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KINASE CHARACTERIZATION AND HTRF ASSAY DEVELOPMENT

A Major Qualifying Project Report

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Andrew Gagnon
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APPROVED:

Yong Jia, Ph.D. Abbot Bioresearch Center Major Advisor	David Adams, Ph.D. Dept Biology and Biotech WPI Project Advisor

ABSTRACT

Abbott Bioresearch Center strives to characterize specific kinases in order to better understand the processes that take place in cells of the human body. The goal is to develop an inhibiting drug for a kinase pathway associated with a particular target disease. The main focus at Abbott is autoimmune diseases, including rheumatoid arthritis and pro-inflammatory diseases. This MQP details the initial process that each kinase goes through in order to understand compound inhibition under conditions that take into consideration the effects of a physiological environment, and shows the application to one specific kinase, COT. An inhibitor of the kinase was identified that acts in an ATP-competitive manner. These processes allow for screening of thousands of compounds against any kinase that can be isolated and characterized.

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ACKNOWLEDGEMENTS

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BACKGROUND

Introduction

Drug discovery makes advances in very different ways than do other consumer-driven markets. With failure rates totaling in excess of 96%, the amount of resources and time that eventually produces one successful drug to market can span over a decade (Clark, 2003). While negative regulation of pathways may seem far easier than activation, most novel drugs are responsible for the inhibition of an over stimulated pathway. Abbott Bioresearch Center focuses on kinase pathways and inhibition at key points that will reduce or eliminate certain autoimmune and inflammatory responses. With large scale operations at major pharmaceutical companies like Abbott Laboratories, high throughput *in vitro* assays under physiological conditions are necessary to accommodate needs.

The MAP Kinases and COT

It is well known that the majority of autoimmune, inflammatory, and cancerous diseases result from abnormal regulation of mitogen activated protein kinase (MAPK) pathways (Karin, 2004). Since such a large number of diseases correlate to activation and inactivation of these complex pathways, pharmaceutical companies are spending time and money to decipher the relationships between individual kinases and entire signaling events involving tiered structures. *Figure 1* is an edited image from the EMD Biosciences website highlighting the complexity of only a few of the MAPK pathways. Cancer Osaka thyroid (COT), referred to in the figure as Tpl-2 (analog), is a MAP3K residing at the top of the tiered structure. All

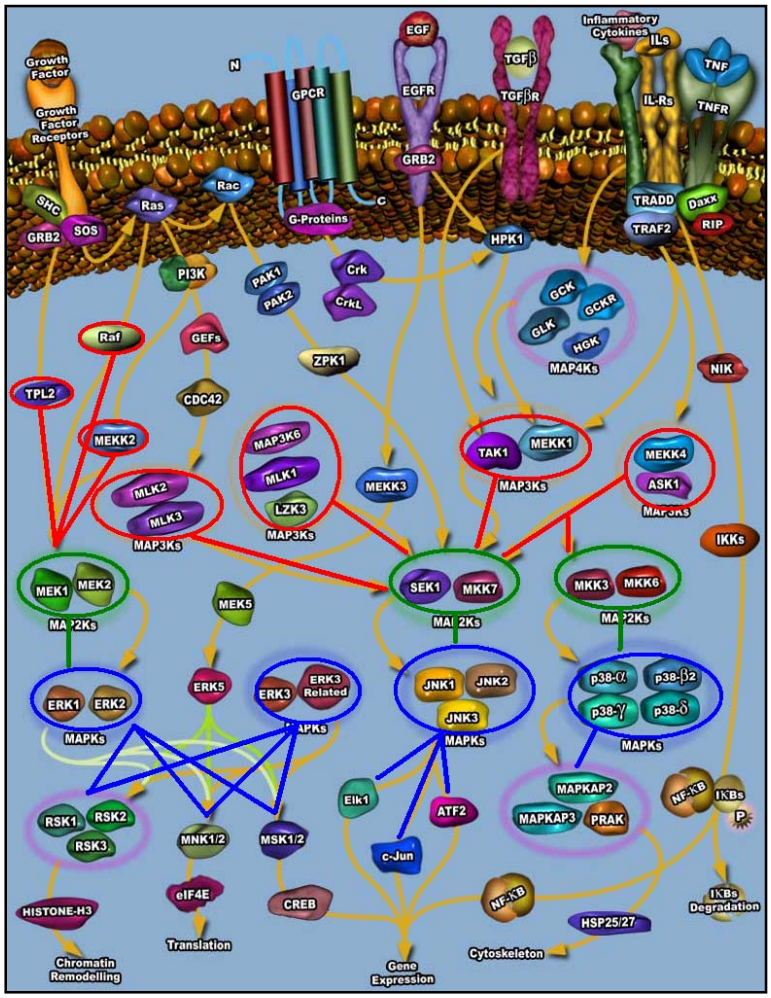


Figure 1: A portion of the kinase signaling pathway; red indicates MAP3Ks, green indicates MAP2Ks, and blue indicates MAPKs (EMD Biosciences website).

MAP3Ks phosphorylate the MAP2Ks on the tier below them at a point in the amino acid sequence near both ATP and substrate binding sites (Clark, 2003). This map makes it clear that cellular signaling pathways are both complicated and finely tuned. Over or under expression at one point can have more than a single resulting effect. The negative regulation of COT is provided through the binding of inhibitor p105. p105, a direct transcription of the gene product from *nfκb1* of the NF-κB family, produces a COT/p105 complex that is inactive and stable within the cell (Waterfield et al, 2003). Activation of

COT requires the dissociation of p105, and consequential degradation; this ensures that the pathway will not remain active indefinitely (Beinke et al, 2003).

COT is itself a MAP3K, showing activity in the COT-MEK-Erk2 cascade (see MAPK tier in *Figure 1*) (Jia et al, 2005). The knockout model in mice, $COT^{-/-}$, shows blocking of the MEK/Erk pathway, an indication of COT playing a vital role in both MEK (MAP2K) and Erk (MAPK) signaling (Jia et al, 2005). The phenotype of the $COT^{-/-}$ knockout mouse is normal, except for the down regulation of pro-inflammatory cytokine production (Jia et al, 2005). For this reason, COT is currently a target for autoimmune disease research.

Fluorescence Resonance Energy Transfer (FRET)

FRET technology provides an accurate interpretation of the signal generated by what are termed the donor and acceptor molecules (*Figure 2*). The simplest design is that of a donor (shown as red) being excited at a specific wavelength that results in an energy transfer to an acceptor (shown as blue).

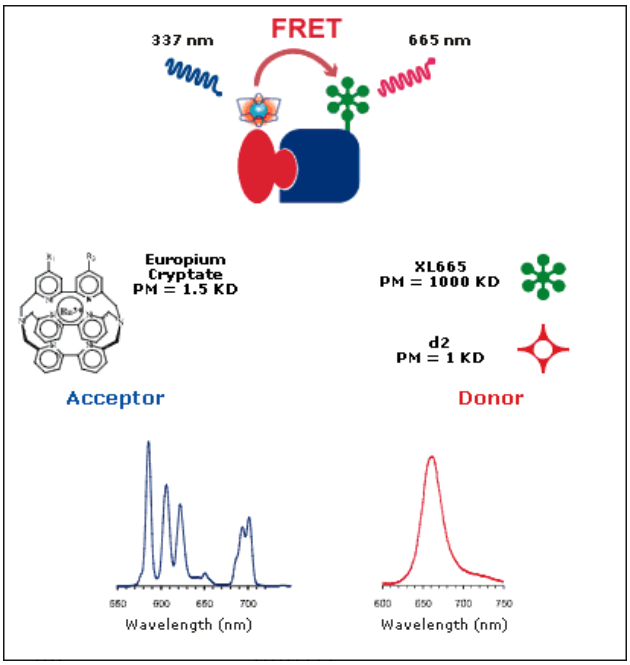


Figure 2: The basic involvement of two molecules in the FRET absorption and emission; since both molecules emit over different wavelength ranges, the interference of Europium cryptate autofluorescence is kept at a minimum (Cisbio, 2002).

FRET will only occur if the donor and acceptor are within a specific proximity, most commonly near 1-7 nm, the range at which energy transfer is 50% efficient (Cisbio, 2002). *Figure 2* illustrates how the process corrects for multiple fluorescing molecules. The Europium cryptate fluoresces at wavelengths (550nm – 630nm and 680nm – 710nm), while the XL665 will fluoresce almost exclusively in the range from 640nm – 680nm (Cisbio, 2002). By emitting over a different range of wavelengths, interference is brought down to a minimum. FRET being the theory of the energy absorption, transfer, and emission, there are many variations that explore various donor-acceptor pairs. The most applicable design for COT involves labeling an antibody donor with Europium cryptate and an acceptor, XL665 (Jia et al, 2005). This optimization of the FRET process was developed and patented by Cisbio International. Termed homogeneous time resolved fluorescence (HTRF), it abolishes some of the major drawbacks of basic FRET (*Figure 3*). Similar to the need to distinguish between emission from the Europium cryptate and

XL665, HTRF emissions readings eliminate short-lived signals from both the media of the experiment and the unbound XL665. This time response property enables a clear isolation and detection of the FRET signal by delaying the reading of emissions, avoiding interference from the solution and individual components of the system (*Figure 3*) (Cisbio, 2002). To account for overall variance in experiments from one to another and day to day, the measurement of 665 nm / 620 nm is used.

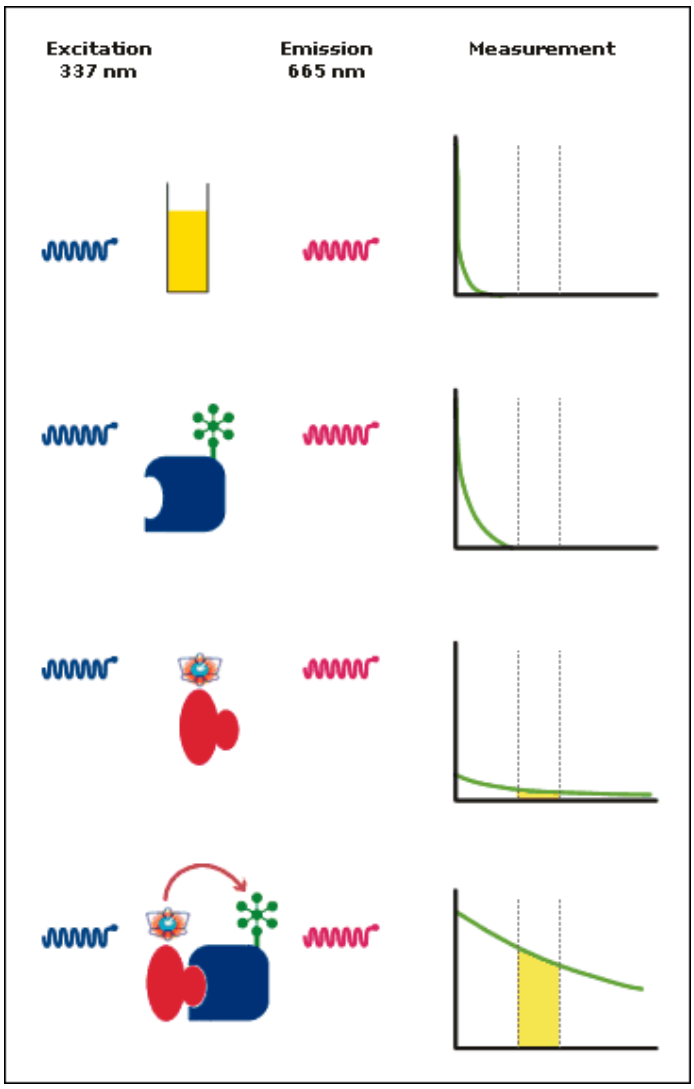


Figure 3: The basics of HTRF. The media and the unbound XL665 both emit short-lived signals that are removed (yellow and blue, respectively). The unbound Europium cryptate emits a long-lived signal that is accounted for in the signal ratio (red). The complexed Europium cryptate and XL665 emit a long-lived, extremely strong signal (Cisbio, 2002).

Homogeneous Time Resolved Fluorescence (HTRF) for COT

As already mentioned, HTRF is an optimization of FRET technology for the COT assay, using Europium cryptate and XL665 labeling. The Europium cryptate labeled antibody (i.e. Eu-Anti-phospho-'substrate') is excited by a laser at 337 nm, producing an emission at 620 nm that is absorbed by the XL665 molecule (Clark, 2003).

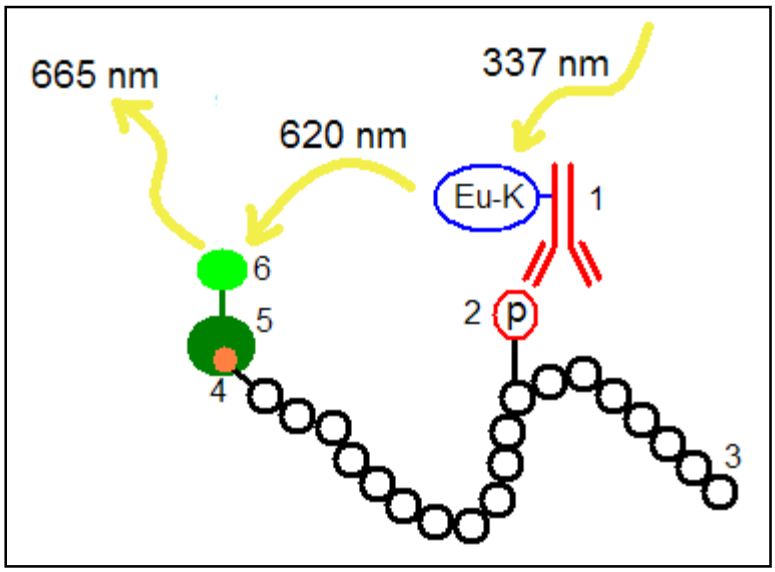


Figure 4: HTRF visualization indicating the Europium cryptate labeled antibody (1) and its affinity for the phosphorylated site (2) of the substrate (3). This brings it into close proximity with XL665 (6) through the interaction of the biotin (4) labeled end of the substrate and the streptavidin (5) labeled XL665.

Before introduction to the reaction, the substrate is biotinylated while the XL665 is bound to streptavidin (SA) (Clark, 2003). *Figure 4* illustrates how all of these individual parts come together, and how the release of an emission occurs. The substrate is phosphorylated at a specific site (2), enabling the attachment of the Europium cryptate labeled antibody (1). By labeling the XL665 with streptavidin, and the substrate molecule with biotin, the XL665 is essentially bound to the substrate itself (*Figure 4*). This ideal donor-acceptor pair leads to an efficiency of 50 – 95% for distances of 5 – 10 nm (compared with standard FRET efficiencies of near 50% and distances of 1 – 7 nm) (Cisbio, 2002).

Enzyme Kinetics (Michaelis-Menton Kinetics)

The pioneers of steady state enzyme kinetics were Leonor Michaelis and Maud Menten; the kinetic constant K_M is named after them, with the subscript 'm' representing their surnames. The most common illustration used for the mechanism of steady state kinetics is that of:

$$E + S \underset{k1}{\overset{k\text{-}1}{\rightleftharpoons}} ES \xrightarrow[k2]{} E + P$$

(Equation 1)

Where E is enzyme, S is substrate, ES is enzyme/substrate complex, and P is the product. k_1 and k_{-1} are the rate constants for formation and degradation of the ES complex. k_2 is the rate constant for the formation of the product P. The rate limiting step in the overall reaction is from ES → E + P, or k_2; this takes into consideration the second assumption: that the substrate concentration, [S], is in excess of the enzyme concentration [E], [S]>>[E] (Willmott, 2005). Thus, the velocity (or rate) of the reaction can be written as $v = k_2[ES]$. Up to this point, it is important to remember that these equations are based upon the assumption that the substrate is in excess and that the system is in steady state. This means that the ES complex is being formed and broken down at the same rate. The mathematical visualization of this is $k_1[E][S] = (k_{-1} + k_2)[ES]$; this leads to:

$$\frac{k_1[E][S]}{k_{-1}+k_2} = [ES]$$

(Equation 2)

At this point, Michaelis and Menten combined all of the constants present in the equation into their own constant:

$$K_M = \frac{k_{-1} + k_2}{k_1}$$

(Equation 3)

When the substitution is completed, the use of the inverse of the K_M allows for a simplified view of *Equation 2*:

$$\frac{[E][S]}{K_M} = [ES]$$

(Equation 4)

In other words, the enzyme concentration multiplied by the substrate concentration, and then divided by the K_M is equal to the concentration of the enzyme-substrate complex.

Next, it is important to mathematically determine the rate at which all enzyme has substrate bound. This is termed V_{max}, or the point at which the velocity is at its maximum. Assuming that the total amount of available enzyme is $[E_T]$ and is equal to the amount of unbound enzyme [E] in addition to bound enzyme [ES], $[E] + [ES] = [E_T]$. Rearrangement and substitution into the equation, and the insertion of $v = k_2[ES]$ results in Equation 5:

$$v = k_2 \frac{[E_T][S]}{[S] + K_M}$$

(Equation 5)

Considering the earlier statement that the substrate concentration is in excess of the enzyme concentration, it is valid to propose that V_{max} can be substituted for v, and that $[E_T]$ can be substituted for [ES]; the resulting form of the original velocity equation now

becomes $V_{max} = k_2[E_T]$ (Willmott, 2005). Substituting this into the more complicated velocity equation derived earlier in this paragraph results in what is formally known as the Michaelis-Menten equation:

$$v = \frac{V_{max}[S]}{K_M + [S]}$$

(Equation 6)

The significance of this equation becomes clear through a visualization of the conditions when the rate of reaction (v) is half of the maximum rate of reaction (V_{max}) (Willmott, 2005).

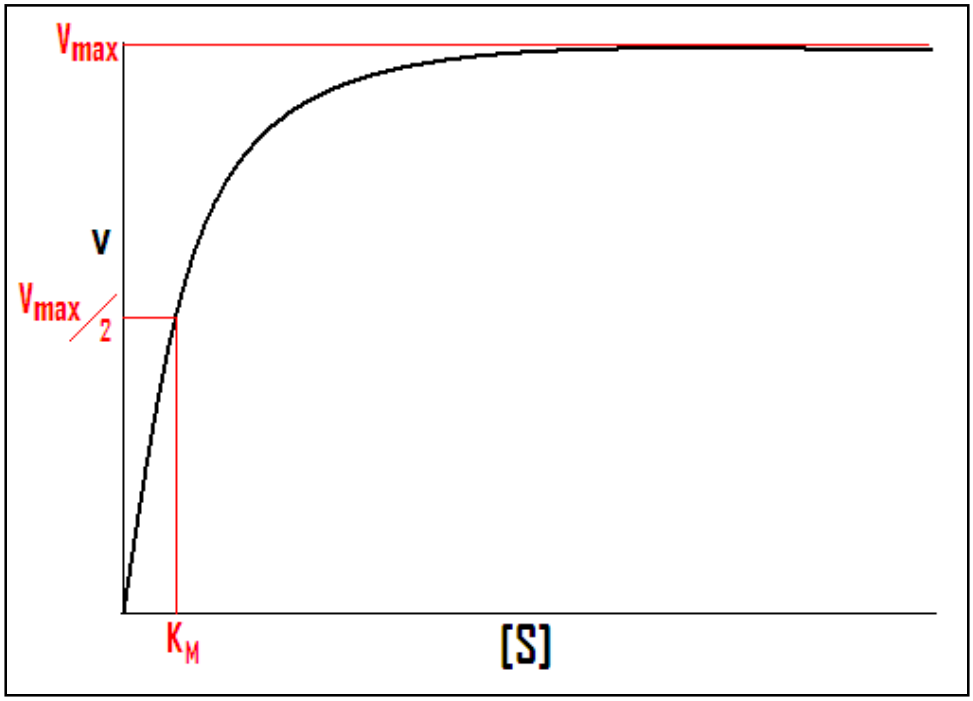


Figure 4: Velocity versus substrate concentration curve showing that the K_M is equal to the substrate concentration at which one half of the V_{max} is reached.

In this case, using $v = V_{max}/2$ as a substitution into the Michaelis-Menten equation, simplifying produces the realization that under these conditions, the K_M of a specific enzyme is the substrate concentration, [S], at half of the maximum rate. Steady state

kinetics requires an equilibrium during which substrate turnover is constant. This catalytic efficiency, referred to as k_{cat}, is equal to $V_{max}/[E]$.

Inhibitor Characterization

For each specific kinase target identified as a key point in a pathway of interest it is important to develop an assay that can be used to verify inhibitor potency/activity. There is a series of experiments that enable the development of an assay for characterization of compounds that may become future drug candidates. With many years of assay development and study, physiologically relevant conditions have been developed and can be applied to the majority of assay development protocols.

When a kinase is first acquired in a useable amount and concentration, it must be tested for activity. From one batch to the next, a kinase may experience drastic variability in robustness. A kinase titration is most often done by testing a series of serial dilutions of the kinase over a set amount of time. If the chosen concentrations extend high enough to reach complete saturation, it is then possible to identify the linear range for a chosen kinase. It is also important to have a signal to background ratio (referred to as the kinetic window) that is 5 to 10 fold (Clark, 2003).

To further understand the kinetics of the target kinase, and to make sure that linearity is available from 0 to 60 minutes at a specific enzyme concentration, a timecourse is needed. In cases where the chosen enzyme concentration is too high, the reaction will run to completion quickly and will not provide an adequate kinetic window. This period of linearity that is desired is referred to as steady state kinetics, and will be discussed in greater detail in the results section.

After an enzyme concentration has been found that adequately supports steady state kinetics, the ATP K_M must be determined in order to assist compound mechanistic studies. The decided upon conditions are used from the previous experiments, with the variable being the ATP concentration. A timecourse is run for a very wide range of ATP concentrations, and the slope calculated from each resulting concentrations becomes a single point on a graph. If done correctly, the graph will proceed to appear similar to *Figure 4*, and the ATP K_M can be mathematically determined. Since the ATP K_M value is a value directly related to the affinity of the target enzyme for ATP, it helps to further design experimental conditions for compound inhibition testing (IC_{50}).

The IC_{50} of a specific compound is identified as the concentration of that compound producing 50% inhibition of the target enzyme. Referring back to the reasoning behind the ATP K_M determination, it can be seen that the inhibition level of the compounds is relative to the experimental conditions used. If a step in the development process, from enzyme to ATP to substrate concentrations is not done correctly, it is likely that the results of the IC_{50} will be invalid. The most common indicator of such an event is inconsistent readings and ratios from experiments, as well as IC_{50} values that are not reliable from experiment to experiment. Based on steady state kinetic equations, it is possible to predict outcomes for most experiments, leading to early notification of incorrectly performed assays.

PURPOSE

With the recent identification of COT as a new target kinase, it is important to develop a useable high throughput assay to produce consistent IC_{50} data. For this reason, a series of experiments was developed to characterize this target kinase, including an understanding of its kinetic parameters. Once it has been isolated and purified, and these kinetic parameters calculated, compound inhibition can be done.

The purpose here is to describe the process used to develop an assay using HTRF technology. This series of experiments is applicable to nearly all kinases, and is used with the hopes of identifying an inhibitor that can become a drug candidate. The entire process is done with the assistance of medium and high throughput technology. Without such advancements as HTRF technology and 96- and 384-well plates, rapid advancement would not be possible.

METHODS

Introduction

All reactions were adapted to a total reaction volume of 40 µL; this reaction volume was quenched with 10 µL of 0.5 M ethylenediaminetetraacetic acid (EDTA), a metal chelator that prevents further reaction. Developing solutions brought the total volume to 125 µL. Each point was performed in duplicate, and all experiments were done using Costar 96 half-well plates at room temperature. Dimethyl sulfoxide (DMSO) is an agent that is able to associate with most proteins, nucleic acids, ionic substances, and even water. For this reason it is commonly used as a stable storing medium for concentrated compounds. 10 µL of each reagent at 4x was used to produce 1x concentration under reaction conditions. For example: 10 µL of 4 mM ATP is added to a total volume of 40 µL, producing a reaction condition of 1 mM ATP.

Enzyme Titration

DMSO was used in the reaction at a volume of 10 µL and a final concentration of 5%. 10 µL of ATP was added, as well as 10 µL of chosen substrate. The enzyme was titrated in 1x RB by performing a serial 1:2 dilution of 10 steps, producing a large range of concentrations. The reaction was started by adding 10 µL of each enzyme to its respective well. After 60 minutes at room temperature, the reaction was stopped by adding 10 µL of 0.5 M EDTA to each well. Development of completed reaction involves addition of 75 µL of Revelation Buffer, consisting of the following components: 2 mM KF, 0.1% BSA, 0.01% Tween-20, SA-XL665, and Europium cryptate labeled antibody. Overnight development was allowed at 4°C. The plate was read using BMG Laboratories' RUBYstar the following morning. Data analysis was done using Excel by Microsoft.

Timecourse

Each well contained 10 µL of DMSO at a final concentration of 5%, 10 µL of ATP, and 10 µL of substrate. 10 µL of 0.5 M EDTA was added to the first well, creating a 0 time point. 10 µL of enzyme at was added to each well, starting the reaction. In order

to cover a wide range, several identical timecourses were run on the same plate with different enzyme concentrations. At time points between 0 and 80 minutes, at room temperature, 10 µL of 0.5 M EDTA was added to the corresponding wells. Development of completed reaction for HTRF reader involves addition of 75 µL of Revelation Buffer consisting of the following components: 2 mM KF, 0.1% BSA, 0.01% Tween-20, SA-XL665, and Europium cryptate labeled antibody. Overnight development was allowed at 4°C. The plate was read using BMG Laboratories' RUBYstar the following morning. Data analysis was done using Excel by Microsoft.

ATP K_M Determination

Prepared 1x RB was used to perform a serial 1:2 dilution of ATP, providing an adequate range of final concentrations. 10 µL of corresponding ATP dilution was added to each well. 10 µL 5% final concentration DMSO was added to each well, as was 10 µL of substrate. 10 µL of enzyme was added to begin reaction. After 60 minutes of reaction time, at room temperature, the process was quenched by addition of 10 µL 0.5 M EDTA. Development of completed reaction involves addition of 75 µL of Revelation Buffer consisting of the following components: 2 mM KF, 0.1% BSA, 0.01% Tween-20, SA-XL665, and Europium cryptate labeled antibody. Overnight development was allowed at 4°C. The plate was read using BMG Laboratories' RUBYstar the following morning. Data analysis was done using Excel by Microsoft.

IC_{50} Determination

Stock 1 mM compound in 100% DMSO was titrated 1:5 by moving 10 µL compound into 40 µL of 100% DMSO. 6 step titration produces 1 mM → 0.064 uM compound concentrations; 100% DMSO without compound is used for control points. Compound dilutions (including controls) were further diluted 1:5 in 1x RB, producing compound concentrations 200 uM → 0.0128 uM, with DMSO at 20%. 10 µL of compound dilution was added to corresponding reaction. 10 µL of ATP and 10 µL of substrate were also added to each well. 10 µL of enzyme was added to all wells containing compound, as well as the positive control wells. 10 µL 1x RB added to negative/background control wells. After 60 minutes at room temperature, all wells quenched with 10 µL 0.5 M EDTA. Development of completed reaction for HTRF reader

involved the addition of 75 µL of Revelation Buffer consisting of the following components: 2 mM KF, 0.1% BSA, 0.01% Tween-20, SA-XL665, and Europium cryptate labeled antibody. Overnight development was allowed at 4°C. Plate was read using BMG Laboratories' RUBYstar the following morning. Data analysis was done using Excel by Microsoft.

RESULTS

Interpreting HTRF Data

As explained in the background section, the RUBYstar plate reader uses a laser to excite the contents in each well of a 96-well plate. The technology allows for a reading process that removes background fluorescence and optical clarity of the medium (Cisbio, 2002). The reader is able to accumulate emissions at two different wavelengths; in this case, they are termed 'A' and 'B' counts. The B count is the emission reading at 620 nm, accounting for the emission from the Europium cryptate. The A count is the emission reading at 665 nm, representing the emission from the acceptor. This measures the FRET transfer, and is proportional to the amount of product that has been formed. The ratio used to plot data from each variation of HTRF assay is the following:

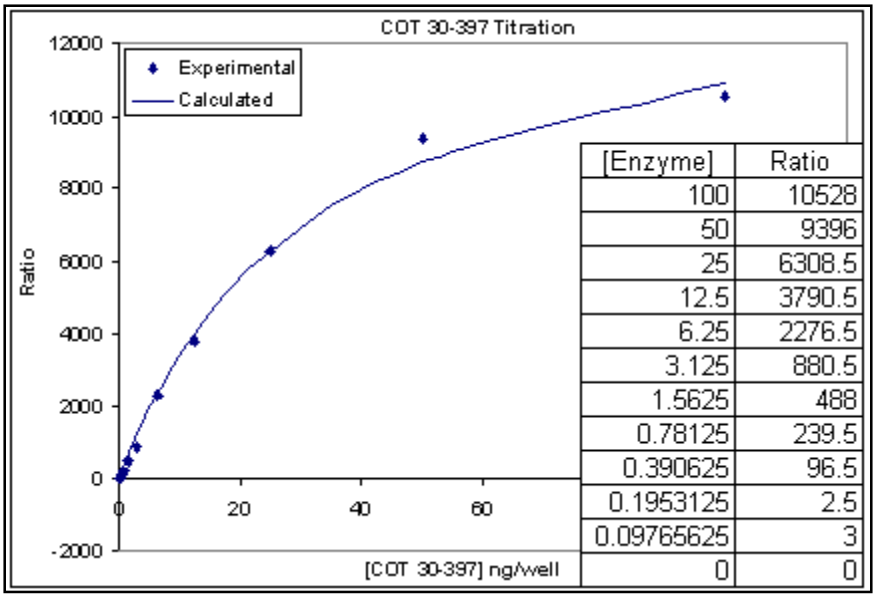
$$(\text{A Count} / \text{B Count}) \bullet 10{,}000 = \text{Ratio}$$

Since A count is the relative number used for quantification, and it is also the numerator of the previous equation, it is favorable to have a large B count. By doing so it means that minor fluctuations in B count will not drastically affect the overall ratio. All wells are duplicated, and the average of the two ratios is taken prior to evaluation.

Enzyme Titration

Assuming an adequate substrate can be obtained, and that physiological conditions can be replicated with the correct buffer, it is important to titrate the enzyme (in this case COT) (*Figure 5*). This will provide indications of the linear range of the

enzyme concentration, show the saturation point, and produce an acceptable range of concentrations to continue with for assay development (*Figure 5*).



[Enzyme]	Ratio
100	10528
50	9396
25	6308.5
12.5	3790.5
6.25	2276.5
3.125	880.5
1.5625	488
0.78125	239.5
0.390625	96.5
0.1953125	2.5
0.09765625	3
0	0

Figure 5: COT titration curve. The ratio was acquired by standard enzyme titration.

Further timecourses are often required to show if the chosen enzyme concentration produces a linear range within a predetermined time period. Most often this is optimized as close to 60 minutes as possible, for reasons of convenience for high throughput screening. By titrating the enzyme it is also possible to save available resources by reducing needed amounts; it is essential, however, to keep the concentration high enough to produce an acceptable signal and signal-to-background (S/B) ratio. From *Figure 5* the chosen COT enzyme concentration for further assay development was 25 ng/well, or a final experimental concentration of 13.9 nM. This is enough to produce a strong signal (see *Figure 5*), but not so much that it would saturate the experiment.

Timecourse

To further evaluate the linearity of the chosen enzyme concentration, it is important to produce a timecourse. This enables the visualization of not just the one enzyme concentration chosen (25 ng/well), but the linearity of that concentration over time. The chosen concentration was performed in duplicate in a horizontal fashion across

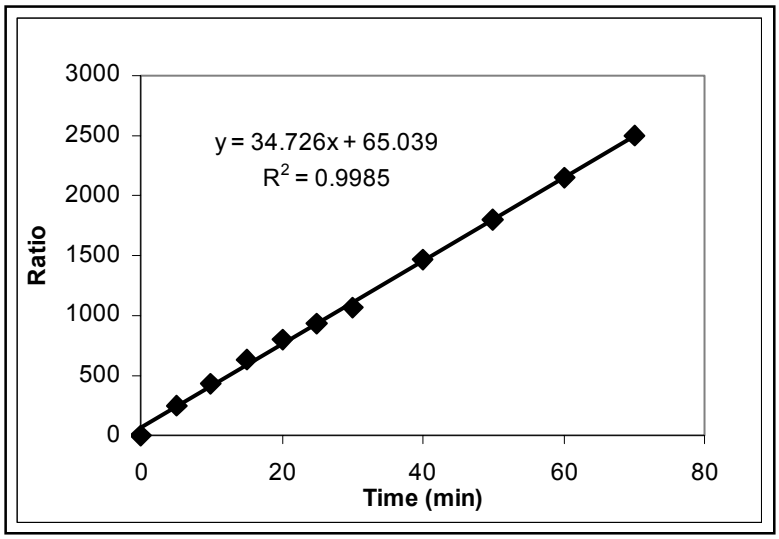


Figure 6: COT timecourse using HTRF format. Experimental data was produced at 25 ng/well, allowing for optimization of the assay.

a 96-well plate (*Figure 6*). Given that there are 12 wells across the plate, there are 12 chosen time points: 0, 5, 10, 15, 20, 25, 30, 40, 50, 60, 70, and 80 minutes. The zero time point is considered the background, as there has been no enzyme catalysis. This was subtracted from all of the values during the analyzing of the data. The timecourse was extended beyond the desired 60 minutes to ensure the linear trend. The resulting data from the experiment produced a graph seen in *Figure 6*; several equations were also simultaneously calculated. A linear trend line was applied to the data point, in order to understand two things: first, the slope of the points; and second, the R^2 value of the line. The slope of the line in y = mx + b form (the 'm' value) is also the rate of the reaction. This information is valuable in determining the ATP K_M, as well as a checkpoint for

consistency between experiments. The R^2 value is a useful tool to quantify exactly how 'linear' the points really are. If the best fitting straight line is able to intersect with every point on the graph, the value will be $R^2 = 1$. The greater the overall deviation of the graphed data points from a straight line, the lower the respective R^2 value. *Figure 6* indicated an R^2 value of 0.9881. In all timecourses, an acceptable R^2 value is usually over 0.9, though in most cases it is better to make sure that linearity is as stringent as 0.97 or higher.

ATP K_M Determination

Once the assay was optimized for enzyme concentration and linearity throughout a specific time period, the ATP K_M was determined to better understand the kinetic properties. Since ATP competitive compounds are of major interest in our studies, it is important to understand the kinase's ATP affinity. As outlined in the methods section of this paper, the ATP K_M is determined through data collected from timecourses done at a wide range of ATP concentrations. For kinase COT, ATP concentrations ranged from 25 uM to 4 mM, with time points adjusted to over three hours to make sure linearity for the lower concentrations was accurately measured. The slope of each linear section from each of the ATP concentrations constitutes a single point on *Figure 7*. The slope, or velocity of the reaction, was graphed vs. the concentration of ATP at which it was determined. A curve fitted to these data points was used to calculate

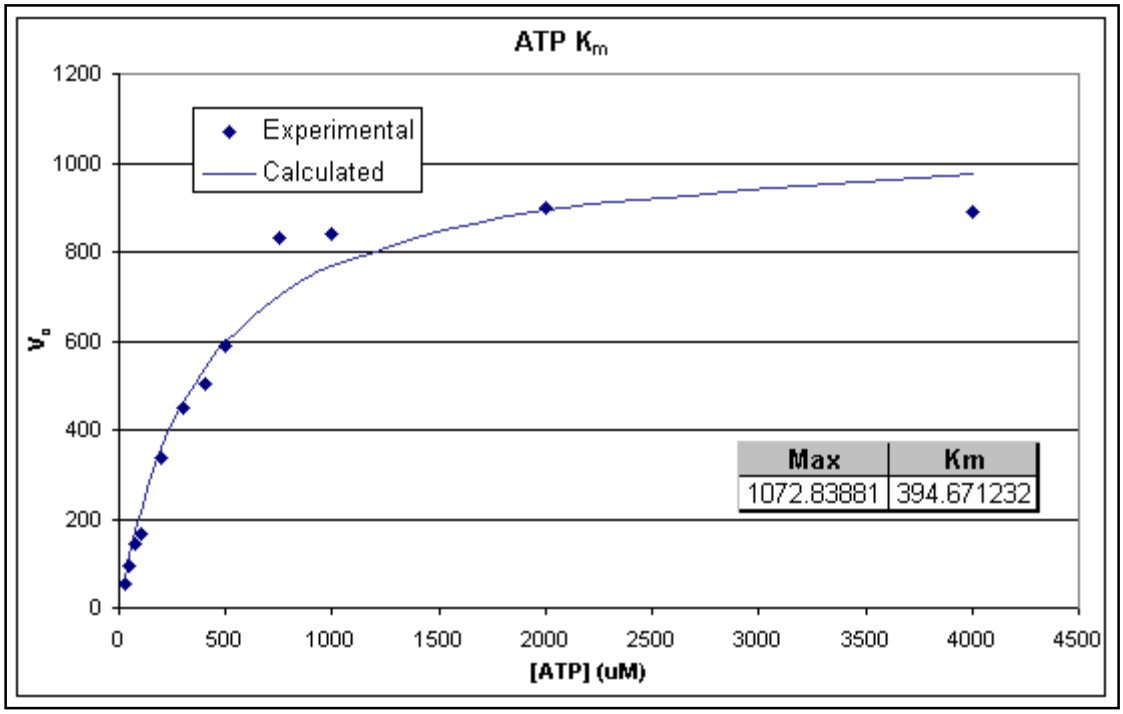


Figure 7: ATP K_M for COT30-397, indicating the V_{max} and K_M values from the calculated trend line based on the experimental data.

the maximum velocity (rate) and the K_M. The K_M was determined using the Michaelis-Menton equation. Calculations resulted in an ATP K_M of 394 uM, meaning that optimum conditions for identifying weakly ATP competitive compounds will be under 400 uM.

IC_{50} Determination

At this point, the kinase (COT) has been characterized to the point where compound inhibition can be accurately tested using the HTRF assay. The experiment used to do this is called an IC_{50} determination. By definition, the IC_{50} is the concentration of a specific compound required to inhibit the enzyme activity by 50%. Since compound potency can vary with experimental conditions, the use of the developed conditions must remain constant in order to understand the relative potency of different compounds. As a positive control that has a low IC_{50} (50% inhibition is achieved at a low compound concentration) is used. Under the developed conditions, the chosen compounds are

titrated and added to the reaction. In *Figure 8*, Compounds 1, 2, 3, and the Control Compound (names withheld for proprietary purposes) were all serially diluted 1:5 in a 6-step process, and then diluted again 1:5. These final concentrations are shown on the X-axis of *Figure 8*, recorded in logarithmic order from 50 uM to 0.0032 uM.

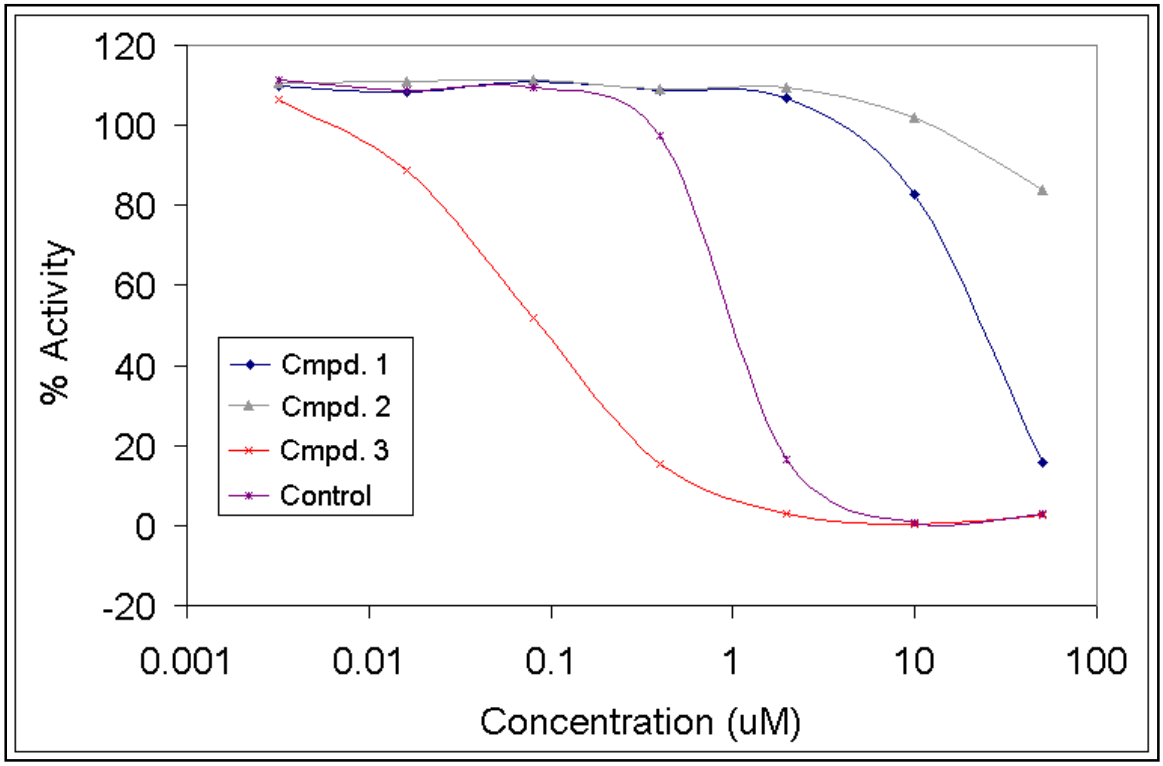


Figure 8: COT IC_{50} comparison for Compound 1, 2, and 3 versus the Control. Calculated IC_{50} for Cmpd. 1 = 18.39 uM, Cmpd. 2 = >50, Cmpd. 3 = 0.0683 uM, Control = 0.481 uM.

The Y-axis indicates the percent activity at the corresponding compound concentration (*Figure 8*). This is determined in relation to both the positive and negative controls built into each experiment. Since the same control compound is used for every experiment, an IC_{50} of around 0.481 uM should be seen each time the experiment is performed. The IC_{50} values experimentally determined against COT in *Figure 8* are as follows:

	Compound 1	Compound 2	Compound 3	Control
IC50 (uM)	18.39	>50	0.0683	0.481

Table 1: COT IC_{50} values determined experimentally under developed conditions for COT.

Compound 2 was calculated to have an IC_{50} of greater than 50 uM (>50) because this is the highest detection limit of the assay. The curve fitting of the data analysis program can predict an IC_{50} value of greater than 50, though this value cannot be reached experimentally, and is of little value anyway. The lower detection limit of the assay is 0.0032 uM, and compounds with a determined IC_{50} of less than 0.0032 uM are considered extremely potent, and most often will warrant further investigation. None of the compounds tested here achieved such a high level of inhibition.

ATP Competition

In order to determine if compounds inhibit the kinases by binding to its ATP site, an ATP competition experiment needs to be performed. To do so, a series of IC_{50} values are determined at varying ATP concentrations. A data analysis program can produce the compound k_i and the assumption of whether or not the compound is ATP competitive. If a compound is not ATP competitive, the values taken at varying ATP concentrations (experimental) will not match the theoretical predictions.

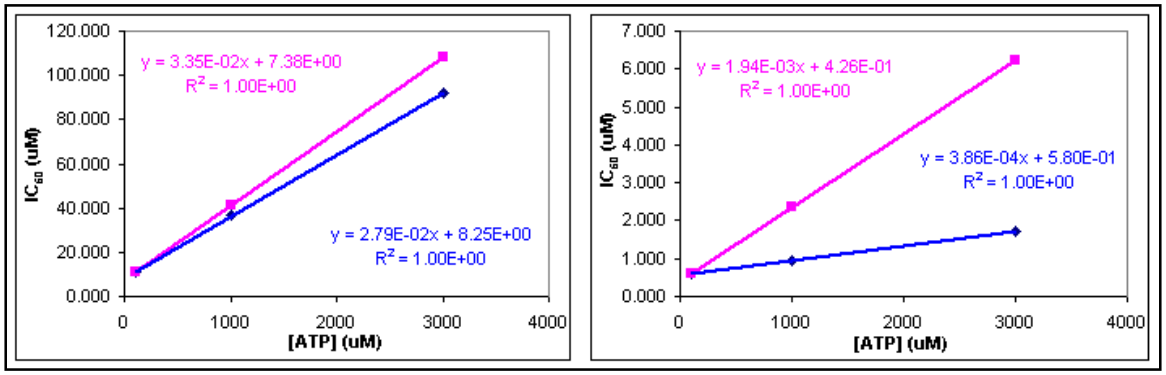


Figure 9: COT ATP competition data analysis. Pink line shows theoretical values for IC_{50} at 100 uM, 1 mM, and 3 mM ATP, blue line shows experimental data at the same ATP concentrations. The left graph indicates a compound that is ATP competitive, while the compound in the right graph is not.

Computer graphing creates a graph of the theoretical points and simultaneously graphs the experimental points. The program is then able to indicate whether or not the

compound is ATP competitive based on a comparison of slopes. If the experimental slope is over 50% of the theoretical slope, it is defined as ATP competitive, while under 30% can be considered non-ATP competitive. The left graph in *Figure 9* has a competitive percentage of 83%, indicating that compound is ATP competitive for COT, while the right graph has only 19%, indicating that compound is not ATP competitive for COT. This experiment defines not only how potent a compound is against a target kinase, but also the mechanism of inhibition by which it is competing with ATP.

DISCUSSION

As stated in the purpose, Abbott Bioresearch Center strives to create compounds that are able to inhibit specific kinases. *Figure 1* in the background section is only a small portion of a much larger kinase signaling pathway, parts of which are still not understood and may be completely unknown. HTRF technology and inhibitor compounds have been adapted to explore relationships between these pathways and the compounds that are introduced. But even with this technology, development of the assay is still necessary for each individual kinase. Precise as this technology may seem to be, and as advanced as it really is, it is still researching processes that are millions of years old.

Years and sometimes decades of work by hundreds of people will be spent pursuing a target kinase. When a target kinase is presented to the Molecular Pharmacology Group, the first thing done is to gather every piece of research previously completed. In some cases there is very little information available, and initial reaction conditions and substrate identification can become tedious. Assay development is a key point in drug discovery, and any mistakes made will only be amplified as the project progresses. Incorrect development of assays can produce not only false positives, but false negatives. If a compound is found to be potent when in actuality it is not, it may be months before such evidence comes back to prove it so. Conversely, an assay may not show how potent a compound really is, and the next great cure might be simply overseen.

For these reasons, each enzyme titration is taken seriously. A difference of one nanogram per well in a 96-well plate may not seem like a large difference, but in small scale biochemistry it can drastically effect the robustness of an assay. Too little of an enzyme will produce lackluster signal ratios, and too much will saturate the experiment.

Not to mention that the number of compounds screened numbers in the thousands, and saving micrograms per plate can result in thousands of savings per year.

Timecourses allow for the manipulation of linearity during development. While titrations produce data relative to the amounts needed to optimize the assay, a timecourse can show the variations in rate for those chosen concentrations. Some enzymes exhibit lag phases and other individual traits, each of which can help identify problems that may occur at a later time. While it may have become standard to have reaction times of 60 minutes, it is not out of the ordinary to stop reactions sooner or allow them to proceed for a longer period of time. It is not so much the actual components of the final assay and their concentrations, but whether the true characteristics of the enzyme are shown in response to compound inhibition experiments. Development of an assay is truly put to the test, as mentioned in the results section, when an ATP competition experiment is performed.

At this point a compound has been identified as being an inhibitor of COT enzyme function, and it was determined to inhibit in an ATP competitive manner. From this point onwards, the compound is handled by *in vivo* labs that will begin to test the compound's toxicity levels.

In the increasingly competitive field of drug discovery and pharmaceuticals, Abbott Bioresearch Center has adapted to the use of HTRF technology in order to increase the number of compounds that can be screened daily. The development of assays is done in singular, bench experiments. It becomes another development and troubleshooting process entirely when upscaling HTRF assays for medium and high throughput use. In many ways robots are more efficient than humans, but in every other way they are only as advanced as we can make them. High throughput science is another

area entirely, and the assay development is become more focused on meeting the needs of such technologically advanced environments. Fortunately, many companies have been successful in discovering new drugs, and for this it is worth the great amounts of time and resources that are required.

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