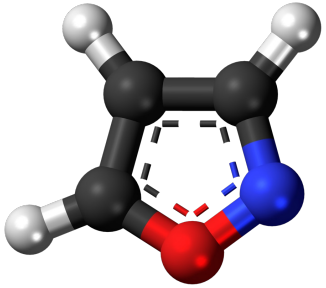
Application of Isoxazole in Chemoproteomics as Novel Photo-cross-linker



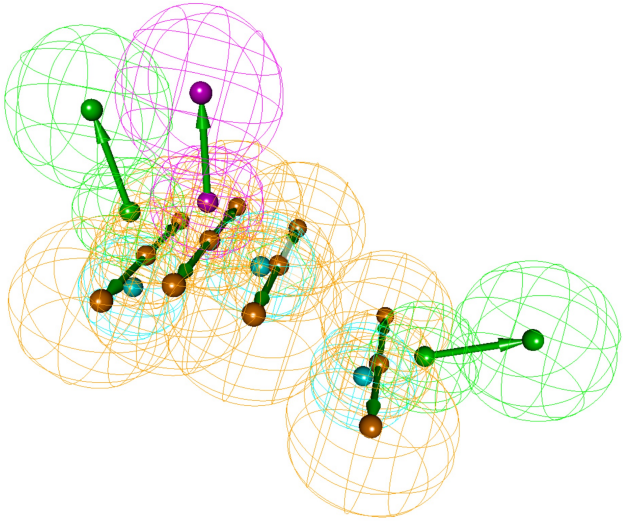
230408

Junhao Fu

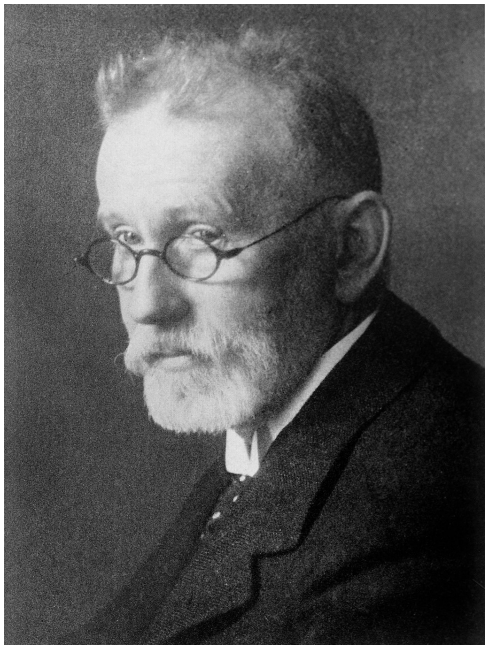
Contents

Pharmacophore & Molecular Interaction

a molecular framework that carries the essential features for a drug's biological activity (1909)



abstraction



Paul Ehrlich (1854~1915)
Nobel Prize in physiology or medicine

an ensemble of steric and electronic features that is necessary to ensure the optimal supramolecular interactions with a specific biological target and to trigger (or block) its biological response (1997, IUPAC)

1. Yang, S.-Y. *Drug Discov. Today* **2010**, *15*, 444.

Pharmacophoric Features

feature type	complementary feature type	interaction type	structural examples
hydrogen bond acceptor (HBA)	HBD	hydrogen bonding	amines, carboxylates, ketones, alcohols, fluorine substituents, …
hydrogen bond donor (HBD)	HBA	hydrogen bonding	amines, amides, alcohols, …
aromatic (AR)	AR, PI	π-stacking, cation-π	aromatic rings
positive ionizable (PI)	AR, NI	ionic, cation-π	ammonium ions, metal cations, …
negative ionizable (NI)	PI	ionic	carboxylates
hydrophobic (H)	H	hydrophobic interaction	halogen substituents, alkyl groups, alicyclic rings, weak or non-polar atoms, …

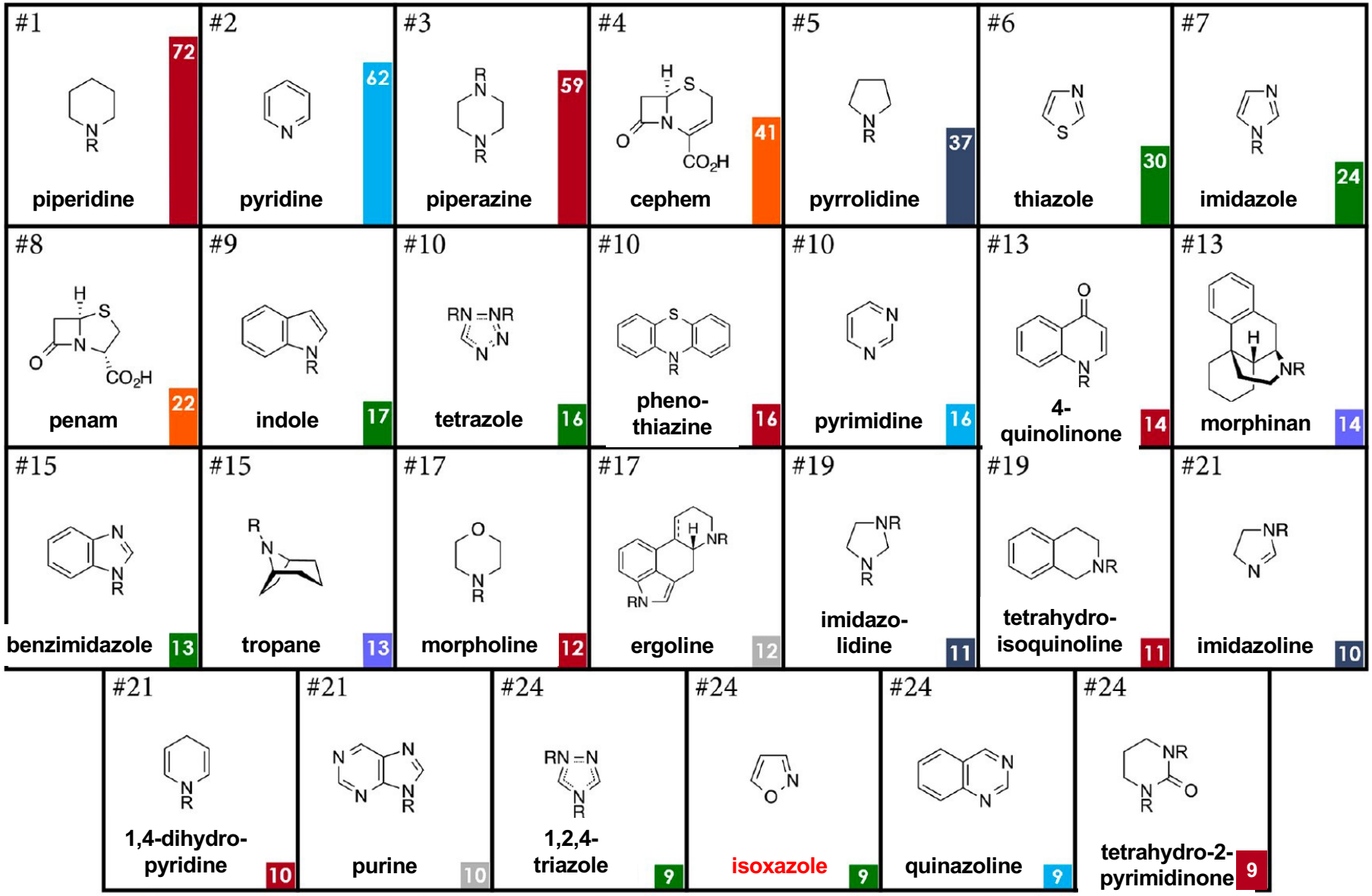
potential ligands of biomolecules generally posses these features

frequent involvement of N atom, especially N-heterocycles

1. Seidel, T.; Wieder, O.; Garon, A.; Langer T. *Mol. Inf.* **2020**, *39*, 2000059.

N-Heterocycles in FDA-Proved Drugs

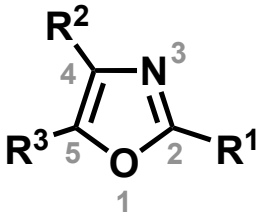
among FDA-proved unique small-molecule drugs: N-atom: 84%, N-heterocycle: 59%



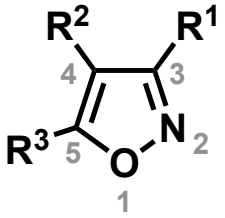
1. Vitaku, E.; Smith, D.T.; Njardarson, J.T. *J. Med. Chem.* **2014**, *57*, 10257–10274.

Isoxazole and Its Occurrence

core structure:



oxazole



isoxazole

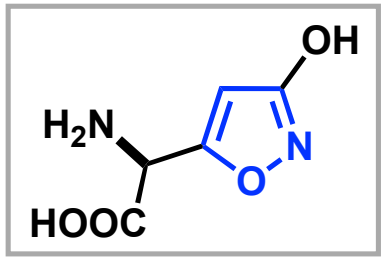
- electron rich aromatic ring with hydrogen bond acceptors
- interaction with biomolecules:
  - ➢ hydrogen bonding
  - ➢ pi-pi stacking
- good solubility
- bio-compatibility

→ **potential in drug discovery**

occurrence in nature: ibotenic acid

Amanita muscaria

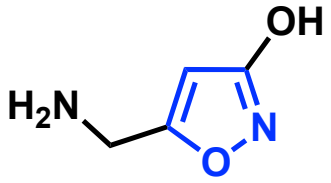
- isolated by Takemoto *et al.* in 1964
- psychoactive
- powerful neurotoxin
- 10-fold stronger 'umami' than glutamic acid (味の素) …



ibotenic acid

glutamate receptor agonist

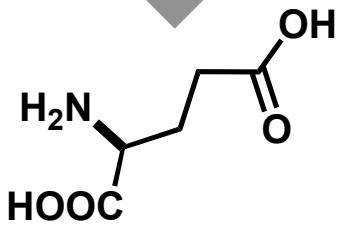
decarboxylation →



muscimol

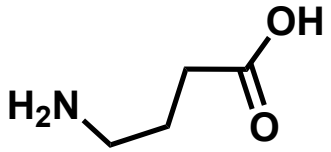
$GABA_A$-rho receptor agonist

similarity ↓



glutamate

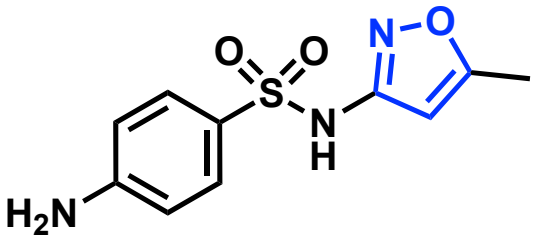
similarity ↓



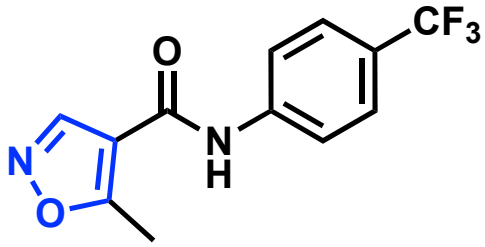
GABA

1. Johnston, G. A. R.; Curtis, D. R.; de Groat, W. C.; Duggan, A. W. *Biochem. Pharmacol.* **1968**, *17*, 2488–2489.

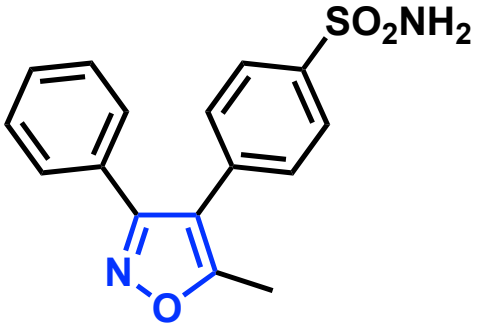
Isoxazole in Bioactive Molecules



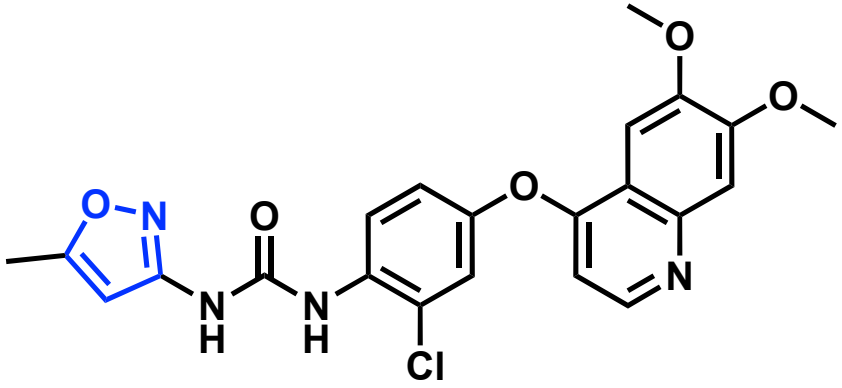
Sulfamethoxazole (1968)
antibacterial



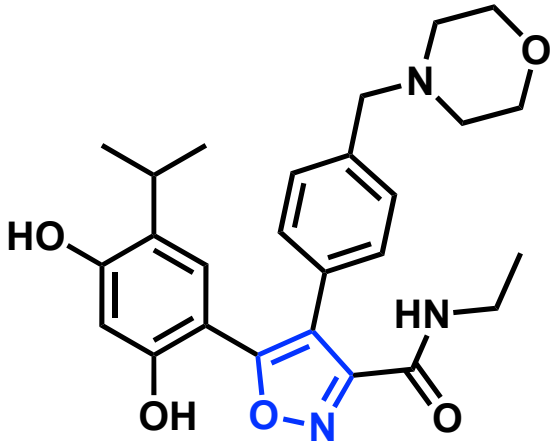
Leflunomide (1998)
anti-rheumatic



Valdecoxib (2001)
anti-inflammatory



Tivozanib (2021)
anti-cancer



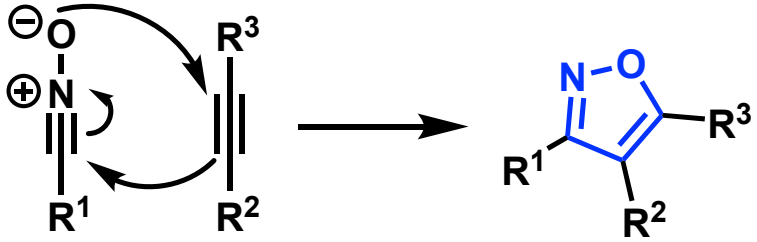
Luminespib (phase II)
anti-cancer

wide variety of bioactivities in isoxazole-containing molecules:
antibacterial, antiviral, anti-rheumatic, anti-inflammatory, anti-cancer, anti-diabetic, …

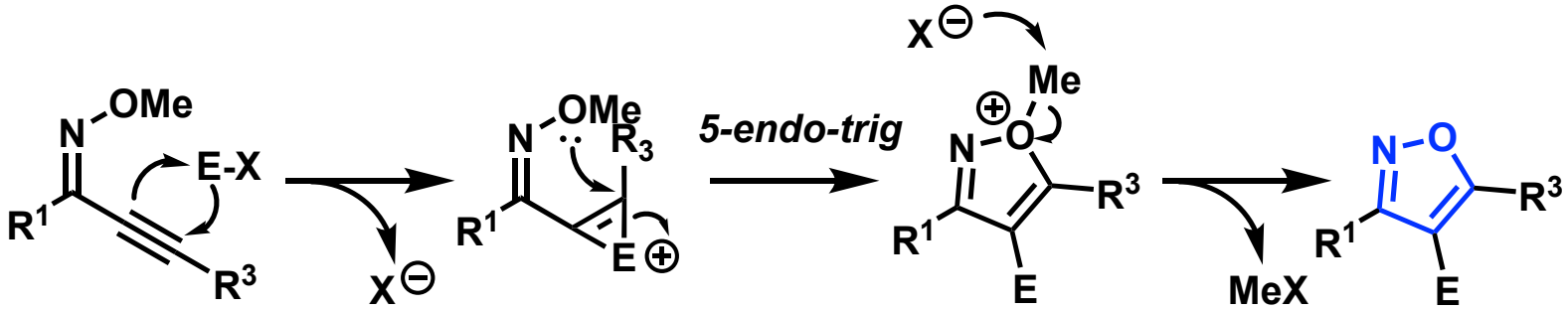
1. Arya, G.C.; Kaur, K.; Jaitak, V. *Eur. J. Med. Chem.* **2021**, *221*, 113511;
2. Sysak, A.; Obminska-Mrukowicz, B. *Eur. J. Med. Chem.* **2017**, *137*, 292–309.

Synthetic Availability of Isoxazole

A. [3+2] cycloaddition: Quilico *et al.* (1950)

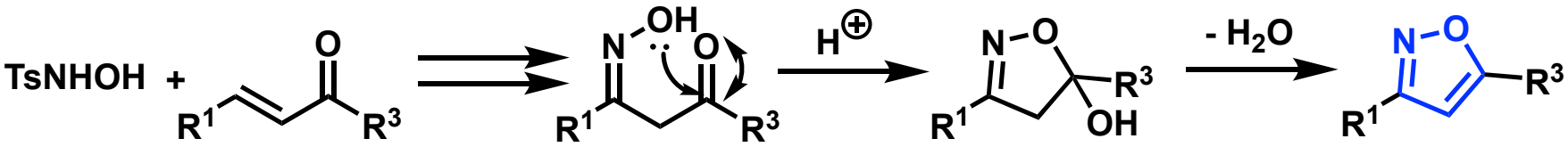


B. electrophilic cyclization: Larock *et al.* 2005



E-X = I_2, ICl, Br_2, PhSeBr

C. condensation: She *et al.* (2009)

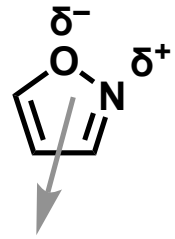


Numerous other routes have also been developed, making it accessible for simple construction of the isoxazole structure.

1. Quilico, A.; Stagno d'Alcontres, G.; Grünanger, P. *Nature* **1950**, *166*, 226–227.
2. Waldo, J.P.; Larock, R.C. *Org. Lett.* **2005**, *7*, 5203–5205.
3. Tang, S..; He, J.; Sun, Y.; He, L.; She, X. *Org. Lett.* **2009**, *11*, 3982–3985.

Synthetic Intermediate Role of Isoxazole

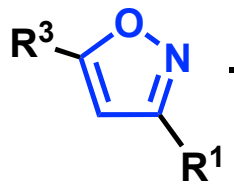
reductive ring opening reaction — key reaction as synthetic intermediate:

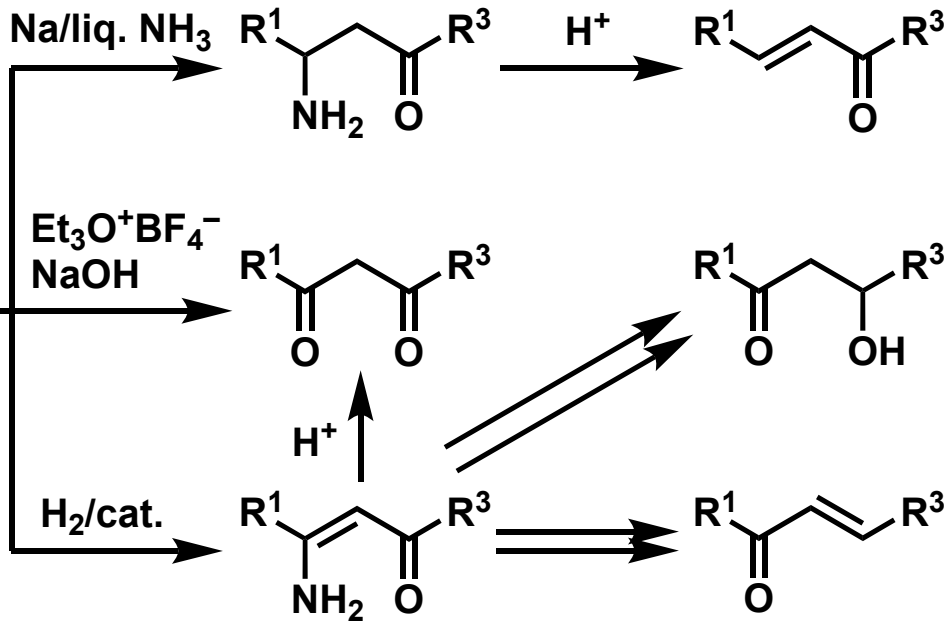


BDE_{N-O} = 630.6 kJ/mol

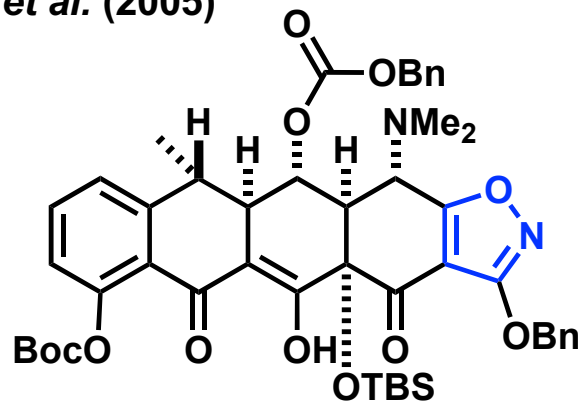
- polarized N-O bond
- ring strain

susceptible to chemical reactions



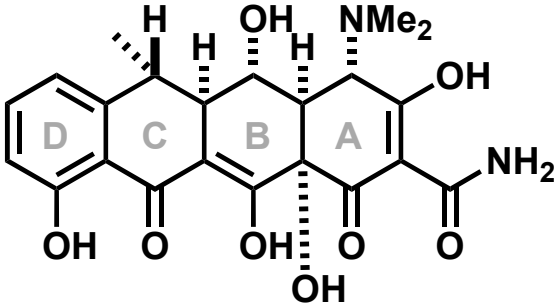


application in the total synthesis of natural product:
Myers *et al.* (2005)



1. HF, MeCN
2. H_2, Pd, THF, MeOH

(90%, 2 steps)



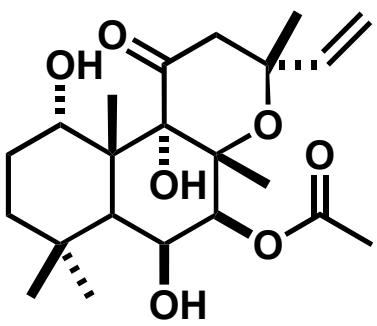
(–)-doxycycline

antibacterial activity against Gram-positive strains

1. Baraldi, P.G.; Barco, A.; Benetti, S.; Pollini, G.P. Simoni, D. *Synthesis* **1987**, *10*, 857–869.
2. Li, Z.; Carmichael, I.; Ptasińska, S. *Phys. Chem. Chem. Phys.* **2018**, *20*, 18271–18278.
3. Charest, M.G.; Lerner, C.D.; Brubaker, J.D.; Siegel, D.R.; Myers, A.G. *Science* **2005**, *308*, 395–398.

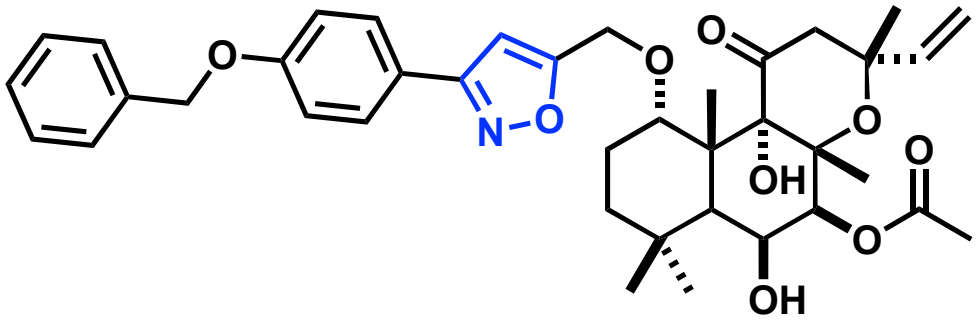
Modification of Natural Products with Isoxazoles

in anticancer natural product:



forskolin

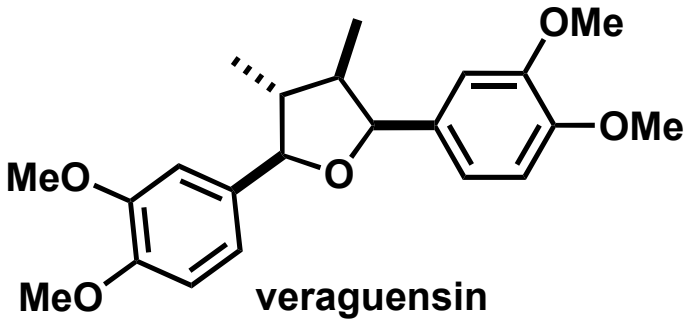
C1-OH substitution



IC_{50} = 63.3 μM against MCF-7,
IC_{50} > 100 μM against BT-474 cells

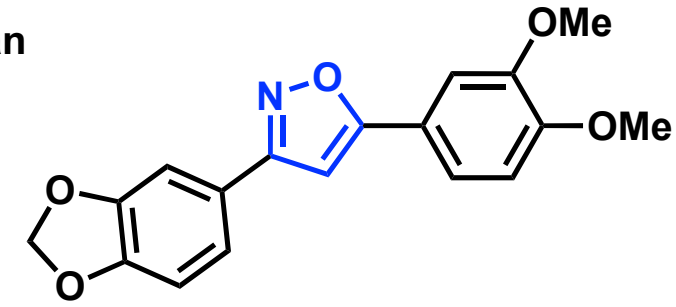
IC_{50} = 0.5 μM against MCF-7,
IC_{50} = 0.5 μM against BT-474 cells

in antimicrobial natural product:



veraguensin

alternation of tetrahydrofuran core



antileishmanial activity: IC_{50} = 48.8 μM (against *L. donovani*)

antileishmanial activity: IC_{50} = 2.0 μM (against *L. amazonensis*)

introduction of isoxazole to natural products could possibly change their biological properties

1. Burra, S.; Voora, V.; Rao, C.P.; Kumar, P. V.; Kancha, R,K.; Krupadanam, G.L.D. *Bioorg. Med. Chem. Lett.* **2017**, *27*, 4314–4318.
2. Baroni, A.C.M. et al. *Chem. Biol. Drug Des.* **2019**, *93*, 313–324.

Contents

Author Information

Dr. Hongyan Sun

2003-2008	**Ph.D. in chemical biology @ National University of Singapore (Prof. Shao Qin Yao)**
2008-2011	**postdoctoral fellow @ Max Planck Institute of Molecular Physiology, Germany (Prof. Herbert Waldmann)**
2011-	**associate professor @ City University of Hongkong, department of chemistry**

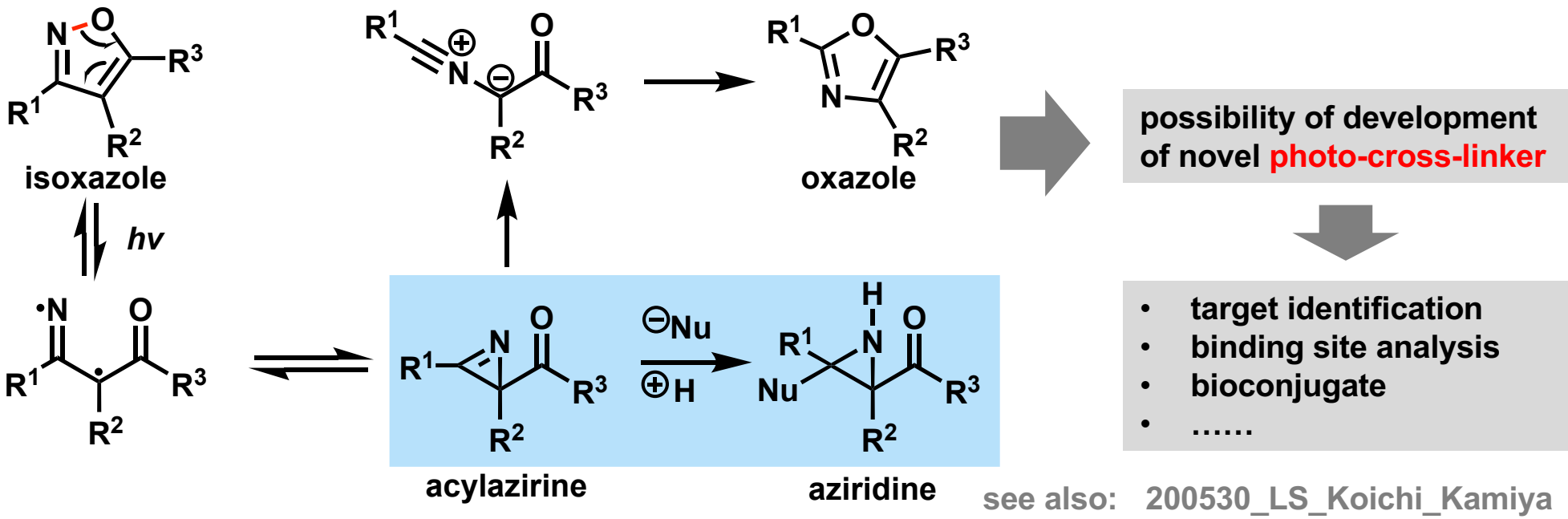
current research topics:

1. **Development of microarray-based screening platform**

2. **Development of novel fluorescent probes for live cell imaging studies**

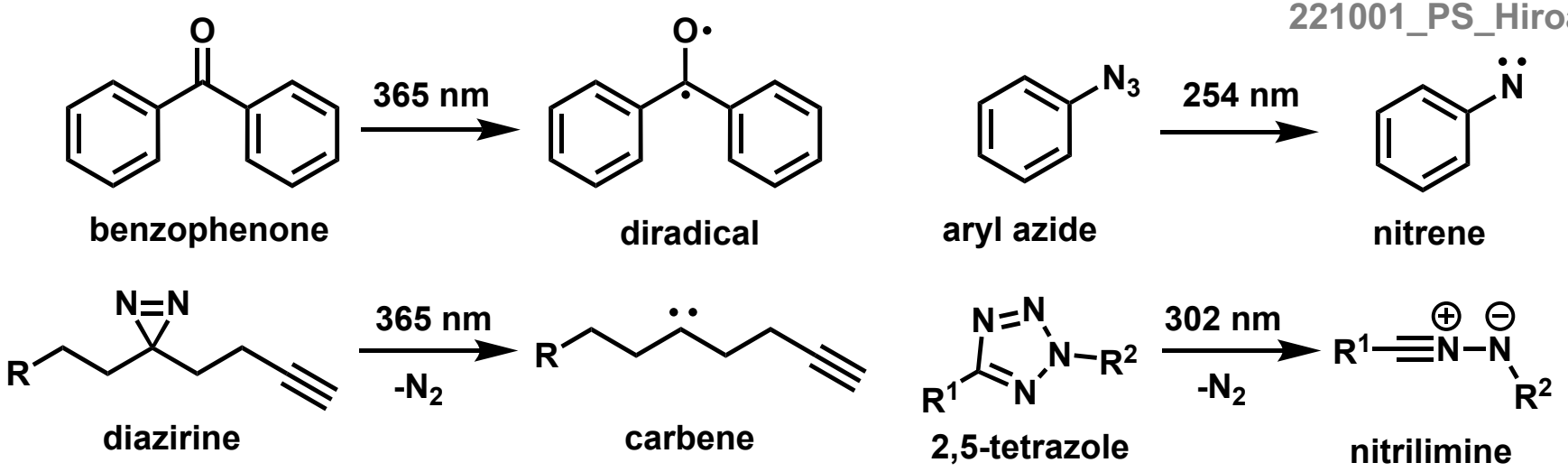
3. **Development of novel biomaterials for biological application**

Photochemistry of Isoxazole



see also: 200530_LS_Koichi_Kamiya
211203_LS_Yuanqi_lin
221001_PS_Hiroaki_Itoh

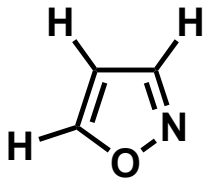
representative photo-cross-linkers:



1. Singh *et al. J. Am. Chem. Soc.* **1966**, *88*, 1844–1845.
2. Meijiler *et al. Bioorg. Med. Chem.* **2012**, *20*, 554–570.
3. Brandon *et al. ACS Chem. Biol.* **2014**, *9*, 2823–2832.
4. Schobert *et al. J. Org. Chem.* **2013**, *78*, 2455–2461.
5. Yao *et al. Angew. Chem., Int. Ed.* **2013**, *52*, 8551–8556.
6. Yao *et al.* Angew. Chem., Int. Ed. 2016, 55, 2002–2006.

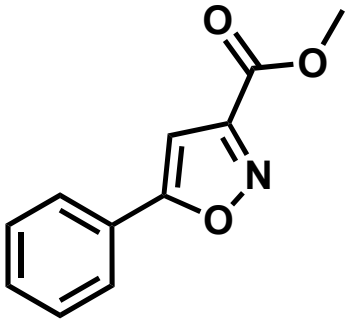
Verification of Photo-Reactivity – Model Study (1)

design of model compound:

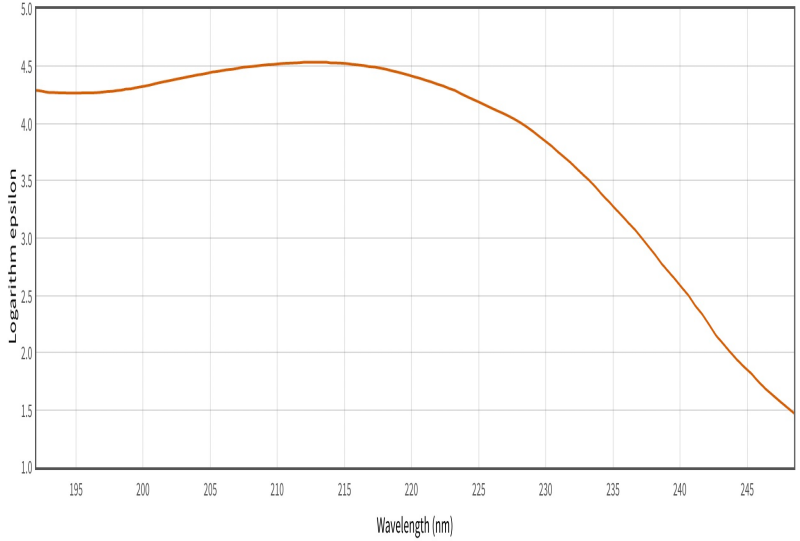


isoxazole core

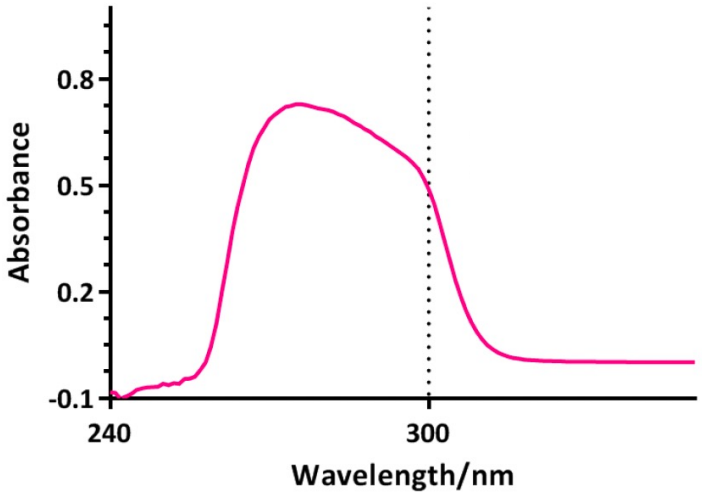
introduction of aryl substitution



methyl 5-phenylisoxazole-3-carboxylate (MPISC)



short-wave UV absorbance (190–250 nm)

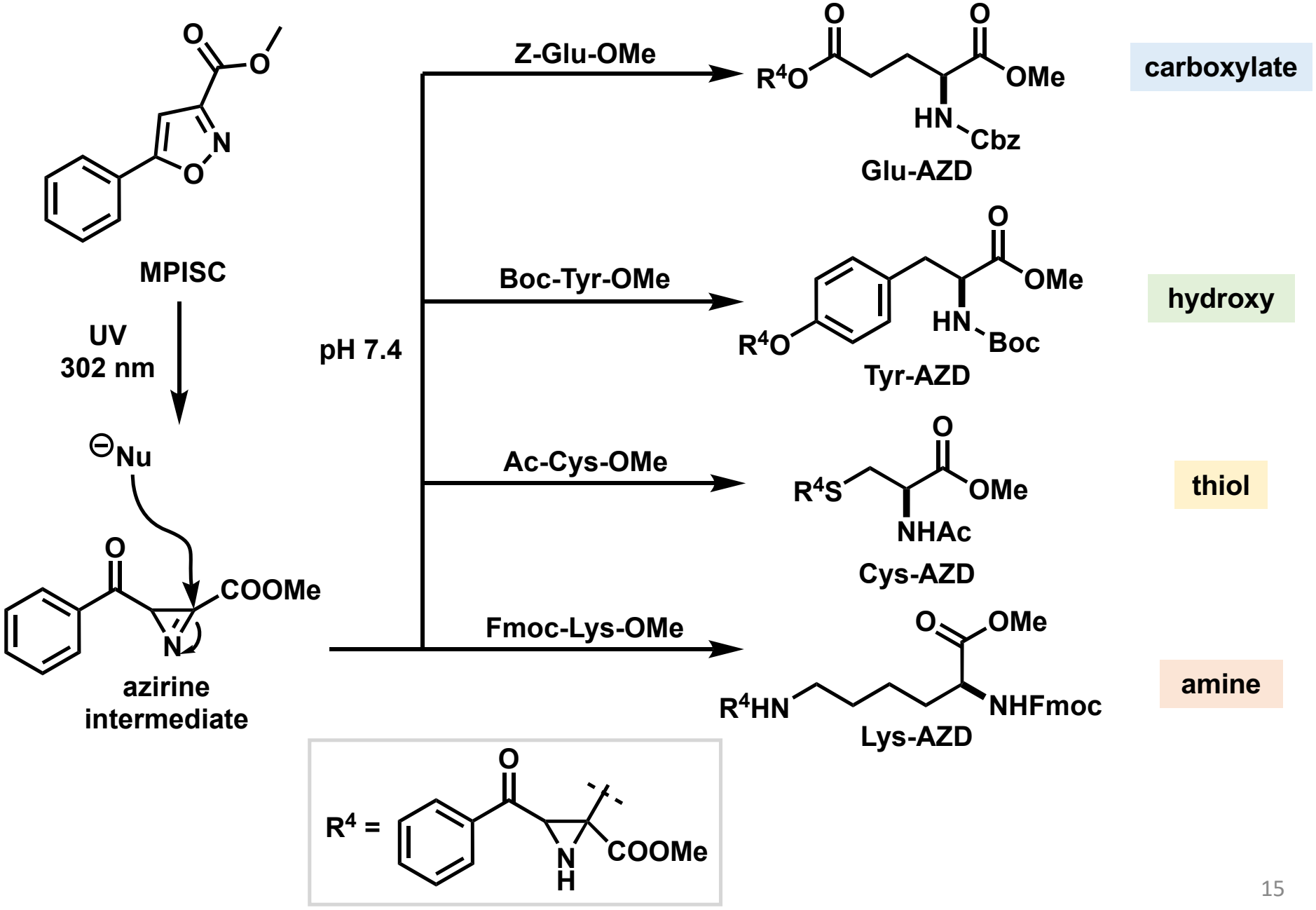


broad absorption (260–310 nm)

302 nm for the irradiation: avoiding damage on cell and proteins by short-wave UV

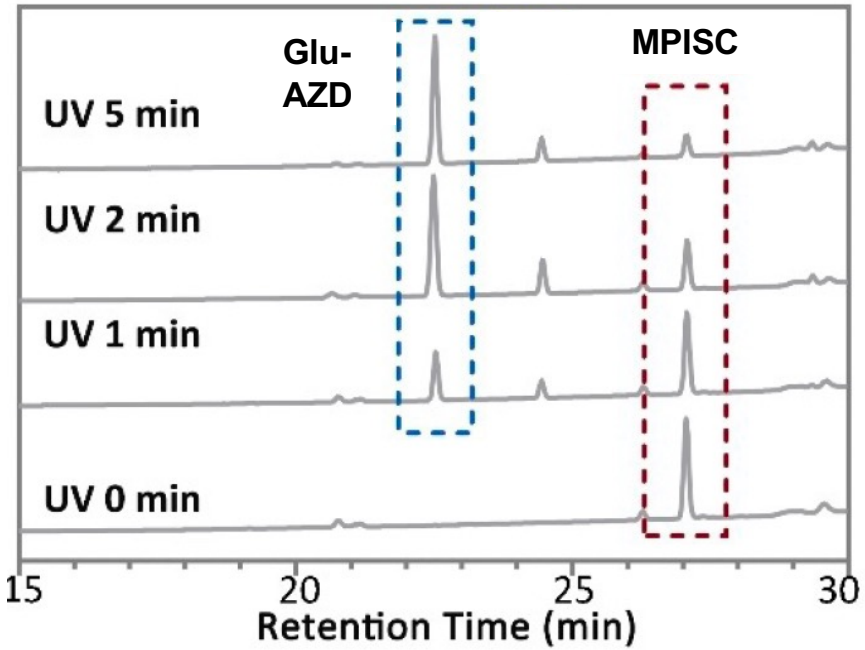
Verification of Photo-Reactivity – Model Study (2)

photo cross-link with nucleophilic amino acids:



Verification of Photo-Reactivity – Model Study (3)

for acidic amino acid (Glu):

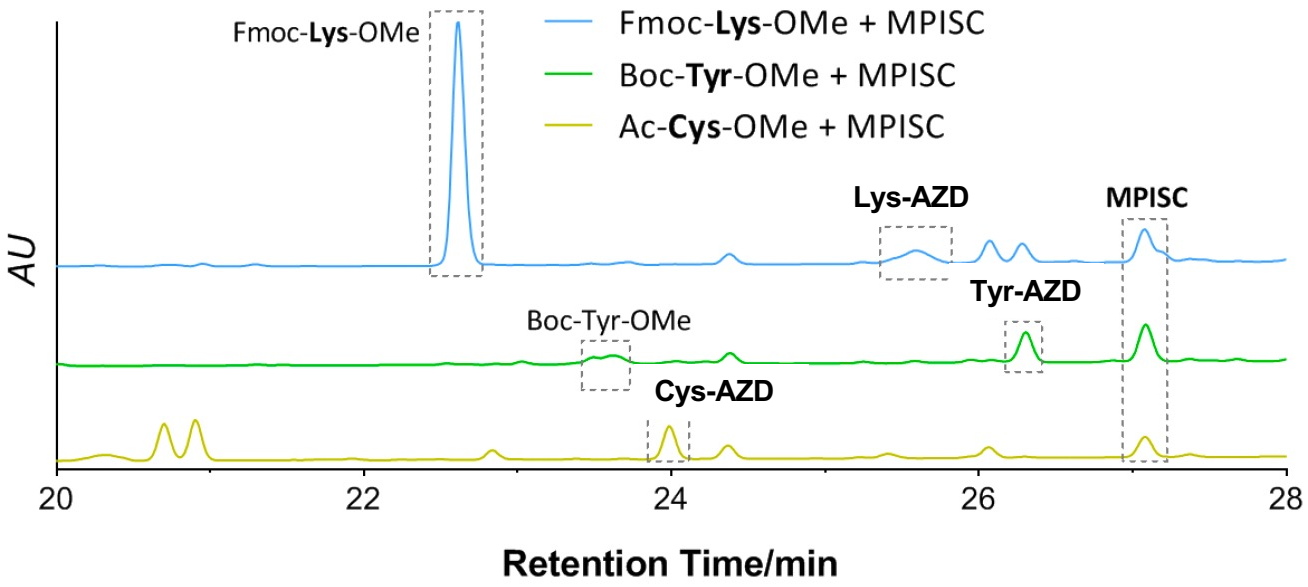


- rapid consumption of MPISC within 5 min
- high yield of Glu-AZD
- no observation of azirine intermediate

preference on acidic amino acid at physiological pH

- relatively lower yields
- possibly influenced by protonation/deprotonation status at pH7.4

for other nucleophilic amino acids:

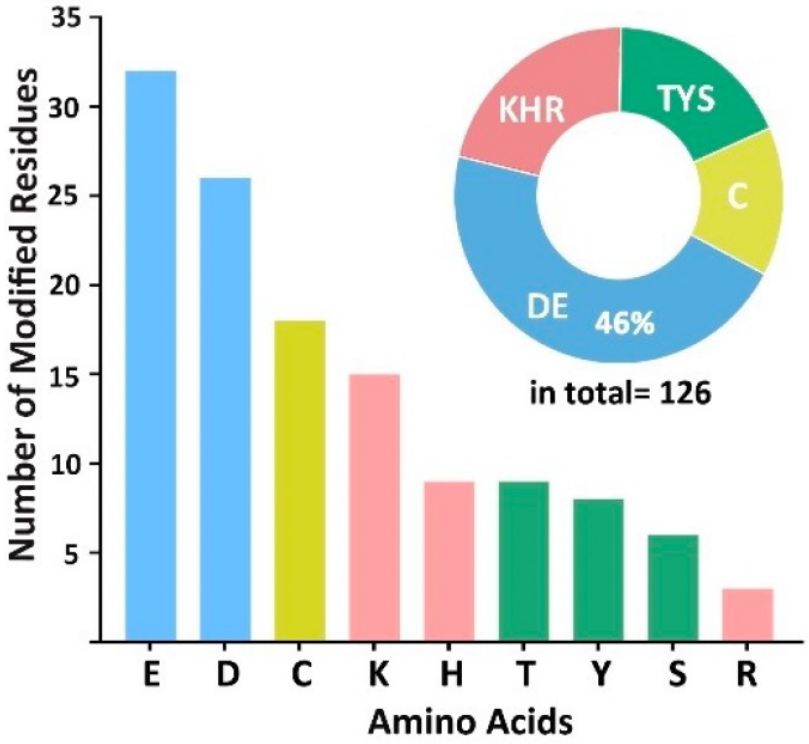


Verification of Photo-Reactivity – Model Study (4)

photo-cross-link site analysis on bovine serum albumin (BSA):

workflow:

results: 126 cross-link sites(in total)



* These results only reflected 126 possible labeling sites, instead of a stoichiometry of MPISC:BSA 126:1

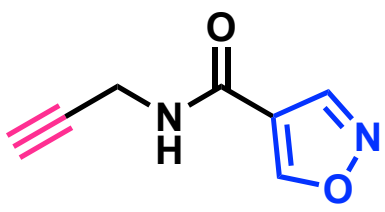
the cross-link preference of MPISC:

- **acidic residues: Glu, Asp (main)**
- **basic residues: Lys, His, Arg**
- **hydroxylic residues: Thr, Tyr, Ser**
- **thiol side chain residues: Cys**

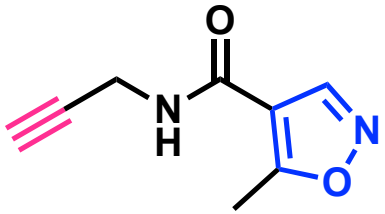
Functionalization of Isoxazole Photo-Cross-Linker

design and synthesis of isoxazole photoaffinity labeling (PAL) probes:

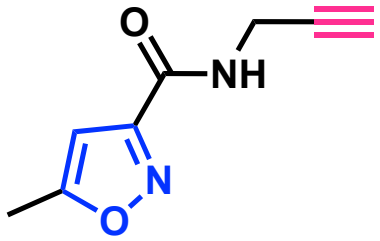
alkyl isoxazole PAL probes:



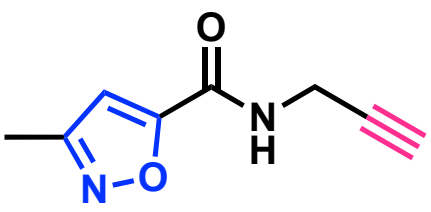
isx1



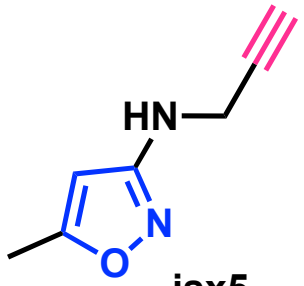
isx2



isx3

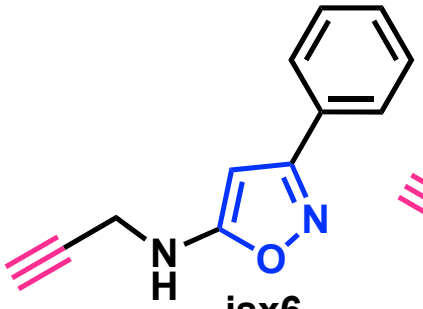


isx4

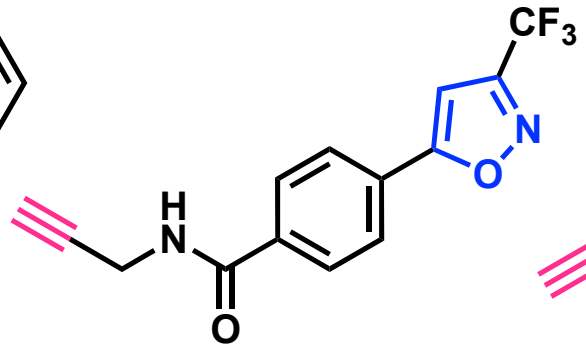


isx5

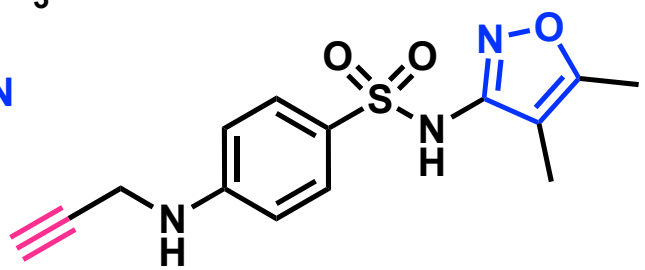
aryl isoxazole PAL probes:



isx6

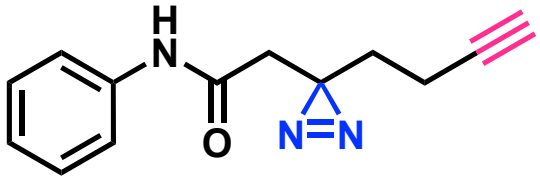


isx7

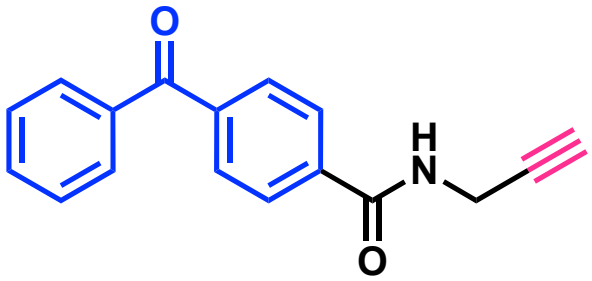


isx8

non-isoxazole PAL probes:



diazirine probe (DZP)



benzophenone probe (BZP)

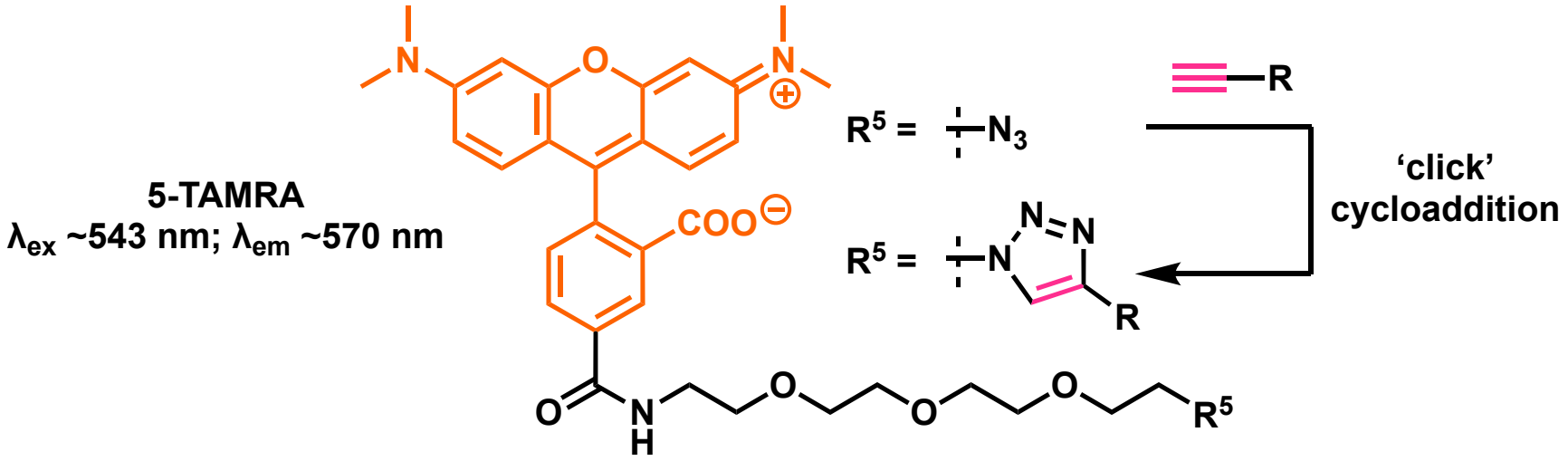
: photo-cross-linker

: biorthogonal reporter unit

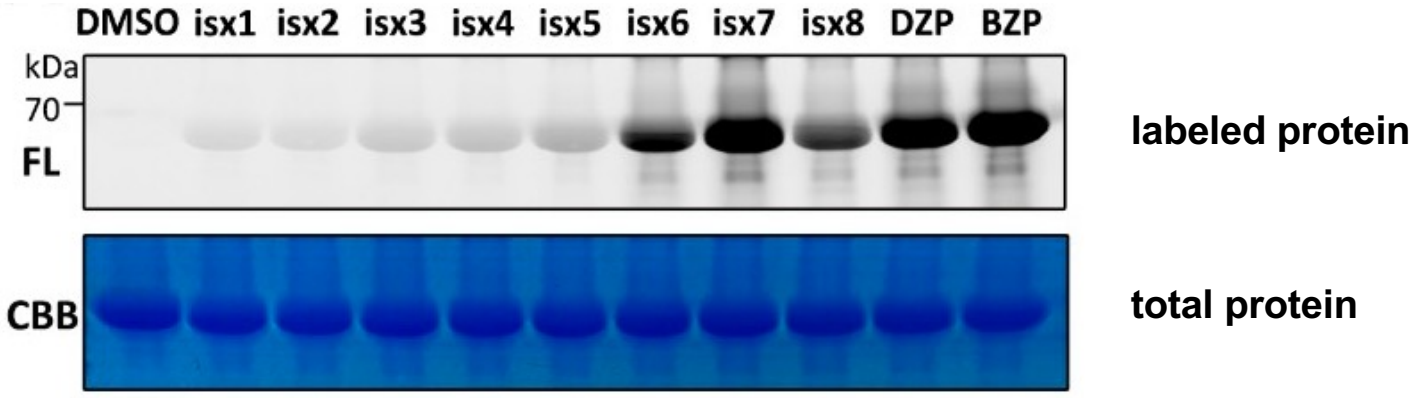
construction could be achieved by condensation or substitution reactions using known compounds

Photoaffinity Labeling Using Isoxazole Probes (1)

detection of labeled proteins: gel fluorescence



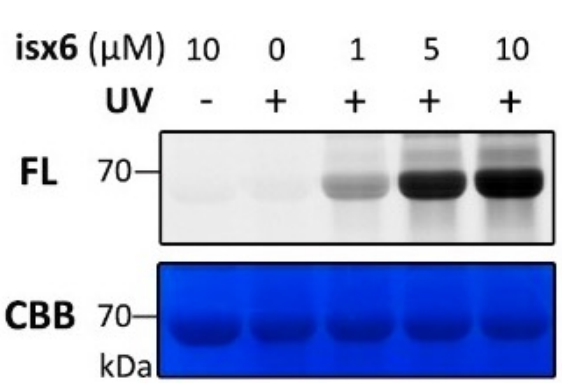
labeling of recombinant BSA:

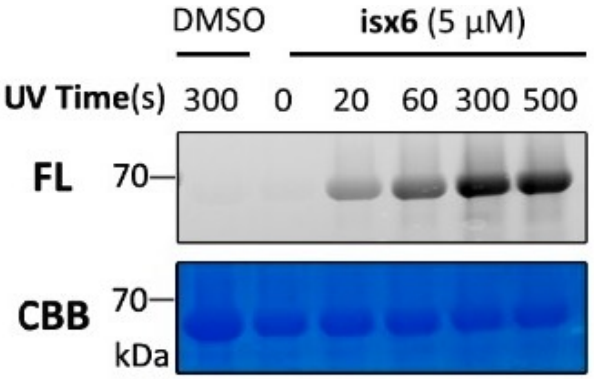


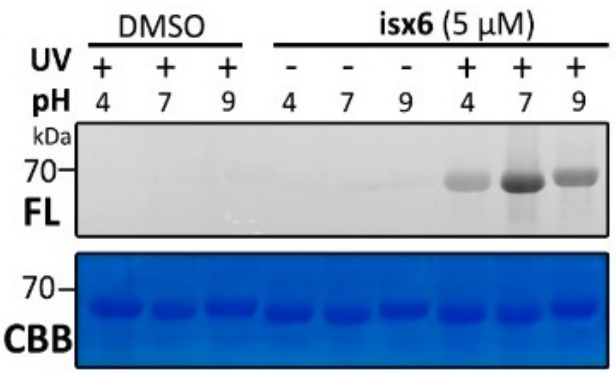
- **alkyl probes (isx1–5) showed moderate labeling, which is a result of its weak absorbance at 302 nm**
- **aryl probes (isx6–8) showed equipotent labeling efficiency with non-isoxazole PAL probes**

Photoaffinity Labeling Using Isoxazole Probes (2)

investigation of influence of conditions on labeling efficiency:



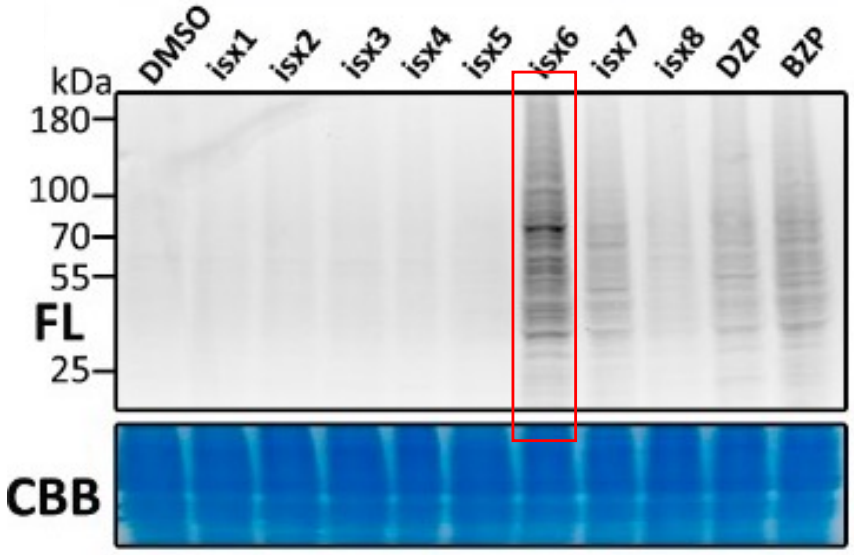


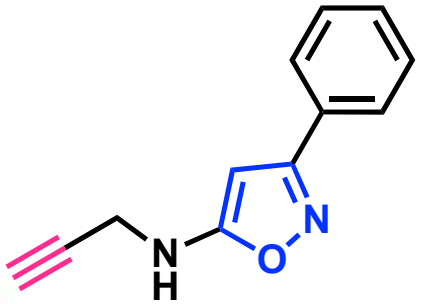


- probe concentration-dependent
- quick reaction within 5 min

- pH-dependent: physiological pH is favored

in situ photoaffinity labeling of live HeLa cell:





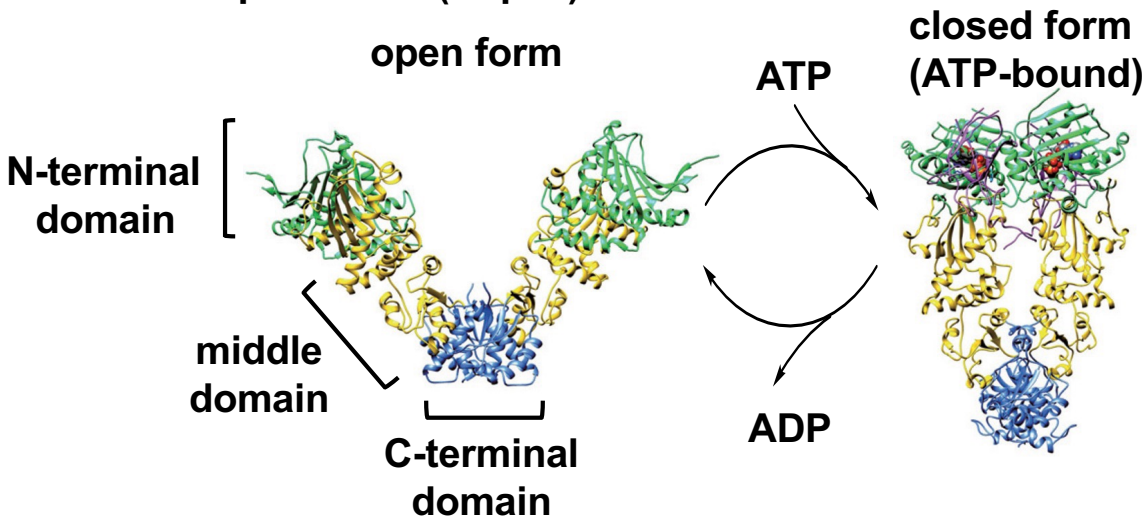
isx6

- labeling of interaction proteins
- isx6 labeled the most proteins even more than diazirine-type probe (DZP)

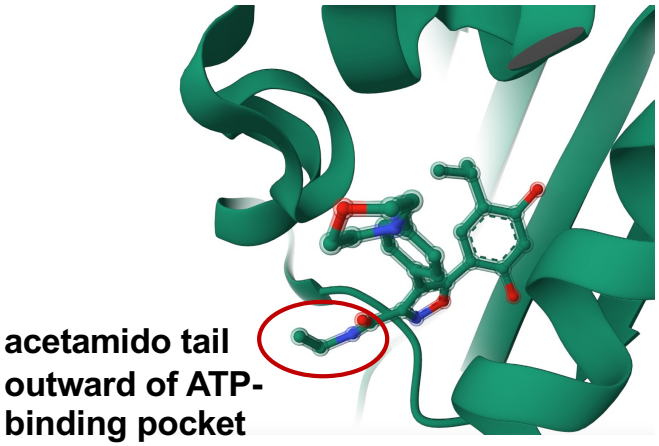
potential of isoxazole as effective photo-cross-linker

Heat Shock Protein 90 (Hsp90) and Luminespib

heat shock protein 90 (Hsp90):

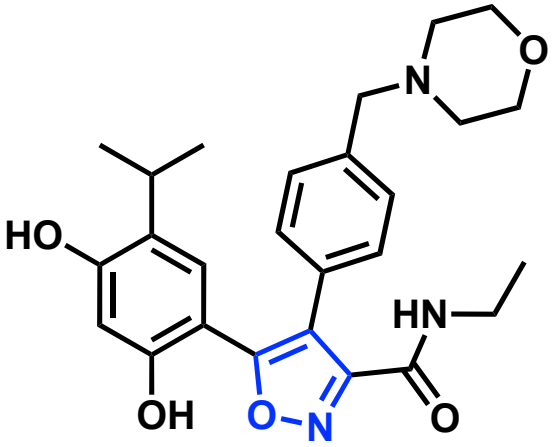


docking of luminespib with Hsp90:
(PDB ID: 2VCI)

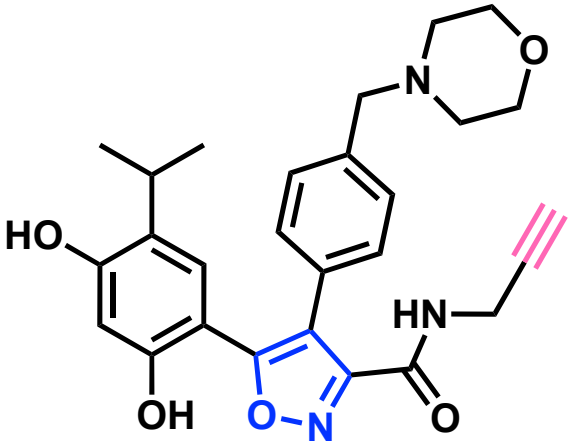


- assistance in protein folding (chaperone function)
- stabilization of proteins against cell heat stress (including those for tumor growth)

Hsp90 inhibitor luminespib and PAL probe design:



Luminespib



isx10

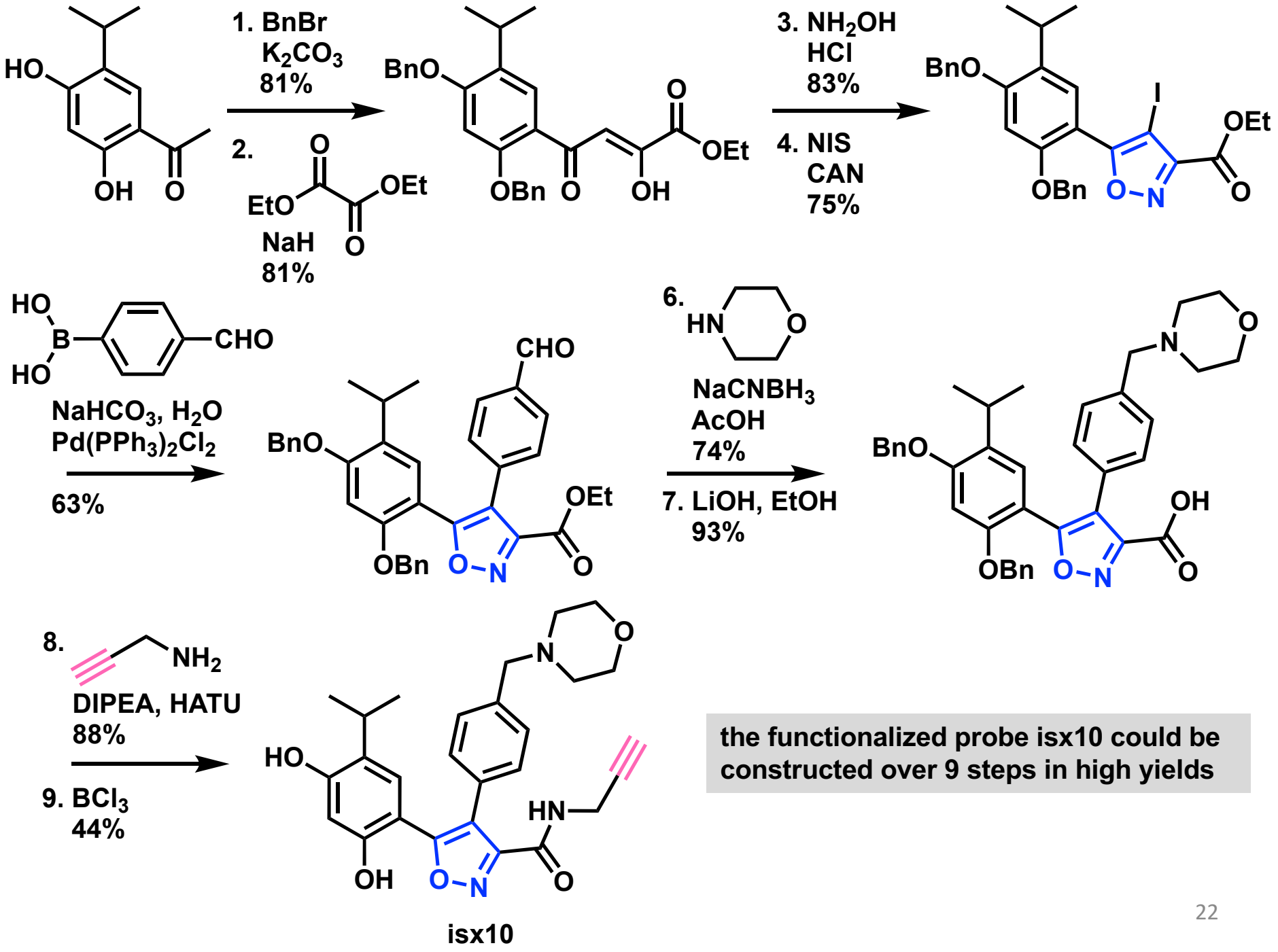
- adverse effects: diarrhea, visual disturbances, and nausea
- necessity of identification of off-targets
- photo-cross-linker embedded

minimal structure modification

- retaining of biological properties
- profiling of interaction proteins

1. Brough, P. A. et al. *J. Med. Chem.* **2007**, *51*, 196–218

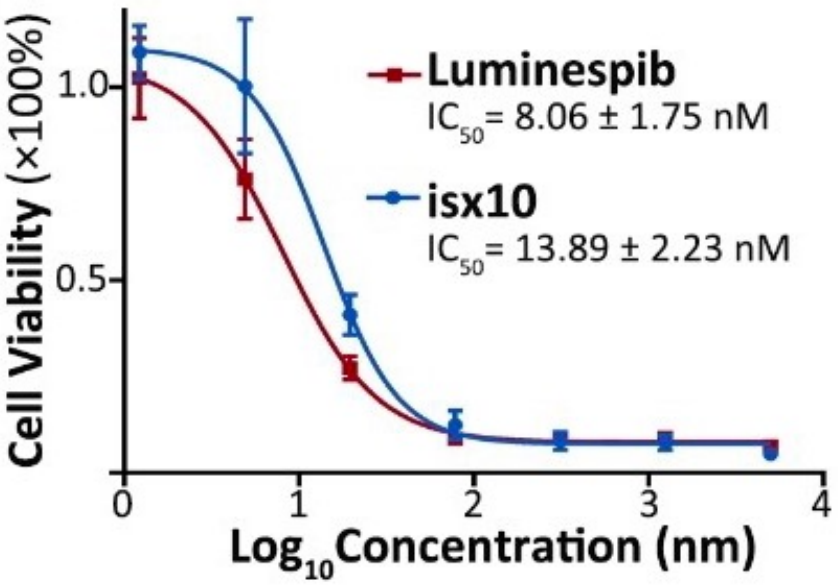
Synthesis of isx10



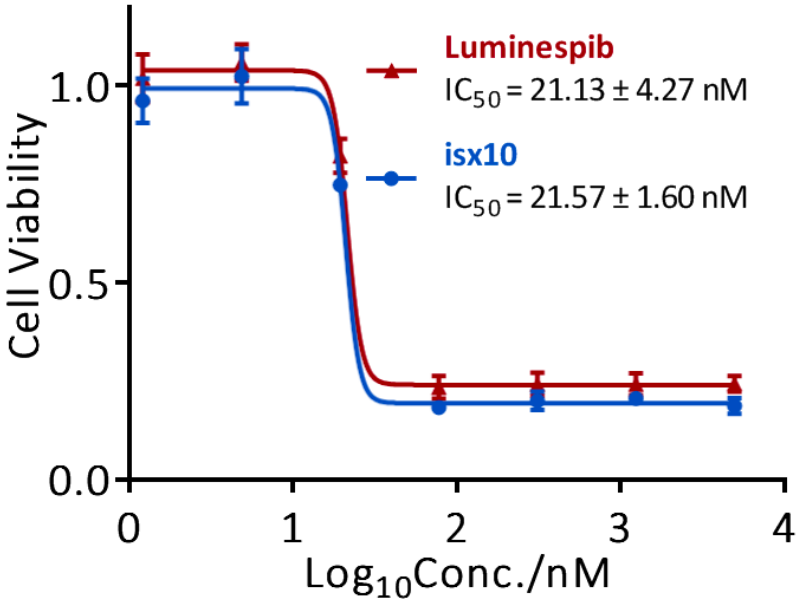
the functionalized probe isx10 could be constructed over 9 steps in high yields

Bioactivities of isx10

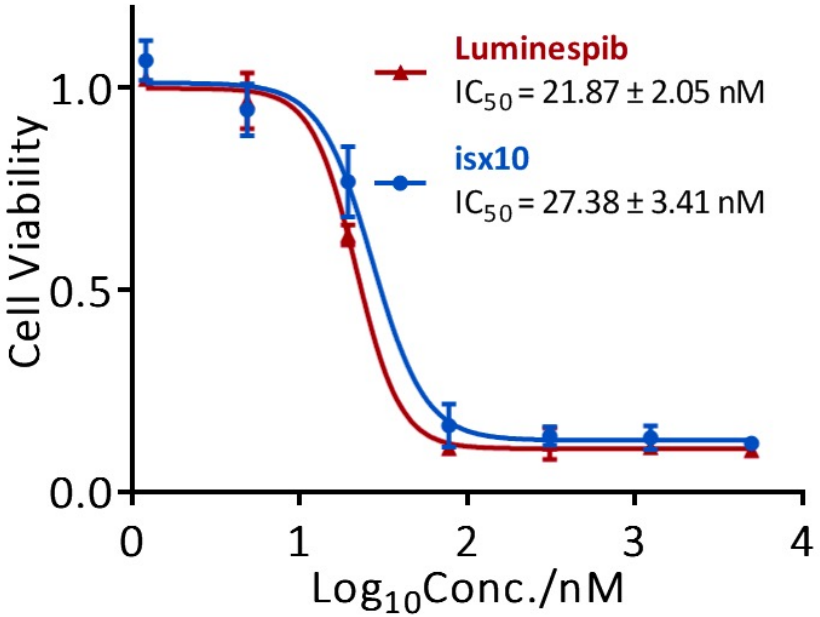
HeLa: cervical cancer cell



HEK293T: human embryonic kidney cell



HCT116: human colon cancer cell



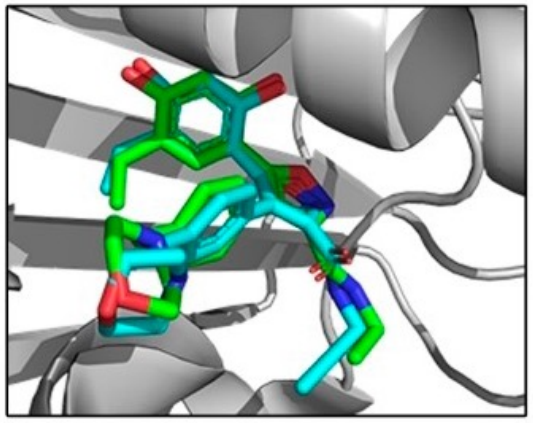
	IC_{50} (nM)		
	HeLa	**HEK293T**	**HCT116**
luminespib	**8.06**	**21.1**	**21.9**
isx10	**13.89**	**21.6**	**27.4**

- **equipotent cytotoxicity**
- **similar cell-line selectivity**

preservation of bioactivities

Interaction of isx10 with Hsp90

docking analysis of compounds with Hsp90:



: Hsp90

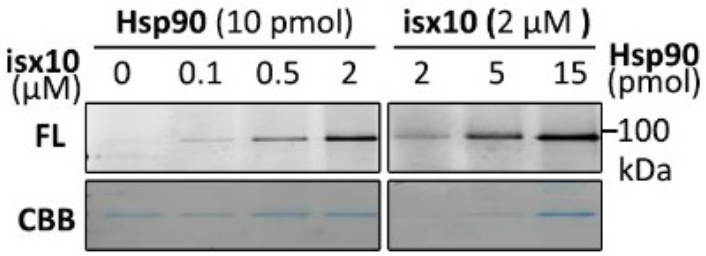
: Luminespib

: isx10

- similar atomic coordinates
- similar Hsp90-binding patterns

photoaffinity labeling of Hsp90 with isx10:

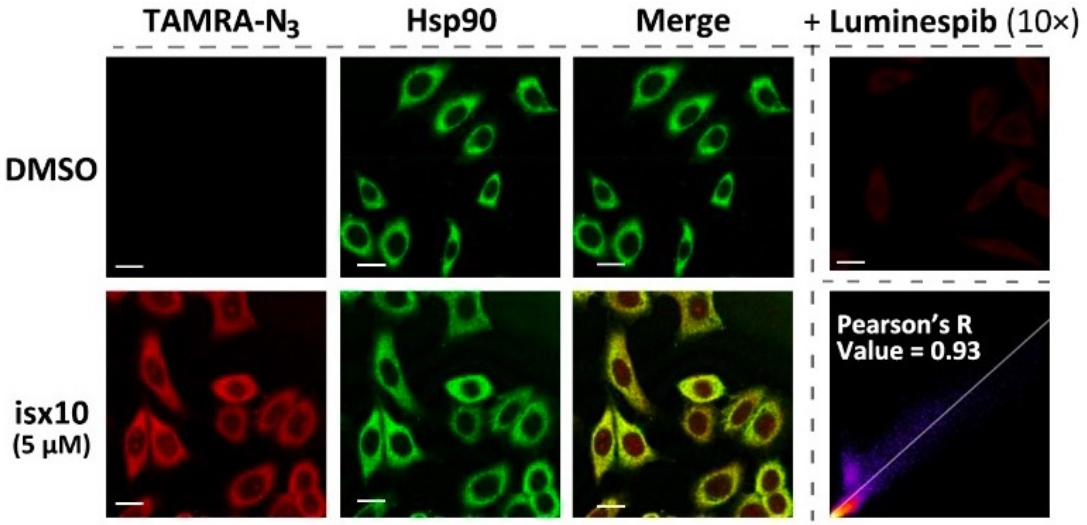
using recombinant Hsp90:



- sensitive labeling of recombinant Hsp90 even at low concentrations

- good efficiency and specificity

in live HeLa cell:



: fluorescence of TAMRA

: fluorescence of Alexa Fluor 488 conjugated on secondary antibody

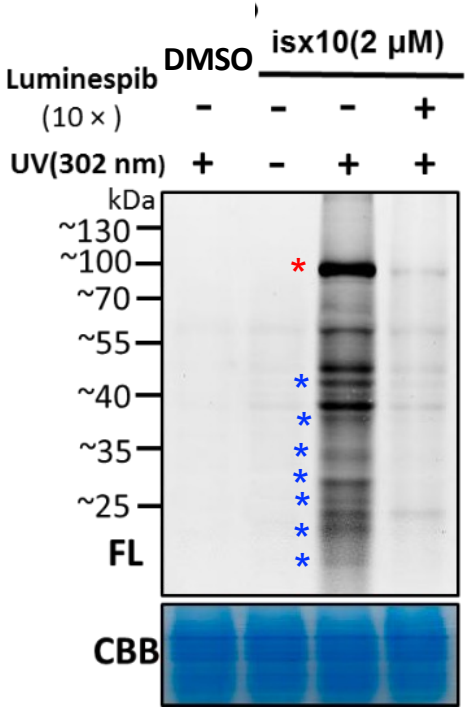
functionalization of luminespib didn't affect the molecular interaction with Hsp90

Photoaffinity Labeling of Interaction Proteins

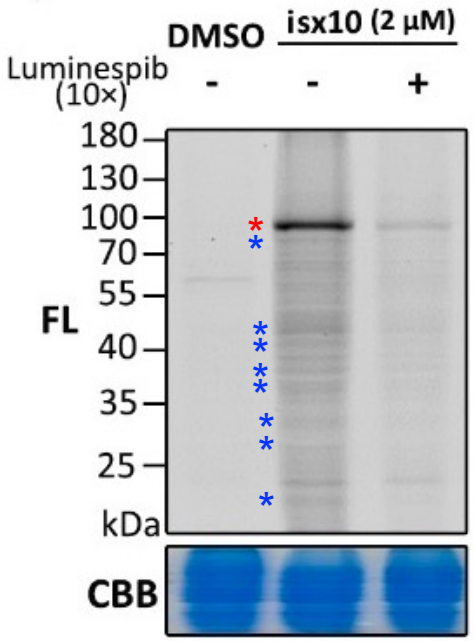
competitive photoaffinity labeling: exclusion of non-specific interactions

parent luminespib used as competitor

using HeLa cell lysate:



in live HeLa cell:



***: Hsp90**

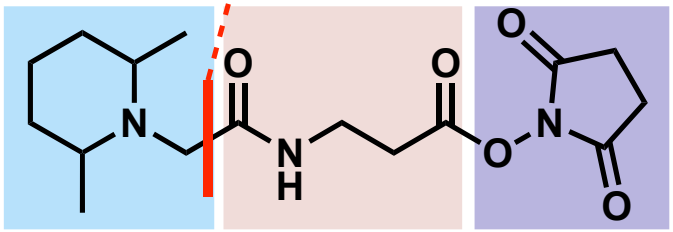
***: specifically labeled proteins**

- **several unknown proteins specifically labeled**
- **existence of possible interaction proteins confirmed**

identification of possible interaction proteins –
tandem mass tag (TMT)-based chemoproteomic experiments

TMT-based Peptide Quantification (1)

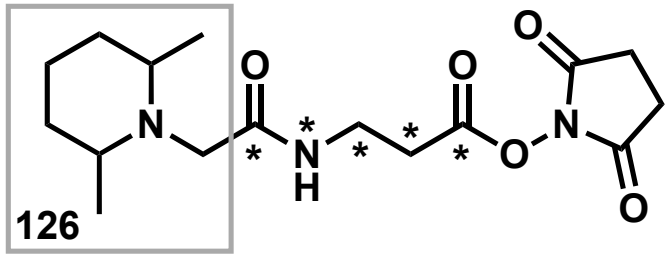
cleavable by higher energy collisional dissociation (HCD)

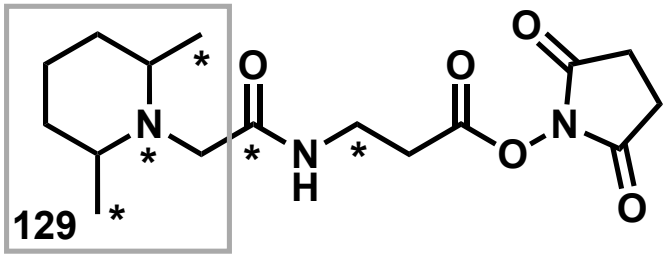


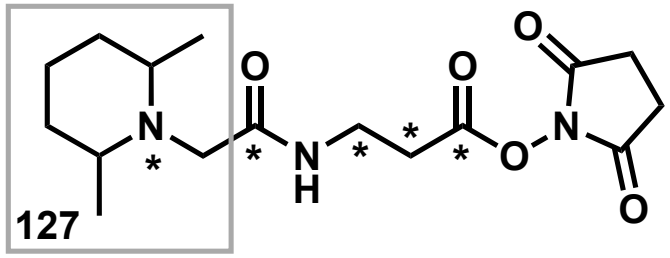
mass reporter | mass normalizer | NH_2-reactive group

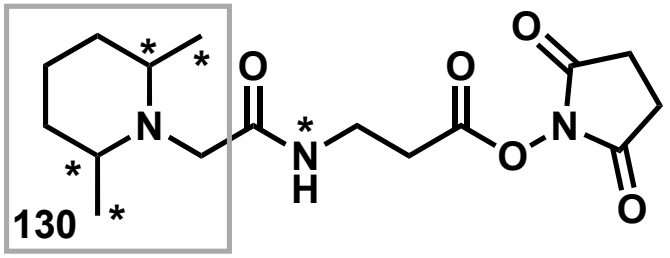
*: heavy atom

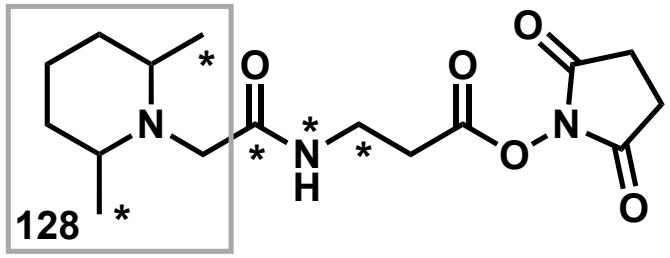
peptides can be tagged through nucleophilic addition of N-terminal NH_2 or Lys side chain NH_2 to TMT

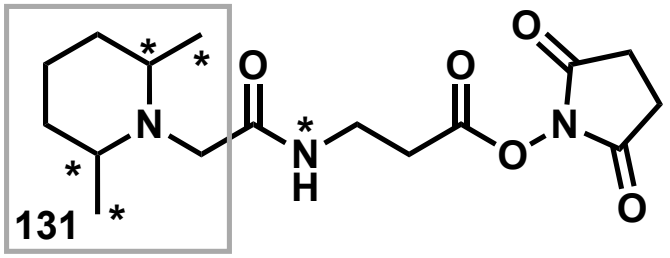








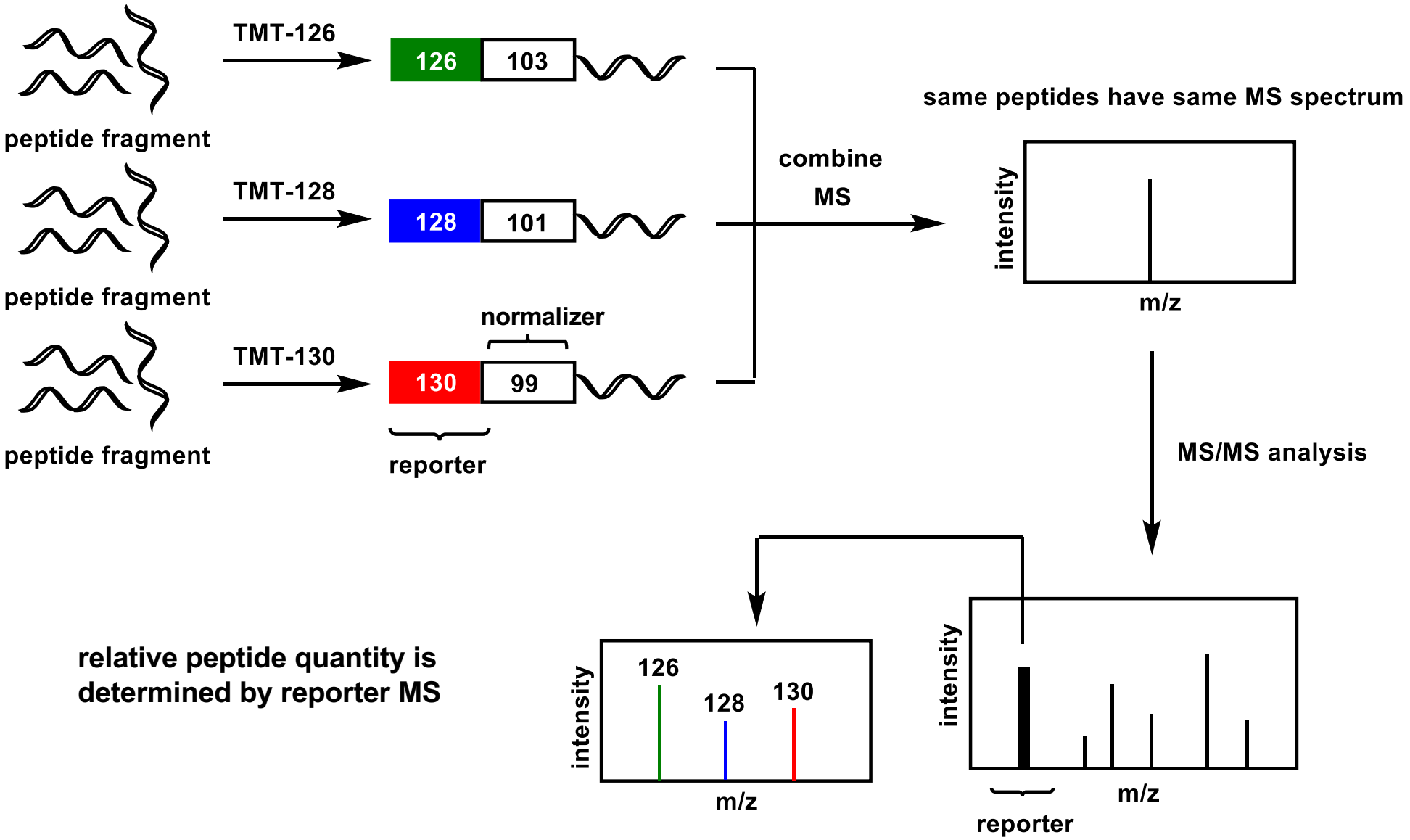




1. Dayon, L.; Hainard, A.; Licker, V.; Turck, N.; Kuhn, K.; Hochstrasser, D. F.; Burkard, P. R.; Sanchez, J. *Anal. Chem.* **2008**, *80*, 1895.
2. see also 200530_LS_Koichi_Kamiya

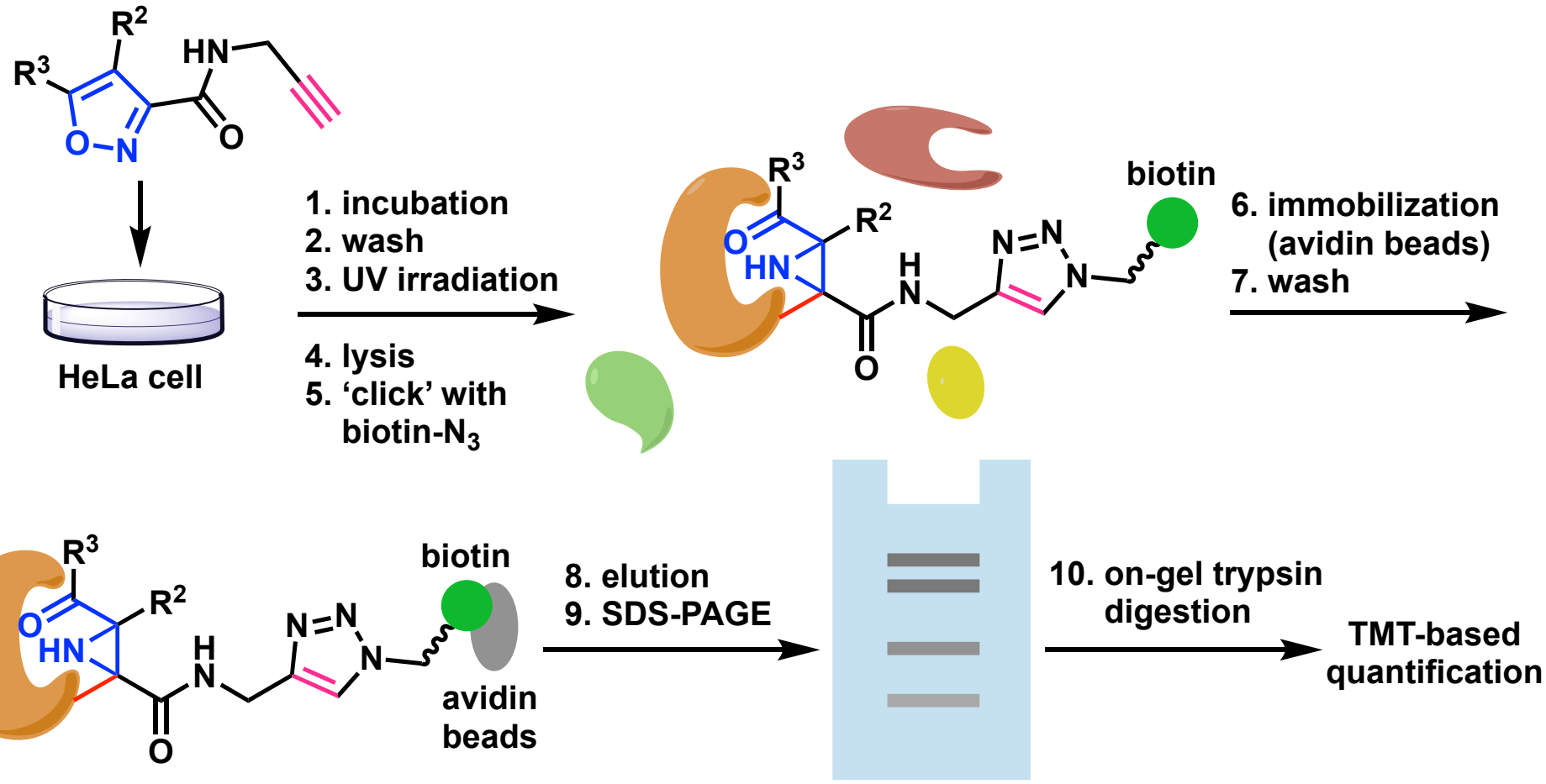
TMT-based Peptide Quantification (2)

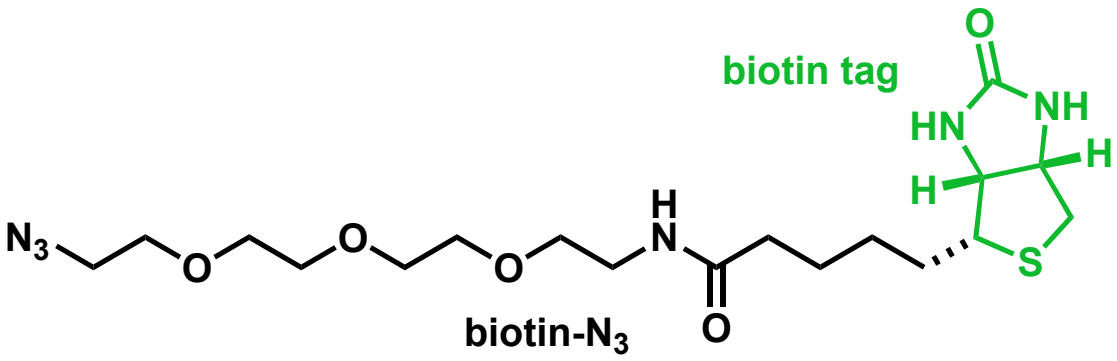
a total *m/z* number of 229 to keep the mass of TMT constant



1. Thompson, A.; Schäfer, J.; Kuhn, K.; Kienle, S.; Schwarz, J.; Schmidt, G.; Neumann, T.; Hamon, C. *Anal. Chem.* **2003**, *75*, 4942–4942.

PAL Pull-Down — Enrichment of Interaction Proteins



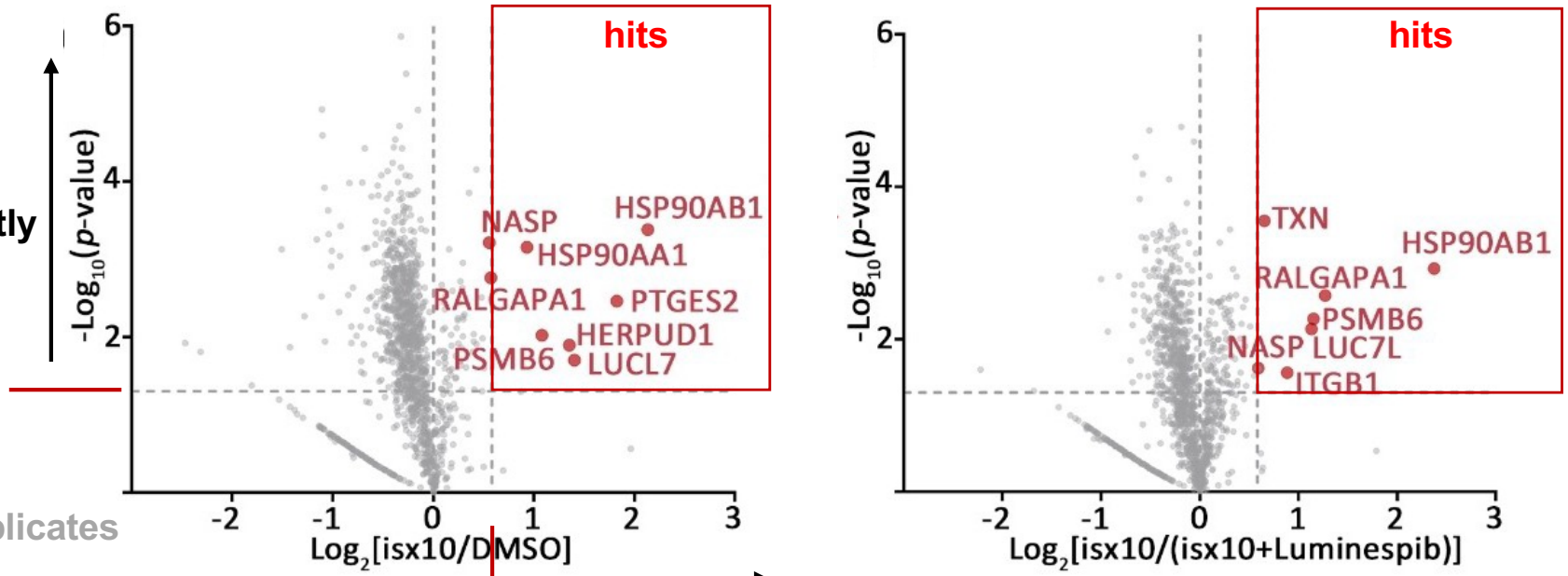


immobilization of labeled proteins: strong biotin-avidin interaction (K_D ~ 10^3 pM)

Identification of Interaction Proteins

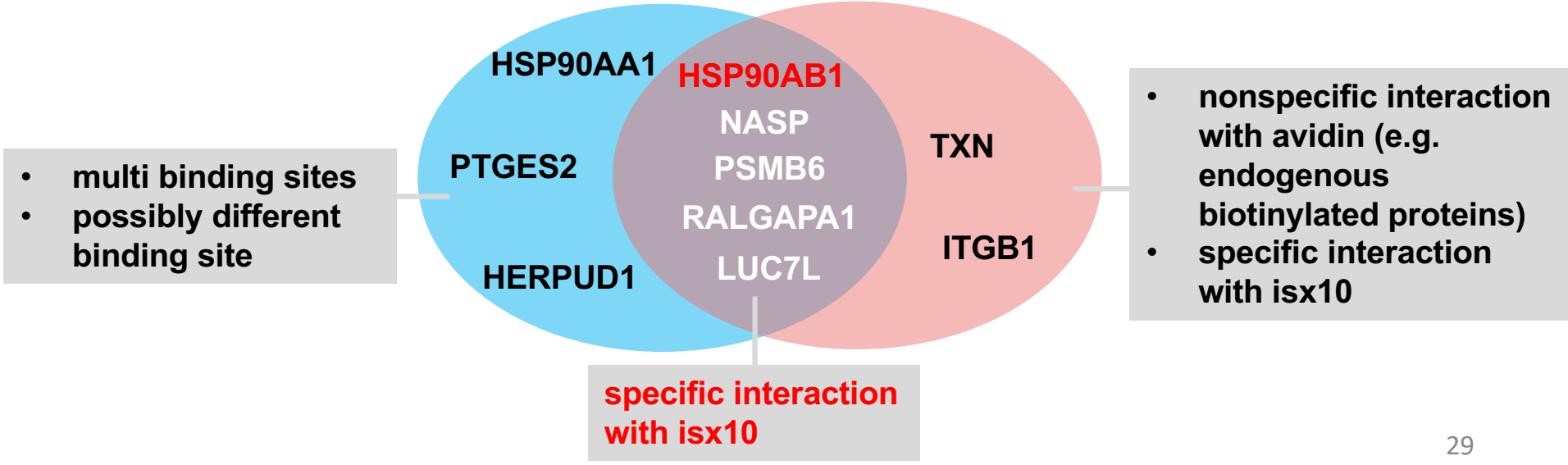
normal:

competitive:



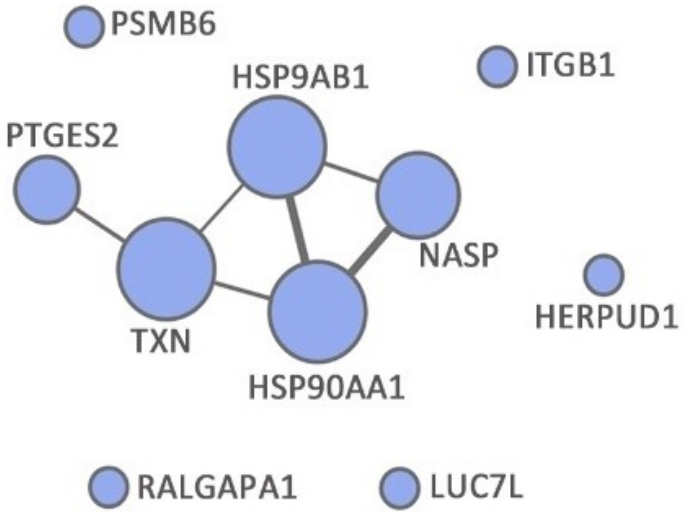
* four replicates

10 enriched proteins:



Investigation on Enriched Proteins

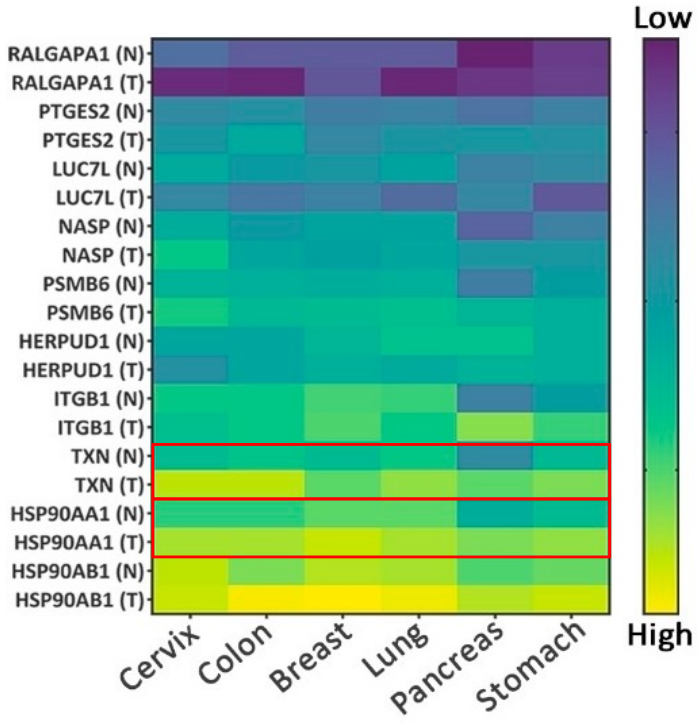
protein-protein interactions (PPIs):



HSP9AB1: Hsp90β isoform
HSP90AA1: Hsp90α isoform
NASP: nuclear autoantigenic sperm protein (histone chaperone and Hsp90 binding protein)
TXN: thioredoxin: reductive Cys residue (anti oxidative stress)
PTGES2: prostaglandin E2 synthase (potential COVID-90 therapeutic target)

three interaction proteins in Hsp90 PPIs network: possible involvement in anti-cancer activity

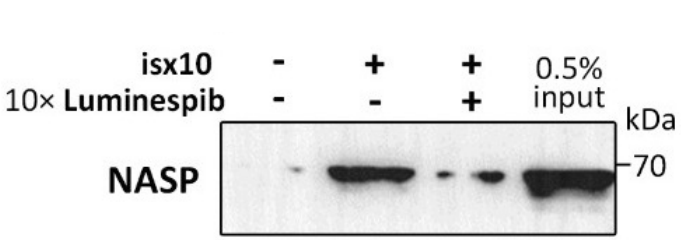
gene expression in normal and tumor tissues:

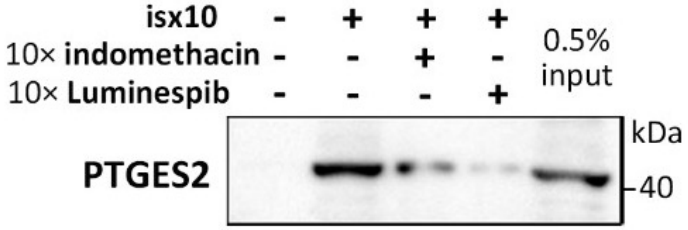


- **Hsp90** and **TXN** showed higher expression in cancer tissues — **cancer therapeutic specificity**
- other interaction proteins have minor changes in expression level — **potential toxicity**

Validation of Protein Binding

competitive PAL pull-down and Western blotting: indomethacin: known inhibitor of PTGES2

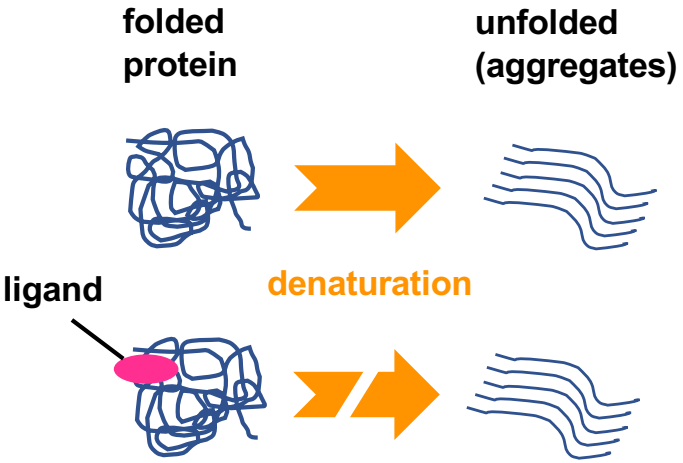




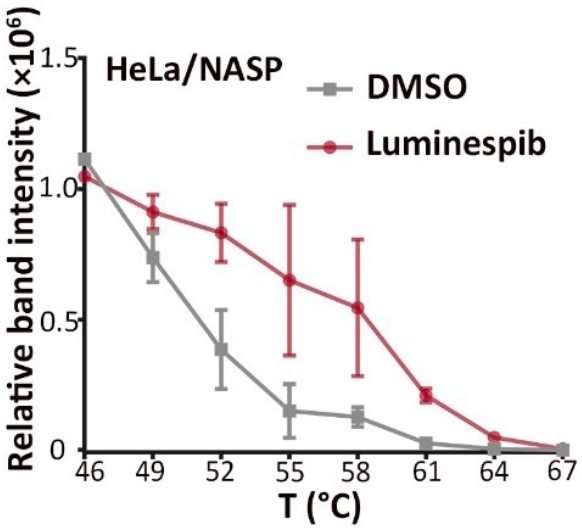
chemiluminescence from enzyme activity of antibody-conjugated HRP

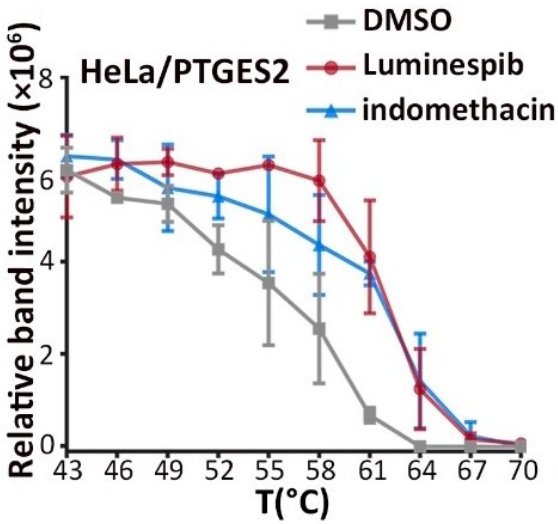
luminespib specifically interacts with NASP and PTGES2

cellular thermal shift assay (CETSA):
influence of binding complex formation on thermostability



melting curve: plot of temperature to soluble folded protein

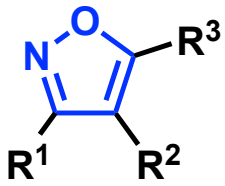




remarkable stabilization of luminespib on NASP and PTGES2 validated the binding affinity

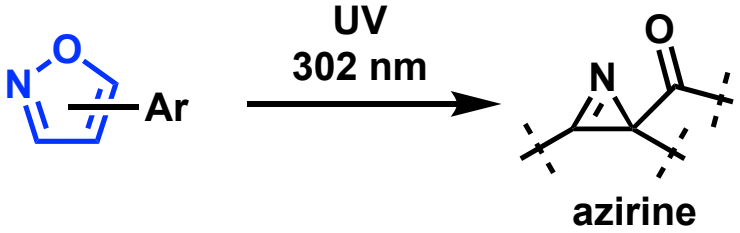
Summary

isoxazole: significant skeleton for bioactive compounds



- **pharmacophoric features**
- **easily synthesized**
- **important synthetic intermediate**

capitalize on photochemistry features



- **efficient labeling of amino acids and proteins**
- **application of luminespib-derived probe in chemoproteomics**

future perspectives

novel photo-cross-linker (embedded/extra):

- **chemoproteomics: identification of interaction proteins**
- **bioconjugate: cross-link with protein/antibody**
- **covalent inhibition: photo therapeutics**