

Covalent Modification of Proteins

Kinetics and High-Throughput Library Screening Using the Echo[®] MS+ system

PRESENTER

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RESEARCH GROUP

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SYMPOSIUM

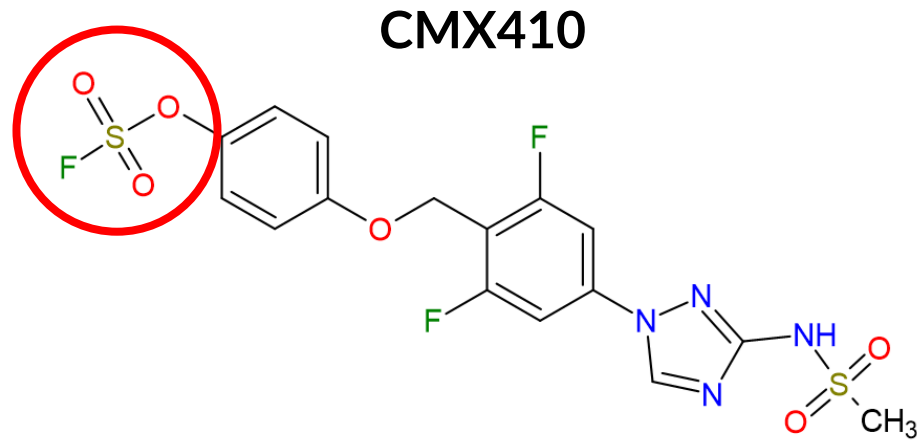
CASSS MS Symposium

Covalent Modification Kinetics

Studying the kinetics of a covalent protein-inhibitor interaction

Covalent Inhibitor CMX410

- Sulfur(VI) fluoride exchange (SuFEx) library screened against *Mycobacterium tuberculosis* (Mtb)
- 24 initial hits, 1 followed up - CEC215
- Further inhibitor optimization led to CMX410
- CEC215/CMX410 target the AT domain of *Mtb* Pks13



SuFEx-based antitubercular compound
irreversibly inhibits Pks13

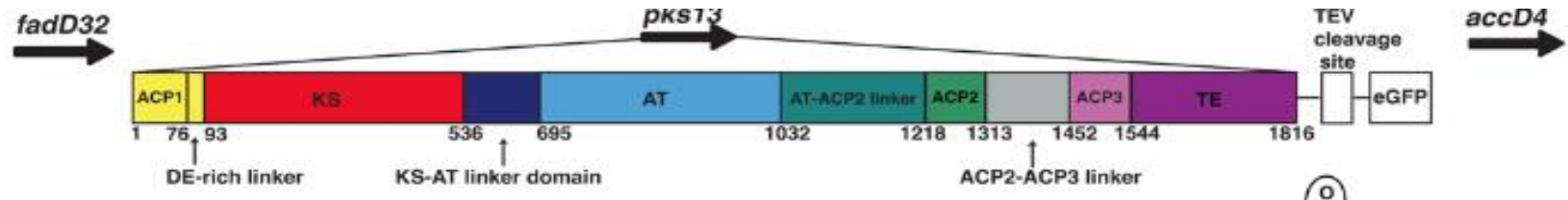
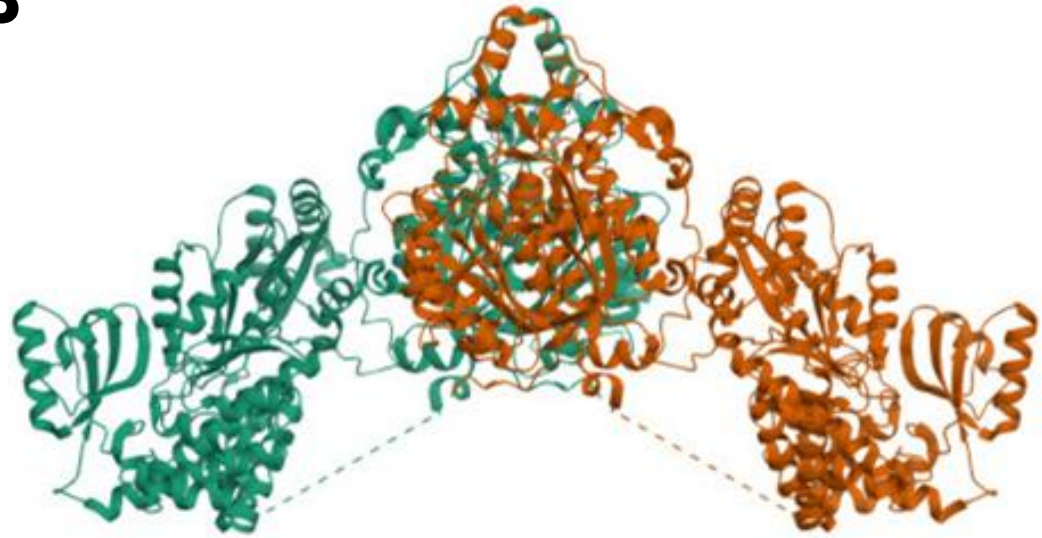
Nature (2025)

Krieger, I.V., Sukheja, P., Yang, B. et al.

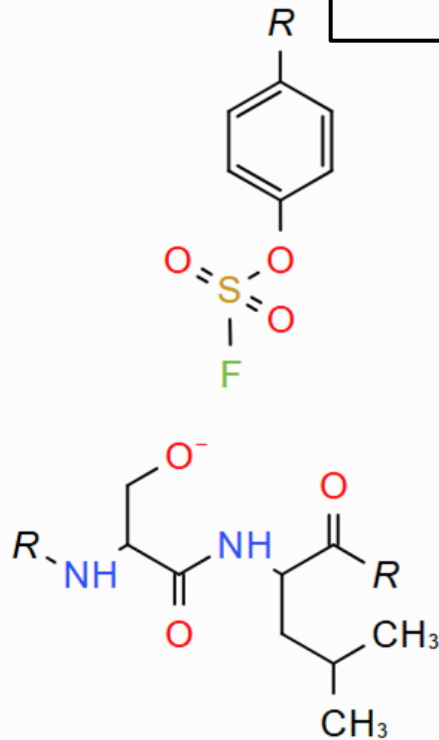
<https://doi.org/10.1038/s41586-025-09286-3>

Background on Pks13

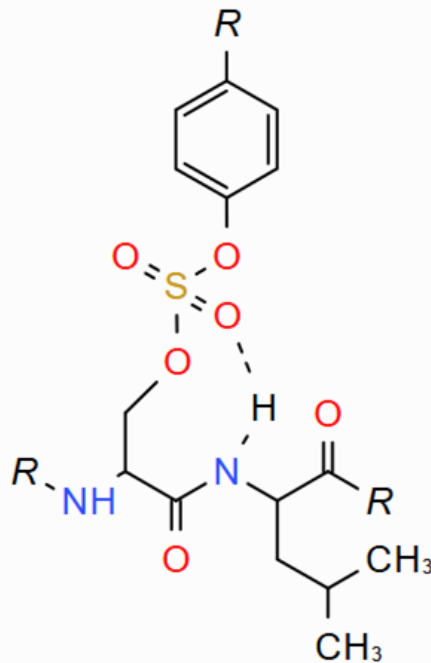
- Polyketide Synthase 13 (Pks13) from Mtb
- Assembly of mycolic acids^{4,5}
- Difficulties in designing traditional activity assays



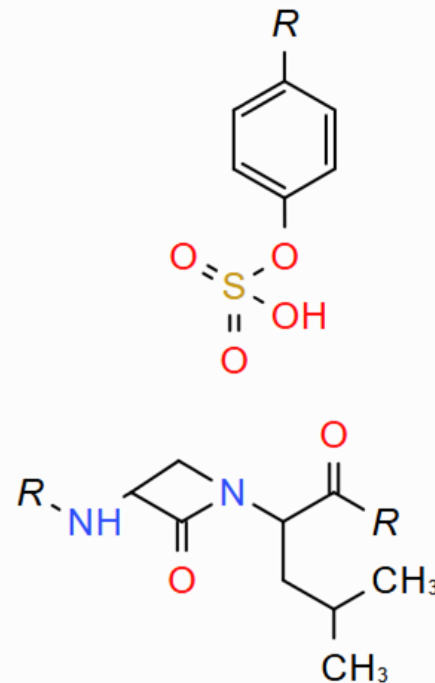
CMX410 Mode of Inhibition



E
Unmodified
Protein



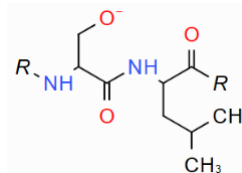
E - I
Covalent
intermediate



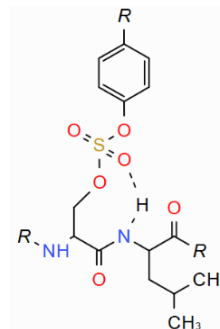
E*
Modified
Protein

Kinetics of Inhibition

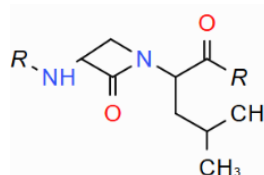
- Unique masses for each inhibition stage
- Turned to a mass-based readout on the Echo® MS+ system
- Kinetic data on the interaction of CMX410 and Pks13-AT
- Changes in 3 protein states over time



Unmodified Protein
53016.98 Da



CMX410-Protein Adduct
53475.3 Da



Modified Protein
52998.58 Da

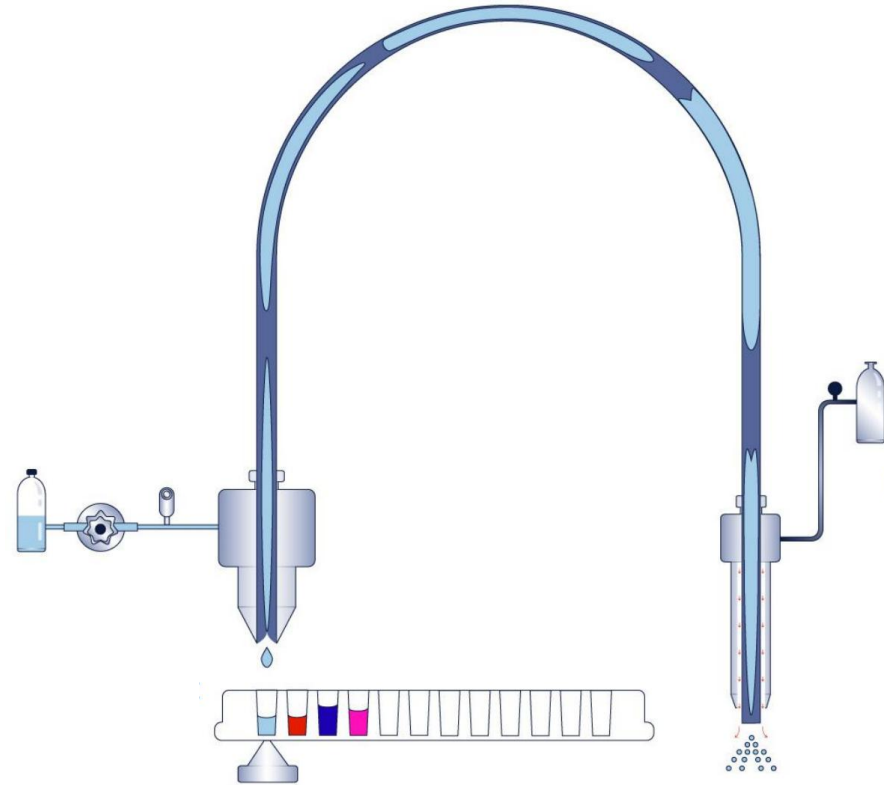
LCMS Kinetics and Intact Protein Analysis

- Kinetics are difficult on conventional LC-MS platforms
 - Rely on discrete stopping points
- Intact protein work is even more technical
 - Extensive protein sample prep
 - Challenging analysis



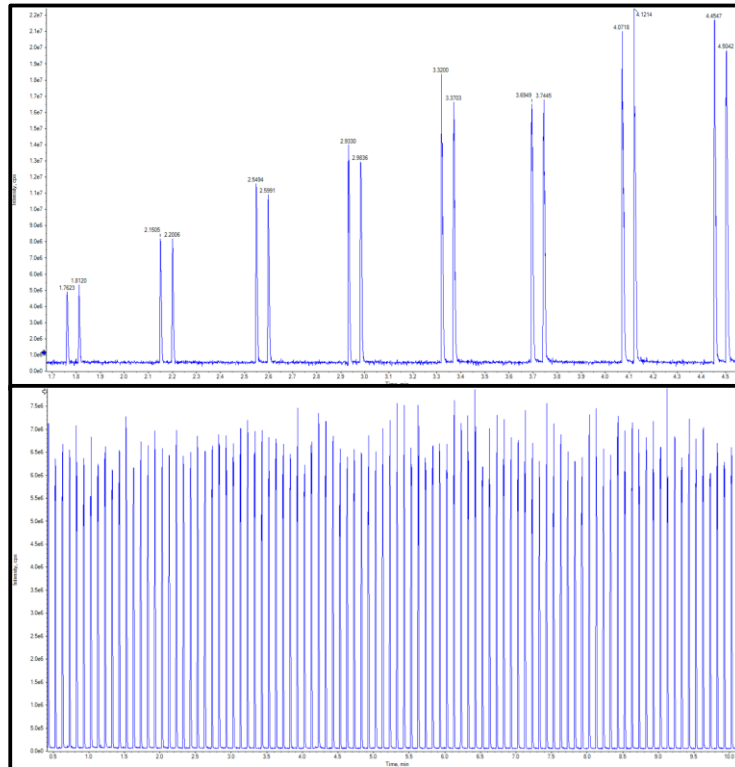
What is the Echo[®] MS+ system?

- High-throughput mass spectrometry
- Acoustic ejection technology
- 2.5 nL droplets ejected into carrier solvent flow
- Quick, accurate, and contactless mass readouts



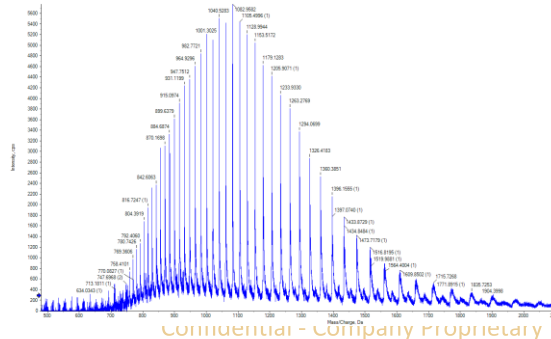
Echo[®] MS+ system Optimization

- Comparatively minimal assay setup for Pks13-AT
- Simple buffer conditions minimize background
- Protein standard curve
- Optimized ejection volume and carrier solvent flow rate



Workflow

- 20 μ M Pks13-AT in 10 mM TRIS pH 7.5
 - Mixed with 20 μ M CMX410
- Ejected replicate samples over 45 minutes
 - 0.1% formic acid in H₂O carrier solvent
 - Flow rate of 225 μ L/min
- Mass deconvolution performed at a resolution of 4,000



Method Parameters

General

Workflow:	Intact proteins
Intact protein mode:	True
Large protein (>70kDa):	False
Decrease detector voltage:	False
Method duration (minutes):	2.86
Total scan time (seconds):	0.024
Estimated cycles:	7118
Actual method duration (minutes):	3.31

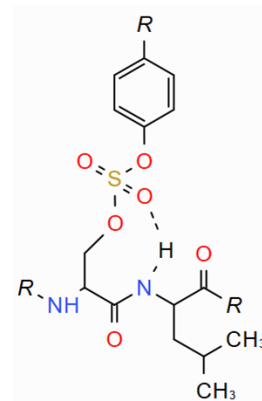
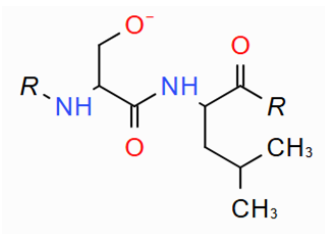
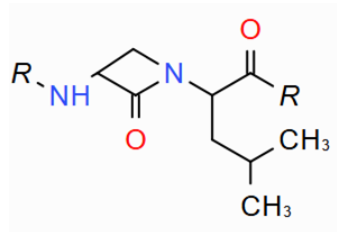
Ion Source

Source name:	Echo® MS probe/MicroCal
Curtain gas:	35
CAD gas:	7
Ion source gas 1 (psi):	90
Ion source gas 2 (psi):	45
Temperature (°C):	450

Experiment

Scan type:	TOFMS
Polarity:	Positive
Spray voltage (V):	5000
Time bins to sum:	80
Channel 1:	True
Channel 2:	True
Channel 3:	True
Channel 4:	True
TOF start mass (Da):	100
TOF stop mass (Da):	4000
Accumulation time (s):	0.02
ITC:	70
Declustering potential (V):	150
Declustering potential spread (V):	0
Collision energy (V):	10
Collision energy spread (V):	0
Override QJet RF value:	False
QJet RF amplitude (V):	212.14142705657

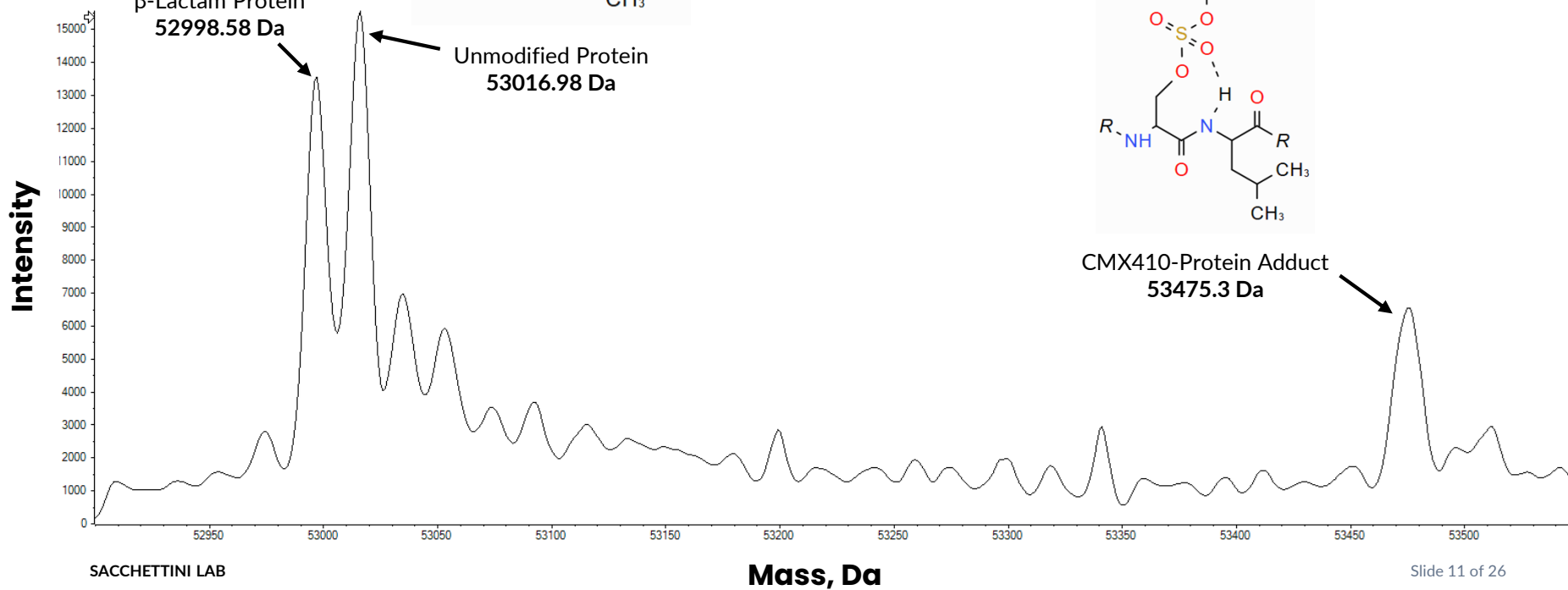
**1:1 20 μ M Pks13-AT and CMX410
10 Minute Incubation**

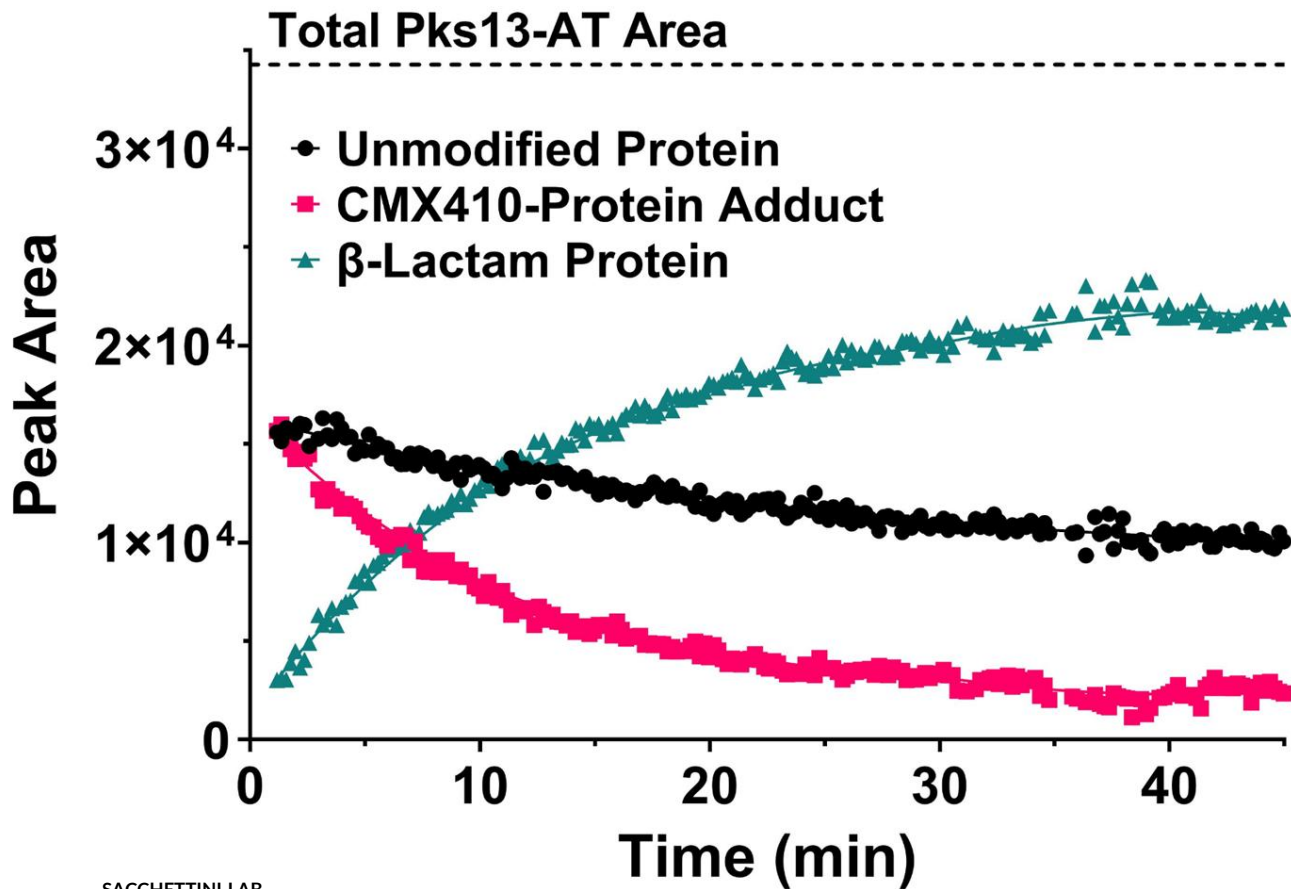


β -Lactam Protein
52998.58 Da

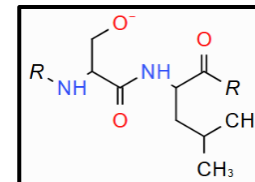
Unmodified Protein
53016.98 Da

CMX410-Protein Adduct
53475.3 Da

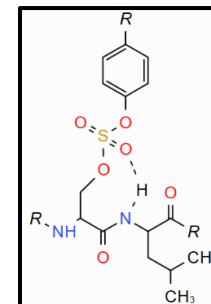




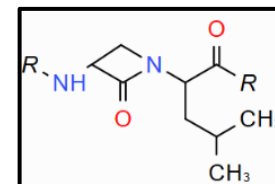
Unmodified Protein



CMX410-Protein Adduct



Modified Protein

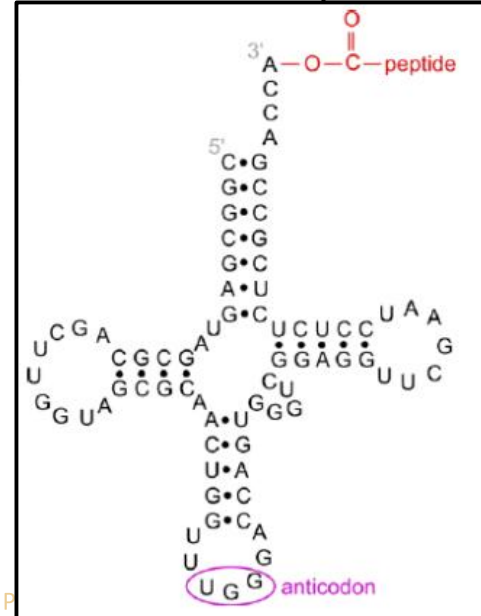
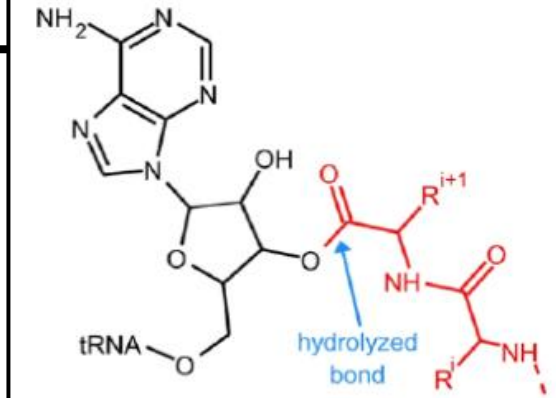


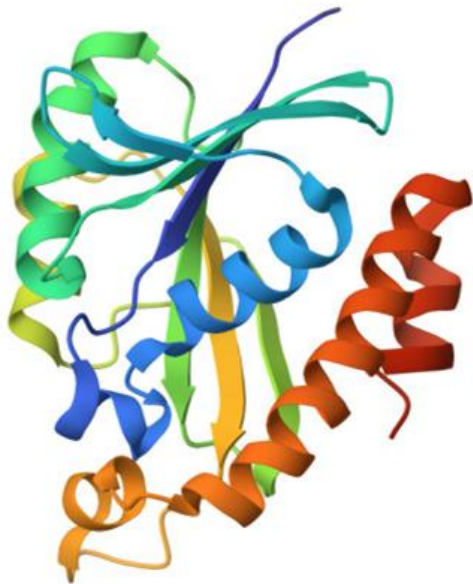
Covalent Libraries in HTS

Taking what we learned and scaling it up to the next level

Background on Pth

- mRNA translation errors result in stalled ribosomes
- Peptidyl-tRNAs can dissociate from the ribosome⁸
- Accumulated free peptidyl-tRNAs can lead to cell death
- tRNA is recycled by Peptidyl tRNA hydrolase (Pth),⁸



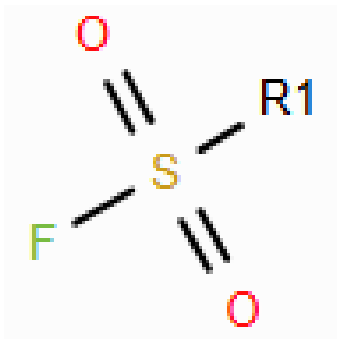


Drug Discovery Complications

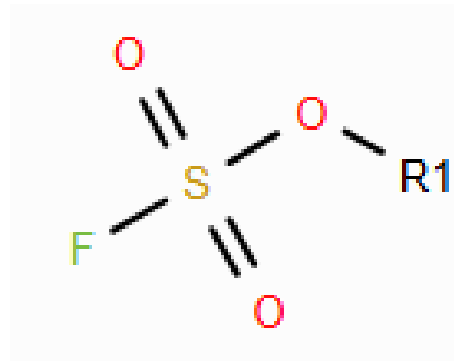
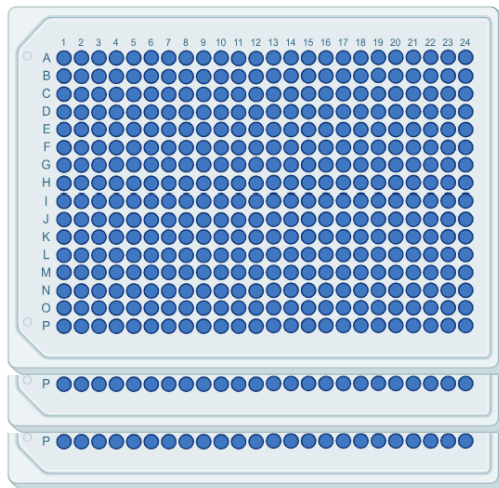
- Natural substrate is complex and unstable
 - Difficult to produce at a large scale for screening
- Crystal structures solved
- Little knowledge on *in vivo* function or activity
- No published inhibitory data to date

Covalent Library Screening

- Basis from Pks13-AT kinetics study on the Echo[®] MS+ system
- Screened reactive warhead SuFEx library containing fluorosulfates and sulfonyl fluorides in a high-throughput 384 well format



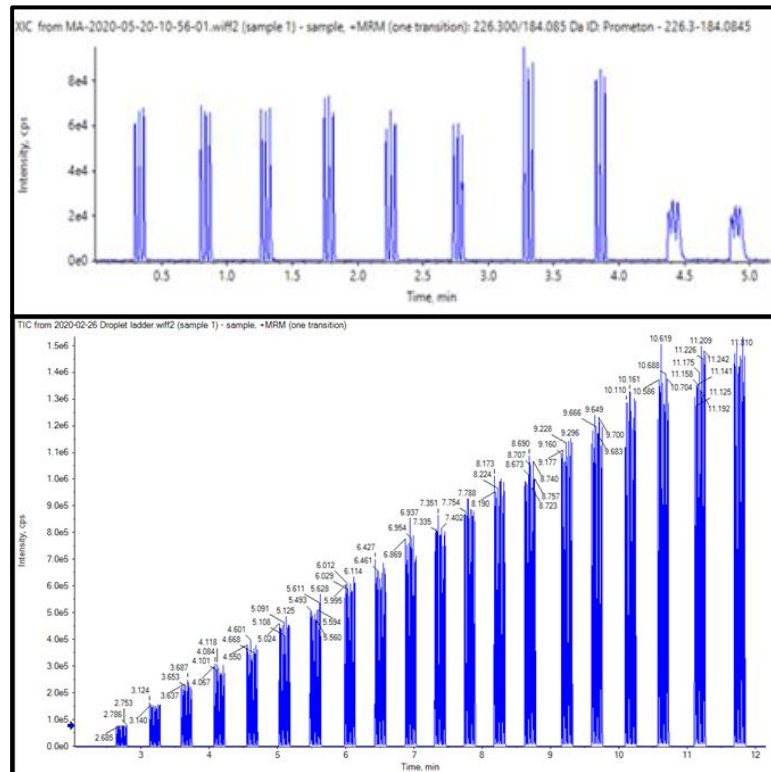
Sulfonyl Fluorides



Fluorosulfates

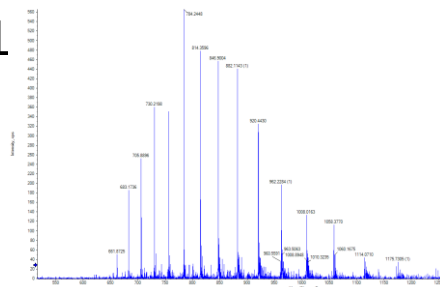
Echo[®] MS+ system Optimization

- Pth optimization similar to Pks13-AT
- Same buffer conditions
- Protein standard curve
 - Important at high-throughput scale
- Optimized ejection volume and carrier solvent flow rate



Workflow

- 15 μM Pth in 10 mM TRIS pH 7.5
 - Mixed with 100 μM or 20 μM compound
- Ejected samples at 45 minutes and 2 hours
 - 0.1% formic acid in H₂O carrier solvent
 - Flow rate of 300 μL
- Mass deconvolution performed at a resolution of 2,500



Method Parameters

General

Workflow:	Intact proteins
Intact protein mode:	True
Large protein (>70kDa):	False
Decrease detector voltage:	False
Method duration (minutes):	12.68
Total scan time (seconds):	0.104
Estimated cycles:	7328
Actual method duration (minutes):	13.45

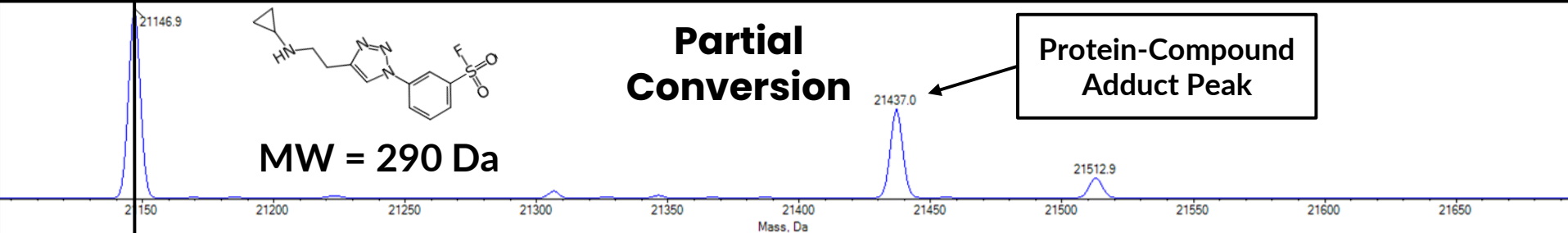
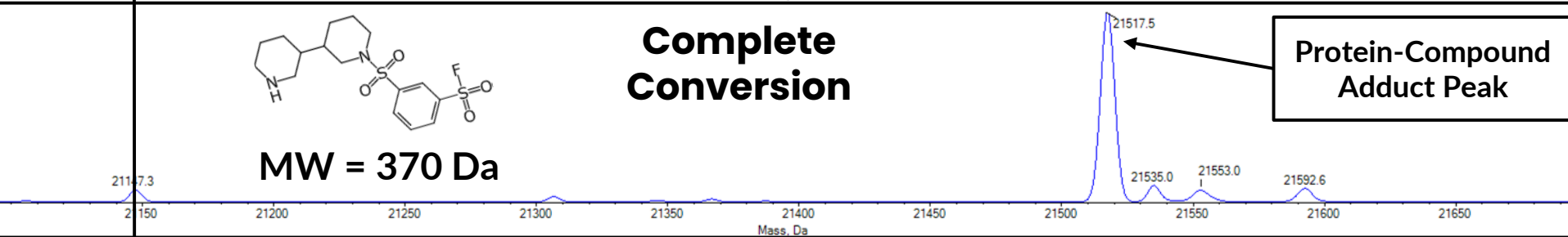
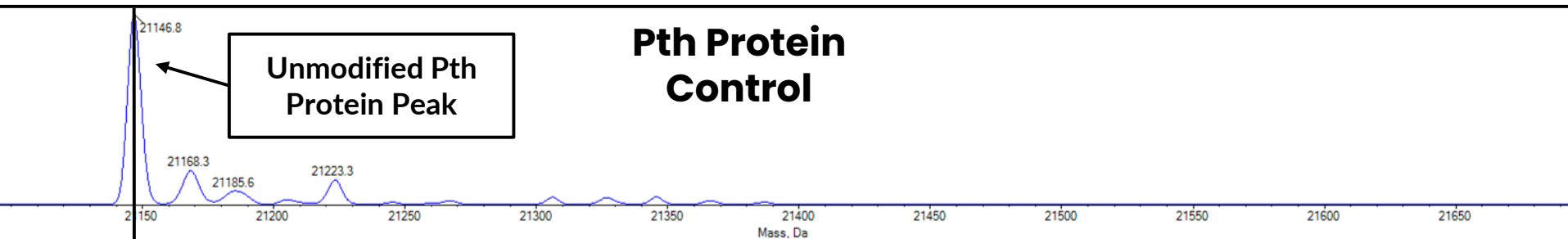
Ion Source

Source name:	Echo® MS probe/MicroCal
Curtain gas:	35
CAD gas:	7
Ion source gas 1 (psi):	90
Ion source gas 2 (psi):	45
Temperature (°C):	450

Experiment

Scan type:	TOFMS
Polarity:	Positive
Spray voltage (V):	5000
Time bins to sum:	80
Channel 1:	True
Channel 2:	True
Channel 3:	True
Channel 4:	True
TOF start mass (Da):	100
TOF stop mass (Da):	4000
Accumulation time (s):	0.1
ITC:	70
Declustering potential (V):	150
Declustering potential spread (V):	0
Collision energy (V):	10
Collision energy spread (V):	0
Override QJet RF value:	False
QJet RF amplitude (V):	212.141427056575

15 μ M Pth : 20 μ M Compound
2 Hour Incubation



Data Analysis

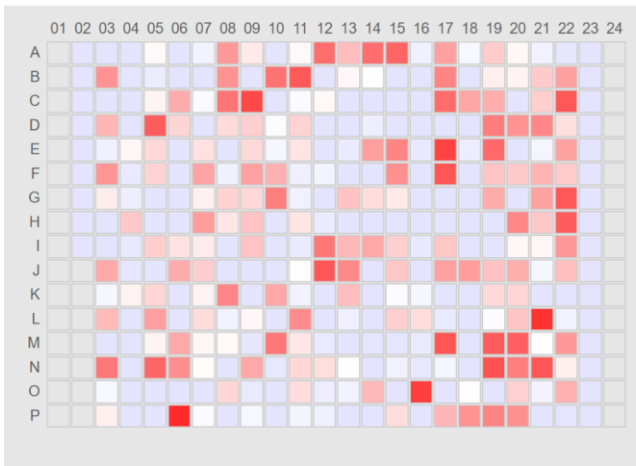
- Identifying covalent adducts with Pth
- Calculated expected peak shift MW
- Separately integrated unmodified and shifted protein peaks
- Compared and assigned percent conversion value:

Unmodified Pth
Protein Peak
21,146 Da

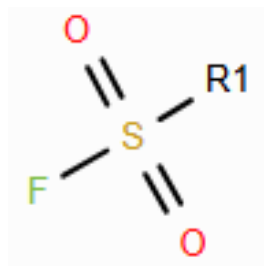
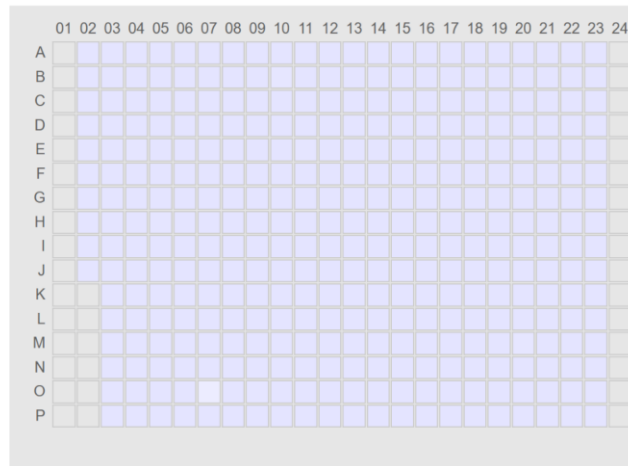
$\frac{\text{Shifted Protein Area}}{\text{Shifted Protein Area} + \text{Unmodified Protein Area}} \times 100$						
Well	Unmodified Protein Peak Area	Shifted Peak Area	Shifted Peak MW		Compound MW	Calculated Shifted MW
A02	28422.47217	0	0			21126.66949
A03	1280.392483	0	0		356.4554	21482.98684
A04	23721.34427	0	0		343.4136	21470.22339
A05	16413.26181	3430.55712	21437.3651		310.3472	21437.16097
A06	22899.59114	0	0		335.3981	21462.30692
A07	20610.73452	1038.52413	21441.3301		314.3359	21441.20941

15 μ M Pth : 20 μ M Compound 2 Hour Incubation

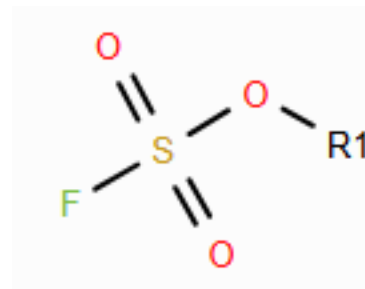
1925944-Y40-01



1925944-Y40-02



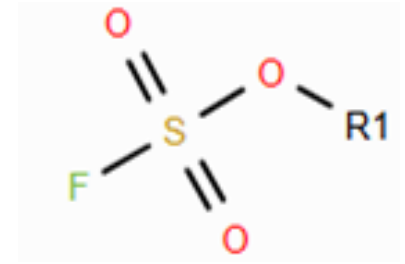
Sulfonyl Fluorides



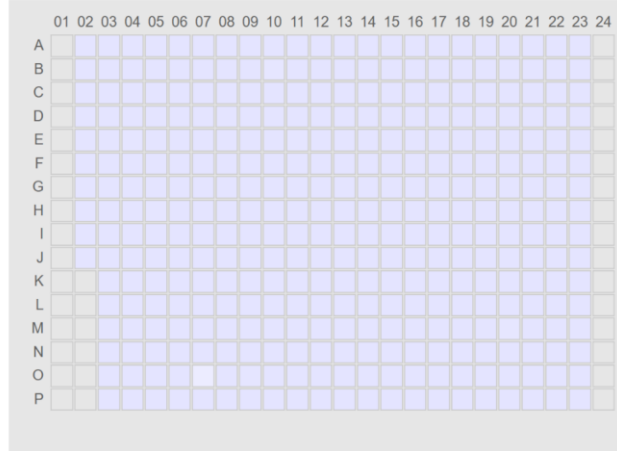
Fluorosulfates

Fluorosulfates

15 μ M Pth + 100 μ M Compound

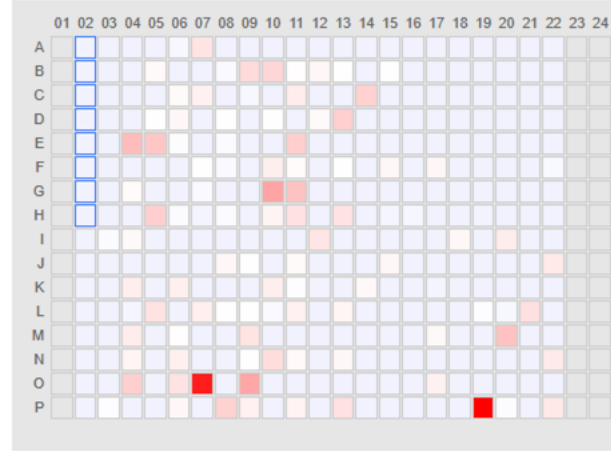


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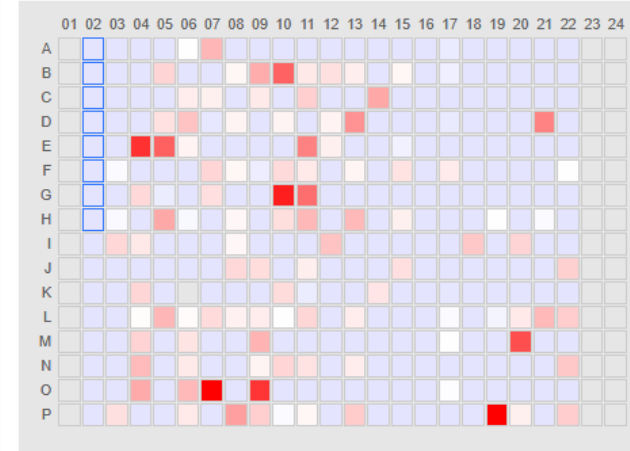
2 Hours
Incubation

1925944-Y40-02



7 Hours
Incubation

1925944-Y40-02



24 Hours
Incubation

A Case for the Echo[®] MS+ system

- Distinct proteins with unrelated substrates
- Traditional assay methods proved ineffective
- Echo[®] MS+ system provided unexplored avenue
 - Starting point for drug discovery on Pth
 - Furthered research on Pks13-AT by enabling kinetic studies



Special thanks to Dr. James
Sacchettini and Dr. Inna Kreiger for
their expertise and years of work
that made this data possible

Questions?

References

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