



**WORLDWIDE  
RESEARCH**

# **Assay Development and Inhibitor Characterization For Protein Kinases Using an LC 3000**



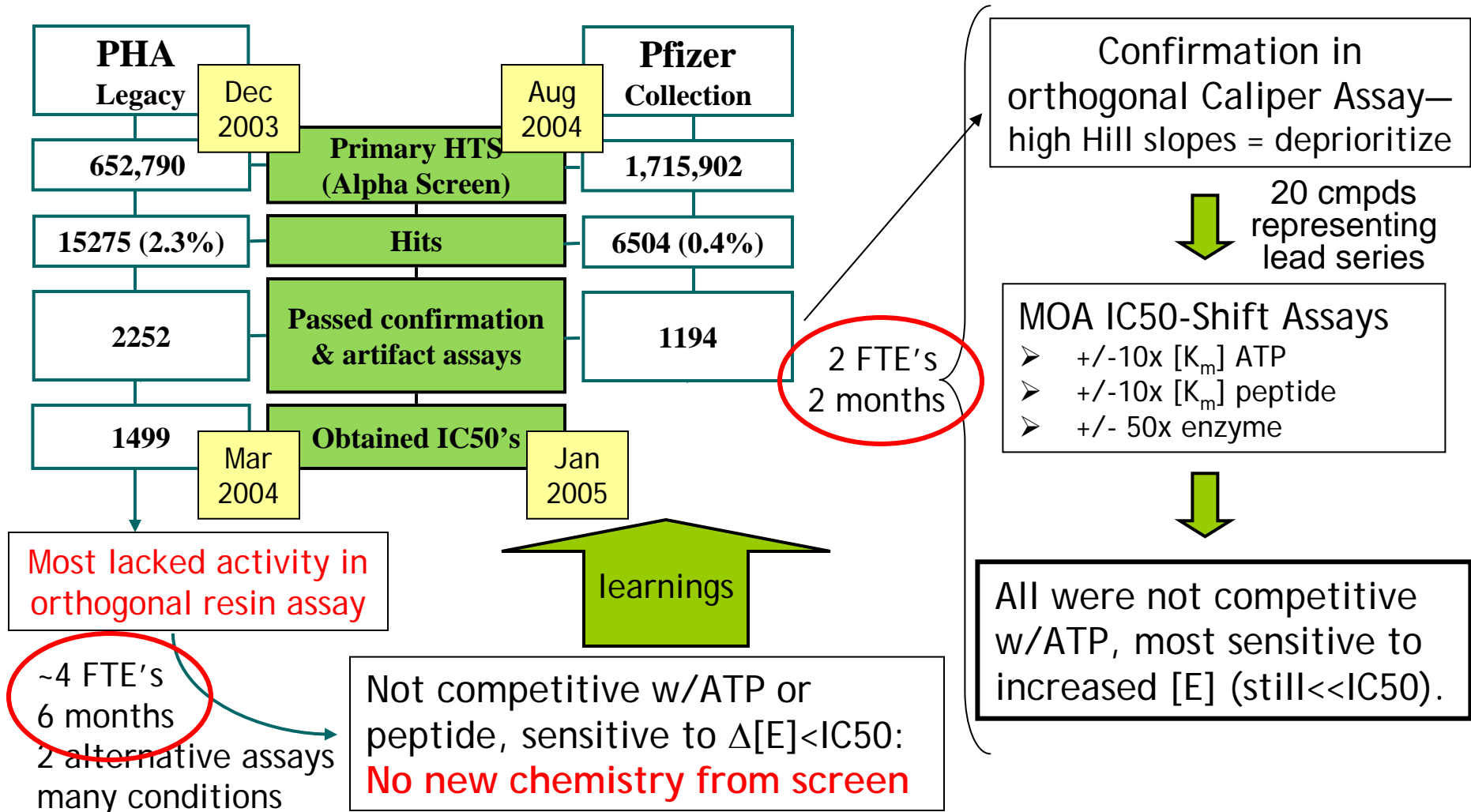
# Impact of Early Mechanism of Action Studies on Kinase Drug Discovery



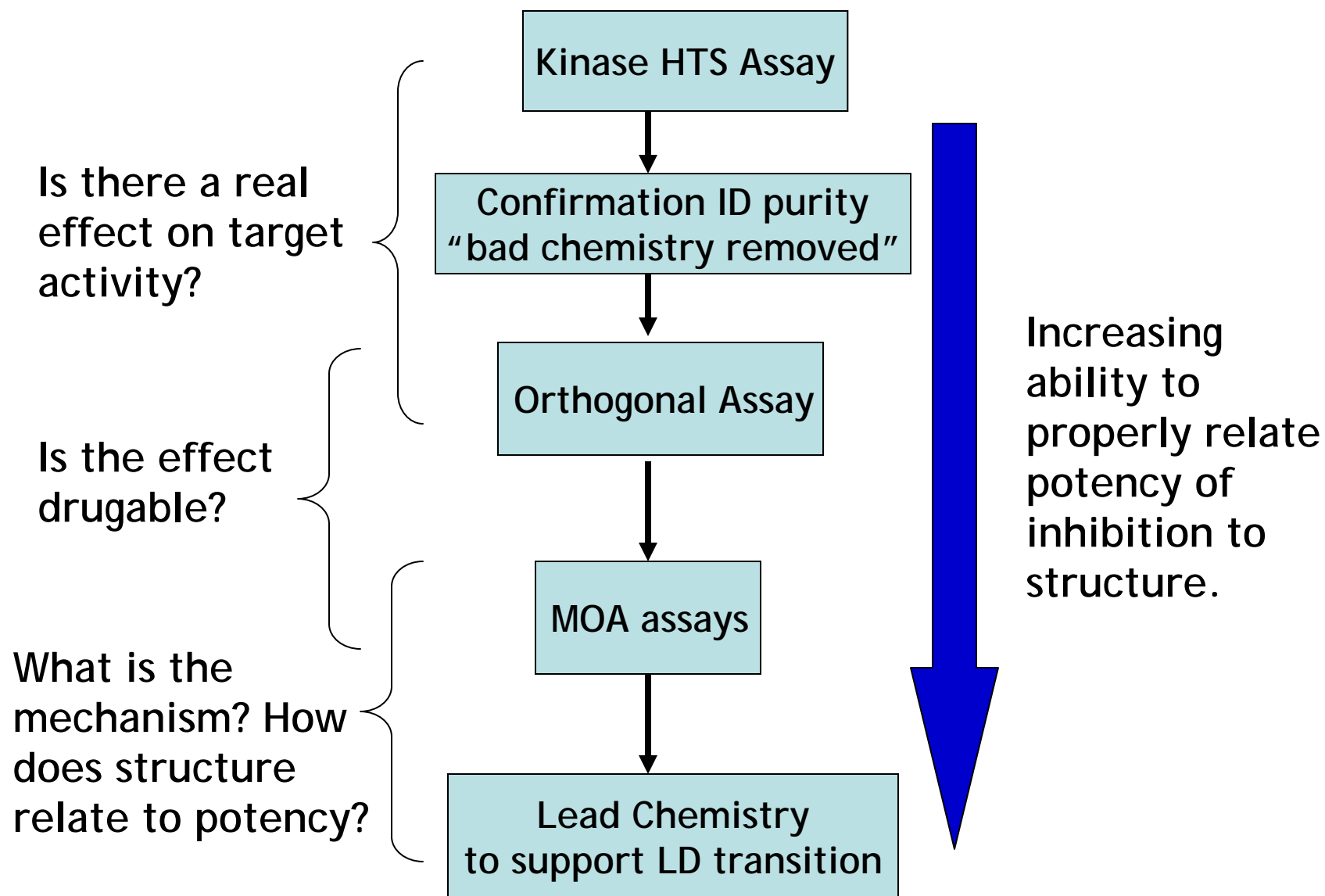
- ◆ **Goal: To quickly relate potencies to structure to drive SAR**
- ◆ *However*, screening hits are not lead chemistry.
- ◆ Even when verified by an orthogonal assay—most do not give mechanism of action (MOA) confidence...we need more than artifact identification.
- ◆ We need to **more quickly** identify chemical lead matter that:
  - ◆ Causes the desired functional effect: inhibition, agonism, etc.
  - ◆ Binds the biological target with a drugable mechanism.
  - ◆ Shows appropriate potential for selectivity.
  - ◆ Has potential for drugable properties and is chemically doable.
- ◆ Quicker evaluation of the results of a screen will
  - ◆ Decrease the time to LD & facilitate SAR if there is viable chemistry
  - ◆ Allow quicker adoption of alternative approaches if there isn't

# Why We Need A Plan...

Consider Kinase A...



## Approach to HTS testing funnel





## Design of HTS and Orthogonal Assays

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### HTS Assay Design Focus:

- ◆ Support full file screen (low volume)
- ◆ Format must provide robust signal (may be indirect measure of product formation)
- ◆ Overcome artifacts from compounds (absorbance or fluorescence)
- ◆ Format may not allow use of natural substrate
- ◆ Design assay to find inhibitors with certain mechanism (competitive, noncompetitive and uncompetitive)

### Orthogonal Assay Focus:

- ◆ Should provide direct measure of product
- ◆ May not support full file screen
- ◆ Must (at least) confirm that target activity is affected (not just assay).
- ◆ Allows for substrate(s) to be varied (enables MOA studies)



## Example of Kinase Assay Formats Available

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### Radiometric

- ◆ Phosphocellulose (Millipore)
- ◆ SAM-filtration (Promega)
- ◆ SPA beads (Amersham)
- ◆ Flashplate (Perkin Elmer)

### Chemiluminescence

- ◆ AlphaScreen (Perkin Elmer)
- ◆ Lumitech (BioWhitaker)
- ◆ KinaseGlo (Perkin Elmer)
- ◆ HitHunter (DiscoverRx)

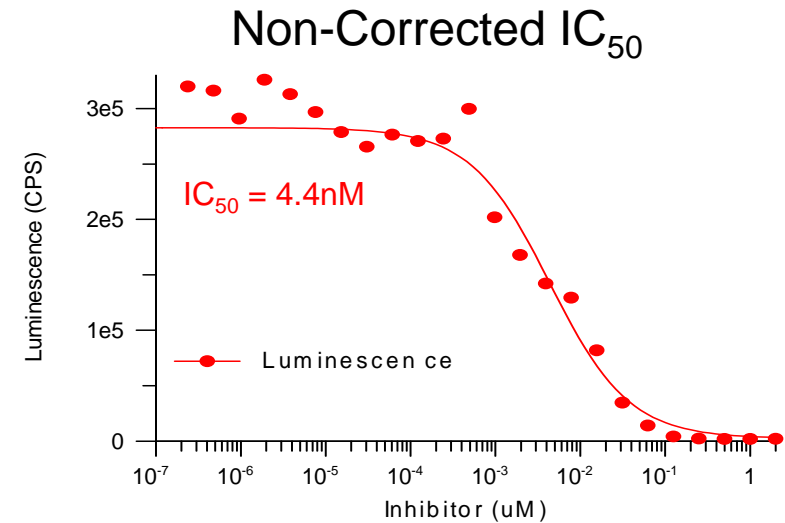
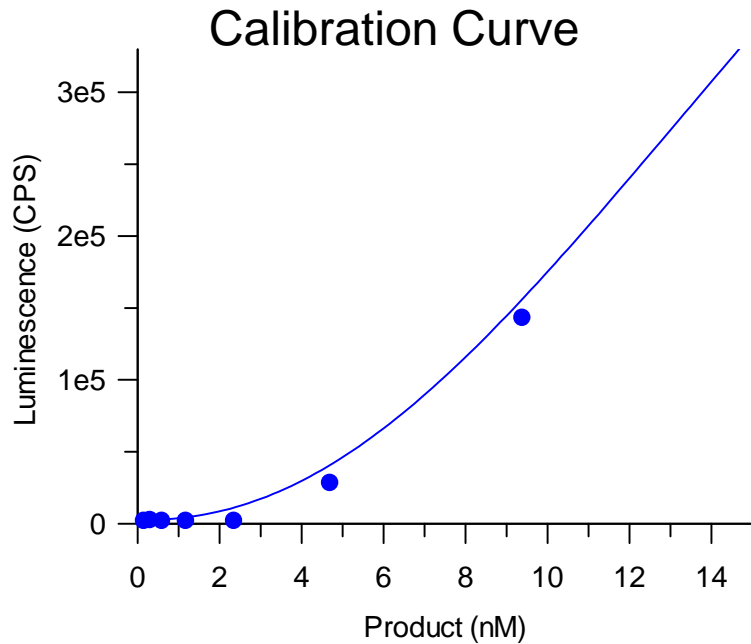
### Fluorescence

- ◆ Z'-LYTE FRET (Invitrogen)
- ◆ LANCE (Perkin Elmer)
- ◆ IMAP (Molecular Devices)
- ◆ Omnia - CHEF (BioSource)

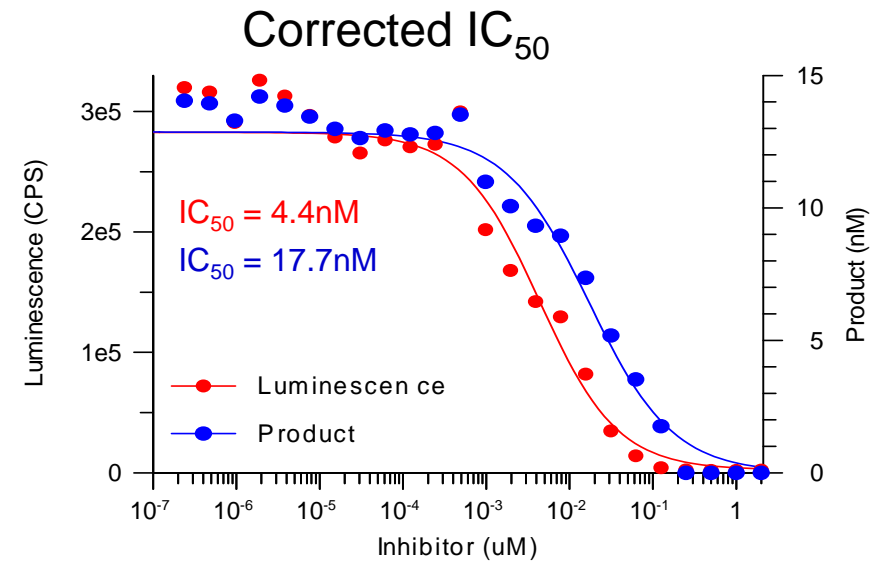
Most kinase HTS assays suffer from not making the relationship between product and signal. The following do make the relationship:

- ◆ LapChip 3000 (Caliper)
- ◆ Veloce (Nanostream)

# Potential Issues with Indirect Assay Readout



- ◆ Coorelation for  $IC_{50}$  poor when compared back to product formation
- ◆ Difficult to perform kinetic analysis and  $K_m$  determination



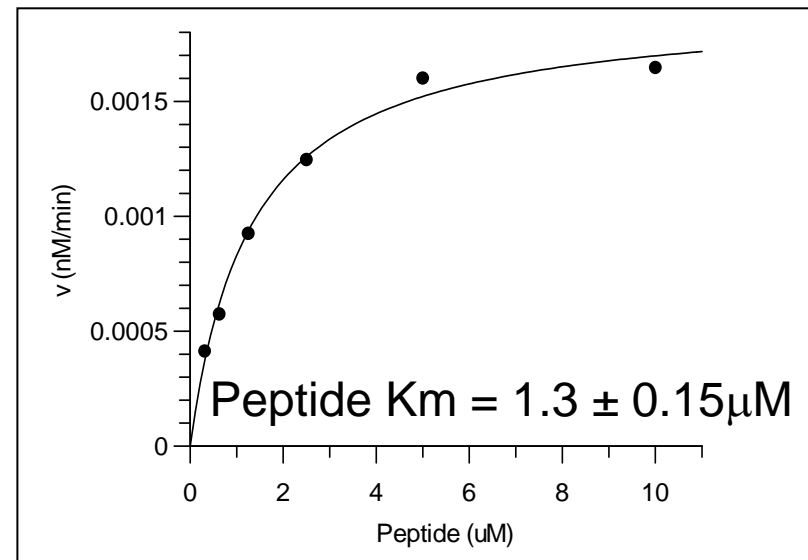
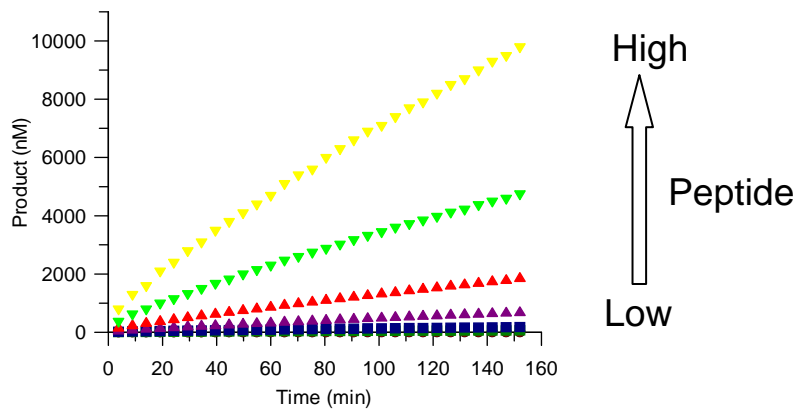
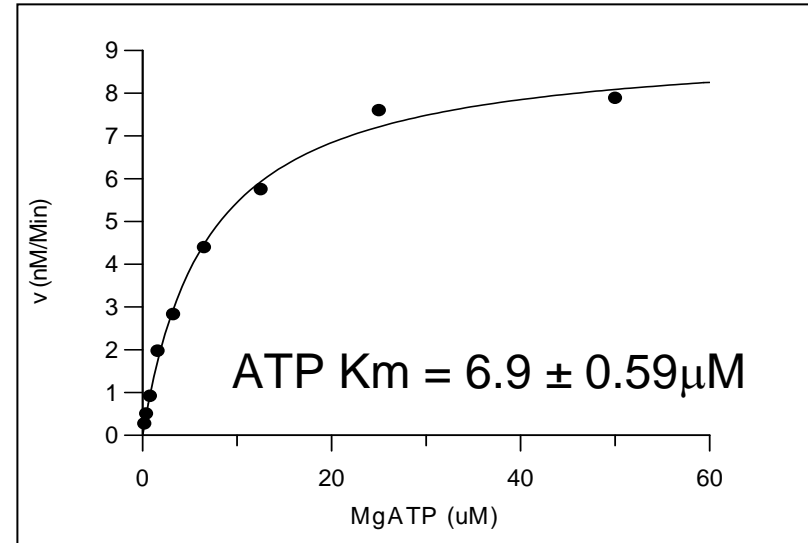
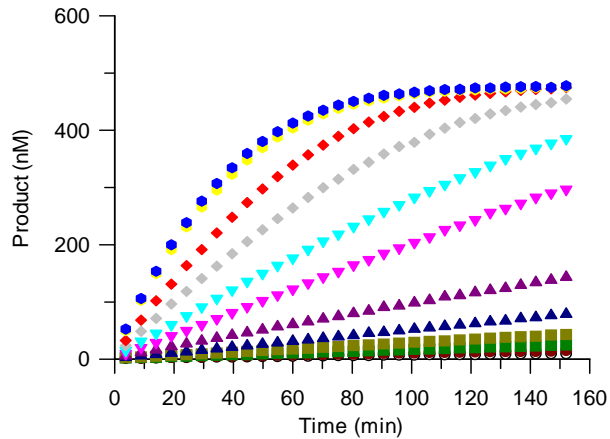


## Assay Development using LC3000

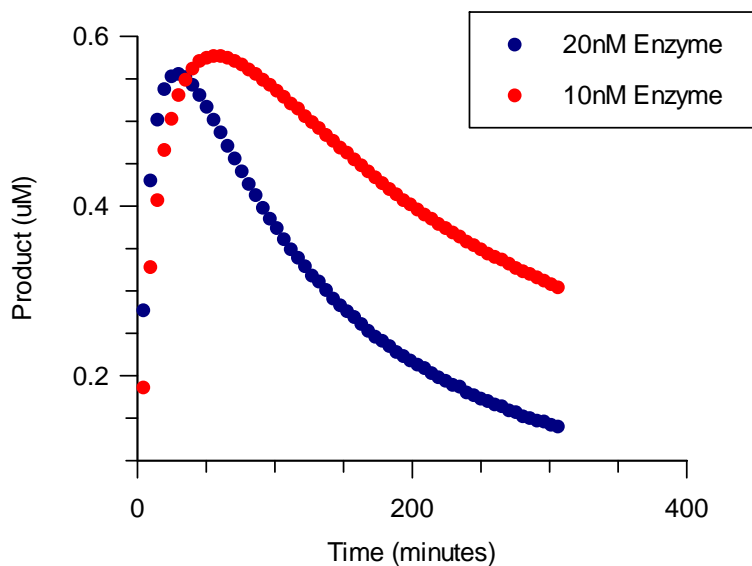
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- ◆ Rapid assay development
- ◆ Can be used for evaluation of kinases, phosphatases and other enzymes
- ◆ Format amenable to mechanism of action studies
- ◆ Easy to identify potential issues

# Apparent Km Values for Ser/Thr Kinase determined using LC3000

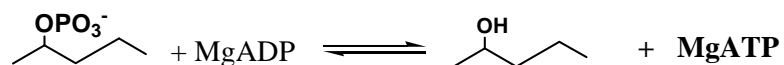


# Discovery of Dephosphorylation Reaction for Kinase-B



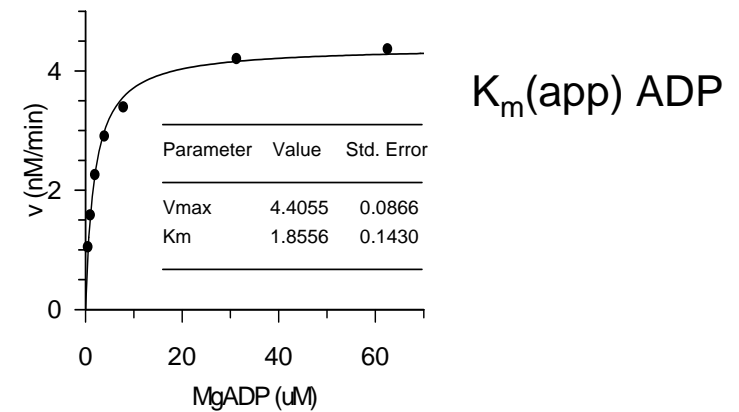
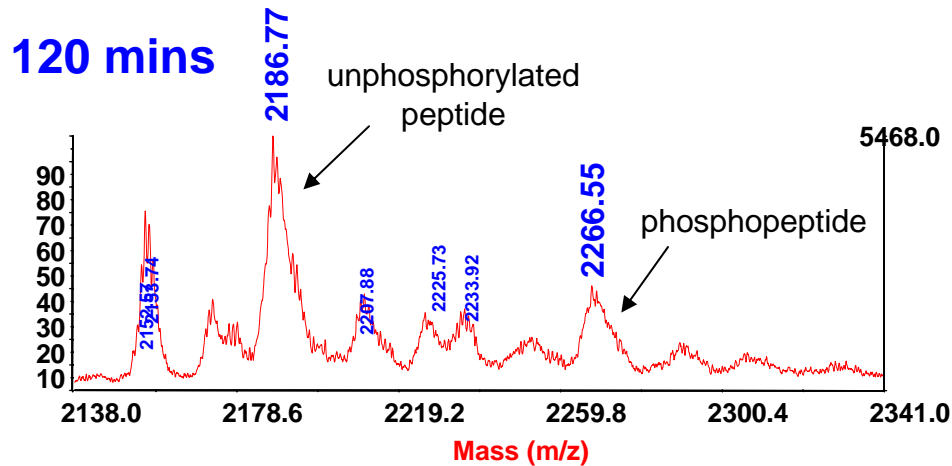
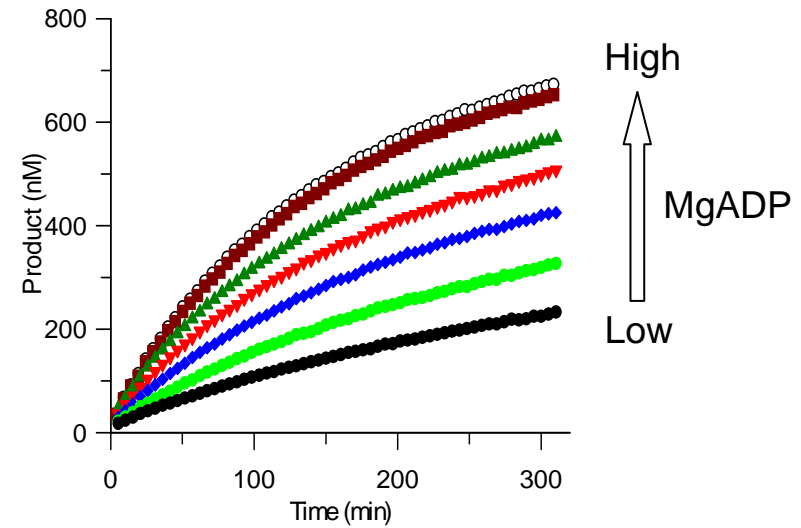
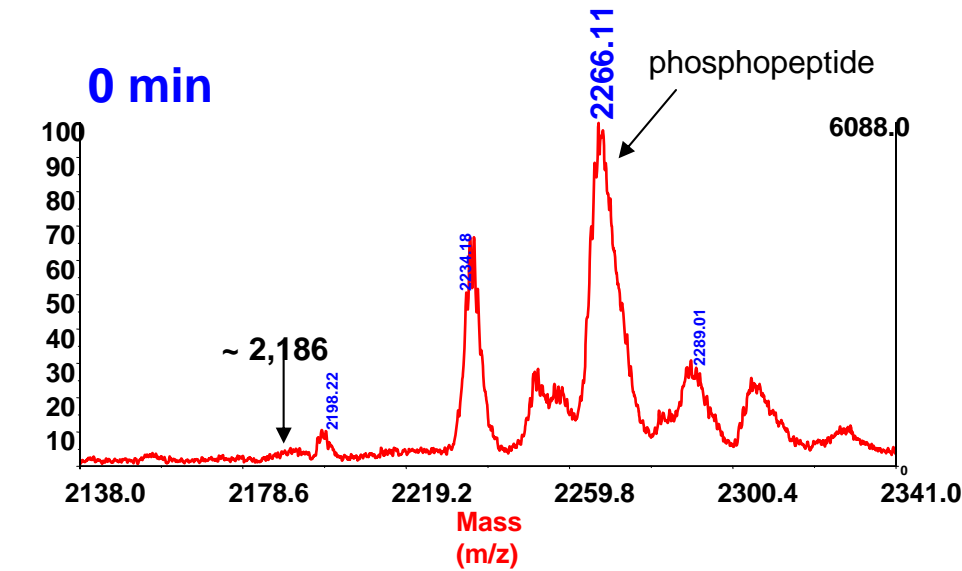
1uM Peptide  
4uM ATP

- ◆ Suggests the phospho-peptide is being dephosphorylated



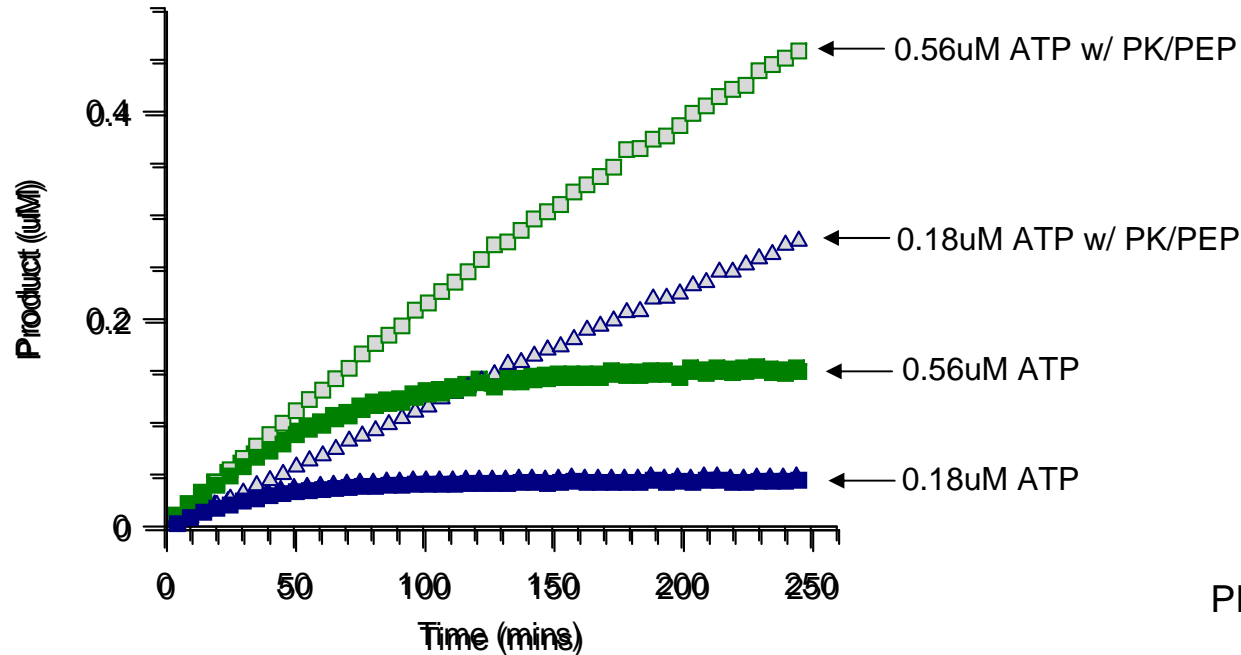
- ◆ Evaluate phenomenon using Mass Spec

# MS Confirmation of Dephosphorylation Reaction

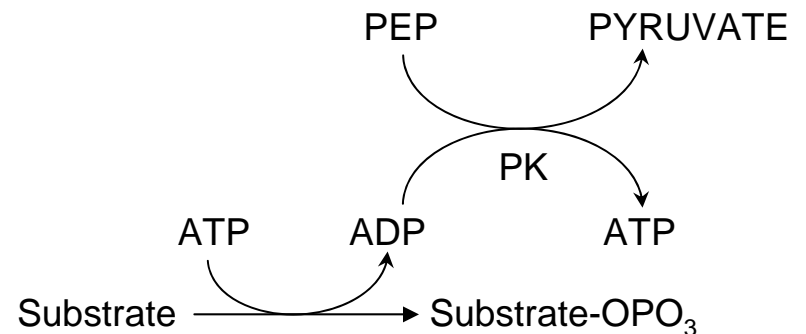


# Assay Development of Kinase C

1uM peptide substrate



- ◆ Technology allowed us to identify potential inhibition
- ◆ Allowed us to design experiments to evaluate if inhibition was due to product inhibition, substrate inhibition, ATPase activity, etc.





## Assay Design Summary

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- ◆ Rapidly determine  $K_m$  for ATP and Peptide
- ◆ Assays can be developed in as little as 3 days
- ◆ Substrate measurement allows us to identify potentially novel events
- ◆ Allows us to design experiments to address the unique events

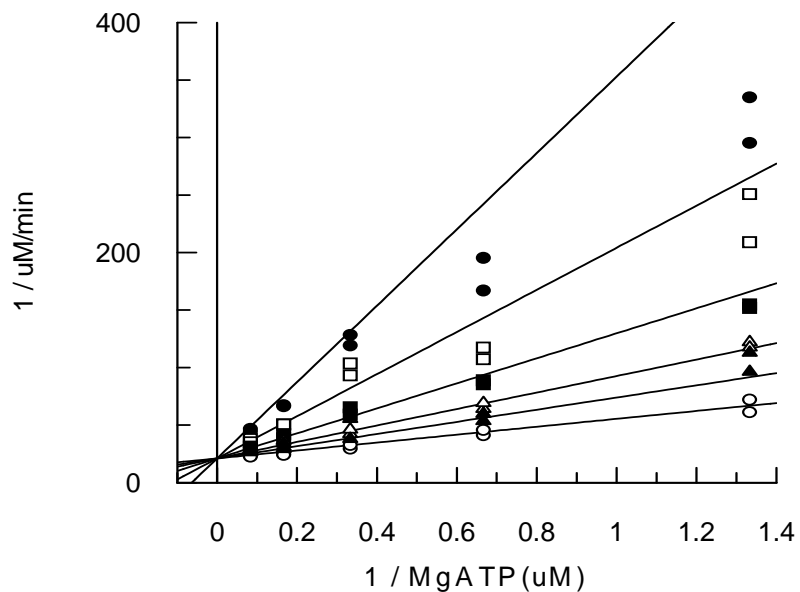


## Mechanism of Action Studies using LC3000

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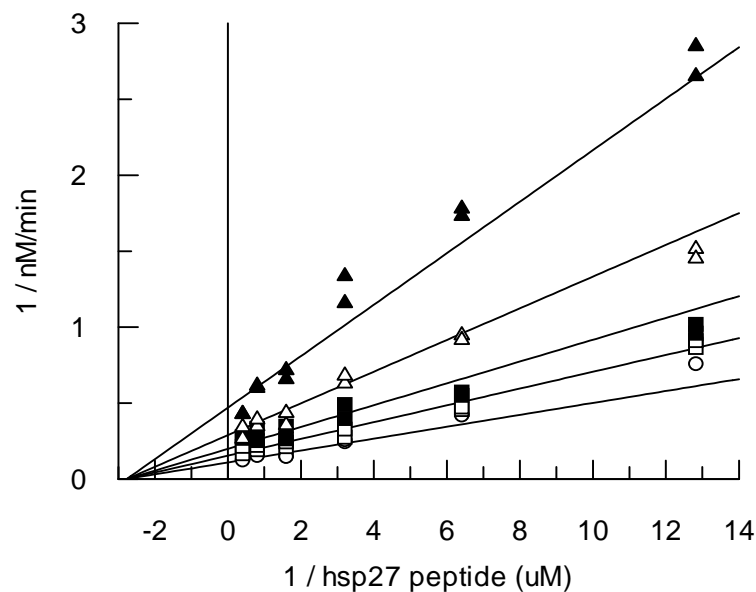
- ◆ Progress curves (on-, off-rates)
  - ◆ Important for projects and is placed high in testing funnels
- ◆ Same assay format used for both screen and MOA studies
- ◆ Assay conditions can be easily varied (i.e. buffer, co-factor)

## Competitive Inhibition vs. MgATP



$$K_{is} = 0.34 \pm 0.032 \mu\text{M}$$

## Non-competitive Inhibition vs. FITC-peptide



$$K_i = 0.45 \mu\text{M}$$

- ◆ Peptide  $K_m$  within dynamic range of instrument
- ◆ Both substrates varied easily



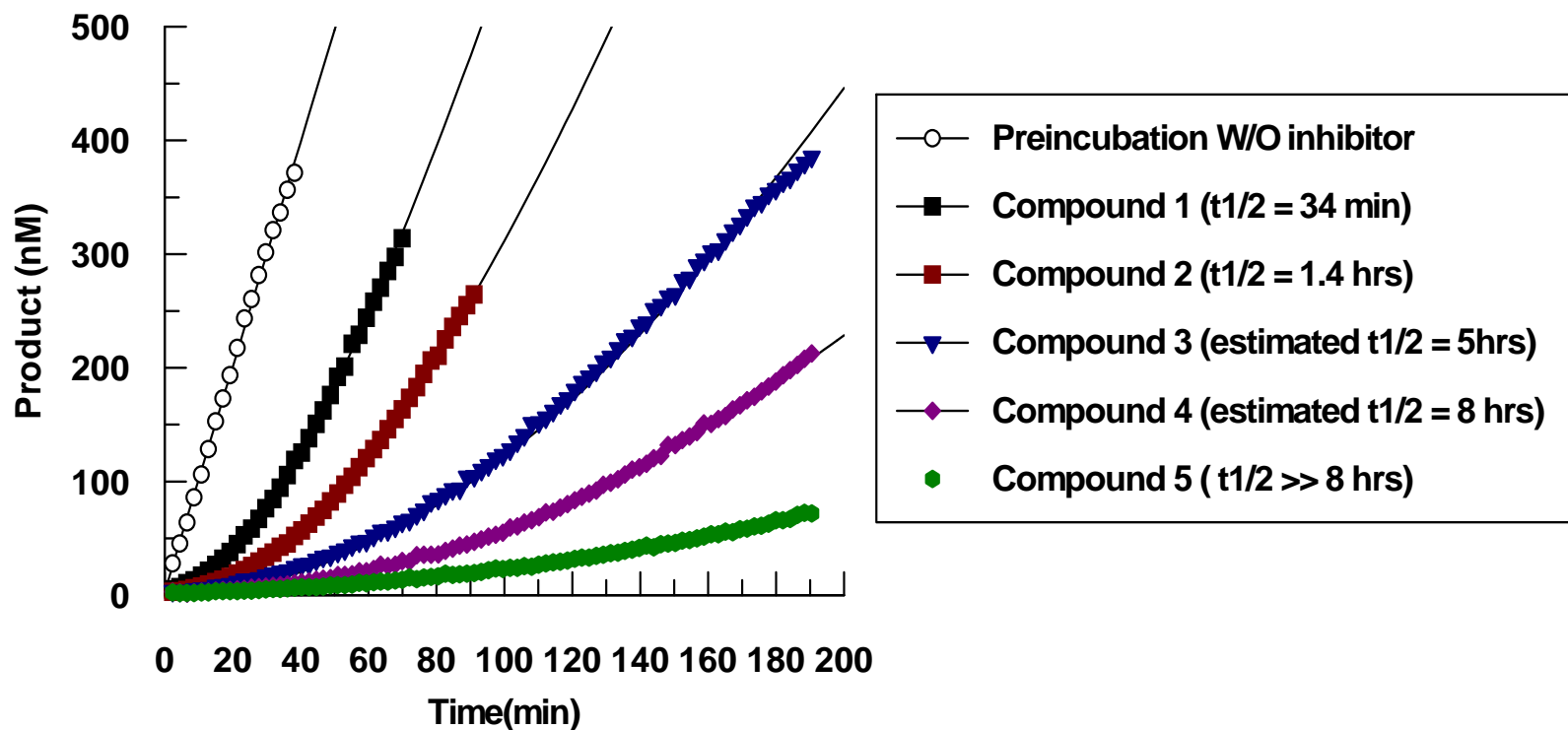
## When Your Assay Bottoms Out: Limited By Enzyme Concentration



Compound	IC <sub>50</sub> (nM)*	IC <sub>50</sub> (nM)* Pre-incubation 1 hour
<b>Compound 1</b>	<b>63 ± 6</b>	<b>10 ± 2</b>
<b>Compound 2</b>	<b>77 ± 12</b>	<b>17 ± 5</b>
<b>Compound 3</b>	<b>82 ± 9</b>	<b>17 ± 4</b>
<b>Compound 4</b>	<b>86 ± 12</b>	<b>21 ± 5</b>
<b>Compound 5</b>	<b>32 ± 3</b>	<b>12 ± 3</b>

\*10nM Enzyme

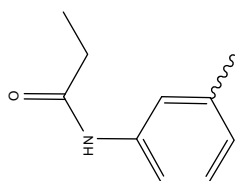
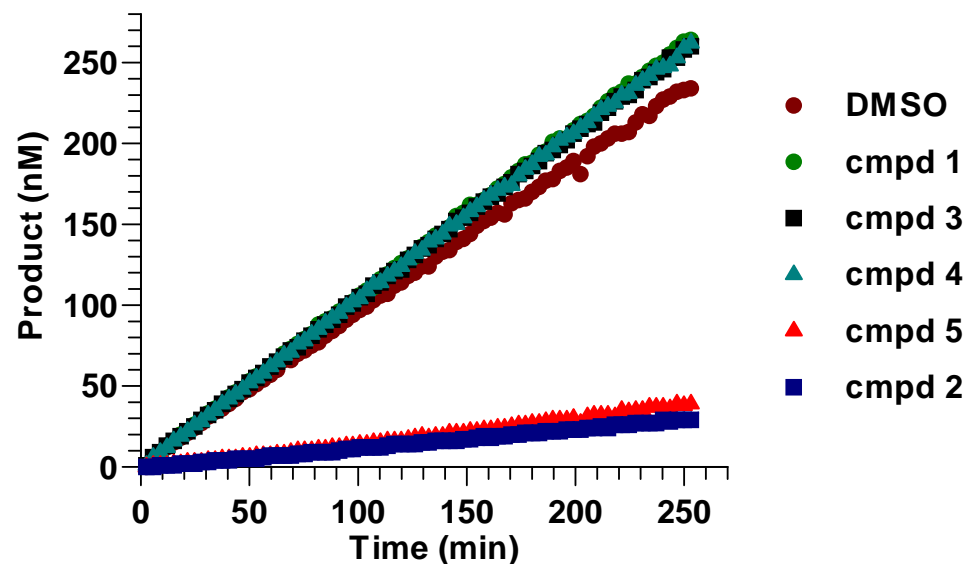
- ◆ In some cases pre-incubation results in lowering IC<sub>50</sub> value (3 to 7-fold)
- ◆ Shift upon pre-incubation is not large; longer pre-incubation times does not result in further decrease in IC<sub>50</sub> values (enzyme = 10 nM)
- ◆ Flat SAR may be due to enzyme concentration ( $IC_{50} = 0.5[E] + K_{iapp}$ )



- ◆  $t_{1/2}$  values imply that  $K_i$  values would vary greatly ( $K_i = k_{off}/k_{on}$ ).
- ◆  $IC_{50}$  values determined in this assay will not drive SAR.
- ◆ As inhibitor potency approaches your  $[E]$  you must determine  $k_{on}$  and  $k_{off}$  values to drive SAR.
- ◆ Caliper allowed team to put  $t_{1/2}$  assay early in testing scheme

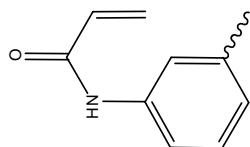
# Designing a Screen for Irreversible Inhibitors

- ◆ Protein Kinase D has cys residue at active site
- ◆ Preincubate kinase and inhibitor for 60 min.
- ◆ Dilute 200-fold into excess MgATP (30X  $\text{app}K_M^{\text{MgATP}}$ )



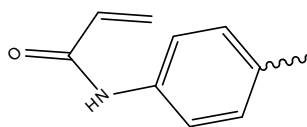
**Cmpd 1**

$$v_i/v^o = 1.1$$



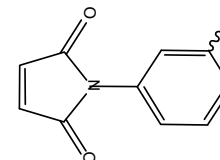
**Cmpd 2**

$$v_i/v^o = 0.13$$



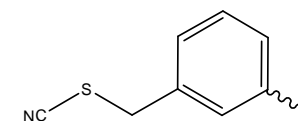
**Cmpd 3**

$$v_i/v^o = 1.1$$



**Cmpd 4**

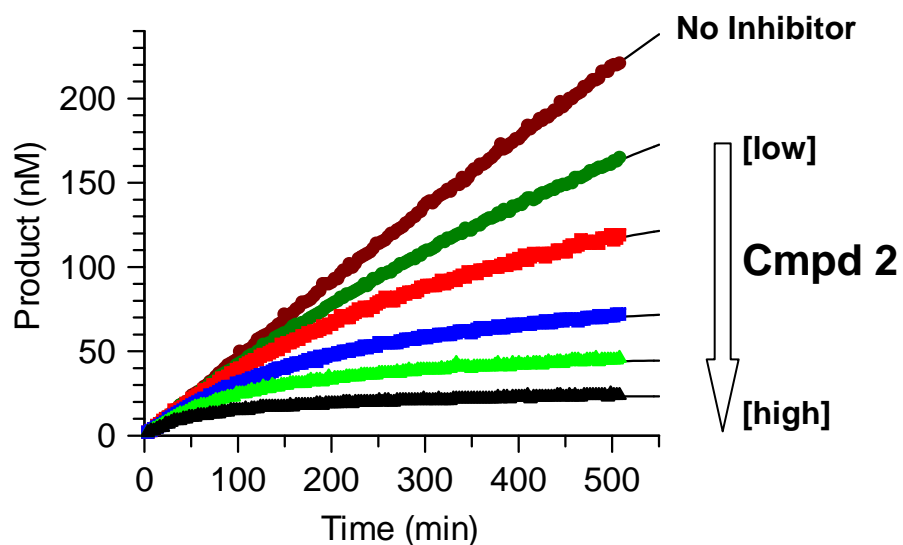
$$v_i/v^o = 1.1$$



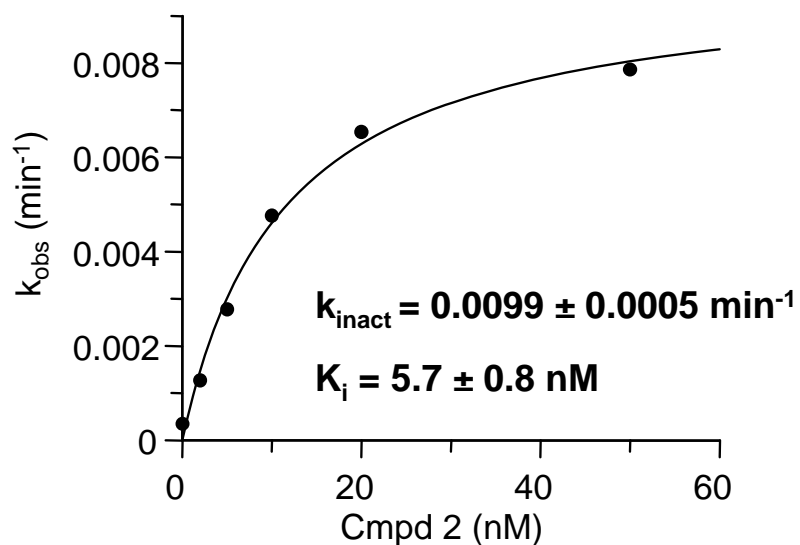
**Cmpd 5**

$$v_i/v^o = 0.17$$

# $K_i/k_{inact}$ for Irreversible Inhibitors to Drive SAR

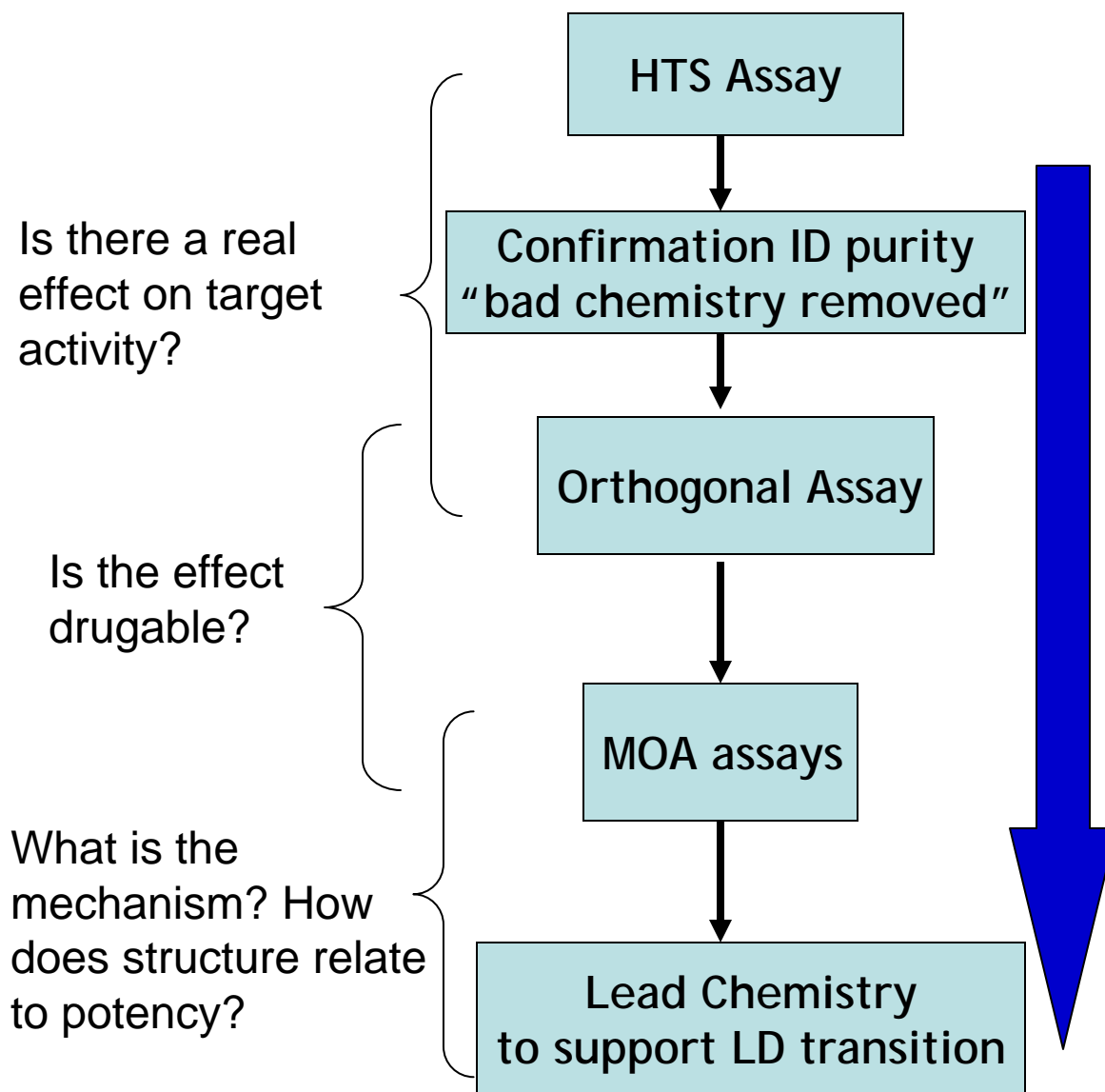


- ◆ SAR for irreversible inhibitors requires more characterization than  $IC_{50}$  +/- preincubation with enzyme
- ◆ Enzyme added last to mixture of MgATP, peptide, and inhibitor
- ◆  $k_{obs}$  were calculated from progress curves



Replot  $k_{obs}$  versus inhibitor concentration

# Based on Learnings: Approach to HTS testing funnel



- ◆ Design the assay based on the desired mechanisms: What inhibitors will you miss?
- ◆ Confirm the target activity is affected (not just the assay)
- ◆ Non-drugable MOA studies.
- ◆ Drugable mechanisms: Caliper allowed team to base compounds on mechanism (competitive, noncompetitive, uncompetitive, time-dependent).
- ◆ Caliper can be used as a tool to develop HTS Assay.
- ◆ Develop deep understanding of how structure is related to potency.



## LC3000 Limitations

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- ◆ Limited fluorescent peptide range (50nM-100uM) without tweaking instrument or adding unlabelled peptide
- ◆ Can not use full length proteins
- ◆ Product conversion required typically higher than desired for enzymology studies (>10%)
- ◆ Limited time run due to evaporation and buffer depletion (typically 6-8 hours)
- ◆ Software capable of only analyzing one product species unless user manually changes software
- ◆ Limited to one phosphorylation site per peptide
- ◆ Area under the curve is not an option



## Acknowledgements

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- ◆ Matt Saabye
- ◆ John Schindler
- ◆ Art Wittwer
- ◆ Mike Davies
- ◆ Molly Hall
- ◆ Heidi Morgan
- ◆ Gary Lange
- ◆ Joe Monahan



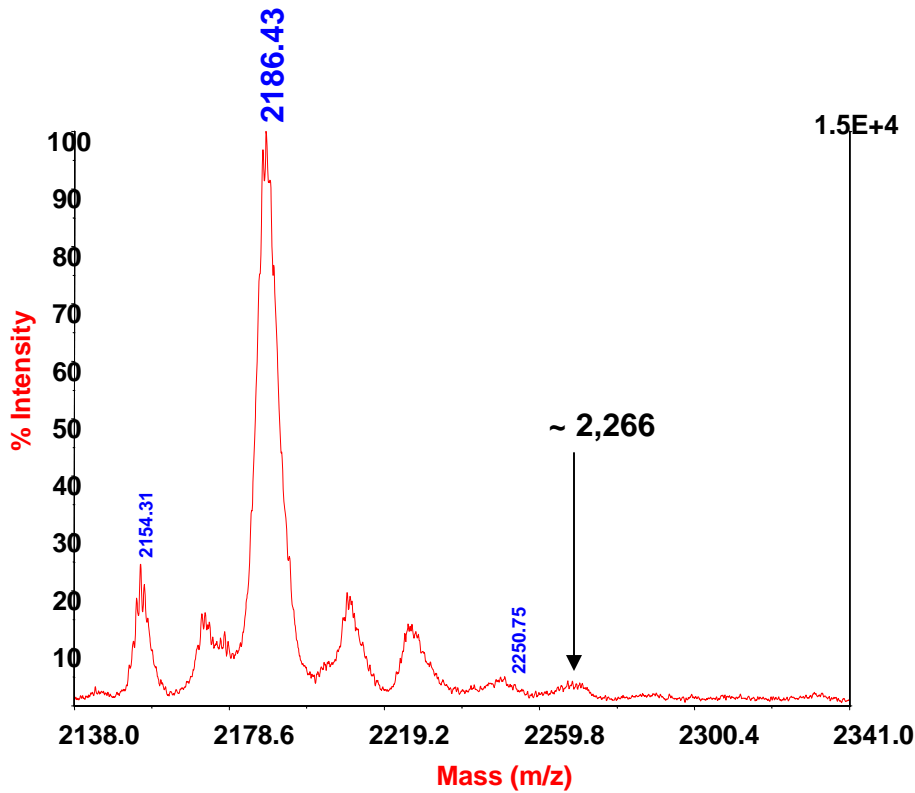
# Backups

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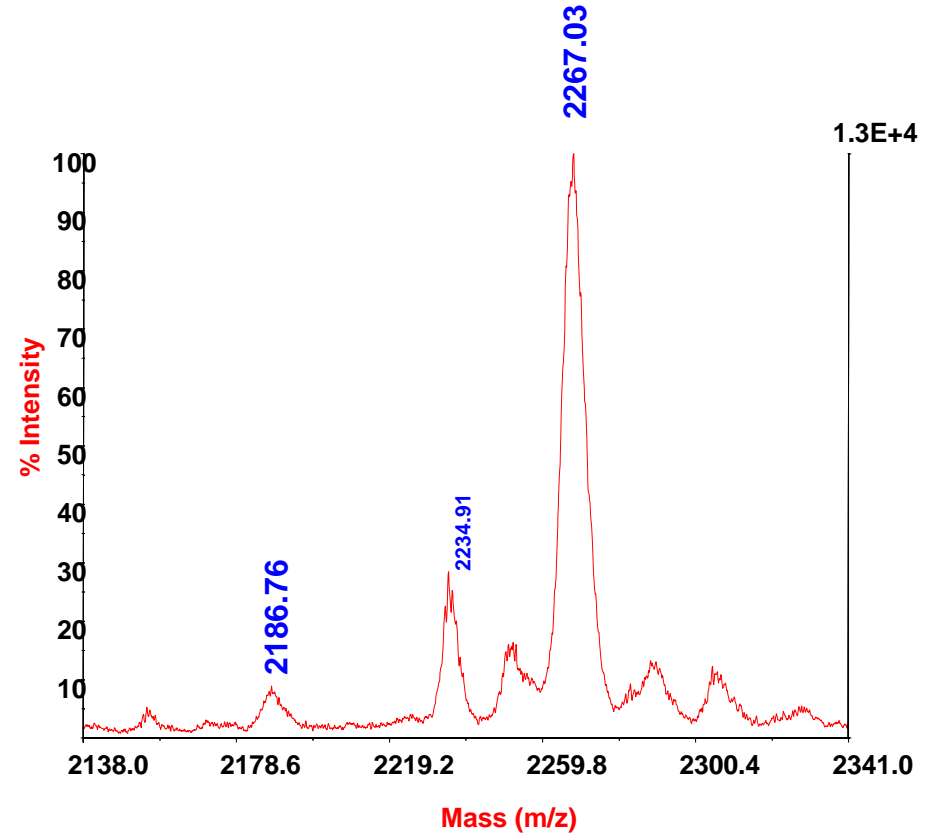


# Mass Spec Data for "Forward" Reaction

## ATP T=0 minutes



## ATP T= 60 minutes



Phosphorylated Peptide = 2,266.4  
Unphosphorylated peptide = 2,186.4



## Kinase Assay Formats that support HTS Full Library

Type of Assay	Readout/Measurement	Pros	Cons
Kinase Glo	Luminescence/Indirect measure of ATP remaining	Inexpensive, can be used for any kinase	50uM ATP concentration, compound interference
Scintillation Proximity Assay	Radioactive/Direct measurement of phosphorylated substrate	Sensitive, used with any substrate	Radioactive, development step slow
IMAP	FP/Measures of flour-phosphorylated peptide tracer	Wide ATP concentration, suitable for numerous Y kinases	Compound Interference, high background from unused reagent
AlphaScreen	Luminensence/Antibody and Bead Based	Detect small amount of product	Interferences – requires artifact assay Releationship between product and single?