDL3280A (by BMS and Genentech). Both PD-1 and PD-L1 antibodies are currently used to treat metastatic melanoma, renal cell carcinoma and non-small cell lung cancer\textsuperscript{55}. Another example is T cell surface molecule cytotoxic T lymphocyte antigen-4 (CTLA-4), which competes with CD28 to bind to B7-1 and B7-2 on APC cell surface. CTLA-4 inhibits downstream Akt and generates an inhibitory signal that blocks T cell response\textsuperscript{56}. Therefore, an anti-CTLA-4 antibody can overcome this inhibitory immune checkpoint. Some examples include ipilimumab (used to treat metastatic melanoma, by BMS) and tremelimumab (by MedImmune LLC)\textsuperscript{57}. Even innate immunity can be enhanced with biologics. Irilumab is a newly licensed, first-in-class antibody by Bristol-Myers Squibb against natural killer cell immunoglobulin-like receptors (KIRs). KIRs are expressed on NK cell surface to interact with MHC I on normal cell surface and prevent NK cell killing of healthy cells\textsuperscript{58}. Irilumab can overcome the inhibitory checkpoint by blocking KIRs interaction with MHC I and increase NK cell activity in killing multiple myeloma cells and acute myeloid leukemia blasts\textsuperscript{59–61}. 
Antibody-based immune modulators are associated with immune related adverse events (irAEs), such as inflammatory infiltration of solid organs by T cells and increased serum inflammatory cytokines. These could have severe side effects and lead to inflammatory pneumonitis, thyroiditis, inflammatory diarrhea/colitis. All antibody-based therapies require intravenous injection, and their dosage is often limited to reduce side effects. Monoclonal antibodies are also limited by their targets to cell surface receptors or soluble cytokines due to their large molecular size and poor membrane permeability. To add on that, antibodies are expensive to manufacture, and their stability and bioavailability in vivo are hard to predict. A recent trend in antibody drug design is to use fragment antibodies such as antigen-binding fragments (Fab), single-chain variable fragments (scFv), and third generation (3G). Due to a lack of Fc domain, fragment antibodies are subject to rapid degradation and short circulating half-life. It also increases the risk of antibody aggregation during production and purification. When used in vivo, fragment antibodies cannot initiate Fc-mediated functions such as antibody-dependent cell-mediated cytotoxicity and complement-dependent cytotoxicity.

Small molecule immune augmentors may be more desirable, because they are easier to administer and cheaper to manufacture. However, they are greatly limited by number. Currently, there are only two small molecule immune modulators available, imiquimod and lenalidomide.

Imiquimod belongs to a group of synthetic small molecule drugs called imidazoquinolones. They were first synthesized in an effort to find antiviral drugs using nucleoside analogs. Imiquimod activates Toll-like receptor 7 (TLR7) and triggers MyD88/NFkB cascade, activating proinflammatory cytokine production of INF-α, TNF-α, and IL-12. These proinflammatory cytokines create a milieu biased towards Th1 cell mediated immunity with enhanced killing of target cells hosting intracellular virus or bacteria. It functions as an innate immune response modifier and is currently used at 5% in a crème to treat external genital warts, superficial basal cell carcinoma, and also some other skin diseases.
Lenalidomide is an analog of thalidomide that was first introduced to treat morning sickness in pregnant women. Due to its association with severe birth defects and neuropathy, it was withdrawn from the market\textsuperscript{76,77}. But later thalidomide was found to have anti-angiogenic properties (which explains its side effects of birth defects) and T-cell costimulatory activities\textsuperscript{78,79}. It was investigated as a treatment for certain cancers. In an effort to look for more molecules with immune augmenting effects, analogs of thalidomide were synthesized and tested\textsuperscript{80}. Lenalidomide and pomalidomide are both structural analogs of thalidomide with potent anti-tumor and immune costimulatory effects\textsuperscript{81,82}. Currently lenalidomide is used to treat multiple myeloma and other B cell neoplasms, and it is used in combination therapy with monoclonal antibodies\textsuperscript{83}. Interestingly, the exact molecular mechanism for lenalidomide remained unknown until recently, which did not interfere with its marketing as an anti-tumor small molecule augmenting immune cell activities. Lenalidomide recruits two zinc finger transcription factors to E3 ubiquitin ligase complex and cause their ubiquitination and subsequent degradation. Both transcription factors are important for B cell differentiation, and multiple myeloma cells require ongoing expression of both for survival\textsuperscript{84}.

Adjuvants work as immune augmenting agents, and when used in combination with vaccines for inoculation, they can greatly enhance the immune system activity. Effects of adjuvants include a depot effect or the slow release of antigens (oil emulsions), and the non-specific activation of phagocytes and all leukocytes (alum precipitate and bacterial toxins). Complete Freund’s Adjuvant is the most potent adjuvant for immunizing experimental animals, and it is a combination of mineral oil and killed \textit{Mycobacteria}. The mineral oil slows down the antigen release, and the bacterial wall peptidoglycan serves as pathogen-associated molecular patterns. Alum precipitate, a combination of aluminum hydroxide and aluminum phosphate, is used in some human vaccines. It has been shown to activate NLRP3 inflammasome, thereby enhancing IL-1 and IL-18 secretion, leading the activated T cells to become Th2 cells, which promote strong antibody responses\textsuperscript{1}. 
Recent efforts in finding new adjuvants are focused on pattern-recognition receptors, such as less harmful versions of lipopolysaccharides (monophosphoryl lipid A and glucopyranosyl lipid A). TLR3 ligand poly I:C (synthetic double-stranded RNA) can activate innate immune response and is investigated in clinical trials for antitumor vaccines. TLR9 ligand CpG DNA (bacterial DNA mimic) preferentially elicits Th1 response for inducing cell-mediated cytotoxicity.

Interestingly, the heat shock protein (HSP) family, known for their functions as molecular chaperon for unfolded proteins under physiological and stress conditions, is found to be involved in antigen processing as well. Certain members of HSP family can augment tumor antigen processing by dendritic cells and enhance NK cell killing. Therefore, HSP is being investigated as an antitumor adjuvant. When incubated with purified tumor antigens under heat shock conditions, HSP binds to the exposed hydrophobic regions and help form a recombinant tumor antigen, such as in the case of breast cancer antigen her-2/neu+HSP110.

Other innate immune stimulators can also be developed into adjuvants. For example, C-type lectin receptors (CLR) such as Dectin-1 and -2, contain carbohydrate-binding domains and can recognize carbohydrate motifs on microbes. Beta-glucan is found in the cell walls of bacteria, yeast and fungi, and it is a known ligand of Dectin-1. Recognition of beta-glucan by Dectin-1 activates the spleen tyrosine kinase-NFκB axis to induce pro-inflammatory cytokine production.

Retinoic acid-inducible gene I (RIG-1)-like receptors (RLRs) are cytoplasmic RNA sensors that can activate interferon promoter stimulator 1 (IPS1) to induce type 1 interferons. There are three members within the family of RLRs: RIG-1, melanoma differentiation associated factor 5 (MDA5), and laboratory of genetics and physiology 2 (LGP2). During viral infection, the repressor C terminal domain of RLRs bind to RNA-PAMP, undergoes a conformational change and releases the caspase activation and recruitment domains (CARD), allowing it to interact with IPS1, and RLRs can enhance antibody development driven by vaccine administration.
Scripps Research Institute holds a patent of several small molecules used for RIG-1 modulation (Patent WO2011115892 (A1)).

Stimulator of interferon gene (STING) is an ER adaptor molecule that can induce type 1 INF production in response to intracellular DNA. They mediate DNA-based adjuvant activity. Cyclic dinucleotides are agonists of STING and are being used as vaccine adjuvants for anti-tumor therapy (Johns Hopkins University and Aduro Biotech, Patent ID US2015010613 (A1)).

Bromodomain-containing proteins (BRD) are components of transcription factor complexes and determinants of epigenetic memory. Several members have been shown to fuse with nuclear protein in testis (NUT) and form novel fusion oncogene BRD4-NUT found in highly malignant form of epithelia neoplasia. Modulating BRD may be useful as a treatment for cancer by altering the epigenetic expression of certain genes in cancer cells. Dana-Farber Cancer Institute has patented the method of combining a BRD inhibitor and an immune modulator for hematological cancer treatment.

The Tufts University holds a patent for a small molecule inhibitor, PT-100, of mammalian dipeptidyl peptidase (DPP) IV activity and/or structural homologues thereof (DASH) serine proteases. DASH is involved in tumor progression, immune system regulation and T cell activation. Recently they have developed the second generation of DASH inhibitor, ARI-4175, which can modulate immune-cell subsets and function in mice.

P-glycoprotein ABCB5, an ATP-binding cassette transporter, is found to be highly expressed in melanoma cells, which may contribute to the resistance of melanomas to chemotherapy by efflux of anti-cancer drugs. Bioxcel Corp. holds a patent of anti-ABCB5 antibody, when used in combination with PD-1 agonist, can induce IL-2 production in the mixed cultures of hPBMCs and melanoma cell lines (Patent WO2016179576 (A1)).
The University of Texas holds a patent of using a small molecule tryphostin to treat immune suppression and enhance T cell functions. Tryphostin induces immunostimulatory cytokines, costimulatory molecules and intracellular signaling in macrophages, and enhanced phosphorylation of Lck and ZAP-70 was observed (WO2008121858 (A1)).

In conclusion, immune modulators, especially small molecules as immune augmentors, are severely limited by numbers. There is a great unmet medical need to look for novel small molecule immune modulators. Traditional basic research aims to study cell phenotypes and their mechanisms, aiming to yield potential molecular targets that underlie the phenomenon. These targets can then be used to design target-based assays to screen for potential drug candidates to modulate the target activity. These candidate molecules will then need to be tested in relevant cell or animal models to observe their effectiveness in modifying the phenotype. In comparison, a phenotypic assay allows us to bypass the intermediate steps and test compounds based on their effects on cell phenotype. Any compound that shows activity in phenotypic screen based on an immune cell assay is an immune modulatory compound. Even with their mechanisms unknown, small molecule augmentors can still be useful in treating tumors as in the case of lenalidomide.

1.4 Immortalized CTLs and their advantages for screening

CTLs serve as an excellent cell model to look for immune modulators. They share similar activation mechanisms as other immune cell types such as T helper cells and B cells. Inhibitors that block the activity of CTLs could also inhibit other immune cells activated by similar mechanisms. Other immune cells are not suitable cell models for HTS. For example, measurement of T helper cell activation through the secretion of IL-2 secretion using ELISA takes hours and multiple washing steps, whereas CTL lytic granule exocytosis takes place within 10 minutes, greatly decreasing the time needed to conduct HTS. Using monensin or brefeldin A can
block cytokine secretion, but the intracellular flow cytometry analysis requires cells to be fixated and permeabilized.

CTLs have a reliable protein marker that can be easily measured in flow cytometer. Lysosome-associated membrane protein-1 (or LAMP-1, also known as CD107a, referred to as LAMP in the rest of the thesis) is located on the lysosome membrane in unstimulated CTL cells. When cells become activated and undergo lytic granule exocytosis, lytic granule membrane fusing with cell membrane. As a result, LAMP gets externalized to the cell membrane, which can be detected via fluorescent antibodies present in the extracellular solution. The binding of LAMP antibody can be measured in flow cytometer without washing away excess antibodies, an important design feature for HTS. The signal for LAMP externalization is robust, giving the assay a large separation band between negative and positive samples to detect any changes.

CTL inhibitors identified through a phenotypic screen could be working on novel molecular targets. By conducting follow up assays, we can test inhibitors against the known signaling pathways involved in CTL activation. As a result, compounds with novel mechanisms could be identified and used as chemical probe candidates to study CTL biology and activation mechanism.

Therefore, our assay serves as a general screen for lymphocyte inhibitors. There is no other easy way of devising an immune cell-based assay looking for immune modulators.

T Acute Lymphoblastic Leukemia (TALL)-104 immortalized cells were used as model cell line for CTLs. TALL cells were first introduced in the early 90s by Santoli group at Wistar Institute of Anatomy and Biology in Philadelphia. TALLs demonstrated cytotoxic effects against a wide range of targets and can sustain tumor killing activity for 2 years in vitro\textsuperscript{100}. TALL cells had been used as cell model system in previous research to investigate signaling events required for CTL activation\textsuperscript{101–104}. TALL-104 cells are αβ T-cells but they kill in MHC non-restricted manner\textsuperscript{100}. In particular, TALL cells can be activated with small molecules that bypass TCR and result in lytic
granule exocytosis\textsuperscript{105}. LAMP externalization as a measurement of CTL cell activation and lytic granule release is also well established in TALL cells\textsuperscript{106}.

LAMP-1 is used as a protein marker for cell activation in our assays. It also has physiological functions. LAMP-1 belongs to the LAMP family. There are also LAMP-2 and -3. LAMP-2 is involved in sorting perforin from the trans-Golgi network into outgoing transport vehicles, with adaptor protein-1 as part of the sorting complex\textsuperscript{107}. In humans, mutations in LAMP-2 cause Danon disease, a fatal myopathy, likely a result of autophagic material accumulation in the striated myocytes\textsuperscript{108}. In mice, LAMP-1 deficiency results in an upregulation of LAMP-2 but no other phenotypic changes\textsuperscript{109}. Evidence from cDNA shows that LAMP-1 and -2 are homologous proteins that share similar structures and sequences, which could explain for their overlapped functions\textsuperscript{110,111}. As a result of this, LAMP-1 function still remains unclear, yet deletions of both LAMP-1 and LAMP-2 cause embryonic lethality in mice\textsuperscript{112}. LAMP-3 is mainly expressed in lungs and activated dendritic cells, hence they are also known as dendritic cell-LAMP\textsuperscript{113}. LAMP-3 is found to be upregulated upon proteasomal inhibition, therefore deleting LAMP-3 increase cell vulnerability to proteasomal inhibition\textsuperscript{114}. Apart from LAMP family proteins that make up \textasciitilde{}50\% of all lysosomal membrane proteins, there are also other membrane proteins such as lysosomal integral membrane proteins, lysosomal membrane glycoproteins, and V-type H+ ATPase pump which is critical to acidify the lysosomal lumen\textsuperscript{110,115,116}.

1.5 Two types of high-throughput screens: phenotypic and target-based approaches

Traditional drug discovery was experience-oriented, mostly based on serendipitous findings or isolation of the active ingredients from traditional remedies. Starting in the 1980s, advancement in technology drove the modern drug discovery campaigns towards an era of high-throughput screening (HTS)\textsuperscript{117}. The process of HTS involves testing large numbers of chemicals from a
library collection against a cell line or a molecular target in an automated fashion, in order to rapidly identify compounds with activities that could alter the phenotype or target activity toward the desired level. These active compounds or mixtures are referred to as “hits”, and they are often the starting points of modern drug development process.

There are two approaches in HTS: the phenotypic approach and the target-based approach. A phenotypic screen is carried out with cells, tissues or even animals. The phenotypic screen is more physiologically relevant and less artificial, because intact cells are used for screening and the interrogated system is its native state. By definition, hits from a phenotypic screen demonstrate activity on the systemic level\(^1\), yet mechanisms of phenotypic hits could remain unknown. To identify the molecular mechanism of action (MNOA) for these hits, follow up experiments are often performed. Effects of hits are tested against known signaling events involved in the onset of the phenotypic change\(^1\). In comparison, a target-based screen is conducted on select molecular targets, such as protein kinases, ion channels or certain genes in a reporter assay. These targets are chosen because they demonstrate a causal link to the disease phenotype. Affecting the target activity is believed to alleviate the disease state. Hits from a target-based screen are active against the target that the assay was designed for. To ensure that these hits can indeed affect the phenotype, they need to be tested in follow up experiments using relevant cell lines or animal models.

In the pipeline of drug discovery, phenotypic screens are often the starting point as they can be conducted with limited understanding of mechanisms\(^2\). Records have shown that phenotypic screens yield more first-in-class medicines\(^1\). Since phenotypic hits could be targeting any molecular targets involved in the altered phenotype, it has the potential of obtaining useful hits as probe candidates to identify novel molecular targets through affinity-based methods. These novel targets, once established as valid targets, can then become candidates for target-based screens in the next stage. The target-based approach is more successful in identifying best-in-class
medicines\textsuperscript{122}, and it offers important support for a molecular target as a therapeutic target, because the molecular target activity can be altered using perturbants. Both approaches are helpful in generating candidates, yet the phenotypic approach in particular contributes to the progression of knowledge.

Historically, phenotypic screening was the classical way of drug screening. For example, as part of anticancer drug screen effort, 60 representative cancer cell lines were selected and screened against NCI library of over 40,000 compounds in a cell proliferation assay\textsuperscript{123}. The phenotypic approach went out of fashion with the emergence of genome sequencing and protein studies, but now it is becoming popular again\textsuperscript{124}. Although the phenotypic screen has less prevalence in drug discovery effort, it has been more successful in identifying new drug candidates, especially those working on novel targets\textsuperscript{122}. Recently, with the advantage of image acquisition and data processing platforms, image-based screen is becoming popular. This is one type of phenotypic approach that involves taking pictures of compound-treated cells or organisms followed by image-based data processing. Since multiple measurements can be collected from each individual image, it is called high-content screen. It is worth noting that with the advances in modern flow cytometry, flow cytometer-based assays can also become high-content screens. A new technique of cell labeling using fluorescent dyes has emerged, also referred to as “fluorescent cell barcoding” (FCB)\textsuperscript{125}. By using combinations of dyes with different fluorescence spectra and at different loading levels, multiple cell samples can be mixed together for compound treatment or phospho-staining, sampled by the flow cytometer, and later deciphered based on dye loading. Combining the powerful FCB technique with many fluorescence channels available on modern flow cytometers enables researchers to investigate more than one parameter of cells at a time\textsuperscript{125}.

The final outputs of phenotypic and target-based approaches in HTS reflect their underlying ideology of holistic and reductionism: the holists believe in polypharmacology, and that a phenotypic approach can reveal drug candidates that interact with multiple targets like “magic
shotguns’. In comparison, reductionists correlate the origin of a disease with a single target, such as an overexpressed protein or a mutant gene, and the target-based approach can identify compounds highly active against one particular target like a “silver bullet”\textsuperscript{126}. Since the target-based approach is a hypothesis-driven process, it could fail to yield any useful drug candidates if the hypothesis is false and proof-of-concept studies fail to reject it.

1.5.1 Why a phenotypic assay?

A phenotypic assay measures the final cell activity as the output, and it can be performed with limited knowledge of underlying molecular mechanisms of action. Compounds identified active through this approach are guaranteed to work on the cellular level, and they are not limited by potential targets. When proper follow up testing was carried out on active compounds to investigate their molecular of action, we could obtain compounds that work on both known and yet to be identified molecular targets. For the purpose of obtaining specific probe candidates, a phenotypic assay is more feasible than a target-based approach.

We choose to use flow cytometry to detect fluorescent antibody binding as a result of granule exocytosis. Flow cytometers take up cell samples in suspension and then focus them into a single cell stream via hydrodynamic focusing. Individual cells are interrogated by a laser beam with minimum amount of bathing solution included. This feature limits the interference from background fluorescence present in the solution, making the flow cytometer a better platform than the plate reader in reducing background noise. The plate reader samples the fluorescence of each well uniformly, and it does not differ cell-associated fluorescence from solution-associated fluorescence. In flow cytometry, cells do not need to be washed to remove excess unbound antibodies, which is also favored for HTS. Flow cytometer can easily measure the fluorescence of thousands of individual cells in under a minute, from which the average cell activity of the population can be determined. This is much faster than microscopy when the same number
measuring the cell fluorescence intensity of several fields in microscopy, and the data analysis is much faster in flow cytometry than fluorescent microscopy.

The invention of the HyperCyt platform enables higher throughput of cell samples in flow cytometer-based assays. HyperCyt uses peristaltic pump coupled with an autosampler, and each well sample is separated by an air gap due to continuous sampling of the sip. A 1536-well plate can be sampled by four sips coupled to four cytometers at the same time to reduce the overall sampling time (<15 min). The software can then deconvolute the flow data for individual well based on the air gap-separated feature\textsuperscript{127}.

1.6 Types of Libraries used in HTS

Chemical libraries are collections of stored chemicals usually used in HTS to look for potential drug candidates. There are two general types of chemical collections for screening: synthetic and natural product (NP). Some drugs are defined botanical mixture, NP-derived, NP mimics or synthetic drugs but with NP pharmacophores, and they belong to the NP category. A review of new approved drugs over 33 years from 1981-2014 (n=1562) showed that 51% are NP, NP-related or NP-inspired synthetic drugs, and only 27% are synthetic drugs\textsuperscript{128}. It has been recommended that more NPs should be incorporated into libraries for screening, as the current medicinal chemistry space does not overlap with the bioactive space\textsuperscript{129}. There are also discussions as to how to increase the biogenic bias in chemical library design, in order to include more diverse ring scaffolds into library collections for screening\textsuperscript{130}.

Over the past 30 years, technology development in monoclonal antibody production has enabled library collections of antibodies to be used for screening, and Boehringer Ingelheim is a leading pharmaceutical company in this area. However, biologics library development requires high-end facility for animal maintenance, and library collections of this type are often proprietary and
inaccessible for outside screening. Therefore, they are not included in the discussion of this section.

Traditional drug discovery relied on NPs, and people in all cultural groups have been looking for therapeutic remedies from natural sources for thousands of years. The earliest written record in traditional Chinese medicine dates back to 200 AD, yet a collection of 365 herbal plants were compiled into Shen-nung Pen-tsao Ching, during a historical time believed to be around 2800 BC. Many remedies are used even nowadays such as ginseng, jimson weed and cinnamon bark. The Indian holy books Vedas (1500-1000 BC) mentioned 107 miracle herbs as plant-based treatment for different ailments, and some ingredients are used today as culinary spices such as nutmeg, cardamom, clove. The Ebers Papyrus (1500 BC) is an Egyptian medical papyrus containing 700 formalas and remedies, and it covers some interesting topics including contraception, intestinal disease and parasites, eye and skin problems, dentistry and the surgical treatment of abscesses and tumors\(^\text{131}\).

NPs are small or intermediate size molecules produced by biological sources. They can be directly isolated from natural sources such as herbal extraction, or they could be secondary metabolites synthesized and secreted by organisms. Compared to synthetic small molecules, NPs have more diverse chemistry and novel structures; they are synthesized by organisms, so by definition they have binding partners in nature; they possess privileged structures and drug likeliness due to the long evolutionary history\(^\text{132}\). With modern technology in extraction and purification, pure compounds can be isolated to achieve higher efficiency in modulating target activity, such as the successful extraction of artemisinin that is used to treat malaria\(^\text{133}\). NP could be difficult for de novo synthesis, so efforts have been put into devising strategies for semisynthesis from NP precursors as starting molecules, such as the synthesis of paclitaxel from 10-deacetylbaccatin\(^\text{134}\).
Libraries of synthetic molecules emerged as new sources for drug screening campaigns in the last century. Synthetic molecules through organic chemistry or medicinal chemistry are usually derived from a common scaffold. The molecular weight range is lower than NP, and they tend to be more hydrophobic. The hypothesis is that most molecular targets are regulated through the active sites or allosteric sites with hydrophobic pockets, and drug-like molecules should have the same hydrophobic feature. Also, hydrophobic small molecules tend to cross the plasm membrane more easily and bind intracellular molecular targets. Indeed, most commercial vendor libraries are dominated by small, simple, sp2-rich heteroaromatic compounds\textsuperscript{135}. Once identified as hits, synthetic molecules are easier to conduct follow up assays such as structure-activity-relationship. If selected as drug candidates, synthetic molecules are cheaper to manufacture, easier to upscale the production. To ensure oral availability, Lipinski’s “rule of five” was proposed for small molecule drug design\textsuperscript{136}: molecular mass <500 Da, number of hydrogen-bond donors <5, number of hydrogen-bond acceptors <10 and calculated octanol–water partition coefficient (LogP) <5. However, synthetic molecules could contain undesirable chemical groups that affect cell viability. Secondary assays are necessary to rule out simple cytotoxicity effect of synthetic molecules.

Recent advancement in computer technology and large chemical databases also allows for in silico screening, or virtual screening, where a ligand-docking assay is performed in the computer software. The assessment of binding affinity between a target protein and compound collections in libraries can be performed without bench work, and it has been shown to improve the accuracy of HTS hit list, such as in the case of angiogenin inhibitor screen for small molecules with anti-angiogenic properties\textsuperscript{137}. This type of screening requires the molecular target to be structurally deconvoluted at high resolution, and prior knowledge must be obtained through crystallography.

Apart from randomized library collections, there are also targeted libraries, such as kinase inhibitor panel, GPCR agonist/antagonist library, PI3K/Akt inhibitor library, ion channel ligand library, etc. SelleckChem offers commercial library collections of different types. Particularly, FDA-
approved drug library (Prestwick Chemical Library) has been routinely used as a small sized library in pilot screening for assay validation or optimization. Since all chemical collections are FDA-approved drugs, this type of libraries serves as a platform for drug repurposing. Some pharmaceutical companies also have a subset collection of chemical libraries with drug-like or lead-like properties, and they can be used for pilot screening in a new assay.

1.7 Goal of my study

The goal of my study is to develop and refine cell-based assays of CTL lytic granule exocytosis so that they have the greatest ability to detect interesting compounds to serve as immunomodulators. To achieve this, I used different methods to trigger CTL activation and increasingly sophisticated designs for assay multiplexing.

The initial version of the assay used small molecule stimulants to achieve the maximal level of cell activation, which allowed for immune inhibitors to be identified. The ultimate purpose of this inhibitor screen was to obtain hits, subject them to mechanism testing, and, by a process of elimination, identify novel inhibitory compounds that could be used as chemical probes for ultimate target identification. An HTS was carried out using this assay format and confirmed hits were put through secondary assays to analyze their MOA. This revealed several novel inhibitors that could be used as chemical probes in target identification. The initial assay was a good way to screen for look for immune inhibitors, but the maximal stimulation of cell responses did provide an opportunity to screen for augmenting compounds.

In the second version of the assay, CD3 antibody-coated beads were used to achieve a submaximal activation level of CTLs, which allowed for immune augmentors screening. In addition, changes in light scatter caused by bead binding allowed us to separately analyze cells bound or not bound to beads in a mixed sample. This allowed us to distinguish between immune stimulators and immune enhancers. We differentiated immune enhancers, compounds that
enhance the cell response after they become activated via anti-CD3 bead binding, from immune stimulators, compounds that stimulate cells on their own. This is the first dimension of multiplexing the assay. A natural product library was screened, and we identified an immune enhancing hit. The hit was shown to contain teleocidin A-1, a known PKC activator, a mechanism that could enhance CTL lytic granule exocytosis. We next screened an NCI diversity set library collection and found more immune augmentors as well as inhibitors, yet they were only active at high concentrations. A short incubation time of cells with compounds could explain why only compounds active at high concentrations were identified in such screening.

In the third version of the assay, we sought to increase the power even more by including multiple treatment times with compounds. An inert fluorescent dye was used at different concentrations to label cells before they are added to compound-containing plates at different time points. Being able to test compounds effects at multiple time points is the second dimension of multiplexing the assay. This allowed compounds with multiple modes of actions to be identified. Immediate effect targets could be calcium release inhibitors, PKC inhibitors, whereas long-term effect targets range from activated PKC degradation to gene activation. The NCI diversity set was re-screened at short and long-time points, revealing several immune augmenting compounds that showed activity after long-term incubation.

With each multiplexing step of the assay, the power is greatly increased to screen for interesting compounds that can act as immune modulators. The assay based on CTL granule exocytosis serves as a quick and robust cell model to reveal active compounds in HTS. Combined with follow up testing for molecular mechanism of action for hits, compounds can be categorized into two groups, those working via known mechanisms that can then be tested in other immune cells, and more importantly, those working via novel mechanisms that can then be developed into chemical probes to identify their targets (Figure 4).
In forward chemogenomics, a cell-based assay is used to screen for small molecules that can alter the phenotypic activities of a cell. By studying their mechanisms of action and ruling out those working on known targets, novel hits can be developed into chemical probes that can then be used to reveal novel molecular targets. This direction answers the question: what is the underlying molecular mechanism that is driving a cell phenotype? Reverse chemogenomics typically uses a target-based assay to screen for small molecules that can alter the target activity. These molecules are then tested in cell systems to determine whether they could also result in phenotypic changes. They serve as tool molecules in the traditional target validation. This direction answers the question: is the molecular target sufficient in causing the cell phenotype?
Chapter 2: Materials and Methods

2.1 Chemicals, Cells, and Solutions

TALL-104 cells were obtained from American Type Culture Collection (Manassas, VA) and grown in Iscoves’s modified Dulbecco’s medium (Sigma I3390) supplemented with 1% pen-strep, 2% L-glutamine, 10% characterized fetal bovine serum and 200 IU/ml human recombinant IL-2 (NCI Frederick). They were grown in a humidified incubator at 37 °C in 10% CO₂. Ringer’s Experimental saline (ES) was composed of 155 mM NaCl, 4.5 mM KCl, 1 mM MgCl₂, 2 mM CaCl₂, 5 mM HEPES, and 10 mM glucose. The pH was adjusted to 7.35-7.45 with NaOH, and 2% (w/v) Bovine Serum Albumin (BSA) was added to make 2% normal Ringer’s solution (2%NR). Powder resupplies including analogs of substance ID (SID) 7977862 were from Chembridge (San Diego, CA) and the University of Kansas. Powder resupply of SID103159345 was synthesized by Dr. Jeff Aubé, then at the University of Kansas. Alexa647-conjugated anti-LAMP antibody (0.5 µg/mL final concentration) was purchased from BD Pharmingen (San Jose, CA). Phorbol 12-myristate 13-acetate (PMA) was from Alexis Biochemicals (San Diego, CA). Thapsigargin (TG) was from AdipoGen (San Diego, CA). Paraformaldehyde 16% (PFA) was from Alfa Aesar (Ward Hill, MA) and was diluted to 2% in phosphate-buffered saline (PBS). Dynabeads coated with anti-CD3 or anti-CD8 monoclonal antibody (Thermo Fisher, Waltham, MA) were washed in ES according to the manufacturer’s protocol, and then resuspended beads were added to cell suspension at a ratio of one bead per cell. Calcein AM (Thermo Fisher, Waltham, MA) at indicated concentrations was incubated with cells in ES, and then washed with dye-free ES solution twice.

2.2 Screening of Libraries

2.2.1 HTS of the Molecular Library Small Molecule Repository (MLSMR)

HTS was carried out by Mark Haynes, Bruce Edwards and Oleg Ursu at the University of New Mexico Center for Molecular Discovery. The detailed procedure is described our publication¹³⁸.
In brief, screening of the library was performed in 1536-well plates. Cells were dispensed into wells containing compounds (10 μM final concentration) or DMSO (1%), and stimulation solution containing 2 μM TG+50 nM PMA+anti-LAMP antibody (final concentrations) were added. Plates were sealed, rotated end-over-end to mix, and incubated for 2 hours at 24°C then overnight at 4°C. On the following day, warmed samples were collected on a custom HyperCyt HTS platform with four sampling probes linked to four Accuri C6 flow cytometers.

A pooled fluorescence intensity histogram of unstimulated cells from all control wells containing DMSO alone (positive controls for inhibitors) was used to establish an intensity threshold above which we detected fewer than 5% of the unstimulated cells. Cells from compound-containing wells with fluorescence intensity exceeding this threshold were considered to be responsive to the TG+PMA stimulation, and subthreshold cells were considered to be unresponsive. Typically, 80–100% of cells from DMSO control wells treated with TG+PMA (negative controls) were responsive.

The response range for the assay was defined as the difference between the averages of % responsive (%resp) cells in the negative control (Ncntrl, stimulated) wells and in the positive control (Pcntrl, unstimulated) wells, respectively. Response inhibition mediated by compounds in sample wells was calculated as follows:

Equation 1 % inhibition in MLSMR screen

\[
100 \times \frac{\text{Ncntrl\%resp} - \text{Sample\%resp}}{\text{Ncntrl\%resp} - \text{Pctrl\%resp}}
\]

Compounds inhibiting 55% or more were considered to be active, a threshold chosen to generate ~2500 active compounds. Wells in which fewer than 10 cells were detected within the viability gate were not analyzed further due to insufficient sample size; 767 of 364,202 tested compounds (0.2%) were labeled undetermined as a result.
2.2.2 Bead Assay and Screening Condition

For tunicate-associated bacterial extract library screening, 1 µL of bacterial extracts (stock concentration of 1 mg/mL in DMSO) or DMSO was added to test plates (Corning 3603) prior to cell addition. One hundred microliters of ES-washed TALL-104 cells (2.5 × 10^6/mL) was added to each well, mixed with DMSO or bacterial extracts, and incubated at 37 °C for 30 min. Ten microliters of stimulation solution (ES supplemented with 20 µM TG and/or 1 µM PMA, anti-CD3 beads, anti-LAMP) or control solution (ES + 4% DMSO, anti-CD8 beads, anti-LAMP) was added and mixed, and the plate was incubated for an additional 50 min in the dark at room temperature with constant rotation. One hundred microliters of 2% PFA was added to each well, and samples were transferred to flow tubes for cytometric analysis. Samples were collected on a two-laser FACSCalibur (BD Biosciences).

2.2.3 Multiple-treatment time protocol for bead assay

For screening, 1 µL of compound at a concentration of 1mM in DMSO or 1 µL of DMSO was added to wells of test plates (Greiner 675076, Kremsmünster, Austria). One hundred microliters of medium-washed TALL-104 cells (1.25 x 10^6/mL) were added to each well, mixed with the compound or DMSO, and incubated at 37°C for 24 hours. This comprised the 24 hour treatment time sample. Cells for the 30 minute treatment time were maintained in medium at 37°C for 23 hours, before being bar-coded with 20nM calcein AM and added to the plate in 10 µL cell solution (1.25 x 10^7/mL). Each well was thoroughly mixed and incubated at 37°C for 30 minutes. To stimulate cells, ten microliters of anti-CD8 bead solution (medium supplemented with anti-CD8 beads, anti-LAMP1, 4% DMSO), anti-CD3 bead solution (medium supplemented with anti-CD3 beads, anti-LAMP1, 4% DMSO), or stimulation solution (medium supplemented with anti-CD3 beads, anti-LAMP1, 1 µM PMA, 20 µM TG) was added to the appropriate wells and mixed by repeated pipetting. All manipulations were performed with digital multichannel pipetters. Plates
were incubated at room temperature for 1 hour with constant rotation (resulting in plate inversion), before it was sampled.

2.3 LAMP and BLT-Esterase Assays

One hundred microliters of ES-washed TALL-104 cells (2.5×10⁶/ml) was added to each well, mixed, and incubated at 37 °C for 30 min. Five microliters of stimulation solution (ES supplemented with 20 µM thapsigargin and 1 µM PMA) or control solution (ES + 4% DMSO) was added and mixed, and the plate was incubated for an additional 90 min in the dark at room temperature. The plate was centrifuged, and 50 µl of supernatant was transferred to a new plate for BLT-esterase assays, which measure the release of granzyme B by monitoring the cleavage of a synthetic substrate, benzylxycarbonyl-L-lysine thiobenzyl ester.11 Meanwhile, 50 µl of ES containing 0.6 µg/ml anti-LAMP antibody was added to the wells containing the pellets, mixed, and incubated for 30 min in the dark at room temperature with constant rotation prior to the addition of 100 µl/well of 2% PFA. The geometric mean value of LAMP fluorescence or the absorbance values at 410 nm were determined from unstimulated (U) or stimulated (S) DMSO-treated or stimulated compound-treated (C) cells, and the percent inhibition of LAMP externalization or BLT-esterase secretion by each compound was calculated as follows:

\[
100 \times (1 - \frac{C - U}{S - U})
\]

2.4 Intracellular Calcium measurement

2.4.1 Fura-2 Protocol for Plate-based assay

Ninety microliters of ES was added to each well of a black flat-bottomed plate laid out with DMSO- and compound-containing wells, then it was mixed, and the fluorescence excited at 340 and 380
nm measured at 510 nm emission (F340 and F380) was acquired (reading 1, blank B). TALL-104 cells were loaded with 0.5 µM Fura-2 AM for 25 min in the dark at room temperature, then washed with ES and incubated for 20 min. 2.5×10⁷ cells in 10 µl ES were added to each well of the test plate and mixed. The plate was incubated for 30 min at 37 °C and read again (reading 2). Finally, 5 µl of stimulation solution (ES supplemented with 40 µM thapsigargin) or control solution (ES + 4 % DMSO) was added and mixed. After 50 min, the third fluorescence reading was taken (reading 3). U calcium levels were reading 2 from DMSO-treated cells. S calcium levels were reading 3 from DMSO- or compound-treated cells. Blanks for each well were subtracted for F340 and F380 prior to computation of the F340:F380 ratio. Percent inhibition of calcium rise was calculated using Equation 2.

2.4.2 Fluo-4 Protocol for Flow Cytometry-based Assay

TALL-104 cells were loaded with 1 µM Fluo-4 AM (Molecular Probes, Eugene, OR) in culture medium at room temperature for 25 min, washed to remove excess dye, and resuspended in ES without 2% BSA. Anti-CD3 or anti-CD8 beads were added, or cells were left untreated, and samples were analyzed cytometrically at selected time points over 1 h.

2.5 Assays for Protein Kinase C (PKC), and Mitogen-Activated Protein Kinase Kinase (MAPKK) Activity

One hundred microliters of ES-washed TALL-104 cells (2.5×10⁶/ ml) was added to Eppendorf tubes containing compound or DMSO and incubated at 37 °C for 30 min. Five microliters of stimulation solution or control solution (as above for LAMP and BLT-esterase measurements) was added and mixed, and tubes were incubated for an additional 50 min in the dark at room temperature with constant rotation. After incubation, cells were fixed with 100 µl of 2% PFA and permeabilized by the addition of 1 ml of ice-cold methanol. Cells were washed with FACS buffer,
incubated with primary antibodies [rabbit anti-active PKC substrate (Cell Signaling 2261; Cell Signaling, Danvers, MA), 1:100; and mouse anti-p44/p42 MAPK (Cell Signaling 9106), 1:1000] in FACS buffer, then incubated with secondary antibodies (6 µg/ml Alexa488-conjugated donkey anti-rabbit and 7 µg/ml Cy 5-conjugated donkey anti-mouse) and washed. The geometric mean value of Alexa488 and Cy5 fluorescence was determined for U or S DMSO-treated or stimulated C cells. The percentage inhibition of PKC substrate phosphorylation or extracellular signal-regulated kinase (ERK) phosphorylation was calculated using Equation 2

$$100 \times \left(1 - \frac{c-u}{s-u}\right).$$

2.5.1 Intracellular p-ERK Staining in Bead Assay

ES-washed TALL-104 cells were treated with anti-CD3 or anti-CD8 beads and incubated for 50 min at room temperature with constant rotation. Immunocytochemistry was carried out as previously described.

2.5.2 PKC Activity Dose Response by Immunocytochemistry

TALL-104 cells were resuspended in culture medium and treated with PMA or compound E1 at 9 concentrations ranging in a threefold dilution series from 0.01 nM to 200 nM for 30 min at 37 °C, followed by 50 min at room temperature with constant rotation. Immunocytochemistry was carried out as previously described. Values of geometric mean of fluorescence at different concentrations were fitted to Equation 3 Hill equation.

2.6 Immunoblotting to Detect Effects on Calcineurin Activity

TALL-104 cells were nucleofected as described previously with CaNAR1 complementary DNA (cDNA). Five hours later, $1 \times 10^6$ ES-washed transfected cells in 400 µl were added to each tube
containing 4 µl test compound at an initial concentration of 3 mM or 4 µl DMSO, mixed, and incubated for an additional 30 min at 37 °C. Six hundred microliters of stimulation solution (ES supplemented with 1.67 µM TG and 83.3 nM PMA) or control solution was added and mixed, and the tube was incubated for another 50 min in the dark at room temperature with constant rotation. Cell pellets were lysed in standard radioimmunoprecipitation assay buffer supplemented with 10 mM EGTA, 10 mM EDTA, protease, and phosphatase inhibitor cocktails, and processed for immunoblotting on nitrocellulose membranes.

2.7 Immunocytochemical Determination of Compound Effects on ERK Catalytic Activity

TALL-104 cells were nucleofected with ERK activity reporter (EKAR) cDNA. Five hours later, 100 µl ES-washed transfected cells (2.5×10^6/ml) were treated with test compound or DMSO and stimulated. Cells were fixed and permeabilized, then stained with rabbit anti-phospho Cdc25c antibody (Cell Signaling 9527), followed by a Cy5-conjugated secondary. FL-1 signals (corresponding to yellow fluorescent protein (YFP) expression level) and FL-4 signals (corresponding to anti-phospho-Cdc25C staining intensity) were collected. Percent inhibition was calculated as described above.

2.8 Plate Reader and Flow Cytometer

All plate measurements for follow-up experiments were performed with a BioTek Synergy 2-plate reader (BioTek). Most flow cytometry experiments were conducted on a FACSCalibur (BD Biosciences) equipped with 488 nm and 640 nm lasers. NCI library screen at two time points was conducted on a five-laser (350 nm, 405 nm, 488 nm, 540 nm and 640 nm) BD Fortessa X-20 with a plate reading unit. Both instruments are housed in the University of Connecticut’s Flow Cytometry Facility. Flow cytometry data were analyzed with FlowJo Software (Tree Star Inc., Ashland, OR).

2.9 Natural Product Library Generation
Tunicate-associated bacterial extract library was constructed by Dr. Marcy Balunas lab at the School of Pharmacy, University of Connecticut. The detailed procedure was described in our publication\textsuperscript{139}.

In brief, tunicate specimens were collected in Long Island Sound, CT, the Bahamas, and the Republic of Panama by scuba diving or from shallow water dock lines. Tunicates were sterilized, homogenated, and biomass was streaked onto plates. Bacteria colonies were isolated from plates, expanded in liquid culture, and extracted via Diaion HP-20 beads with consecutive washes of methanol and acetone. The resulting bacterial extract was suspended in DMSO in 96-well plates for storage and screening.

\textbf{2.9.1 Isolation and Identification of Teleocidin A-1}

The detailed procedure was described in our publication\textsuperscript{139}.

In brief, the augmenting hit was fractionated via C\textsubscript{18} reversed-phase solid-phase extraction chromatography with gradient methanol:water elution. Each eluted fraction was tested in bead assay, and the most active fraction was subject to liquid chromatography-mass spectrometry characterization. NMR spectra for \textsuperscript{1}H and \textsuperscript{13}C were collected for structural deconvolution.

Teleocidin A-1 was isolated from \textit{Streptomyces} sp. strain AVP053U2, isolated from the tunicate \textit{Styela clava}, collected from shallow water dock lines at Avery Point, CT, in May 2011 (41° 18.975 N, 72° 3.647 W). The bacterium was isolated from ISP4 agar, replacing water with Instant Ocean (United Pet Group, Blacksburg, VA), and cultured at room temperature. The morphology of AVP053U2 resembled an actinobacterium, with a yellow base and green spores.
Chapter 3: HTS of CTLs Granule Exocytosis Led to Identification of Novel Immunosuppressants

3.1 Overview

This chapter describes the first version of CTL lytic granule exocytosis assay used in an HTS campaign screening for novel immunosuppressants. Findings from this project were published in Journal of Biomolecular Screening. CTLs kill cancerous and virus-infected cells via lytic granule exocytosis, and they are involved in transplant rejection and autoimmune diseases. Current small molecule immunosuppressants are not specific to T cells and have several side effects, so there is a need to look for novel immunosuppressant compounds to serve as drug candidates. CTLs lytic granule exocytosis serves as a good cell model, because the process is fast, the detection is robust and signaling pathways for CTL activation are shared by other immune cell types. CTLs were activated with small molecules that bypass T cell receptor, and the externalization of LAMP to cell membrane was detected by fluorescent antibody present in the extracellular solution. Antibody binding was measured in flow cytometer in a no-wash fashion, suitable for HTS. We reasoned that a phenotypic assay would allow us to identify compounds with either known or unknown mechanisms of actions, and the latter could lead to valuable probes for target identification. The screen >300,000 small molecules in the collection of Molecular Library Small Molecule Repository (MLSMR) from National Institute of Health led to the identification of 31 novel immunosuppressant candidates working through known mechanism(s) and 8 inhibitors with novel molecular mechanisms of action. Two novel inhibitors were confirmed from powder resupply and are being developed into probe molecules.

3.2 Results

3.2.1 LAMP assay of TALL-104 cells
Previously a phenotypic assay was devised based on CTL lytic granule exocytosis, where the translocation of LAMP onto the cell surface can be detected by fluorescent antibody present in extracellular solution\textsuperscript{142}. The assay can be performed without washing away excess antibodies in 96- and 384-well plates (Figure 5A). The fluorescence of cells was collected on the flow cytometer and the background fluorescence interference was reduced to minimum. Eliminating the washing step in LAMP assay is advantageous for HTS, where plate washing can be a laborious step and increases the cost of the screen. The plate uniformity testing included maximally stimulated cells treated with TG+PMA, half stimulated cells treated with TG only, and unstimulated cells. Z’ factor was calculated between unstimulated cells and maximally stimulated cells by TG+PMA treatment. Control samples repeatedly showed Z’ factor > 0.6, which indicated excellent assay quality\textsuperscript{143}.

![Figure 5 LAMP assay can detect the level of lytic granule exocytosis.](image)

Detecting exocytosis via anti–lysosome associated membrane protein (LAMP) antibody binding without washing. (A) Histograms of anti-LAMP antibody fluorescence for cells that were washed (top) or not washed (bottom). Unstimulated cells are represented by a dashed line, whereas stimulated cells are denoted by a
solid line. Note the shift in the fluorescence of the unwashed unstimulated cells compared with the washed unstimulated cells. (B) Plot of the geometric mean of anti-LAMP antibody fluorescence from a test conducted in a 96-well plate. PMA, phorbol 12-myristate 13-acetate; TG, thapsigargin. (C) Illustration of the experiment procedure for a no-wash LAMP assay.

3.2.2 HTS of MLSMR library

In collaboration with the University of New Mexico (UNM) Center for Molecular Discovery, we performed the assay in 1536-well plate to conduct a HTS of the compound collection of 364,202 small molecules from MLSMR. At UNM, a clustered HyperCyt was used to sample each plate in four quadrants. Prior to screening the library compounds, control plates were set up to downscale the assay volume for 1536-well plate. Figure 6B shows a column and a quadrant from a control plate, where half of the cells were stimulated with TG+PMA and the other half were left unstimulated. Each well was sampled for 900ms and an interwell air gap was 400ms. This allowed a 1536-well plate to be finished in ~10.5min. In the validation runs, this resulted in the acquisition of ~1150 cells/well.

As seen in Figure 6C, the assay showed a bivariate nature in anti-LAMP fluorescence profile for unstimulated versus stimulated cells, and therefore we explored two different ways of analyzing data: geometric mean and percent positive. Geometric mean calculates the average of a logarithmically distributed data, so it directly reflects the amount of antibody bound to cells and the level granule exocytosis. Percent positive calculates the percentage of cells among a population with a fluorescence intensity above the predefined threshold, which was decided based on the level of cell responses in negative and positive controls. Analyzing data by determining the percentage of responding cells was found to give better reproducibility than the MFI analysis, and it was therefore used to analyze primary screening data. Using that analysis, 1195/1208 plate quadrants (98.9%) had Z values > 0.5, consistent with the idea that the assay had appropriate statistical reliability for HTS. Hits from plates that had Z < 0.5 were not excluded.
Seven hundred and sixty-seven wells had fewer than 10 cells in the live cell gate and were scored as inconclusive. A low event number may not reflect compound toxicity; a number of issues, such as problems with dispensing cells or acquiring samples, can reduce the event number.

Figure 6D shows a representative row of data from a plate in the primary screen where a potential inhibitor is indicated. Within each plate, two columns on the left are left unstimulated and therefore serve as positive control for inhibitor screening, and one column on the right is stimulated with TG+PMA and serves as negative control. They are indicated by arrows in Figure 6E with the color scale corresponding to percent positive cells in each population. The lines across the plate indicate the sampling by four cytometers simultaneously.

We wanted to select ~2500 compounds for follow-up. Using an arbitrary threshold of 55% inhibition yielded 2404 hits. One thousand four hundred and sixty of these substances were available and did not have obvious chemical liabilities (reactive groups, dye moieties, or unsuitable properties for chemical optimization). These were obtained and retested. One hundred and sixty-one substances were confirmed active (11% confirmed), and these were selected for dose–response testing. Possible reasons for the lower confirmation rate include: the liquid handling system used for ultra-HTS can be problematic at times where no stimulation solution was added to the well; inter-cytometer difference where a lower response level was observed from the 3rd quadrant of the sample plate (Figure 6E), yet a uniformed threshold was used for hit selection across all samples; low cell numbers in some wells (~50 cells) were not excluded from the analysis.

Dose–response measurements were performed by testing the effects of compounds at 9 concentrations ranging in a threefold dilution series from 7 nM to 15 µM. Data were fitted to Hill equation as in Equation 3.

Equation 3 Hill equation
\[
\text{% Inhibition} = \frac{\text{top} - \text{bottom}}{1 + 10^{(\text{logEC50} - x) \times \text{Hillslope}}}
\]

where \text{top} is the maximum inhibition; \text{bottom} is the response of stimulated cells in the absence of compound; \text{logEC50} is the logarithm of the \text{EC}_{50} (\text{in \mu M}); and \text{Hillslope} is the Hill coefficient. Forty-five compounds exhibited acceptable dose-dependent inhibitory curves (monotonic dose dependence with \text{EC}_{50} < 10 \text{ \mu M} and least 1 point defining an intermediate region of the curve) when analyzed using the percent positive analysis strategy described above. Additional compounds demonstrated acceptable dose–response behavior, however, when curves were fit to the MFI measurement. Based on these considerations and the availability of compounds, a resupply of 75 substances was obtained for further analysis. Figure 7 outlines the decision points leading from 364,202 substances screened to the 75 that were selected for follow-up.

**Figure 6** Screening the National Institutes of Health (NIH) Molecular Libraries Small Molecule Repository (MLSMR) in a 1536-well format with high-throughput cluster cytometry.
(A) Plot of forward scatter versus side scatter from an experiment validating the 1536-well format of the assay. The circled region represents live cells, which were selected for analysis. (B) Representative data from row A (top) and the entire first quadrant (bottom) of a test plate in which half the columns contained unstimulated cells, whereas the other half contained stimulated cells. (C) Histogram of FL-4 fluorescence for all live-cell events in a validation plate showing the bivariate nature of the distribution of fluorescence corresponding to unstimulated (to the left of the line) and stimulated (to the right of the line) cells. (D) Representative data from 1 row of a library screening plate. (E) Heat map for a representative plate from the primary screening campaign. Arrows in the upper left indicate columns that were not stimulated to serve as positive controls. Arrow in the upper right designates stimulated negative controls. The color scale used to display data (indicated to the right) ranges from 0% response (purple) to 85% response (red). Dashed lines demarcate quadrants. (F) Histogram of Z values for the 1208 quadrants analyzed.
Figure 7 Flow chart showing hit selection process from the primary screening.

The number at each step indicates the number of compounds that are excluded from or included into the hit selection.

3.2.3 Confirming the activity of selected hits

We first confirmed the activity of the substances using a protocol that combined a repeat of the LAMP assay with BLT-esterase assays, a standard means for measuring granule exocytosis (Figure 8). We combined the 2 measures to minimize compound use and reduce the chance for error. Compounds were tested at 30 µM to achieve maximal inhibition of exocytosis. Such a high concentration was chosen because our major goal was to identify compounds with
unknown MMOA, and we felt that using a relatively high concentration would likely reveal any effects on known MMOA. In flow data, we did not observe striking effects on the fraction of cells in the live cell gate in these experiments, suggesting that toxicity in the short term was not a problem.

![Graph](image)

Figure 8 Confirming compound activity in a combined format of the primary assay and orthogonal assay.

Plot of inhibition measured in flow cytometer-based LAMP assay versus inhibition measured in the plate reader-based BLT-esterase assay. The dashed line has a slope of 1 and passes through the origin, indicating the behavior expected for a perfect correlation between the 2 measures. The solid line is a best-fit regression line for the data from 75 cherry-picked hits. Compounds with negative numbers in % inhibition are those that resulted in slightly higher cell responses than DMSO-treated controls.

Cells were pretreated with compounds or DMSO, then, except for control wells, stimulated with TG+PMA. Fifty minutes after stimulation, plates were centrifuged, and samples of the supernatant were collected for BLT-esterase assays. The pelleted cells were stained with anti-LAMP antibodies for 15–20 min, then fixed and analyzed via flow cytometry. We have shown previously that staining cells after stimulation yields essentially similar results as stimulating them in the presence of the antibody\textsuperscript{105}. 

44
We found that 48 substances blocked granule exocytosis by >50% as measured by LAMP staining. BLT-esterase measurements reported on average ~20% less inhibition of exocytosis than LAMP staining. Despite this, 41 substances also inhibited lytic granule exocytosis >50% as measured with the BLT-esterase assay. For 7 compounds, there was a sufficient discrepancy between the 2 measures of exocytosis that compounds scored as active on the basis of LAMP externalization were scored as inactive based on BLT-esterase assays. A number of factors, including a modest degree of compound toxicity, could be responsible for this. Those compounds were further investigated.

3.2.4 A strategy to identify MMOA of confirmed hits

Follow-up experiments were intended to determine the mechanism by which hit compounds block exocytosis (Figure 9). We envisioned 7 testable known MMOAs that could block lytic granule exocytosis. Sustained calcium influx, which is required for exocytosis, could be inhibited by two MMOAs: (1) blockade of store-operated calcium channels, which are known to mediate calcium signals in CTLs; or (2) blockade of K+ channels, which maintain a favorable driving force for calcium entry. (3) Inhibition of PKC could block exocytosis, as could (4) inhibition of the activation of the MAPK ERK by upstream MAPKKs or (5) block of ERK catalytic activity, (6) calcineurin activity could be inhibited, either directly or (7) as a result of inhibition of calmodulin or of calmodulin binding to calcineurin. We reasoned that it would be most efficient to put assays that could be conducted entirely in plate format early in our experimental design. When possible, we interrogated multiple processes simultaneously. In addition, we reasoned that we might not need to test each known MMOA individually, provided we could interrogate a common output. For example, inhibition of calcium influx can result from block of calcium channels or block of K+ channels; either will be revealed by assessing intracellular calcium increases. Similarly, block of either calmodulin or calcineurin’s catalytic activity will result in decreased calcineurin-dependent dephosphorylation; both can be assessed by examining dephosphorylation of calcineurin.
substrates. In the end, 4 sets of experiments allowed us to test all of the MMOAs described above. All experiments to determine MMOA described below were performed at least twice, and average results are reported.

Figure 9 Rationale behind experiments testing the MMOA of hit compounds.

(A) Diagram (modified from a previous paper) indicating known signal transduction components triggered by TG and PMA treatment (bold), and the order in which tests were conducted (red). (B) Flow chart illustrating the order of follow-up assays employed to identify MMOA of compounds, and the results of those experiments.

3.2.5 Testing for inhibition of [Ca^{2+}]_i increases

To assess effects on intracellular [Ca^{2+}] (Figure 10), a calcium sensitive dye Fura-2 was used to load TALL cells. The excitation spectral shift due to intracellular calcium level increase results in a ratio increase of the dye fluorescence when excited with two different wavelengths. To obtain information about any potential compound fluorescence and avoid their influence on our ability to measure intracellular calcium level, we measured the fluorescence of substance-containing
solutions at 340 and 380 nm excitation prior to adding Fura-2 loaded cells. We then dispensed Fura-2 loaded cells into the wells and incubated them for 15 min with test substances. We measured fluorescence at 340 and 380 nm excitation to estimate resting [Ca^{2+}]i levels, then stimulated cells with TG and measured fluorescence again after 50 min, a time point at which [Ca^{2+}] elevations depend entirely on Ca^{2+} influx. We found that 8 of the substances that blocked lytic granule exocytosis had sufficiently high fluorescence at 340 and/or 380 nm excitation that we were not able to acquire meaningful Fura-2 signals from cells treated with them. Of the substances with sufficiently low fluorescence that we could acquire Fura-2 signals, 15 inhibited increases in the Fura 340:380 ratio by 50% or more. We retested the highly fluorescent substances with the related dye Fura-Red in flow cytometry, because this dye has the same K_d for calcium as Fura-2 but different spectral characteristics. We loaded cells with Fura-Red, then treated them with substances for 15 min at 37 °C. Half of the substance-treated cells were stimulated with TG+PMA, and half were left unstimulated. Samples were analyzed 50 min later. We found that 6 out of the 8 substances decreased the Fura-Red fluorescence of unstimulated cells in at least 1 of the trials, an effect that could be an artifact related to leakage of the dye from cells or that could reflect elevation of resting calcium levels. Because these possibilities cannot be distinguished with a single wavelength indicator, we were able to measure with confidence only the blocking effects of 2 substances, neither of which inhibited calcium signals >50%. Figure 10B plots the percent inhibition of LAMP responses versus the percent inhibition of Fura signals for substances that decreased exocytosis by >50% (filled squares). The 6 substances for which we could not reliably measure effects on [Ca^{2+}]i with Fura-2 or Fura-Red were not pursued further.
Figure 10 Measuring effects of compounds on calcium influx.

(A) Pseudo color representations of Fura-2 ratios prior to (top) and after (bottom) TG stimulation. Black indicates wells for which fluorescence measured prior to the addition of cells was sufficiently high that ratios could not be acquired. The leftmost and rightmost columns contain DMSO-treated cells. Half of these wells were stimulated, whereas half were left unstimulated. The color scale used to display data (indicated to the right) ranges from a ratio of 0.04 (purple) to 0.5 (red). (B) Filled squares plot the inhibition of LAMP responses as a function of the inhibition of the Fura-2 signal for all test substances that inhibited exocytosis >50% in the experiments shown in Figure 8. Open circles are plots of LAMP responses versus inhibition of Fura-2 signals caused by varying the concentration of extracellular Ca2+, normalized to the responses at 2 mM Ca2+. The solid curve is a fit of a sigmoidal function to the data in the open circles. The dashed lines outside the solid curve represent 99% confidence bands for the fit. The straight lines denote 50% levels for Fura-2 and LAMP inhibition, respectively.

Overall, 15 of 48 exocytosis-inhibiting substances blocked Fura signals by >50% (Figure 10 and Table 1), a level that we thought was appropriate to use as a cutoff to discriminate compounds likely to work by blocking Ca2+ increases [all hits are listed by their SIDS, and information about them can be found by searching PubChem Substance: http://www.ncbi.nlm.nih.gov/pcs Substance]. To confirm that this level of inhibition of
Ca^{2+} mobilization is sufficient to block exocytosis, we measured Fura-2 ratios and lytic granule exocytosis while varying extracellular Ca^{2+} from 0 to 2 mM (Figure 10, open circles). We plotted the relative amount of exocytosis at each concentration of Ca^{2+} versus the Fura-2 ratio we measured, allowing us to estimate the dependence of granule exocytosis on Fura-reported measures of intracellular [Ca^{2+}]. Inhibiting the increase in Fura ratios by 50% or more reduced lytic granule exocytosis by >50%. Thus, we considered compounds that block exocytosis by >50% but inhibit Ca^{2+} increases <50% as likely to have an alternate MMOA.

Table 1 Summary of MMOA Testing for confirmed inhibitors from MLSMR screen.

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3.2.6 Testing for Effects on PKC and Activation of ERK MAP Kinases

We next tested compounds whose inhibitory effects on granule exocytosis were unlikely due to their effects on calcium rise in cell assays that measure PKC activity and the activation of ERK using immunocytochemistry (Figure 11A). Cells were incubated with test substances before treated with PMA to activate PKC, and then they were fixed, permeabilized and stained for intracellular kinases. To test for PKC activity, active PKC substrates were measured, and simultaneously to test for MAPKK activity, p-ERK was measured using different fluorophore-conjugated secondary. As a control, we treated cells with the compound Ro31-8220, which blocks PKC and thus PMA-stimulated activation of MAPKKs\textsuperscript{103}. Ro31-8220 inhibited PKC substrate phosphorylation by 82% and ERK phosphorylation by 95%. Seven of the exocytosis-blocking compounds tested inhibited ERK phosphorylation by >50%, but none significantly reduced the
fluorescence intensity of anti-phospho PKC substrate staining (see Table 1). We conclude that 7 compounds likely inhibit granule exocytosis by inhibiting MAPKK activation downstream of PKC.

**3.2.7 Testing for Effects on Calcineurin Activity**

We tested the effects of substances whose effects on exocytosis were not due to effects on Ca\(^{2+}\), PKC, or ERK for their ability to inhibit the activity of the Ca\(^{2+}\)-dependent phosphatase calcineurin, which is required for granule exocytosis. To measure calcineurin activity, we used a genetically encoded reporter construct\(^{151}\) based on the transcription factor NFAT, the best-known calcineurin substrate. Because NFAT is highly phosphorylated in resting cells, dephosphorylation resulting from calcineurin activity increases the reporter construct’s apparent mobility on SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) gels from \(~125\) kD to \(~110\) kD. Four sets of gels and transfers were required to run all of the samples; Figure 11B is a montage of the results (see legend for details of its assembly). In unstimulated cells (column 1), immunoreactivity was present as 2 bands. As expected, stimulation of cells caused a shift in the pattern of immunoreactivity to a single band of low molecular weight (column 2). Treatment of cells with the calcineurin inhibitor cyclosporin A prior to stimulation caused immunoreactivity to shift exclusively to the high-molecular-weight form (blot 4, column 5). One of the substances we tested, SID 92764285 (blot 3, column 7), completely blocked the shift of the sensor to lower molecular weight, resulting in a single band of high molecular weight, identical to the effect of cyclosporin A. Other substances were without apparent effect.

**3.2.8 Testing Effects on ERK Catalytic Activity**

Last, we tested whether compounds for which an MMOA had not yet been determined blocked the catalytic activity of ERK (Figure 11C), because this possibility was not excluded by our experiments with the phospho-ERK antibody, which detects activation of ERK by upstream MAPKKs. We used cyto-EKAR\(^{152}\), a genetically encoded sensor of ERK activity that contains the
phosphorylation site of Cdc25c and an ERK docking motif (Figure 11C). We used an anti-phospho Cdc25c antibody to detect activity. In unstimulated cells, increasing expression of the sensor (as detected by YFP fluorescence) was accompanied by higher levels of anti-pCdc25c staining. Stimulating cells with TG+PMA increased anti-pCdc25c staining specifically in the YFP-positive cells (Figure 11Ci). This increase in anti-phospho-Cdc25c staining was inhibited when cells were treated with the PKC inhibitor Ro31-8220 (Figure 11Cii), a result consistent with the involvement of PKC activation in activating ERK. We found that none of the compounds inhibited the increase in anti-pCdc25c staining by >30%, whereas Ro31-8220 blocked by 83%. This suggests that no compound is likely to block lytic granule exocytosis solely by inhibiting ERK catalytic activity.
Figure 11 Testing the effects of compounds on PKC and MAPKK activity, NFAT dephosphorylation, and ERK catalytic activity.

(A) Histograms of anti-phospho PKC substrate antibody staining (left) and anti-phospho ERK staining (right) are shown for unstimulated control cells, stimulated control cells, cells treated with Ro31-8220, and cells treated with SID 56324549, 1 of 7 substances (see Table 1) that we conclude inhibit exocytosis by blocking MAPKK activity. (B) Western blotting to assess calcineurin activity was performed on control and compound-treated lysates from cells transfected with CANAR. The image displayed is a montage. Each row is from a single blot. Columns corresponding to molecular weight standards have been digitally removed, and lanes to their right have been moved horizontally to the left. In each row, samples in column 1 are from unstimulated DMSO-treated cells, and samples in column 2 are from DMSO-treated cells stimulated with TG+PMA. In blot 4, the sample in column 5 was prepared from cells treated with the calcineurin inhibitor cyclosporin A, which serves as a positive control, whereas the sample in column 4 was treated with Ro31-8220, serving as an additional negative control. Note the single experimental sample (blot 3, lane 7) with high-molecular-weight immunoreactivity corresponding to inhibition of calcineurin. (C) Flow cytometry was performed on EKAR-transfected cells to assess ERK catalytic activity. (i) Contour plots of anti-phospho Cdc25C staining intensity versus yellow fluorescent protein (YFP) fluorescence for unstimulated control cells (top) and for cells stimulated with TG+PMA (bottom). The vertical line indicates the cutoff that was used to gate YFP-positive cells. (ii) Histograms of anti-phospho Cdc25C staining intensity for YFP-positive cells from the samples shown in (i), and also for stimulated cells treated with the positive control Ro31-8220 and with a test substance, SID 16952891. Stimulation with TG+PMA causes a shift to higher anti-phospho Cdc25c fluorescence intensity that is inhibited by Ro31-8220.

3.2.9 Two compounds were obtained from powder and their activities were confirmed

Sixteen compounds did not inhibit a single pathway tested by >50%. Eight of those, however, exerted effects on multiple pathways that, although separately of insufficient magnitude to account for the block of granule exocytosis, could be imagined to exert summed effects large enough to inhibit release. The compounds with significant multiple inhibitory effects are detailed in Table 1. Because we know relatively little about how varying the strength of the different signals affects
exocytosis, it is difficult to determine whether the effects of these compounds on known MMOAs completely account for their ability to block exocytosis or whether they have additional effects. Our choice to use a relatively high concentration of compounds for MMOA testing may play a role in generating cases of apparent multiple activities. The final 8 compounds had, however, summed inhibitory effects that seemed unlikely to be of sufficient magnitude to account for blocking.

To develop novel inhibitors into molecular probes that could be used to identify their binding partners in cells, we obtained powder resupplies for two of the novel inhibitors with novel MOA from Chembridge Corporation (San Diego, CA): SID7977862 and SID103159345. Compound powders were reconstituted with anhydrous DMSO and tested in LAMP assay to confirm their inhibitory activity. Both showed >75% inhibitory at 30 uM on cell granule exocytosis when stimulated with TG and PMA (Figure 12 and Figure 13). Dose responses of compounds showed IC50 within micromolar range (4.6uM for SID7977862 and 6.9uM for SID103159345). To confirm the compounds do not affect known signaling events from powder resupply, each compound was subject to all the mechanism testing assays. Consistent with previous results, two compounds from powder resupply did not block intracellular calcium rise, ERK activity or calcineurin activity.

We next tested the cytotoxicity of compounds using PrestoBlue reagent (Life Technologies, Carlsbad, CA), which could be reduced by metabolically active cell and show a fluorescence change. In Figure 12D, a standard curve of cell number was used to compare compound- and DMSO-treated samples. When cells were treated with 100uM SID7977862 for 1.5 hours, a much higher concentration than its IC50, there was no change in live cell number. In Figure 13D, dose responses of SID103159345 was carried for the incubation time of 1.5 hours or 24 hours. Prestobluue was added in the last one hour. Although higher concentrations (30 and 100uM) at 24 hours resulted in cell death, there is no dose dependent cell killing at 1.5 hours, the usual time span of LAMP assay. In conclusion, neither compound had acute toxic effects on cells within 1.5 hours, so their inhibition on lytic granule exocytosis is unlikely to be a result of killing the cells.
Figure 12 Confirming the effects of SID 7977862 from powder.

(A) Structure of SID 7977862. (B) Histograms of anti-LAMP fluorescence for unstimulated control (blue), stimulated control (red), and SID 7977862–treated cells. Experiment was conducted in ES without 2% BSA. (C) Dose–response testing using LAMP externalization. Data are the average of 2 separate experiments conducted in ES. IC50=4.6 uM, hill rate=2. (D) Assessing toxicity using the PrestoBlue reagent (Life Technologies, Carlsbad, CA). Cells were pretreated with 100 µM SID 7877962 (triangle, 250,000 cells) or DMSO (circles, different numbers of cells), and PrestoBlue fluorescence was measured. Different numbers of DMSO-treated cells treated with Triton X-100 (open circles) were used as an additional positive control. (E) Testing for inhibition of calcium influx. (F) Measuring effects on ERK phosphorylation. Experiments were conducted as in Figure 11A. (G) Measuring effects on ERK catalytic activity. Experiments were conducted as in Figure 11C. (H) Testing for inhibition of calcineurin. Experiments were conducted essentially as in Figure 11B.
Figure 13 Confirming the effects of SID 103159345 from powder resupply.
(A) Structure of SID 103159345. (B) Histograms of anti-LAMP fluorescence for unstimulated control (blue), stimulated control (red), and SID 103159345-treated cells (green). Experiment was conducted in cell culture medium. (C) Dose response testing using LAMP externalization. Data are representative of two independent repeats in cell culture medium. IC50=6.9uM, hill rate=1.3. (D) Dose response testing short-term and long-term cytotoxicity of SID 103159345 using PrestoBlue reagent. Cells were pretreated with DMSO or different concentrations of the compound for 30 minutes or 24 hours in culture medium, before PrestoBlue was added and incubated for 1 hour, and the fluorescence was measured. (E) Testing for inhibition on calcium influx. Fura-2 loaded TALLs were incubated with DMSO or 30uM of SID 103159345 for 30 minutes, before 2uM TG was added and a kinetics reading of Fura-2 fluorescence was measured. (F) Measuring effects on ERK phosphorylation. (G) Measuring effects on ERK catalytic activity. (H) Testing for inhibition of calcineurin.

3.2.10 Structure-Activity Relationship Study of SID7977862

To obtain structure-activity-relationship of SID7977862, 8 analogs were ordered from ChemDiv Corporation and 21 analogs were synthesized by University of Kansas Center of Excellence in Chemical Methodologies and Library Development (Table 2). Except for 1-(4-Methoxyphenyl)-2,5-pyrrolidinedione (analog 1), all other analogs showed >90% inhibitory at 30uM. Analog 1 is a partial structural breakdown of the parent compound, and its loss of inhibition indicates that keeping the sulfide bond in the core structure is important for its inhibitory activity. All other analogs are substituted with different chemical groups at R1 and R2 positions, and 16 analogs showed >75% inhibition at 1uM.

Dose responses were carried out for all analogs to test their potency in blocking lytic granule exocytosis. When polar substitutions are introduced to R1 position, the presence of an ortho-hydroxyl group decreased analog potency (analogs 2-6). Yet this decrease was reversed when a para-bromobenzene is introduced to R2 position (analog 5). Next, we examined what effects other polar substitutions on R2 position could have on the analog potency. When R1 position remains the same as the parent compound, the presence of 3,4-dichloro-benzene on R2 greatly increased
the potency (analog R), which turned out to be the most potent analog among all others, showing an IC50<0.03uM and over 150-fold increase over the parent compound (IC50=4.6uM). Two other analogs sharing the same R2 substitution as analog R showed slightly lower potency when R1 was changed (para-chlorobenzene for S and para-methylbenzene for T). When the number of chlorides on R2 benzene ring is reduced to one, analog potency is decreased (analogs E-H), yet it is still higher than the parent compound. In comparison, a benzene ring for R2 resulted in a loss of potency regardless of the functional groups for R1 (analogs A-C), and this suggests that polar substitutions on R2 are important for efficient inhibition. In conclusion, a polar substitution on R2 with a nonpolar substitution on R1 could improve analog potency.

Next we wanted to know how the analog inhibitory activities compare to their cytotoxicity. To test long-term cytotoxicity, cells were incubated with analogs at different concentrations for 24 hours, before PrestoBlue reagent was added and fluorescence was measured. Culture medium was used to ensure cell survival for 24 hours. To compare these results to LAMP assays, dose response testing LAMP externalization was redone in culture medium. Surprisingly, most analogs showed improved IC50 in culture medium compared to Ringers solution supplemented with 2% bovine serum albumin, likely a result of serum protein-assisted uptake. However, there is no apparent correlation between TC50 and IC50 of analogs. Analog R had the highest ratio between TC50/IC50, indicating an improvement of therapeutic window for drug development. However, within the scope of this study, none of the analogs were intended to be used as drug candidates, and compound cytotoxicity is not the primary concern in probe development.

To examine lipophilicity of analogs, logP value was calculated by Molinspiration Cheminformatics Website based on SMILES. Some analogs had values close to 5 (analogs S and H), and others were within the range of 2-4. A high value of logP indicates lipophilic compound and usually poor absorption. All analogs are within the desirable range according to Lipinski’s rule-of-five\textsuperscript{136}. Another parameter, LiPE\textsuperscript{153}, was used to examine the ligand-lipophilic efficiency of analogs, which
takes into account the analog potency in relative to its lipophilicity. A high LiPE indicates high quality of compounds, and analog R had the highest value among others.

Table 2 Structure activity relationship (SAR) study of SID7977862

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1Powder resupply of the parent compound was from Chembridge Corporation. Analogs 1-8 were purchased from ChemDiv Corporation, and analogs A-U were synthesized by the University of Kansas Center of Excellence in Chemical Methodologies & Library Development.

2Dose response parameters were from fits to the Hill equation. Compound concentrations ranged from 0.1-30uM (1-8) and from 0.03-10uM (A-U). Dose response testing was performed twice in 2%NR, and once in complete culture medium. Compound cytotoxicity testing was performed once. Cells were treated with analogs for 24 hours in complete culture medium.

3LogP value was predicted by Molinspiration Cheminformatics based on SMILES.

4LiPE was calculated as pIC50-LogP

3.3 Discussion
Our initial HTS campaign worked essentially as anticipated. Using clustered cytometers, we were able to miniaturize the assay to a 1536-well format, resulting in a substantial decrease in the amount of reagents—primarily, cells and antibody—required. Overall, we had 161 confirmed hits, a number that seemed low to us considering the complexity of the cellular phenotype we were interrogating. This hit rate, however, appears consistent with results from the most directly comparable screening campaign conducted on the MLSMR that we were able to identify. That screen looked for inhibitors of thrombin-stimulated platelet dense granule release using luciferase to detect adenosine triphosphate release accompanying granule exocytosis. Primary screening [assay ID (AID) 1663] of 302,517 compounds identified 661 that were active. Subsequent confirmation (AID 1189) and counterscreening for compounds that inhibit luciferase activity (AID 1891) yielded ~250 active compounds that inhibited platelet granule release. This effort resulted in the discovery of a probe that targeted protease-activated receptor 1. Surprisingly, given what we would anticipate would be many similarities between exocytosis in CTLs and platelets, including the involvement of signaling pathways like PKC and calcium influx, as well as the involvement of SNARE (soluble N-ethylmaleimide-sensitive factor activating protein receptor) proteins, there was only 1 compound active in that screen that we also found to be active. SID 17414945, which we found blocked lytic granule exocytosis and MAPKK activation, inhibited lytic granule exocytosis with an EC$_{50}$ of ~0.3 µM, but it apparently inhibited platelet granule release much less potently, with an EC$_{50}$ of 19 µM. We cannot account for these results. It seems unlikely to result from our use of a cell line, because TALL-104 cells recapitulate key features of CTL function. A second potentially comparable phenotypic screening campaign used a luciferase reporter construct to look for inhibitors of NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells) activation in Jurkat cells stimulated with PMA and ionomycin. That effort identified 735 confirmed active compounds (initial screen in AID 435003, and confirmation in AID 435020). Although the hit rate in that screening campaign was ~5 times higher than ours, the phenotype interrogated includes the entire transcriptional and translational apparatus required to
generate the reporter, together with the proteins required for ubiquitination\(^\text{157}\), which may account for the larger number of hits.

One of our hypotheses was that a phenotypic cellular endpoint assay followed by appropriately designed mechanism testing could be used as a high-content screen for important targets. Consistent with this idea, our experiments revealed a number of substances that act on important pathways, including 15 inhibitors of calcium influx, 7 MAPKK inhibitors, and 1 calcineurin inhibitor. Were any of these activities defined in previous screens of the MLSMR? Determining this is, unfortunately, not straightforward and was not facilitated by the structure and query capabilities of the PubChem web interface. To the best of our knowledge, no screen has looked for inhibitors of calcium influx in nonexcitable cells. We were, however, able to identify 3 screens of >100,000 MLSMR compounds conducted on calcium-permeable channels and 5 conducted on potassium channels. Blockers of these channels could be envisioned to block calcium or potassium channels in CTLs, although this is by no means certain. None of the substances we defined as inhibiting calcium signals were reported to block calcium-permeable CAV3 (confirmation in AID 489005), TRPC4 (confirmation in AID 2247), or TRPC6 (confirmation in AID 488961) channels. One substance that blocked Fura-2 signals, SID 49721823, was reported to inhibit KCNK9 potassium channels (confirmation in AID 492992). KCNK9 (also known as TASK-3) channels have been found in lymphocytes, and inhibitors reduce proliferation and cytokine production\(^\text{158}\). SID 49677461 blocked lytic granule exocytosis and KIR2.1 potassium channels (confirmation in AID 2032), but it gave inconclusive results in our Fura assays. Similarly, SID 4964129 blocked lytic granule exocytosis and KCNK3 potassium channels (confirmation in AID 651638), but it also gave inconclusive results in our Fura assays.

There has been a screen of MLSMR compounds on ERK activation by MAPKKs. That effort (AID 1454) used AlphaScreen technology to assess levels of phosphorylated ERK in lysates of cells treated with epidermal growth factor. Of the 8 MAPKK inhibitors we found, only 2 appear to have
been tested in that screen, which tested an earlier version of the MLSMR containing fewer compounds. Of the 2 substances both active in our assay and tested in theirs, they found SID 24810923 to be inactive, and SID 26727153 gave inconclusive results. We were unable to identify any screens of the MLSMR that might have been expected to reveal calmodulin or calcineurin inhibitors. The substance that we found to inhibit NFAT dephosphorylation [SID 92764285, 2-chloro-3-(3,5-dimethylmorpholin-4-yl) naphthalene-1,4-dione] was, as expected based on its chemistry, fairly broadly active in deposited assays, but it had no confirmed effects that suggested to us possible effects on either calmodulin or calcineurin.

In addition to revealing important actions of compounds on known targets, we suspected that a screen of lytic granule exocytosis could reveal substances that might work via an unknown MMOA. This appears to be the case because we identified SID 7977862 and SID 103159345, both blocking lytic granule exocytosis without inhibiting any of the pathways we tested. Inspecting the reported effects of this compound does not lead to well-supported hypotheses about its MMOA. A high-content screen (AID 1381) found that SID 7977862 inhibited dynein motor protein activity, but whereas dynein has been reported to be involved in reorientation of lytic granules to the site of contact with target cells\textsuperscript{159}, there is no evidence for dynein involvement in TCR-independent exocytosis. There was a confirmatory assay and a dose response study conducted for active hits of dynein inhibitors, but SID 7977862 was not included. Furthermore, >1000 other compounds were active in AID 1381, but none of them inhibited lytic granule exocytosis in our screen. If dynein were a common target in both assays, we would have expected more extensive overlap. SID 103159345 was shown to be an inhibitor of isocitrate dehydrogenase 1 (AID 686970), which results in the production of onco-metabolite 2-hydroxyglutarate and is associated with acute myeloid leukemia\textsuperscript{160,161}. In another assay (AID 743279), it was shown to be an inhibitor inflammasome signaling. However, inflammasome signaling is activated in antigen-presenting cells but not CTLs\textsuperscript{162}, it remains unknown how the compound is inhibiting lytic granule exocytosis.
Examination of the confirmed activities reported in the PubChem database for each of the 6 other substances for which we were unable to determine a MMOA also failed to suggest plausible mechanistic hypotheses for any of their actions. In addition to the MMOAs we were able to test, other possible targets include SNARE proteins and associated components of the exocytic machinery (see Schwarz et al. for a recent review). Note that in preliminary experiments, we tested the effects of DMSO stocks of all 8 substances with undefined MMOA on CD3-stimulated interleukin-2 secretion by Jurkat human leukemic T lymphocytes, and we found that all inhibited, suggesting that each is likely to have broad immunosuppressive activity rather than being a specific inhibitor of lytic granule exocytosis (J. Doucette and A. Zweifach, unpublished observations).

Our assay was configured specifically to detect inhibitors. One way we achieved that was the use of very strong artificial stimulation with TG and PMA. We therefore did not—and did not expect to—detect enhancers of exocytosis. A recent study with human NK cells screened the Prestwick Compound Library (PCL) for agents modulating FCGRIII-stimulated lytic granule exocytosis, integrin activation, and protein synthesis. This study found 56 inhibitors and 12 enhancers. Our screen of the PCL identified several of the inhibitors they found, including amoxapine, desloratidine, thimerosol, and zotepine. Differences in assay conditions—particularly protein concentration in the buffer used—likely account for incomplete overlap of inhibitors.

Despite an emphasis in recent years on target-based approaches, a recent analysis demonstrates that a majority of first-in-class new drugs released between 1999 and 2008 were discovered by phenotypic screens. Our screen of lytic granule exocytosis identified a number of previously undescribed actions of compounds on known targets that could serve as leads for immunosuppressants, and it also identified several compounds that may work via an unknown MMOA. We determined that at least 2 of these have probe-like properties. This confirms that our phenotypic screen based on lytic granule exocytosis may be a good means of identifying
immunosuppressive compounds. Furthermore, we suggest that our basic strategy of screening a complex cellular phenotype that incorporates multiple potential targets and then defining MMOAs for active compounds may be a useful means of identifying interesting biologically active small molecules in compound collections.

3.4 Future Direction for novel inhibitors - Target deconvolution using affinity-based approach

Three approaches for target deconvolution include direct biochemical method (affinity-based purification and isolation)\textsuperscript{165}, genetic interaction (yeast or mammalian three-hybrid systems)\textsuperscript{166}, and computational inference method\textsuperscript{167–169}. For the scope of our research, I’ll focus on affinity-based target identification approach.

It is currently in collaboration with Dr. Dennis Wright Lab to develop SID 103159345 into a chemical probe for affinity-based target identification. The affinity-based method involves chemical modification of the small molecule ligand by attaching a functional group to the structure without affecting its activity. The functional group is known as a linker, which could later link the small molecule to a solid matrix for isolation purpose. If the linker is too short, it could generate steric hinderance and prevent the ligand to engage the molecular target. If the linker is too long and hydrophobic, it could result in auto-aggregation or nonspecific binding\textsuperscript{170}. Ideally, the specific interaction between the ligand molecule and its target ensures only the protein of interest remains on the column after sufficient washing. The bound protein can be recovered by increasing amount of ligand and subject to mass spectrometry for protein identification.

Advantages for affinity-based method: it uses cell lysate containing protein complexes, allowing evaluation of polypharmacology. Also, cell lysate retains the molecular target in its original conformation including all post-translational modification. Testing small molecule efficacy also gives information about its toxicity and offers support for its target as druggable. Affinity-based
approach is the classical way of target deconvolution\textsuperscript{171}. From the earliest insulin receptor isolation from liver homogenates, to FK506 binding protein identification from lymphocytes, researchers have relied on the high affinity and specificity of probe to its molecular targets\textsuperscript{172,173}. Thalidomide teratogenic effect is also elucidated by affinity-based method\textsuperscript{174}.

The premise for affinity-based method is a high-affinity ligand that binds a relatively abundant target protein. If the binding is weak, or if the target protein is not abundant, the affinity purification will not work, as the target protein will be washed away in the process. The washing step also inevitably generates bias towards highest-affinity interactions. However, physiological interactions of compounds with proteins often occur at much lower concentration in cellular contexts\textsuperscript{175}.

Higher-affinity probes can be synthesized to overcome the affinity barrier. The binding efficiency of probe to its molecular target is not determined by the on rate but rather the off rate\textsuperscript{176}. Dissociation constant is determined by the strength of the compounds. Both our compounds are general affinity ligands with EC50 within micromolar range, which corresponds to a retention time of seconds. To improve the probe efficiency, analogs of much higher efficiency need to be synthesized and tested. If EC50 could be improved to nanomolar range, they become high affinity ligands and the retention time will be extended to minutes, which represent a manageable length of time to work with.

Recent advances to overcome the affinity barrier include UV light-induced cross-linking that covalently links the chemical probe to its binding partner under UV light\textsuperscript{177,178}. It works great for low-abundance proteins or low-affinity targets. However, UV-clickable chemical probes generate high, nonspecific background noise due to cross-linking. Also, if the cross-linking is on the functional group of small molecule probe, it will generate false negative results. Click chemistry can also be used to covalently bind the probe to its molecular target, such as the use of cycloadditions of azides and acetylenes\textsuperscript{179}. 
Enriching the relevant protein species can be achieved by pre-purification of samples. Our phenotypic hits are presumed to be targeting signaling molecules involved in CTL granule exocytosis. Pre-washing cell lysate with the probe molecule could be carried out to reduce nonspecific binding. Previously, to identify the binding partner of quinoline drug used to treat malaria, purine binding proteome were obtained first based on structure similarity to quinone, and this led to the identification of quinone reductase as a molecular target\textsuperscript{180}.

To verify the on-target binding of the chemical probe, comparison variant such as an inactive analog of the ligand could be used to indicate nonspecific protein binding\textsuperscript{181}. Competition assays using excess un-conjugated ligands can also be performed, and they should result in less target protein binding\textsuperscript{182}. By running the isolation process in parallel with the original probe, the comparison variant or excess un-conjugated ligands, probe-target protein binding can be validated. For example, to ensure prohibitin as a true target for melanogenin, a comparison variant of melanogenin was used and resulted in less prohibitin protein binding\textsuperscript{183}. A purine derivative QS11 was shown to bind to the GTPase activating protein of ADP-ribosylation factor 1 and modulate Wnt/β-catenin signaling through an effect on protein trafficking. The target protein band disappeared when the affinity column was run in the presence of excess QS11 in a competition assay\textsuperscript{184}.

Two new approaches that circumvent chemical probe immobilization: thermal/protease stability shift upon small molecule binding, also known as DARTs (drug affinity responsive target stability)\textsuperscript{185}, and characteristic shift in retention time upon compound binding, also known as TICC (target identification by chromatographic co-elution)\textsuperscript{186}. DARTs involves incubating cell lysate with free ligand molecules and then subjecting them to protease catalytic activity or temperature increase. The binding of ligand to protein target can stabilize the protein in dynamic equilibrium towards more stable conformation and therefore protect proteins from degradation or proteolysis. It has been used to elucidate or confirm ligand-target interaction in cases such as FK506-
Calcineurin, rapamycin-TOR etc. TICC uses HPLC to elute samples and measure protein concentration in the fractions. When free ligands bind target proteins in treated cells, they cause a shift in the elution profile of cell lysate (compared to untreated cell lysate) and allow the target protein peaks to be identified subject to mass spectrometry. It was used to identify methotrexate with its primary target dihydrofolate reductase, radicicol and sordarin, trichostatin A and nucleosome remodelling and histone deacetylation (NURD) complex.

Recently, a newly emerged methodology of compound-centric quantitative proteomics has been applied to reveal protein targets for small molecules\textsuperscript{187}. It combines high-resolution mass spectrometry with bioinformatics analysis. Cell lysate was treated with compound, but there is no clickable reaction, allowing bound proteins to be eluted and subject to SDS-PAGE or shotgun proteomics analysis. Compared to traditional target identification, chemical proteomics is unbiased and applies to the whole proteome, great for proteins with post-translational modifications and proteins at their natural expression level. However, it is subject to high background and gives no indication of compound IC50. It uses two types of labeling to achieve protein quantification and demonstrate ligand-target protein interaction: metabolic labeling as in stable-isotope labeling by amino acids in cell culture (SILAC)\textsuperscript{188} and chemical labeling as in isotope-coded affinity tag (ICAT)\textsuperscript{189}. SILAC allows sample pooling early in the process, eliminates quantification errors due to sample handling, but it is limited to immortalized cell lines; ICAT and iTRAQ can label more versatile sample types, but they rely on peptides to generate labels and are prone to more variation and less accuracy later in the proteomic workflow.

In conclusion, there are many affinity-based methods that allow for protein target to be identified with a chemical probe.
Chapter 4: Enhanced Phenotypic Screen of CTLs Identified Augmenting Compounds

4.1 Overview

This project modified the CTL assay to achieve submaximal cell activation for detecting augmenting effects. Results from this project were described in two publications\textsuperscript{190,191}. One more paper is currently in revision. Immune modulation, especially augmentation by small molecules (referred to as IMiDs, immune modulatory drugs), is of therapeutic importance in tumors or cancers, viral or bacterial infections and vaccination adjuvants\textsuperscript{192–194}. A limited number of IMiDs are available and there is a demand for modulators with novel mechanisms\textsuperscript{83,195,196}. However, the signaling pathways leading to immune cell activation are not fully understood, making it hard to devise target-based screens. In the modified assay, anti-CD3 coated beads were used to activate CTLs in a T cell receptor-dependent manner. The assay was used to screen >300 NPs and >900 small molecules. It led to the isolation and identification of teleocidin A-1 and seven small molecules with augmenting effects only after prolonged incubation.

In the previous chapter, a CTL-based assay was used to screen for novel immunosuppressants in a HTS campaign. The assay used small molecules that bypass the TCR to achieve maximal cell stimulation and granule exocytosis. This worked well for inhibitor screen, however the maximal stimulation did not leave any room for augmenting effect. In addition, the use of small molecule stimulants did not interrogate signaling events upstream of IP3 and DAG. In this chapter we modified the CTL assay so that it is able to achieve submaximal level of CTL activation \textit{in vitro}. Submaximal activation is important for the augmentor screen because it leaves room to observe any augmenting effects. There are two ways of activating CTL submaximally \textit{in vitro}, target cells that engage T cell receptor (TCR) and co-receptor on the cell surface, and immobilized anti-CD3 antibodies that engage TCR\textsuperscript{145}. In the first method, target cells would significantly complicate a
cell-based assay because cytotoxic killing is also affected by the ratio of target:effector\textsuperscript{197}, and the liquid handling system must be very accurate to achieve the same ratio throughout a plate in a HTS setting. In the modified assay, I used microscopic dynabeads coated with anti-CD3 antibodies to activate CTLs through TCR engagement. CD3 is the major component of TCR, and immobilized anti-CD3 can cause TCR to cluster on T cell surface, thus activating CTL as a target cell would have (Figure 14). Anti-CD3 immobilized to test plates can also achieve TCR stimulation, but in this case, cells remain attached to the plate and would require a detaching step prior to flow cytometric measurement. One additional benefit of using dynabeads is that their binding changes the scatter profile of cells in flow cytometer. This allows us look at both TCR-activated cell response and unstimulated cells in a mixed population. The simultaneous assessment of compound effects on activated cells and resting cells is one step in multiplexing the assay towards high-content screening.

\textit{Figure 14 Activation of CTL lytic granule exocytosis through TCR engagement by anti-CD3 beads.}

\textit{T cell receptor becomes clustered and activated when CTL contacts anti-CD3-coated bead. Lytic granules reposition near site of contact and undergo exocytosis. As a result, LAMP is externalized to cell membrane and can be detected by fluorescent anti-LAMP present in extracellular solution.}

\textbf{4.2 Results}
4.2.1 Light scatter profile allows for the identification of cells bound by beads

In order to achieve a submaximal level of cell stimulation that would allow for augmenting compound screening, we replaced small molecule stimulants with antibody coated beads. Cell samples treated with beads showed different side scatter profiles on flow cytometer, and this feature allowed us to differentiate the population of cells bound by beads from cells left unbound from the same sample (Figure 15A). Increasing the ratio of bead:cell resulted in new populations with higher side scatters, reflecting the increased granularity of multiple beads bound to one cell. Since the dynabeads are made of supramagnetic material that likely has autofluorescence, we examined the fluorescence intensity of bead-treated cells in multiple channels and found that the it is maximal in FL3 (Ex488/Em670LP on FACS Calibur). In Figure 15B, cells bound by beads (black) were well correlated to bead alone (blue) for their fluorescence intensity in FL3 channel. In fact, the linear relationship between SSC and FL3 fluorescence remains the same when examining cells treated with more beads (Figure 15B, right). Therefore, we devised the following gating strategy to obtain cells bound by different number of beads: first using FSC-SSC profile to obtain two separate populations (no-bead and one-bead population), then using FL3 fluorescence to refine the two populations. Because dynabeads are autofluorescent in multiple channels (data not shown), we need to compensate for their fluorescence contribution in other channels. By treating them as a separate fluorophore, we were able to generate the compensation matrix that removes their autofluorescence (<20%) in FL1 (Ex488/Em530) and FL4 (Em635/Em661) channels. All histograms and calculations shown in this chapter are based on compensated flow data.

To ensure that anti-CD3 bead binding can activate cells, we measured intracellular calcium level and PKC activation following bead treatment, two signaling events necessary for granule exocytosis. As a negative control, anti-CD8 coated beads were used to treat cells, since CD8 molecules lack intracellular ITAM domains for PLC activation. We hypothesized that if anti-CD3
beads can activate cells through ITAM domains, and given that bead-bound cell population can be gated on flow files based on scatter profile and fluorescence in FL3 channel, we should see cell activation in only anti-CD3 bead-bound population but not anti-CD8 bead-bound population or unbound cell population in anti-CD3 bead-treated sample. In Figure 15C, cells loaded with calcium sensitive dye were treated with beads and measured on flow cytometer at indicated time points, and only anti-CD3 bead-bound cell population showed an increase in intracellular calcium level upon bead binding. Unbound cells from the same anti-CD3 bead-treated sample did not show calcium level rise. Anti-CD8 beads did not activate T cells as there was no increase in calcium level whether cells were bound by beads or not. The level remained the same through the time course as the untreated samples (black). To test for PKC activation, cells were treated with different beads, fixed and permeabilized, and then stained for intracellular kinase using phospho-specific antibodies against p-ERK, a downstream signaling molecule of PKC activation. As shown in Figure 15C middle panel, cells bound by anti-CD3 beads showed an increase in p-ERK level but not cells bound by anti-CD8 beads. In anti-CD3 bead treated sample, cells that were never bound by a bead did not show any ERK phosphorylation. Finally, the level of granule exocytosis was assessed for cells treated with beads. Anti-LAMP was used to detect LAMP externalized onto cell membrane in cells treated with either beads without permeabilization. In anti-CD3 bead-treated cell sample, the one-bead population showed a submaximal increase in cell response, whereas the no-bead population from the same sample showed no response. In anti-CD8 bead-treated cells sample, neither population showed any exocytosis (Figure 15C bottom). In conclusion, anti-CD3 beads can bind to cells and activated exocytosis through both calcium signaling and PKC activation.
Figure 15 Changes in scatter allow detection of cells bound to antibody-coated beads.

(A) Plots of forward scatter vs. side scatter from cells (top) and cells treated with increasing amounts (middle, bottom) of anti-CD3 beads. Arrows indicate cells alone and cells bound by beads from a mixed sample. (B) Left: Plot of FL3 fluorescence vs. side scatter of two samples overlaid: cells + anti-CD3 beads (black) and beads alone (blue). The bead sample includes some doublets. Right: Plot (linear scale) of side scatter vs. FL-3 fluorescence for the population shown in A, bottom. (C) Top: Plot of Fluo-4 fluorescence vs. time cells bound to anti-CD3 beads (red triangles), unbound cells from the same population (red open triangles), cells bound to anti-CD8 beads (blue squares), and unbound cells from the same population (blue open squares). Untreated cells are shown in black circles. Middle: Anti-phospho-ERK staining from bead-bound and bead-free cells from populations to which anti-CD3 or anti-CD8 beads were added. Bottom:
Anti-LAMP staining from bead-bound and bead-free cells from populations to which anti-CD3 or anti-CD8 beads were added. Experiments were representative of more than three independent repeats.

4.2.2 Design of bead assay for immune modulator screening

Anti-CD3 beads activate cells through TCR, whereas small molecules TG and PMA can bypass TCR and activate downstream signaling events. We hypothesized that in the presence of small molecule stimulants TG and PMA, CD3-triggered response could be increased to a higher level.

To assess the level of granule exocytosis in these conditions, anti-CD8 and anti-CD3 beads were used to treat cells in the presence of TG and/or PMA. As shown in Figure 16A, cells responded to anti-CD3 bead (red) binding but not anti-CD8 bead (blue) binding as previously observed. When PMA is added together with anti-CD3 beads to cells (orange), cells bound by beads showed an increase in LAMP externalization, whereas cells not contacted by beads remained unstimulated. Since PMA can activate PKC, and kinase activation alone is not enough to trigger granule exocytosis, cells from no-bead population did not show any response. In one-bead population, cells were activated through TCR, and with the addition of PMA, kinase signaling was maximized and therefore generating a higher response compared to anti-CD3 beads alone. When TG is present in anti-CD3 bead-treated samples (green), there is a slight increase in granule exocytosis level in one-bead population, and no-bead population also showed increased level of exocytosis. TG can trigger calcium release, and increased calcium can activate conventional PKC isoforms. When both TG and PMA are present (pink), cells responded maximally in both one-bead and no-bead population, supporting our hypothesis.

With anti-CD3 bead-stimulated response serving as a baseline for screening, we expect to observe three kinds of immune modulatory actions: immune inhibitors that decrease the level of granule exocytosis when TCR is activated, and anti-CD8 bead-treated sample serve as a control for this type of modulation; immune enhancers that increase the level of granule exocytosis in one-bead population but not no-bead population, and anti-CD3 bead+PMA serve as a control for...
this type of modulation; immune stimulators that increase the cell response in both one-bead and no-bead populations, and anti-CD3 bead+TG or anti-CD3 bead+TG+PMA serve as a control.

To assess the plate uniformity and assay robustness, we tested cells treated with beads in different conditions on 96 well plate (Figure 16B). Each column of the plate contained wells treated with the same conditions in repeats, and the plate was incubated with anti-LAMP with constant rotation for one hour, before being fixed with PFA and sampled on flow cytometer. The cells were not washed prior to sampling, because our prior experience with LAMP assay showed that fluorescence signal by LAMP staining acquired on flow cytometer was robust enough without washing away the excess antibody present in the solution. There is no apparent drift in cell activity level over the course of sampling, indicating a stable baseline for LAMP measurements. We tested a total of three control plates, each on a different day. A standard statistical method Z' factor was used to assess the assay robustness, and repeated plate testing yielded Z' > 0.5 between anti-CD8 bead- and anti-CD3 bead-bound cell samples, as well as between anti-CD3 bead-bound cells and anti-CD3 bead+TG+PMA, indicating excellent assay quality for both inhibitor and augmentor screening.

Figure 16 Anti-CD3 beads trigger submaximal exocytosis facilitating a robust plate-based assay format.

(A) Histograms of anti-LAMP fluorescence for the conditions indicated. (B) Plots of the geometric mean of anti-LAMP fluorescence from a 96-well plate containing cells and anti-CD8 beads (circles), anti-CD3 beads
(squares), anti-CD3 beads + PMA (diamonds), and anti-CD3 beads + TG + PMA (triangles). Responses from cells assessed as bound to beads by scatter are shown on top. Responses from cells that are not bound to beads are on the bottom. Experiments were conducted more than three times. TG and PMA were used at final concentrations of 2 µM and 100 nM, respectively.

4.2.3 Bead assay can detect inhibitory compounds of granule exocytosis

In our previous study, a total of 161 inhibitors of granule exocytosis were identified from screening the MLSMR library. We made use of the cherry-picked 75 inhibitory hits from the primary screen and tested their effects on cells stimulated by anti-CD3 beads. Not all of the inhibitors were confirmed on retesting with TG+PMA stimulation (Figure 8), so we expected to see a wide range of compound activity in bead assay. Shown in Figure 17A is a comparison of compounds effects on cells in response to anti-CD3 bead stimulation between LAMP assay and BLT esterase assay, a standard measurement of granzyme release from lytic granule exocytosis. By conducting the two assays in a combined procedure on the same cells as previously described, we hope to minimize the difference between testing and offer a direct comparison between the two measurements. The dotted line indicates linear regression fit of data, and it shows a good correlation between LAMP measurement and the esterase release measurement in bead stimulated samples ($r=0.88$). When collecting supernatant for esterase assay, there was no differentiation between esterase released from bead-bound cells and random release from unbound cells. Whereas all LAMP measurements were cells bound by beads from gated samples. This could explain some discrepancies in data.

We next compared cell response to anti-CD3 beads and TG+PMA using LAMP assay to assess how the modified assay performs (Figure 17B). We found that the two assays correlate for most compound activities ($r=0.77$). Some inhibitory compounds showed higher % inhibition in TG+PMA stimulated cells than anti-CD3 bead stimulated cells, probably due to the more upstream signaling events initiated by anti-CD3 bead binding. Similar result is obtained when comparing compounds
effects on cells stimulated with anti-CD3 beads vs TG+PMA using BLT esterase assay (Figure 17C, r=0.72). We concluded that bead assay can reliably detect inhibitory compounds based on LAMP staining, and BLT esterase served as an orthologous biochemical testing.

Finally, we chose two inhibitory compounds (SID 7977862 and SID 103159345, Figure 12 and Figure 13) that were confirmed from powder resupply to test in the bead assay, and compared their dose response curves to LAMP assay with TG+PMA stimulation (Figure 17D). Both compounds block lytic granule exocytosis without affecting any known signaling pathways, and prior dose responses conducted with TG+PMA stimulation indicated that SID 7977862 is more effective. In anti-CD3 bead-treated samples, SID 7977862 showed similar dose-dependent effects on cell response, with the IC50 lower than the other compound. Dose responses obtained by using different types of stimulation yielded similar results for each compound, indicating good correlation between the bead assay and TG+PMA stimulation.
Figure 17 Detection of exocytosis-inhibiting compounds.

(A) Plot of inhibition of anti-LAMP fluorescence vs. inhibition of BLT esterase release for cells that were treated with 75 compounds from the NIH’s MLSMR, 48 of which were confirmed to inhibit lytic granule exocytosis, then stimulated with anti-CD3 beads. All compounds were tested at a final concentration of 30 µM. Numbers are compound identifiers. (B) Plot of the inhibition of anti-LAMP responses measured from A vs. inhibition of responses measured previously for cells stimulated with TG + PMA. (C) Plot of the percent inhibition of BLT esterase activity for cells stimulated with anti-CD3 beads vs. percent inhibition of BLT esterase activity measured previously for cells stimulated with TG + PMA. For A–C, data represent the mean of three independent experiments conducted with anti-CD3 beads and four experiments conducted with TG + PMA. Lines are regressions, fit using Minitab software. (D) Dose–response curves for inhibition of anti-LAMP staining for cells treated with SID7977862 (closed symbols) or SID103159345 (open symbols) stimulated with anti-CD3 beads (triangles) or TG + PMA (circles). Data are the mean ± SEM of three separate experiments. Dashed lines represent fits to the Hill equation, with IC50 = 0.6 and 0.7 µM for TG + PMA–stimulated and anti-CD3 bead–stimulated inhibition by SID7977862, and IC50 = 3.4 and 10 µM for TG + PMA–stimulated and anti-CD3 bead–stimulated inhibition by SID103159345.

4.2.4 Screening of tunicate-associated bacterial extract library led to the isolation of an immune enhancer identified as Teleocidin-1

Using the bead assay, we conducted a screen of natural product library obtained from Dr. Marcy Balunas lab from the School of Pharmacy. Tunicate specimens were collected from shallow water dock lines in three different locations and tunicate-associated bacteria were isolated for culture in lab. Bacterial products were extracted using bead binding followed by consecutive washing. By screening bacterial product library, we hope to identify some novel immune modulatory NPs.

Tunicates are marine invertebrate animal, and they belong to Chordata-Tunicata. Tunicates can replicate by budding and become colonies. Adult tunicates are often permanently attached to hard surfaces such as rocks on the ocean floor, though some tunicate species can also swim freely in the ocean. Tunicates feed on planktonic particles by filtering sea water through their tube-like
bodies with two openings, known as siphons. They are closely related to vertebrates because of their tadpole-like form during the larval stage.\textsuperscript{198} The name tunicate comes from their unique outer covering or "tunic", which is formed from proteins and carbohydrates, and acts as an exoskeleton. In some species, it is thin, translucent, and gelatinous, while in others it is thick, tough, and stiff. Previously, didemnins were isolated from tunicates collected in the Caribbean Sea. The cyclic depsipeptide compound was investigated in several phase II clinical trials for various cancer treatment, but it was eventually withdrawn due to its high cytotoxicity and anaphylactic reactions.\textsuperscript{199}

Tunicates possess both cell mediated immunity and humoral immunity. Their immune cells express histocompatibility molecules and demonstrate cytotoxicity. They can also secrete soluble molecules such as defensins and cytokine-like molecules to inactivate certain antigens.\textsuperscript{200} Therefore, bacteria associated with tunicates are likely to synthesize and secrete molecules to modulate the host immune system. In addition, marine NPs are favored over terrestrial NPs, because they possess chemical and biological novelties.\textsuperscript{201} Given the earth is largely covered by ocean, and that marine animals constant live in an environment filled with bacteria, fungi and other microorganisms, it is not surprising that ~70% of molecular scaffolds are used exclusively by marine agents.\textsuperscript{202} One common problem in natural product study is the resupply of materials. In our study, tunicate-associated bacteria are first cultured in laboratories and processed to obtain extracts, so resupply of natural product can be achieved by expanding bacterial culture.

A total of 320 extracts were screened at the final concentration of 10\(\mu\text{g/ml}\), and we found two extracts that increase cell response to a higher level when cells are bound by anti-CD3 beads. Figure 18A shows the screening result of one of the library plates that contained the more enhancing extract. Each black circle represents cell response to anti-CD3 bead binding in the presence of an extract. The arrow indicates an augmenting extract which was later confirmed using LAMP assay. Further testing of cells incubated with the extract in the presence of anti-
LAMP also showed that it did not stimulate granule exocytosis on its own. Since each extract potentially contains multiple compounds, we further fractionated the extract based on polarity and tested each fraction on cell response. The activity-guided fractionation helped isolate the most active fraction (Figure 18B). It can augment the level of cell response to five-fold of that of anti-CD3 bead binding. From the most active fraction, a purified compound E1 was isolated using RP-HPLC with a retention time of ~7 minutes. A dose response of purified compound E1 indicated augmenting activity within the nanomolar range (Figure 18C, EC50=1.2nM).

Next we wanted to identify MMOA for E1 by conducting follow up assays. As mentioned previously, both calcium rise and protein kinase activation are required for lytic granule exocytosis. We felt it unlikely that E1 is acting on cells through calcium signaling, because it had no stimulatory effect on cells by its own. This was confirmed using cells loaded with calcium sensitive dye Fura-2 and treated with E1 for live cell imaging (data not shown). We then tested E1 effects on kinase signaling using phospho-specific antibody against active PKC substrate. E1 was found to be an activator of PKC, and the activating effect was independent of calcium. To measure E1’s activating potency, a dose response was carried for cells treated with E1 alone and then subject to permeabilization and antibody staining (Figure 18D). Compared to PMA as a positive control for PKC activator, E1 demonstrated similar activity range with an EC50 of 6.9nM (3.6nM for PMA).

Structural elucidation of E1 was carried out using 1D and 2D-NMR as well as mass spectrometric analysis. These led to the identification of E1 as teleocidin A-1, a known PKC activating metabolite also known as lyngbyatoxin A203. Further testing using optical rotation and circular dichroism confirmed the configuration of E1 to be teleocidin A-1204.
Figure 18 Detection and activity-guided isolation of an exocytosis-enhancing PKC-activating compound.

(A) Screening a plate of bacterial extracts at the final concentration of 10 µg/mL. The geometric mean of anti-LAMP fluorescence is plotted for cells treated with anti-CD3 beads and bacterial extracts (black), cells treated with anti-CD8 beads (blue), or cells treated with anti-CD3 beads (red). The arrow indicates an enhancing hit. (B) Fractions (A–F) of the extract in well A2 tested for anti-CD3 bead–stimulated lytic granule exocytosis. (C) Dose–response measurement of the effects of compound E1 on lytic granule exocytosis (normalized anti-LAMP fluorescence to anti-CD3 bead–stimulated response). EC50 = 1.3 uM, hill rate = 1.2. (D) Dose–response measurement of PKC activation by compound E1 isolated from fraction E of the extract in well A2 (circles) and PMA (squares). Lines indicate fits to the Hill equation. Experiments were conducted three times in C and twice in D.
4.2.5 Screening of NCI diversity set V plates

We wanted to use the enhanced assay to identify more active compounds so that we can subject them to the secondary assay flow, such as testing compound effects on cytokine production by helper T cells, antigen presentation and immunoglobulin secretion by B cells. The enhanced assay was used to screen NCI diversity set V (Figure 19). This set prioritized molecules with rigid formations and pharmacologically desirable features.

The primary screen was carried out over 15 days for 12 plates. Compounds were screened at the final concentration of 100 µM in complete culture medium. The concentration was determined to identify compounds that could work on cells in a short time (30 minutes). When normalizing data to anti-CD3 bead controls treated with DMSO, we observed an offset in cell responses to the majority of the library compounds (Figure 19A). This is likely due to different DMSO used in compound resuspension. Since the library plate received was in DMSO form without excess solvent in control columns, we used our own DMSO as controls. One way to compensate for the offset is by normalizing compound-treated samples to the median cell response in each plate. However, some plates are not normally distributed and it requires more labor to select the median value for each plate. Therefore, we resolved to use a more stringent threshold for inhibitor selection and a more flexible one for augmentor selection. Among 960 compounds tested, 42 inhibitors (>90% inhibition) and 14 augmentors (150% compared to anti-CD3 bead control) were identified. Two augmenting compounds were activating cells on their own and were categorized as stimulating hits. Confirmation run resulted in 12 inhibitors at 10 µM and 8 augmentors at 100 µM. A lower concentration was chosen for inhibitory compounds in the confirmation run to reduce the number of inhibitors for followup assays. In comparison, augmenting compounds are more interesting and in greater demand regardless of their mechanisms of actions.

Dose response testing was carried out for all confirmed hits (Figure 19C). All of the augmenting hits were active at 30 µM or 100 µM, but not at lower concentrations. Compounds active only at
higher concentrations were more likely to be activating cells in non-specific ways. Their dose responses cannot be fitted to Hill equation properly due to missing data points within the high concentration range. However, the highest concentration obtainable was restricted by the library, which contained 10 mM stocks of compounds. At 1% DMSO the final concentration in cell solution is 100 µM. Inhibitory compounds showed similar dose responses, with most activities observed only at 10 µM. Two inhibitors even lost their activity at 10 µM likely due to compound degradation from repeated freeze-thaw cycles.

Figure 19 Screening NCI diversity set V with bead assay led to both augmentors and inhibitors.

(A) Compounds in diversity set were screened at the final concentration of 100 uM in complete culture medium. Cells bound by beads were gated in flowjo software and analyzed for APC fluorescence. The ratio of APC to CD3 control was plotted for the whole library as a histogram, with the line indicating CD3 control.

(B) Formula molecular weight of compound was collected from library map and plotted against ratio of APC. The horizontal lines indicate the arbitrary thresholds set for selecting augmentors (150% of CD3) and
inhibitors (90% below CD3). (C) Dose response was carried out for confirmed compounds. The augmentors concentrations ranged from 0.1-100 uM and inhibitors from 0.01-10 uM in 3-fold serial dilution. Dose responses were fitted to Hill equation for 8 inhibitors.

We wondered whether longer incubation time with augmenting compounds could shift their dose responses. We tried 4 hours of incubation with augmenting compounds over the same concentration range, and augmenting activities were obtained only at the highest concentrations. One compound caused cell death according to FSC-SCC profile at 100 µM. There was no clear shift in compound potency with prolonged incubation time (data not shown).

4.2.6 Incorporating Multiple Treatment Times in Bead Assay

The diversity set screening indicated that the length of compound treatment time can determine what type of compounds can be identified through the screen. A short incubation time could yield less specific compounds that are only active at high concentrations. Treating cells with compounds at lower concentration for prolonged time could lead to more potent compounds being revealed. To facilitate identification of compounds with a wider variety of MOAs, it may be desirable to devise an assay format incorporating varying lengths of compound treatment time. We envisioned that compounds active after short-time incubation are likely to target immediate effects in cell activation, such as calcium release and PKC activation; compounds active after long-time incubation may affect PKC degradation, IL-2R gene activation.

Fluorescent cell barcoding technique was incorporated to enable multiple treatment time. The technique was described previously by Nolan group to reduce antibody consumption in 96-well plated phospho-specific flow cytometry. Here we used it to multiplex the bead assay in the time dimension. In the process, cells were first labeled with an inert fluorescent dye, Calcein AM, prior to being added to the plate containing test compounds at different times. Different levels of dye labeling allowed cell mixtures to be resolved later for different treatment time (Figure 20A). Calcein AM dye was chosen because its fluorescence spectrum has the least overlap with that of LAMP
antibody or bead autofluorescence. Once taken in by cells, Calcein fluorescence is retained by cells for a long time (Figure 20B). Mixing cells barcoded with different levels of dye for up to 6 hours showed clear separation of each individual populations. We determined on using the highest level of dye loading to barcode the cells for 30-minute treatment time, intermediate level for 6-hour, and unloaded cells for 24-hour to avoid potential dye leakage. Cell testing showed that calcein dye, even at its highest loading concentration, does not interfere with cell response to different stimulants (Figure 20D).
Figure 20 Multiple treatment time with compounds is achieved by fluorescent cell barcoding.

(A) FSC-SSC of a mixed cell population barcoded with different concentrations of Calcein dye at 0, 50 nM, 200 nM. Cell populations were washed in dye-free medium before being combined. FITC fluorescence reveals three individual populations of cells indicated by different fluorescence intensities. (B) Calcein dye fluorescence is retained by cells for 6-hour after labeling. Square: 0 uM, open circle: 50 nM, closed circle: 200 nM. (C) Proper compensation matrix removes fluorescence spillover between the channels for Calcein dye, bead autofluorescence, and LAMP antibody. (D) Mixed cell population from A were treated with small
molecule stimulants TG+PMA versus control DMSO, or bead stimulant anti-CD3 beads versus control anti-CD8 beads, in the presence of LAMP antibody. Samples were resolved based on Calcein fluorescence intensity and results shown are compensated LAMP histograms.

We first compared MOAs of imiquimod and PMA using the multiple treatment time (Figure 21A). Previously we have shown that imiquimod is able to enhance the level of CTL granule exocytosis in response to anti-CD3 bead treatment after 6-hour incubation time, whereas PMA has stimulatory effect on CTLs within one hour. Here we treated cells with imiquimod or PMA for different time in 96 well plate to test the multiple treatment time protocol in screening format. After resolving cell populations from mixed samples in repeat wells, we compared the LAMP fluorescence of bead-bound subset between different treatment and between different incubation time. As expected, the stimulatory effect of PMA on anti-CD3 bead-treated cells was most clear in 30-minute cell population, and it disappeared in 24-hour cell population. In comparison, the augmenting effect of imiquimod was not observed in 30-minute but started showing in 24-hour cell population. The different modes of action would have been missed if we carried out the screen at only one time point.

We implemented the multiple treatment time protocol to the re-screening of diversity set library at 10 uM. A lower concentration of library compound was chosen to decrease chances of cytotoxicity over long incubation time. We anticipated more authentic hits would be identified using lower concentration with longer treatment time. To simplify the screening procedure, we decided on a 2-time point protocol, 30-minute for short term effects, and 24-hour for long term effects. We reasoned that compounds showing long-term effects could be analyzed in time course experiment to determine the onset of their effects. The re-screen of the NCI diversity set V library led to the identification of 14 augmenting compounds (conducted by Liza Henowitz), and seven augmentors were confirmed from powder resupply (Table 3). All but one showed augmenting activities on
bead-triggered lytic granule exocytosis after 24-hour treatment with cells, and one compound (NSC 11643) showed augmenting activities in 30-minute treatment as well (Figure 22).

Figure 21 Multiple treatment time allows for detecting compounds with different mode of actions.

(A) Cells were treated with DMSO control, 10 uM Imiquimod or 100 nM PMA for indicated time. After incubation, cells were treated with anti-CD3 beads or anti-CD8 beads in the presence of LAMP antibody. Data were resolved from barcoded cell mixture. Each data point represents an individual repeat with average and standard error shown. (B) Dose response of one augmenting hit NSC 125095 identified from diversity set screening and found to be active only after 24 hours of compound treatment. Geometric mean of LAMP fluorescence for cells+anti-CD3 bead was compared to that of DMSO treated control.

Table 3 Augmenting hits of CTL lytic granule exocytosis identified from NCI diversity set over long-term incubation.

<table>
<thead>
<tr>
<th>NSC</th>
<th>MW</th>
<th>CAS</th>
<th>LogP</th>
<th>SMILES</th>
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<tr>
<td>159632</td>
<td>236</td>
<td>53043-28-0</td>
<td>-0.72</td>
<td>C(=O)C1=C2C=C(C)OC=C2C(O)C(C)(O)C1=O</td>
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<td>7420</td>
<td>298</td>
<td>6284-50-0</td>
<td>3.64</td>
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<tr>
<td>9782</td>
<td>282</td>
<td>3129/8/6</td>
<td>3.47</td>
<td>CC12CC3C(C=Cc4cc(O)cc(C)c34)C1CCC2=O</td>
</tr>
<tr>
<td>11643*</td>
<td>288</td>
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<tr>
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<tr>
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<td>375</td>
<td>80789-71-5</td>
<td>4.98</td>
<td>Oc1cc(nc2ccc(cc12)C(=O)OCC)c3ccc(cc3)S(F)(=O)=O</td>
</tr>
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</table>
*Compound was augmenting bead-triggered cell response in both 30-minute and 24-hour treatment time.

LogP was predicted by Molinspiration based on SMILES.

Figure 22 Augmenting compounds of CTL granule exocytosis from NCI diversity set V library screen.

Structures for augmenting compounds confirmed from powder resupply with their NSC numbers shown. Refer to Table 3 for detailed information.

4.3 Discussion

We have shown that we can identify TALL-104 cells bound to anti-CD3 or anti-CD8 beads in cytometry via changes in light scatter. This makes it possible to study events associated with
quasi-physiological activation specifically in cells that have received the activating stimulus. Importantly, it also makes it possible to conduct two-way screens in which compounds that either inhibit or enhance lytic granule exocytosis can be detected. When using the assay, comparison of responses in cells that have bound a bead to responses in cells that have not bound a bead makes it possible to distinguish between compounds that stimulate exocytosis on their own and compounds that enhance receptor-stimulated exocytosis (Figure 16A). Thus, the modified version of the assay is capable of identifying three classes of compounds: (1) inhibitors; (2) stimulators, compounds that trigger responses; and (3) augmenters, compounds that enhance receptor-stimulated responses but are not themselves capable of producing responses. We believe that the strategy could facilitate studies of signal transduction and, although we have not tested this here, suspect the strategy could likely also be applied to other cell types, including primary T lymphocytes and other cell types that respond to stimuli presented on a solid phase. Similar beads are routinely used to stimulate cells for functional experiments, and so we think it highly likely that the method will be more generally applicable. Note that the requirement that cells respond to solid-phase stimulation can also be seen as a limitation of the method, as some cells may respond only to soluble stimuli. Also, some types of bead may not produce optical scatter properties that allow their identification. The assay is suitable for screening natural compound collections, and can be used as part of an activity-guided isolation scheme. In preliminary experiments, we found that further miniaturization to the 384-well format is possible (B. E. Edwards, A. Zweifach, unpublished observations.) If, as we strongly anticipate should be possible, the assay can be translated to the 384-well format, it will be possible to conduct HTS for immunologically active compounds. As it is, a 96-well format enables screening of moderately sized collections.

Our assay has both similarities to and differences from an assay that used anti-LAMP antibodies to assess exocytic responses in NK cells stimulated with plate-bound activating antibodies. Both assays use flow cytometry to assess exocytosis via binding of anti-LAMP
antibodies. However, that assay was not able to distinguish responses from cells that did or did not receive the activating stimulus. Thus, while they were able to identify a number of stimulatory compounds in the Prestwick compound library—along with a number of inhibitors—they were unable to distinguish whether those compounds cause exocytosis on their own or augment receptor-stimulated responses. One powerful feature of their assay was that they multiplexed detection of exocytosis with measurements of protein synthesis and inside-out changes in cell adhesion. It is possible that we could enhance our assay by adding additional antibodies able to detect other cellular events. However, the fluorescence of the beads would limit the additional channels that could be used.

In another study, AID 2052 used primary murine CD8+ T cells in a cell-based HTS to look for small molecules that could affect the interaction of LFA-1 and ICAM-1 and therefore achieve immune inhibition of CTLs. The assay was also multiplexed by combining different measurements of vehicle binding to T cells. More interestingly, they proposed for a combined analysis of high-content screening, in that arbitrary coefficients were assigned to each parameter and a combined activity score was calculated for each compound. They screened 326,161 molecules and identified 221 active hits (0.07% hit rate) that inhibited LFA-1 and ICAM-1 binding at 7uM. Potential mechanisms for inhibitors are inside-out signaling and protein-protein interactions. The assay was within 2 hours, so gene transcription level change is unlikely to be a mechanism.

Our assay’s ability to identify inhibitors of lytic granule exocytosis was confirmed by the fact that we were able to detect a number of inhibitory compounds with different MMOA that we originally identified with the prior version of the assay (Figure 17). Just as important, many compounds that ultimately proved not to be inhibitory using the prior assay also did not inhibit using the new format. Agreement between the results with the old and new assays was not perfect, but that might be explained by degradation of compounds in storage over time or by differences in the dependence of granule exocytosis on strength of signals when cells were stimulated with soluble TCR-
independent chemicals compared to stimulation through their TCRs. Little is known about how the strength of signals is translated to lytic granule exocytosis, and so it is not clear that perfect correspondence in the degree to which exocytosis is inhibited by compounds should always be expected. Supporting the ability of the new assay to identify compounds that enhance lytic granule exocytosis, the compound we identified from bacterial extracts, teleocidin A-1, is a known PKC activator, and has been shown to enhance granule-dependent cytotoxicity in primary human lymphocyte preparations^{203,205–207}.

The assay we developed was successfully utilized to screen 320 natural product extracts from tunicate-associated bacteria. Compared to screening synthetic compound collections, screening of natural product extract libraries presents several difficulties in the search for novel compounds with biomedically relevant activities^{208}. Extracts are complex mixtures of compounds that often range in polarity and solubility. Active natural product compounds may be present in only minute quantities, and their activity may be overshadowed by other bioactive components or interfering nuisance compounds within the mixture. Crude extracts often contain pigmented compounds and may also contain autofluorescent metabolites. In addition, compounds may be less soluble when isolated and/or be unstable when not in the extract matrix.

That our assay is suitable for screening both synthetic compound collections and complicated natural product mixtures for exocytosis-suppressing and exocytosis-enhancing compounds suggests it might be a powerful means of discovering immunologically active small molecules from diverse sources. Using short compound treatment times, as we did with tunicate-associated bacterial extract library, we suspect that the assay is most likely to find compounds that, like teleocidin A-1, enhance signals leading to lytic granule exocytosis. In addition, we could likely detect compounds with other mechanisms of action with longer treatment times as in diversity set library screen. In a trial compound testing, we found that treating cells with the known immunomodulator imiquimod increased anti-LAMP staining by ~75% in cells bound to anti-CD3
beads after a 6 h incubation. Compounds that affect lytic granule exocytosis would affect immune functions that involve lytic granule exocytosis, and on this basis might be expected to modulate antiviral and antitumor immunity. Furthermore, we would anticipate that many compounds identified as active in a screen based on lytic granule exocytosis would also affect the activity of other immune cells. Thus, the assay we describe here may provide a general means of screening compound collections for immunologically active small molecules. We surmise that one reason there are so few immunomodulatory small molecules is that to date, there has not been a convenient way to screen for them. With our assay, the ease of screening both synthetic and natural extract libraries should make for more rapid discovery of new small-molecule immunomodulators.

4.4 Future direction of novel immune augmenting compounds

Seven compounds emerged from the NCI mechanistic set screening with multiple treatment time. They are all augmentors of CTL lytic granule exocytosis after long-term incubation. Does this mean they are all immune augmenting compounds? Not necessarily. As discussed in detail in this section, augmenting lytic granule exocytosis may not lead to increased target cell killing.

To test whether these compounds possess general immune augmenting activities, a future direction is to develop a set of secondary assays with other immune cell types. CTL hits that show activity in other immune cell types are broadly immune modulatory compounds, and they could have immense therapeutic values in treating complicated diseases, such as reverting the immune inhibitory effect by the tumor microenvironment. If broadly immune modulatory compounds work via novel mechanism, they are potential chemical probes that can be exploited to investigate novel regulators conserved across multiple immune cell types. Once characterized, these protein targets can be used to design target-based screenings to look for pharmaceutics that can regulate immune cell activity.
I selected the following cell models to test *in vitro* with a focus on adaptive immunity, and I compiled assays that measure each cell activity individually (Figure 23). These cell types are chosen because their assays are well developed and relatively easy to perform. Additionally, assays in this section are designed to prioritize hits, instead of selecting hits. All hits at this stage are worth follow-up. The purpose is to prioritize hits that could affect more than one immune cell types.

*Figure 23* Follow-up testing of immune augmentors in other cell types.

*(left)* Adaptive immune response to virus infection\(^{209}\). *(right)* Selected cell types will be tested in cellular assays according to the flow chart.

**Target cell killing assay by CTLs to select physiologically relevant hits** – Hits identified through LAMP externalization can affect lytic granule exocytosis, but they may have no effect or detrimental effects on target cell killing activity of CTLs. CTLs are capable of serial killing, and only \(\sim 15\%\) of granules are needed per killing. Releasing more granules upon the first target cell encounter may reduce the number of granules available for subsequent target cells\(^{210,211}\). Calcein
release assay is a standard measurement for target cell killing efficiency. Raji B cells serve as target cells for CTLs. Raji will be loaded with calcein dye, then treated with bispecific antibody CD19/CD3 to allow CTL recognition, and last mixed with TALL-104 cells pre-treated with hits. The release of fluorescent calcein into supernatant can be measured on plate reader, and the remaining calcein in cells can be measured after cells are lysed with Triton. % of calcein release can be calculated as supernatant/(supernatant+cell lysate).

*Testing hits effects on helper T cells using ELISA to measure IL-2 secretion* – Helper T cells can produce cytokines to regulate other immune cell activity, so they are important regulators in adaptive immunity. The standard method to detect helper T cell activity is through enzyme-linked immunosorbent assay (ELISA) on interleukin-2 (IL-2) secretion by Jurkat cell line\(^2\). First Jurkat cells will be incubated with hits or equal amount of DMSO in test plates. Then soluble anti-CD3 and anti-CD28 will be added to Jurkats in the presence of cross-linking goat-anti-mouse IgG. After 6 hours, supernatant will be collected, and cells will be lysed to release the remaining IL-2. Standard ELISA will be carried out to measure the amount of IL-2 present in supernatant and cell pellet. The addition of the two represents total IL-2 synthesized and released by Jurkat, and it could be affected by hits on the protein synthesis level. The ratio of supernatant/(supernatant+cell lysate) represents the fraction of IL-2 secreted by Jurkats, and the ratio could be affected by hits on the level of granule secretion. To find a concentration of antibody for submaximal cell activation, I will titrate anti-CD3 from 0.1~10 µg/ml.

*Testing hits effects on natural killer cells using antibody binding to measure lytic granule exocytosis* – NK cells share similar effector functions as CTLs. Active hits identified through CTL-based screen are likely to be active in NK cells. To assess NK cell activity, I will measure LAMP
externalization using fluorescent antibody in flow cytometry\textsuperscript{213}. NK-92 cell line will be used because they can be stimulated by TG+PMA or a target cell line such as K562 cells\textsuperscript{214}. To achieve a submaximal cell response for augmentor testing, target cell K562 will be used in this followup assay. Effector:target ratio will be investigated from 5:1 to 20:1 to identify a submaximal activation condition. NK-92 cells will be loaded with Calcein dye to differ from target cells, pre-treated with hits, then incubated with target cells in the presence of anti-LAMP, and sampled on the flow cytometer.

\textit{Testing hits effects on B cells using endocytosis assay} – B cell is an important component of adaptive immunity. It can secrete antibodies specific for antigens to neutralize pathogens. To measure B cell activity, flow cytometer will be used to measure the amount of protein B cells can endocytose upon cell activation. Ramos B cell line can be activated by anti-IgM, and activated Ramos display increased endocytosis activity\textsuperscript{215–217}. The amount of anti-IgM will be titrated to identify a submaximal level of B cell activation. Anti-CD40 will be used in addition to anti-IgM to achieve maximal cell activation. FITC-labeled Ovalbumin protein will be used to for B cell-mediated endocytosis. Cells will be treated with hits, then activated for 3 hours and read on flow cytometer. The amount of FITC fluorescence indicates the level of cell activation.

\textit{Testing hits effects on dendritic cells using antibody binding to measure antigen presentation} – Antigen processing and presentation by dendritic cells, although belonging to innate immune response, is necessary to elicit the adaptive immunity. In order to assay for dendritic cell activity, cross-presentation of ovalbumin peptide (SIINFEKL) on the cell surface will be monitored by flow cytometry. So far there is only one human dendritic cell line MUTZ-3, but it requires days to present a given antigen\textsuperscript{218}. Murine DC2.4 cells will be used as a cell model for dendritic cells,
since they are established dendritic cell line that can take up soluble ovalbumin *in vitro*\(^{219}\). Upon endocytosis, ovalbumin is digested by proteasomes and presented on MHC I molecules. Activated dendritic cells can be stained with antibody against ovalbumin peptide when bound to MHC I (H-2Kb-SIINFEKL, eBioscience), and the amount of fluorescence can be measured on flow cytometry\(^{220}\). I will titrate ovalbumin from 10-1000µg/ml. The concentration that generates a submaximal level of cell activation will be used for hit testing. I envision that active hits could affect antigen presentation via affecting cytoskeleton-mediated phagocytosis, the maturation of endosome to antigen-processing compartment, or the protease activity in the compartment.
Chapter 5: Discussion on Modern Drug Discovery and Development

5.1 NPs vs. Synthetic Molecules: Learning Lessons from Nature

In traditional cancer drug discovery, almost half of drugs were either NPs or directly derived from them\textsuperscript{128,221}. A recent study listed several important drugs derived from NPs and their clinical uses\textsuperscript{222}. NPs are synthesized or secreted by organisms, so by definition they have binding partners and are more likely to be biologically relevant. They also have more diverse structures and cover larger chemical space, especially the biologically relevant space\textsuperscript{223,224}. NPs are often underrepresented in numbers by most libraries, yet they have higher success rates to become drug candidates\textsuperscript{134,225}. Despite the advantages of NPs, they are not favored by modern therapeutics discovery programs, because they don’t follow easy chemistry or possess drug-likeliness (according to Lipinski’s rule of five, even though he himself noted that these rules do not apply to NPs\textsuperscript{226}) and often material resupply is an issue especially for mass production. Fast dereplication process is also needed for natural product in order to avoid repeated effort of isolating previously known molecular entities.

In our cell-based assays, natural product libraries tend to give a higher hit rate than small molecule libraries. Balunas lab had a natural product library composed of $\sim$300 tunicate-associated bacterial extracts, from which 2 augmenting hits were identified and confirmed (hit rate is $\sim$0.7%). In another screening conducted with mushroom extracts, we identified and confirmed 6 inhibitory fractions from only four plates of $\sim$340 extracts (hit rate $\sim$1.8%). Although both hit rates were calculated using small sample sizes from in-house natural product libraries, it shows the rich activities in NPs compared to small molecules. The hit rate is observed to be lower in libraries of synthetic small molecules (MLSMR screen had a hit rate of $\sim$0.04% with 161 hits confirmed from 364,202 compounds). The main reason for this low hit rate could be attributed to the poorly curated library, where chemical liabilities often exist. Especially in the case of MLSMR, after we identified 2404 hits in the primary screen, 40% (944 hits) were not available or had chemical
liabilities and were therefore not included in the confirmatory screening. The purpose of large non-proprietary libraries is to include as many chemicals as possible, instead of assembling drug-like or lead-like chemicals for drug screening campaigns. Even so, it fails to represent the chemicals in the ratio of their success rates in drug discovery and development efforts, as NPs only account for ~1% of total MLSMR of 2010 version\textsuperscript{135}. Chemicals shown as hits could be unavailable from source depositor after several years, and chemicals that routinely showed activities (likely to be nonspecific effects when those activities were observed in different types of assays screened) were not excluded from the library collection. In another effort of screening the BROAD Institute diversity-oriented synthetic (DOS) library, we identified and confirmed the inhibitory activity of 26 compounds among ~10,000 (hit rate ~0.3%), and all hits showed inhibition in either calcium signaling or MEK activity. In addition to library composition, different assay types could account for the differences in hit rates. Bead activation used for the natural product library screen interrogated pathways downstream of TCR activation, whereas small molecules used for MLSMR and BROAD-DOS screen lie downstream of IP3 and DAG. One recent study looked at subsets of chemical libraries and analyzed effects of different chemical forms on the screening result retrospectively. They found that NPs subset, being the most diverse compound class, had significantly higher hit rates compared to traditional synthetic libraries and combinatorial libraries\textsuperscript{227}. A historical review of all new approved drugs from 1981 to 2010 showed that among 1355 drugs, only 29% (387) are purely synthetic compounds, 50% are NPs (including natural product mimics and derivatives) and synthetic molecules with a natural product pharmacophore. The rest 21% belong to vaccines and biologicals\textsuperscript{225}. The reason for this higher hit rate and success rate could be explained by the long evolutionary history of NPs, and there are efforts of biology-oriented synthesis that try to enrich for compounds that resemble the structures of NPs\textsuperscript{228}. Given the higher molecular weights of NPs, they are very good candidates to regulate protein-protein interactions (PPI), which are typically hard to access with small molecules\textsuperscript{228}. In more complicated phenotypic screening assays based on pathways, systems or even organisms, where PPI plays
an important role, NPs may show higher hit rates because they could access and interfere PPI sites better than small molecules. For example, a microbial product tacrolimus (also known as FK506) can bind with FKBP12 and inhibit calcineurin activity\textsuperscript{43}. A fungal metabolite Chlorofusin was identified as an inhibitor of p53-MDM2 interaction\textsuperscript{229}. A closer look at the chemical structures and ring structures of the compounds found in commercially available libraries and the generated database of 26 million compounds revealed that a lot of molecules with higher molecular weights actually resemble their NPs and metabolite neighbors. This bias towards biogenic molecules also resulted in the success rates of screening, and 12,977 (83\%) of the ring scaffolds found in NPs are unrepresented in commercial molecules\textsuperscript{130}. In fact, if synthetic chemistry is striving to synthesize chemicals that resemble NPs, what is the reason of not using natural product library directly in the screening?

The risk of isolating previously known compound is one major concern of using NPs in screening campaigns. NPs libraries used for a screen are often crude extracts or pre-fractionated mixtures, so the process of isolating and identifying the active ingredient(s) from hit wells is necessary following the screening process. This is also known as the dereplication process. In collaboration with Balunas lab, we identified two augmenting bacterial extracts on granule exocytosis process. We investigated the hit with the higher augmenting level, and activity-guided fractionation led to the isolation of an active compound, which later became identified to be teleocidin A-1 using 1D and 2D NMR. The family of teleocidin is a known tumor promoter as it was previously isolated and shown to activate PKC\textsuperscript{206,207,230}. Our work identified a new source of teleocidin A-1 synthesized by marine tunicate-associated bacteria \textit{Streptomyces sp.} strain. With the advances of NMR technology, chemical structures can be determined with raw materials as few as micrograms\textsuperscript{231,232}. To allow for fast dereplication, modern metabolomics workflow also couple LC-NMR stations, analysis software and databases such as Dictionary of Natural Product and Chemspider. Results from such workflows are subject to principal component analysis and pattern
recognition allow for fast comparison of chemical structures to the known chemicals in the database. A recent review introduced the common procedures of dereplicating microbial products from colony isolation, spectroscopic description, re-fermentation and purification, to compound structure identification. Proper use of these procedures should help avoid investing efforts in studying previously known compounds in early stage of HTS using natural product libraries. Importantly, pre-fractionation methods combined with UHPLC-MS profiling of library for library construction also simplifies the dereplication process following the identification of a hit.

One important aspect to consider when constructing a natural product library is to remove nuisance chemical, such as polyphenol tannins from plant extracts. Chemists and biologists also need to consider the possibility of a hit that showed activities in multiple assays be contaminations from culture broth or the leachable from pre-fractionation procedure, such as mycotoxins from fungal culture and plasticizers from equipment coating.

Another concern with NPs is the resupply of more materials. Due to the extreme complex structures of NPs, it is considerably hard to conduct de novo synthesis. In the case of tunicate-associated bacterial product library screening, our collaborators from Balunas lab collected tunicates from different geographical locations, and then cultured tunicate-associated bacteria in laboratories. Since bacterial culture was processed to obtain extracts used in the screen, resupply of natural product can be achieved by expanding the bacterial culture. Natural product material supply issue can also be solved by genome mining. Some databases also support the virtual screening of NPs, such as Chinese Natural Product Database and Database from historical and medicinal plants DIOS.

With the current libraries where NPs are under-represented, chemicals tested in screenings often do not reflect bioactive space. NPs are born with the advantages in the field of antimicrobial and PPI perturbant screening. There is an urge to explore more chemically and structurally diverse NPs in the upcoming era, and there are grants that encourage synthetic chemists to donate their
results including intermediate molecules to the national library, in order to enrich the compound composition\textsuperscript{135,240,241}. It’s my strong belief that more drug candidates and even lead molecules can come from largely unexplored natural sources. Coupled with modern technology that allows for fast dereplication of natural product material, using an in-house chemical library with a smaller size yet more biological relevance should increase the hit rate in a screening campaign. With more researchers coming to realize that NPs are valuable sources for potential candidates, the success rate of discovery effort would greatly improve.

5.2 Phenotypic vs. Target-based Screens: Grow Your Own Fruits in Novel Target Discovery

Historically, a lot of screenings were conducted using cell lines, especially the early anticancer drug discovery efforts, where cancer cell proliferation rate was measured after compound incubation\textsuperscript{123}. Recently the HTS efforts shifted to focus on target-based assays, as more is learned about how cells function, and upregulated proteins or mutated kinases are often thought to have caused the disease. Modern technology even enabled screenings based on biologics entities\textsuperscript{242,243}. I think the phenotypic approach is undervalued at present. In long term, the phenotypic approach is more efficient in generating the first-in-class medicines and it serves as the driving force to the drug discovery field in future\textsuperscript{244}. A phenotypic screen could identify useful compounds as tool molecules for target validation studies. It could also reveal novel molecular mechanisms of biological systems.

Current efforts in cancer drug discovery and development are focused on target-based screenings. One reason for favoring the target-based approach is that most molecular targets are readily available through academic research results. Identification of a novel target without prior knowledge could be years of work, and even once it is identified, target validation process requires tool molecules, knockout experiments and rescue experiments. The pharmaceutical industry only
rewards the first one to hit the market with a drug. In this scenario, researchers are reluctant to make the time-consuming efforts to reveal novel targets when existing targets (although they may not be validated well yet) could yield drug candidates much faster.

Another reason for phenotypic-based screen to be less prevalent is that it may be hard to identify the molecular targets for novel hits, if possible at all. After all, is knowing the mechanism of action really important for disease treatment? In fact, we don’t need to know the targets of medicines as long as they work. Lead candidates could also be approved without mechanisms being identified, and there are drugs on the market without known mechanisms. Valproic acid and clozapine are commonly used to treat neurological disorders, and they have been the most successful drugs even though their targets have only been revealed recently.126

Since a phenotypic assay detects the final output of a biological system, it is not limited by mechanism of targets or pathway-hypothesis, making it capable of identifying compounds with polypharmacological effects. Polypharmacology has been shown to be more important than one particular molecular target in treating complex diseases, such as cancer, cardiovascular, and psychiatric disorders. It has been accredited for the efficacy of many drugs, and targeted polypharmacology could help identify drugs that treat more complex, heterogeneous diseases245. In these cases, drugs working like “magic shotguns” could be more effective than a “silver bullet”.

If phenotypic screens are useful in finding compounds with novel mechanisms, why is there not more phenotypic-based screens? Why are we chasing after the targets that are supposed to be involved in disease onset, rather than setting out to look for compounds that can affect the phenotype directly? For example, within the field of cancer drug discovery, if anti-proliferation is the desired therapeutic effect of compounds, we could simply conduct phenotypic screenings with cell-proliferation as the readout. In fact, NCI routinely conducts anticancer screen using cell proliferation assay.123 Although their library is not featured to be drug-like or lead-like due to chemical properties and cytotoxicity, the mechanisms of actions by which compounds work can
yield further information about molecular targets that are important for cell proliferation. This is also known as chemical library profiling.

Chemical library profiling can be applied to both phenotypic and target-based screening results. We are in an era of generating data from screenings faster than the processing speed. Millions of compounds are screened in multiple assays constantly. With the information we have already gathered, it is possible to construct a knowledge network that can reveal important aspects of biological systems. For example, when a common library is screened in multiple phenotypic assays, one could gather the activity scores of compounds from different assays, and use them to generate matrices comparing chemical structures to phenotypic activities\textsuperscript{246}. One could even multiply two matrices with different information to obtain knowledge such as predicting molecular targets of small molecules\textsuperscript{167}, generating a protein binding profile and correlate it to their cellular function\textsuperscript{247}, guiding chemical synthesis to target desired biological functions\textsuperscript{248}.

One advantage of the phenotypic screen is the identification of tool compounds that can be used for target validation. With thousands of compounds selected through different screening campaigns as probe candidates, how to prioritize their usefulness in target validation? Recently a research group at Novartis proposed for a tool score system\textsuperscript{249}. They used machine learning to conclude a mathematical model, combining strength and selectivity parameters for each compound based on available information. Using the calculated tool score helps prioritize compounds as target validation probes. More studies like this should be encouraged, so we could also devise a similar strategy to prioritize protein targets in future drug discovery campaigns.

In the current fast pace screening, we should never stop with the results obtained from one single screen. Instead, we should constantly seek corroboration between multiple results from independent research groups, as this helps validate the target for a phenotype. A well curated chemical library will facilitate the discovery of novel mechanisms of biological systems.
5.3 Target validation

Target validation is a crucial step before any target-based HTS is conducted. The high cost seen in pharmaceutical industry recently is driven by the high attrition rate\textsuperscript{250,251}. Given that the mainstream of drug discovery effort has been target-based approach, why do so few molecules make it successfully to the market? One reason is that “low-hanging fruits” such as easy targets have already been explored in such approach. Novel targets can result from phenotypic screens that yield compounds with novel mechanisms of actions. Another reason is that targets used for target-based approaches may not be well understood. Classical methods used in target validation involve protein knockout or knockdown experiments, which could affect the structural functions of the target protein other than its enzymatic activity. Small molecules that are routinely used as tool compounds (inhibit the target protein activity without affecting its expression level) to demonstrate proof-of-concept also have limitations, because their activities could result from their off-target effects. This applies especially to preclinical cancer research, where tool compounds are often not specific for just one target and down assays are used for screening.

Kaelin recently pointed out several caveats in preclinical cancer target validation\textsuperscript{252}. The most often observed mistake is to draw a causal link when simply observing a correlation between two factors. Even in cases where a causal link does exist between a phenomenon and the proposed underlying mechanism, it does not rule out the potential involvement of other molecular targets. For example, many gene mutations result in tumor formation, and not one single gene mutation is sufficient in causing the cell transformation. Therefore, they are all be necessary (blocking one mutation product is enough to stop cell proliferation) but not sufficient. Another example is the subcutaneous xenograft model to assess the in vivo performance of anti-tumor drug candidates, however it can be problematic: a xenograft model requires tumor cells to have angiogenic property which pre-select them for anti-angiogenic perturbants; immune compromised mice do not offer normal immunity, and the tumor microenvironment is not representative of human's; the system
focuses on tumor cell proliferation instead of host immunity or tumor metastasis. More careful experimental designs need to be carried out for target validation.

Most preclinical cancer research are based on down assays, that is, evidence shows a loss of cell fitness or tumor growth rate as a result of drug treatment. However, down assays are prone to false positives, as there are more uninteresting ways to make a system work worse than ways to make it work better. Inhibitory compounds could simply be cytotoxic or disturbing the plasma membrane potential. To demonstrate the target as a valid one for the phenotype, rescue experiments are often performed to assess the compounds effects. For example, cells affected by a particular kinase inhibitor can be transfected with cDNA that codes for a mutant kinase resistant to the inhibitor. If the mutant kinase cDNA rescued cells and restored normal phenotype, it suggests that the target kinase is important for the phenotypic change. However, this has a caveat that cDNA could result in constitutive kinase expression and would not apply for cell cycle-related kinases (often presumed to be targets for tumor cell proliferation). Even when the expression is controlled by an inducible promoter, the level of kinase expression could be much higher than the endogenous level. Therefore, the results of rescue experiments may not be physiologically relevant. Also, even if the rescue experiments show results as expected, it does not eliminate the possibility that a second off-target mechanism is involved in causing the phenotype. In other words, the sufficiency of the on-target effect by the perturbant is hard to demonstrate. This scenario often leads to the failure of drug candidates in phase II clinical trials, as cells in vivo could have other mechanisms to rescue the inhibited pathway.

In appendix, I will be discussing a novel approach towards target validation. Instead of relying on a couple of tool compounds in particular to demonstrate the sufficiency of target, we could gather an ensemble of compounds via a combined screen measuring both the phenotypic change and the target activity.

5.4 Quality Control of HTS Assays: Univariate Z’ Factor is Not Enough
The standard statistical tool of assessing the quality and robustness of an assay is the Z’ factor, first introduced by Zhang et al. The equation takes the simple mathematical ratio of the separation band over difference of means for two groups of samples (both are presumed to have normal distribution). Z’ factor is calculated when the equation is applied to negative and positive control samples, and Z factor when applied to control samples and compound treated samples. An arbitrary threshold is set for assays with excellent quality (Z’ >0.5), double assay (0~0.5) and unacceptable assay (<0).

Since the definition of data variability band in Z’ factor is dependent on the normal distribution of sample groups and relatively small standard deviation, Z’ factor can be affected in screens of focused libraries where more compounds are presumed to have an effect on the assay readout. Z’ factor can also be affected by variable controls with a large standard deviation. In our assay using anti-CD3 beads to activate TALL cells, we have seen plates with variable controls that resulted in Z’ factor being 0~0.5. In those cases, cell activities in response to compound treatment were compared to the average of the plate instead of the control wells, and it was assumed that majority of compounds in the library did not have an effect. Ultra-HTS conducted in 1536-well plates may result in lower Z’ factors than those conducted in 96- or 384-well plates, due to inter-well cell number variability and extremely small volume liquid handling.

To solve the problem of low Z’ factor, it has been proposed to replace it with robust Z’ factor. In the robust Z’ factor calculation, standard deviation is replaced with robust standard deviation, and mean is replaced with median, both measurements are less subject to outliers. This adjustment in calculation eliminates the need to go through each control data point and hand-pick outliers, which is a subjective and labor-intensive process. Robust standard deviation=median absolute deviation*1.483 (the factor 1.483 comes from the adjustment of MAD to SD in normal distributed data set), and MAD comes from median of absolute (data point-median of data set).
Z’ factor or robust Z’ factor only applies to one-dimensional data where only one parameter is collected from the screening. In cases such as high-content screening or multiplexed assay, a more sophisticated quality control is required to assess the robustness. One way around this is the multivariate Z’ factor proposed by Kummel254. Combining multiple parameters in high-content screening for a multivariate Z’ factor involves linear transformation of all parameters: individual signal intensities are added up (as long as they change in the same direction, otherwise they are subtracted), therefore reducing the multiple dimensions to one-dimensional data. In cases where a signal is much larger than the other signals, a weighed linearization is used: coefficient*individual signal before adding up (coefficient is different depending on signal scale, determined by linear discriminant analysis).

However, reducing multiple dimensions of the signals into one-dimensional data will lose the benefits and rich information of a high-content screen. As Kummel later pointed out in a review, machine learning should be used to generate cell classifiers, and multidimensional sample profiling should be used to record data from a high-content screen, such as matrix description of cells255. Pattern recognition used for image-based screening and principal component analysis could be adopted to analyze high-content data.

5.5 Bridging Academic Research & Pharmaceutical Industry

There is raising awareness that the high attrition rate in clinical trials is due to selecting poor targets in pharmaceutical industry250. Target validation in preclinical cancer research is often misleading when people mistake correlation for causation252. In the pharmaceutical industry driven by profit, only the first runner gets the prize. Research and development department is affected by the rush to move the chemicals forward into trials and registrations.
There is a lack of funding for academic labs conducting research related to cell-based assays and phenotypic screenings. As Eggert pointed out, traditional hypothesis-driven research has been the focus for funding, whereas HTS, especially phenotypic screening is not hypothesis-driven, the study outcomes from a screening project are not guaranteed; also, some early phenotypic screens generated probes that were not useful due to low-potency or nonselectivity\textsuperscript{119}. Given the uncertain nature of a phenotypic screen, most funding agencies are reluctant to take on the risk.

Collaboration effort could offer financial support and additional resources to academic labs to pursue more hits from their screening. Right now, all research results are protected by intellectual property laws and they belong to the university, especially when federal funding is used to sponsor the research labs. To further pursue any hits for chemical probe development, lead optimization or even clinical trials, both money and multi-disciplinary team are needed, and no research lab alone is resourceful enough see through the process. Many academic drug discovery centers have initiated the effort to establish collaborations and seek alternative funding\textsuperscript{256}. Pharmaceutical companies also have in-house libraries that are often proprietary and used for internal screenings exclusively. Collaborations could allow academic screening centers to use those libraries as alternative resources.

The future of academic drug discovery still relies on the basic research and careful experiment design. Most importantly, we shall collaborate with the pharmaceutical industry to advance our understanding of disease onset and to improve the medicine. The future of drug discovery and development lies beyond its boundaries today.
Appendix: A novel FRET-based assay revealed aerobic glycolytic inhibitors and provided support that glycolysis is a strong anticancer target in leukemic cells

6.1 Overview

The appendix describes a novel approach using an ensemble of compounds to offer unbiased strong small molecule support for a potential anti-cancer pathway. The manuscript from this project is in submission. Preclinical cancer drug discovery depends on small molecules as tool compounds to validate the molecular target and to establish the target's druggability\textsuperscript{257}. However, early tool molecules are not optimized and have multiple effects, so they provide only weak support\textsuperscript{250}. One area in which this is especially apparent is the Warburg effect, the adoption of aerobic glycolysis by cancer cells, which is currently thought to represent an antiproliferative target in many kinds of cancer\textsuperscript{258–260}. We developed a Forster Resonance Energy Transfer (FRET)-based assay using an ATP sensor expressed in K562 leukemic cells to look for metabolically active compounds\textsuperscript{261}. The screen of ~800 small molecules from National Cancer Institute (NCI) mechanistic set yielded 27 inhibitors of glycolysis and oxidative phosphorylation-dependent ATP production. We then mined the NCI’s data set for growth inhibitory effects of the library compounds, and compared the effects of our ensemble of compounds to the whole library set. Compounds selected based on glycolytic inhibition were significantly more anti-proliferative than the overall compound collection, while inhibitors of oxidative phosphorylation-dependent ATP production were not. We calculated the probability of obtaining compounds as antiproliferative as the glycolysis inhibitors from the library purely by chance, and found that it was extremely low. Based on these results, we conclude that glycolytic inhibition is likely a good strategy for blocking leukemic cell proliferation. We propose that, rather than relying on the use of a tool compound for target validation, a screen such as ours— a target validation screen— should be adopted routinely as part of the drug discovery process.
6.2 Background

6.2.1 Cancer drug discovery trend and high attrition rate

The process of biopharmaceutical research and development is time consuming and expensive. For a new molecular entity to be approved by FDA and put on market, it usually takes 12-14 years and almost 2 billion US dollars\(^{262}\). It is estimated that less than 12% of candidate medicines entering phase I clinical trials will make it to the approval stage (http://www.phrma.org/advocacy/research-development/clinical-trials). The high attrition rate in clinical trials is often due to lack of efficacy or safety\(^{251,263}\). The later into discovery and development stage the candidate fails, the more money and resource it costs to pull it out of the pipeline.

Cancer drug discovery in early times was conducted with phenotypic screens looking for anti-proliferative compounds. Then the effort shifted to target-based screens for easier pharmaceutics development and pharmacokinetics study. However, recent cancer drug discovery efforts fail too often\(^{263}\). The current industry model puts much of the effort and resources in lead optimization and conducting phase clinical trials, whereas the process of target selection and validation is not emphasized enough\(^{262}\). The lack of a valid target often leads to failures of drug candidates in phase clinical trials and greatly increase the cost of drug development\(^{264}\). Intuitively, a target-based screen would not result in any useful drug molecules if the target is not effective in blocking tumor cell proliferation. It has been pointed out that target selection and validation is the most important factor in transforming pharmaceutical industry productivity\(^{250}\).

6.2.2 Aerobic glycolysis as a potential anticancer strategy

One area in which this is especially apparent is the study of aerobic glycolysis, also known as the Warburg effect, a phenomenon first described by Warburg in 1926\(^{265}\). Often observed in tumor cells and other proliferating cells, it refers to upregulated glucose uptake and the dependence on
glycolysis for ATP production, even in the presence of functional mitochondria and oxygen\textsuperscript{260,266}. The Warburg effect recently reemerged as an interesting anticancer strategy\textsuperscript{267–270}. Many enzymes involved in the Warburg effect are overexpressed in tumor cells and have been studied extensively as anticancer targets. However, small molecules used to regulate the target protein activity and to demonstrate the Warburg effect validity often have off-target effects and therefore do not serve as strong support for the Warburg effect as effective anti-proliferative strategy.

There are four proposed benefits of the Warburg effect on cancer cells: in the tumor microenvironment with limited energy source, increased level of glucose uptake by cancer cells leaves healthy cells less glucose for energy production or immune function; the end product of glycolysis pyruvate can be converted to lactate and secreted out of cells, which lowers tissue pH and promotes acid-mediated tumor invasion; glycolysis supplies cancer cells with extra carbon source for anabolic activity to produce biomass; altered oxidative phosphorylation in mitochondria changes the reactive oxygen species (ROS) balance and signaling pathways\textsuperscript{266}.

\textbf{6.2.3 Three pillars for a valid target}

Typically there are three approaches towards target validation in anticancer drug discovery\textsuperscript{244,257}:

1) \textbf{Target is overexpressed or mutated in cancer tissues}. Proteomics or genomics studies of patient samples and healthy tissues usually lead to the identification of a protein target being overexpressed or a mutated gene that results in gain-of-function or loss-of-function for certain kinases or channels. In aerobic glycolysis, glucose transporter 1, hexokinase 2 (HK2), pyruvate kinase M2 are found to be upregulated and important for tumor cell growth\textsuperscript{271–274}.

2) \textbf{Altering target expression level reduces proliferation}. Overexpressed or mutated proteins in cancer cells can be knocked out or knocked down in cell lines or animal models with siRNA
or genetic engineering, and researchers can use these *in cellulo* or *in vivo* models to study the effects of target proteins on cancer cell proliferation or cancer development.

However, knocking out or knocking down a protein is not the same as regulating its activity. For example, HK2 is the first rate-limiting enzyme involved in aerobic glycolysis, yet it is also localized at the voltage-dependent anion channels on mitochondrial outer membrane and involved in cytochrome c release and cell apoptosis, as well as ADP/ATP exchange activity. Therefore, knocking out HK2 will likely result in anti-proliferative effects on cells due to its multiple biological functions.

3) **A small molecule that inhibits the target blocks proliferation.** Small molecules are used as tools in preclinical studies to verify the target effectiveness without affecting target protein expression level. They are also important in establishing the druggability of the target.

Due to the nature of pharmaceutics discovery process, small molecules used for target validation are not optimized for their targets and therefore often have off-target effects. For example, 3-bromopyruvate (3-BrPA) and 2-deoxyglucose (2-DG) can inhibit HK2 activity and they were used to support HK2 as an anticancer target. However, 3-BrPA is an alkylating agent that crosslinks key thiols in the cell, generating ROS and resulting in oxidative stress, whereas 2-DG inhibits N-linked glycosylation process, induces ER stress and stimulates autophagy. Their anti-proliferative activities could result from their off-target effects. Even though HTS have been conducted to look for more specific inhibitors of HK2, researchers failed to test the inhibitors on other potential targets such as glucose transporter in cells.

Therefore, small molecules represent the weakest support for a valid target and they fail too often due to their non-specific nature and off-target effects. These observations led to my proposal of conducting a target validation screen to improve small molecule support. The idea is to screen a chemical library against a potential target and identify an ensemble of hits with similar activities:
if a target is valid, all compounds that inhibit it will be anti-proliferative; if a target is invalid, compounds would have the same efficacy as the library as a whole. This approach offers much stronger support than a tool molecule for target validation.

In this project, we used K562 cells with long-term expression of a Forster Resonance Energy Transfer (FRET)-ATP sensor in a multi-read assay to measure ATP production via glycolysis or oxidative phosphorylation (OXPHOS)\(^{281}\). The assay was used to screen compounds from NCI mechanistic set, and we identified 10 glycolytic inhibitors and 7 OXPHOS-dependent ATP production inhibitors. After comparing their anti-proliferative activities, we found that glycolytic inhibitors are more anti-proliferative than the library and the OXPHOS inhibitors. We concluded that inhibiting aerobic glycolysis is a good strategy to block cell proliferation. We also propose to replace the use of a tool compound with a target validation screen, which should be routinely adopted in drug discovery efforts.

6.3 Methods

6.3.1 Cells, constructs, and reagents.

K562 cells were obtained from ATCC (Manassas, VA) and cultured in Iscove’s medium containing 10% newborn calf serum, 1% penicillin-streptavidin and 1% L-glutamine. Additional cell lines tested in Figure 25 were provided by Drs. Charles Giardina and Kenneth Campellone of the University of Connecticut. CDNAs encoding the ATeam FRET sensor\(^{281}\) and the FLII\(^{12}\)-pGLU-600\(\mu\)delta\(\delta\) glucose sensor\(^{282}\) were obtained from Addgene (Cambridge, MA). Cells were transfected using nucleofection and sorted based on YFP fluorescence as described previously\(^{283}\). Flow cytometric measurement of FRET signals were described previously\(^{283}\). Experiments with ATeam-expressing cells were conducted in Ringer’s solution with or without added glucose as indicated. Glucose-free Ringer's contained the same components as ES
without glucose. Where indicated, bovine serum albumin (BSA, Sigma) was added at a final concentration of 0.12% to simulate the protein level in cell culture medium. Sodium azide and glucose were from Fisher (Fair Lawn, NJ). The Cell Titer-Glo 2.0 kit used for luciferase confirmation of effects on intracellular ATP levels was purchased from Promega (Madison, WI). The colorimetric hexokinase assay kit was purchased from BioVision (Milpitas, CA). Both kits were used according to the manufacturer’s recommended protocols. Mechanistic Set III and powder resupply of hit compounds was obtained from the National Cancer Institute’s Developmental Therapeutics Program (Bethesda, MD).

6.3.2 FRET screening.

A Molecular Devices SpectraMax i3x plate reader (Sunnyvale, CA) was used to measure the fluorescence of cells expressing ATeam and FLII\textsuperscript{12}pGLU-600µ in three channels: CFP (450/9 nm excitation, 485/15 nm emission), FRET (450/9 nm excitation, 535/15 nm emission), and YFP (485/9 nm excitation, 535/15 nm emission). For screening with ATeam, cells were washed in glucose-free Ringer’s and added to flat-bottomed Greiner half-area 96-well plates, with ~100,000 cells in 100 µL per well. 1 µl of DMSO or 1 µl test compounds at 1 mM were added to wells, producing a final compound concentration of 10 µM. After addition of cells, plates were vortexed and incubated for 30 minutes at 37 degrees in a humidified CO\textsubscript{2} incubator.

The following equation was used to calculate FRET ratios

\[ \text{FRET ratio} = \frac{\text{FRET}_c - \text{FRET}_b}{\text{CFP}_c - \text{CFP}_b} \]

where FRET\textsubscript{c} and CFP\textsubscript{c} are the fluorescence intensities of cells, while FRET\textsubscript{b} and CFP\textsubscript{b} are background fluorescence intensities of wells containing 100 µL Ringer’s solution.
FRET ratio was determined from cells treated with glucose but not Na azide (P), treated with compounds (C), or treated with Na azide but not glucose (N), and percent inhibition is defined as Equation 2 % inhibition of down assay.

6.3.3 Hit confirmation by Cell-Titer Glo.

Cells were washed in glucose-free Ringer’s and incubated with DMSO or test compounds for 30 minutes at 37 degrees. 50 µl of Titer Glo reagent was added to 50 µl of cells (10,000 cells per well), mixed, and incubated for 10 minutes. Luminescence was measured on the Spectramax i3x plate reader.

6.3.4 Hexokinase (HK) activity assay.

K562 cells (50,000 cells per condition) were lysed with supplied lysis buffer, and 25 µl of cell lysate was incubated with DMSO or test compounds for 10 minutes at room temperature. 25 µl reaction mix (freshly prepared according to manufacturer’s protocol) was added and absorbance at 450nm was measured on the plate reader in kinetic mode. A standard curve of NADH was used to convert deltaOD_{450} to HK activity.

6.3.5 Dose-response measurements.

Compounds that were confirmed active in a repeat of the primary assay were resupplied from NCI in powder and dissolved in DMSO. Concentrations between 0.1-10 µM were tested at 2-fold dilutions on cells. Percent inhibition data were fitted to Equation 3 Hill equation.

6.4 Results

6.4.1 Establishing a stable FRET sensor cell line to measure intracellular ATP level

K562 leukemic cells were transfected with a FRET sensor cDNA which responds to intracellular ATP level change^{281}. There are two reasons for choosing K562 cells: K562 cells display the Warburg effect, as they can maintain high ATP level in the absence of OXPHOS activity but in
the presence of glucose (confirmed by luciferase-based ATP assay on cell lysate Figure 25); K562 belongs to the panel of 60 tumor cell lines chosen by NCI to conduct an anticancer drug screen with their libraries\textsuperscript{123}, and the anti-proliferative data of all compounds are readily accessible for K562 cells.

K562 cells were isolated from a 53-year-old female with chronic myelogenous leukemia in terminal blast crises and established by Lozzio. It hosts the Philadelphia chromosome and can undergo unlimited proliferation\textsuperscript{284,285}. It has routinely been used as a target for natural killer cells due to a lack of MHC I antigens on the cell surface\textsuperscript{286,287}. It is highly undifferentiated cell line of granulocyte lineage, with the potential of becoming erythrocytic, granulocytic or monocytic cell line\textsuperscript{288}.

After two rounds of cell sorting, stable cells were obtained and named ATP cells. Majority of ATP cells express the FRET sensor at a high level according to YFP fluorescence profile in flow cytometry (Figure 24A), and this led us to investigate the use of plate reader to measure FRET signals. Plate reader test was carried out with ATP cells treated with four control conditions in repeats (Figure 24B), and ~10-fold fluorescence signal over background was observed in FRET and CFP channels. Variations between wells could result from different cell numbers, and taking the ratio of FRET/CFP normalized the signals and showed uniformed performance in the plate. FRET ratio change between low and high ATP levels resulted in $Z'>0.5$ repeatedly, demonstrating good assay quality to screen for inhibitors of ATP production.

To find positive and negative controls for our cell-based assay, we tested ATP cells in different conditions and measured their fluorescence intensities (Figure 24C). When cells are in their native solution with plenty of glucose and no inhibitor of OXPHOS-dependent ATP production, their FRET ratio represent the high level of ATP; when cells were washed in glucose-free Ringer’s solution, and sodium azide was added to inhibit the respiratory complex IV, there is a significant drop in ATP level; when cells were treated with sodium azide in the presence of glucose, they can
restore ATP to high level via aerobic glycolysis; and surprisingly, when cells were washed with glucose-free solution, they can still maintain a high level of ATP for 3 hours, probably due to alternative energy source such as stored amino acid and fatty acid.

Figure 24 FRET measurements using a genetically-encoded ATP sensor in K562 cells.

(A) Measuring ATeam FRET with flow cytometry. (i) Plot of forward vs. side scatter for ATeam K562 cells. The live-cell gate is indicated. (ii) Histogram of YFP fluorescence for long-term transfectants expressing ATeam sensor. The blue trace represents the YFP fluorescence of untransfected K562 cells. (iii and iv) Plots of FRET ratio vs. YFP for cells washed in glucose free Ringer’s solution and treated with sodium azide (iii) or in the presence of glucose (iv). FRET ratio was calculated as described in Materials and Methods.

(B) Measuring ATeam FRET using a plate reader. Plots of CFP fluorescence (i), FRET fluorescence (ii), and FRET ratio (iii) for ATeam cells in a 96-well plate treated with indicated conditions. (C) K562 cells can generate ATP in the absence of glucose or functional mitochondria but not both. (i) Average FRET ratios for cells from (B). Data are the mean ± S.D. from 24 wells per condition. Similar results were obtained in multiple independent experiments. (ii) Time dependence of changes in ATeam FRET ratio after cells were washed in glucose-free Ringer’s solution (filled circles), washed in glucose-free solution and treated with...
sodium azide (open squares), treated with sodium azide and glucose (filled squares), or glucose (open circles). Sodium azide and glucose were used at final concentrations of 5 mM and 20 mM, respectively.

The transfection of K562 with ATeam sensor did not affect their catalytic activities or ATP production, as orthogonal assay using Cell-Titer Glo based on luciferase activity confirmed similar ATP level changes in conditions as in Figure 24C. In addition, the Warburg effect was observed in multiple tumor cell lines other than K562 leukemic cells, and most cell lines can maintain a high level of ATP production even when glucose was absent (Figure 25).
Figure 25 Using luciferase to characterize metabolism in various cell lines.

Cells were treated with indicated conditions and ATP level was measured with CellTiter-Glo 2.0 assay kit according to manufacturer’s protocol. Sodium azide and glucose were used at final concentrations of 5 mM and 20 mM, respectively. Data are mean ± S.D. from 3 wells per condition.
6.4.2 Multiple-read assay design and the screen of NCI library led to the identification of several inhibitors of ATP production

The use of plate reader led us to come up with the following assay design which involves multiple readings with addition-only treatment of cells (Figure 26A): 1) ATP cells were first washed in glucose free Ringer's solution to reversibly block ATP production from glycolysis. Cells were incubated with test compounds for 30 minutes and FRET was measured. A decrease in FRET ratio indicates the inhibitory effects on OXPHOS-dependent, glucose-independent ATP production; 2) sodium azide was added to all cells to block OXPHOS-dependent ATP production. FRET was measured and a decrease in FRET ratio was observed for all conditions; 3) glucose was added to all cells to initiate glycolysis. After 30 minutes of incubation FRET was measured, and a decrease in FRET ratio indicates inhibitory effects on ATP production via glycolysis. The sequential treatment of ATP cells and the addition-only steps allowed us to separate ATP production from aerobic glycolysis from OXPHOS. We anticipated four types of effects compounds could have on cells (Figure 26B).

We used this assay to screen ~800 small molecules from National Cancer Institute (NCI) mechanistic set. The library was chosen because all of the compounds have been tested at several concentrations for their effects on cell growth against the panel of NCI60 cancer cells\(^{123}\). The library also includes diverse representative compounds from each cluster of >37,000 open compounds, so it should yield inhibitors with different mechanisms of action. From the initial screen, we identified 50 inhibitors (6% hit rate) of glycolysis and/or OXPHOS with % inhibition>75% at 10uM. In confirmatory assays using FRET measurement, 5 inhibitors showed significant compound fluorescence in more than one runs and we were unable to obtain meaningful measurement of FRET ratio. They were not included in further testing. We then used an orthogonal assay based on luciferase activity to confirm compound activity (TiterGlo 2.0 from Promega). 35 compounds were confirmed for their inhibitory activity (70% confirmation rate).
We chose 30 most active inhibitors to order powder resupply from NCI for further testing, but 1 was not available in powder. Among the 29 compounds reconstituted from powder resupply, all but two compounds were confirmed with % inhibition >75%. We categorized inhibitors into two categories and found 14 OXPHOS inhibitors and 13 glycolysis inhibitors.

Immediate cytotoxicity was tested for all inhibitors as plate-reader based initial screen does not offer any information on % live cell in each well. To test for cytotoxicity, cells were incubated with compounds and regular assay flow was used to obtain confirmatory FRET measurement and achieve the same incubation time. After the third read, PI solution was added to each well and fluorescence 535/617 was obtained on the plate reader. Control samples were treated with DMSO or Triton. One compound (NSC 306864) showed 67% cell killing compared to controls, and the rest of the compounds were < 10% cytotoxic in 2 hours.

We chose 30 most active inhibitors to order powder resupply from NCI for further testing, but 1 was not available in powder. Among the 29 compounds reconstituted from powder resupply, all but two compounds were confirmed with % inhibition >75%. We categorized inhibitors into two categories and found 14 ox-phos inhibitors and 13 glycolysis inhibitors.

It is important to point out that, our screen of the NCI library was not to look for drug candidates or lead molecules. Instead, it was to select a group of compounds based on their inhibitory effects of glycolysis or OXPHOS. This is only the first step towards our target validation approach, and next we hope to compare these two groups of inhibitors to the library set as a whole in their effectiveness in blocking leukemic cell proliferation.
Figure 26 A multiple-read plate reader protocol enables detection of inhibitors of both glycolysis and OXPHOS-dependent ATP production.

(A) Design and performance of an assay with three reads. ATeam cells were washed in glucose free solution and put into a 96-well plate. After 30 minutes of incubation, fluorescence signals were measured and FRET ratio was calculated (1). Sodium azide was added to all but 8 control wells in column 12 and...
incubated for 30 minutes, before the second measurement was taken (2). Glucose was added to all but 8 control wells in column 1 and incubated for 30 minutes, before the third measurement was taken (3). Sodium azide and glucose were used at final concentrations of 5 mM and 20 mM, respectively. (B) Four potential compound effects and the ATeam response produced in the multiple read protocol. (1) No effect on ATP production. Cells were washed with 0 glucose solution, then treated with sodium azide, then glucose; (2) Inhibition of OXPHOS-dependent ATP production. Cells were treated with 5 mM oligomycin, then sodium azide, then glucose; (3) Glycolysis inhibition. Cells were washed with 0 glucose solution, then treated with sodium azide; (4) Inhibition of both glycolysis and OXPHOS-dependent ATP production. Cells were treated with 5 mM oligomycin, then sodium azide. (C) Heat maps of FRET ratios of ATeam-expressing cells incubated in a representative plate that contained compounds from NCI mechanistic set in columns 2-11. Columns 1 and 12 contained control wells as described in (A). The scaling of heat maps is displayed to the right. One well that contained a fluorescent compound is represented in white. (D) Screening 813 compounds from NCI mechanistic set. Data are presented as percent inhibition of FRET ratio compared to control wells in each plate. Inhibition of OXPHOS-dependent ATP production and glycolysis are obtained from readouts 1 and 3, respectively.

6.4.3 Molecular mechanism of action for glycolytic inhibitors

To gain confidence in glycolytic inhibitors activities, we wanted to assign a molecular mechanism of action to at least some of them. Glucose uptake through glucose transporter is the first step in glycolysis, and GLUT1 is found to be upregulated in K562 cells. Glycolysis inhibitors were tested on glucose uptake. To do this, we constructed another FRET-based K562 cell line using pFLIIP glucose sensor. Transfected cells were first washed in glucose free solution and then treated with test compounds. Then 20 mM glucose was added and FRET fluorescence was measured on the plate reader. As a positive control, cytochalasin B was used and showed 100% inhibition of glucose uptake at 100 µg/ml. Only one glycolytic inhibitor (NSC 18268) blocked glucose uptake by 100%, so it was not included in the other mechanism testing. The compound is found to be actinomycin D, a known antitumor chemotherapeutic under the trade name
Cosmegen. Some compounds showed increased level of intracellular glucose, likely due to the build-up of intracellular glucose as a result of inhibited glycolysis.

With only one GLUT inhibitor identified among the mechanistically diverse library was surprising, so the library was screened using Glucose cells and 9 inhibitors were identified to block glucose uptake. Surprisingly, NSC 18268 was not identified in that screen. Instead, NSC 3053, an analog of actinomycin D was identified. None of the other GLUT inhibitors overlapped with glycolytic inhibitors identified in ATP screen. However, a closer examination of ATP cells dose-dependence on glucose level showed that ATP level in cells is less sensitive to extracellular glucose level drop. Combined dose responses showed that [ATP]i can be maintained at high level via glycolysis even when [glucose]o was dropped to 1 mM. We wanted to find out whether other GLUT inhibitors could also inhibit ATP production via glycolysis using ATP cells. In order to observe effects of GLUT inhibitors on ATP levels in cells, we lowered the glucose used to only 0.5 mM, and found three GLUT inhibitors that blocked ATP production by glycolysis by >75% at 10 µM (NSC 61811, 96932, 260610). None of them was identified through the ATP cell screen, likely due to the much higher glucose concentration (20mM) used in ATP assay. Unfortunately, when powder resupplies of GLUT inhibitors were obtained, only one was confirmed (NSC 96932).
Figure 27 Discrepancy of GLUT inhibitors identified from ATP cells and Glc cells screens could be a result of different dose dependence on extracellular glucose.

(A) (left) Plot of FRET ratio vs. YFP for cells washed in glucose free solution (blue) and treated with 20 mM glucose (red). (right) Average FRET ratios obtained from cells in 96-well plate treated with indicated conditions in repeats. Fluorescence signals were measured on the plate reader. Data are the mean ± S.D. from 16 wells per condition. Similar results were obtained in multiple independent experiments. (B) Structures of NSC 18268, actinomycin D (MW3808), an inhibitor identified through ATP screen; and NSC 3053, Dactinomycin (MW1255), an inhibitor identified through GLUT screen. (C) Dose-response testing glucose and ATP levels in FRET cell lines. Cells expressing FLII12pGLU-600µdelta6 were bar coded using 0.1 uM calcein red-orange dye before being mixed with ATeam-expressing cells. The mixed cells were washed in glucose free solution and treated with 5 mM sodium azide. Glucose was added at indicated final concentrations and the fluorescence signal was measured on flow cytometer after 30 minutes.
Since Dactinomycin was characterized as a GLUT inhibitor, it was not included in the powder resupply. None of the other glycolytic inhibitors blocked glucose uptake when tested from powder resupply. HK2 is the first enzyme in glycolytic metabolism that converts glucose to glucose-6-phosphate, and it is found to be upregulated in cancer cells and associated with poor prognosis. We tested glycolytic inhibitors on HK activity using a commercial kit that measures the production of NADH when HK reaction was coupled with G6PDH reaction. Four inhibitors showed >75% inhibition of HK. However, one of them was found to inhibit G6PDH from the kit. Dose responses of the other three HK inhibitors showed µM range activities.

Figure 28 Determining molecular mechanism of action for select glycolytic inhibitors.

(A) Measuring effects of compounds on glucose influx. Long-term transfected lines expressing a FRET sensor for intracellular glucose were washed in glucose-free solution and incubated with indicated glycolysis inhibitors for 30 minutes. Glucose was added at a final concentration of 20 mM and plates incubated for an additional 30 minutes. Fluorescence signals were measured on the plate reader and FRET ratio was calculated. Dotted lines indicate control wells and their FRET ratios. (B) Measuring effects of compounds on hexokinase activity. Top: K562 cell lysates were incubated with indicated glycolytic inhibitors in vitro for 10 minutes before hexokinase colorimetric assay kit was used to detect hexokinase activity. The negative control received no lysate, and the positive control received cell lysate treated with DMSO. Bottom: hexokinase inhibitors were tested for their effects on G6PDH. 100 µM glucose-6-phosphate was added directly to the reaction mix. (C) Dose response curves for HK inhibitors. Filled circles, NSC 30916, open circles NSC 400944, closed triangles NSC 68634. Data are from a single experiment, representative of two.
6.4.4 Probability of obtaining anti-proliferative compounds based on glycolytic inhibition

We obtained the proliferation measurements of NCI compounds at 10uM (growth inhibition or GI10), the same concentration used in ATP screen. The proliferation assay was conducted by incubating cells with compounds for two days in culture medium, followed by the addition of a protein binding dye\textsuperscript{123,290}. Data showed the amount of total protein as a result of cell proliferation in each compound treated well, with 100% representing normal cell growth, 0% for no cell growth, and negative % meaning the well contained less cells than when started (Figure 29A). The mechanistic library had a wide range of compound effects on cell proliferation, with the median value around 49% cell growth.

To compare our metabolic inhibitors to the results from NCI proliferation test, we tested our compounds in the presence of BSA to simulate the serum condition in culture medium, as serum is very fluorescent and interferes with FRET measurement. 10 glycolytic inhibitors and 7 OXPHOS-dependent ATP production inhibitors were confirmed in the presence of BSA (Figure 30 and Table 4), as BSA may bind to compounds and reduce efficiencies. When comparing confirmed inhibitors to the library as a whole for their anti-proliferative activities, OXPHOS inhibitors (squares in Figure 29B) had a wide spread, with the mean of 40%, which is not significantly different from the median of the library. It is as if random compounds were chosen from the library and they all happened to be inhibitors of OXPHOS-dependent ATP production. Screening for OXPHOS inhibition did not enrich for anti-proliferative compounds. Particularly, one OXPHOS inhibitor did not affect cell growth at all (GI10=75.8), which eliminates inhibition of OXPHOS-dependent ATP production as a valid strategy for blocking cell proliferation. On the contrary, all glycolytic inhibitors (circles in Figure 29B) are effective in blocking leukemic cell proliferation. There is a significant difference between glycolytic inhibitors as a group and the library as a whole. Choosing compounds based on glycolytic inhibitory effects enriched for anti-
proliferative activities among the hits. Even the least effective glycolytic inhibitor had GI10 of 6%, representing a 94% inhibition on cell proliferation. This is a very high threshold, and only 26.5% of library compounds are as effective or more effective than this compound.

What is the chance of obtaining all glycolysis inhibitors from the top anti-proliferative compounds among the library? The probability is given by the binomial distribution. As more hits are identified with anti-proliferative activity, the probability for this event to happen at random decreases. For a library with 26.5% compounds being anti-proliferative, finding 10 glycolytic inhibitors also with anti-proliferative activity had $1.7/1000000$ chance of being a random event (Figure 29C). This very unlikely event by chance provided unbiased strong support for inhibition of aerobic glycolysis as an effective anti-proliferative strategy.

Figure 29 Confirming glycolysis as a valid target for tumor cell killing.

(A) Histogram of percent cell growth of NCI mechanistic set compounds tested at 10 uM (G10) in K562 cells. (B) Cumulative distribution for compounds from (A). Dotted line represents the median activity. G10
values for inhibitors of glycolysis and OXPHOS-dependent ATP production that were active in the presence of BSA are indicated by circles and squares, respectively. The mean and standard deviation for each group are indicated. (C) Plot of the probability of obtaining the indicated number of hits for libraries with indicate percentage of antiproliferative compounds. (D) Proposed scheme for conducting target validation screens.

6.5 Discussion

Based on what we observed with the ATP screen, we propose for a target validation screen to replace the use of tool molecules (Figure 29D). Given a library collection of diverse molecules, compounds could be tested on cell proliferation in anticancer study, then a target of interest could be chosen to conduct the target-based assay on the library. When an inhibitor of the target is identified, it will be determined whether it belongs to anti-proliferative group according to a predefined threshold. Any inhibitor on the target yet without anti-proliferative activity will remove the target from the screening effort. Otherwise the target-based screen can carry on. The number of inhibitors required to establish high level of confidence depends on the library property. For a library where all compounds are anti-proliferative, the chance of obtaining inhibitors of the target with anti-proliferative activity is 100%, and vice versa, a library with no anti-proliferative compounds would be of no use to the screen as the chance is always 0%. The more phenotypically active compounds there are in the library, the higher the chance of obtaining inhibitors with the desired activities, and according to binomial distribution, the more hits one would need to identify and confirm before drawing the conclusion on target validity.

The chemical library (target validation set or TV set) does not need to contain compounds that are lead-like or pharmaceutically desirable, because the purpose of a TV screen is not to find lead compounds or probe molecules but rather verifying the target with an ensemble of compounds. However, the library must be first phenotypically characterized, and the compounds in the library need to have a wide range of activities. Compounds could be screened for anti-proliferation or
other processes such as cell differentiation. We previously used granule exocytosis assay to screen MLSMR, which could be used for future target-based assays as a phenotypically characterized library for CTL function. The amount of work of screening a library against a cell phenotype is huge investment upfront, especially when dose responses of individual compounds are involved. However, on the long run, the effort in the beginning will be worthwhile in improving target selection process and increasing the success rate of drug candidates.

About the confidence level: The chance of getting random event is not set to a threshold. The model gave four scenarios of different library properties, and people could determine how many hits they need to identify before drawing the conclusion with high level of confidence. To us, 1.7/1000,000 is a very rare event by chance, given by 10 glycolytic inhibitors as a group. If the chance of 1/10000 is good enough, the screen can stop when 7 inhibitors were identified to be anti-proliferative. It is true that any given compound from the inhibitor group could have off-target effects and have anti-proliferative activity as a result of their inhibition on other cellular targets. However, as a group, an ensemble compound approach offers unbiased support for a target.

About the hit rate: the Warburg effect covers the entire glycolytic pathway, and the hit rate (6%) is higher than a single molecular target-based screen. Pathway-based screen is between target-based screen and phenotypic screen. If the target validation approach were to be tested against a glycolytic enzyme, ie HK2 (0.37% hit rate), more compounds would need to be screened in order to obtain enough compounds to reach the desired probability.

About artificial sensor: instead of relying on what the cells offer (LAMP in CTLs), we introduced sensors for interesting targets to relevant cell models, and therefore create targeted phenotypic screening. This can be done with any target of choice. Other studies have used luciferase-based gene reporter assay as a model to screen for activators of certain genes. Previously we used kinase activity sensors in a multiplexed flow-based assay. Here we introduced ATP sensor to leukemic cell line that has the Warburg effect. Although there are commercial kits that detect ATP
based on luciferase activity, cells would need to be lysed to release ATP, and we won’t be able to obtain any information on the live cell functions and processes. By using FRET sensor, we can monitor intracellular ATP level in real-time fashion and study metabolism pathways in intact cells.

Multiplexed assay vs. multiread assay: both approaches can yield multiple cell measurements in one screening, so they are both high-content assays. Multiplexed assay employs cell barcoding technique and reads the mixture of cell samples at one-time point, and different barcodes represent different subsets of cells, so multiple protein targets can be interrogated at the same time. Information about how compounds work in cells could be gathered as the initial screening is being completed. Multiread assay uses sequential treatment of cells to achieve the measurement of different cell activities, and they yield hits that target different cell processes. To identify the molecular targets of hits, follow-up assays must be conducted following the initial screen. However, a plate-reader based multiread assay can be combined with long-term cell viability/proliferation test, and this can generate the library phenotypic profile at the same time of target validation screening.

6.5.1 Future Direction: Target validation approach applied to an alternative target

In this appendix, we proposed for a target validation approach to offer strong support for a molecular target. Instead of depending on the traditional RNAi knockout or knockdown and a handful of tool compounds that could all have multiple targets, we sought to combine our target-based screening results with the phenotypic information of a characterized library. However, this validation screen theory is not tested. One of the future direction that we can take to verify the theory is by library profiling and data mining. There have already been efforts by NCI to predict potential targets for library compounds. If we could categorize those targets as either causing or not causing tumor cell proliferation based on published literature, we would hope to find that all compounds presumed to share the same valid target would have anti-proliferative effects on cells as well. For an invalid target, the group of compounds would have both anti-
proliferative and no effects. One caveat is that all potential mechanisms and targets are predicted but not tested. If experiments were to be carried out to determine the effects of library compounds on individual molecular target, less targets can be examined due to expense and time of screening efforts.

Within the scope of this study, how to further verify that aerobic glycolysis is a good target for blocking tumor proliferation? Theoretically, if we could generate cell lines that are resistant to Warburg effect, we can treat them with glycolytic inhibitors and expect to see no ATP change and no effect on cell proliferation rate. Replacing glucose with glutamate in culture medium could enable the cells to grown independent of glucose. It is unclear how glycolytic inhibition could result in anti-proliferation effect. Build-up of intermediate metabolites could initiate transcription factors to activate genes related to cell apoptosis. From the standpoint of tumor microenvironment, this could be a strategy of the tumor cell colony to preserve energy resource.
Figure 30 Structures of glycolytic inhibitors (1-13) and oxidative phosphorylation-dependent ATP production inhibitors (14-27).
For more information see Table 4. Compounds are displayed in the same order.

**Table 4 Metabolically-Active compounds in NCI Mechanistic Set III**

<table>
<thead>
<tr>
<th>NSC Number</th>
<th>Inhibiton of glycolytic ATP production</th>
<th>Inhibition of non-glycolytic ATP production</th>
<th>IC50 (uM)</th>
<th>Hill slope</th>
<th>G10</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>136037: 111 ± 3; 113 ± 6</td>
<td>3 ± 2; 4 ± 5</td>
<td>1.5 ± 0.3</td>
<td>2.1 ± 0.8</td>
<td>-4.9</td>
</tr>
<tr>
<td>2</td>
<td>105808: 107 ± 3; 110 ± 5</td>
<td>8 ± 5; 2 ± 3</td>
<td>3.3 ± 0.2</td>
<td>4.8 ± 1</td>
<td>-34.6</td>
</tr>
<tr>
<td>3</td>
<td>111041: 110 ± 3; 97 ± 14</td>
<td>8 ± 3; 28 ± 6</td>
<td>4.4 ± 0.4</td>
<td>5.2 ± 2.8</td>
<td>-49.4</td>
</tr>
<tr>
<td>4</td>
<td>697923: 99 ± 13; 86 ± 2.2</td>
<td>9 ± 9; 2 ± 2</td>
<td>&gt;10uM</td>
<td>-24.2</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>328587: 108 ± 3; 101 ± 7</td>
<td>13 ± 8; 3 ± 3</td>
<td>2 ± 0.4</td>
<td>1.9 ± 0.5</td>
<td>6.3</td>
</tr>
<tr>
<td>6</td>
<td>400944*: 89 ± 11; -10 ± 2</td>
<td>20 ± 7; 3 ± 3</td>
<td>1.7 ± 0.3</td>
<td>5.4 ± 2.9</td>
<td>47.4</td>
</tr>
<tr>
<td>7</td>
<td>686349*: 90 ± 9; 90 ± 6</td>
<td>36 ± 24; 7 ± 5</td>
<td>4.2 ± 0.5</td>
<td>2.8 ± 0.8</td>
<td>-36.6</td>
</tr>
<tr>
<td>8</td>
<td>30916*: 106 ± 6; 73 ± 13</td>
<td>51 ± 20; 43 ± 11</td>
<td>4 ± 0.4</td>
<td>3.6 ± 1</td>
<td>-35.7</td>
</tr>
<tr>
<td>9</td>
<td>635448: 98 ± 9; 43 ± 12</td>
<td>71 ± 16; 59 ± 10</td>
<td>5.6 ± 1</td>
<td>4.2 ± 3.1</td>
<td>-41.3</td>
</tr>
<tr>
<td>10</td>
<td>659997: 118 ± 4; 107 ± 6</td>
<td>87 ± 6; 45 ± 10</td>
<td>1.1 ± 0.2</td>
<td>2.2 ± 1</td>
<td>-19</td>
</tr>
<tr>
<td>11</td>
<td>631529: 102 ± 4; 70 ± 13</td>
<td>99 ± 8; 25 ± 19</td>
<td>&gt;5uM</td>
<td>-38</td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>168597: 113 ± 3; 86 ± 11</td>
<td>120 ± 11; 112 ± 3</td>
<td>2.2 ± 0.4</td>
<td>1.4 ± 0.3</td>
<td>-2.2</td>
</tr>
<tr>
<td>13</td>
<td>164914: 85 ± 8; 45 ± 8</td>
<td>124 ± 11; 113 ± 7</td>
<td>2.8 ± 0.4</td>
<td>1.5 ± 0.3</td>
<td>-54.3</td>
</tr>
</tbody>
</table>

**Compounds are numbered in the same order as their structures are displayed in Figure 30.** *indicates hexokinase inhibitors.

*Measured from read 3, expressed as percent control. Data are mean + S.E.M. from 4 measurements from powder resupply. The first value was obtained in the absence of BSA, the second in its presence.
\* Measured from read 1, expressed as percent control. Data are mean ± S.E.M. from 4 measurements from powder resupply. The first value was obtained in the absence of BSA, the second in its presence.

\* From fits to the Hill equation for data from 3 separate experiments including doses ranging from 0.1-10uM. When dose-response curves cannot be fit, range of inhibitory concentrations is indicated. Performed with resupplied compounds.

\* Compiled from NCI data. In some cases, multiple reported values were averaged.
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