9. Identifying Unknown Proteins

Activity-based Profiling

1. Serine protease inhibitors
2. Enzyme inhibitor discovery

Cravatt et al. *Nat. Chem. Biol.* **2008**, 405

Niphakis, Cravatt *Annu. Rev. Biochem.* **2014**, 341–377

Target-centric approach:

1. Serine protease inhibitors

Chemocentric approach:

2. Enzyme inhibitor discovery

Serine proteases

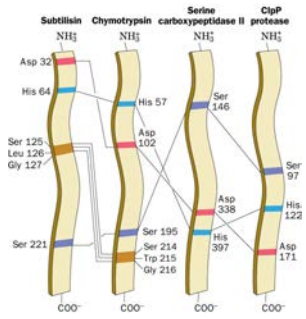
Found widely in vivo: eukaryotic and prokaryotic **digestive enzymes**, **thrombin**, **acetylcholinesterase**. They are very similar conceptually to **cysteine** and **aspartate proteases** and some dehydrogenases.

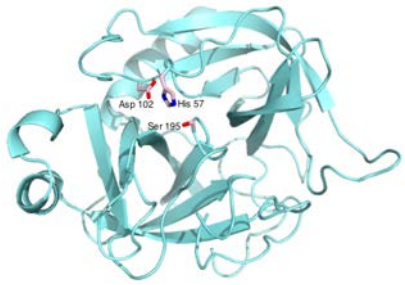
- 16 superfamilies x 1 to 14 families (which several homologs)
- Categorised accroding to substrate specificity: **trypsin**-like, **chymotrypsin**-like or **elastase**-like.

Enzyme	Source	Function
Trypsin	Pancreas	Digestion of proteins
Chymotrypsin	Pancreas	Digestion of proteins
Elastase	Pancreas	Digestion of proteins
Thrombin	Vertebrate serum	Blood clotting
Plasmin	Vertebrate serum	Dissolution of blood clots
Kallikrein	Blood and tissues	Control of blood flow
Complement C1	Serum	Cell lysis in the immune response
Acrosomal protease	Sperm acrosome	Penetration of ovum
Lysosomal protease	Animal cells	Cell protein turnover
Cocoonase	Moth larvae	Dissolution of cocoon after metamorphosis
α-Lytic protease	*Bacillus sorangium*	Possibly digestion
Proteases A and B	*Streptomyces griseus*	Possibly digestion
Subtilisin	*Bacillus subtilis*	Possibly digestion

Source: Stroud, R.M., *Sci. Am.* **231**(1), 86 (1974).

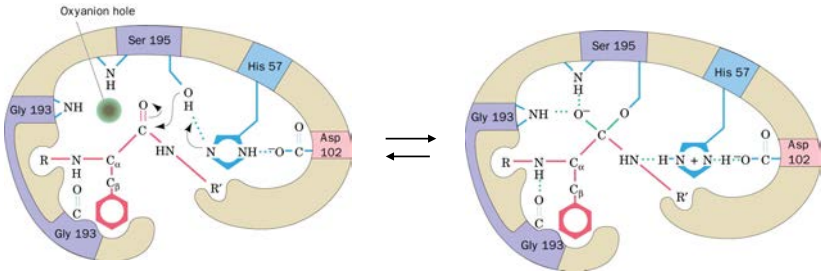
- Catalytic site requires the triad of **Asp/His/Ser** — that are *not* contiguous AA in the primary sequence.





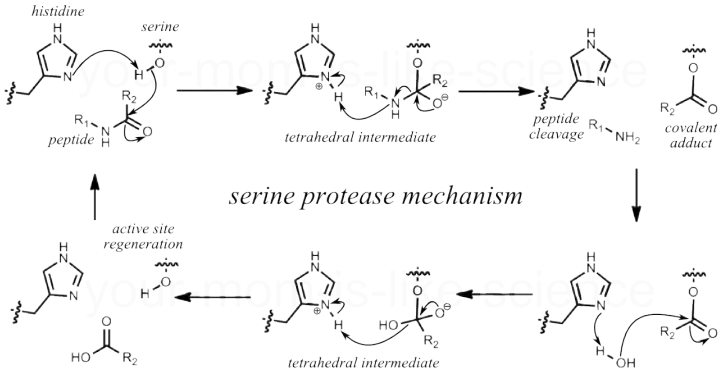
Distinctive structure, consisting of two beta-barrel domains that converge at the catalytic active site.

- Catalytic site requires the triad of **Asp/His/Ser** — that are *not* contiguous AA in the primary sequence.

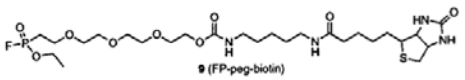


• Enhanced nucleophility of SerOH by His: the His: -> HisH+ that is stabilised by Asp-
• SerOH attacks >C=O to give tetrahedral intermediate with O- stabilised on oxyanion hole
• Restoration of C=O to acyl enzyme and amine leaves but needs the proton from HisH+.

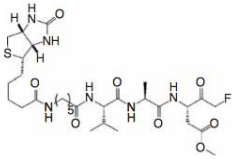
• His now activates a water molecules H-OH by attracting H+ to give "HO-" that attacks C=O to give tetrahedral intermediate stabilised by oxyanion hole.
• restoration of C=O give acid and SerOH as leaving group (Ser gets H+ from HisH+).



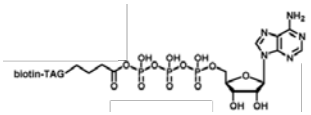
Serine protease - inhibitors ***diversity***



Cravatt, B.F. Biochemistry 40, 4005–4015 (2001).

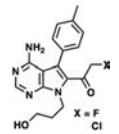


Bogyo, Nat. Chem. Biol. 1, 33–38 (2005)

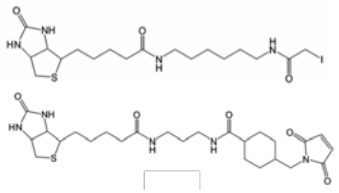


Biochemistry 46, 350–358 (2007).

Src	[276]	LGQGCFGEVWMGTWNG
RSK1	[424]	IGVGSYSVCKRCVHKA
RSK2	[428]	IGVGSYSVCKRCIHKA
RSK3	[421]	IGVGSYSVCKRCVHKA
RSK4	[432]	IGVGSYSVCKRCIHAT
MSK1	[432]	LGEGSFSICRKCVHKK
MSK2	[395]	LGQGSFSVCRRCRQRQ
PLK1	[59]	LGKGGFAKCFEISDAD
PLK2	[88]	LGKGGFAKCYEMTDLT
PLK3	[29]	LGKGGFARCYEATDTE
NEK2	[14]	IGTGSYGRCQKIRRKS
MEKK1	[1300]	IGLGAPSSCYQAQDVG



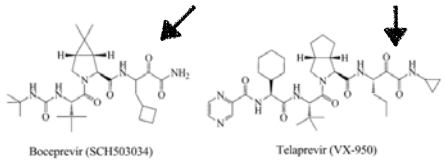
Shokat, et al. Science 308, 1318–1321 (2005).



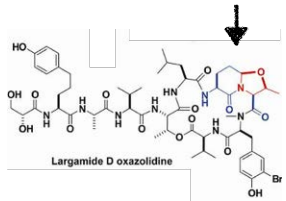
Chem. Res. Toxicol. 20, 859–867 (2007)

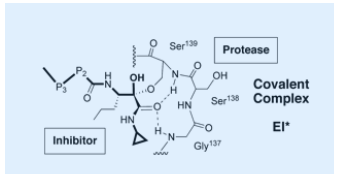
Serine protease - inhibitors

Most drugs are not specific…



hepatitis C virus (HCV)



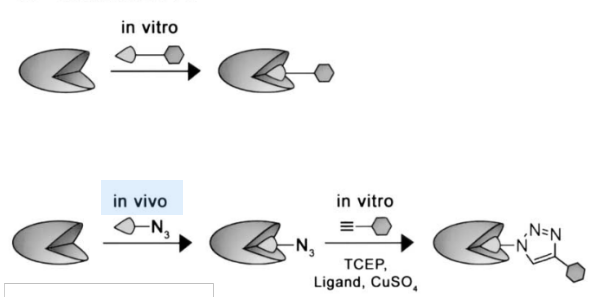


The challenge:

How can we test (in vivo) to find **specific** inhibitors?

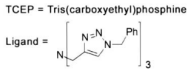
1. Find how many **off-target proteins** interact with your drug,
2. Analyze **structural features** for common elements / differences,
3. **Design new drug** candidates based on specific features.

Comparison of **standard** and **click chemistry** ABP



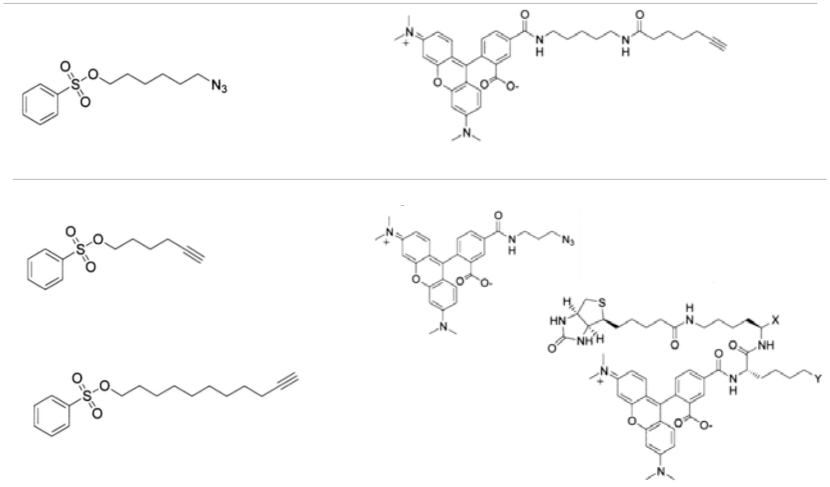
In contrast to standard ABPP, **click chemistry ABPP allows for the profiling of living cells and organisms** by treating these specimens with tag-free azide- or alkyne-modified probes, which are then conjugated *in vitro* to the complementary alkyne- or azide-modified tag to visualize probe-labeled proteins.

TCEP = Tris(carboxyethyl)phosphine



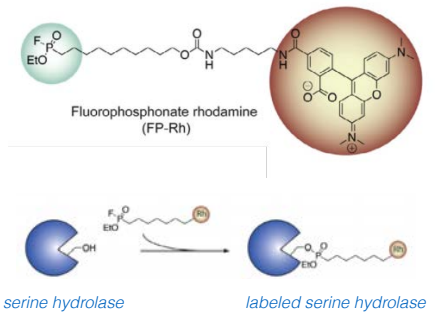
Seepers, Cravatt *Chem. Biol.* **2004**, 535.

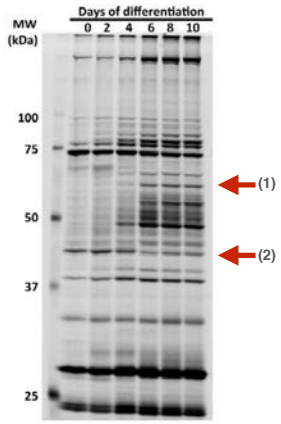
Probes **Tags**



Seepers, Cravatt *Chem. Biol.* **2004**, 535.

Example: **Serine hydrolase**





Reporter-tagged **fluorophosphonate probes react covalently** only with the active form of serine hydrolases. They can be used to profile the pattern of serine hydrolase activity by gel electrophoresis. **Upregulation** (**1**) and **downregulation** (**2**) of proteins.

Cravatt *et al. Methods Enzymol.*, Chp. 9, 151–169 (2014)

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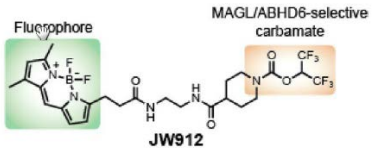

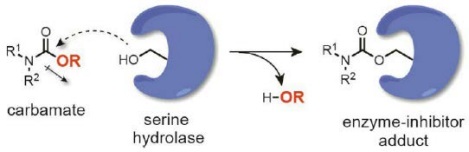

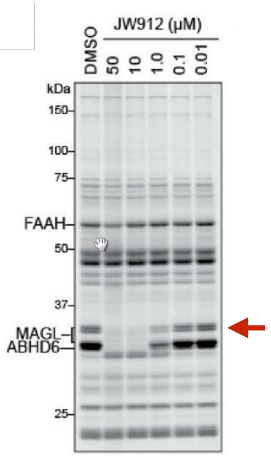

Cravatt *et al. ACS Chem. Biol.*, **2013**, 1590

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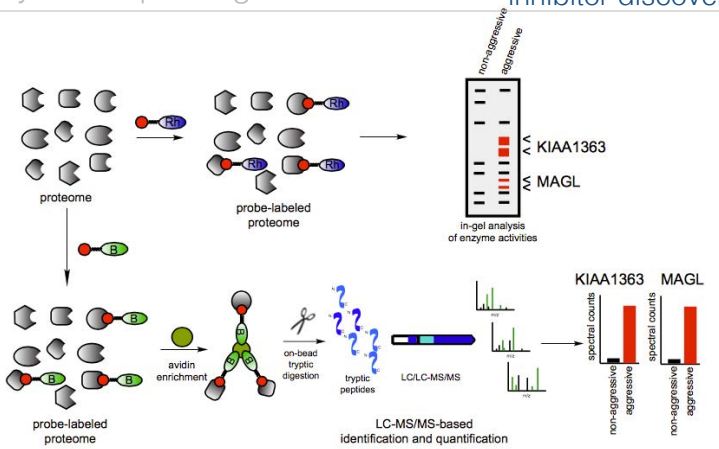
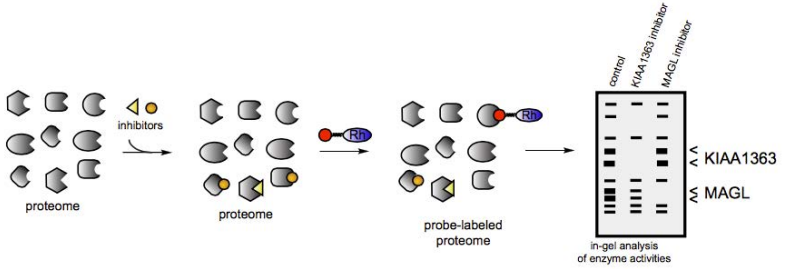


Activity-Based Protein Profiling Coupled with Untargeted Metabolomics in Annotating Dysregulated Enzyme Activities in Aggressive Cancers. ABPP uses active site-directed probes to assess the functional state of large numbers of enzymes directly in complex proteomes. In a typical ABPP experiment, a proteome is reacted with the activity-based probe and readout either by fluorescence on a SDS-PAGE gel (top path), or by avidin enrichment, on-bead tryptic digest, and identification and quantification of peptides (bottom path).

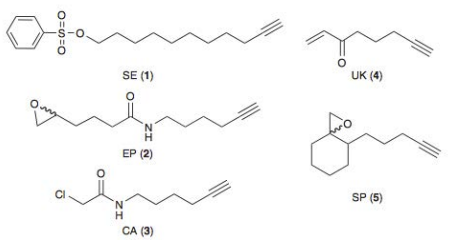
Cravatt *et al. Cell Metabol.* **2012**, 565



Competitive ABPP for enzyme and inhibitor discovery. Activity-Based Protein Profiling can also be used in a competitive format to assess potency and selectivity of inhibitors in complex proteomes. Inhibitors can compete with the ABP and enzyme inhibition will be read out by loss of fluorescence on a SDS-PAGE gel or loss of spectral counts by mass spectrometry.

Cravatt *et al. Cell Metabol.* **2012**, 565

Disparate proteome **reactivity profiles of carbon electrophiles**



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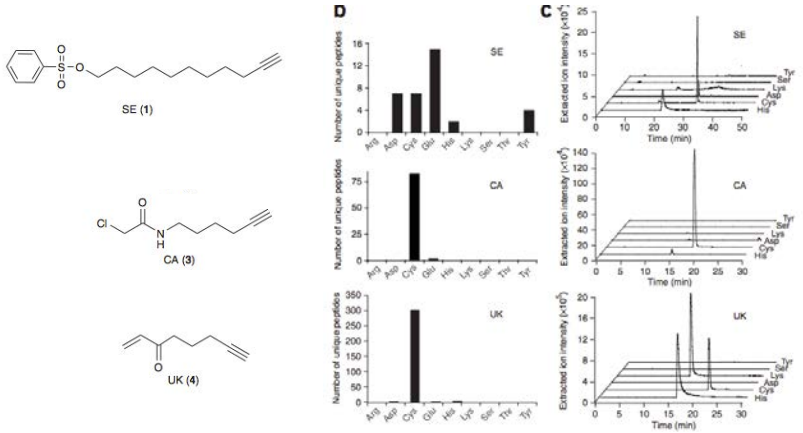
Disparate proteome **reactivity profiles of carbon electrophiles**



Cravatt et al. *Nat. Chem. Biol.* **2008**, 405

Disparate proteome **reactivity profiles of carbon electrophiles**

Description	Peptide	UK	SE	CA	Site of labeling	Function
δ-3,5-δ-2,4-dienoyl-CoA isomerase	EVDMGLAAD*VGTLQR	–	755	–	Asp204	Active site proton donor
Long-chain specific acyl-CoA dehydrogenase	GFYYLMQELPQE*R	–	26	–	Glu291	Active site proton acceptor
Corticosteroid-11 β-dehydrogenase 1	MTQPMIAPY*SASK	–	4	–	Tyr183	Active site proton acceptor
UDP-glucose 6-dehydrogenase	ASVGFGGSC*FQK	–	–	18	Cys276	Active site nucleophile
Nitrilase 2	VGLGIC*YDMR	–	–	125	Cys153	Active site nucleophile
Chloride intracellular channel protein 4	AGSDGESIGNC*PFSQR	–	–	74	Cys35	Site of nitrosylation
Alcohol dehydrogenase 1	IDGASPLDKVCLIGC*GFSTGYGSAVK	13	–	–	Cys175	Zinc binding
	VIPLFSPQC*GECR	18	–	–	Cys98	Zinc binding
	VIPLFSPQCGEC*R	22	–	–	Cys101	Zinc binding
L-lactate dehydrogenase B chain	ITVVGVGQVGMAC*AISILGK	45	–	–	Cys36	NAD binding domain
Ubiquitin-conjugating enzyme E2 D2	IYHPNINSNGSIC*LDILR	29	–	–	Cys85	Active site glycyl thioester intermediate

Asterisks after residues denote probe-labeled residues; numerical values denote total spectral counts for each probe across all proteomic samples.

Cravatt et al. *Nat. Chem. Biol.* **2008**, 405

Activity-based profiling

Activity-based protein profiling probes are available for a variety of enzyme classes

2-Deoxy-2-fluoro glycoside	Exo- and endoglycosidases	Vocadlo and Bertozzi (2004) and Hekmat et al. (2005)
Acyloxymethyl ketone	Cysteine proteases	Kato et al. (2005)
E-64 based	Papain-like cysteine proteases	Bogyo, Verhelst, Bellingard-Dubouchaud, Toba, and Greenbaum (2000)
Photoreactive benzophenone-hydroxamate	Metalloproteases	Saghatelian, Jessani, Joseph, Humphrey, and Cravatt (2004); Chan, Chattopadhaya, Panicker, Huang, and Yao (2004)
Photoreactive benzophenone-hydroxyl-ethylene	Aspartyl proteases	Li et al. (2000)
Quinolimine methide	Proteases	Zhu, Girish, Chattopadhaya, and Yao (2004)
Quinone methide	Tyrosine phosphatases–glycosidases	Lo et al. (2002) and Tsai et al. (2002)
Sulfonate ester	Dehydrogenases–glutathione *S*-transferases–sugar kinases–epoxide hydrolases–transglutaminases	Adam, Sorensen, and Cravatt (2002)
Vinyl sulfone	Proteasome	Bogyo (2005); Verdoes et al. (2006)
Vinyl sulfone	Ubiquitin-specific proteases	Borodovsky et al. (2005)
Wortmannin analogues	Lipid and protein kinases	Yee, Fas, Stohlmeyer, Wandless, and Cimprich (2005); Liu, Shreder, Gai, Corral, Ferris, and Rosenblum (2005)
α-Bromobenzylphosphonate	Tyrosine phosphatases	Kumar et al. (2004)
Acyl-phosphate	Kinases/ATPases	Patricelli et al. (2007)

Cravatt *et al. Methods Enzymol.*, Chp. 9, 151–169 (2014)

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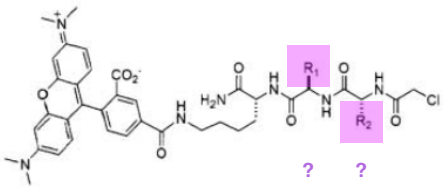
Activity-based profiling — inhibitor discovery

Example: **Enzyme activities differentially expressed in lean and obese mice**



Cravatt *et al. Chem. Biol.*, **2004**, 1523

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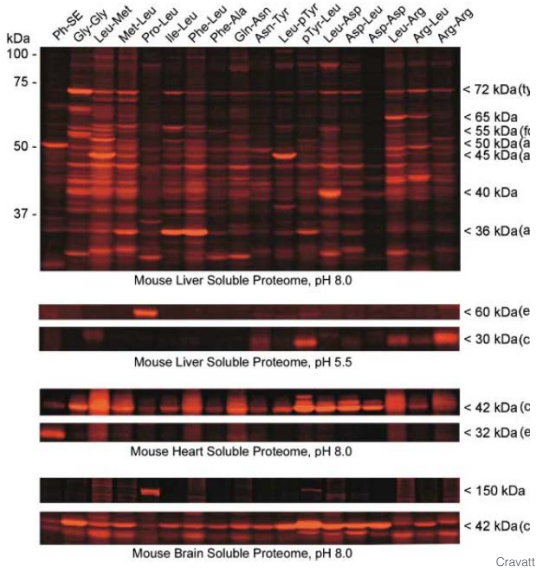
Activity-based profiling — inhibitor discovery



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24

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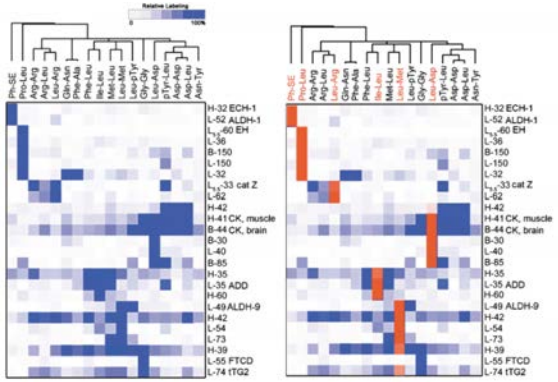

Cluster Analysis of Proteome Reactivity Profiles of -CA Dipeptide Probes. A representative probe was selected to form an optimal probe set for biological experiments (profiles of the six selected probes are shown in red).

Cravatt *et al. Chem. Biol.*, **2004**, 1523

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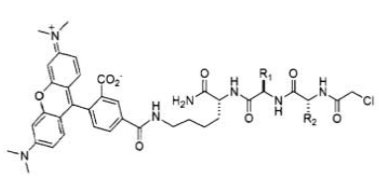

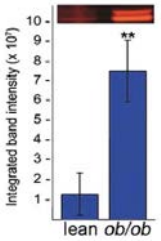

Quantitation of the labeling intensity of HPR with the Leu-Asp--CA probe in wt and ob/ob liver.

Hydroxypyruvate reductase was 6-fold upregulated in obese livers. HPR participates in the conversion of serine to glucose, suggesting that this unusual metabolic pathway may contribute to gluconeogenesis selectively in states of obesity.

Cravatt *et al. Chem. Biol.*, **2004**, 1523