Synthesis of Small Molecule Inhibitors of Protein Kinase D and Botulinum Neurotoxin A1

James Johnson

Wipf Group Topic seminar 9/7/13

Synthesis of Small Molecule Inhibitors of Protein Kinase D and How I learned to Love PMB Protecting Groups.

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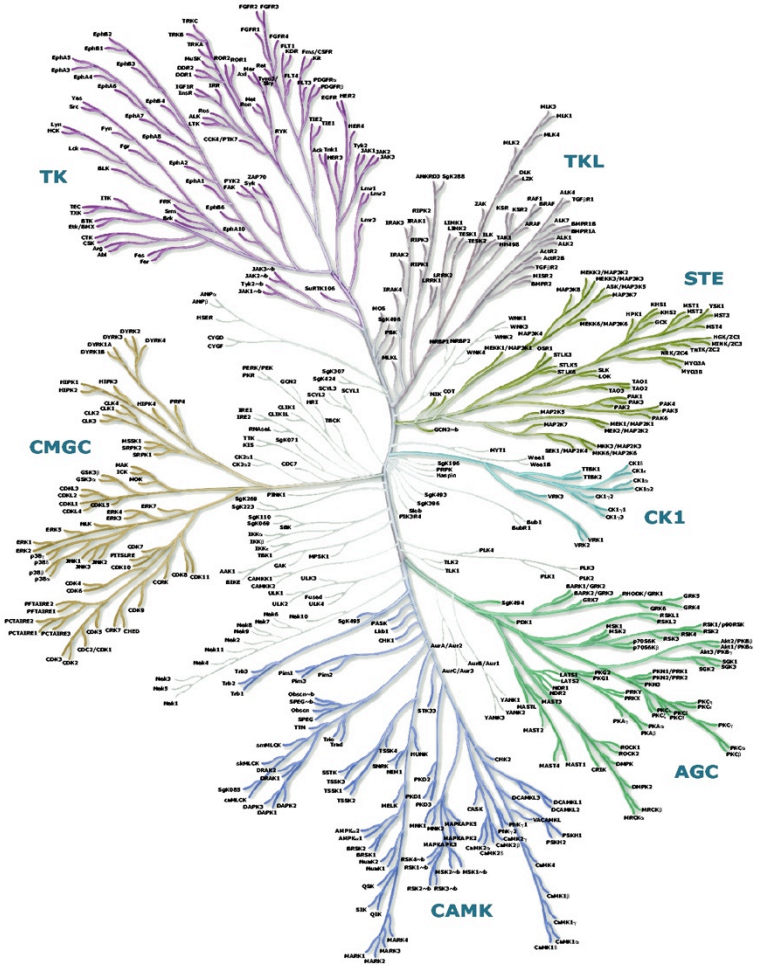
Wipf Group Topic seminar 9/7/13

Presentation format

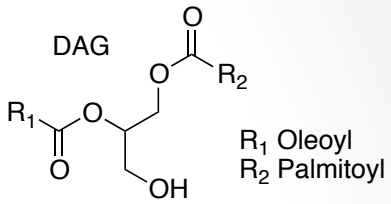
- Protein Kinase D
  - Background
  - The Protein
  - Synthesis of Targets and Analogs.
  - Summary
- Botulinum Neurotoxin
  - Background
  - The Protein
  - Synthesis of Targets
  - Summary

Protein Kinases

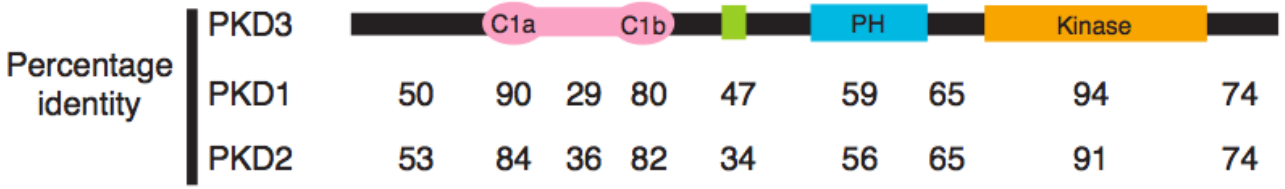


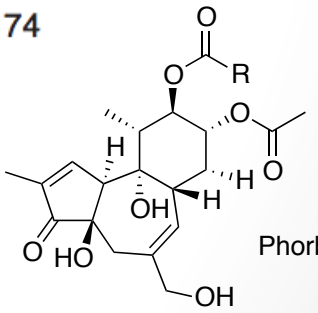
- Protein Kinases (PK) are used in cell signal transduction by the phosphorylation of proteins.
- Popular pharmaceutical targets
- PK's involved in cell growth, proliferation, or apoptosis
- Gleevec, Sutent, Tykerb, Sprycel, Tasinga are approved Protein Kinase inhibitors

Protein Kinase D



- Protein Kinase D (PKD) is a family of diacylglycerol (DAG)-stimulated serine/threonine kinases.
- Part of superfamily of Ca^{+2} / calmodulin Kinases (CAMK) and are highly homologous to the myosin light chain kinase.
- Originally classified as a subfamily of the Protein Kinase C family.
- PKD isoforms: PKD1 (PKCμ), PKD2, PKD3 (PKCν)

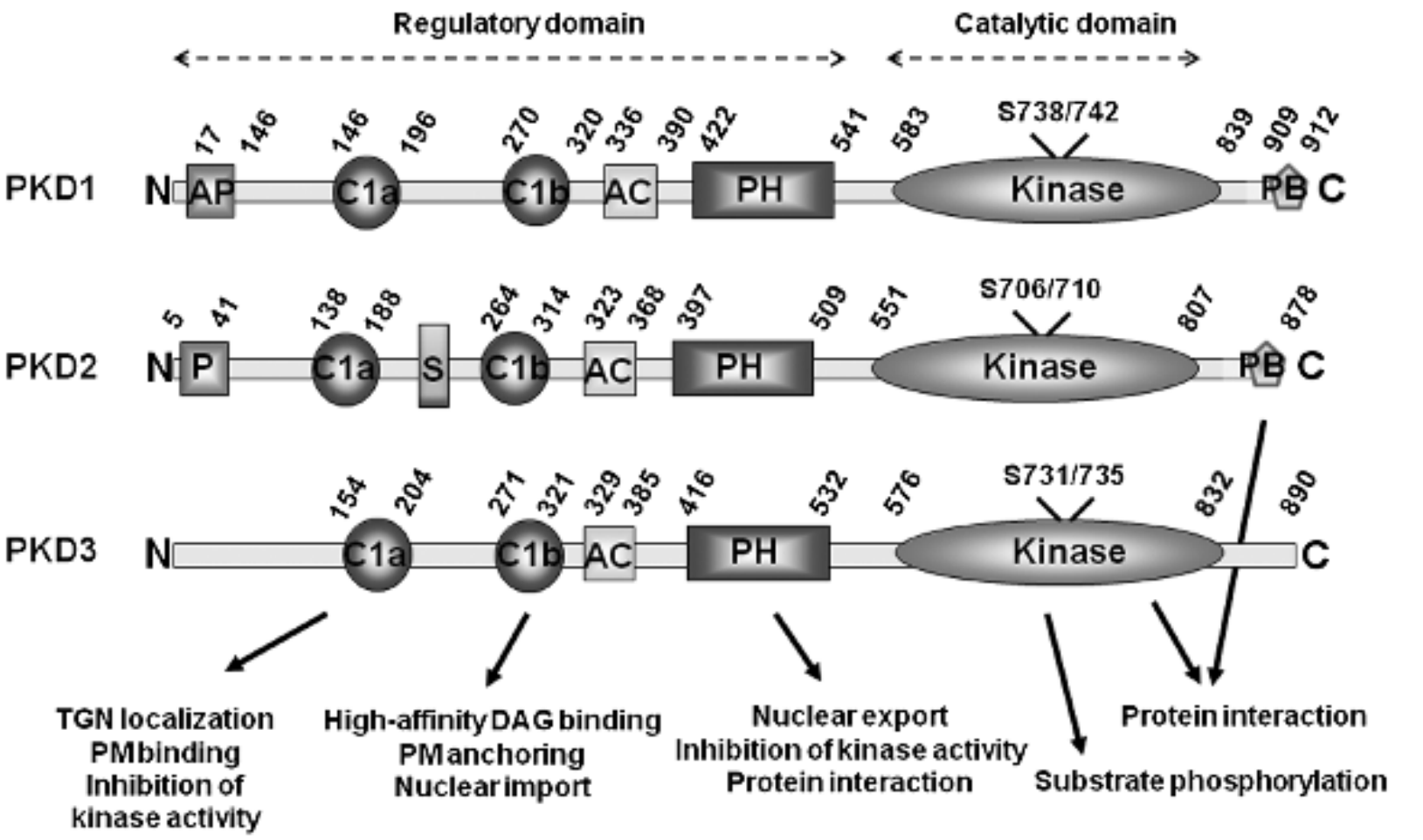




Phorbol ester

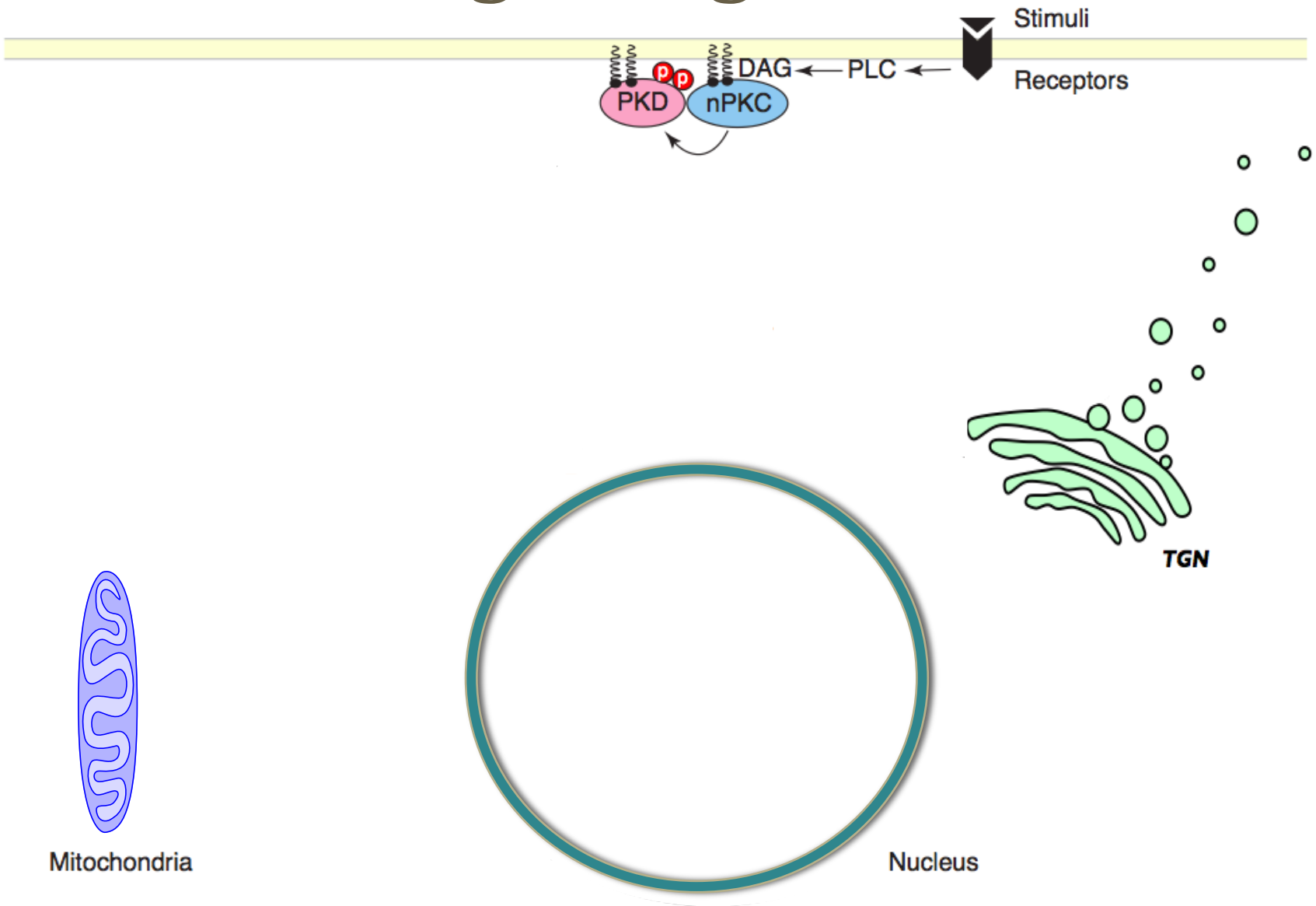
Biochem. J. **2010**, 429, 565-572
Science **2002**, 298, 1912-1934

PKD



Mol Cancer Res 2011 9:985-996

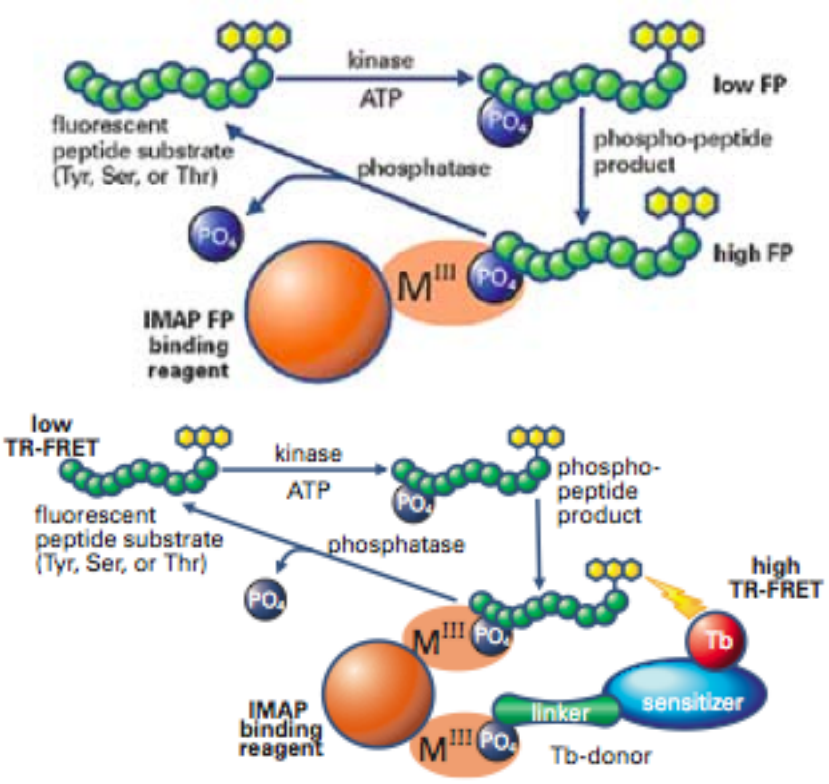
PKD and signaling



Trends Pharmacol. Sci. **2006,** *27* (6), 317-323. EMBO reports 2011, 12, 785-796

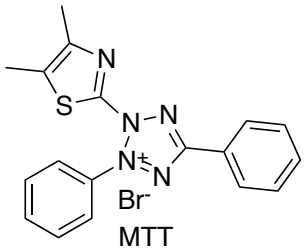
HTS IMAP-FP Assay

- IMAP (Immobilized Metal Affinity Phosphorylation)-FP or TR-FRET.
- Small trivalent Nanoparticles bound to ATP
- Non radioactive. Can be analyzed by FP or TR-FRET
- Higher FP with bound peptide due to lower anisotropy.

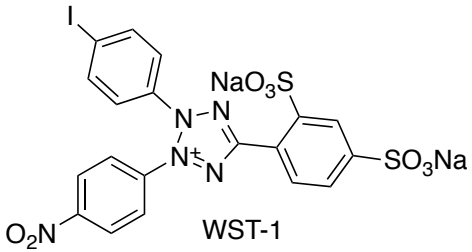


Biological Assay

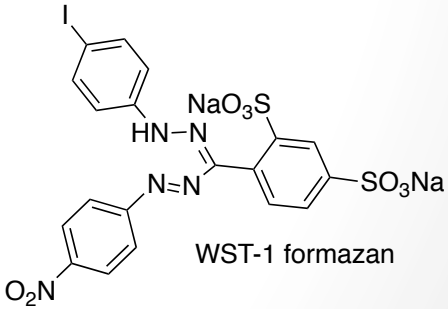
- In Vitro Radiometric Kinase Assay
  - Measures Kinase binding using P^{32} labeled ATP.
- Cell based:
  - MTT ((3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)
    - Measures cell viability
  - WST-1 (water soluble tetrazolium)
    - Measures cell viability



MTT



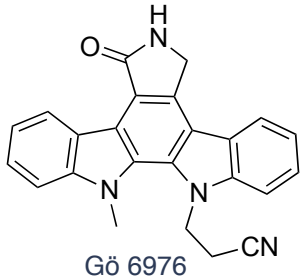
WST-1



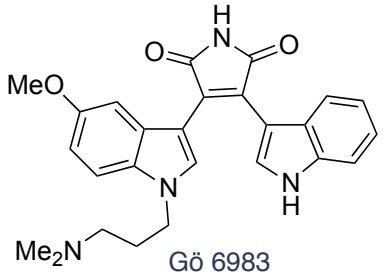
WST-1 formazan

Clontech: WST-assay,

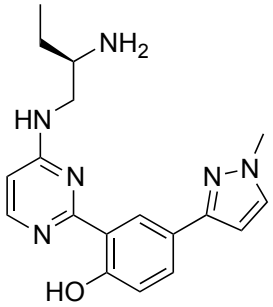
Known PKD Inhibitors



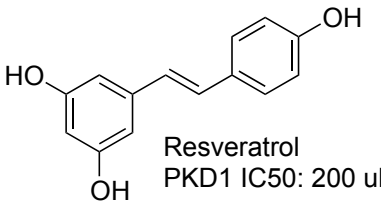
Gö 6976
PKD1 IC50: 20 nM



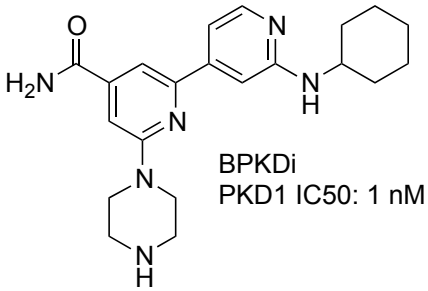
Gö 6983
PKD1 IC50: 20 uM



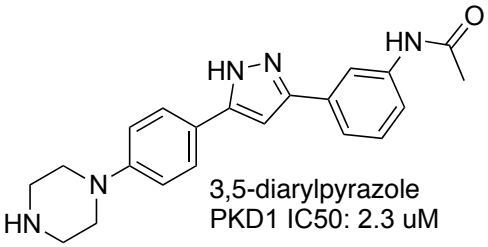
CRT0066101
PKD1 IC50: 1 nM



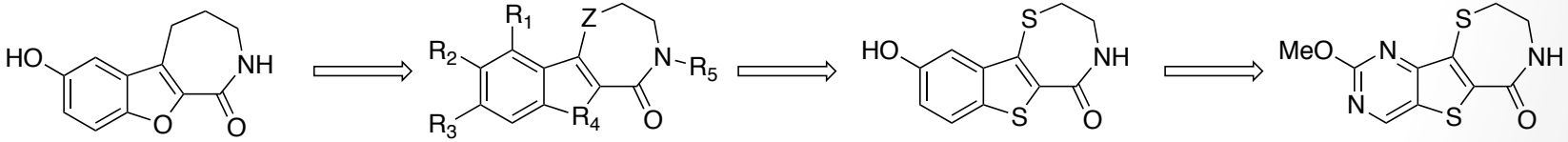
Resveratrol
PKD1 IC50: 200 uM



BPKDi
PKD1 IC50: 1 nM



3,5-diarylpyrazole
PKD1 IC50: 2.3 uM



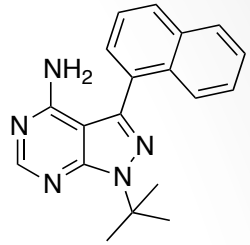
CID755673
IC50:
Rad .18 uM
Cell 11.8 uM

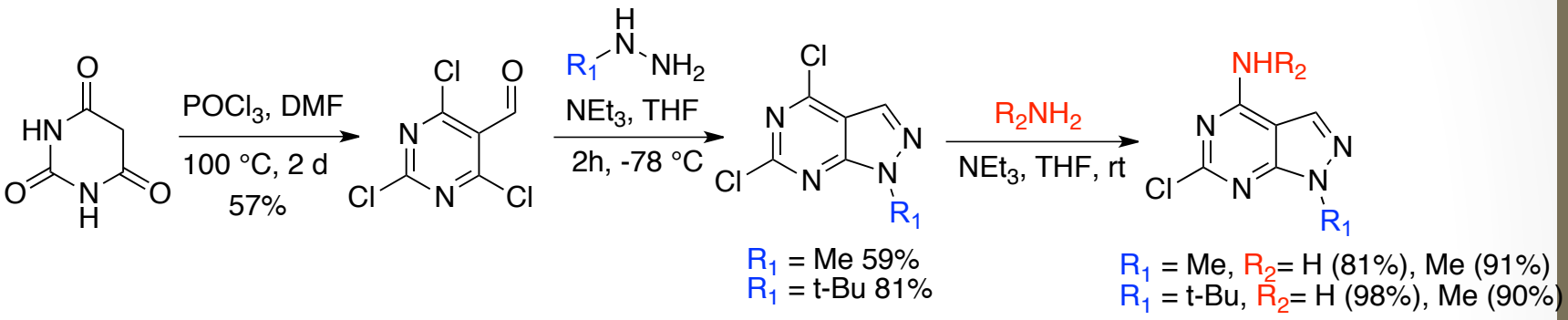
kb-NB142-70
IC50:
Rad .028 uM
Cell 2.2uM

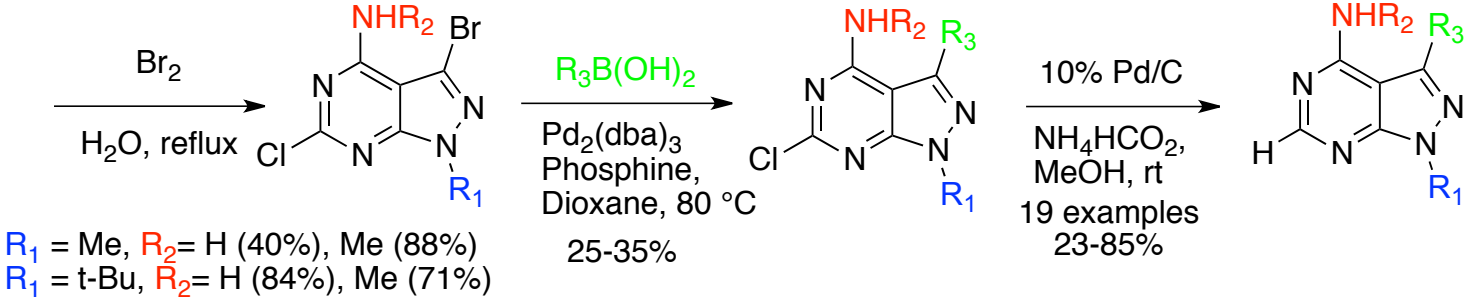
kmg-NB4-23
IC50
Rad .12 uM
Cell 6.8 uM

(BBA) Reviews on Cancer **2010,** *1806* (2), 183-192.
J. Biol. Chem. ***2008****, 283:33516-33526, ACS Med. Chem. Lett.* ***2011****, 2, 154-159.*

Synthesis of 1-NA-PP1

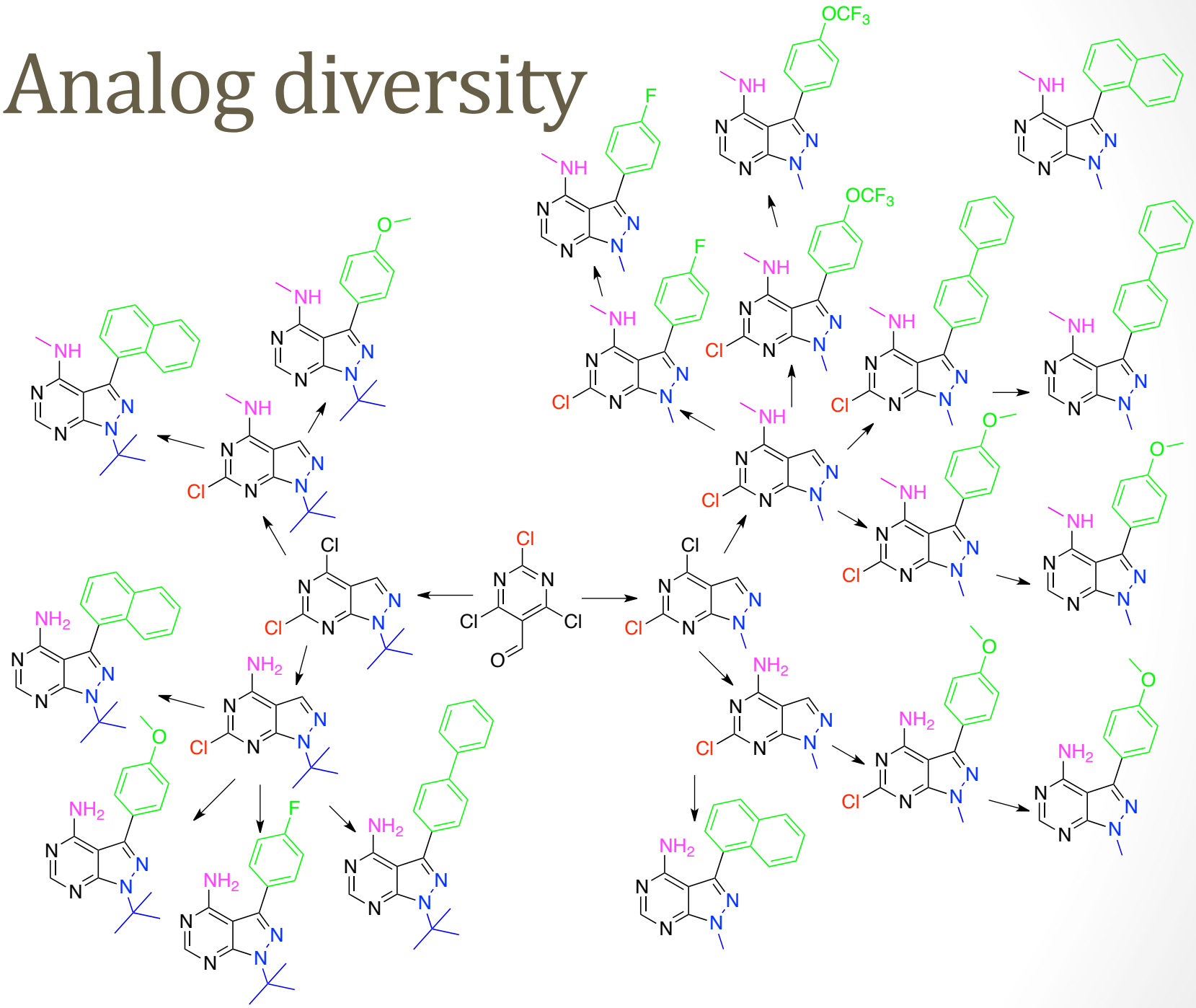




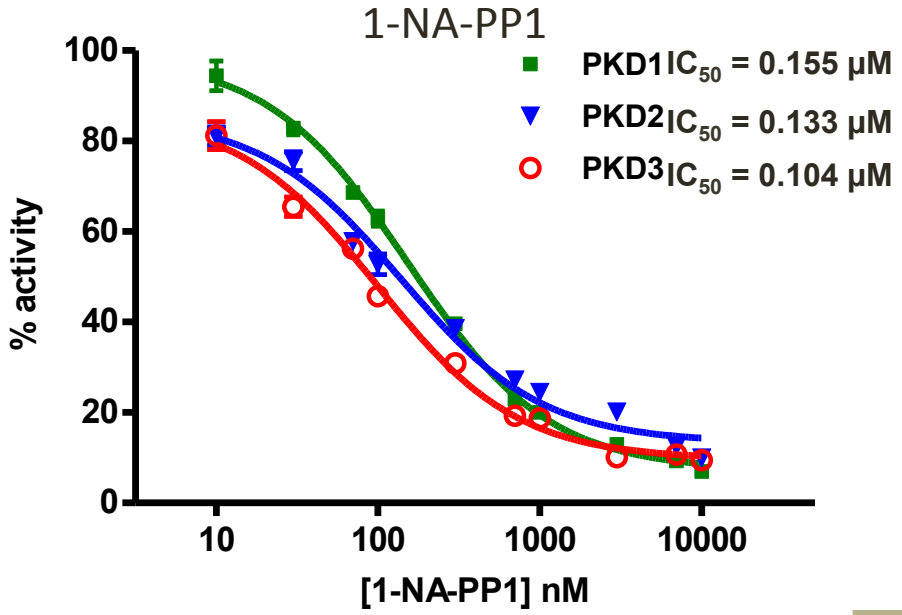


PLoS ONE **2013**, In Press

Analog diversity



1-NA-PP1 Activity



Cells (PKD2)	%Kinase Inhibition: IC50 uM
HCT116	0% : 11.5 ± 1.6
RKO	0% : 6.3 ± 0.6

Manuj Tandon PKD activity in 1-NA-PP1 using a radiometric in vitro kinase assay
John Schmitz cellular data and activity for PKD2 WST-1 viability assay

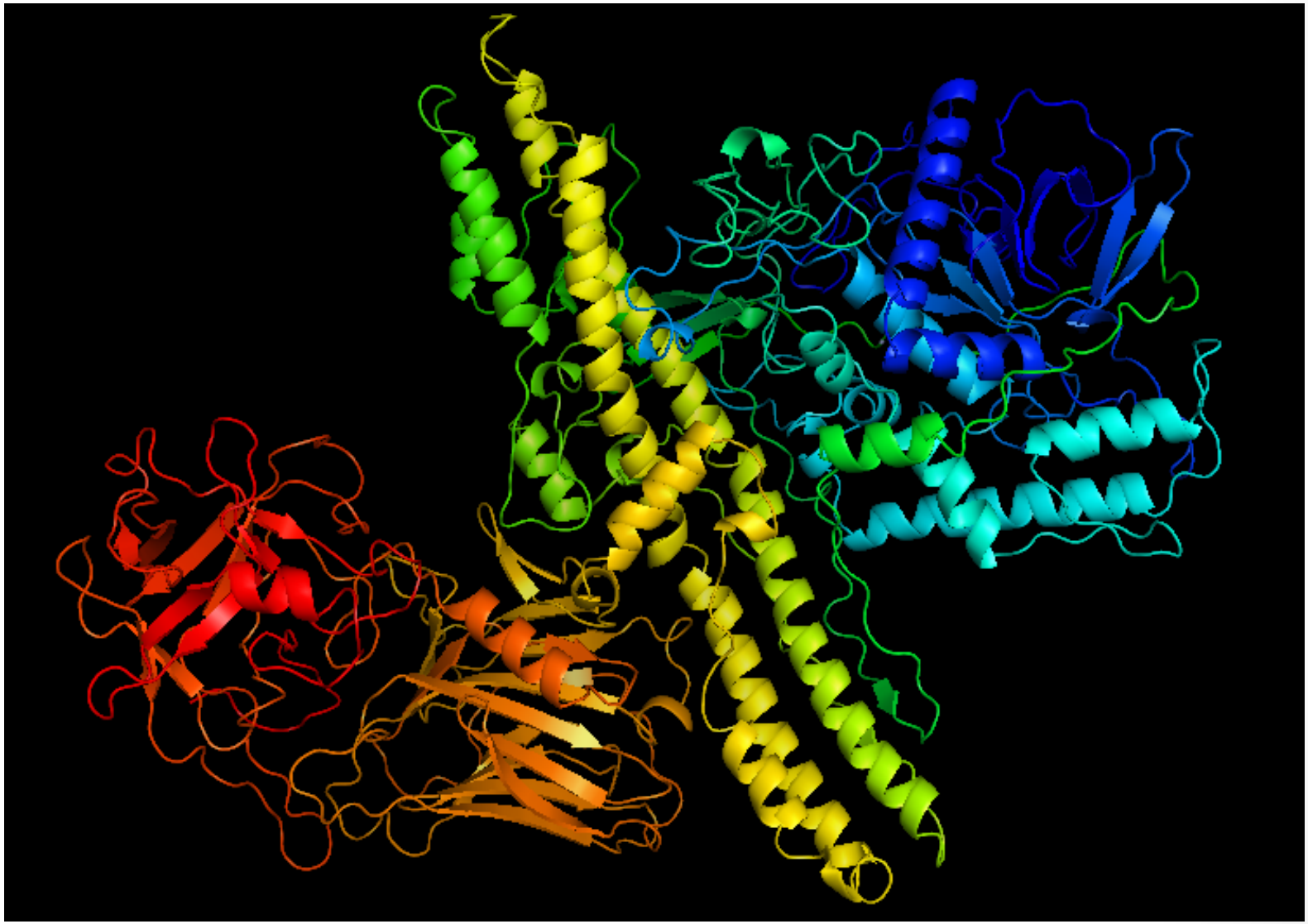
Future goals

- New Scaffold Development through homology modeling and in-silico ligand screening.
- Produce a potent, isoform specific PKD inhibitor.

Presentation format

- Protein Kinase D
  - Background
  - The Protein
  - Synthesis of Targets and Analogs.
  - Summary
- Botulinum Neurotoxin
  - Background
  - The Protein
  - Synthesis of Targets
  - Summary

Botulinium Neurotoxin A1



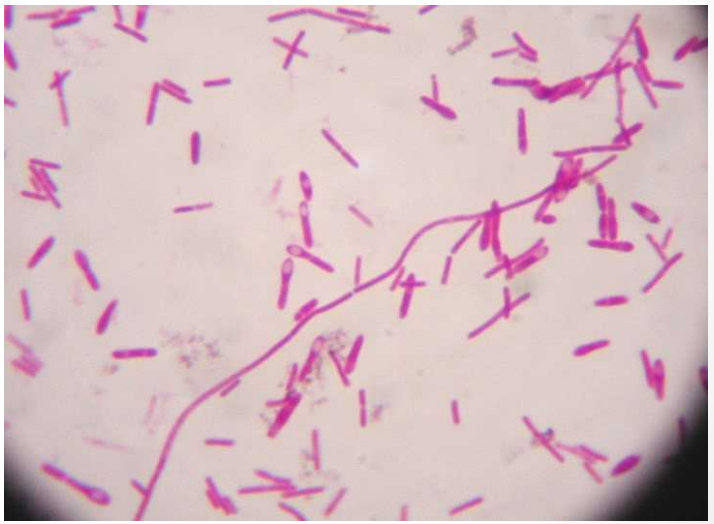
DOI:10.2210/pdb3bta/pdb

Overview

- Background
  - History
  - Intoxication
  - Uses
- Protein
  - MOA
- Previous Inhibitors
- Development of inhibitors around pharmacophore
- New inhibitors
- Synthesis of New Inhibitors

Botulinum Neurotoxin

- Produced by the spore forming gram-positive bacteria Clostridium Botulinum.
- Toxicity less than 1 ng/kg in humans is the lethal dose
- From a family that contains 7 serotypes A-G.
- The genus of Clostridium contains several toxic members i.e. C. tetani and C. perfringens.
- Typical symptoms include:
  - Muscle Weakness
  - Flaccid Paralysis
  - Respiratory Failure



Mov. Disord. 2004, 19: S2–S6..

History

- First discovered by J. Kerner in 1822 as “Sausage Poisoning” or “Fatty” Poisoning. Became known as “Kerner’s disease”
- 1895 E. Ermengem isolated and anaerobic rod-shaped bacteria gave the name Clostridium Botulinum
- 1944 E. Schantz discovers methods for large scale purification of BoNT/A1
- 1989 BoNT/A first biological toxin to be approved by the FDA as BOTOX

Perspect Biol Med, 1997, 40, 317-327
Mov. Disord. 2004, 19: S2–S6..

BoNT's

- Therapeutic:
  - Botox, Dysport, Myobloc are forms of Botulinum A and B approved by the FDA in 1991
  - Must be applied intramuscular
  - Can be used to treat MS, urinary incontinence, Chronic Migraine, Upper limb spasticity, Axillary hyperhidrosis, and Blepharospasm.
- Biological Warfare:
- Saddam Hussein produced large quantities of the C. Botulinum during Gulf War
- ~300g could kill the entire population in the United States

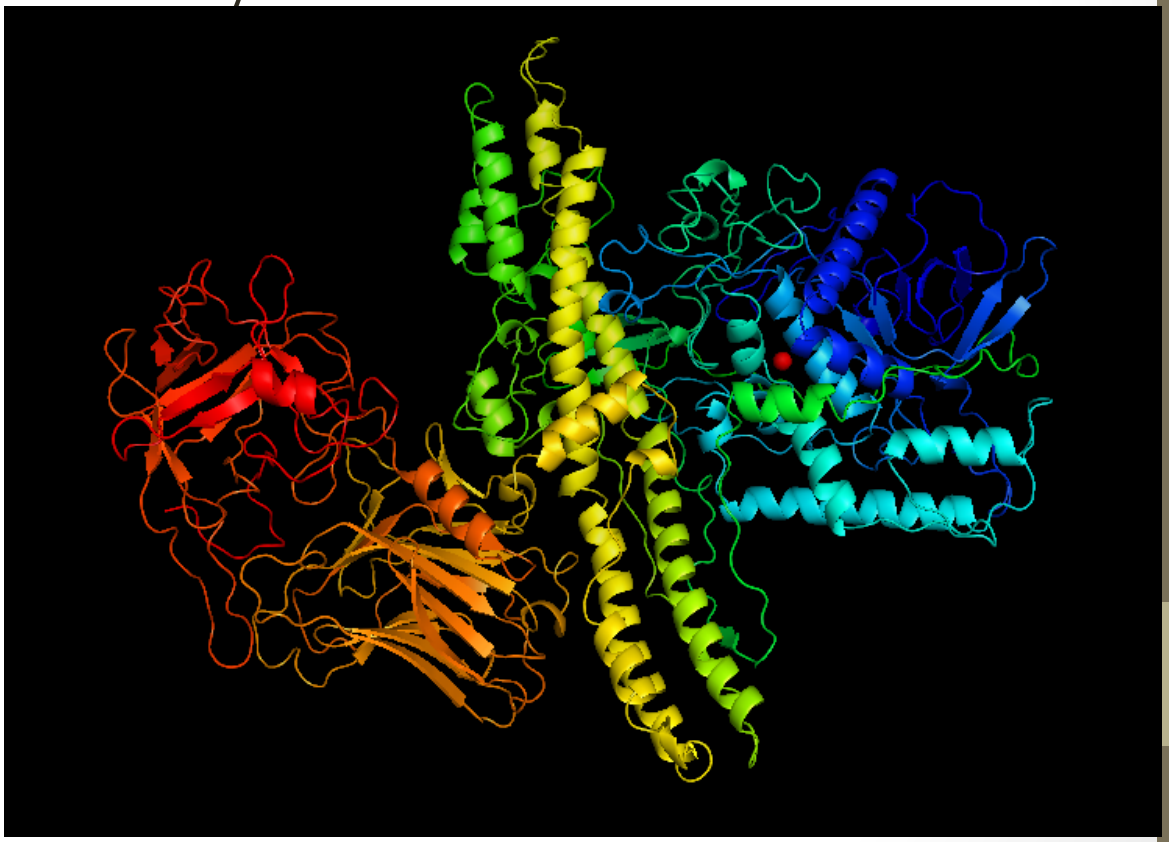
http://www.fda.gov/Drugs/DrugSafety/
Movement Disorders ***1997,*** 12, 1013-1018

Methods of Intoxication

- Food-borne
- Infant botulism/hidden botulism
- Wound botulism
- Inadvertent botulism
- Intentional botulism

BoNT/A the Protein

- 1296 amino acids long – divided into 3 domains each 50kDa
  - Catalytic domain 50kDa Light chain
  - Translocation domain 50 kDa Heavy chain N-terminal domain
  - Binding domain 50kDa Heavy chain C-terminal domain
  - Stable to pH 3-5
  - LC and HC linked disulfide bridge



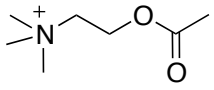
Nat. Struct. Biol. **1998**, 5, 898 –902, Trends Biochem. Sci. 2002, 57, 552

Protein Binding site

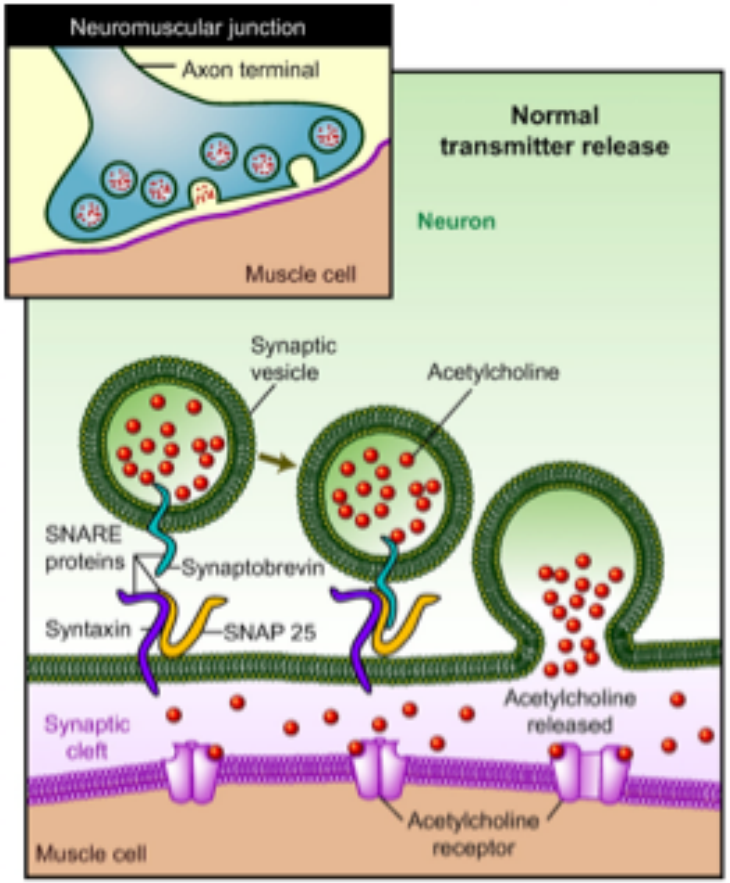
- Binding site on LC
- Each serotype of BoNT cleaves a SNARE protein
  - VAMP(vesicle associated membrane protein) 1+2 (synaptobrevins
    - BoNT B, D, F, G
  - SNAP-25 (Synaptosomal associated protein-25)
    - BoNT A, C, E
  - Syntaxin
    - BoNT C

Science 2012, 335, 928-929

Mechanism of Action



Acetylcholine

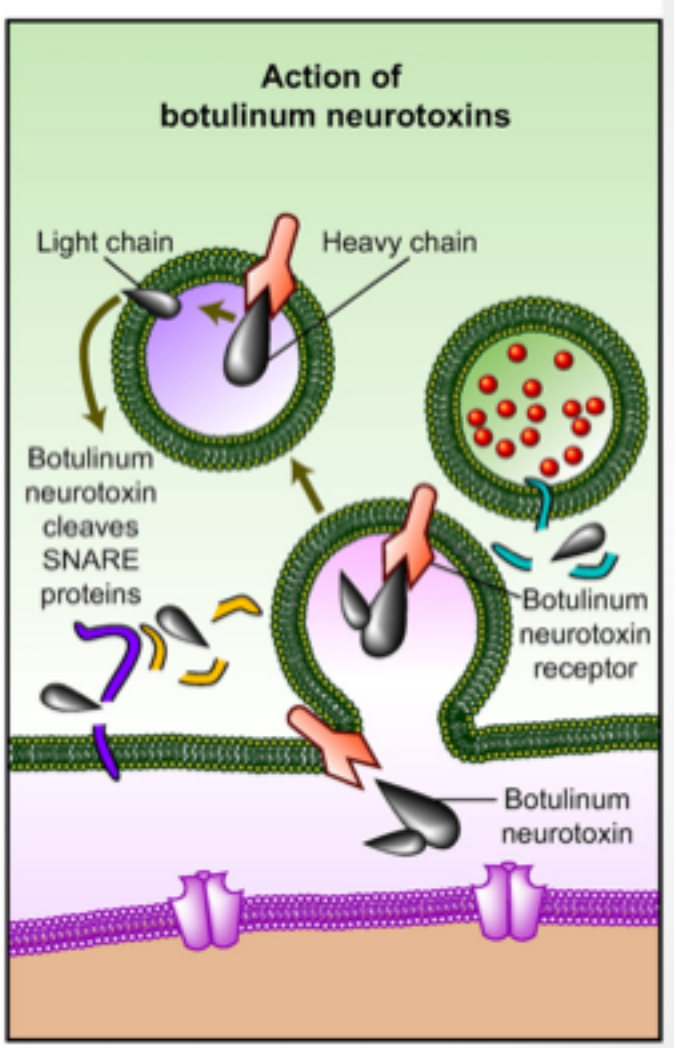


Normal Neurotransmitter release.

- Acetyl Choline (ACh) is a neurotransmitter involved in muscle contraction
- Stored in vesicles that upon polarization move to the membrane SNARE proteins that facilitate exocytosis to the neruomuscular junction in the peripheral nervous system.
- SNARE proteins are **S**oluble **N**-ethylmaleimide-sensitive factor **A**ttachment protein **Re**ceptor.
- SNAREs consist of:
  - SNAPs, Syntaxin, and Vamps

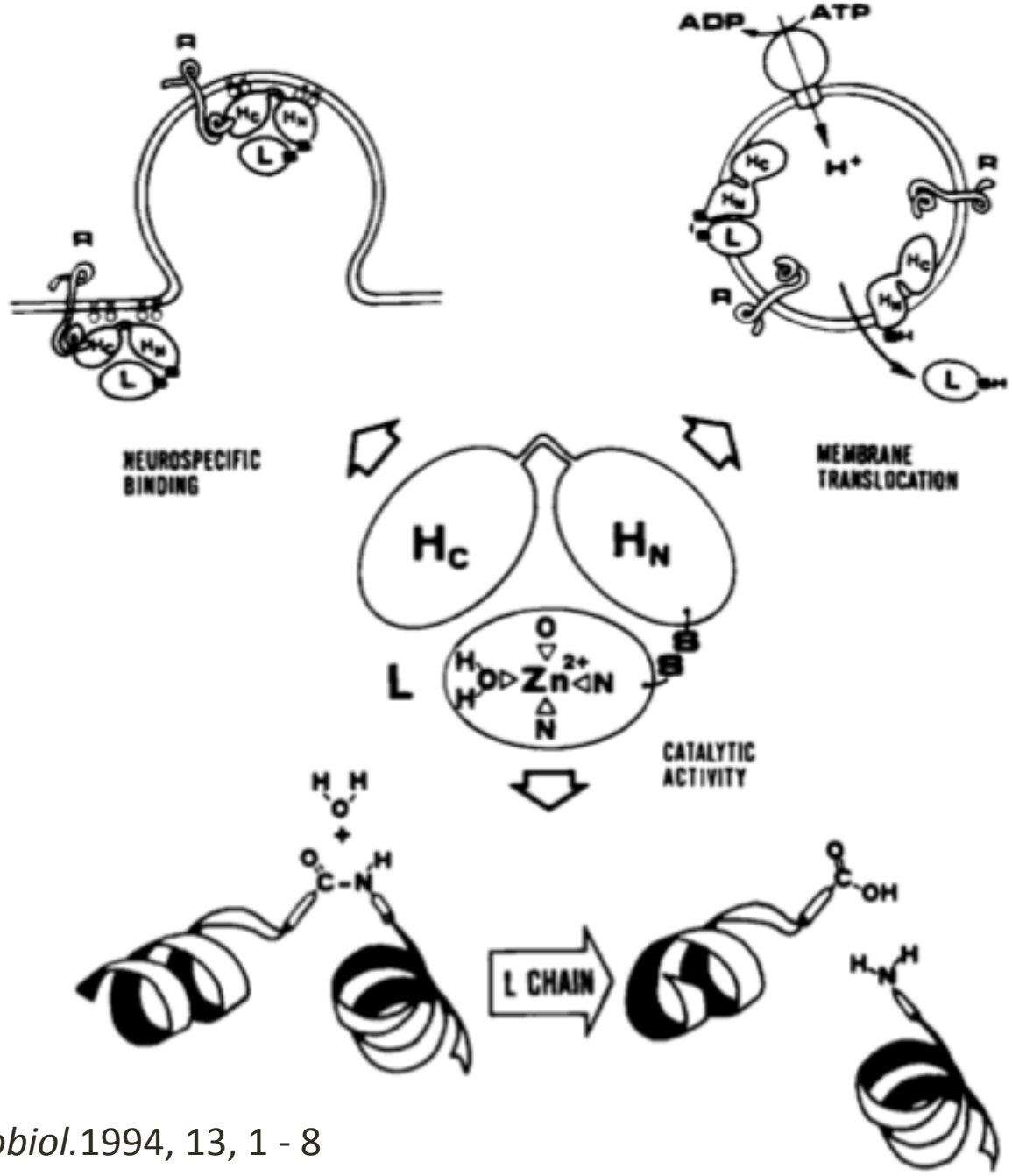
ACS Chem. Biol. 2006, 1, 359-369.
Annu. Rev. Cell Dev. Biol. 2003. 19:493–517

Mechanism of Action



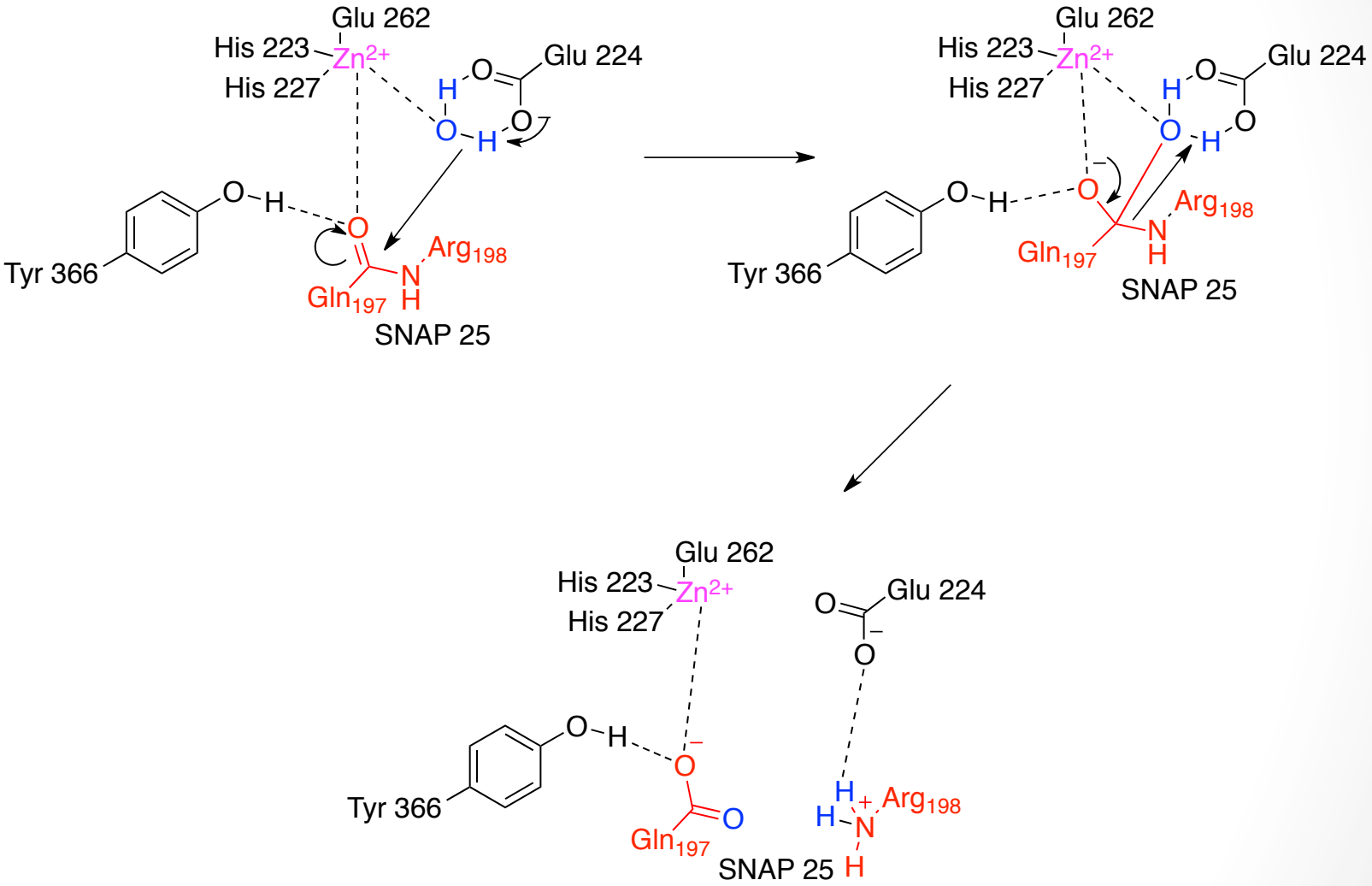
ACS Chem. Biol. 2006, 1, 359-369.

1) Binding to ganglioside G1b (GD1b, GT1b, and GQ1b)
2) Internalization of toxin receptor complex into the synaptic vesicle
3) Acidification of the synaptic vesicle causes translocation domain HN to insert into Membrane
4) Translocation of BoNT LC across membrane
5) Reduction of disulfide in cytosol
6) Hydrolysis of SNAP 25 – inactivation of snare complex leads to inability for Neuronal exocytosis of acetylcholine



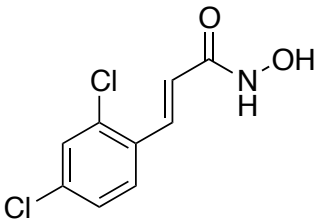
*Mol. Microbiol.*1994, 13, 1 - 8

Hydrolysis of SNAP-25 at Gln^{197}-Arg^{198}

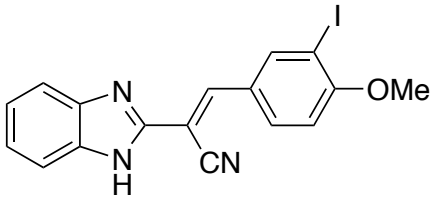


Adapted from Structure **2008**, 16, 1588–1597
JBC 2008, 283, 18883-18891

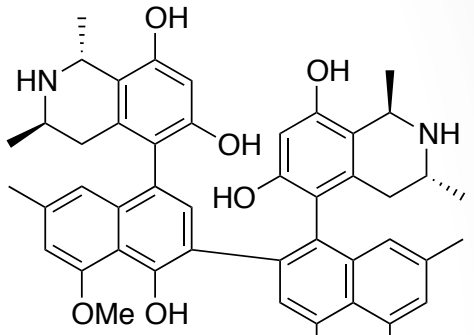
1st Gen. Inhibitors of BoNT's



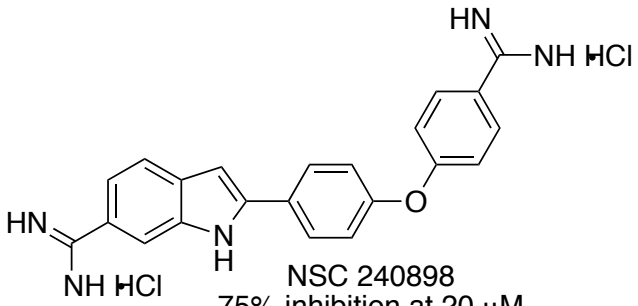
2,4-Dichlorocinnamic
hydroxamic acid
75% inhibition at 20 μM



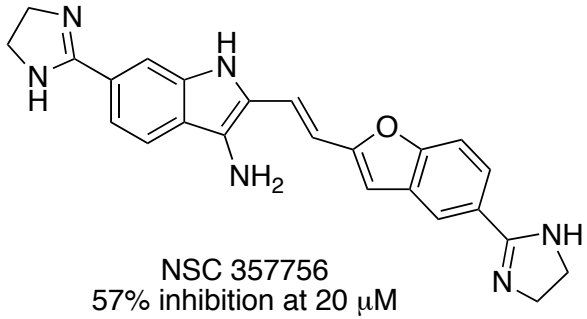
Benzimidazole
acrylonitrile
IC50 = 7.2 μM



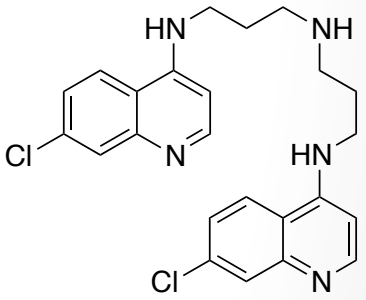
Michellamine B
62% inhibition at 20 μM



NSC 240898
75% inhibition at 20 μM
Ki = 10 μM



NSC 357756
57% inhibition at 20 μM



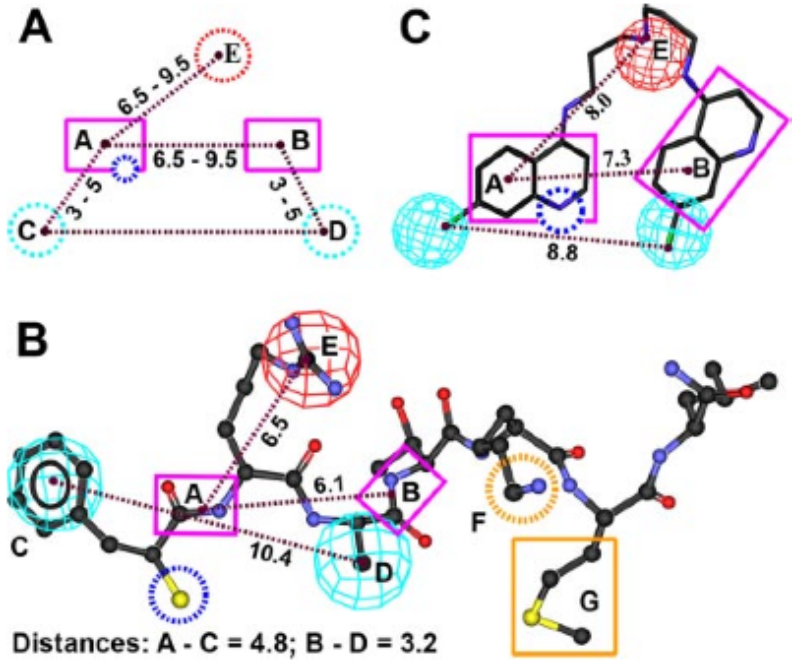
Q2-15
60% inhibition at 20 μM

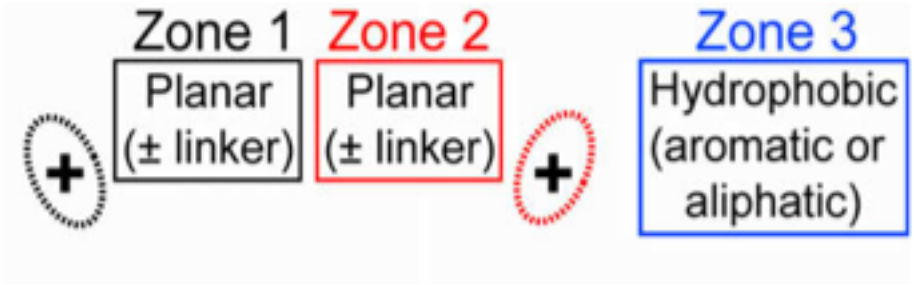
- Inhibition of BoNT monitored by HPLC analysis to observe hydrolysis of SNAPtide at residues Gln197 and Arg198. Or FRET screening of large library.
- HPLC assay using SNAP-25 (141-206)

Heterocycles, 2009, 79, 487-520

Eur. JOC 2012, 53, 374-379

Development around Pharmacophore

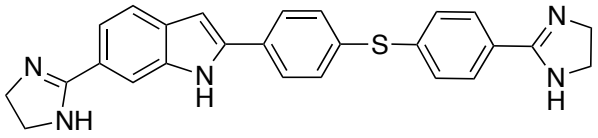




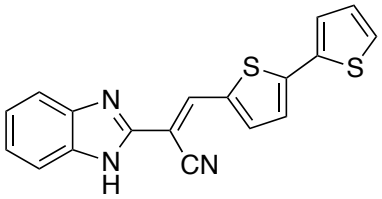
- Molecular docking of pseudo-peptide like mpp-RATKML (Ki=331nM) have helped identify key interactions in the binding site.
- Key components:
  - Planar linkers
  - Polar chelating function
  - Cationic caps
- Recent developments in non-metal-chelating ligands has shown a new hydrophobic binding pocket that can be accessed by different aromatic linkers

JBC **2007**, 282, 5004-5014
Bioorg. Med. Chem. Lett. **2009**, 19, 5811-5813

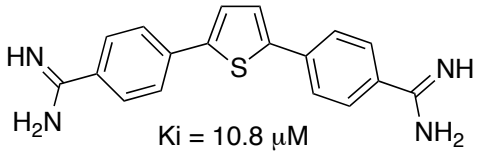
2nd Gen. Inhibitors of BoNT/A



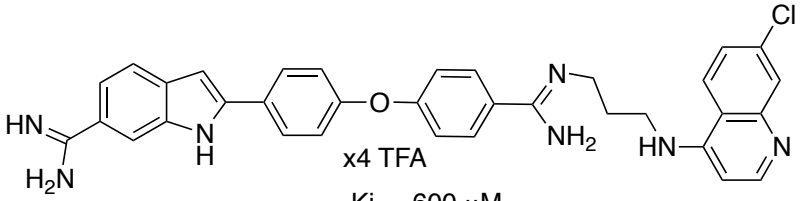
CWD-021
80% inhibition at 20 μM



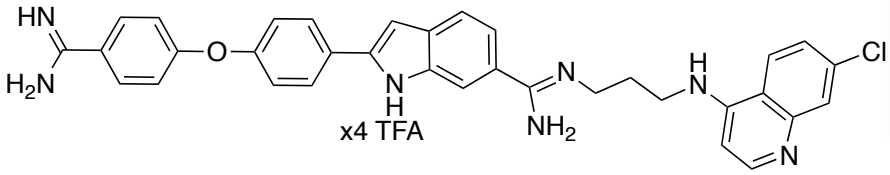
Non competitive
inhibitor of BoNT/A
IC50 26 μM



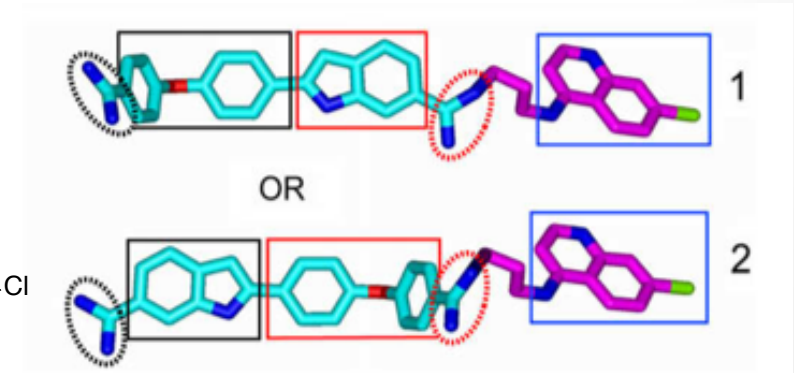
Ki = 10.8 μM



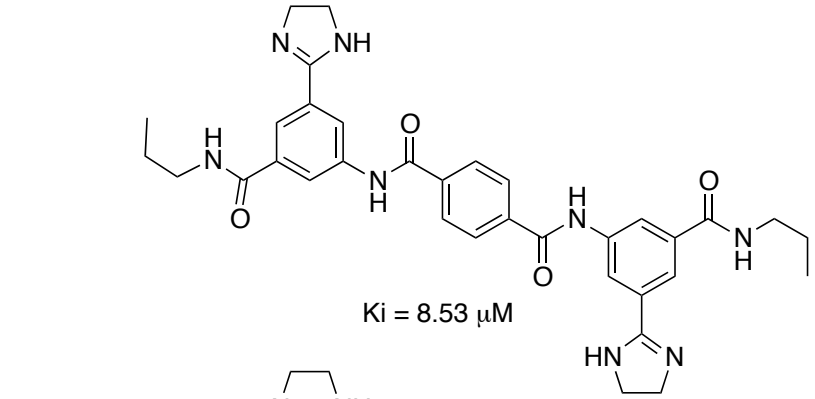
x4 TFA
Ki = .600 μM

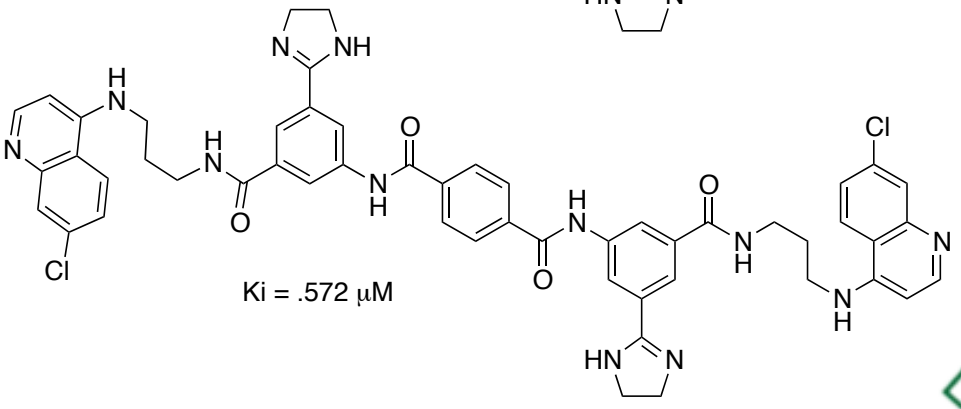


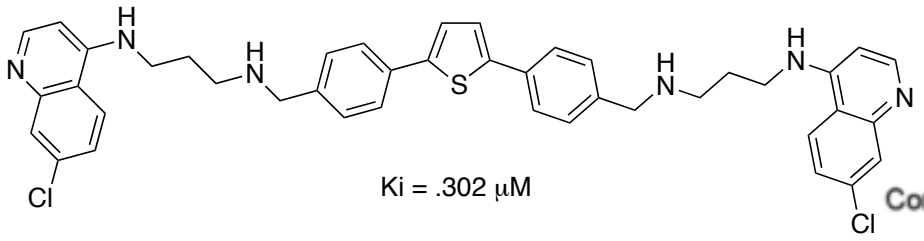
x4 TFA

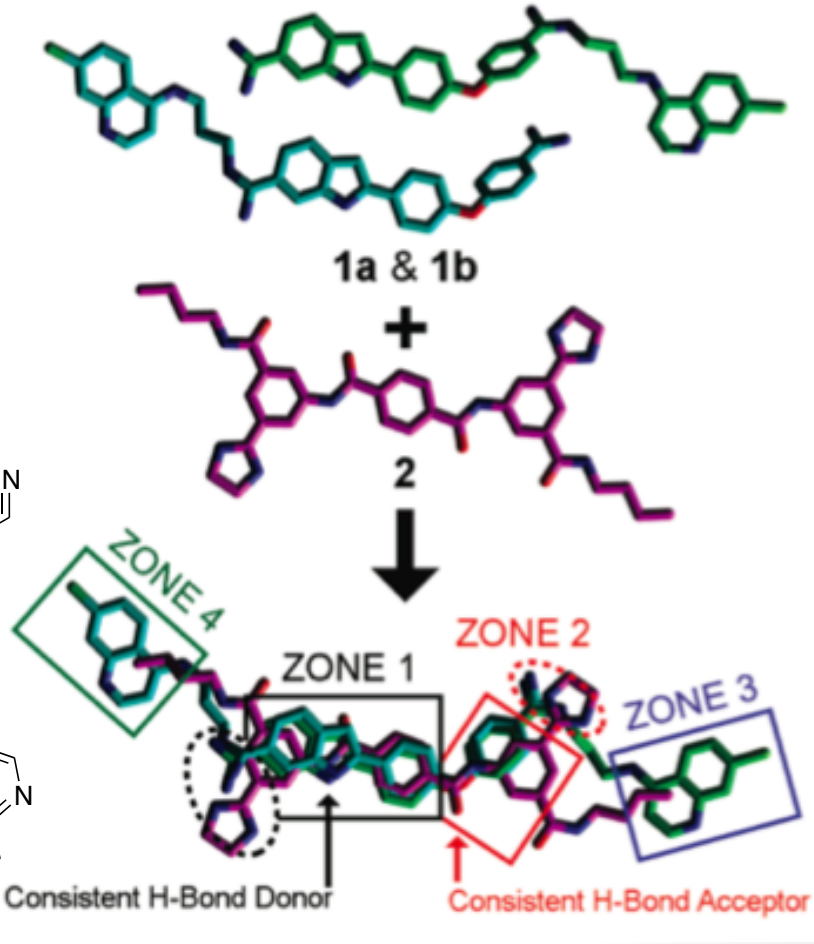


Further Development 3rd Gen.









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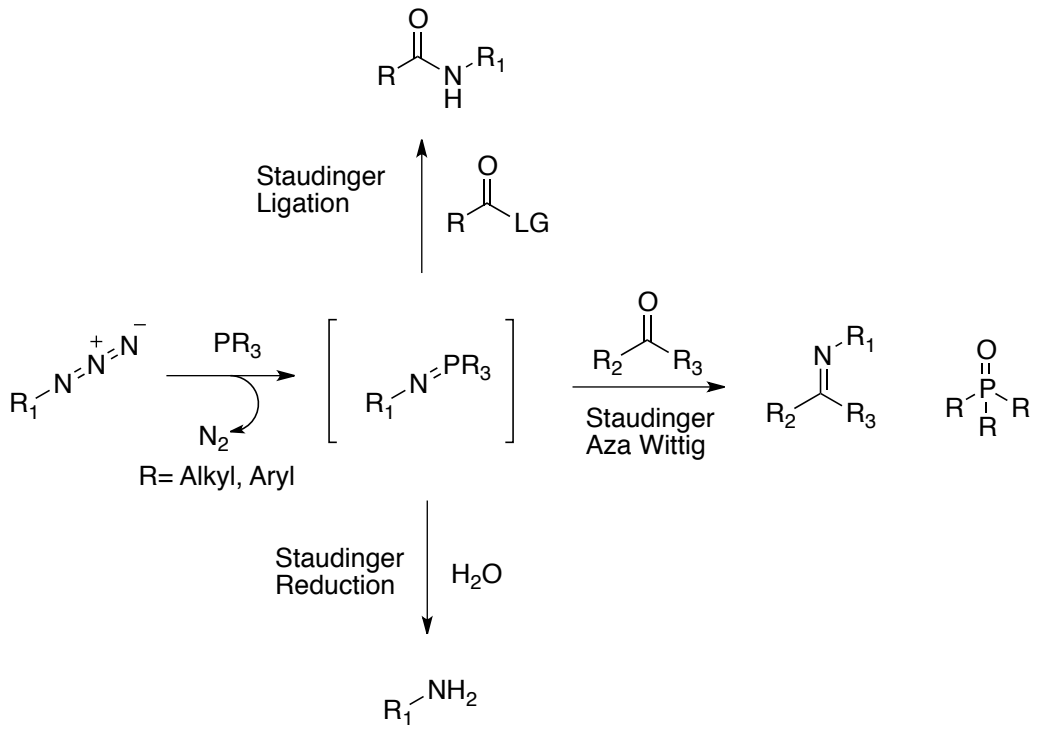

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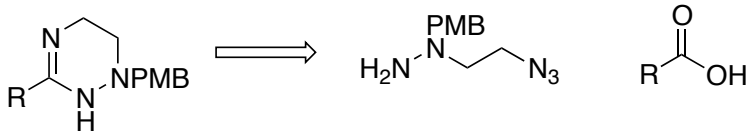
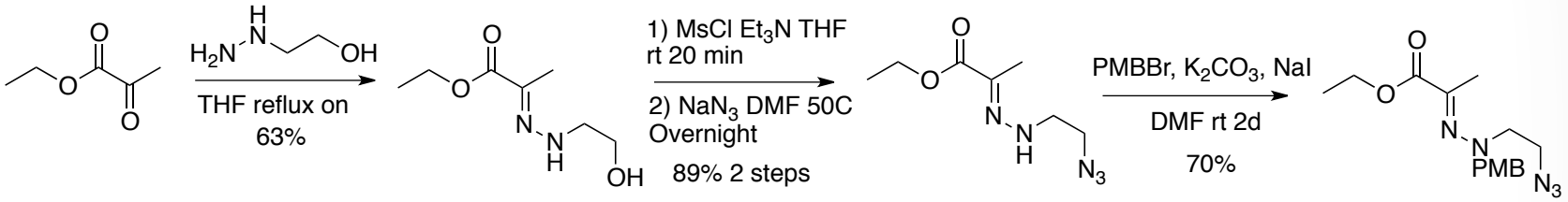
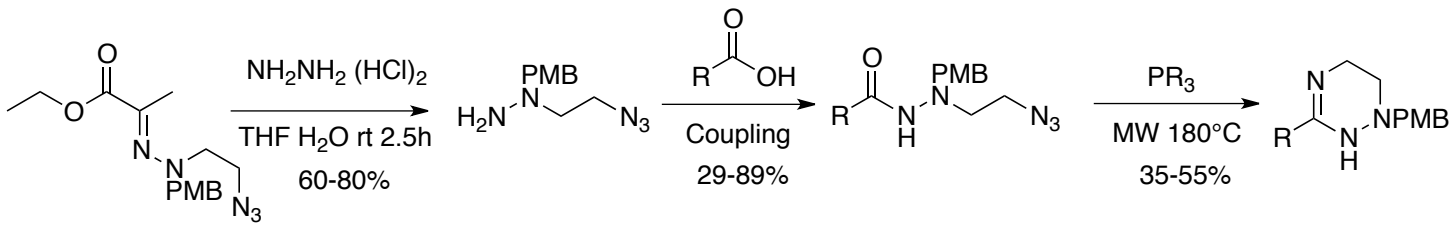
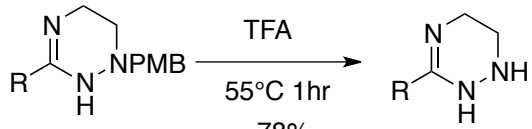
From hydrazine to triazine









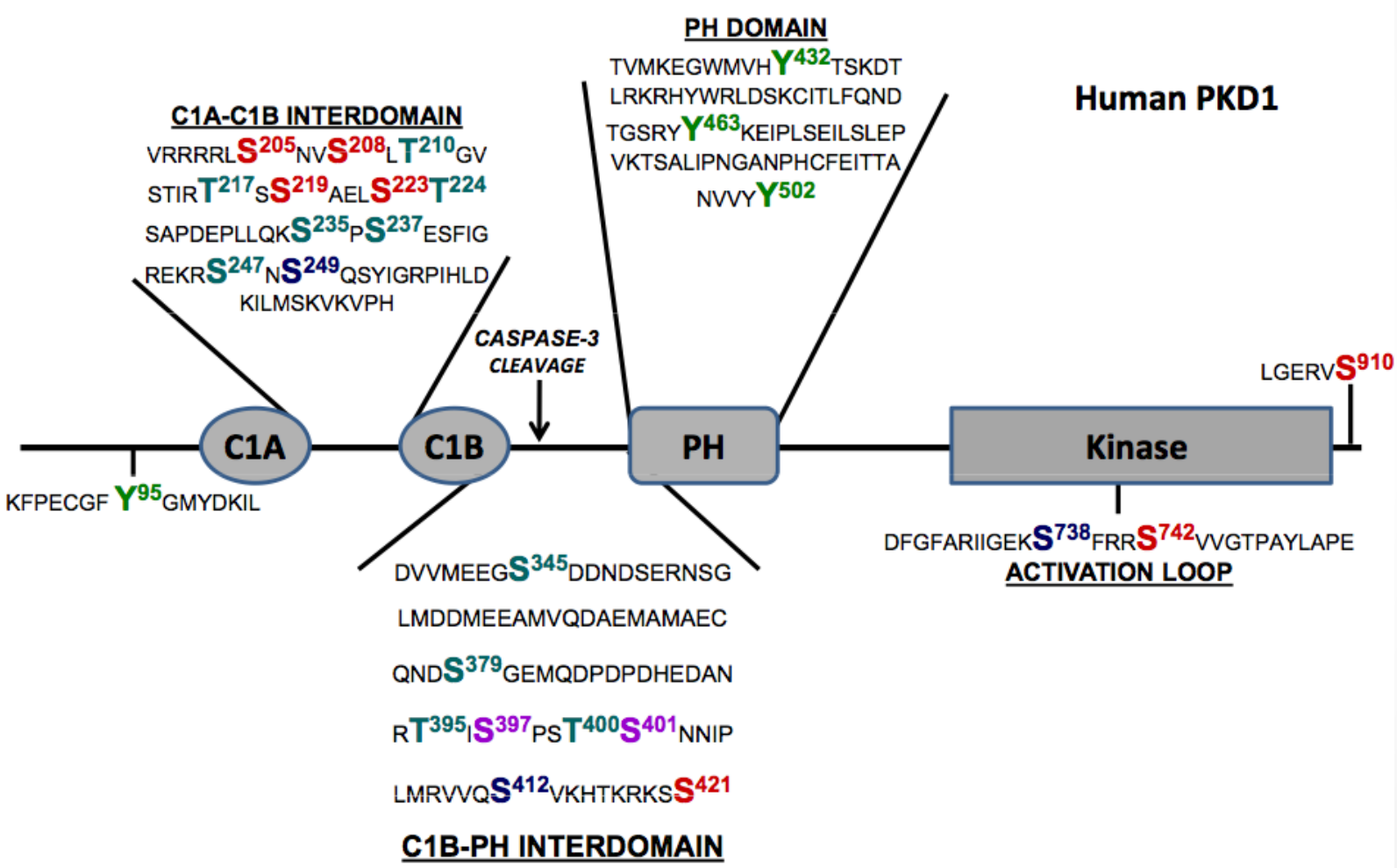
Future Goals

- Finish the synthesis of both targets.

Acknowledgements

- Dr. Wipf
- Pete Chambers, Sage Bowser
- Collaborations
  - Dr. Jane Wang, Dr. Manuj Tandon, and Dr. John Schmitz PKD
  - Dr. Sina Bavari, Dr. Jonathan Nuss, Dr. James Burnett, and Dr. Rick Gussio BoNT
- Funding: SAIC/NIH
- Wipf group past and present

PKD1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VKTSALIPNGANPHCFEITTA
NVVYY502
Human PKD1
C1A-C1B INTERDOMAIN
VRRRRLS205NVS208LT210GV
STIRT217SS219AELS223T224
SAPDEPLLQKS235PS237ESFIG
REKRS247NS249QSYIGRPIHLD
KILMSKVKVPH
CASPASE-3
CLEAVAGE
LGERVS910
C1A
C1B
PH
Kinase
KFPECGF Y95GMYDKIL
DVVMEEGS345DDNDSERNSG
LMDDMEEAMVQDAEMAMAEC
QNDS379GEMQDPDPDHEDAN
RT395IS397PST400S401NNIP
LMRVVQS412VKHTKRKSS421
C1B-PH INTERDOMAIN
DFGFARIIGEKS738FRRS742VVGTPAYLAPE
ACTIVATION LOOP

Tetazolium reduction mechanism