

**DEVELOPMENT OF SYNTHETIC PEPTIDE
SUBSTRATES TO STUDY TYROSINE KINASE
ACTIVITY**

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Abstract

Kinases are key targets for cancer therapeutics due to their role in development and progression of cancer. Despite this, the majority of new kinase inhibitors and studies remain focused on a small subset of target kinases, leaving much of the kinome understudied. To aid researchers in kinase studies and inhibitor design, we implemented a streamlined phosphoproteomics pipeline to determine substrate profiles of kinases and used this information to design synthetic peptide substrates to study kinase activity. Using Bruton's tyrosine kinase (BTK) as a proof-of-concept, we used phosphoproteomics to identify in vitro substrates of BTK, and input these substrates into our KINATEST-ID V2.1 R-package to identify amino acid preferences. We designed and characterized novel BTK synthetic substrates, as well as established a correlation between the assigned KINATEST-ID score for each substrate and biochemical performance. To increase applicability of the BTK substrates, we integrated a known terbium-chelation motif into the substrate sequence for use in a time-resolved terbium luminescence assay to monitor BTK activity over time. These workflows were then applied to the understudied TAM family of receptor tyrosine kinases, Tyro3, Axl, and Mer. We designed a set of synthetic substrates for each TAM kinase, which can be used in activity assays. We also found that Tyro3 had differential phosphorylation patterns of the synthetic substrates, allowing for a Tyro3 synthetic substrate to be specific for Tyro3 over Axl or Mer. Overall, we demonstrate optimized workflows for studying substrate profiles of tyrosine kinases and design and characterization of synthetic peptide substrates. This work can be applied towards in vitro kinase activity studies and inhibitor screening and characterization.

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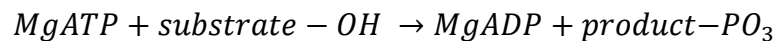
Chapter 1. Introduction

Naomi Widstrom wrote this chapter in its entirety

1.1. Kinases

Protein kinases are enzymes that catalyze the transfer of a phosphate group from adenosine triphosphate (ATP) to a protein or peptide substrate. The addition of a phosphate group to a protein can initiate conformational changes, creating or removing docking sites for other proteins, or alter the activity of an enzyme. Protein phosphorylation is a key element of cellular signaling, mediating major pathways of cell proliferation, survival, migration, and other cell specific functions. Kinases therefore have large roles in regulating important cell signaling pathways and represent a diverse array of functions.

For catalysis, ATP and a protein or peptide are required as substrates, as well as a divalent ion, typically magnesium. The protein or peptide typically contains a residue with an alcohol group that can be phosphorylated: serine (S), threonine (T), or tyrosine (Y). During catalysis, the gamma-phosphate group of ATP is transferred to the protein substrate, creating the phosphorylated product and adenosine diphosphate (ADP) (Figure 1.1).



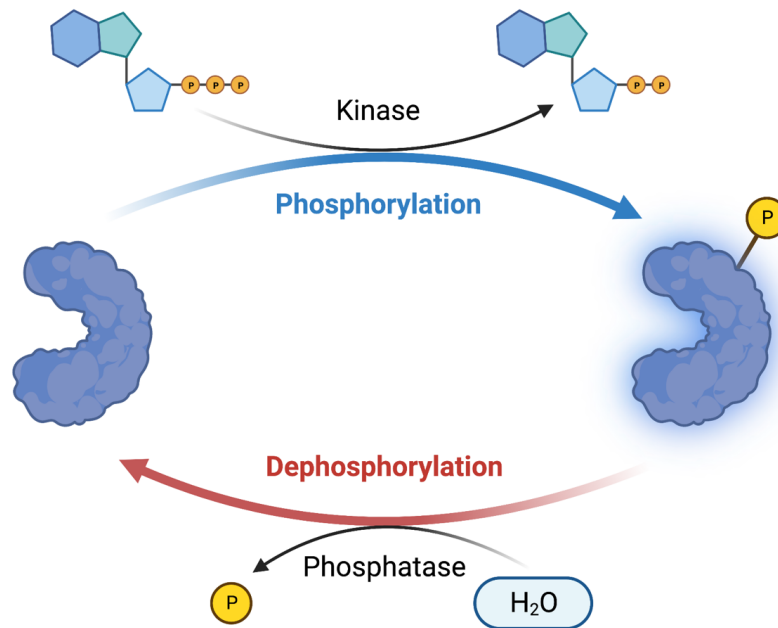


Figure 1.1. Protein phosphorylation. Protein kinases phosphorylate substrates using ATP. This process is reversed by protein phosphatases, which catalyze the removal of a phosphate group. Created with BioRender.com

1.1.1. Structure of protein kinases

The catalytic domain of protein kinases is highly conserved, formed of two lobes with an ATP-binding pocket in a cleft between lobes. The N-terminal lobe is smaller, mainly composed of beta-sheets and the larger C-terminal lobe primarily helical, with an α C-helix linking the two lobes. The active site is composed of several structural elements: the activation loop, catalytic loop, and the α C-helix. The activation loop begins with a conserved DFG (Asp-Phe-Gly) motif and ends with a conserved APE (Ala-Pro-Glu) motif.

Active kinases undergo conformational changes to position both substrates for the transfer of the phosphate group from ATP to the protein substrate. When the kinase is catalytically active, the DFG motif in the activation loop is oriented toward the ATP-

binding site, where it coordinates with Mg^{2+} for catalysis.¹ This conformation is referred to as “DFG_{IN},” whereas when the DFG motif points away from the ATP binding site when the kinase is inactive is called “DFG_{OUT}” (Figure 1.2). Phosphorylation of the activation loop typically initiates this change in orientation.^{2, 3} The catalytic loop interacts with the hydroxyl group on the side chain of the S/T/Y residue of the protein substrate and transfers the gamma-phosphate group of ATP to this substrate. The α C-helix forms a salt bridge with a lysine-containing motif within the N-terminal lobe when the kinase is active, a conformation referred to as the “ α C_{IN}” conformation.⁴ This allows formation of hydrogen bonds between the lysine side chain and the ATP molecule. Dephosphorylation of the activation loop destabilizes the interactions between these components, favoring the “DFG_{OUT}” conformation of the activation loop, and shifting of the α C-helix out of the active site.³

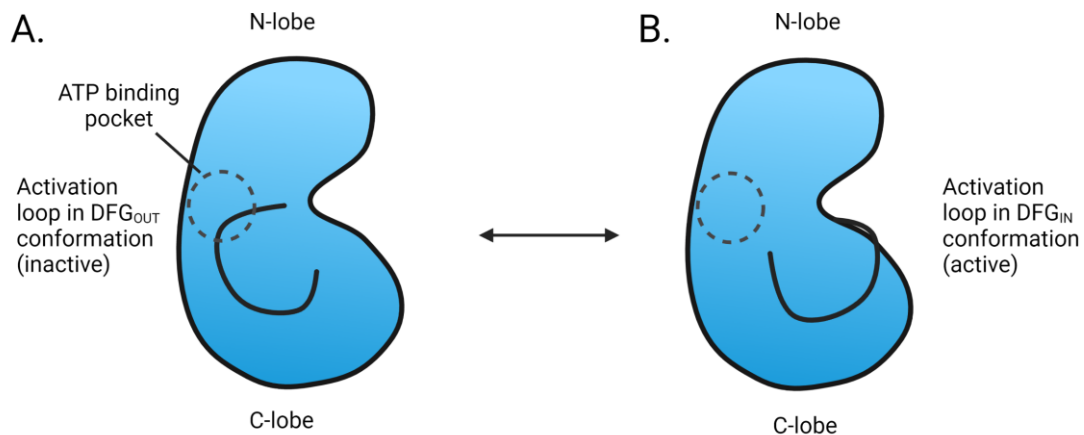


Figure 1.2. Schematic of kinase structure. Schematic of kinase in A.) inactive conformation and B.) active conformation. Created with BioRender.com

Kinases also contain a hydrophobic skeleton that spans both lobes consisting of the catalytic spine (C-spine), which aligns with bound ATP, and the regulatory spine (R-spine).^{3, 5} The R-spine contains four nonconsecutive hydrophobic residues that align to

form an intact spine in active kinases; additionally, several shell residues help stabilize the formation of this spine, including the gatekeeper residue.^{3, 6} The gatekeeper residue sits near the hinge of the kinase, close to the adenine binding pocket, and controls access to a hydrophobic pocket. The size of the gatekeeper residue dictates the size and accessibility of this hydrophobic pocket, which is important for ATP-competitive inhibitor design.

In general, kinases share these conserved structural elements allowing activation and phosphorylation of protein or peptide substrates. However, additional domains and regulatory motifs add diverse function and specificity to the kinome by modulating kinase activity or localization.⁷

1.1.2. Human protein kinome

The kinase portion of the proteome, called the kinome, includes 518 kinases.⁸ Broadly, the kinome can be divided into tyrosine kinases and serine/threonine kinases. Tyrosine kinases phosphorylate proteins on tyrosine (Y) residues, while serine/threonine (S/T) kinases phosphorylate proteins on serine or threonine kinases, with a few dual functionality kinases able to phosphorylate all three residues. The tyrosine kinase family can be further subdivided into receptor tyrosine kinases (RTK) and nonreceptor tyrosine kinases (NRTK) while the larger S/T kinase family is divided into a number of different families.

1.1.3. Receptor tyrosine kinases

Receptor tyrosine kinases (RTKs) are a small subset of the kinome yet are some of the best studied kinases. RTKs have both an extracellular and intracellular component connected by a single-pass transmembrane domain. The extracellular domain(s) interact

with ligands to initiate activation, while the intracellular component contains the kinase domain. In general, ligand binding to the extracellular domain initiates dimerization of the receptors, leading to activation of the intracellular kinase domains via trans autophosphorylation (Figure 1.3A).^{9, 10} Additional domains may also be involved in activation. For example, some RTKs have an intracellular juxtamembrane domain, such as EGFR. This domain of EGFR has been shown to stabilize the formation of an asymmetric dimer of the kinase domains, promoting kinase activation.^{11, 12} Similarly, the juxtamembrane domain of Axl may be required for full activation of the kinase domain.¹³ Phosphorylated residues on the intracellular domains provide docking sites for downstream signaling proteins, such as scaffolding or adaptor proteins containing Src-homology (SH2) domains.

1.1.4. Non-receptor tyrosine kinases

In contrast to RTKs, non-receptor tyrosine kinases are entirely intracellular. The mode of activation and regulation varies across the nine NRTK families.¹⁴ For example, Syk family kinases contain SH2 domains that bind phosphorylated ITAM sequences found on immune receptors, which relieves the autoinhibitory state and activates the kinase (Figure 1.3B).¹⁵ Similarly, Src family kinases are autoinhibited when a site on their C-terminus is phosphorylated and activated when their activation loop is phosphorylated by upstream RTKs (Figure 1.3C).¹⁶

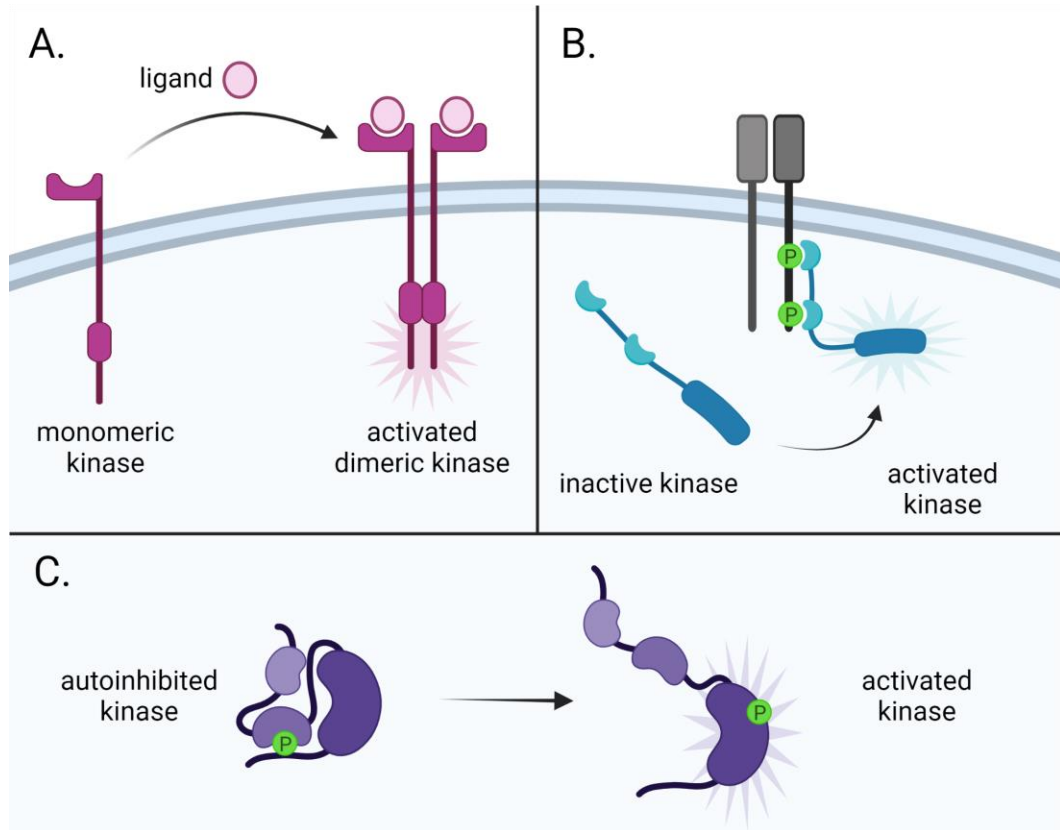


Figure 1.3. Tyrosine kinase activation. A.) Activation of receptor tyrosine kinase via ligand binding and dimerization. B.) Activation of nonreceptor tyrosine kinase by binding to a phosphorylated motif. C.) Autoinhibited kinase conformation relieved by dephosphorylation of the C-terminal tail and phosphorylation of activation loop. Created with BioRender.com

1.1.5. Serine/Threonine kinases

Serine/threonine kinases make up the majority of the kinome, representing a greater diversity and number of kinases compared to tyrosine kinases⁸ While a small subset of S/T kinases are very well studied, a larger proportion remains poorly understood. S/T kinases can be divided up into 6 families based on sequence homology and domain structure.^{8, 17} S/T kinases can be both receptors or non-receptors and are critical to a large variety of different cell signaling pathways.

1.1.6. *The dark kinome*

While the generic structure and function of kinases are broadly defined, the precise biological function of many kinases remains unknown.^{18, 19} In 2010, it was noted that clinical trial efforts and academic publications alike focused on a narrow range of well understood kinases, with large portions of the kinome entirely ignored.²⁰ In some cases, the lack of knowledge about the function of a kinase means that commercially available kinase assays are not available, further hindering research.¹⁸ These poorly studied kinases with few chemical probes available have been termed ‘dark kinases’ and form the dark kinome. Due to well-established efforts, kinases are considered a druggable family, meaning that while the exact physiological function of these dark kinases remains unknown, they represent potential targets for new disease therapeutics. As such, understudied kinases fall under the NIH Illuminating the Druggable Genome program (<https://commonfund.nih.gov/idg>) which aims to improve understanding and develop chemical probes for potential targets. This work helped outline parameters to define which kinases fell under the definition of ‘understudied’ and create a platform to aid researchers to investigate and update findings.²¹ Further work specifically investigating the dark kinome determined that 24% of the kinome is understudied.¹⁹ Of the 8% of kinases that are targets for FDA-approved small molecule inhibitors, 70% of these belong to the TK family, highlighting the disparity between TK and S/T kinases.¹⁹ Analysis of publicly available differential-gene expression, clinical datasets, and other information reveals that some understudied kinases would likely be relevant cancer therapeutic targets, but overall, the lack of chemical probes and activity assays impede research and drug development.¹⁹

1.2. Kinases and the hallmarks of cancer

As kinases are intricately connected with crucial cell signaling pathways in physiologically normal cells, they are just as intertwined with cancerous cell signaling. At its most basic, cancer refers to cells displaying abnormal growth and invasive properties. As a disease, cancer can be subdivided into numerous categories, depending on tissue of origin, specific genetic changes, metastatic potential and more. However, at their core, cancers share key hallmarks as defined by Hanahan and Weinberg in 2000 and 2011.^{22, 23} They originally outlined six hallmarks: sustained proliferative signaling, evasion of growth suppressors, activation of invasion and metastasis, enabling of replicative immortality, induction of angiogenesis, and resistance to cell death.²² In their follow up review, the additional hallmarks of dysregulation of cellular energetics, avoidance of immune destruction, genome instability and mutation, and tumor-promoting inflammation were added.²³

Kinases play a role within most of these classic hallmarks. Many of the best studied kinases are involved in proliferative signaling pathways. Cancer cells activate proliferation and growth pathways through several different mechanisms, such as production of growth-ligands by the cancer cells themselves, alteration of cells in the environment to produce more ligands, and increased number of receptors at cell surface. Additionally, cancer cells can be growth-ligand independent, where structural changes in receptors or downstream molecules result in constitutive activation of the pathway.

Dysregulation of kinase signaling through mutation or expression changes in cancer alter proliferation and growth. For example, signaling through epidermal growth factor receptor (EGFR) promotes cell proliferation through downstream pathways of

ERK MAPK, SRC, PI3K-AKT among others.²⁴ EGFR is commonly mutated in cancers, with the somatic mutation L858R in the kinase domain causing increased kinase activity resulting in constitutive downstream pathway activation.²⁵ EGFR is just one example of a RTK implicated in cancer cell proliferation signaling, and alterations in downstream kinases can initiate similar changes in signaling as well. B-Raf V600E is a classic mutation commonly found in melanoma, where the residue substitution results in constitutive active kinase and downstream activity.²⁶

Advanced stage cancers typically have a poorer prognosis as cancer cells gain invasive properties. Invasive cancer cells can migrate and invade secondary tissue sites, known as metastasis. Focal adhesion kinase (FAK), a non-receptor tyrosine kinase, has been implicated in numerous cancer types for its role in cancer invasion and motility. FAK is involved in responding to signaling from integrins, receptors that bind the extracellular matrix (ECM) and intracellular actin cytoskeleton networks. Following activation, FAK signals through a number of different pathways, influencing cell migration, and when overexpressed or activated in cancer cells, invasion and metastasis.²⁷ FAK also has a role in mediating the epithelial-mesenchymal transition (EMT), which is the process in which epithelial cells lose characteristic properties, such as cell-to-cell adhesion, and gain mesenchymal properties, such as motility and migration.²⁸ This process allows primary tumor cells to migrate from the original tumor site and form metastases at distant sites.

Increasing research suggests that the tumor microenvironment (TME) has a crucial role in tumor survival and expansion. The TME is the immediate environment surrounding a tumor, typically a heterogeneous collection of cells, blood vessels, and

extracellular matrix (Figure 1.4). The immune cells present within the microenvironment contribute to two hallmarks: tumor-promoting inflammation and evasion of immune destruction. These two hallmarks seem at odds with each other, indicating the immune system can both promote and inhibit cancer growth. Acute versus chronic inflammation accounts for the differences in effects; while acute immune action can lead to the destruction of cancer cells, chronic inflammation is associated with tumor progression.²⁹ Among the mixture of infiltrating immune cells in the TME are tumor-associated macrophages. Macrophages are innate immune phagocytes that have different functions and roles depending on external stimuli. Typically, macrophages can be roughly divided into two subpopulations: pro-inflammatory and anti-inflammatory.³⁰ Tumor-associated macrophages are generally more similar to the anti-inflammatory macrophages, with properties that shift the TME toward a more anti-inflammatory state.^{31, 32} This anti-inflammatory shift can help tumor cells avoid immune surveillance as well. Cytotoxic T-cells and natural killer (NK) cells recognize tumor-specific antigens and mark the cells for destruction, helping prevent further growth of tumors.³³ In order to survive, tumors must either avoid or negate this immune surveillance. The immunosuppressive microenvironment maintained by the tumor-associated macrophages aids tumors in prevention of immune-mediated death. The release of anti-inflammatory cytokines and other factors also help inactivate infiltrating cytotoxic cells.³² Additionally, tumors can alter their external markers to prevent recognition. Cells display markers to indicate they are “self” to immune cells, which prevent immune cells from destroying healthy cells. For example, cytotoxic T-cells express the programmed cell death protein 1 (PD1), which acts as an inhibitory receptor when bound to its ligand PDL1, which is widely expressed

across cell types.³⁴ This prevents the T-cells from destroying healthy cells, but tumor cells can upregulate expression of PDL1 preventing detection by T-cells. Checkpoint blockade immunotherapies aim to treat cancers by blocking this and similar interactions, re-invigorating the immune surveillance function of immune cells against tumors. The TAM family of RTKs, discussed in depth in Chapter 3, have been found to play a large role in modulating the TME affecting the efficacies of these types of treatments.

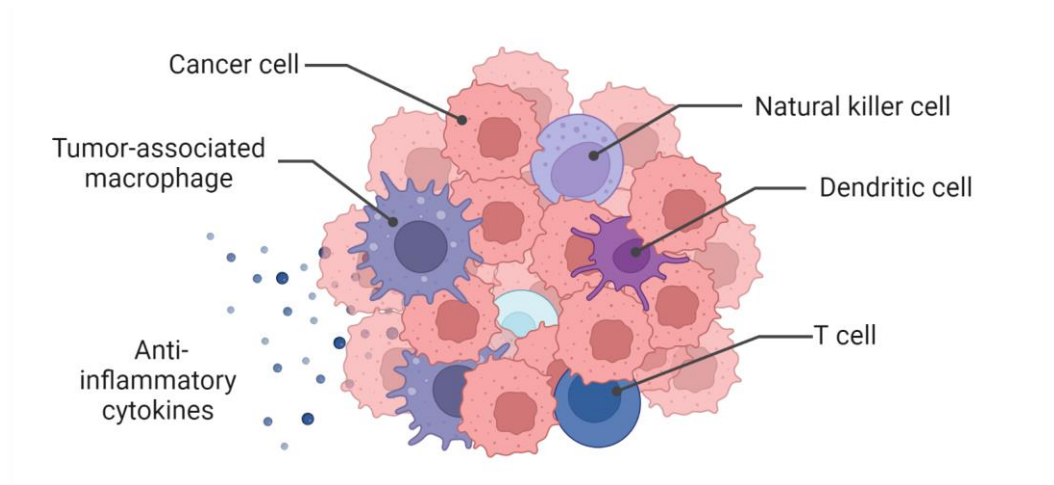


Figure 1.4. Schematic of tumor microenvironment. Tumor-associated macrophages secrete tumor-promoting cytokines and express immune-suppressing receptors, contributing to an immunosuppressive environment. Any dendritic cells present are inactivated and prevented from presenting tumor-specific antigens, aiding evasion of immune destruction. Similarly, natural killer cells and infiltrating T-cell cytotoxic activity is suppressed. Created with BioRender.com

1.2.1. Kinase dysregulation mechanisms

Kinases involved in cancer development or progression are dysregulated by a range of different mechanisms. Mutations in a kinase may cause constitutive activity, such as the case of EGRF and BRAF. These mutations shift the conformation of the kinase, leading to constitutive activation. In the case of BRAF V600E, the negatively charged glutamate

mimics the phosphorylation necessary for activation, stabilizing the active conformation of the kinase.³⁵

A larger scale mutation can occur during chromosomal translocations, resulting in fusion of two genes. A classic example is the BCR-ABL fusion kinase, formed following the reciprocal translocation of chromosomes 9 and 22, called the Philadelphia chromosome. As a result, a portion of the BCR gene is fused to the ABL1 kinase gene, creating the fusion kinase. The fusion between the two genes removes an autoinhibitory component of the Abl kinase, resulting in constitutive downstream activity.³⁶ This translocation commonly occurs in chronic myeloid leukemia (CML).

Not all oncogenic kinases are dysregulated due to gene mutations, some kinases are overexpressed in cancer, typically due to gene amplification. Human epidermal growth factor receptor 2 (HER2) is a receptor tyrosine kinase overexpressed in breast and other cancers, caused by amplification of the HER2 gene.³⁷ Similarly, overexpression of a ligand for a kinase can also result in constitutive activity. While these different mechanisms typically produce a similar result of constitutive activity and activation of downstream pathways, the mechanism of dysregulation is important when considering strategies to inhibit target kinases.

1.3. Kinase inhibitors

Given the multitude of roles kinases play in cancer, they make obvious targets for cancer therapeutics. The billion-dollar kinase inhibitor industry was launched by the success of the small molecular kinase inhibitor Gleevec, also known as imatinib, which targets the fusion kinase BCR-ABL in chronic myeloid leukemia.³⁸ Most of the effort to design kinase inhibitors are focused on small molecules, with a few monoclonal antibodies

(mAbs) and alternative methods, such as proteolysis targeting chimera (PROTAC) inhibitors being explored.

1.3.1. Small molecule kinase inhibitors

To date, there are 71 FDA-approved small molecule kinase inhibitors, with many more in clinical trials. The majority of these inhibitors are approved for treatments of various cancer types and stages, with a few approved for use in other diseases, such as autoimmune disorders.⁴

1.3.1.1. Classification of small molecule kinase inhibitors

Small molecule inhibitors are further subdivided into classes, depending on where the inhibitor binds and the conformation of the target kinase when the inhibitor binds (Figure 1.5). Type I and II inhibitors are ATP competitive inhibitors, meaning they bind to the same site as ATP. Type I inhibitors bind to the active conformation of the kinase.

Structurally, the DFG motif is in the active conformation pointing inwards (DFG_{IN}) and the α C-helix is pointed inwards, while the activation loop is open.^{39, 40} Type II kinase inhibitors also bind to the ATP binding site, but when the kinase is inactive with DFG_{OUT}, and the activation loop is in a closed conformation.³⁹⁻⁴¹ Another class occupies the niche in between these two subtypes, defined as type I ½ inhibitors. These small molecules bind the ATP binding site of a kinase that is inactive yet in the DFG_{IN} conformation.^{40, 42}

Both type I ½ and type II can be further subdivided into whether or not the inhibitor binding site extends beyond the ATP binding pocket into other hydrophobic pockets.⁴

Type III and IV inhibitors are allosteric inhibitors, which alter the activity of the kinase by binding in a location outside of the active site. These types are differentiated by the location of the inhibitor binding site; type III inhibitors bind in a pocket adjacent to the

ATP binding site while type IV bind to an alternative location distal from the ATP binding site.^{40, 43} Type V inhibitors are bivalent inhibitors, meaning that they span across two different kinase domain regions.⁴⁴ Unlike types I-V, type VI inhibitors are irreversible inhibitors. They covalently bind to a residue in the active site of the kinase, typically a cysteine.⁴⁰ The majority of FDA approved inhibitors are type I, I 1/2, or II.⁴⁵

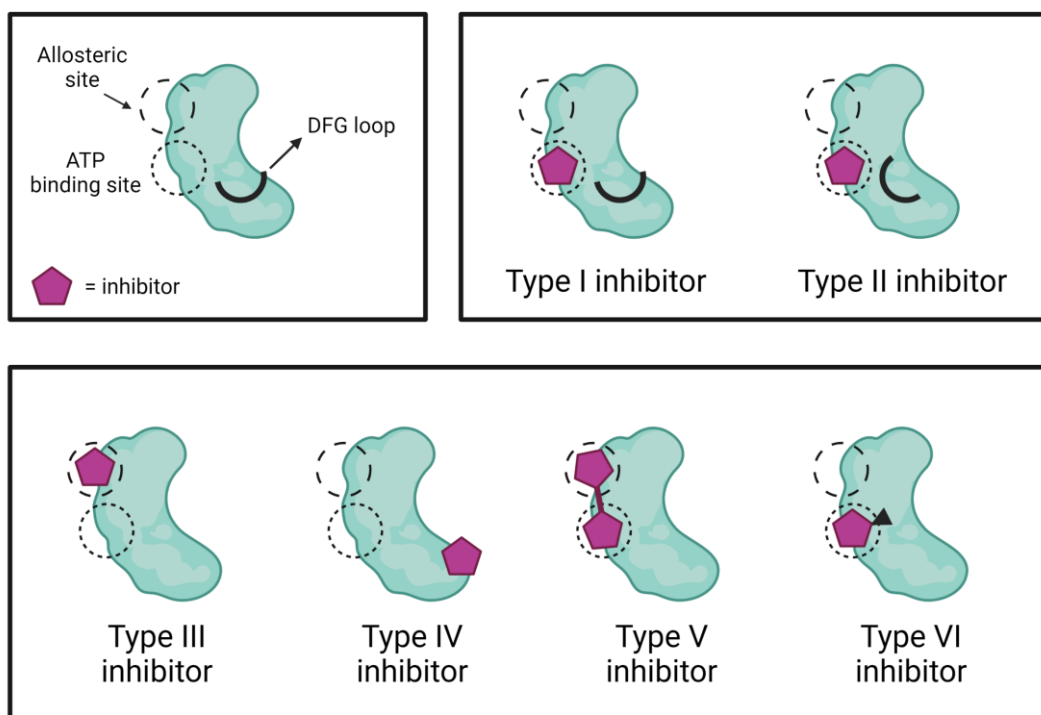


Figure 1.5. Classification of small molecular kinase inhibitors. Created with BioRender.com

1.3.1.2. Specificity of small molecule kinase inhibitors

An important consideration in the design and testing of small molecule kinase inhibitors is the specificity and potential clinical side effects that arise from off-target inhibition. Although many inhibitors were specifically designed to inhibit one particular target kinase, off-target inhibition of other kinases is a common complication, often due to the similarity in kinase active sites across the kinome. This can be advantageous, allowing

for multiple targets to be inhibited simultaneously and expanding the use of the inhibitor. However, these off-target effects can also cause side effects preventing clinical use.

The type I inhibitors are well-known to have off-target effects, as the portion of the ATP-binding pocket these inhibitors compete for is highly conserved amongst kinases.^{46, 47} Type II inhibitors are thought to be more selective, as inactive conformations of kinases are more diverse. Off-target effects can cause a range of side-effects, the toxicity of which may limit the clinical use of the inhibitor.⁴⁸ Inhibitors are constantly being re-designed to improve specificity and efficacy against a target. The initial inhibitor is referred to as the “first-generation” inhibitor, with subsequent inhibitors targeting the same kinase either referred to as “second generation” or “next generation.”

These next generation inhibitors are designed not only to improve specificity, but also to treat patients who have developed resistance to the first line of treatment. For example, first-generation imatinib targets BCR-ABL in CML, but is known for off-target effects against KIT and other kinases, and many patients develop resistance. Second generation inhibitors nilotinib, dasatinib, and bosutinib all have increased activity against BCR-ABL, although with varying off-target effects. These inhibitors are also effective at treating some imatinib-resistant cancers.^{49, 50} While next generation inhibitors aim to treat inhibitor-resistant cancers as they develop, the constant evolution of cancer to bypass inhibition presents a major roadblock to the efficacy of cancer therapeutics.

1.3.1.3. Clinical resistance to small molecule kinase inhibitors

Although some small molecule inhibitors show high initial success, intrinsic or acquired mutations can prevent long-term remission of cancer. Intrinsic resistance is caused by mutations already present in a tumor that prevent a treatment from being effective,

whereas acquired resistance arises from changes or mutations gained by the tumor that allow it to become desensitized to the treatment.

For example, some lung cancer patients have no response to EGFR first generation inhibitors, characterized as intrinsic resistance.^{51, 52} In other cases, patients develop EGFR mutations following treatment with EGFR inhibitors. The most common acquired mutation is T790M, which prevents binding of first- and second-generation EGFR inhibitors, leading to decreased efficacy and disease progression.⁵³ EGFR T790M is an example of a gatekeeper mutation in a kinase. The gatekeeper residue is the amino acid near the regulatory spine that controls access to a hydrophobic pocket near the adenine binding site.^{42, 54, 55} Mutation of this residue alters the size and chemical functionality of the binding pocket, modifying the binding properties of inhibitors.⁴² Other common gatekeeper mutations in cancer-related kinases include BCR-ABL T315I, BTK T474M, c-Kit T670I and more, with all these mutations documented to cause resistance to inhibitors.⁵⁶ Point mutations of other residues can also lead to inhibitor resistance by altering kinase conformation or inhibitor binding contacts.⁵⁷

Beyond mutation of the target kinase itself, cancers have other mechanisms they employ to evade inhibition. Cancers can bypass the inhibited kinase by activating a different kinase. For example, lung cancers resistant to EGFR inhibitors often have hyperactivated the kinase Met and bypass the need for EGFR activity allowing for remission and continued growth.^{58, 59} Similarly, proteins downstream of the target kinase can be activated, again rendering the inhibition of the kinase inconsequential. This has been documented in melanoma patients resistant to BRAF inhibitor Vemurafenib; the MAPK pathway is reactivated by mutations to downstream kinase Met.⁶⁰

1.3.2. Monoclonal antibodies

Several monoclonal antibodies (mAbs) have also been approved for cancer treatment. Monoclonal antibodies are designed to specifically bind a target molecule; they might inactivate the target by blocking ligand binding, or initiate an immune response leading to cell destruction.⁶¹ Several mAbs against RTKs have been FDA approved for cancer treatment including Trastuzumab which targets HER2, Cetuximab for EGFR, and Ramucirumab for VEGFR2.⁶¹ The size of mAbs prevents them from crossing the cell membrane and so they can only target the extracellular domains of RTKs. mAbs are more specific compared to small molecule inhibitors, as the antibody precisely recognizes an exact epitope. However, there are still a number of undesirable adverse side-effects associated with mAb treatment, due to the activation of the immune system and or to affects to the normal physiological function of the kinase in healthy cells.

1.3.3. Proteolysis Targeting Chimeras (PROTACs)

Another method for kinase inhibition is through use of a Proteolysis Targeting Chimera (PROTAC) which induces degradation of a target protein, thereby inhibiting activity. The PROTAC approach was first described in 2001, and while none have been fully FDA-approved, there are several contenders currently undergoing clinical trials.^{62, 63}

PROTACs function by exploiting the cellular protein degradation machinery. Within a cell, ubiquitin ligases recognize and mark proteins for degradation by modifying them with ubiquitin molecules. A PROTAC is a heterobifunctional molecule, consisting of a component that interacts with an E3 ubiquitin ligase, linked to the second component that interacts with the protein of interest.⁶² The PROTAC then brings the E3 ubiquitin ligase in close contact with the target protein, which is subsequently ubiquitinated. The

ubiquitinated target is then degraded in the cellular proteasome and the PROTAC molecule is freed to bind another molecule of protein.⁶³ This system has some advantages over small molecule inhibitors as the component linking the PROTAC to the kinase doesn't have to inhibit catalytic activity, allowing for greater diversity in the recognition site, and therefore potential specificity. This system does require the cellular E3 ubiquitin ligase to function, presenting a potential limitation. Preliminary work exploring the use of PROTACs in inhibitor-resistant cancers is underway, including targeting BTK in ibrutinib-resistant lymphoma and BCR-ABL in imatinib resistant CML.⁶⁴⁻⁶⁶

As the initial PROTAC compounds progress through clinical trials, the preliminary results may shed light on the potential pitfalls of this newer technology or add these molecules into the increasing repertoire of cancer therapeutics.

1.4. Assays to study kinase activity

Prior to the clinical trials that establish efficacy and potential side effects of kinase inhibitors, inhibitor screening is done on a large scale to test large numbers of chemical compounds against the target kinase of interest. This screening relies on kinase assays which test the activity level of the kinase, demonstrating whether a putative inhibitor compound prevented the kinase from catalyzing the phosphorylation of a substrate. Inhibitor screening is typically done on a large scale, referred to as high-throughput screening. In contrast, other assays are designed to only look at a few conditions at a time, such as to validate candidate inhibitors or examine biological functions of a kinase.

1.4.1. Radiometric assays

First described in the 1970s, radiometric kinase assays use [γ -³²P]-ATP which is incorporated into the product protein (Figure 1.6A). The labeled product is then captured

by a membrane and measured with scintillation spectrometry.^{67, 68} This assay is commonly used because of several advantages, including high sensitivity, relatively simple set-up, and a direct measurement of the final product rather than an intermediate.⁶⁸ However, the safety concerns regarding use and disposal of radioactive material are major disadvantages to this assay, and many researchers are not equipped or may be unwilling to use this assay due to these concerns. As an alternative, there are a variety of fluorescent-based kinase assays.

1.4.2. Fluorescent intensity assays

Fluorescent intensity assays typically rely on a secondary component to recognize or convert a product to a fluorescent molecule that can be measured. For example, the Promega ProFluor™ assays use Rhodamine 110, which is a fluorescent molecule. Rhodamine is conjugated to the peptide substrate, which significantly attenuates its fluorescence. Upon addition of a protease, rhodamine is cleaved, restoring the fluorescent properties. When the substrate is phosphorylated, the peptide cannot be cleaved so there is no resulting fluorescence.⁶⁹ This assay demonstrates high signal to noise ratio allowing for sensitive detection.

Another example is R&D Systems™ Universal Kinase Activity Kit. This assay uses the enzyme CD39L2 which converts the ADP created in the kinase reaction to inorganic phosphate, which is then detected by malachite green (Figure 1.6B).⁷⁰ Both this assay and the ProFluor™ assay rely on a coupled enzymatic reaction for a readout of kinase activity. This means the conditions for the coupled reaction must also be optimized, adding a layer of complexity to the kinase assay. Additionally, these are indirect readouts of activity, as the product is not directly measured.

1.4.3. Fluorescent polarization assays

Fluorescence polarization (FP) assays are rapid and quantitative and are used to study the interactions between many types of molecules, not just kinases. FP requires a fluorescently labeled component, which is excited by polarized light, meaning the light waves are all aligned on a single plane. As the labeled molecule moves around in the solution via Brownian motion, it emits light in different directions relative to the original excitation light. The rotation of the labeled molecule is related to the size of the molecule, so a smaller molecule rotates rapidly, with photons emitting in a randomized motion, resulting in more depolarization of the light. A larger molecule rotates more slowly, with less random motion, resulting in less depolarized light.⁷¹

One type of FP kinase assay uses a fluorescently labeled peptide substrate, which is detected with an anti-phospho antibody. If the peptide is phosphorylated by the kinase, the antibody binds the phosphopeptide. The binding of the large antibody causes the complex to rotate more slowly and resulting in a corresponding change in emitted light.⁷² This has the advantage of directly measuring the phosphorylation state of the peptide substrate without additional reactions required and can easily be scaled up for high-throughput applications. Additionally, only one component needs to be fluorescently labeled which is an advantage over many other fluorescent assays. However, the use of the anti-phosphoantibody limits the applications of this assay. While anti-pY antibodies are fairly robust and typically only recognize the phosphoresidue itself without too much specificity in sequence requirements, anti-pS/T antibodies are typically sensitive to the sequence of the peptide. Therefore, generic antibodies are not available and individual antibodies would need to be developed for studying S/T kinases.⁷³

To avoid the use of specific antibodies, another version of the FP kinase assay is to use a labeled anti-ADP antibody. The antibody is initially bound to a fluorescent tracer with low affinity but binds to ADP with higher affinity. This is called a competitive FP assay, as the ADP produced by the kinase assay competes with the fluorescent tracer on the antibody. Increased turnover from ATP to ADP therefore results in most of the antibody being bound to ADP, freeing the tracer, and decreasing its molecular size.⁷⁴ The commercially available Transcreener® ADP2 FP Assay (BellBrook Labs) uses this principle (Figure 1.6C).⁷⁵ This assay can be easily used for high-throughput applications and removes the reliance on kinase substrate-specific antibodies. Additionally, the peptide substrate doesn't have to be conjugated to the fluorophore. This prevents concerns about the bulky fluorophore group altering or blocking substrate recognition by the kinase of interest.

1.4.4. Förster Resonance Energy Transfer (FRET) kinase assays

Förster resonance energy transfer (FRET) refers to the transfer of energy between a donor and acceptor fluorophore. When the donor fluorophore is excited at a certain wavelength, it emits light at a second wavelength which excites the acceptor fluorophore, which in turn emits at another wavelength. This transfer can only occur when the molecules are in close proximity to each other, so the emission ratio of the two fluorophores is used to determine if the molecules were in close enough proximity for FRET to occur.

For these assays, two fluorophores must be used. The Invitrogen Z'-LYTE® biochemical assay uses a peptide substrate that is labeled at both ends. Following the kinase assay, a protease is added. If the substrate is phosphorylated, the protease is unable to cleave the peptide, resulting in FRET between the donor and acceptor. If the substrate

is not phosphorylated, the protease cleaves the peptide, so there is no transfer between donor and acceptor fluorophores (Figure 1.6D). Invitrogen has a variety of fluorophore conjugated peptides available for use in this assay which are well-documented and easy to use as a readout for the available kinases. The use of the protease may limit some of the available substrates, as preliminary experiments verifying protease cleavage of the substrate are required.

1.4.4.1. Time-resolved FRET assays

Time-resolved (TR) FRET assays use similar principles as previously described fluorescent assays except they use fluorophores with longer lifetimes. This allows for a delay in emission measurement, which helps decrease background as any fluorescence from other assay components will quickly decay prior to measurement. Invitrogen's LanthaScreen™ employs this TR-FRET technology, using a terbium-labeled phosphosite specific antibody and a GFP-labeled substrate peptide or protein of interest. Terbium is a lanthanide ion, which can fluoresce when chelated to a sensitizing ligand. Lanthanides have the advantage of longer emission lifetimes compared to other fluorophores. Following the kinase reaction, the terbium-labeled antibody binds to the phosphosite. Terbium then acts as the donor to the fluorescently labeled substrate (Figure 1.6E).⁷⁶ For this method, the substrate needs to be fluorescently labeled, and a phosphosite-specific labeled antibody is needed.

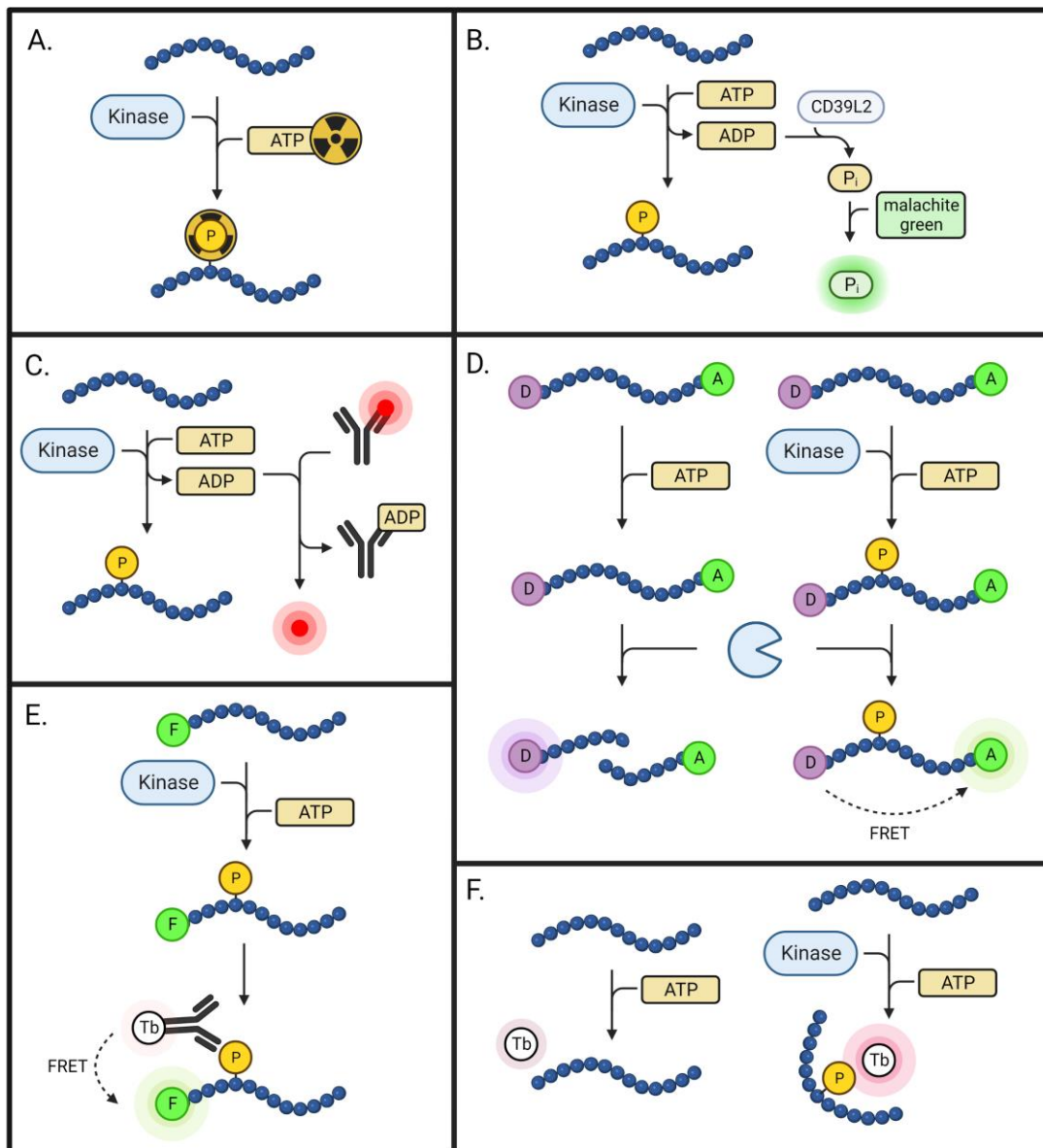


Figure 1.6. Overview of kinase activity assays. A.) Radiometric assay. B.) Fluorescent intensity assay (R&D Systems™ Universal Kinase Activity). C.) Competition fluorescence polarization assay (BellBrook Labs Transcreeper® ADP2 FP Assay). D.) Competition FRET assay (Invitrogen Z'-LYTE®). E.) TR-FRET assay (Invitrogen LanthaScreen™). F.) Terbium luminescence assay. Created with BioRender.com

1.4.5. *Time-resolved luminescence assays*

Similar to the TR-FRET assays, lanthanide-based time-resolved luminescence assays take advantage of the longer emission time of lanthanides, but without reliance on antibodies. Additionally, the lanthanide luminescent signal is measured directly, instead of via FRET to another fluorophore. Lanthanides such as terbium absorb light poorly on their own, but with a sensitizing chromophore such as phospho-tyrosine or tryptophan have high luminescence with a long lifetime.⁷⁷ The chemical environment must also be conducive for this luminescence, namely the exclusion of water molecules to prevent quenching. For select tyrosine-containing peptides, terbium is able to chelate with higher affinity to the phosphorylated peptide, causing an increase in luminescent intensity (Figure 1.6E).⁷⁸⁻⁸⁰ A time-resolved approach allows for the background signal from any unphosphorylated peptide to be gated out, demonstrating high sensitivity and signal to noise for this assay.⁷⁸ This method was employed in Chapter 2 for creation of novel BTK peptide substrates for time-resolved luminescence assays.

Terbium-luminescence assays are not limited to tyrosine kinases. Since pS/T is not capable of sensitizing terbium due to the lack of an aromatic side chain, these peptides incorporate the amino acid tryptophan to act as the donor fluorophore.^{81, 82} Prior to phosphorylation, these peptides have weak terbium fluorescence, but phosphorylation improves terbium chelation, resulting in increased signal.⁸¹

These lanthanide assays have the advantage of being completely antibody free, saving cost and production time. They also have the potential to be used for high-throughput screening due to the sensitivity of the assay, and the simplicity of the assay components. Unfortunately, not all peptide substrates can incorporate the terbium-

chelation motif, which is highly acidic.^{78, 79, 81, 83} Depending on the kinase of interest, the substrate may or may not be compatible with this particular assay, limiting the applicability.

1.4.6. Enzyme-linked Immunosorbent Assay (ELISA)

Enzyme-linked immunosorbent assays (ELISAs) can also be used as a readout for kinase activity. For this platform, the peptide substrate must be modified to allow for affinity capture. We have used peptides with a biotinylated lysine successfully; this allows for the peptide to be captured by streptavidin coated plates.⁸⁴ Following capture and washing, the peptide phosphorylation state can be probed using an anti-pY antibody conjugated to HRP enzyme. HRP catalyzed oxidation of a fluorogenic substrate produces a fluorescent molecule, providing a readout for the phosphorylated peptide (Figure 1.7). For tyrosine kinases, this method is highly sensitive and is relatively quick. However, the multiple washing steps required make this method low-throughput and less ideal for screening. Additionally, without a universal anti-pS/T antibody this method remains limited to tyrosine kinases.

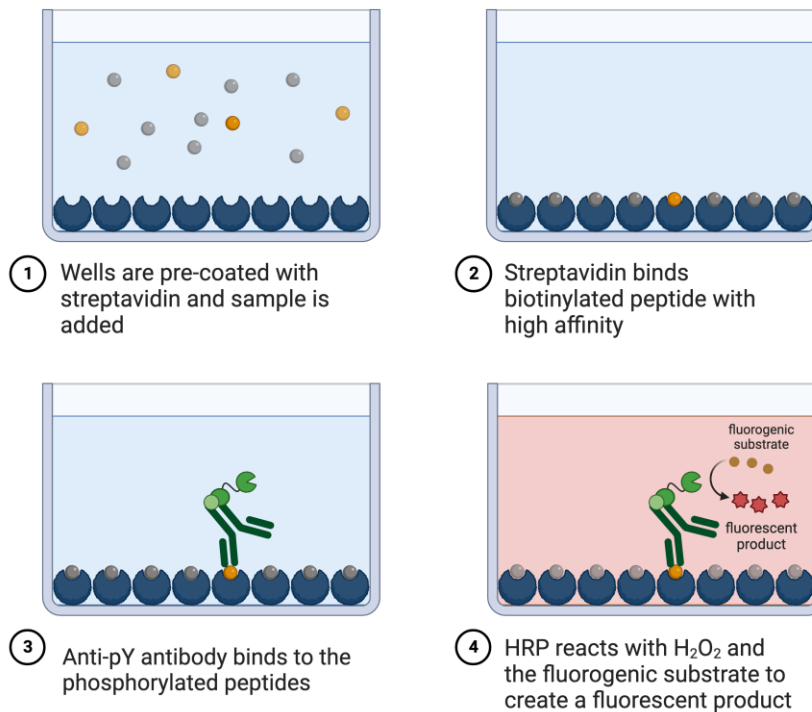


Figure 1.7. Steps of an ELISA. A streptavidin coated plate (1) is used to affinity capture biotinylated sample (2). Phosphorylated peptide is detected with anti-pY antibody (3) and detected via conversion of added fluorogenic substrate to product (4). Created with BioRender.com

1.5. Kinase substrate discovery

Across the kinase activity assays discussed, all require a peptide or protein substrate to be phosphorylated by the kinase of interest. For well-studied kinases, many substrates are known, easily allowing for a suitable candidate to be selected. However, for understudied kinases few substrates are known, creating a hurdle for activity assays, and further hindering research and drug development. Therefore, many techniques have been developed to determine the substrate profile of a kinase. These are the substrates known to be phosphorylated by the kinase of interest, under physiological or in vitro conditions. Large-scale substrate discovery often relies on a peptide library which is then assessed to determine which peptides were phosphorylated. These techniques differ in the method

of creating the peptide library to be assessed and the readout of whether each peptide was phosphorylated.

The design and method of kinase substrate discovery depends on the experimental objective. For determining physiological substrates of a kinase, careful consideration is needed on the experimental conditions and substrates present in the library. Conversely, for experiments examining the amino acid preferences of a kinase, the total library size and relative diversity may be more important.

1.5.1. Synthetic peptide libraries

To assess a library of putative substrates and determine if any are phosphorylated by the target kinase, the library must first be assembled. Synthetic peptide libraries were one of the first techniques used to examine the substrate profile of a kinase. These positional scanning peptide libraries have a fixed amino acid (S/T/Y) and a set number of degenerate positions on either side. Radiolabeled ATP has been used to determine which peptides are phosphorylated, whether the library is fixed on beads⁸⁵ or in solution.⁸⁶⁻⁹⁰ Many of these methods use smaller libraries, as synthetic libraries can be difficult and expensive to synthesize. Smaller libraries can have advantages, as there is less concern that the signal will be lost in the noise of all the unphosphorylated peptides. However, the smaller library size also limits the applicability, as only a small number of putative substrates are assessed. Other methods employ much larger libraries, creating a more comprehensive view of the substrate profile of a kinase.

1.5.2. Library-based display technologies

Display technologies use molecular biology techniques to present large libraries of peptides that can be subjected to affinity selection. While library generation

techniques are different for each display technology, they all have the conserved theme of linking the peptide (phenotype) to its encoding DNA (genotype). For kinase substrate discovery, displayed peptide libraries are incubated with the kinase of interest, and the subsequent phosphopeptides are enriched. The sequences of the enriched peptides are subsequently identified via DNA sequencing. A number of different techniques make use of genetically encoded libraries, such as phage and mRNA display.

Phage display presents peptides of interest fused to coat proteins of a bacteriophage which is a virus that infects bacteria. The phenotype of the peptide displayed is therefore linked to the genotype encoded in the phage genome. This method has been successfully used to identify substrates of kinases.⁹¹⁻⁹³ Following construction, the cDNA library, comprising all the sequences of interest, is cloned into a phage expression vector. The resulting phage ‘displays’ peptides from the library on its coat proteins. The phages can then go through a selection cycle, where the phages displaying peptides that were phosphorylated by the kinase of interest are selected. To select tyrosine phosphorylated peptides, 4G10 anti-phosphotyrosine (pY) antibody has been used.⁹¹ These peptides can then be re-entered into the selection cycle with increased washing stringency or additional mutagenesis to continue to advance the desired phenotype.⁹⁴ Finally, only robust substrates remain, and the genes encoding those substrates are isolated and sequenced. Phage display libraries can be large and diverse, providing the kinase with a broad range of sequence space. This means prior knowledge of substrate preferences is not required. The selection step also represents a potential hurdle in the phage display process, as the modified peptides need to be selected with each round. While 4G10 has been used to select for pY peptides, there is not a

comparable anti-phosphoserine/threonine antibody, limiting the applicability of this technology to tyrosine kinases.

mRNA display is a completely in vitro display technology that has previously been used to discover substrates for v-Abl kinase.⁹⁵ Each library member consists of a peptide covalently linked to its encoding mRNA. To create the genotype-phenotype linkage, the DNA library is transcribed and then ligated to the 5' end of a synthetic DNA oligonucleotide with a puromycin moiety on the 3' end. The mRNA is translated until the mRNA-DNA junction is reached, where the ribosome stalls. The puromycin mimics the amino-acyl moiety of a tRNA molecule, which allows it to enter the ribosome and be covalently bound to the peptide chain. This results in the newly translated peptide being directly linked to its encoding mRNA.⁹⁶ This peptide library can then be exposed to selection methods, and enriched peptides of interest easily identified by reverse transcribing and sequencing the linked mRNA. To find substrates of v-Abl, a large, randomized synthetic DNA library comprised of five variant amino acids on either side of a central tyrosine (e.g. X-X-X-X-X-Y-X-X-X-X-X) was created.⁹⁵ 4G10 was used to enrich any phosphorylated substrates after each round of selection. Ultimately, the identified sequences closely matched the previously reported consensus motif and were efficiently phosphorylated in follow up assays by v-Abl kinase.

Similar to phage display, mRNA display requires a selection agent like 4G10, limiting current substrate discovery efforts to tyrosine kinases. mRNA display libraries can be larger than phage display libraries, as mRNA display does not require cellular transformation, which is a limiting factor in library generation.^{94, 97} However, the mRNA

display process is laborious, requiring multiple gel purification steps and is more expensive.

Overall, display technologies have the advantage of very large, diverse peptide libraries, and substrates identified are not limited by biased initial libraries created from known substrates. These methods have the potential to be very useful in determining kinase substrate preferences but would not be suitable for examining physiologically relevant substrates. The peptides also lack modifications that can be present in cells, which may affect kinase substrate preferences. Additionally, expensive affinity reagents (typically antibodies) must be available to filter the phosphorylated peptides.

1.5.3. Mass spectrometry-based approaches

Many modern methods use mass spectrometry-based approaches to identify kinase substrates. These methods can be roughly divided into whether the phosphorylation is at the protein or peptide level (Figure 1.8) For protein-level phosphorylation, the library of potential substrates are undigested proteins, typically still folded. For peptide-level phosphorylation, proteins are protease-digested, creating a natural library of peptide substrates. Protein versus peptide level phosphorylation can result in large differences of identified substrates, with differing information on the physiological relevance of the identified substrates. Protein libraries can be used to ask physiologically relevant questions, but substrates discovered are also influenced by other factors, such as protein-protein interactions, endogenous modifications, and loss of cellular localization following lysis. Peptide libraries are larger and more diverse, but also consist of peptides that may not be naturally available to the kinase of interest, or from internal sections of proteins not typically exposed for phosphorylation.

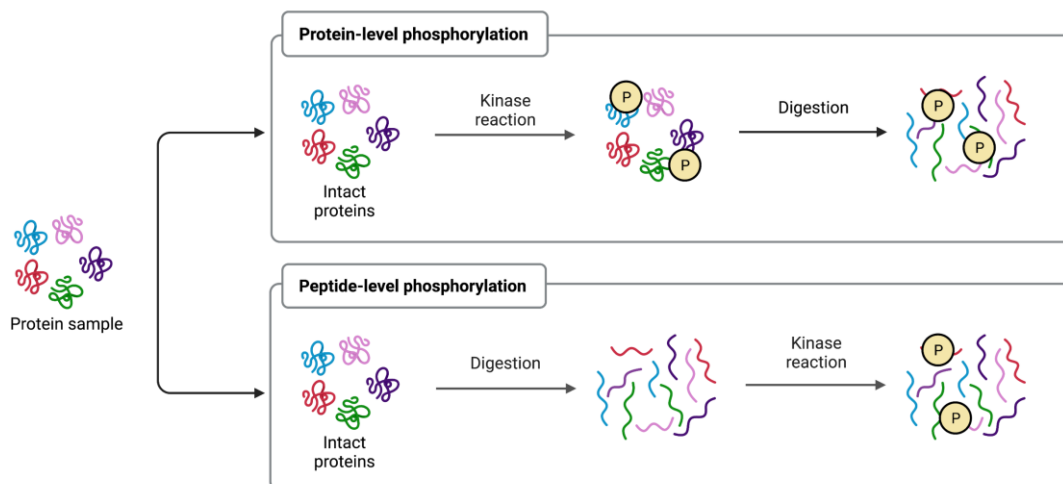


Figure 1.8. Differences in sample preparation for protein vs peptide-level phosphorylation experiments. Created with BioRender.com

1.5.3.1. Additional considerations for phosphoproteomics experiments

Phosphoproteomics is a subset of proteomics that specifically focuses on identification of phosphorylated proteins within the proteome. Beyond the typical factors influencing a proteomics study, phosphoproteomics experiments have additional complexity due to the stability and abundance of phosphorylation sites. As phosphorylation is a highly dynamic modification, often only small numbers of proteins are phosphorylated which can result in phosphoproteins being obscured by the high abundance of non-phosphorylated proteins. Therefore, a few different phosphoenrichment strategies are employed to enrich for the phosphoproteins or phosphopeptides and improve MS detection. While anti-pY antibodies can be used for selection, this strategy is not universal, as it ignores the pS and pT sites.

Most commonly, modern techniques rely on inorganic ion affinity chromatography.^{98, 99} Immobilized metal ion chromatography (IMAC) uses chelators on a resin to fix the ions in place. Phosphopeptides then bind to the immobilized metal ions,

allowing other peptides to be washed away. The metal ion choice may influence phosphopeptide selection, influencing the final pool of analyzed peptides.⁹⁹ Metal oxide affinity chromatography (MOAC) applies similar principles, using metal oxides to bind the phosphate groups of phosphopeptides. TiO₂ is most commonly used, although other metal oxides have successfully demonstrated phosphoenrichment.⁹⁹ Like IMAC, MOAC selection of phosphopeptides is influenced by the metal oxide used. Commonly, multiple metal oxides are used in combination, to enrich larger numbers and more diverse phosphopeptides. Sequential metal oxide affinity chromatography (SMOAC) method combines TiO₂ and Fe-NTA enrichment: the sample is first enriched using the TiO₂ and the wash and flowthrough retained are enriched using FeNTA.¹⁰⁰ Using a sequential enrichment strategy provides a more complete selection of the available phosphopeptides, reducing some of the selection bias. For all phosphoproteomics studies, the experimental workflow must consider the transitory nature of the modification and the extra requirements to accurately capture the phosphorylation state of a sample.

1.5.3.2. Protein-level phosphorylation

Many of the applications that use intact, folded proteins as the substrate library are searching for physiologically relevant substrates under a particular context, and typically validate their findings using other techniques. The Kaibuchi group described the kinase interacting substrate screening (KISS) where GST-tagged kinase domain of Rho kinase is immobilized by an affinity column and exposed to undigested cell or tissue lysate.^{101, 102} From this method, 300 interacting proteins were identified. Follow up work expanded to find the substrates of PKA, MAPK1, CDK5, CaMK1, PAK7, PKN, LYN, and FYN.¹⁰³ This method aims to identify biologically relevant substrates of the target kinases. By

using cell lysate that has not been denatured, the structure of the proteins and interactions between proteins is preserved, allowing closer mimicry of the biological environment. However, the lysis of the cell itself disrupts localization and regulation which may affect protein availability. Additionally, using the catalytic domain of the kinase means that no regulatory domains on the kinase itself are available to aid substrate selection. Given these caveats, these studies provide initial substrate lists that provide launching points for further research.

A potential issue whenever using cell lysates of any source is the endogenous modifications remaining on the proteins or peptides. A dephosphorylation step is commonly included to remove as many endogenous phosphorylation sites as possible prior to analysis. However, dephosphorylation is never 100% efficient, and some phosphosites almost always remain. One strategy to avoid incorporation of these background phosphorylation sites into the identified substrate list is the kinase-catalyzed biotinylation with inactivated lysates for discovery of substrates (K-BLIDS).¹⁰⁴ This method uses biotin-ATP, which is a modified ATP molecule with biotin on the gamma phosphate group. When this phosphate group is transferred to the substrate by the kinase, the biotin label is transferred as well, so any phosphorylated substrates by the kinase are now biotin-tagged. K-BLIDS uses non-denatured cell lysate, where the endogenous kinases are inactivated with pan-kinase inhibitor FSBA, and active kinase plus the biotin-ATP added to the lysate. The modified peptides are then enriched using streptavidin resin and analyzed using LC-MS. The use of the biotin-capture avoids accidental enrichment of background phosphorylation sites; however, use of a modified ATP molecule may

affect the kinetics of the kinase or substrate preferences, limiting the applicability of this method.

An alternative method commonly used to avoid background phosphorylation is to measure phosphopeptides in a negative control sample that is not treated with kinase and subtract any identified peptides during data analysis. Sugiyama *et al.* used dimethyl labeling to differentiate between the kinase treated and negative control samples, to allow pooling of the two conditions during LC-MS analysis.¹⁰⁵ Dimethyl labeling adds mass onto the peptides without significantly affecting the retention time, allowing for differentiation of two conditions and relative quantification of peptides.¹⁰⁶ They used a high-throughput method to identify the substrates of over 300 recombinant kinases. Non-denatured HeLa cell lysate was used for the peptide library, and high numbers of phosphosites were identified across the kinases analyzed, the labeled control used to remove any background. However, this study used alkaline phosphatase for their dephosphorylation treatment, which preferentially removes phosphosites on tyrosine over serine or threonine. This may have affected their analysis, as higher numbers of background phosphoserine/threonine were likely present, potentially obscuring motifs.

1.5.3.3. Peptide-level phosphorylation

Peptide-level phosphorylation with mass spectrometry readout methods can use peptide libraries derived from a variety of sources. Some methods use synthetic peptide libraries while others use natural peptide libraries, that are created by digesting cell lysate. While the proteins expressed in the cell at the time of lysis may differ, digestion of these proteins enables sufficient variety in the peptides available to kinases as substrates.

The kinase activity assay for kinome profiling (KAYAK) uses 90 synthetic peptides sourced from the MAPK and PI3K pathways as the library.¹⁰⁷ Rather than testing a specific kinase, treated lysate was added to the library to determine how different treatments affected phosphorylation of the peptides. This method is a small-scale substrate profile, that only reports select pathways and identifies how they change in response to treatments, limiting the scope of the findings. Additionally, synthetic peptides can be expensive, limiting the scale of this method.

Using a natural peptide library expands the applicability of the method and reduces the cost of library formation. Natural peptide libraries are formed by protease digestion of a cell lysate, creating an array of natural peptides. Many strategies combine a natural peptide library with a kinase treatment to identify substrates. The substrate profile of the Plk family kinases, Haspin, Bmpr2, and Camkk2b were determined by using digested HeLa cell lysate treated with phosphatase, separated into fractions by strong-cation exchange chromatography, and treated with the kinase of interest.¹⁰⁸ Following phosphopeptide enrichment and LC-MS peptide identification, multiple independent motifs were identified, demonstrating that this is a sensitive technique to probe substrate profiles and motifs.

A similar method, Kinase Assay LInked with Phosphoproteomics (KALIP), aims to identify genuine kinase substrates within the cell type used.¹⁰⁹⁻¹¹¹ Using Syk and both B-cells and breast cancer cells as a proof of concept, they identified 64 and 23 direct substrates, respectively.¹¹¹ To determine these substrates, they compared both the substrates found from the in vitro kinase reaction and the in vivo phosphoproteome. For the in vitro reaction, they treated the cells with a phosphatase inhibitor and

phosphoenriched the digested peptides to create the peptide library. These peptides were then treated with a phosphatase followed by Syk kinase, and the phosphopeptides enriched and identified via mass spectrometry. Simultaneously, they explored the phosphoproteome of the cells under two conditions, with and without Syk expression. The substrates identified in both the *in vitro* kinase reaction and the *in vivo* phosphoproteome were termed genuine substrates, reasoning that neither of these sets of substrates on their own represent substrates without significant caveats. A similar strategy using proteins instead of peptides, proKALIP, has also been published.¹¹⁰ These strategies were combined to explore direct substrates of Abl kinase in CML cells treated with various TKIs.¹¹² These methods use stringent standards to define bona fide substrates for the kinases of interest, allowing for physiologically relevant substrates to be identified compared to other peptide-based methods alone. Additionally, they provide a streamlined method to investigate amino acid preferences of a kinase, providing insight into the different substrate motifs preferred by various kinases. However, as with the other protein-level phosphorylation-based method discussed, the substrates identified depend on the proteins expressed at the time of lysis, which may limit the total number of substrates identified. Even with the peptide libraries, substrates may be missing due to lack of representative sequences.

An alternative proteome-wide serine-oriented human peptide library (SERIOHL) has been described and postulated to avoid the bias of using a library derived from cell lysate.¹¹³ This library was designed by converting known serine phosphorylation sites on proteins into a cDNA library with 15 amino acids on either side of the serine, which can then be transformed and expressed in *E. coli*. Following the *in vitro* kinase reaction, the

resulting phosphopeptides were enriched and identified using mass spectrometry. Using PKA as a proof of concept, they identified 519 unique phosphopeptides. Additionally, they repeated these experiments with PKC β and mutants and observed changes in substrate preferences across the mutants. This technique has the advantage of using a pool of defined potential substrates consistent across experiments, allowing for easier comparison between different kinases or mutations. However, using the pre-selected library of serine phosphosites may filter out unknown substrates, limiting the final identified substrates. Additionally, this method would need to be expanded to identify substrates of tyrosine kinases or serine/threonine kinases that favor threonine phosphorylation.

1.6. Thesis objectives

Despite the clear role kinases play in cancer development and progression, many kinases remain understudied. This issue is further perpetuated by lack of kinase activity assays, many of which require a known substrate of the target kinase. This gap in kinase knowledge is circular in nature: for the understudied kinases, few substrates are known, meaning kinase assays may not be available, further preventing study of the kinase. To address this, we design short synthetic peptide substrates for kinases of interest that provide a readout of kinase activity. These substrates can then be used to study kinase activity in vitro and are applicable to high-throughput inhibitor screening and discovery. This thesis discusses the process of substrate discovery of target kinases, design of synthetic substrates, and characterization of selected synthetic substrates.

Chapter 2 contains a published manuscript describing the application of this process to Bruton's tyrosine kinase (BTK), which is implicated in hematopoietic cancers.

While several BTK inhibitors are FDA approved, inhibitor resistance remains a common issue. Despite the known role BTK plays in lymphomas and leukemias, the total number of known substrates was insufficient to design synthetic substrates. We used phosphoproteomics to identify in vitro substrates of BTK, which were used input in our newly updated KINATEST-ID V2.1 R-package. We selected eight synthetic substrates for characterization and demonstrated a correlation between the KINATEST-ID score and biochemical performance in an in vitro kinase assay. We further increased the applicability of the BTK substrates by incorporating a known terbium-chelation motif, adapting the substrates for a time-resolved terbium luminescence assay.

Chapter 3 focuses on the study and development of synthetic substrates for the TAM family receptor tyrosine kinases: Tyro3, Axl, and Mer. All three of the TAM family kinases are implicated in a number of different cancer types, commonly associated with pro-survival signaling, metastasis, and chemoresistance. Despite this, no FDA-approved inhibitors target the TAM family, and large gaps in the understanding of their biology remain. We used our phosphoproteomics pipeline to determine the in vitro substrate profile of each TAM family kinase. Following input of this data in the KINATEST-ID workflow, we designed synthetic substrates for each TAM family kinase. Characterization of the substrates indicates several are highly efficient substrates for their target kinase, applicable for studying kinase activity. This set of synthetic substrates allowed for further characterization of the TAM kinases in vitro substrate profile, demonstrating differences in Tyro3 preferences compared to Axl and Mer. We further characterized select Tyro3 synthetic substrates and found them to be specific for Tyro3 over Axl and Mer.

Chapter 2. Novel Bruton's Tyrosine Kinase (BTK) substrates for time-resolved luminescence assays

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Minervo Perez who performed the phosphoproteomics experiments, did the initial analysis and designed BTK synthetic substrates A-D, along with John Blankenhorn who designed BTK synthetic substrates F, H, and K (Figure 2.3B). Lindsay Breidenbach and Hannah Peterson performed the initial analysis of these synthetic substrates (Figure S2.1). Erica Pratt wrote the updated and consolidated KINATEST-IDv2.1.0 R package. Naomi Widstrom re-analyzed the phosphoproteomics data using KINATEST-IDv2.1.0 and re-scored the synthetic peptides (Figure 2.3A, B). Naomi Widstrom worked with Jason Heier to measure the initial rate of phosphorylation of the synthetic substrates (Figure 2.3C, D). Naomi Widstrom designed and tested the synthetic substrates with an incorporated terbium motif (Figure 2.4). Naomi Widstrom made all figures except Figure 2.2, assembled all data and methods, and wrote the entire chapter.

2.1. Summary

Bruton's tyrosine kinase (BTK) is a well-documented target for cancer therapeutics due to its role in B-cell signaling pathways. However, inhibitor design is hindered by lack of tools to assess kinase activity. We used *in vitro* phosphoproteomics to determine BTK's substrate preferences and applied this information to our updated data processing pipeline, KINATEST-ID 2.1.0. This pipeline generates a position-specific scoring matrix for BTK and a list of candidate synthetic substrates, each given a score. Characterization of selected synthetic substrates demonstrated a correlation between KINATEST-ID 2.1.0 score and biochemical performance in *in vitro* kinase assays. Additionally, by incorporating a known terbium-chelation motif, we adapted synthetic substrates for use in an antibody-free time-resolved terbium luminescence assay. This assay has applications in high-throughput inhibitor screening.

2.2. Introduction

Nonreceptor Bruton's tyrosine kinase (BTK) sits at a node of immune signaling pathways, controlling both adaptive and innate immune cell function, proliferation and other key signaling pathways.¹¹⁴ In B-cells, BTK activity is required for maturation and B-cell receptor signaling, as well as mediating signals from chemokine receptors.¹¹⁵⁻¹¹⁷ In myeloid cells, BTK has a role in toll-like receptor signaling and modulating the innate immune response.¹¹⁸ Dysregulation of BTK is linked to a number of different B-cell leukemia and lymphomas.¹¹⁹ Additionally, BTK expression in tumor-associated macrophages within the tumor microenvironment is linked to promotion of cancer cell migration and metastasis.¹²⁰ Due to these roles, BTK has been an attractive target for cancer therapeutics. To date, there are three FDA-approved BTK inhibitors for treatment

of various B-cell malignancies: ibrutinib, acalabrutinib, and zanubrutinib.⁴ All three are irreversible inhibitors that covalently bind to cysteine 481 in the kinase domain of BTK, effectively inhibiting BTK activity. Although these inhibitors show promising results,¹²¹⁻¹²⁴ disease relapse due to resistance remains an issue.^{125, 126} Therefore, there is a need to continue BTK inhibitor development, which relies on sensitive, high-throughput screens to determine kinase activity. Kinase activity can be assessed by a variety of means, such as radiometric or fluorescent based approaches, but these methods are higher cost or reliant on antibodies, making them less ideal for screening.¹²⁷

Lanthanide luminescence assays are an option for kinase activity detection that have the advantage of being antibody free. Lanthanides such as terbium absorb light poorly on their own, but in the presence of a sensitizing chromophore, such as phosphotyrosine, terbium exhibits high luminescence with an increase in luminescent lifetime.⁷⁷ To take advantage of these properties, multiple approaches have been described using a peptide incorporating a terbium-chelation motif and appropriate sensitizer to detect phosphorylation.^{81, 83, 128, 129} Previously, we have adapted a sensitive time-resolved terbium luminescence assay into a format that works well for high-throughput drug screening.⁷⁸⁻⁸⁰ In this assay, a synthetic peptide substrate with a central tyrosine residue is phosphorylated by the kinase of interest. When phosphorylated, the peptide can better chelate the Tb³⁺ ion allowing tyrosine to sensitize the Tb³⁺ luminescence. Tb³⁺ chelation and sensitization greatly enhances the luminescence intensity, which is especially pronounced when using time-resolved luminescence measurements. This allows for sensitive differentiation of the phosphorylated form of the synthetic substrate, providing a fast readout of kinase activity through straightforward

liquid addition steps (without requiring complex handling). The peptide substrate used in this assay must contain a terbium-chelating motif, while also maintaining the required substrate motif to be phosphorylated by the kinase of interest. Therefore, we developed an *in silico* pipeline called KINATEST-ID to assist in designing such substrates by identifying kinase preferences and providing information on which positions are amenable to substitution of amino acids that facilitate chelation.⁷⁹ However, design of these synthetic substrates was hindered by the lack of known BTK substrates. Here, we describe our work to determine the substrate profile of BTK using *in vitro* phosphoproteomics, updates to the tools used to analyze the substrate profile and design synthetic substrates (KINATEST-ID 2.1.0), and the optimization of novel synthetic BTK substrates for use in the time-resolved luminescence assay.

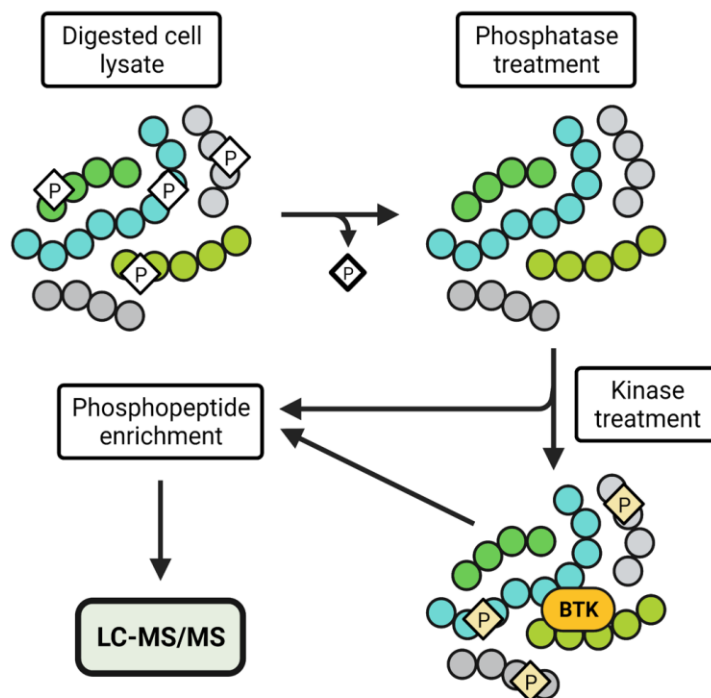


Figure 2.1. Schematic of phosphoproteomic workflow. KG-1 cells were lysed, trypsin-digested, and treated with alkaline phosphatase to create a natural peptide library. The peptide library was treated with recombinant BTK and phosphoenriched prior to LC-MS/MS analysis. Created with BioRender.com.

2.3. Results and Discussion

Design of BTK synthetic substrates. The KINATEST-ID approach uses information about a kinase's known substrates to identify novel optimal substrate candidate sequences.⁷⁹

However, very few substrates for BTK have been reported, and we required a larger knowledgebase in order to design novel substrates effectively. Therefore, we employed a modified phosphoproteomics workflow designed to determine the substrate profile of a kinase (similar to our previous reports for developing FLT3 substrates).^{110, 130, 131} In brief, we treated trypsin-digested cell lysate with phosphatase to remove endogenous modifications, followed by reaction with recombinant BTK for two hours.

Phosphopeptides resulting from this reaction were enriched and identified using an

Orbitrap Fusion mass spectrometer (Figure 2.1). The identified substrates were input into our updated data processing workflow, KINATEST-ID 2.1.0 (Figure 2.2). KINATEST-ID 2.1.0 is an updated R package of our previous KINATEST-ID workflow, comprising a full set of data handling and analysis steps. The updated v2.1.0 uses a Fisher Odds-based positional scoring matrix to calculate the odds ratio and significance of each amino acid at each position relative to the central phosphorylation site, as opposed to the standard deviation model we used previously.⁷⁹ It also includes amino acids from -7 to +7 flanking the phosphorylation site, in order to provide information about whether residues on either end (-7 to -5 and +5 to +7) can be substituted easily with acidic residues to improve chelation of terbium for the time-resolved luminescence assay (however, it only uses positions -4 to +4 for predicted substrate scoring, since that region is typically employed in studies of tyrosine kinase substrate recognition using peptides).¹³² From the 4,684 unique peptides confidently identified across all three replicates, we found 314 phosphotyrosine sites, with 68 nonredundant sites in common after subtraction of sites found in control samples (see Supplemental Materials file for data processing, output details, and a discussion of the caveats of this analysis). Representing the most robust observations, these sequences were used to calculate the position-specific odds ratio of each amino acid and generate a heatmap that illustrates the substrate motif identified through this *in vitro* phosphorylation experiment (Figure 2.3A).

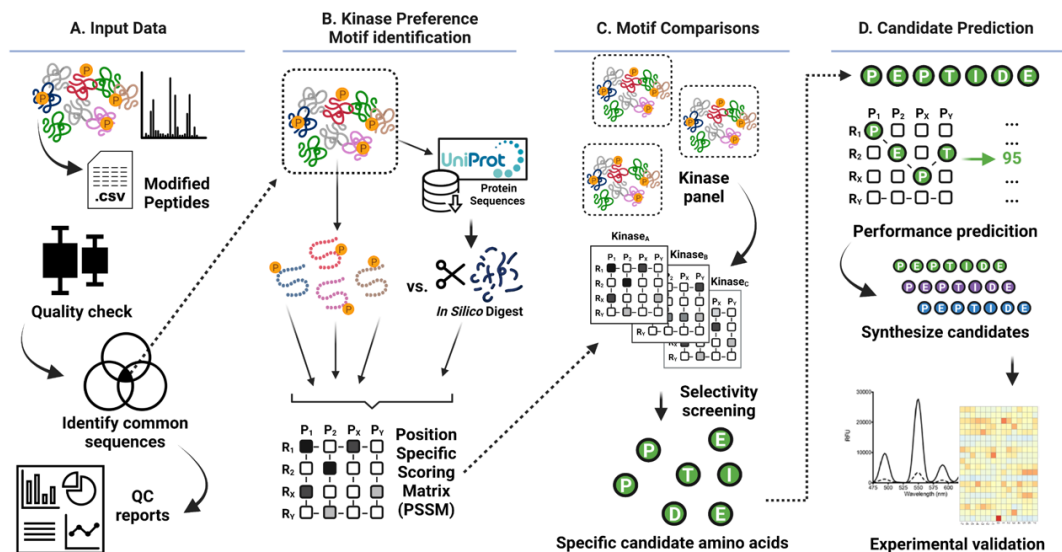
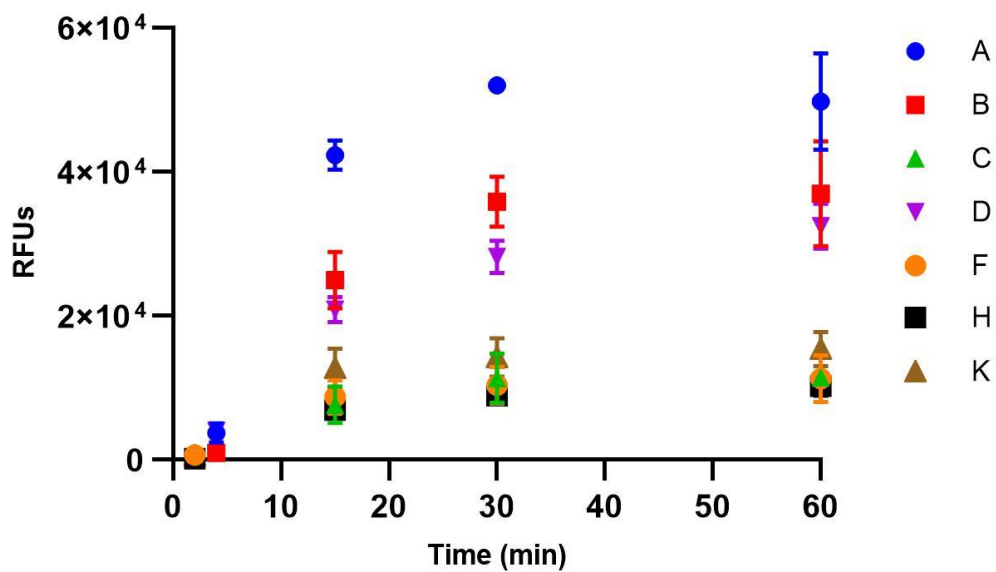


Figure 2.2. Schematic of KINATEST-ID 2.1.0. A.) Results lists of the phosphopeptides identified in the in vitro phosphoproteomics experiment are filtered to select the sequences robustly observed in common between replicates, as well as generate quality control reports. B.) Robustly observed sequences are used in combination with a surrogate “background” consisting of an in silico tryptic digest of protein sequences drawn from the FASTA file using UniProt identifiers linked to phosphopeptides observed, to generate a position-specific scoring matrix for the kinase of interest. C.) The target kinase preferences are screened against a panel of off-target kinases’ preferences to determine motif elements most likely to provide a selective substrate, identifying candidate amino acids and refining selectivity-focused motifs. D.) A table of sequences representing all permutations of candidate amino acids for the -4 to +4 positions is generated, each with a score for predicted biochemical performance. From here, candidate sequences are selected for synthesis and experimental characterization. Created with BioRender.com.

Using the KINATEST-ID 2.1.0 *Generator* function, we generated candidate sequences according to BTK’s identified substrate motif. We selected seven candidate sequences to synthesize. The motif comparison *Screeener* module (Figure 2.2C) was not considered in the design of these peptides, due to the goal of developing an *in vitro* assay in which only a single kinase would be present. Four sequences represented -4 to +4 from the central phosphorylation site (the core recognition motif¹³²), and three were extended to -7 (and in one case also to +7) to incorporate chelating residues. Sequences were chosen to have a

range of KINATEST-ID 2.1.0 scores (calculated just for the -4 to +4 segment, as mentioned previously, using a scoring function derived from the Fisher Odd's table, Eq. 2) to test for a correlation between the sequence's score and performance in a BTK kinase assay. The candidate BTK synthetic substrates were given letter names (A, B, C, D, F, H, K) and each synthesized with glycines flanking the substrate portion, and a biotinylated lysine near the C-terminus, to allow affinity capture of the peptides, followed by additional glycines (Figure 2.3B). An initial *in vitro* kinase assay was performed with recombinant BTK, collecting and quenching four aliquots over 60-min incubation, with phosphorylation detected using an anti-phosphotyrosine antibody in an ELISA-based assay (Supplemental Figure S2.1). As a follow-up to that initial progress curve experiment, we used LC-MS to more thoroughly monitor linear product formation from each substrate from 0 to 10 mins (Figure 2.3C). We compared the KINATEST-ID 2.1.0 scores with the initial slope of phosphorylation using a Pearson correlation analysis and found that there is a statistically significant correlation between the score and the initial rate of phosphorylation of the substrates (Figure 2.3D). This suggests that when a relevant *in vitro* phosphoproteomics dataset is used to determine the Fisher's Odds table and scoring function, the scores generated by KINATEST-ID 2.1.0 are useful to estimate the likely efficiency of a substrate. While all synthetic substrates would still need to be validated using a kinase assay, this information is useful in deciding what range of scores are most likely to indicate an efficient synthetic substrate. These findings demonstrate that we can successfully design synthetic substrates for BTK that perform well in *in vitro* BTK kinase assays.



Supplemental Figure S2.1. Kinase assay with ELISA-based readout. Progress curves for phosphorylation of BTK synthetic substrates A, B, C, D, F, H, and K (37.5 μM) in the presence of recombinant BTK. Aliquots of the reaction mixture were quenched 1:1 in EDTA at timepoints 4, 15, 30, 60 mins (A, B, C, D) or 2, 15, 30, 60 mins (F, H, K). Data points from A, B, C, D average of 3 replicates, data from F, H, K average of 6 replicates \pm SEM. RFU, relative fluorescent units.

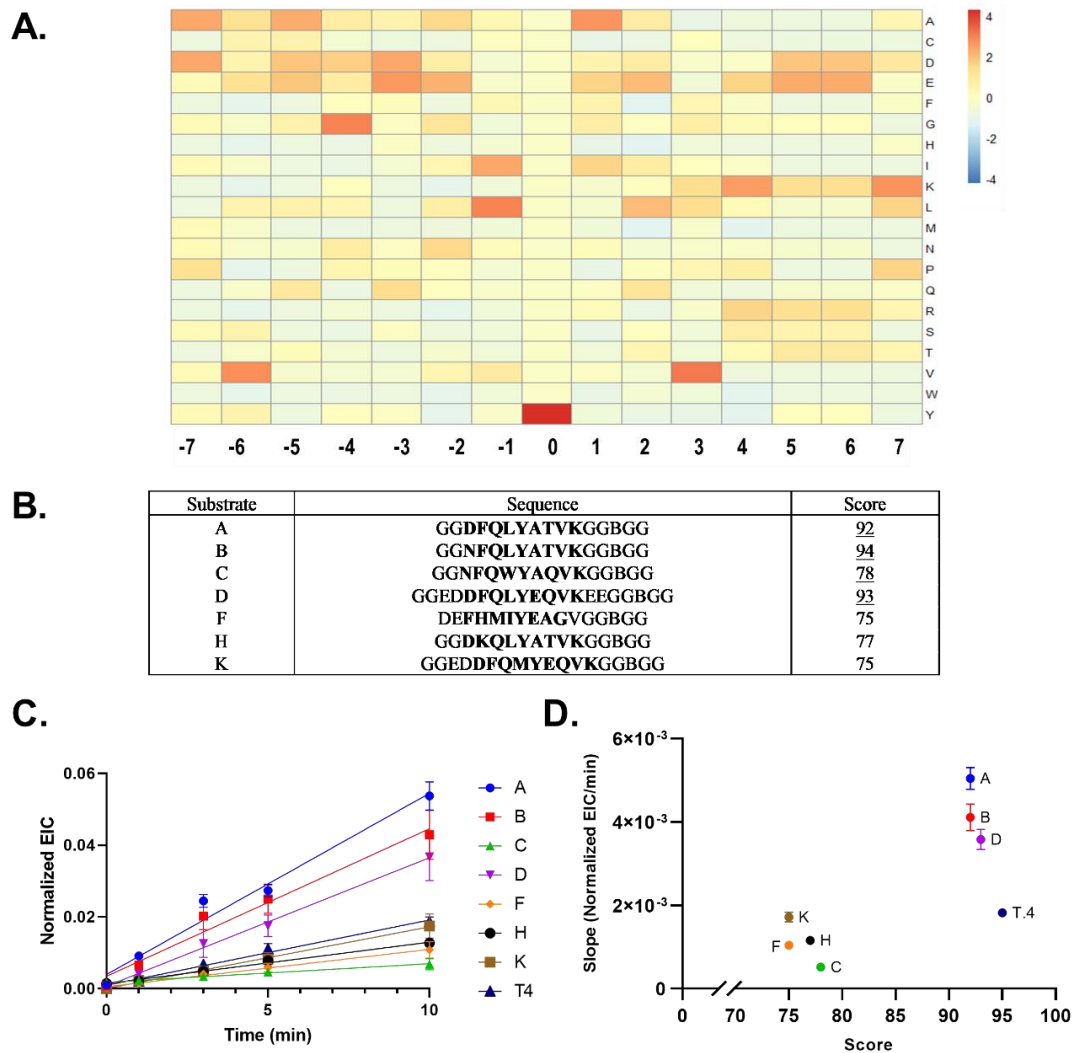


Figure 2.3. BTK phosphoproteomics results and synthetic substrate performance. A.) Heatmap showing amino acid frequency across positions relative to central tyrosine. B.) Synthetic substrate sequences and scores. B denotes biotinylated lysine; bold text denotes designed substrate portion containing the phosphorylation site. Underlined text denotes predicted active substrates. C.) Kinase assay with LC-MS-based readout of the 8 synthetic substrates in the presence of recombinant BTK. Product EIC (P) was normalized with substrate EIC (S) with Eq. 3. Data points are average of 3 replicates \pm SD. D.) Comparison of KINATEST-ID score for each substrate vs. the slope of normalized EIC. Data points are average of 3 replicates \pm SEM. Pearson correlation coefficient $r = 0.7421$ with a p-value of 0.0350. EIC, extracted ion chromatogram.

Design of terbium-chelating substrates. While several of the synthetic substrates showed promising results in the ELISA-based assay, the ELISA itself is not ideal for inhibitor screening, as its multiple wash and incubation steps make high-throughput testing difficult. Although sequence D was capable of chelating and sensitizing Tb³⁺ when phosphorylated, its signal to background for unphosphorylated vs. phosphorylated was too low to achieve useful dynamic range in the assay. Therefore, we focused attention on specifically designing BTK synthetic substrates for use in the sensitive, high-throughput terbium luminescence assay. Previously, we described the characterization of Syk Artificial Substrate peptide (SAS tide), which facilitates terbium chelation when the tyrosine is phosphorylated, leading to luminescence enhancement.⁷⁸ SAS tide's terbium binding motif was designed based on a the alpha synuclein sequence surrounding position Y125 (DPDNEAYEMPSEEG), which sensitizes terbium when phosphorylated.⁸³ We noted that BTK prefers acidic amino acids at a number of positions, making it an ideal candidate to incorporate into the terbium motif. We chose one sequence frame based on the alpha synuclein sequence⁸³, with a sequence predicted by KINATEST ID to be a BTK substrate (DEQIYEGKK) as the central -4 to +4 substrate portion, and altered the total number of acidic residues by substituting in glutamic acid residues for glycine to investigate the role of acidic amino acids in this motif. These synthetic substrates were designated 'T' with variants 1 through 4: synthetic substrates T.1, T.2, T.3, and T.4 (Figure 2.4A). We tested the signal to background ratio by synthesizing a phosphorylated version of each substrate and mixing the two versions together in different ratios to create a standard curve from 0 to 100% phosphorylation (Figure 2.4B). These experiments were done in the same kinase assay reaction buffers as the actual reactions, to mimic

conditions and background of a standard kinase assay. We found that while the background level of each substrate remained similar, by increasing the number of acidic residues we were able to increase the signal to background ratio for detecting phosphorylation and achieve a wide dynamic range. As BTK synthetic substrate T.4 was found to have the best dynamic range of the terbium-chelating synthetic substrates, we next tested its efficiency as a BTK substrate. We performed a kinase assay by adding 20 μ M synthetic substrate T.4 to a kinase reaction mixture containing 20 nM recombinant BTK and quenching aliquots in urea at specific time points. Following addition of TbCl_3 , the time-resolved spectra were immediately measured (Figure 2.4C). Area under the curve (AUC) was calculated from the emission spectra (Figure 2.4D) and a standard curve containing 0 to 100% phosphorylated T.4 and used to interpolate the percent phosphorylation (Figure 2.4E). A clear progression of increased phosphorylation was observed over time and by 60 mins, approximately 50% of the synthetic substrate was phosphorylated, demonstrating that T.4 acts as an efficient substrate for BTK. In the subsequent experiment comparing initial rates for the non-Tb-sensitizing peptides and T.4 (Fig. 2.3C), its phosphorylation was not as efficient as some of the best substrates—however, T.4 was the preferred terbium-binding peptide with the highest signal to noise ratio (S/N) of the four peptides tested (3.34 for T.4, which was 2.5-fold higher than 1.29 for T.2). We felt this trade-off in substrate efficiency in order to achieve a viable lanthanide luminescence assay was acceptable. Overall, these findings demonstrate that the BTK synthetic substrate T.4 can be effectively used with the time-resolved luminescence assay to rapidly determine the activity of BTK.

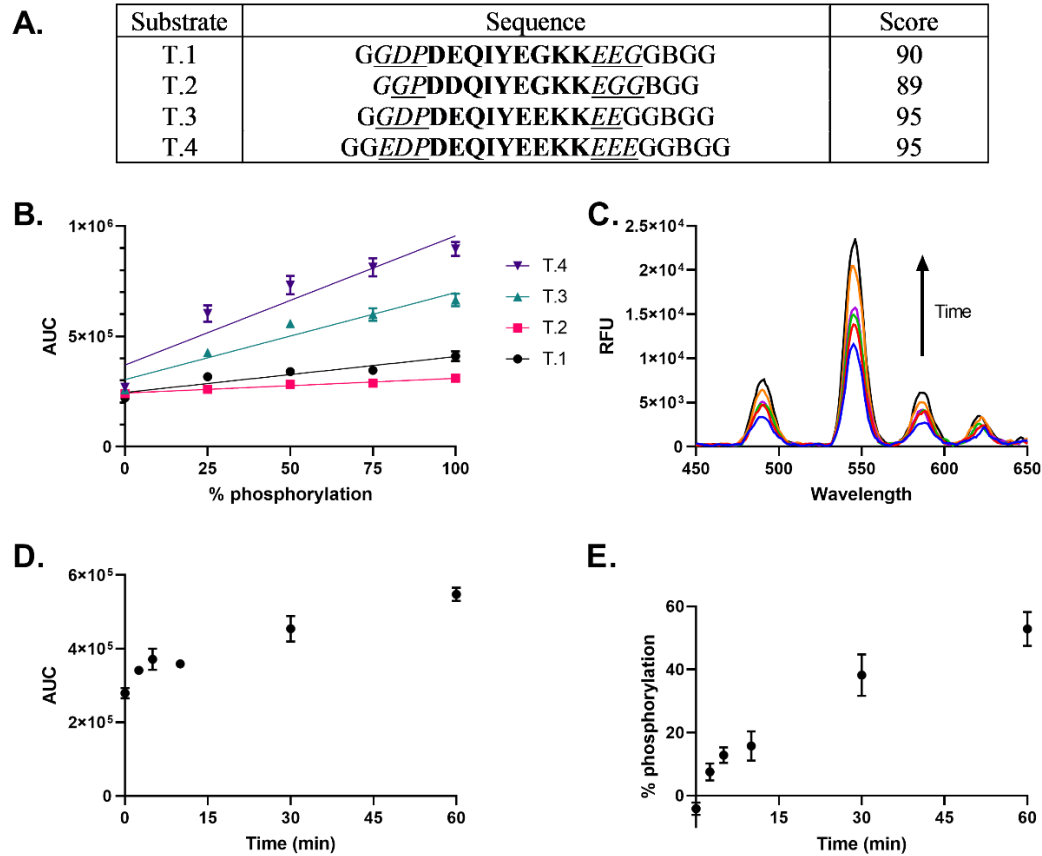


Figure 2.4. Characterization of terbium-chelating BTK synthetic substrates. A.) Sequences of terbium-chelating substrates and their KINATEST-ID V2.1.0 scores. B denotes biotinylated lysine; Bold text denotes designed substrate portion containing the phosphorylation site; Italic text denotes the -7 to +7 positions evaluated for compatibility of acidic residues with BTK's substrate preferences. B.) Dynamic range of four synthetic substrates. Each peptide mixed with different ratios of its phosphorylated form to test the relative range and sensitivity. Data are shown as an average of six technical replicates \pm SEM. C-E.) Terbium luminescence spectra (C) from *in vitro* BTK kinase assay with BTK synthetic substrate T.4 and area under curve (AUC) for each spectra (D). Percent phosphorylation of BTK synthetic substrate T.4 across time interpolated from regression line from standard curve (E). Kinase assay data points represent 6 replicates \pm SEM. RFU, relative fluorescent units; AUC, area under curve.

In summary, we used phosphoproteomics to determine the substrate preference of BTK and optimized tools to use this information to design efficient synthetic substrates for BTK. We found a correlation between our KINATEST-ID 2.1.0 score and substrate performance, establishing that our process can approximate which synthetic substrates are likely to be efficient substrates of BTK, aiding in sequence selection for substrate design. BTK synthetic substrate A proved to be an effective substrate with both the ELISA and LC-MS readout. For designing terbium-chelating synthetic substrates for use in our time-resolved luminescence assay, we demonstrated that increasing the number of acidic residues outside the motif of amino acids preferred by BTK resulted in an increase in the dynamic range for time-resolved lanthanide luminescence detection of phosphorylation, without affecting the background signal. BTK synthetic substrate T.4 proved to be an efficient BTK substrate as well. This novel substrate provides an antibody-free assay that is rapid, sensitive, and requires only simple liquid addition steps, qualities ideal for the high-throughput screening necessary for BTK inhibitor testing. Additionally, the process described here of substrate discovery and synthetic substrate design can be easily applied to other tyrosine kinases to provide useful tools for kinase assay development.

2.4. Materials, Methods and Details of Proteomic Results

Cell culture and natural peptide library preparation. KG-1 cells (ATCC) were maintained in IMDM media (Gibco) supplemented with 20% heat inactivated fetal bovine serum (FBS), 1% penicillin/streptomycin in 5% CO₂ at 37°C. Endogenous peptide sample preparation was carried out as described previously.¹³¹ In brief, cells were lysed, alkylated, and trypsin digested prior to being treated with alkaline phosphatase to remove

endogenous phosphate groups. For three replicate samples, 1.5 μg recombinant BTK (SignalChem) was added to half of the endogenous peptide sample in a kinase reaction mixture (50 mM Tris HCL, pH 7.5, 10 mM MgCl_2 , 1 mM DTT, 1 mM Na_3VO_4 and 2 mM ATP) and incubated for two hours at 37°C . The other half of the sample was processed and analyzed as a control for effectiveness of the phosphatase reaction. All samples were phosphoenriched using PolyMac kits (Tymora Analytical) prior to mass spectrometry analysis.

LC-MS/MS data acquisition. LC-MS/MS data acquisition was carried out as described previously with a modification to the LC elution time interval.¹³¹ In brief, samples were reconstituted in LC/MS solution (98/2/0.5%; $\text{H}_2\text{O}/\text{ACN}/\text{formic acid (FA)}$) and loaded on a ThermoScientific Easy NanoLC LC 1000 system. The mobile phase consisted of 0.1% formic acid in ultra-pure water (Solvent A) and 0.1% formic acid in acetonitrile (Solvent B). Samples were run over a linear gradient (5-30% solvent B; 80 minutes) with a flow rate of 200 nL/min into a high resolution Orbitrap Fusion Tribrid Mass Spectrometer, using the same parameters as previously described.¹³¹ Of note: replicate 1 for the “minus kinase” (phosphatase-treated control) and “plus kinase” conditions was run twice on the LC/MS; both data files were processed through PEAKS Studio X pro and PEAKSModExtractor as described below, followed by concatenation of their results lists for further processing.

Data processing. PEAKS Studio X pro (Bioinformatics Solutions Inc) was used to process and identify the sequences of the modified peptides against the full reviewed UniProt/SwissProt human proteome database (54niport.org), including carbamidomethylation of cysteine as a fixed modification, and oxidation of methionine,

deamidation of glutamine or asparagine, and phosphorylation of serine, threonine or tyrosine, as variable modifications. Following sequence identification, we exported the protein-peptides.csv lists, which contain the results for all peptide identifications for each sample. The protein-peptides.csv lists were used for input into the PEAKSModExtractor script¹³³ (according to its instructions, available in the repository housing the script <https://gitlab.com/jackbrennan07/peaks-modextractor>) which combines all input files into a single output file containing a modification-centered list of sequences from all of the identified peptides, providing the UniProtIDs, modification site and A score (confidence score for modification identity and localization). This sequence list includes separate entries for each modification observed on each identified peptide, thus many modified peptides are represented multiple times (i.e., for each modification). This file was used as the input for the R package KINATEST-ID 2.1.0. Results for unique peptides are summarized in the table below, with detailed lists provided in supplemental file “BTK_uniquepeptides_summary.xlsx.” Of the peptides described in Supplemental Table S1 below, 71 were observed in all three kinase reaction replicates but not in any of the no kinase controls.

Supplemental Table S2.1. Unique peptide summary.

Sample	Replicate	Unique peptides	pY peptides (Ascore \geq 30)
BTK_MINUS (no kinase)	1 (concatenated runs 1 and 2)	2162	72
	2	4595	54
	3	1882	51
BTK_PLUS (kinase reaction)	1 (concatenated runs 1 and 2)	3968	252
	2	1758	195
	3	2398	247

Building on earlier work^{79, 131}, KINATEST-ID 2.1.0

(<https://github.com/lparkerumn/KINATESTIDv2>) integrates curated collections of known endogenous substrate sequences and data from phosphoproteomics-informed kinase assays to develop an *in silico* screening library. The position-specific amino acid preferences contained in the library inform the design of high-performance artificial peptide substrates. Using KINATEST-ID 2.1.0, the initial modified site-centered sequence list (n = 5647) was filtered for sequences centered on modified phosphotyrosine (n = 314). For increased robustness and specificity, the sequence list was additionally filtered for sequences present in all three kinase-treated technical replicates but absent in negative controls (n = 68). The difference in unique tyrosine-centered sequences here (68) vs. the number of unique phosphopeptides identified by PEAKS Studio X pro in all three kinase reaction replicates but not any of the controls (71) can likely be explained by the manner in which PEAKSModExtractor only considers the central modification when

extracting and recording sequences, and ignores other modifications within the -7 to +7 range of each listed modification; this results in more sequences being considered redundant than when the internal modifications are treated as unique. We recognize the caveat that this is a potential limitation of the current analysis, given that it is possible that these other modifications contribute to the physiochemistry of kinase-substrate recognition, and future development of these tools will aim to address this in the code. In the current study, the sixty-eight input sequences were used to construct a position-specific scoring matrix (PSSM). The frequency of each amino acid was calculated for flanking residues -4 to +4 from the central phosphotyrosine. Frequencies were converted to odds ratios applying the Fisher's Exact Test and used to generate the PSSM. A peptide P can be described by the PSSM as follows:

$$P_{PSSM} = \begin{bmatrix} a_{11} & a_{12} & \dots & a_{1j} \\ a_{21} & a_{22} & \dots & a_{2j} \\ \vdots & \vdots & \dots & \vdots \\ a_{i1} & a_{i2} & \dots & a_{ij} \end{bmatrix} \quad (1)$$

Where a_{ij} represents the Fisher odds ratio for amino acid i at flanking position j . The background frequencies for calculating the odds ratios were based on the input peptides. The full sequences for each protein from which a phosphotyrosine peptide was observed were downloaded for each UniprotKB/Swiss-Prot ID found in the 68 sequence input dataset (<https://www.uniprot.org/help/uniprotkb>). *In silico* tryptic digestion was used to generate a control list of tyrosine-containing polypeptides from those proteins ($n = 3301$) which were likely present in the original endogenous peptide library (assuming no alternative splicing or truncation occurred) but were not modified by the kinase of interest. Candidate amino acids with $a_{ij} > 1$ were selected at each flank position based on:

1) $p \leq 0.05$ or odds ratio > 2) the number of kinases in the screening library which *disfavored* that residue (odds ratio < 1).

Candidate amino acids were then permuted to generate a list of potential kinase-specific artificial peptide substrates. The total score s for each peptide P is the product of the odds ratio of each amino acid in the sequence:

$$s_p = \prod_{j=1}^J a_{ij} \text{ where } i = (A, C, \dots, Y) \quad (2)$$

ROC analysis was also performed using the input peptides and control list to estimate a cutoff score for predicted peptide activity. Samples were bootstrapped using the R package ‘cutpointr’ (maximize metric) to calculate a more robust cutoff estimate.

Generally, potential artificial peptide substrates scores above the cutoff are predicted to be biochemically active (i.e. phosphorylated) for that kinase. For interpretability, reported scores are log-transformed and scaled between 0 to 100.

Peptide synthesis and purification. Peptides were synthesized on Symphony X Peptide Synthesizer (Protein Technologies) on a 50 μmol scale with Rink amide resin (Protein Technologies). Fmoc-protected amino acids (Protein Technologies) were coupled in activating solution of HCTU in N-methylmorpholine (NMM) and dimethylformamide (DMF) in two 10-minute couplings. For biotinylated lysine the coupling time was 2 hours, and phosphotyrosine the coupling time was 8 hours. Fmoc group deprotected using 20% piperidine in DMF. Peptides were cleaved from resin using a cleavage cocktail of 94% trifluoroacetic acid (TFA), 2.5% H_2O , 2.5% ethane dithiol and 1% Triisopropylsilane (TIS) followed by precipitation in ice cold diethyl ether and lyophilization. Peptides were then purified by reverse phase HPLC (Agilent 1200 Series

Infinity LCMS) to >90% purity (Supplemental Figure 2). Synthetic substrates were dissolved in 100 mM (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) (HEPES) pH 7.5 for LC-MS readout assays, and in phosphate buffered saline (PBS) for ELISA readouts and terbium time-resolved assays.

Synthetic substrate *in vitro* assay with HPLC-MS readout. Reactions (230 μ L total volume) using recombinant BTK (SignalChem) were performed in triplicate at 25°C in kinase buffer (25 mM HEPES pH 7.5, 10 mM MgCl₂, 100 μ M ATP, 3 mM DTT, 3 μ M Na₃VO₄) with a BTK concentration of 10 nM. After incubating BTK in kinase buffer for 15 min., reactions were initiated with the addition of substrate (20 μ M). Reaction aliquots (50 μ L) were withdrawn and quenched with 10 μ L 20% TFA (3.3% TFA in the quenched solution). Sample aliquots were run on HPLC (Agilent 1200 series) using a C18 Agilent Zorbax column (2.1 x 250mm, 5-micron) at 0.25 mL/min with acetonitrile increasing from 10% to 30% over 40 min. Reaction progress was analyzed via MS (Agilent 6130A) through integration of extracted ion chromatograms (EIC) corresponding to products (P) and substrates (S) (Agilent ChemStation) and normalized according to equation:

$$\text{Normalized EIC} = \frac{\text{product EIC}}{(\text{product EIC} + \text{substrate EIC})} \quad (3)$$

Slope of the timepoints was calculated using GraphPad Prism, and a Pearson correlation analysis used to calculate the correlation between slope and KINATEST-ID 2.1.0 score.

Synthetic substrate *in vitro* assay with ELISA readout. Recombinant BTK (SignalChem) was diluted 10X in (20 mM MOPS pH 7.5, 1 mM EDTA, 0.01% Brij-35, 5% Glycerol, 0.1% beta-mercaptoethanol and 1 mg mL⁻¹ bovine serum albumin (BSA)). Diluted BTK was incubated with reaction mixture (25 mM HEPES pH 7.5, 10 mM MgCl₂, 100 μ M ATP, 3 mM DTT, 3 μ M Na₃VO₄) to a final concentration of 20 nM for

15 mins at 37°C. Peptide substrate was added to a final concentration of 37.5 μM . Sample aliquots were quenched by combining 1:1 with 30 mM EDTA at timepoints. Chemiluminescent detection of phosphorylation was performed as previously described.¹³¹

Synthetic substrate *in vitro* assay with terbium time-resolved readout. Recombinant BTK (SignalChem) was added to a final concentration of 20 nM in kinase reaction mixture (25 mM HEPES pH 7.5, 10 mM MgCl_2 , 1 μM Na_3VO_4 , 100 μM ATP, 0.05 μg μL^{-1} BSA) and incubated at room temperature for 15 mins. The reaction was started by adding the synthetic substrate at a final concentration of 20 μM to the reaction mixture. At timepoints 2.5, 5, 10, 30, and 60 mins a 20 μL aliquot was removed and quenched 1:1 in 6 M urea in a 384-well black plate. 10 μL of terbium luminescent mix (final concentration 100 mM NaCl, 200 μM TbCl_3) was added to each well for a final well volume of 50 μL . The “zero” time point contained BTK storage buffer only (no enzyme). Time-resolved emission spectra were collected on Synergy Neo2 (Biotek) plate reader with monochromator excitation of 266 nm, time-resolved delay of 50 μsec and collection time of 1000 μs . Emission spectra collected between 450-650 nm with 1 nm step and a gain of 230. Area under the curve calculated using GraphPad Prism for each spectrum. Signal to noise ratio (S/N) was calculated by taking the average value of 100% phosphorylation AUC divided by the average value of 0% phosphorylation AUC for each substrate.

Standard curves for terbium time-resolved assay. Synthetic phosphorylated and unphosphorylated peptide were added in ratios of 0, 25, 50, 75, and 100% phosphorylation with a final concentration of 20 μ M peptide. Procedure and buffer solutions used were the same as for the kinase assay, minus the addition of the kinase itself.

Chapter 3. Evaluation of the substrate preferences of the TAM family of receptor tyrosine kinases

Naomi Widstrom wrote this chapter in its entirety

3.1. Summary

The TAM family of receptor tyrosine kinases is implicated in a number of different oncogenic signaling pathways. However, to date there are no FDA-approved small molecule inhibitors for the TAM kinases. Inhibitor design and screening rely on tools to study kinase activity. To address this gap, we designed a set of synthetic peptide substrates for each of the TAM family members: Tyro3, Axl and Mer. We used an in vitro phosphoproteomics workflow to determine the substrate profile of each TAM kinase and input the identified substrates into our data processing pipeline, KINATEST-ID. This pipeline produces a position-specific scoring matrix for each target kinase and a list of candidate synthetic peptide substrates. We designed and characterized synthetic substrates for each TAM kinase, and using in vitro kinase assays, demonstrated they could be used to measure kinase activity. Additionally, we used an LC-MS-based approach to systematically examine the initial phosphorylation rate of all the synthetic substrates with each TAM kinase. This revealed common trends in phosphorylation between Axl and Mer, suggesting similarities in these kinase substrate preferences compared to Tyro3. Follow up experiments reveal a Tyro3 synthetic substrate specific to Tyro3 over the other TAM kinases. This work establishes a set of synthetic substrates that can be used to probe TAM family activity with in vitro kinase assays.

3.2. Introduction

The TAM family of receptor tyrosine kinases consists of three members: Tyro3, Axl, and Mer. They are expressed across of wide range of different tissue types, with their function best studied in immune cells.^{134, 135} The TAM receptors are activated by growth-arrest specific gene 6 (Gas6) and Protein S, among other ligands. The main roles of the TAM

family kinases are as mediators of immunosuppressive signaling and the phagocytosis of apoptotic cells, termed efferocytosis. Despite the similarities, these kinases have non-redundant roles in efferocytosis and different expression patterns across innate immune cells.^{136, 137}

Although the TAM family kinases have not traditionally been studied as mediators of cancer, an overwhelming amount of evidence suggests that they play a key role in supporting cancer progression. The TAM receptors are overexpressed or ectopically expressed in a large variety of solid and hematological cancers, and their expression has been correlated to different aspects of the varying cancers, such as decreased survival, metastasis, and chemoresistance.¹³⁸ While their specific role often varies depending on the cellular context, the TAM receptors are typically associated with pro-survival signaling, invasion, and migration. Axl has been implicated in metastasis, migration, and invasion in a number of different cancers.^{139, 140} Mer activity has also been tied to motility and survival signaling under varying contexts.¹⁴¹⁻¹⁴⁴ In tumor types where multiple TAM receptors are expressed, they may have both overlapping and non-overlapping roles, suggesting independent mechanisms supporting cancer survival and growth.¹⁴⁵⁻¹⁴⁷ Studies on the individual role of Tyro3 are fewer compared to Axl and Mer, but Tyro3 remains an important target in various cancers.¹⁴⁸⁻¹⁵² The TAM kinases are often treated interchangeably, but these studies reveal subtle differences in signaling leading to differential downstream effects. Additionally, due to the physiological role the TAM family plays in immune suppression, their expression in innate immune cells within the tumor microenvironment (TME) may help cancers grow and evade immune detection.¹⁵³ Therefore, even when not expressed within cancer cells, TAM activity can

still aid cancer growth. Preliminary studies on combination therapies suggest dual inhibition of TAM kinases and checkpoint blockade therapeutics may increase efficacy of treatments.¹⁵⁴⁻¹⁵⁷

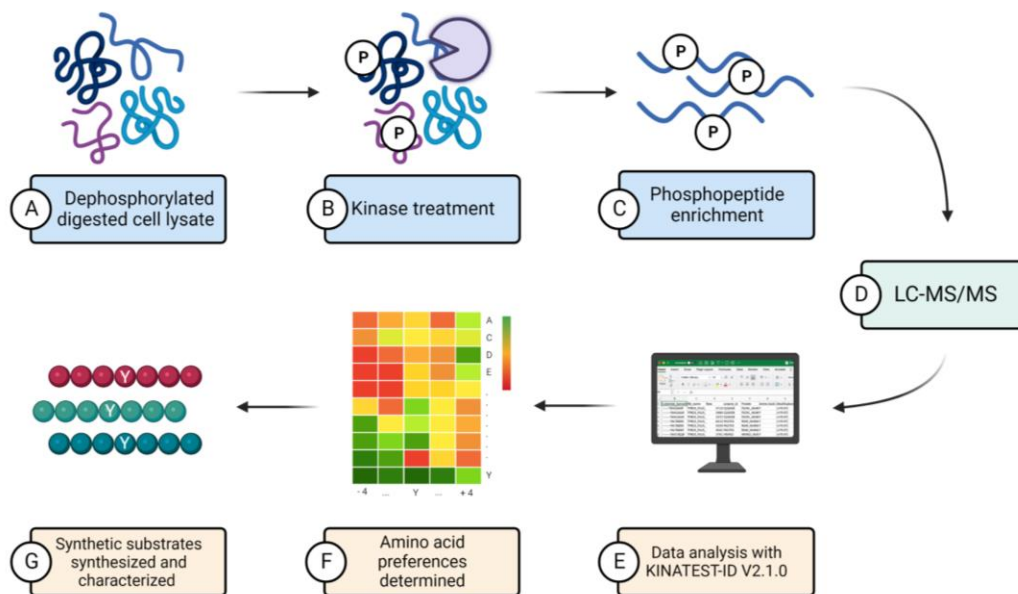


Figure 3.1. Schematic overview of workflow. A.) Dephosphorylated digested cell lysate used as a natural peptide library treated with B.) recombinant kinase (Tyro3, Axl, or Mer) for 2 hours and C.) subsequent phosphopeptides enriched and D.) identified via LC-MS/MS. E.) Identified peptides processed and input into R-package KINATEST-ID, which F.) determines amino acid preferences used to generate an in-silico library of putative substrates. G.) Synthetic substrates are chosen and synthesized. Created with BioRender.com

Despite these studies, much about the physiological and oncogenic function of the TAM family kinases remains unknown. To aid the study of these kinases and inhibitor screening efforts, we designed synthetic peptide substrates capable of providing a readout of TAM family kinase activity for in vitro kinase assays. Using a previously established phosphoproteomics workflow, we determined the substrate profile for each TAM family kinase and used our KINATEST-ID V2.1.0 R-package to analyze the amino acid preferences of each kinase. This allowed us to design a set of synthetic peptide substrates for each kinase and evaluate the in vitro amino acid preferences of the TAM family

(Figure 3.1). We further analyzed the substrate preferences of the TAM family by testing a panel of 15 synthetic substrates against each kinase and found differential preferences of the three kinases.

3.3. Results

Despite the advances made in elucidating the function of TAM family kinases in both physiologically healthy and oncogenic contexts, the total number of known TAM family substrates remains low, with a total of 26 known substrate between all three kinases.¹⁵⁸ Previously, we have successfully used a modified phosphoproteomics workflow to determine the substrate profile of kinases and design synthetic peptide substrates for each target kinase.^{131, 159} To determine the substrates of each TAM family kinase, dephosphorylated digested cell lysate was used as a natural peptide library, which was reacted with recombinant kinase. Following phosphopeptide enrichment, samples were identified on an Orbitrap Velos mass spectrometer. The identified phosphopeptides were used as input into our KINATEST-ID 2.1.0 pipeline.¹⁵⁹ In brief, KINATEST-ID creates a Fisher Odds-based positional scoring matrix for each amino acid at positions surrounding a central phosphorylation site. To ensure that the most robustly observed peptides were used for these calculations, the phosphopeptides identified in each of three replicates per kinase were filtered so that only peptides found in all three replicates were included, while removing any sequences found in the control reactions. We then used the *Generator* function of KINATEST-ID to permute the preference motifs and create candidate synthetic substrate sequences for each TAM family kinase. Each sequence produced is scored for predicted activity against the kinase of interest, as well as a panel of off-target kinases to predict potential specificity (*Screeener* function).

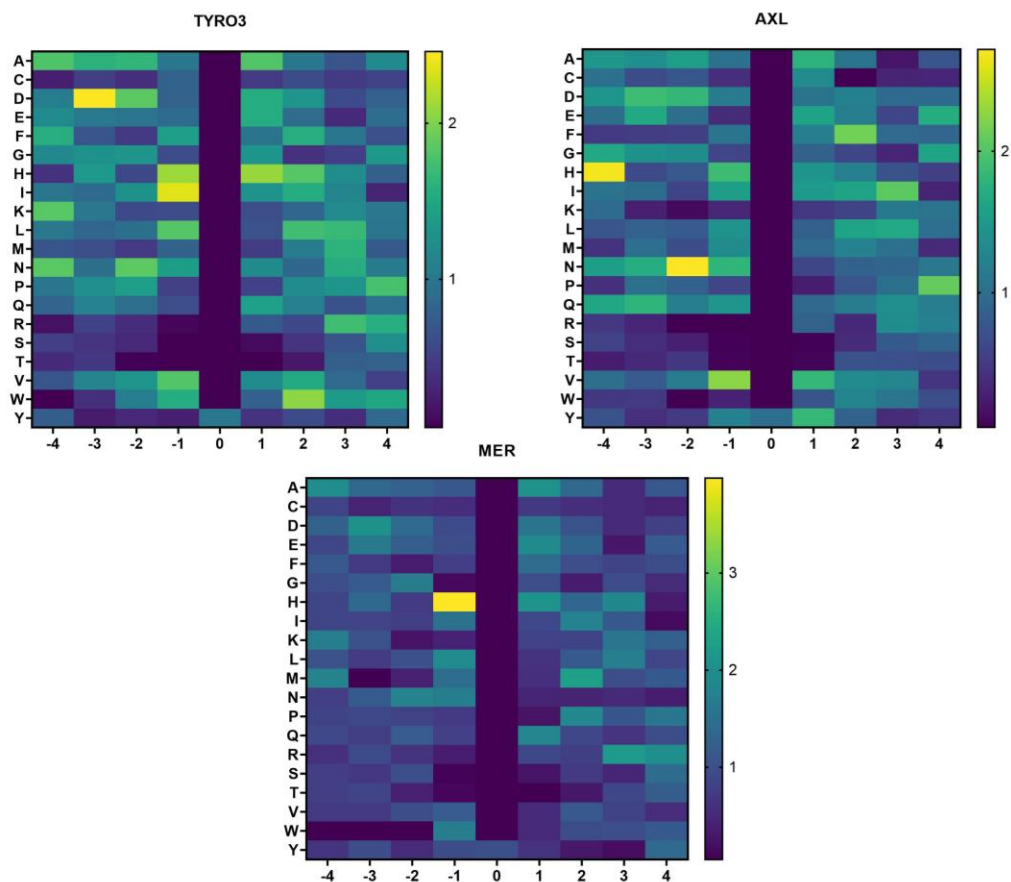


Figure 3.2. Heatmaps of Fisher Odds values for Tyro3, Axl, and Mer. Fisher Odds values above 1 indicate overrepresentation of the amino acid at the position, and values below 1 indicate an underrepresentation.

While the TAM kinases show overlapping substrate profiles based on this *in vitro* phosphorylation experiment, they each have unique substrates identified as well. Comparison of the amino acid preferences of the three TAM kinases reveals both common and unique amino acid preferences (Figure 3.2). Specifically, all three kinases prefer aspartic acid (D) at -3, asparagine (N) at -2, histidine (H) at -1, alanine (A) and glutamic acid (E) at +1, and leucine (L) at +3, relative to the central tyrosine. Each kinase also has unique amino acids preferred at positions as well, suggesting substrates can be designed that could be preferentially phosphorylated by an individual TAM kinase.

We designed fifteen synthetic substrates in total, six based on the observed Tyro3 preference motif, four on the Axl motif, and five on the Mer motif (Table 3.1). Each substrate was synthesized with biotinylated lysine on the C-terminus, flanked by glycine spacers, to allow for affinity capture of the peptides. Although the candidate substrates were ultimately scored from -4 to +4, some of the substrate candidates have amino acids extended beyond to -7 to +7, based on preliminary data that examined amino acid preferences in a larger frame around the central tyrosine.

Table 3.1 Synthetic substrate (syn.) sequences and scores. Bold text indicates portion of peptide used to score each substrate. Underlined scores indicate score is above threshold for target kinase. B denotes biotinylated lysine.

Synthetic substrate name	Sequence	Tyro3 score	Axl score	Mer score
Tyro3 syn. A	EGLYHHRNHPGGBGG	<u>89</u>	<u>90</u>	<u>90</u>
Tyro3 syn. B	HTIYHHKNHPGGBGG	<u>68</u>	77	74
Tyro3 syn. C	HQNYDHKNHPGGBGG	<u>81</u>	81	<u>80</u>
Tyro3 syn. D	HQNYTHKNPRGGBGG	68	65	54
Tyro3 syn. E	HGHYGHNPHPGGBGG	<u>86</u>	<u>80</u>	<u>83</u>
Tyro3 syn. F	HQNYTHKNPPGGBGG	58	65	54
Axl syn. A	NDENNYYYRGGRRGGBGG	73	<u>100</u>	75
Axl syn. B	NDENNYAFRRGRRGGBGG	<u>90</u>	<u>105</u>	<u>93</u>
Axl syn. C	NDENNYYYRGGRRGGBGG	61	<u>97</u>	70
Axl syn. D	NDENYYYYTGRRGGBGG	56	<u>93</u>	63
Mer syn. A	NEGKHGHYAILKDDRGGGBGG	<u>93</u>	<u>89</u>	<u>109</u>
Mer syn. B	NEGKHGFYDARKDDKGGGBGG	<u>86</u>	79	<u>94</u>
Mer syn. C	NFAKHGFYDARRADRRGGGBGG	<u>89</u>	80	<u>98</u>
Mer syn. D	NFGEEGFYAARKEDKGGGBGG	<u>83</u>	<u>88</u>	<u>93</u>
Mer syn. E	DHGHYAILPGGBGG	<u>93</u>	<u>96</u>	<u>108</u>

Characterization of Tyro3, Axl, and Mer synthetic substrates.

The Tyro3 synthetic substrates (syn.) were designed with an aim for specificity over efficiency of phosphorylation. We included substrates with lower scores, below predicted threshold of activity, that had higher predicted specificity (i.e., would favor Tyro3 over the others despite being less efficient substrates). To test the putative Tyro3 substrates, we performed an in vitro kinase assay with recombinant Tyro3, quenching aliquots at time points to monitor reaction progress across 60 minutes (Figure 3.3A) We used an ELISA-based approach with anti-phosphotyrosine antibody 4G10 to detect phosphorylation of the peptides. We found Tyro3 syn. A had the highest rate of phosphorylation and overall signal compared to the other substrates. Of the other substrates, Tyro3 syn. D and E showed moderate signal by 60 minutes, B and F low signal, while syn. C remained unphosphorylated.

The four Axl synthetic substrates were tested with an in vitro kinase assay across a 60-minute time course (Figure 3.3B). Compared to either Tyro3 or Mer, the Axl amino acid preferences indicated additional tyrosine residues near the central phosphosite were preferred. This adds an additional caveat onto data analysis, as different tyrosine sites could be phosphorylated. With the ELISA-based readout method, phosphorylation of different tyrosine sites is indistinguishable, as the anti-pY antibody used will bind to any pY sites present. Of the substrates, Axl syn. C and D both had rapid initial signal, while syn. A and B had much slower initial phosphorylation but did have signal by 60 minutes.

For Mer, four initial substrates were designed based on the KINATEST-ID results while the fifth substrate (Mer syn. E) is a modified shorter substrate adjusted to be similar to universal peptide 5 (U5)¹⁶⁰, which was observed to be very rapidly phosphorylated by

Mer. The in vitro Mer kinase assay shows Mer syn. E and A had very rapid initial phosphorylation, syn. B with very minimal signal after 60 mins, and the rest with moderate to high phosphorylation by Mer (Figure 3.3C).

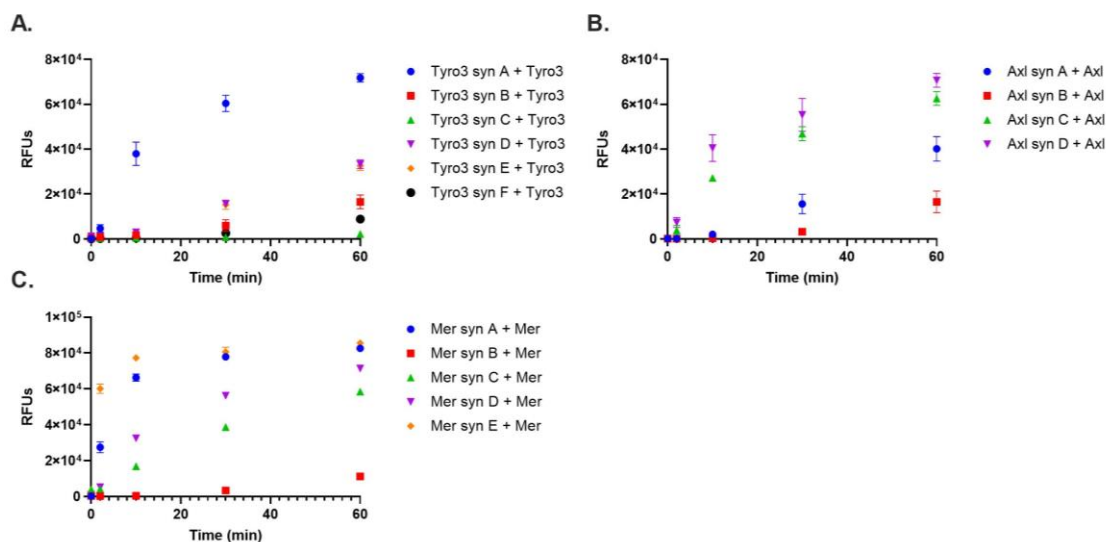


Figure 3.3. TAM kinase reaction progress curves. In vitro kinase reactions for phosphorylation of A.) Tyro3 synthetic substrates in the presence of recombinant Tyro3, B.) Axl synthetic substrates in the presence of recombinant Axl, and C.) Mer synthetic substrates in the presence of recombinant Mer. Aliquots of each mixture quenched 1:1 in EDTA at timepoints 2, 10, 30, and 60 minutes. The zero-time point was quenched prior to addition of substrate. Data points are the average of 6 replicates (A, B) or 3 replicates (C) \pm SD. RFU, relative fluorescent units.

Evaluation of TAM family specificity of the synthetic substrates. Next, we evaluated off-target phosphorylation by the TAM kinases of each of the substrates to gain insight into the TAM specificity of each substrate, and into the individual substrate preferences of each TAM family member. To do this, we performed a one-pot reaction with all 15 synthetic substrates, which were incubated with either Tyro3, Axl, or Mer. Aliquots from the reaction were quenched at 0, 1, 3, 5, 10, and 20 minutes and the product formation of each substrate was monitored with LC-MS. To compare the substrates, the slope of the timepoints was taken as a measurement of phosphorylation efficiency and plotted in a

heatmap (Figure 3.4). In general, the reactions of each TAM kinase plus the matching substrates (e.g., Tyro3 kinase plus Tyro3 substrates) recapitulate earlier results.

For the Tyro3 substrates, Tyro3 syn. A had the highest rate of phosphorylation by Tyro3, and Tyro3 syn. D and E with lower levels of phosphorylation, similar to previous results. Off-target reactions show that Mer also phosphorylates Tyro3 syn. A, and more notably phosphorylates Tyro3 syn. E at a higher rate than Tyro3. Axl also phosphorylated Tyro3 syn. E, to a comparable level as the Tyro3 reaction, but the rest of the substrates seemed to be poor substrates for Axl (Figure 3.4A).

The multiple tyrosine residues in Axl syn. A, C and D added complexity to analysis as there is the possibility of any of the three (Axl syn. A) or four (Axl syn. C and D) tyrosine residues being phosphorylated. We differentiated these products, which have the same mass, based on retention time. Axl syn. A only has one major product, as the other possible products had very low to non-detectable background signal. Axl syn. C has both a distinguishable major and minor product, while Axl syn. D has two major products, suggesting two different tyrosines were phosphorylated roughly equally. We also observed very minor double phosphorylation of syn. C and D but not triple or quadruple phosphorylation. Overall, we found the Axl substrates were phosphorylated at a higher rate by Tyro3 or Mer compared to Axl (Figure 3.4B). However, it's possible that the Axl enzyme is overall less active than Tyro3 or Mer under these conditions. Of the Axl substrates, Axl syn. D had the highest rate of phosphorylation by Axl, followed by Axl syn. C. Tyro3 phosphorylated Axl syn. C at the highest rate, and with a higher rate than any of the Tyro3 substrates. Tyro3 also phosphorylated both Axl syn. D products fairly equally. Interestingly, while Mer also phosphorylated both Axl syn. D products,

one of the sites (major product 2) was apparently phosphorylated more efficiently than the other site (major product 1). Overall, none of these substrates appear to be specific for Axl, although Axl syn. A has potential for Tyro3 specificity.

Of the Mer synthetic substrates, Mer syn. E had the highest rate of phosphorylation by all three kinases (Figure 3.4C). Although Mer syn. E lacks specificity, it still shows a rapid initial rate of phosphorylation very useful for in vitro kinase assays or TAM family screening efforts. Mer syn. A is also phosphorylated well by Mer, and minorly phosphorylated by Axl. At these timepoints, none of the rest of the substrates demonstrate significant phosphorylation.

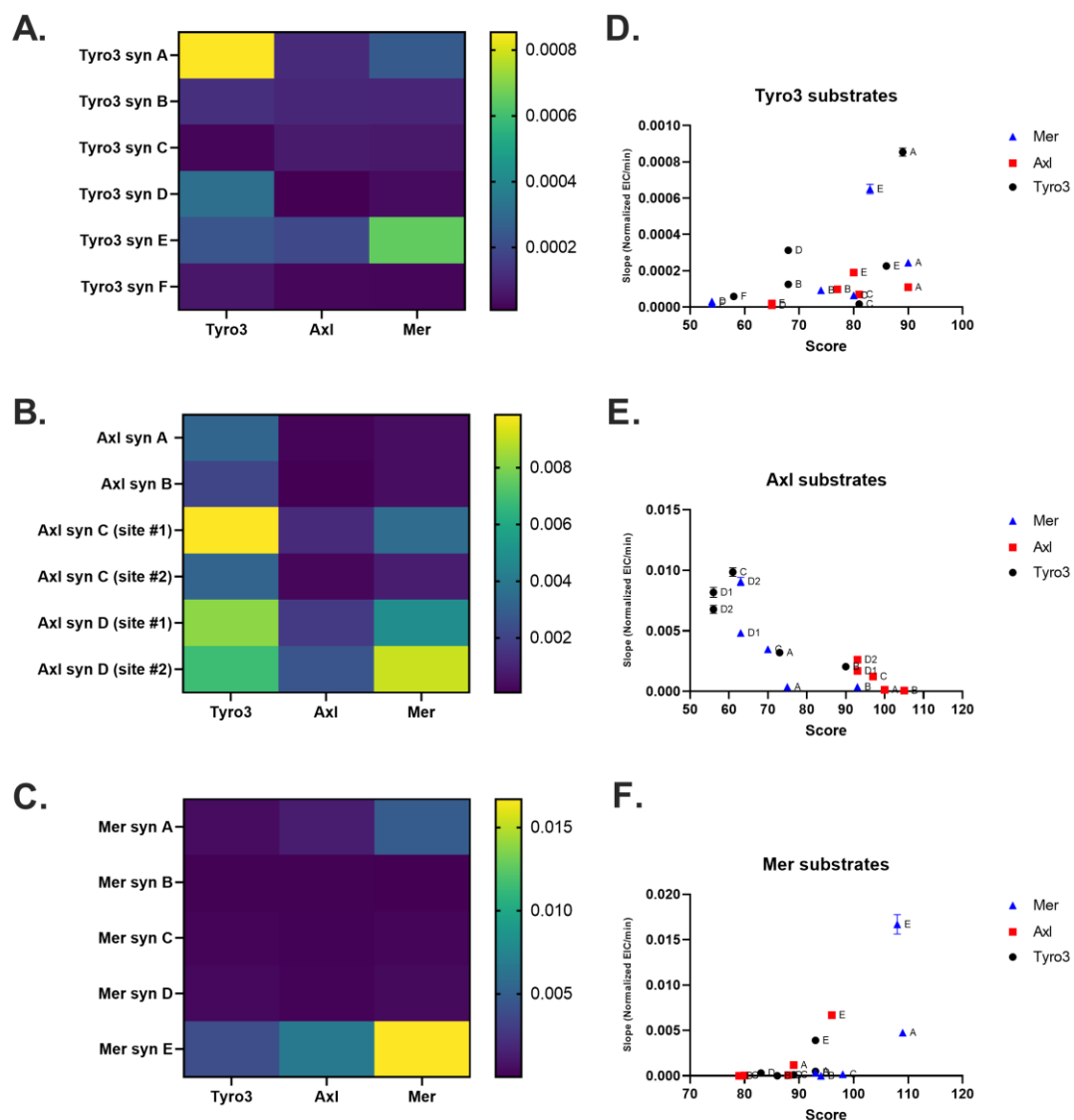


Figure 3.4. Initial phosphorylation rates of TAM synthetic substrates by Tyro3, Axl, or Mer. In vitro kinase assay with the 15 synthetic substrates in a one-pot reaction with either recombinant Tyro3, Axl, or Mer. Timepoints were collected at 0, 1, 3, 5, 10, and 20 minutes in triplicate and analyzed with LC-MS. Product EIC (P) was normalized with substrate EIC (S) and values used to calculate initial rate of phosphorylation for each combination of substrate and kinase. Major and minor products of Axl substrates represent different single phosphorylation sites, identified by differences in LC-MS retention time. Heatmaps show initial rate of phosphorylation signal by Tyro3, Axl, or Mer and A.) Tyro3 synthetic substrates, B.) Axl synthetic substrates and C.) Mer synthetic substrates. Each KINATEST-ID score plotted against initial rate of phosphorylation for reaction with recombinant Tyro3, Axl, and Mer for D.) Tyro3 synthetic substrates, E.) Axl synthetic substrates, and F.) Mer synthetic substrates. Data represented as slope \pm standard error.

Comparing all three sets of substrates, the phosphorylation rate trends by Mer and Axl appear to match, although Axl phosphorylation is consistently lower. This may suggest that Axl itself is less active under these conditions compared to Mer. Considering the amino acid preferences determined by KINATEST-ID, the Axl substrate sequences contained multiple positions with amino acids favored by all three TAM kinases. This may have contributed to the non-specific nature of these synthetic substrates. In contrast, the Tyro3 synthetic substrates sequences contained fewer pan-TAM favored amino acids, instead including amino acids favored only by Tyro3 at specific positions. This may have helped the specificity of these substrates. However, the Tyro3 substrates are all phosphorylated at a lower rate compared to the other substrates. These substrates were designed with specificity in mind, which may have been a tradeoff with efficiency. This comparison of 15 different substrates reveals subtle differences in the substrate preferences of the three highly related kinases. Mer and Axl appear to match more closely in their trends, while Tyro3 has a distinct pattern of phosphorylation.

Next, we evaluated the correlation between slope of the phosphorylation signal and each sequence's KINATEST-ID score (Table 3.1). Both the Tyro3 (Figure 3.4D) and Mer (Figure 3.4F) synthetic substrates had a significant positive correlation between substrate score and performance. However, we did not observe a correlation between score and initial slope for the Axl synthetic substrates (Figure 3.4E). It is possible due to the multiple tyrosine residues present on these substrates, the KINATEST-ID score does not accurately represent the major phosphosite, resulting in an inaccurate score.

Evaluation of Tyro3 synthetic substrate specificity. To further evaluate substrate specificity, Tyro3 syn. A and Tyro3 syn. B were chosen to screen against additional

kinases. Of the Tyro3 substrates, Tyro3 syn. A had the highest rate of phosphorylation by Tyro3, and B was predicted to be specific, although it had a much lower rate of phosphorylation by Tyro3. First, the off-target TAM family results from Figure 3.4A were validated by performing in vitro kinase reaction with each substrate and kinase separately. The substrates were incubated with Tyro3, Axl, or Mer and three timepoints taken across a 30-minute time course with an ELISA-based readout of phosphorylation (Figure 3.5A, B). Tyro3 syn A was rapidly phosphorylated by Tyro3 as expected, with minor signal from Mer by 30 minutes. While Tyro3 syn. B had lower signal all around, by 30 minutes, Tyro3 syn. B showed phosphorylation by Tyro3, but no phosphorylation by Axl or Mer. Despite the lower efficiency of phosphorylation, Tyro3 syn B is specific to Tyro3 over the other TAM family members.

Then, to examine non-TAM family member specificity, Tyro3 syn. A and B were each incubated with Abl, Hck, Src, or Syk and three timepoints taken across a 30-minute time course. Tyro3 substrate A shows moderate phosphorylation by Hck and low phosphorylation by Abl (Figure 3.5C). Tyro3 syn. B, which has a much lower signal, is not phosphorylated by any of the tested kinases (Figure 3.5D). Activity of each kinase was verified by using universal substrate 5 (U5) as a control. This data shows that Tyro3 syn. A may have some off-target phosphorylation by other kinases but Tyro3 syn B has potential to be specific for Tyro3 above other tyrosine kinases.

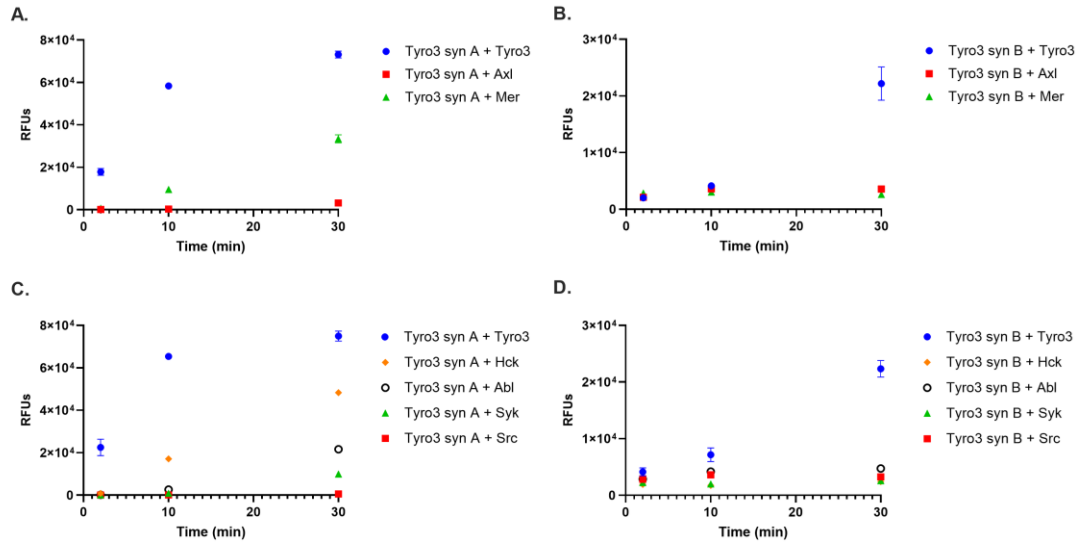


Figure 3.5. Off-target phosphorylation of Tyro3 synthetic substrates A and B. In vitro kinase reaction in presence of recombinant Tyro3, Axl, or Mer and A.) Tyro3 synthetic substrate A or B.) Tyro3 synthetic substrate B. Kinase reaction with Tyro3, Hck, Abl, Syk, or Src and C.) Tyro3 synthetic substrate A or D.) Tyro3 synthetic substrate B. Aliquots of each mixture quenched 1:1 in EDTA at timepoints 2, 10, and 30 minutes. Data points are the average of 3 replicates \pm SD. RFU, relative fluorescent units.

3.4. Discussion.

The TAM family of kinases are implicated in several different cancers, typically for roles in migration, metastasis, and chemoresistance. As regulators of immune suppression, overactivation of the TAM receptors may contribute to an immunosuppressive tumor microenvironment, which aids tumor growth and progression. Their activity may also prevent infiltrating immune cells from initiating immune destruction of tumor cells.¹⁵³ TAM inhibitors therefore may be effective at both treating tumor progression and in combination with other therapeutics to increase efficacy of treatments. However, to date there are no FDA-approved TAM kinase small molecule inhibitors. Additionally, despite the interest in the TAM family for cancer related activity, few substrates of each kinase

are known, adding difficulty to studying the activity of the kinases for in vitro kinase assays or inhibitor screening assays.

To develop tools to improve study of the TAM kinases, we employed an established workflow to determine the substrate profile of Tyro3, Axl, and Mer, and design synthetic peptide substrates to examine their substrate preferences in vitro. We used a modified phosphoproteomics workflow based on the Kinase Assay Linked with Phosphoproteomics (KALIP) method, where a natural peptide library is treated with the target kinase and subsequent phosphopeptides identified with LC-MS/MS.¹¹¹ The natural peptide library is obtained by lysing and digesting cell lysate, resulting in an array of peptides for phosphorylation. While the natural peptide library is relatively inexpensive and easy to create, identified substrates might not be physiologically relevant, as localization, regulation, and structure of all proteins is lost in the lysing and digestion process. However, this is a robust strategy for identifying the amino acid preferences of a target kinase.

Following substrate identification for Tyro3, Axl, and Mer, we used our KINATEST-ID pipeline to analyze the data and determine the amino acid preferences of each kinase. These identified residues were permuted to create an in-silico library of candidate sequences, scored, and screened against an in-silico panel of other kinases. Using this information, we designed a total of 15 synthetic substrates: six targeted for Tyro3, four for Axl, and five for Mer.

Initial in vitro kinase assays showed differential phosphorylation rates for each of the substrates with their target kinase, overall demonstrating successful creation of synthetic substrates for each TAM kinase. To further examine the substrate preferences

of the TAM kinases, we performed a one-pot reaction where all 15 synthetic substrates were tested in combination in an in vitro kinase reaction with Tyro3, Axl, or Mer. This profile revealed similar patterns of phosphorylation for Axl and Mer compared to Tyro3, although Axl was less active under the conditions tested. In comparison, Tyro3 showed high phosphorylation rates for several of the substrates designed for Axl. While Tyro3 and Mer both have substrates with the potential for specificity against the other TAM kinases, none of the Axl substrates tested are specific for Axl alone. Of the tested substrates, Mer syn. E shows the highest phosphorylation and could be used as a universal TAM family substrate, although Mer phosphorylation rate is higher than the rates by Axl and Tyro3. In contrast, further testing of Tyro3 syn. B shows phosphorylation only by Tyro3 and none of the other kinases tested. Additional in-depth specificity testing would be needed to characterize Tyro3 syn B as a specific substrate and would be required prior to use in a cellular environment.

This process demonstrates the difficulty in designing specific substrates and highlights a potential trade-off between efficiency and specificity. One difficulty is the lack of known substrates for other kinases to compare the putative sequences against. As more substrates are identified for a larger range of kinases, it may become easier to select sequences more likely to be specific. However, due to the conserved nature of kinases, especially between family members, total substrate specificity may not be achievable for every kinase. On top of substrate binding, the active site of a kinase must allow for coordination of ATP and Mg^{2+} , and active orientation of the activation loop and αC helix. As a result, kinase active sites are very similar, limiting the varieties of substrate that can bind.

Overall, we successfully designed a set of synthetic peptide substrates that can be used to probe the activity of the TAM family kinases using in vitro kinase assays. We further delineated the differences between Tyro3, Axl, and Mer by demonstrating distinct amino acid preferences and by providing direct comparison of synthetic substrate preferences. Our work shows similar trends for Axl and Mer synthetic substrate phosphorylation, while we were able to design Tyro3 synthetic substrates specific for Tyro3 over Axl and Mer. This work provides a set of tools to further study the TAM kinases in vitro, which could be applied towards inhibitor screening efforts and provides evidence for differences in the TAM family kinase substrate preferences.

3.5. Materials and Methods

Cell culture and phosphoproteomics sample preparation. K562 (ATCC) cells were grown and maintained in IMDM (Gibco) supplemented with 10% fetal bovine serum (FBS), 1% penicillin/streptomycin in 5% CO₂ at 37°C. Cells were washed in phosphate buffered saline (PBS) and resuspended in lysis buffer (7M urea, 2M thiourea, 0.4 M Tris pH 7.5, 20% acetonitrile, 4 mM tris(2-carboxyethyl)phosphine (TCEP), 5 mM ethylenediaminetetraacetic acid (EDTA), 10 mM sodium fluoride, 1 mM solidum orthovanadate, 1X Halt Phosphatase inhibitor cocktail (ThermoFisher)). Lysed cells were probe sonicated and alkylated by adding chloroacetamide (Sigma-Aldrich) to a final concentration of 8mM. Samples were centrifuged at 15,000 x g at 4 °C for 15 minutes to remove insoluble cellular debris. For lysate digestion, samples were diluted 5-fold by adding 25 mM Tris, pH 8.0 and trypsin protease (ThermoFisher) added in a 1:40 ratio and incubated overnight at 37°C. Digestion was quenched by adding an equal volume of 0.6% trifluoroacetic acid (TFA) to solution and samples desalted with Oasis HLB 1 cc

Vac Cartridge (Waters). To remove endogenous phosphate groups, samples were reconstituted in phosphatase reaction buffer (50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 100 mM NaCl, 2 mM Dithiothreitol (DTT), 1 mM MnCl₂, pH 7.5) and 4000 U lambda protein phosphatase (New England Biolabs) added per 1 mg digested lysate. Samples incubated at 30°C overnight and deactivated by heating at 65°C for one hour. Each sample was equally split in two, one half to be used as the (+) kinase treatment and the other as the (-) kinase control. Kinase reaction buffer added to each sample (final concentrations of 50 mM Tris pH 7.5, 10 mM MgCl₂, 1 mM DTT, 1 mM Na₃VO₄, 2 mM ATP) and 2 ug of recombinant kinase (Tyro3, Axl, or c-Mer, Signal Chem) added to the (+) samples. The (-) sample had buffer only added in place of kinase. All samples incubated at 37°C for 2 hours, and the reaction quenched by adding 0.5% TFA by reaction volume. The samples were desalted as previously described and vacuum dried. Phosphopeptide enrichment was carried out according to manufacturer's instructions. Samples were enriched first using High-Select™ TiO₂ Phosphopeptide Enrichment Kit (ThermoFisher), and the flowthrough and wash fractions retained, dried, and enriched using the High-Select™ Fe-NTA Phosphopeptide Enrichment Kit (ThermoFisher).

LC-MS/MS data acquisition. Samples were reconstituted in 0.1% formic acid (FA) in H₂O and loaded onto a Dionex UltiMate 3000 RSICnano system and run over a linear gradient (5-30% acetonitrile; 65 minutes) with a flow rate of 300 nL/min into LTQ Orbitrap Velos Mass Spectrometer (ThermoFisher). The MS was operated using data dependent mode with a resolution of 30,000 with a scan range of 380 – 1800 m/z.

MS/MS triggered for the top six abundant ions using high collision dissociation (HCD) and mass analyzer parameters set between 2 and 7 charge states.

Data processing. Data was processed as previously described.¹⁵⁹ In brief, peptides were identified using PEAKS Studio Xpro (Bioinformatics Solutions Inc.) and exported protein-peptides.csv lists were used for input into the PEAKSModExtractor, which combines all the input lists into a concatenated output file, containing a modification-centered list of all peptides.¹³³ Table 3.2 shows a summary of the total number of unique modified peptides and phosphorylated peptides with A-score ≥ 30 identified for each kinase and replicate.

Table 3.2. Unique peptide summary for each TAM kinase.

Sample	Replicate	Unique Peptides	pY peptides (Ascore ≥ 30)
Tyro3 No kinase control	1	2634	370
	2	789	141
	3	1082	108
Tyro3 Kinase reaction	1	4635	1482
	2	3639	1315
	3	3527	1221
Axl No kinase control	1	822	123
	2	1044	135
	3	3489	1088
Axl Kinase reaction	1	4754	1551
	2	4715	1633
	3	3347	1151
Mer No kinase control	1	668	60
	2	651	85
	3	789	84
Mer Kinase reaction	1	1053	453
	2	1113	449
	3	1068	447

This data was input into KINATEST-ID V2.1.0. and filtered to include only nonredundant substrates found in all three kinase reaction replicates for each kinase, but

not in any of the controls (Tyro3: n = 425; Axl: n = 270; Mer: n = 252). These filtered substrates were used as input to generate the Fisher Odds position-specific scoring matrix (PSSM) for each kinase. The candidate amino acids at each position were then permuted to generate the in-silico library of putative substrates. Each peptide sequence was given a score, calculated based on the odds ratio of each amino acid in the sequence. For each kinase, ROC analysis was performed using the input peptides lists to establish a threshold value for predicted peptide activity. Sequences with scores above this value are predicted to be substrates for the target kinases. Final synthetic substrates were scored and normalized as previously described.¹⁵⁹

Peptide synthesis and purification. Peptides were synthesized as previously described.¹⁵⁹ In brief, Symphony X Peptide Synthesizer (Protein Technologies) was used to synthesize peptides with an activation solution of HCTU in N-methylmorpholine (NMM) and dimethylformamide (DMF). 20% piperidine in DMF was used to deprotect Fmoc groups, and a cleavage cocktail of 94% trifluoroacetic acid (TFA), 2.5% H₂O, 2.5% ethane dithiol and 1% Triisopropylsilane (TIS) used to cleave peptides from resin. Reverse phase HPLC (Agilent 1200 Series Infinity LCMS) was used to purify peptides to >90% purity.

In vitro synthetic substrate assays. Recombinant Tyro3, Axl, or Mer (SignalChem; Tyro3 (aa 455-end): T22-11G, Axl (aa 473-end): A34-11G, Mer (aa 528-end): M51-112G) was incubated with reaction mixture (25 mM HEPES pH 7.5, 10 mM MgCl₂, 100 μM ATP, 1 μM Na₃VO₄ and 0.05 μg/μL BSA) to a final concentration of 10 nM for 15 minutes at room temperature, and the reaction started by adding peptide substrate to a final concentration of 20 μM. Timepoints were taken by quenching sample aliquots 1:1 in

EDTA. The zero-minute timepoints were taken by adding substrate to pre-quenched kinase mixture. Quenched sample aliquots were incubated for 1 hour in streptavidin coated plates (ThermoFisher Scientific) in Tris-buffered saline with 0.05% Tween-20 (TBS-T) with 5% w/v milk. Following sample incubation, the wells were washed with TBS-T and incubated with 4G10 Anti-Phosphotyrosine Antibody horseradish peroxidase conjugate (Millipore Sigma; 1:5000 dilution in TBS-T 5% milk) for 1 hour. A solution of 0.1 mM Amplex Red (ThermoFisher Scientific) and 2.225 mM hydrogen peroxide in 50 mM sodium phosphate buffer, pH 7.4 was added to the wells and incubated in the dark for 30 minutes. Fluorescent measurements were taken on a Synergy Neo2 plate reader (Biotek) with an excitation wavelength of 532 nm and emission wavelength of 590 nm. Off-target kinase assays were performed as above with 10 nM recombinant kinases Abl, Hck, Syk, and Src (SignalChem; Abl: A03-18H, Hck: H02-11G, Syk: S52-10G, Src: S19-18G).

Synthetic substrate *in vitro* assay with HPLC-MS readout. Reactions (300 μ L total volume) using recombinant kinase (Tyro3, Axl, or Mer) were performed in triplicate at 25°C in kinase reaction buffer (25 mM HEPES pH 7.5, 10 mM MgCl₂, 100 μ M ATP, 1 μ M Na₃VO₄ and 0.05 μ g/ μ L BSA) with a kinase concentration of 10 nM. After incubating for 15 minutes, reactions were initiated with the addition of pooled substrate (final concentration 20 μ M). Reaction aliquots (50 μ L) were withdrawn and quenched with 10 μ L 20% TFA (3.3% TFA in the quenched solution). Sample aliquots (50 μ L) (3) were run on HPLC (Agilent 1200 series) using a C18 Agilent Zorbax column (2.1 x 250mm, 5-micron) at 0.25 mL/min with acetonitrile increasing from 5% to 20% over 90 min. Reaction progress was analyzed via MS (Agilent 6130A) through integration of

extracted ion chromatograms (EIC) corresponding to products (P) and substrates (S)
(Agilent ChemStation) and normalized according to equation:

$$\textit{Normalized EIC} = \frac{\textit{product EIC}}{(\textit{product EIC} + \textit{substrate EIC})}$$

Chapter 4. Discussion

Naomi Widstrom wrote this chapter in its entirety

Protein kinases are critical signaling enzymes, involved in cell growth, survival, motility, and range of other key pathways. When kinases are dysregulated through mutation or other mechanisms, these signaling pathways go awry, contributing to cancer development and progression. Due to this, kinases are widely recognized as crucial targets for cancer therapeutics. Imatinib was the first FDA-approved small molecule kinase inhibitor, approved in 2001 for treatment of chronic myeloid leukemia. Imatinib's success paved the way for a massive industry in kinase drug discovery efforts. However, over 20 years later, approval of small molecule kinase inhibitors has lagged, with just over 70 currently approved. One of the reasons so few inhibitors have made it through the development process to approval is that much of the kinome remains understudied. This issue is augmented by the lack of tools to study kinase activity. Measuring the activity of a kinase can be achieved by a number of different methods, but most require a peptide or protein substrate for the kinase to phosphorylate. With many kinases of interest understudied, effective substrates are often unavailable. To that end, substrate profiling techniques have been used to establish known *in vitro* or physiologically relevant substrate lists for kinases of interest. These substrates can then be used as tools to aid kinase studies and inhibitor screening efforts.

In this work, we optimized a phosphoproteomics workflow to obtain the substrate profile of tyrosine kinases. Applied for BTK, Tyro3, Axl, and Mer, our method uses a natural peptide library, obtained by lysing and digesting cancer cells and performing an *in vitro* kinase reaction with the subsequent library. Following phosphoenrichment, the peptides phosphorylated by the kinase of interest are identified using LC-MS/MS. We have also established an optimized data analysis pipeline, KINATEST-ID V2.1.0, that

integrates the identified substrates of a kinase and calculates the amino acid preferences. These are permuted to generate a library of synthetic peptide substrates, which can be sorted for potential efficiency and specificity. Known physiological substrates could also be used as inputs for this process, but for many kinases this is a short list. Additionally, some of the known substrates are influenced by other cellular factors, such as protein localization, regulatory or chaperone proteins, and other modifications. This means they may not accurately reflect the amino acid preferences of the kinase. While the substrates identified with this method might not all be physiologically relevant, they provide a large list of confirmed substrates that can be used to delineate the amino acid preferences of a kinase.

In Chapter 2, we show a significant correlation between the KINATEST-ID score of a BTK synthetic substrate and its biochemical efficiency, measured as initial rate of phosphorylation. This demonstrates that our pipeline can be used to generate synthetic substrates for kinase activity assays. Additionally, we inserted a terbium-chelation motif into the sequence, expanding the applicability of the substrates for time-resolved terbium luminescence assays. This establishes our BTK synthetic peptide substrates as tools that can be used in several different activity assays, which can aid inhibitor screening efforts.

In Chapter 3, we expand the application of our pipeline to examine the substrate profile differences of a small family of kinases, the TAM receptor tyrosine kinases. Similar to Chapter 2, we demonstrate that the designed synthetic substrates can be used in individual kinase assays to study the activity of each of the TAM kinases. Further examination of the differences in phosphorylation rates of the synthetic substrates by each of the kinases reveals that Mer and Axl have similar patterns, while Tyro3 appears

to have slightly different preferences. This allowed for the Tyro3 synthetic substrates to be specific for Tyro3 over Axl or Mer. The TAM kinases are commonly discussed interchangeably, despite evidence of non-redundant roles. The observed variation in substrate preferences provides further confirmation of distinct substrate profiles and roles for the TAM kinases.

Our pipeline connects substrate discovery with synthetic substrate design, creating an uninterrupted pipeline to create tools for any tyrosine kinase of interest. These processes can be applied towards different types of assays, with an aim to improve inhibitor screening efforts, or towards biological study of kinase families to probe differences in substrate preferences. Overall, the results obtained provide tools that aid efforts to study the full complement of kinases, which in turn promotes inhibitor screening efforts with the goal of expanding the repertoire of kinase inhibitors for cancer treatment.

Chapter 5. References

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