Recent advance of photo-crosslinker using tetrazole

Literature seminar #2
M1 Shinpei Takamaru
2023/06/22 (Thu)

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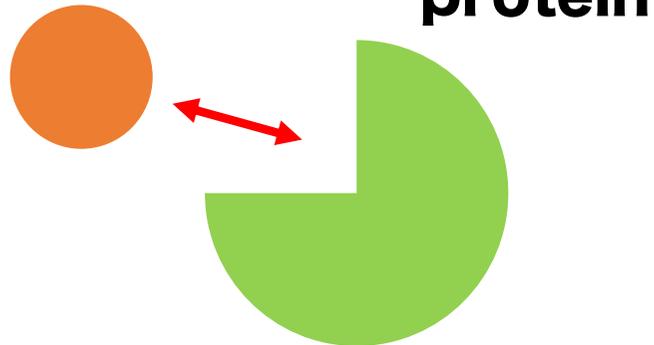
> ## Introduction
>> Standard method to identify target protein
>> Conventional photo-crosslinker

> ## Main
>> Tetrazole as photo-crosslinker
>> 2-acyl-5-carboxytetrazole (ACT)
>> PPI mapping by using ACT in living cells

> ## Summary

Introduction

**drug /
protein**

protein

What ? (identification)

How ? (interaction mechanism)

identifying binding protein or binding site
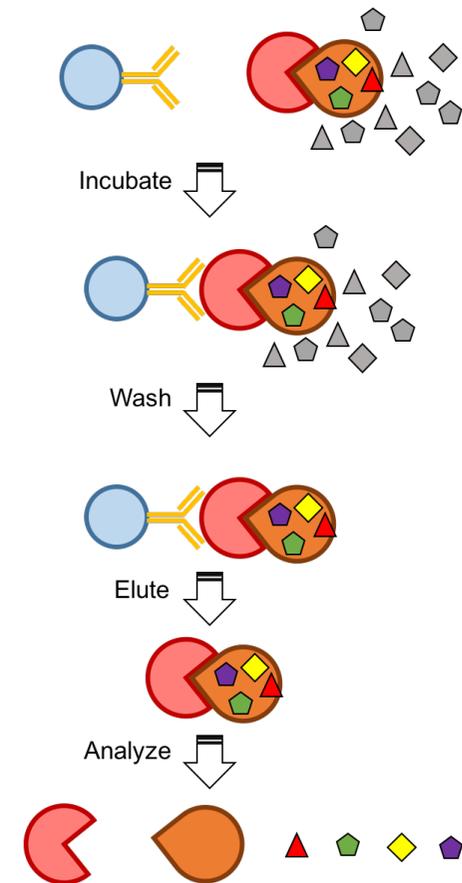
→ understand the mechanism of action

→ improve the drug

Standard method for identifying target protein

Major two methods to identify the target protein

① Co-IP
② crosslinking

➢ ① **Co-IP**

Incubate

Wash

Elute

Analyze

https://www.lifeasible.com/custom-solutions/plant/analytical-services/gene-function-analysis/co-immunoprecipitation-co-ip-assay/

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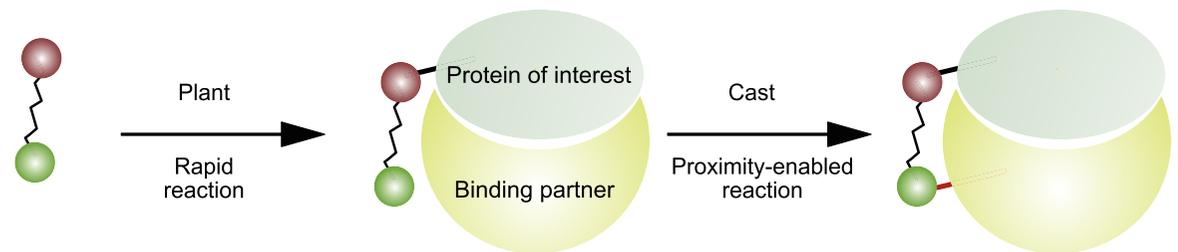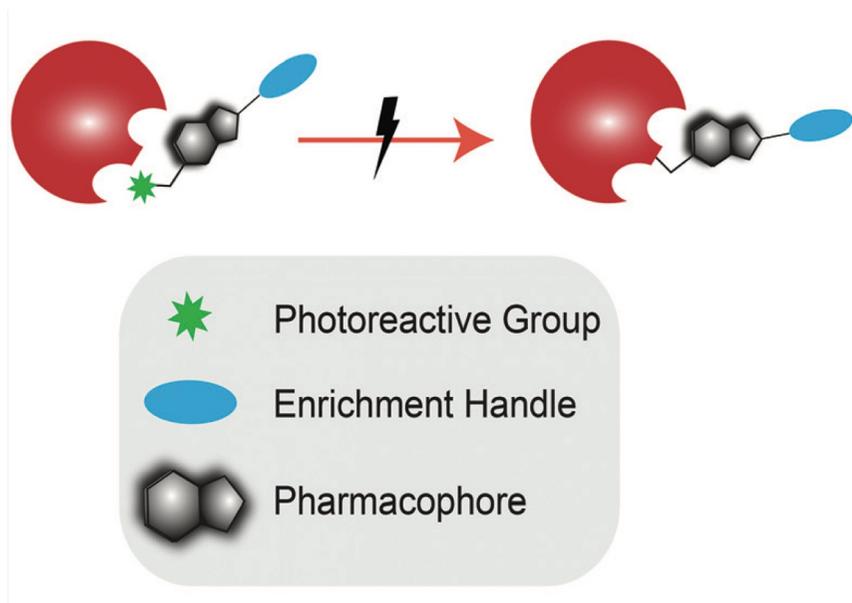# Standard method for identifying target protein

> ② **crosslinking**

Major two methods to identify the target protein

① **Co-IP**
② **crosslinking**

Plant
Rapid reaction

Protein of interest

Binding partner

Cast
Proximity-enabled reaction

Liu, J., Yang, B., & Wang, L. *Current Opinion in Chemical Biology* **2023**, *74*, 102285.

enrichment tag

pharmacophore

photo-reacting group

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Photo-crosslinker and Chemical crosslinker

➢ **Photo-crosslinker**
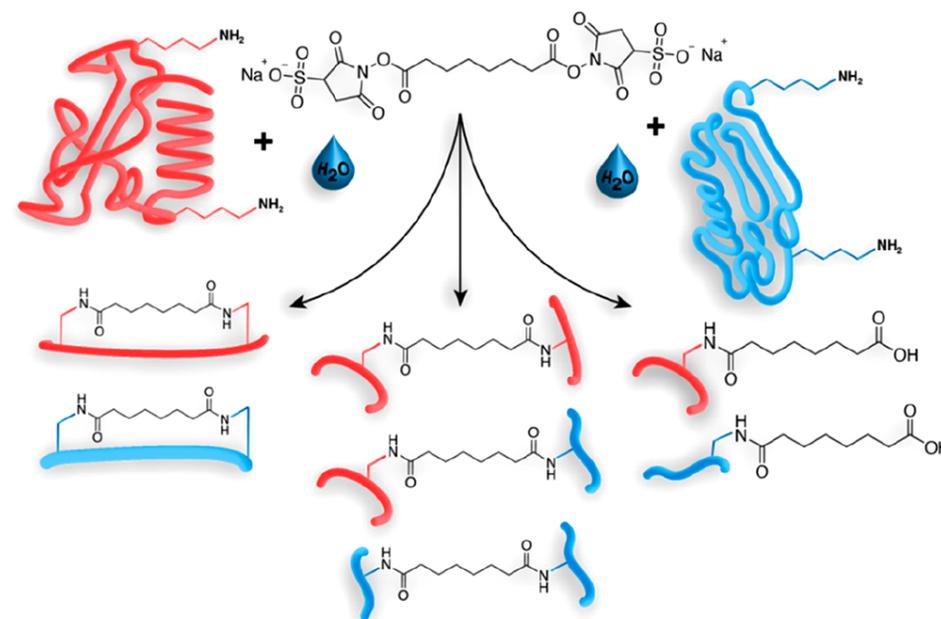

★ Photoreactive Group

⬭ Enrichment Handle

⬡ Pharmacophore

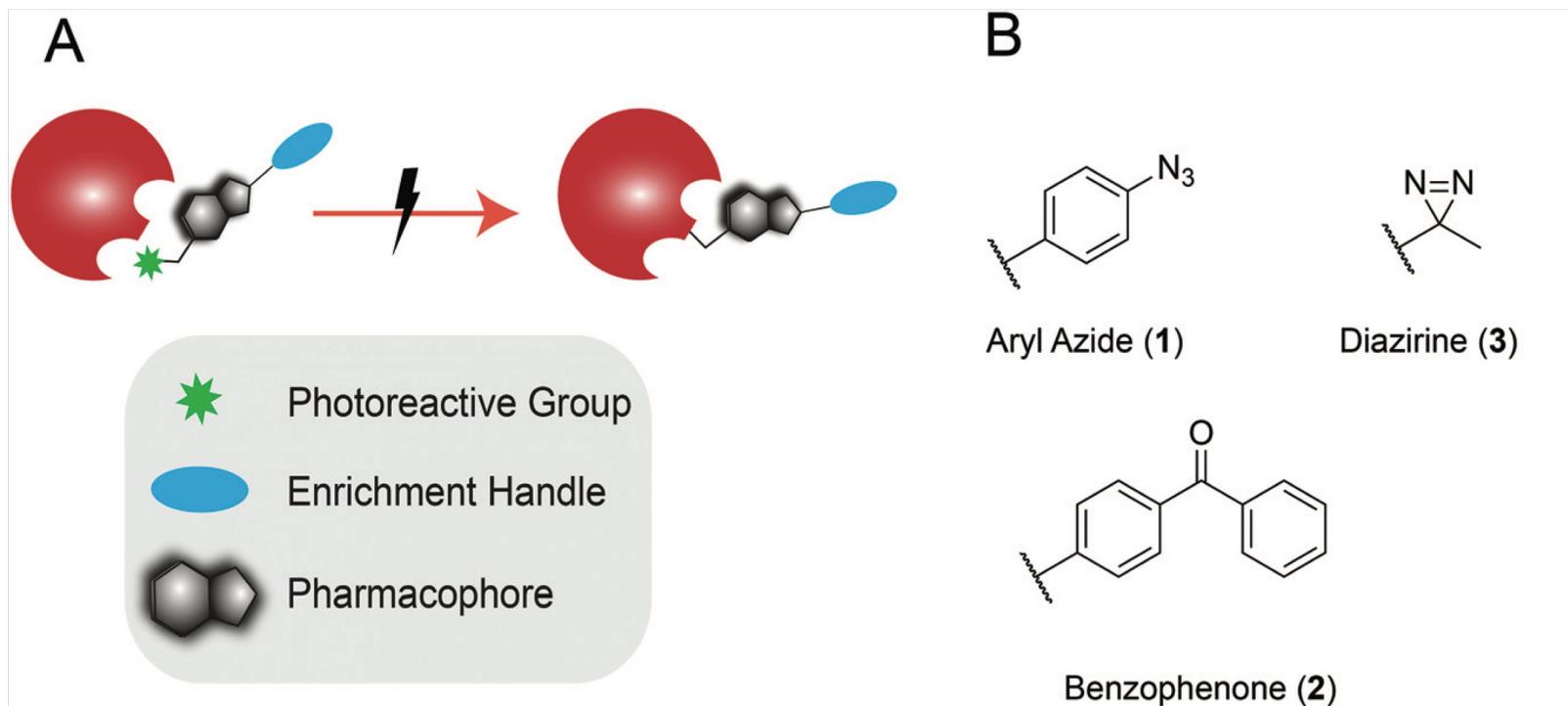Burton, N. R., Kim, P., & Backus, K. M.
Organic and Biomolecular Chemistry **2021**, *19*(36), 7792-7809.
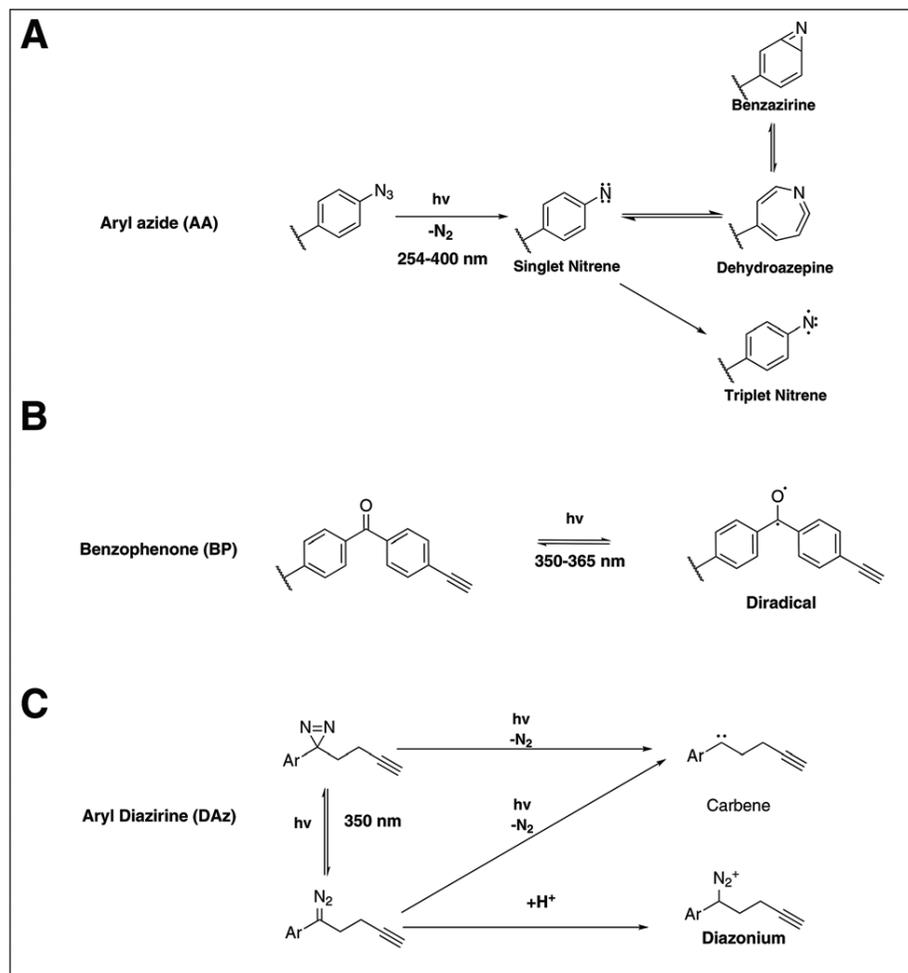
➢ **Chemical crosslinker**

Piersimoni, L., et al. *Chem. Rev.* **2022**, *122*, 7500-7531.

# Conventional photo-crosslinker

Conventional photo-crosslinker

Problem

➤ extremely short half-lives
→ very low target capturing yields

➤ the nitrone, carbene, and diradical intermediates
→ react non-selectively with any proximal C–H/X–H bonds (X = N, O, S)
→ high background

Burton, N. R., Kim, P., & Backus, K. M.
Organic and Biomolecular Chemistry **2021**, *19*(36), 7792–7809.

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Non-radical intermediate photo-crosslinker

c

Tetrazole

d

2-Nitrobenzyl alcohol

e

Photocaged quinone methide
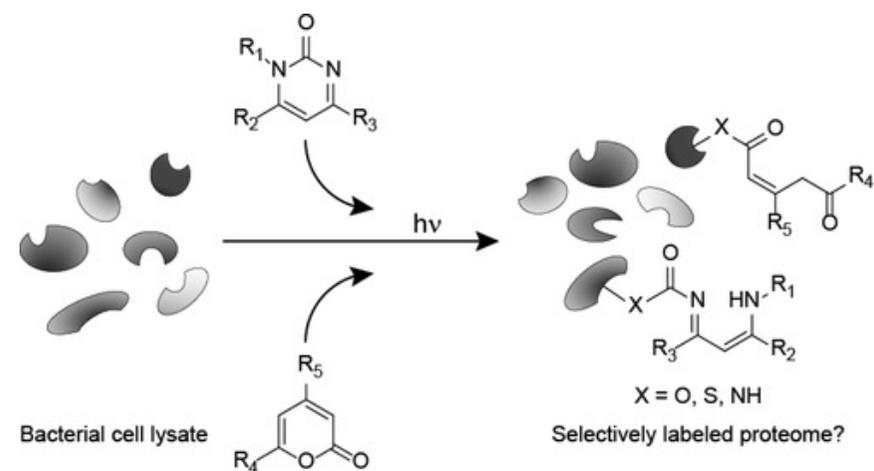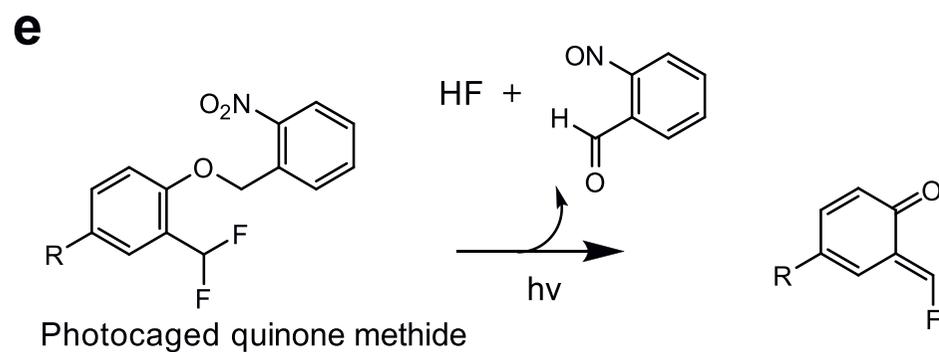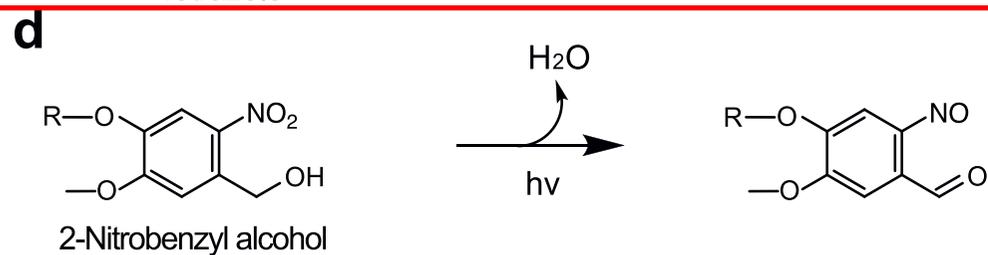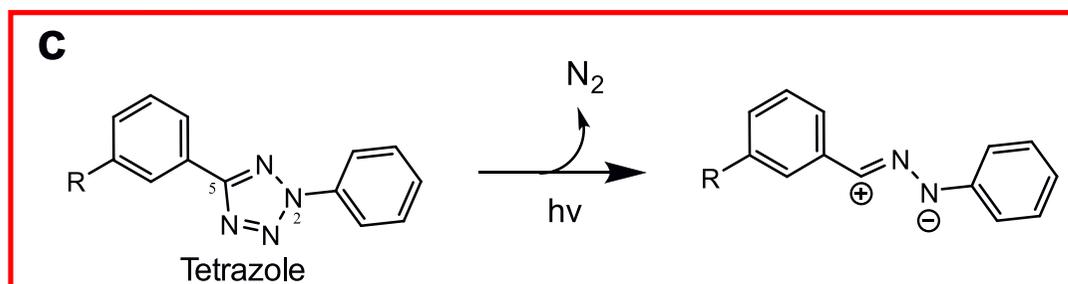
Battenberg, O. A., Nodwell, M. B., & Sieber, S. A. *Journal of Organic Chemistry* **2011**, *76*(15), 6075–6087.

Liu, J., Yang, B., & Wang, L. *Current Opinion in Chemical Biology* **2023**, *74*, 102285.

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- ➢ Introduction
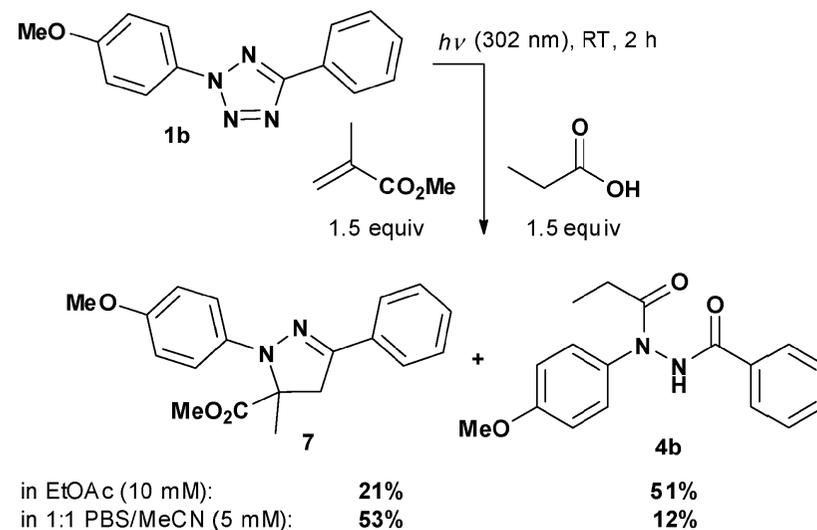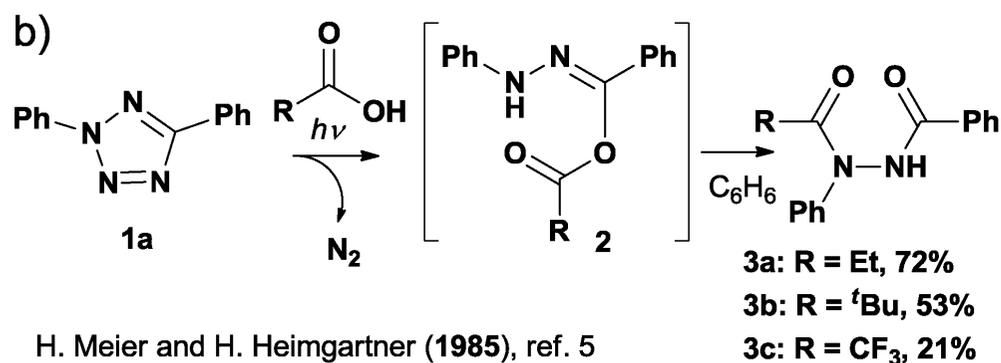 - ➢ Standard method to identify target protein
  - ➢ Conventional photo-crosslinker

- ➢ **Main**
  - ➢ **Tetrazole as photo-crosslinker**
  - ➢ 2-acyl-5-carboxytetrazole (ACT)
  - ➢ PPI mapping by using ACT in living cells

- ➢ Summary

Tetrazole photo-click chemistry

a)

aqueous solution **Lin's work**

b)

3a: R = Et, 72%
3b: R = ^tBu, 53%
3c: R = CF₃, 21%

H. Meier and H. Heimgartner (**1985**), ref. 5

hν (302 nm), RT, 2 h

1b

1.5 equiv   1.5 equiv

7   +   4b

	7	4b
in EtOAc (10 mM):	**21%**	**51%**
in 1:1 PBS/MeCN (5 mM):	**53%**	**12%**
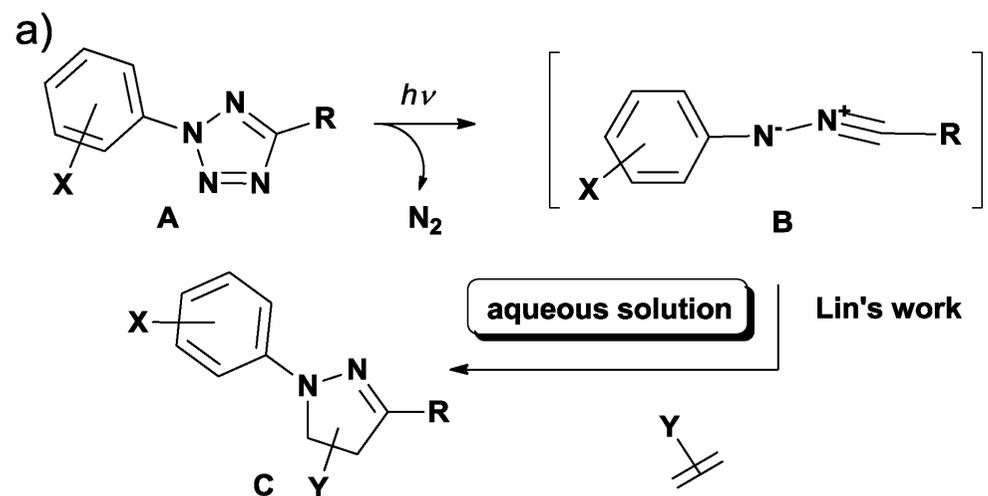
✓ nitrile imine intermediate **B**
could react with a carboxylic acid

Zhao, S., et al. *Chem. Commun.* **2016**, *52*, 4702-4705.
Li, Z., et al. *Angew. Chem. Int. Ed.* **2016**, *55*, 2002-2006.

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Optimized di-aryl-tetrazole

Table 1 Kinetic study of photo-induced reactions of tetrazoles with propionic acid[a]
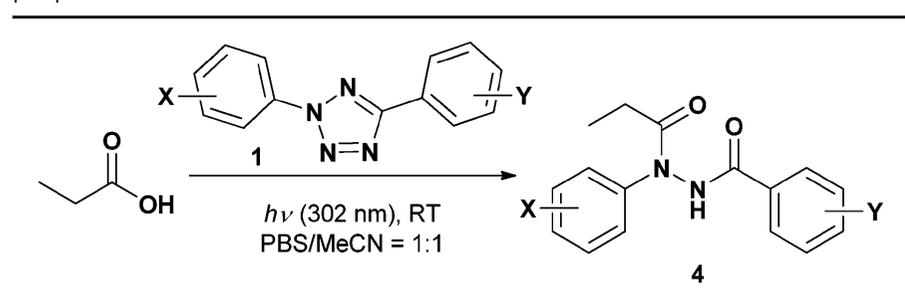

Tetrazole	X	Y	k_{COOH} [M^{-1} s^{-1}]	Yield[b] (%)
1a	H	H	2.8	45
1b	p-OMe	H	5.5	56
1c	H	p-OMe	7.4	87
1d	p-CO$_2$Me	H	0.28	5.9
1e	H	p-CO$_2$Me	0.05	2.8
1f	p-CO$_2$Me	p-OMe	0.88	19
1g	p-OMe	p-CO$_2$Me	0.21	4.7

desired product

cycloaddition product

hydrolysis product

Fig. 2 The effect of pH on the product distribution of reaction of propionic acid with tetrazole 1b. (a) Structures of the three observed products in **PBS/MeCN (1:1) solution**. (b) Reactions carried out at pH values ranging from 2.5 to 10. The yields of products were quantified using HPLC-MS. The data shown are an average of three replicate experiments.

✓ tetrazoles with only electronic-donating group give high product yield

✓ the highest yield of the desired product **4b** was observed under neutral or slightly acidic conditions (pH = 7.1 or 5.5)

Zhao, S., et al. *Chem. Commun.* **2016**, *52*, 4702-4705. (Fig. modified) 12

Amino Acid Selectivity

Table 2   Photo-induced reaction of tetrazole **1b** with acid **8**[a]
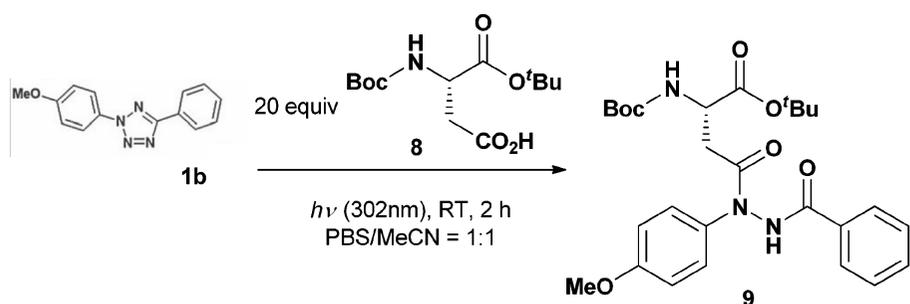
Entry	Additive	Yield[b] (%)
1	None	68
2	Boc-Asn-ONp[c]	74
3	Boc-Gln-ONp	74
4	Boc-Ser-OMe	64
5	Boc-Thr-OMe	69
6	H-Arg-OMe	61
7	H-His-OMe	66
8	Boc-Trp-OSu[d]	66
9	Boc-Met-OSu	78
10	Boc-Tyr-OMe	40
11	H-Lys-OMe	40
12	H-Cys-OEt	33
13	n-C_4H_9CCH	64
14	CH_3CONHOH	65

[a] Reaction mixture of tetrazole **1b** (100 μM), **8** (2 mM) and additive (2 mM) in PBS/MeCN (1:1) within a quartz test tube was irradiated using a handheld UV lamp (302 nm, 6 W) for 2 h. [b] HPLC yield of **9** after 2 h. [c] Np = *p*-nitrophenyl. [d] Su = succinimide.

✓ The complete conversion of **1b** was observed in all reactions

✓ most additives did not interfere with the photo-induced reaction.

✓ in the presence of Tyr, Lys or Cys, the yield of **9** decreased to a different degree (entries 10-12) → presumably due to the reactions between the **nitrile imine intermediate** and **phenol, amino or thiol groups**.
→ Asp, Glu, Cys, Lys selectivity

✓ Interestingly, of all functional groups tested, **CO₂H** appeared **most reactive at physiological pH**, better than other common nucleophiles including thiols, amines, and alcohols.

Li, Z., et al. *Angew. Chem. Int. Ed.* **2016**, *55*, 2002-2006.

Zhao, S., et al. *Chem. Commun.* **2016**, *52*, 4702-4705. (Fig. modified)   13

Photo-crosslinking of myoglobin
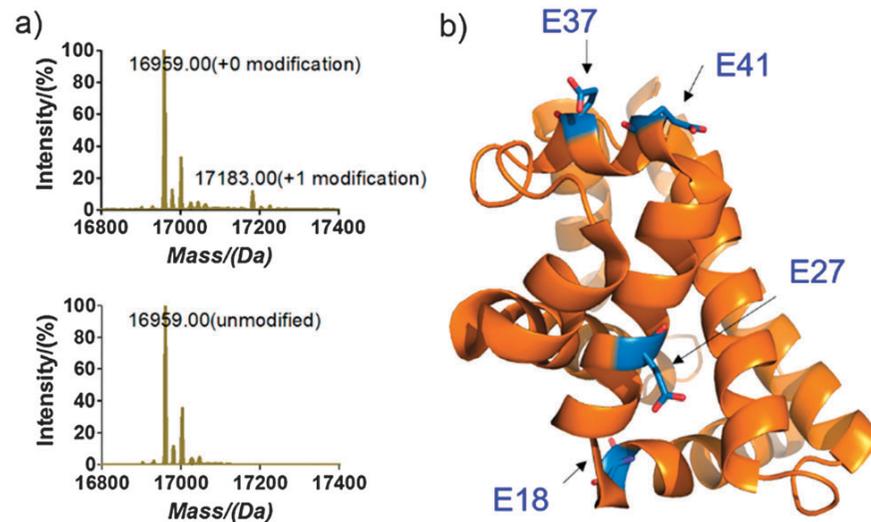
Fig. 4 Photo-labelling of myoglobin using tetrazole 1b. (a) ESI-MS analysis indicating the formation of the anticipated tetrazole 1b adduct (expected Da = 17 813). (b) Residues E18, E27, E37 and E41 identified as the modification sites (blue), the locations of which were indicated by the arrows. View derived from a crystal structure of myoglobin (PDB ID: 3WI8).

Zhao, S., et al. *Chem. Commun.* **2016**, *52*, 4702-4705.

✓ Analysis using ESI-MS indicated the formation of a new species (+1 modification)
✓ Subsequent tryptic digestion and tandem mass spectrometry
   → confirmed the presence of modified-carboxyl groups at positions E18, E27, E37 and E41
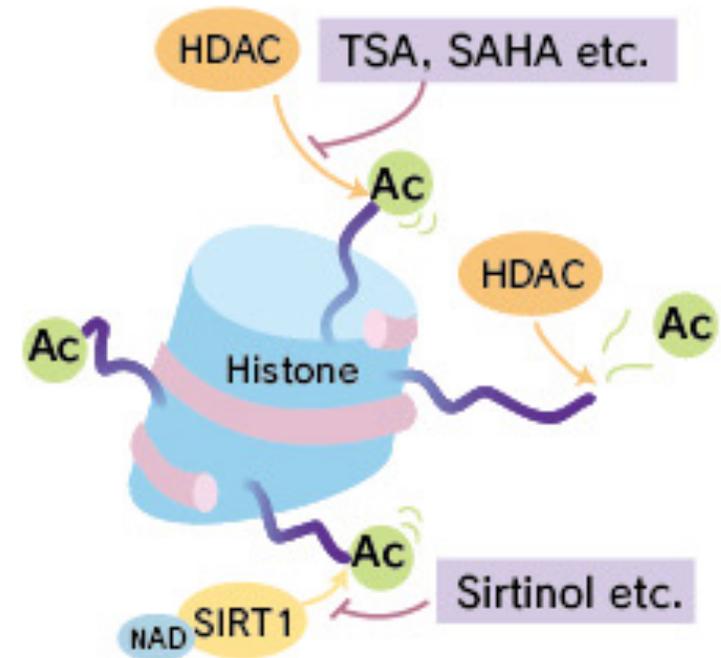
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SAHA and HDAC

➢ As a proof-of-concept study, small molecule inhibitor SAHA was chosen

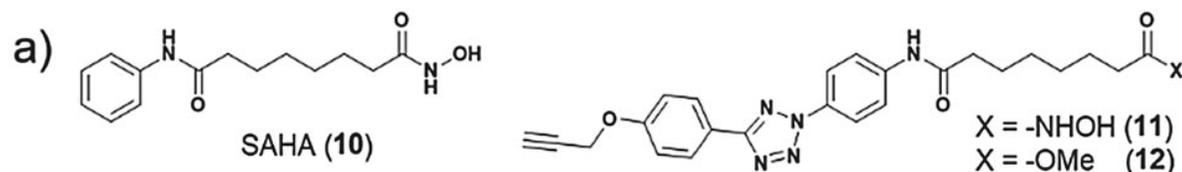✓ SAHA (suberoylanilide hydroxamic acid) inhibits HDAC

→ inhibits cell proliferation and exhibits anti-tumor effects
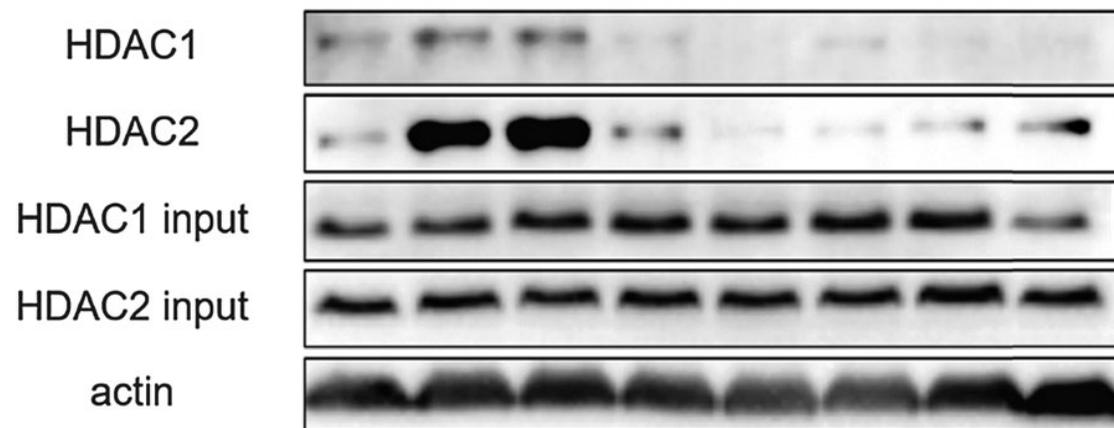→ effective in the treatment of acute myeloid leukemia

https://ruo.mbl.co.jp/bio/product/epigenome/pickup/deacetylase.html

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SAHA-probe can detect HDAC1 and HDAC2

a)

SAHA (10)

X = -NHOH (11)
X = -OMe (12)

b)

| Lane	1	2	3	4	5	6	7	8
Probe 11/μM	0.1	1	10	10	10	-	10	10
Pseudo-probe 12/μM	-	-	-	-	-	10	-	-
SAHA/μM	-	-	-	10	200	-	-	-
UV/302nm	+	+	+	+	+	+	-	+
Cell lysates	A	A	A	A	A	A	A	IA

HDAC1

HDAC2

HDAC1 input

HDAC2 input

actin

> **Proof-of-concept study**

✓ Tetrazole-SAHA probe (**11**) was designed

✓ Pseudo-probe (**12**) lacked the strong Zn^{2+} chelating group (hydroxamic acid)

✓ Tetrazole-SAHA probe (**11**) can modify HDAC1 and HDAC2

✓ Tetrazole-SAHA covalently linked to HDAC1 and HDAC2

Zhao, S., et al. *Chem. Commun.* **2016**, *52*, 4702-4705. 16

Successful labeling was realized in living cells

➤ HDACs enriched from in situ HepG2 cell treated with tetrazole-SAHA probe **11**

c)

Probe **11**/μM	10	10
SAHA/μM	0	200

HDAC1

HDAC2

HDAC1 input

HDAC2 input
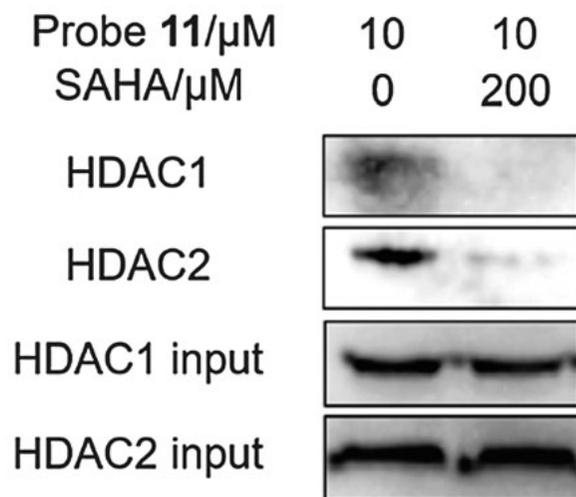
✓ successful labeling was realized by photolysis of probe **11** in living cells

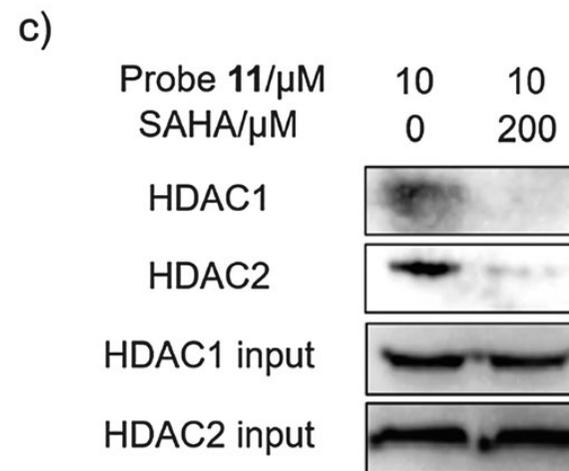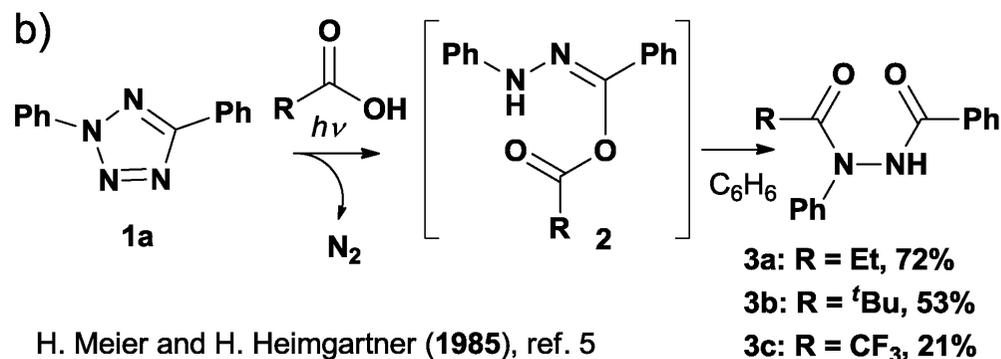➤ MS/MS spectrum
(**in vitro**; HDAC1 and SAHA-probe **11**)

YGEYFPGTGDLR

Res.	mass	b ion	b++	y ion	y++	z	m/z
Y	163.1	164.1	82.5			2	895.9129
G	57.0	221.1	111.0	1629.8	815.4	3	597.2752
E$_Z$	101.0	768.3	384.7	1572.7	786.9	4	447.9564
Y	163.1	931.4	466.2	1025.5	513.3		
F	147.1	1078.5	539.7	862.4	431.7		
P	97.1	1175.5	588.3	715.4	358.2		
G	128.1	1232.5	616.7	618.3	309.7		
T	101.0	1333.6	667.3	561.3	281.2		
G	128.1	1390.6	695.8	460.3	230.6		
D	115.0	1505.6	753.8	403.2	202.1		
L	113.1	1618.7	809.9	288.2	144.6		
R	156.1			175.1	88.1		

✓ from MS/MS Spectrum, E203 close to SAHA was modified

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Zhao, S., et al. *Chem. Commun.* **2016**, *52*, 4702-4705.   17

Short summary

✓ photo-induced diaryltetrazole-acid coupling reaction as a biochemical conjugation strategy

✓ diaryltetrazole can react with selective amino acids (Asp, Glu, Cys, Lys)

✓ successfully profiling HDACs in cell lysates and living cells

✓ this coupling reaction could be extensively applied to protein labeling (in vitro & in vivo)

b)

1a   N$_2$ 2

H. Meier and H. Heimgartner (**1985**), ref. 5

3a: R = Et, 72%
3b: R = ^{t}Bu, 53%
3c: R = CF$_3$, 21%

c)

| Probe **11**/µM | 10 | 10 |
| SAHA/µM | 0 | 200 |

HDAC1

HDAC2

HDAC1 input

HDAC2 input

Zhao, S., et al. *Chem. Commun.* **2016**, *52*, 4702-4705.   18

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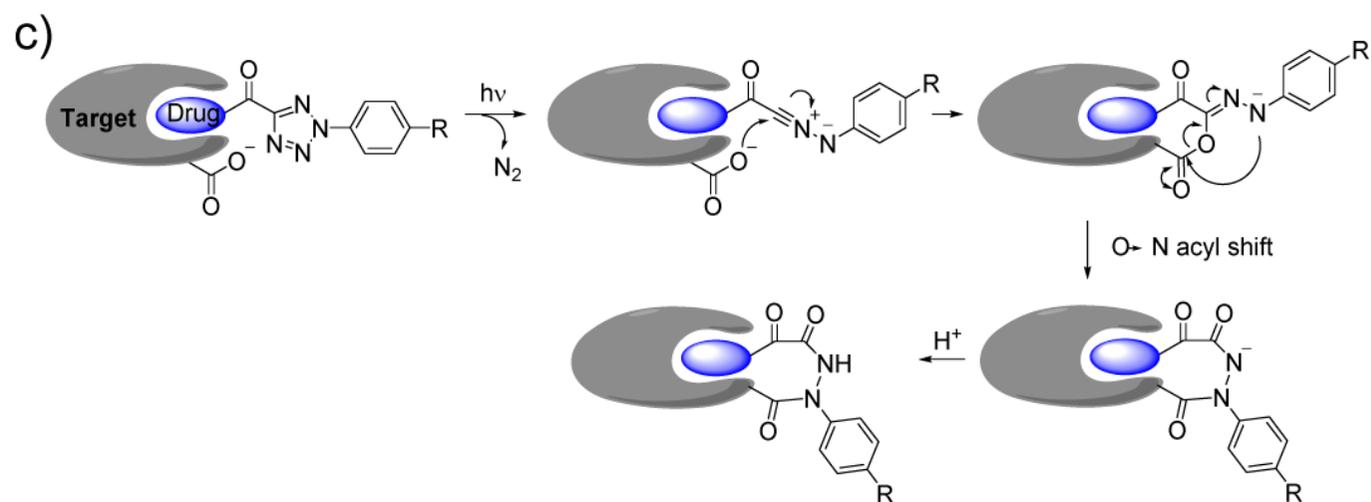 ➤ PPI mapping by using ACT in living cells

➤ Summary

Advance of photo-crosslinker using tetrazole

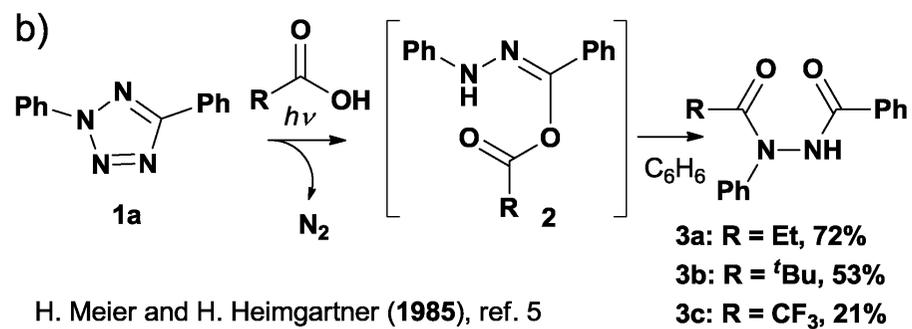- ACT (2-**a**ryl-5-**c**arboxy**t**etrazole): non-radical intermediate
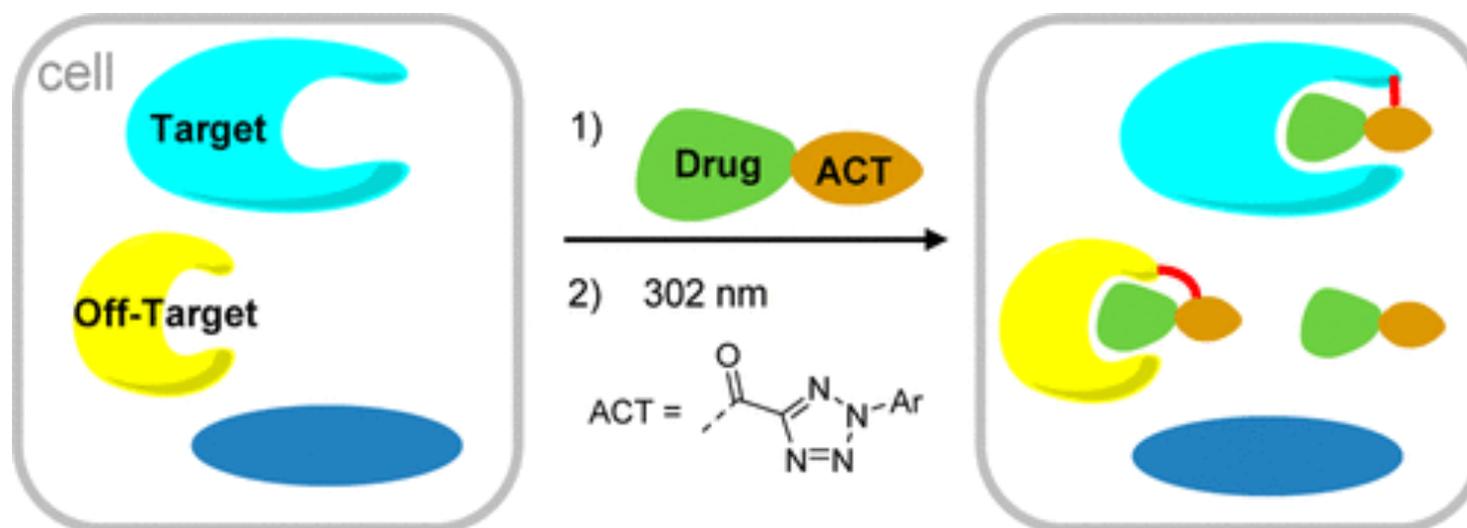
Herner, A., et al. *Journal of the American Chemical Society* **2016**, *138*(44), 14609–14615.
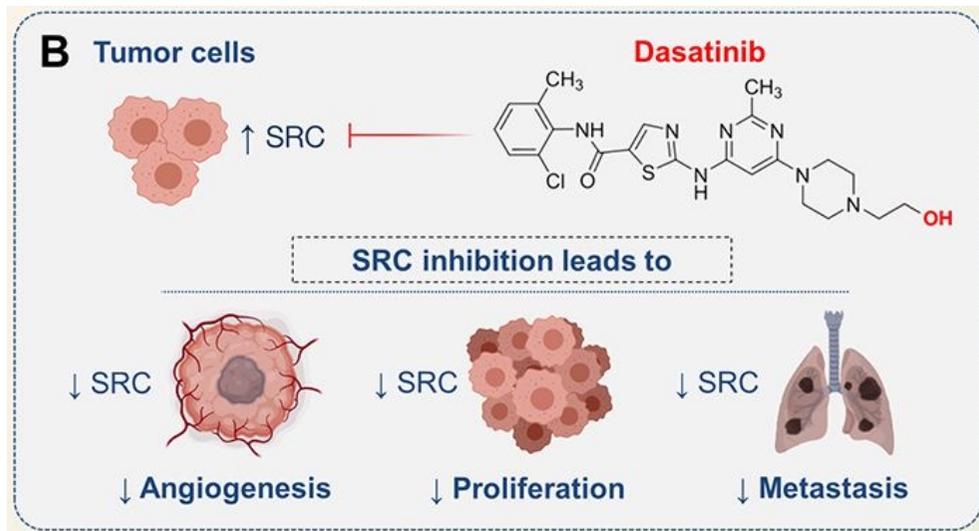
Reaction mechanism

c)

Herner, A., et al. *Journal of the American Chemical Society* **2016**, *138*(44), 14609–14615.

b)

1a

H. Meier and H. Heimgartner (**1985**), ref. 5

3a: R = Et, 72%
3b: R = ᵗBu, 53%
3c: R = CF₃, 21%

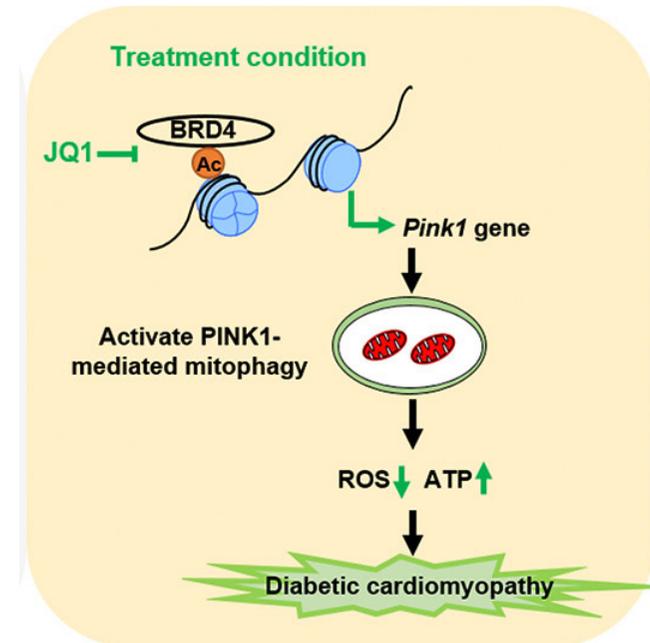Zhao, S., et al. *Chemical Communications* **2016**, *52*(25), 4702–4705.

Dasatinib and JQ-1

➢ **Dasatinib**

Krebs, S., et al. *Journal of Nuclear Medicine* **2020**, *61*(11), 1580-1587.
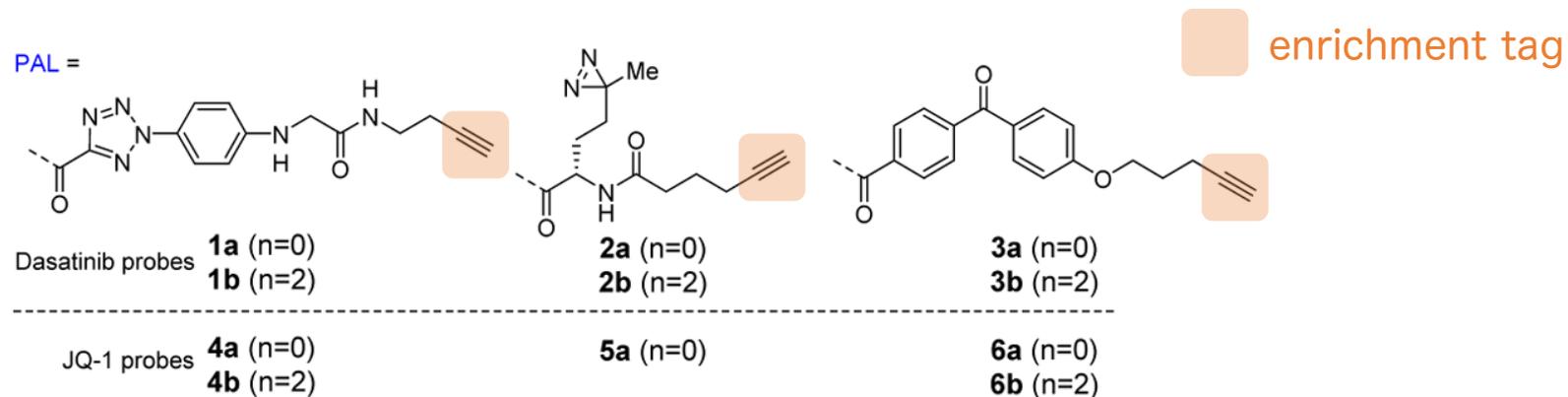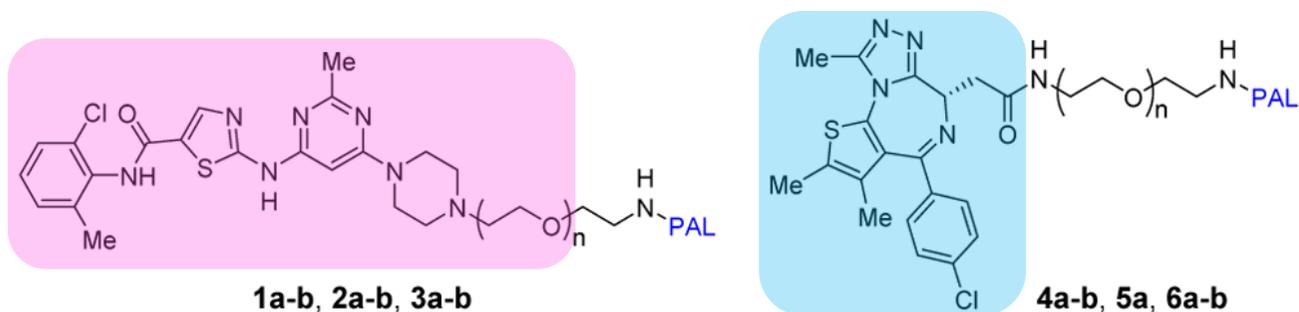
➢ **JQ-1**

Mu, J., et al. *Journal of Molecular and Cellular Cardiology* **2020**, *149*,1–14.
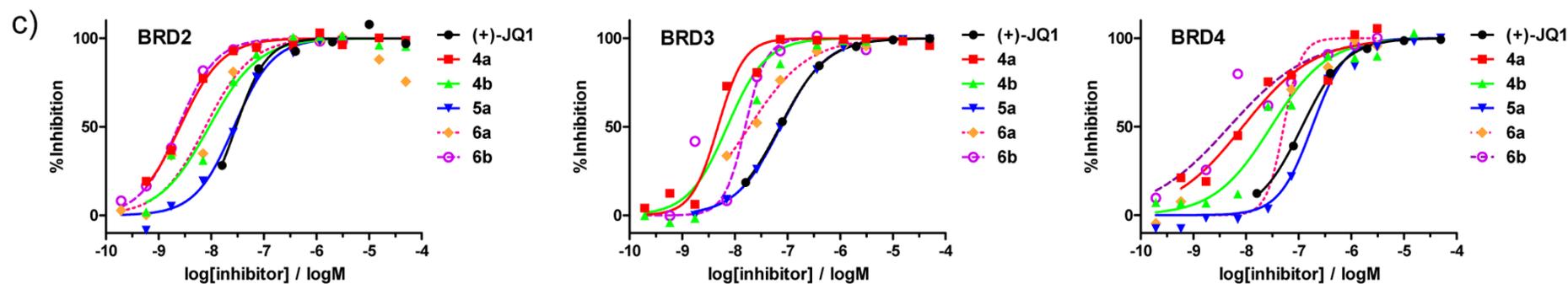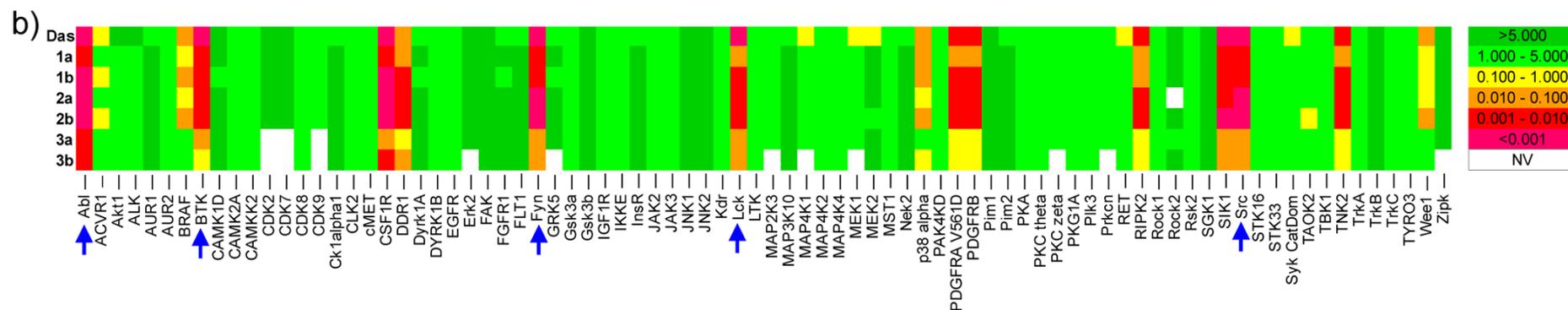
Dasatinib and JQ-1 conjugated with PAL.

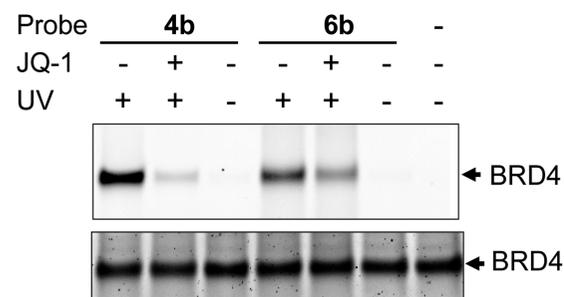enrichment tag

tetrazole   diazirine   benzophenone

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Binding affinity assay

b) Heatmap showing binding affinity values for compounds Das, 1a, 1b, 2a, 2b, 3a, 3b against kinase panel (Abl, ACVR1, Akt1, ALK, AUR1, AUR2, BRAF, BTK, CAMK1D, CAMK2A, CAMKK2, CDK2, CDK7, CDK8, CDK9, Ck1alpha1, CLK2, cMET, CSF1R, DDR1, Dyrk1A, DYRK1B, EGFR, Erk2, FAK, FGFR1, FLT1, Fyn, GRK5, Gsk3a, Gsk3b, IGF1R, IKKE, InsR, JAK2, JAK3, JNK1, JNK2, Kdr, Lck, LTK, MAP2K3, MAP3K10, MAP4K1, MAP4K2, MAP4K4, MEK1, MEK2, MST1, Nek2, p38 alpha, PAK4KD, PDGFRA V561D, PDGFRB, Pim1, Pim2, PKA, PKC theta, PKC zeta, PKG1A, Plk3, Prkcn, RET, RIPK2, Rock1, Rock2, Rsk2, SGK1, SIK1, Src, STK16, STK33, Syk CatDom, TAOK2, TBK1, TNK2, TrkA, TrkB, TrkC, TYRO3, Wee1, Zipk). Legend: >5.000, 1.000–5.000, 0.100–1.000, 0.010–0.100, 0.001–0.010, <0.001, NV.

c) Dose-response curves (% Inhibition vs log[inhibitor]/logM) for BRD2, BRD3, BRD4 with (+)-JQ1, 4a, 4b, 5a, 6a, 6b.
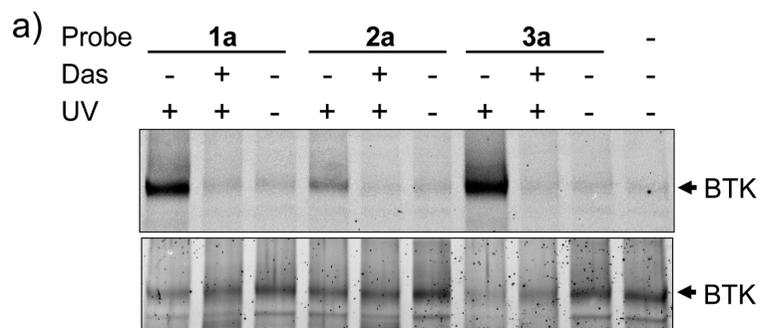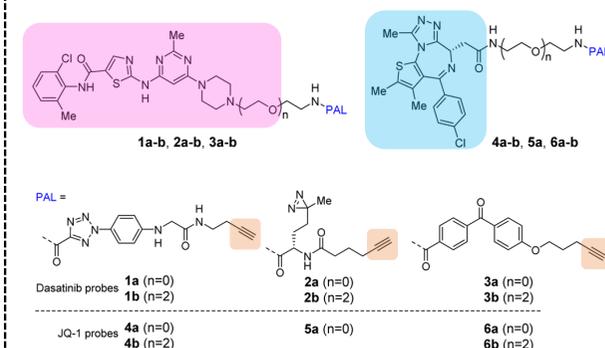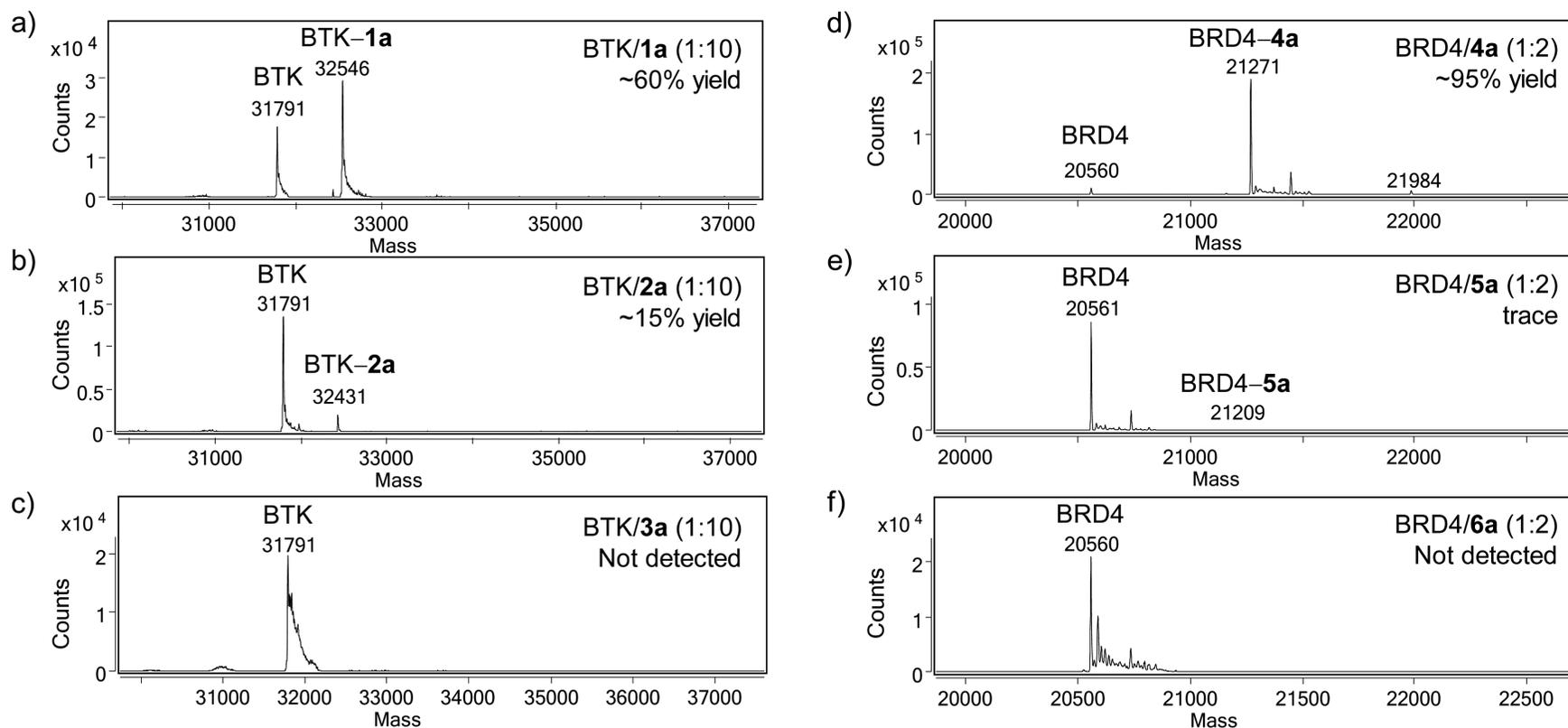
- synthesized probes did not strongly inhibit ligand-POI affinity
- → BTK, BRD4 were used
- For JQ-1, inhibition efficiency was improved

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Herner, A., et al. *Journal of the American Chemical Society* **2016**, *138*(44), 14609–14615.

Evaluating the efficiency and selectivity of photoaffinity-labeling

➢ For BTK, all probes showed irradiation and ligand-dependent labeling

➢ For BRD4, irradiation and ligand-dependent labeling other than **6a/6b**
→ exhibited strong background labeling

a)

b)

Herner, A., et al. *Journal of the American Chemical Society* **2016**, *138*(44), 14609–14615.
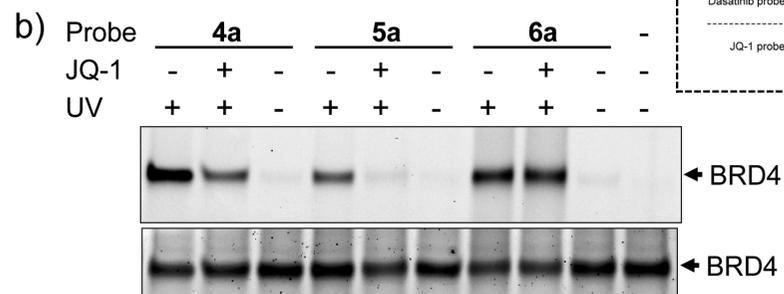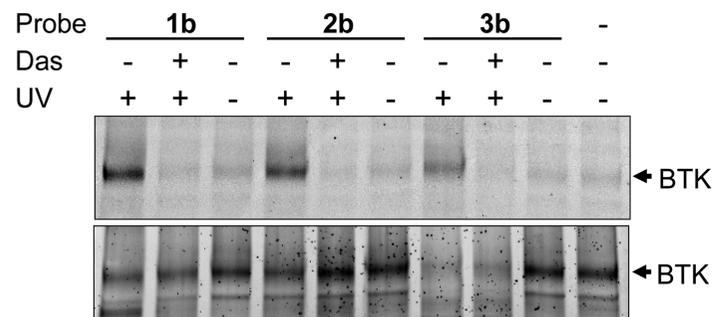
ACT showed higher photo-crosslinking yields

a) BTK/**1a** (1:10) ~60% yield — BTK 31791, BTK–**1a** 32546

b) BTK/**2a** (1:10) ~15% yield — BTK 31791, BTK–**2a** 32431

c) BTK/**3a** (1:10) Not detected — BTK 31791

d) BRD4/**4a** (1:2) ~95% yield — BRD4 20560, BRD4–**4a** 21271, 21984

e) BRD4/**5a** (1:2) trace — BRD4 20561, BRD4–**5a** 21209

f) BRD4/**6a** (1:2) Not detected — BRD4 20560

✓ ACT-based probes **1a** and **4a** showed robust photo-crosslinking with their targets, while DA-based probes **2a** and **5a** gave crosslinked products in much lower yields

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Quenched product yield was almost the same

a)

b)
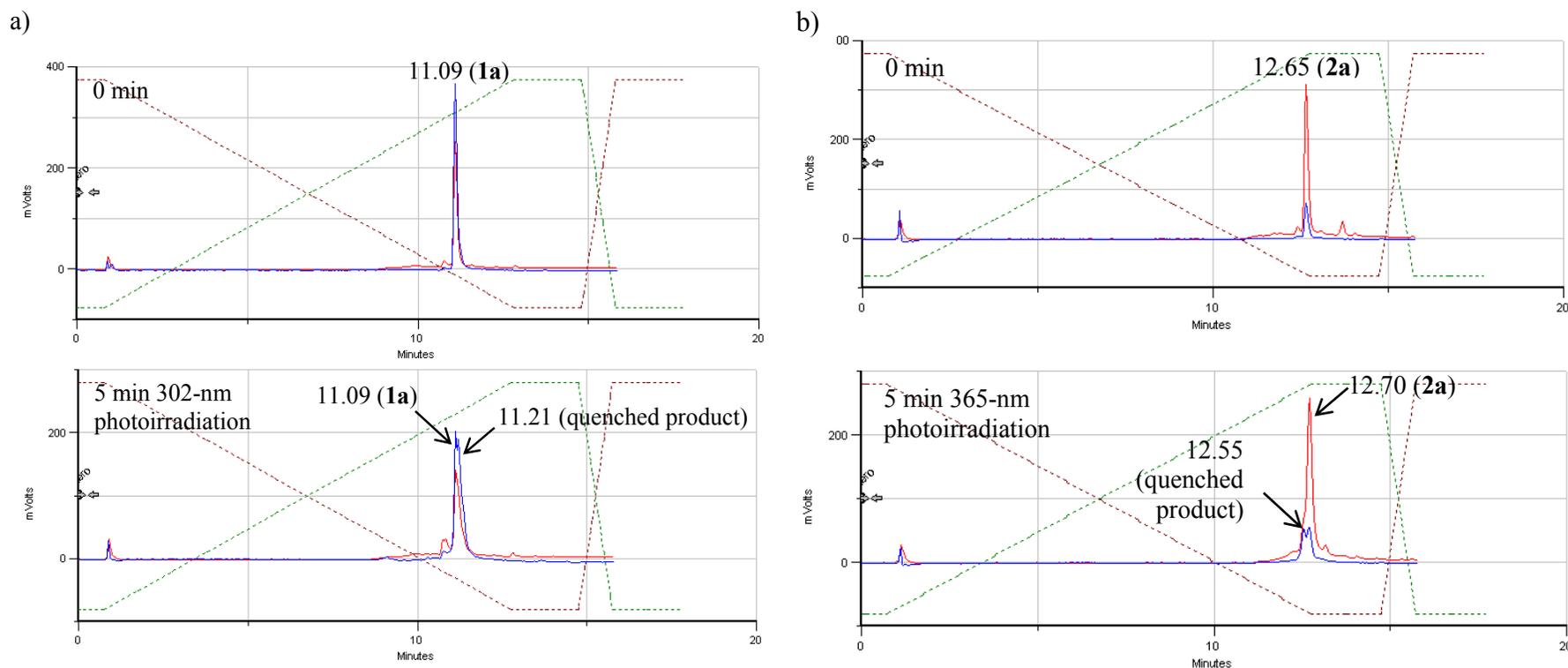
Figure S6. HPLC and LC-MS analyses of the photoactivation of (a) 1a and (b) 2a (100 μM) in PBS. Red trace = absorbance at 254 nm; blue trace = absorbance a 365 nm. The percent conversion was calculated to be **38% for 1a** and **44% for 2a** based on absorbance at 365 nm.

✓ quenched product's yields from **1a** and **2a** were almost the same

Herner, A., et al. *Journal of the American Chemical Society* **2016**, *138*(44), 14609–14615

Determination of the cross-linking site on BTK protein

➢ **MS/MS spectrum**

a)
```
  1    MGHHHHHHEN  LYFQGTAGLG  YGSWEIDPKD  LTFLKELGTG  QFGVVKYGKW
 51    RGQYDVAIKM  IKEGSMSEDE  FIEEAKVMMN  LSHEKLVQLY  GVCTKQRPIF
101    IITEYMANGC  LLNYLREMRH  RFQTQQLLEM  CKDVCEAMEY  LESKQFLHRD
151    LAARNCLVND  QGVVKVSDFG  LSRYVLDDEY  TSSVGSKFPV  RWSPPEVLMY
201    SKFSSKSDIW  AFGVLMWEIY  SLGKMPYERF  TNSETAEHIA  QGLRLYRPHL
251    ASEKVYTIMY  SCWHEKADER  PTFKILLSNI  LDVMDEES
```


E(ACT)-M-R
MW 1188.463 Da
MS/MS 397.158^{3+}
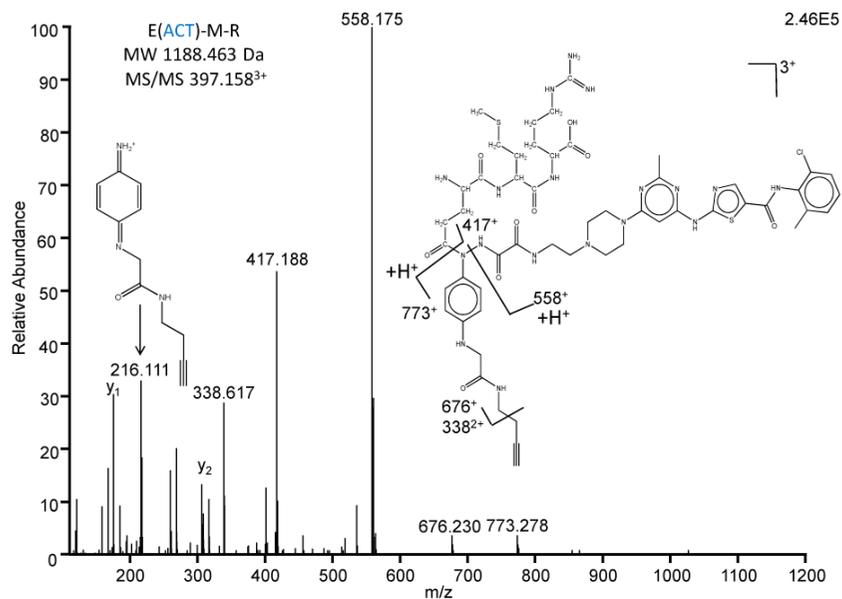
✓ only Glu-488 was detected as labeled
(BTK has 25 Glu, 14 Asp)

➢ **binding model**

b)
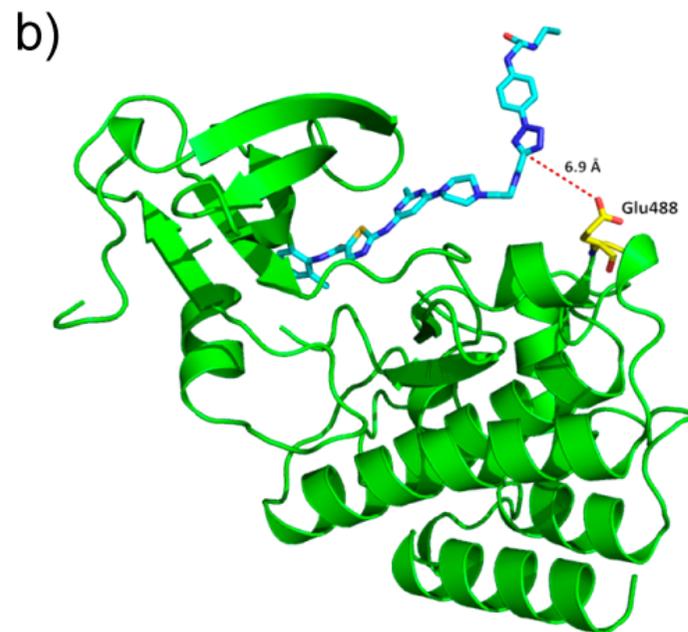
✓ Glu-488 is the only nucleophilic side chain within 9.0 Å from the electrophilic site

Herner, A., et al. *Journal of the American Chemical Society* **2016**, *138*(44), 14609–14615. 28

Proposed mechanism

➢ **reaction mechanism**
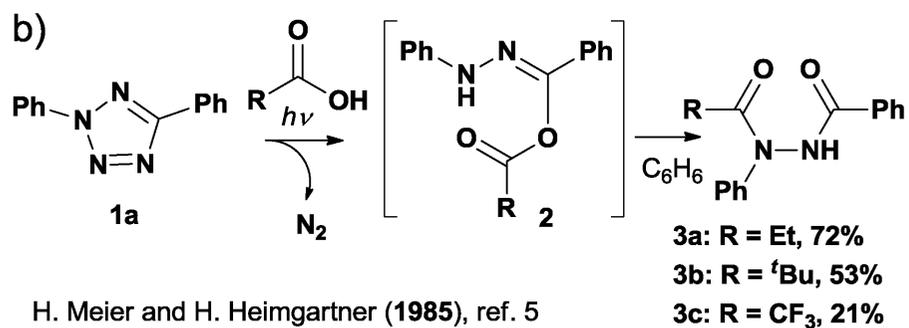
b)

H. Meier and H. Heimgartner (**1985**), ref. 5

3a: R = Et, 72%
3b: R = ^{t}Bu, 53%
3c: R = CF$_3$, 21%

Zhao, S., et al. *Chemical Communications* **2016**, *52*(25), 4702–4705.

c)

O→N acyl shift

H$^+$

*em. Commun.,* 2016, **52**, 4702–   .6
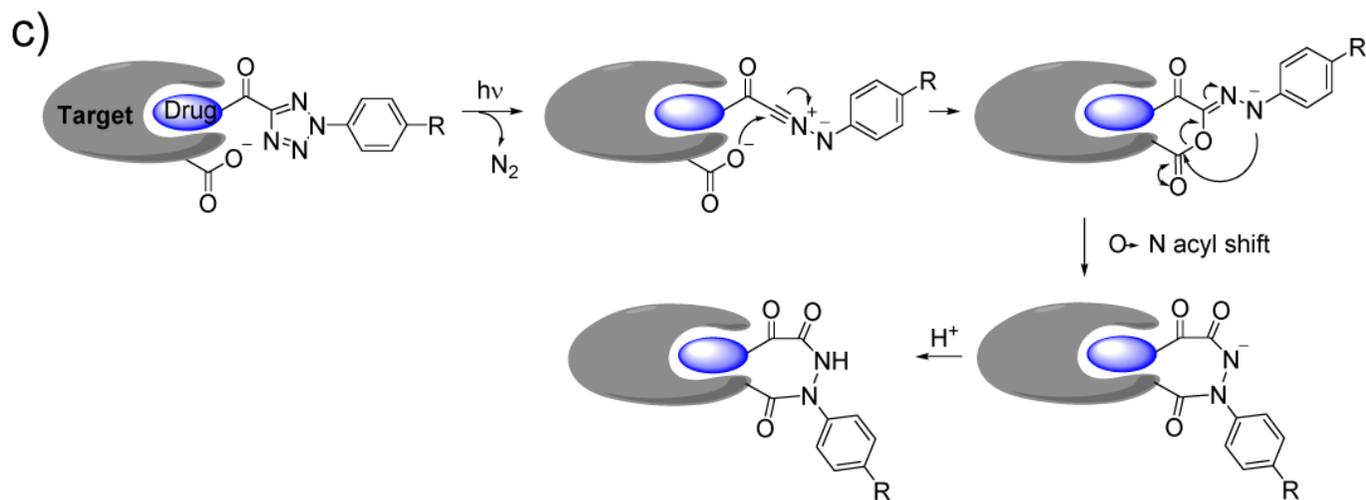
Herner, A., et al. *Journal of the American Chemical Society* **2016**, *138*(44), 14609–14615.

ACT-enabled in situ target identification
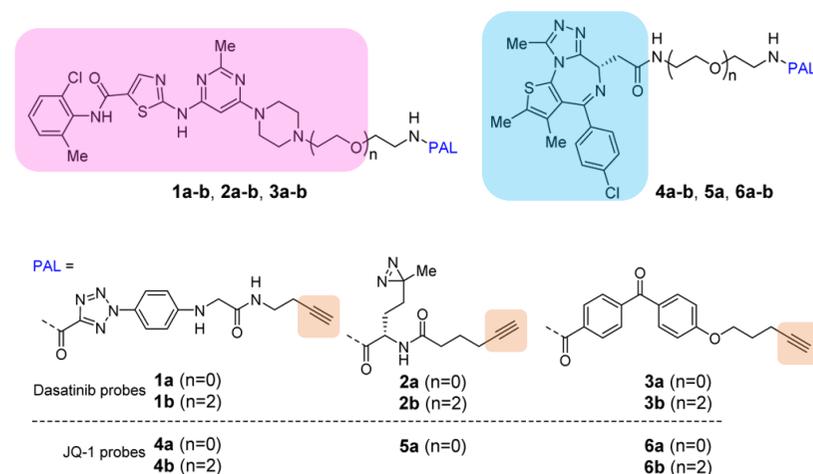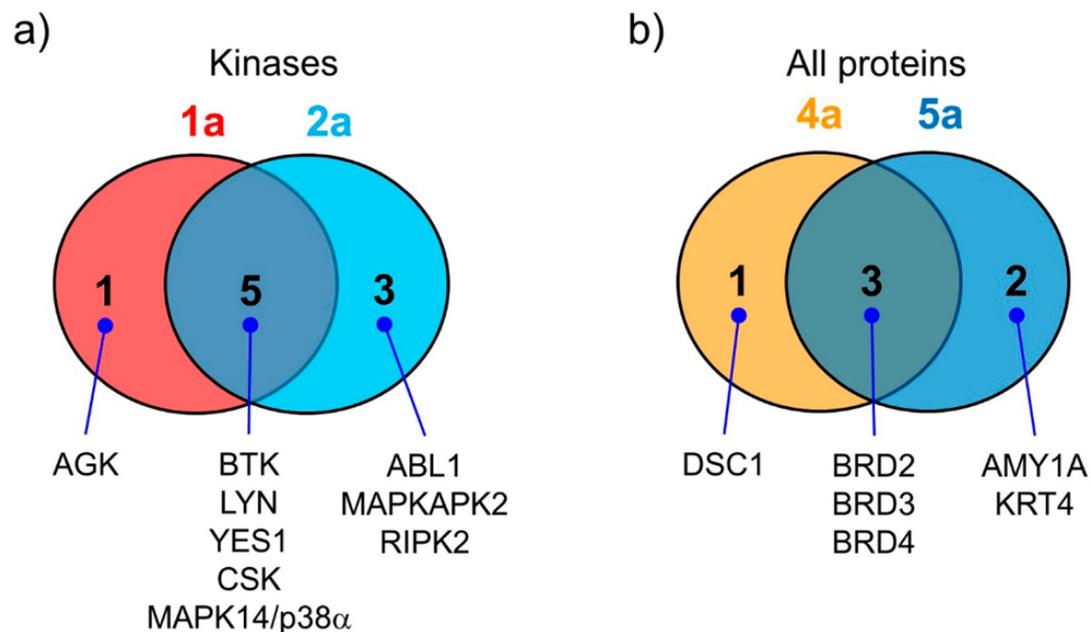
✓ both ACT (2-acyl-5-carboxytetrazole) and diazirine are efficient in the target identification (K562 cell)

✓ ACT did not improve identification regardless of higher crosslinking yield than diazirine but both ACT and DA are efficient in the in situ target identification.

Herner, A., et al. *Journal of the American Chemical Society* **2016**, *138*(44), 14609–14615. (Fig. modified)

Abl and Dasatinib interaction
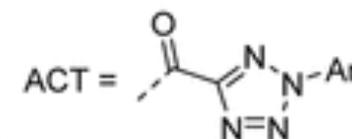

Abl

Dasatinib

Dasatinib
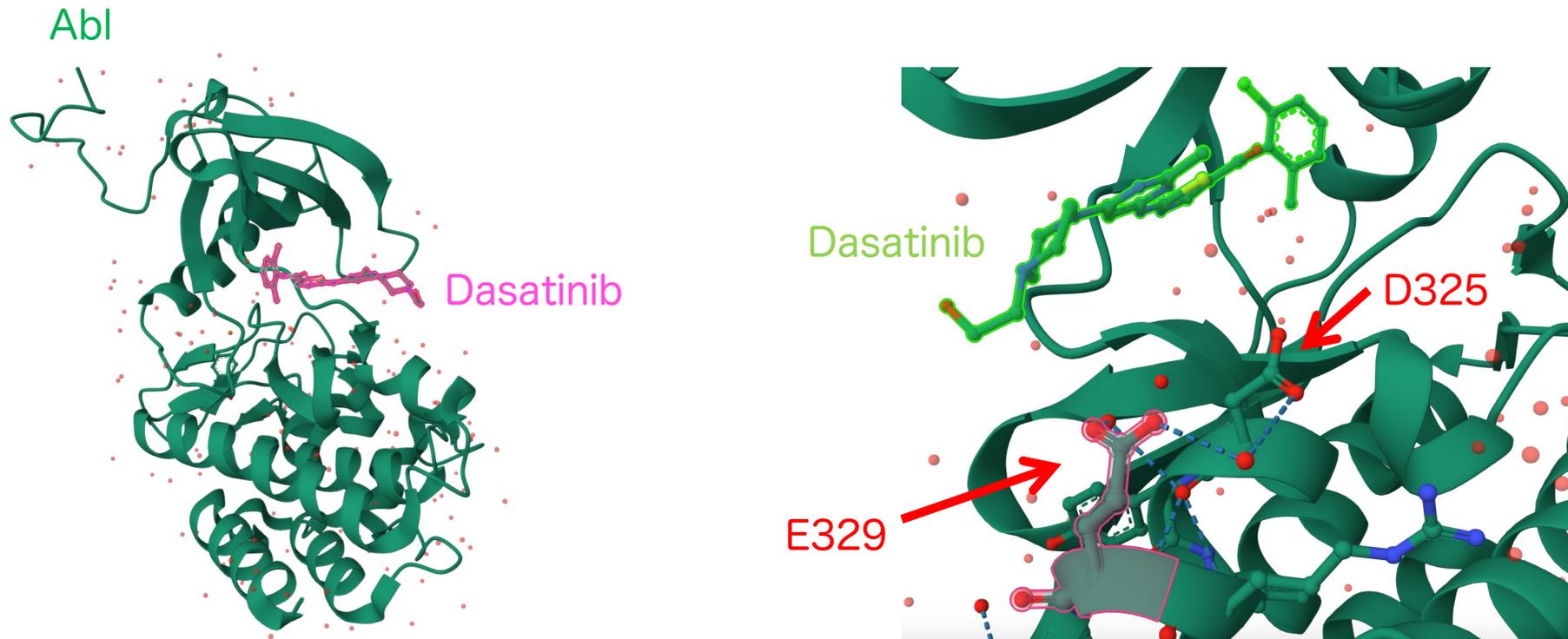
D325

E329

✓ D325 and E329 are present near Dasatinib → probably crosslinking was occurred

Vajpai, N., et al. *THE JOURNAL OF BIOLOGICAL CHEMISTRY* **2008**, *283*(26), 18292–18302.
(PDB 2GQG) https://www.rcsb.org/structure/2gqg (Fig. modified)

Short summary

ACT = (structure)

✓ Tetrazole has selectivity to amino acids (Asp, Glu, Cys, Lys)

✓ ACT (2-acyl-5-carboxytetrazole) can serve as an effective photoaffinity label for target identification both in vitro and in living cells

✓ unique photo-crosslinking mechanism (non-radical)
→ lead to reduced background reactions with nonspecific targets
→ facile mapping of the ligand-binding site

✓ Compared to DA and BP, ACT showed higher cross-linking yields with the desired targets in vitro

✓ achieve efficient in situ target capture and subsequent identification

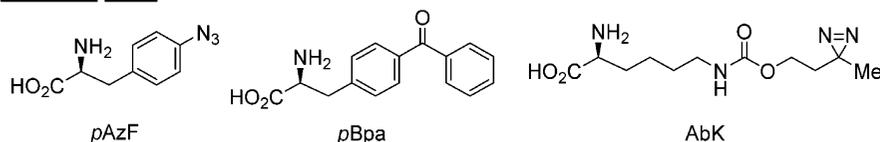Herner, A., et al. *Journal of the American Chemical Society* **2016**, *138*(44), 14609–14615.

Contents

➢ Introduction
    ➢Standard method to identify target protein
    ➢Conventional photo-crosslinker

➢ **Main**
    ➢Tetrazole as photo-crosslinker
    ➢2-acyl-5-carboxytetrazole (ACT)
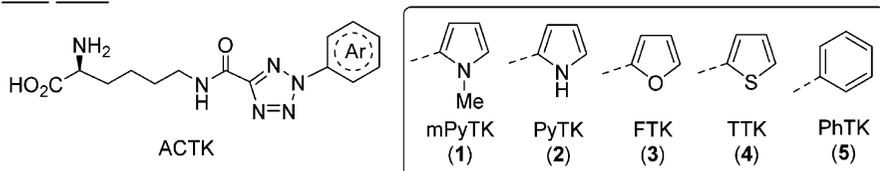   ➢**PPI mapping by using ACT in living cells**

➢ Summary

ACT incorporation to cells as unnatural amino acids
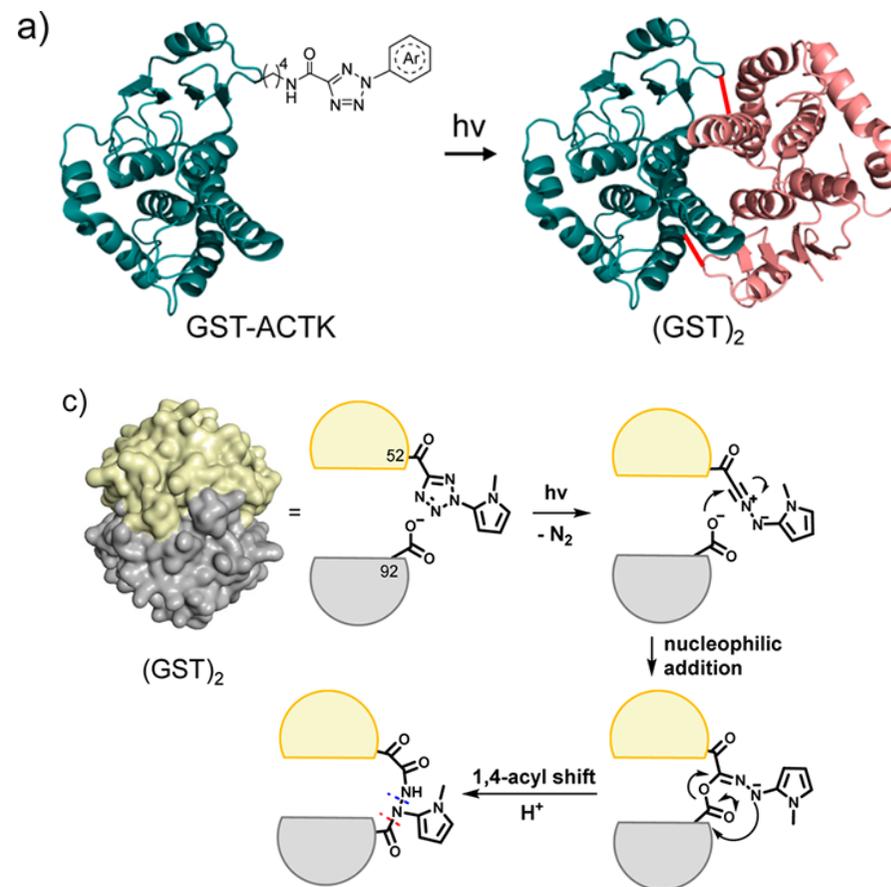
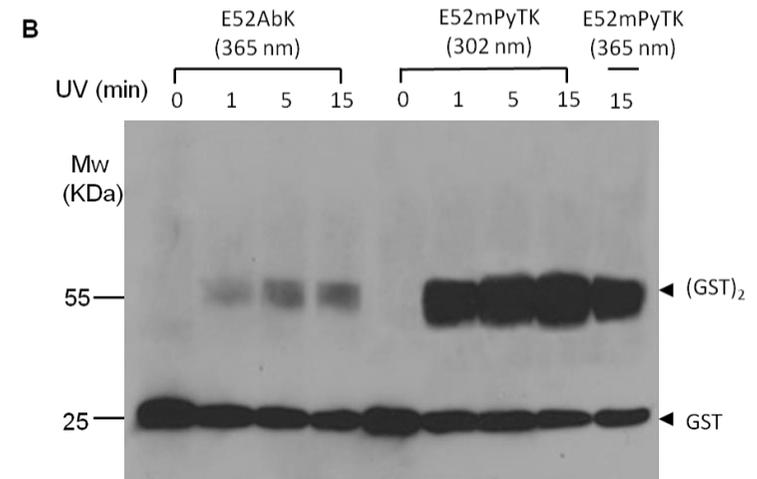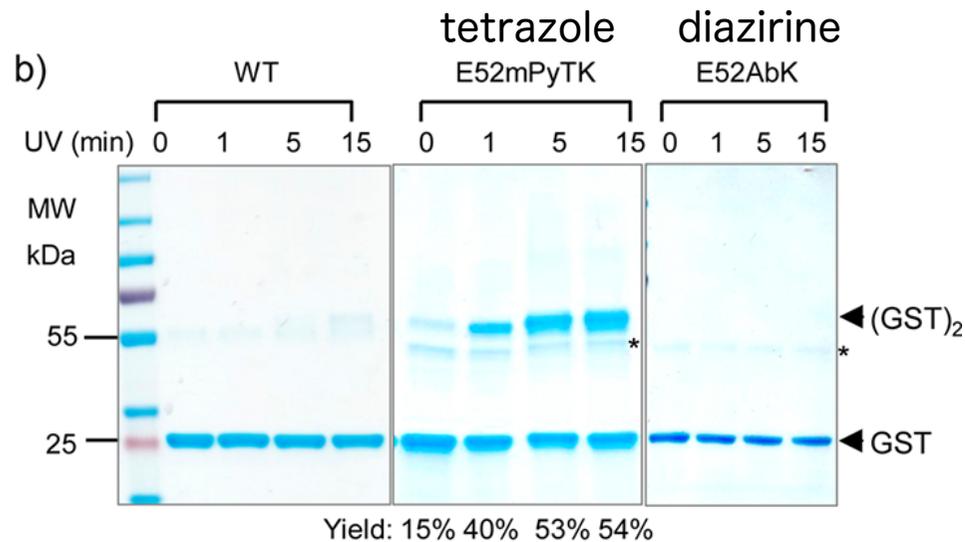**Chart 1. Genetically Encoded Photo-Cross-Linkers**

- Genetically encoded ACT (2-aryl-5-carboxytetrazole)

- give robust and site-selective photo-crosslinking reactivity

Tian, Y., et al. *Journal of the American Chemical Society* **2017**, *139*(17), 6078–6081.

Tian, Y., & Lin, Q. *Chemical Communications* **2018**, *54*(35), 4449–4452.

GST photo-crosslinking by mPyTK

b)

	WT	tetrazole E52mPyTK	diazirine E52AbK

UV (min) 0 1 5 15   0 1 5 15   0 1 5 15

MW kDa

55 ◄──────────────────────────────── ◄ (GST)₂
                                              * *
25 ──────────────────────────────────── ◄ GST

Yield: 15% 40% 53% 54%

B

	E52AbK (365 nm)	E52mPyTK (302 nm)	E52mPyTK (365 nm)

UV (min) 0 1 5 15   0 1 5 15   15

Mw (KDa)

55 ── ◄ (GST)₂

25 ── ◄ GST

✓ GST dimer formation for the E52mPyTK mutant with ~53% yield at 5 min, but not for WT
→ ACT moiety is responsible for dimer cross-linking

✓ AbK exhibited very weak reactivity as the dimer band was detected only by Western blot
→ higher yield by using tetrazole

Identifying the mPyTK photo-crosslinking site in GST

a)

b)

c)
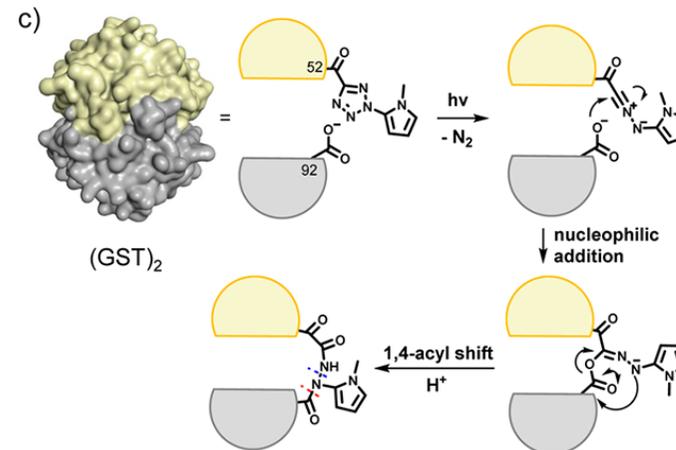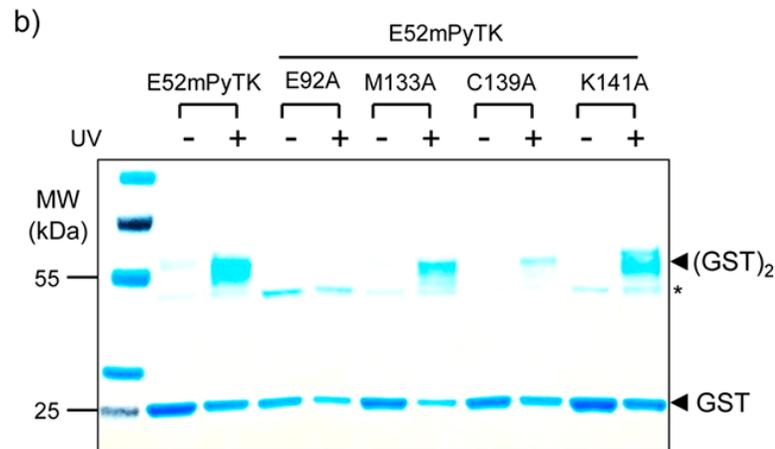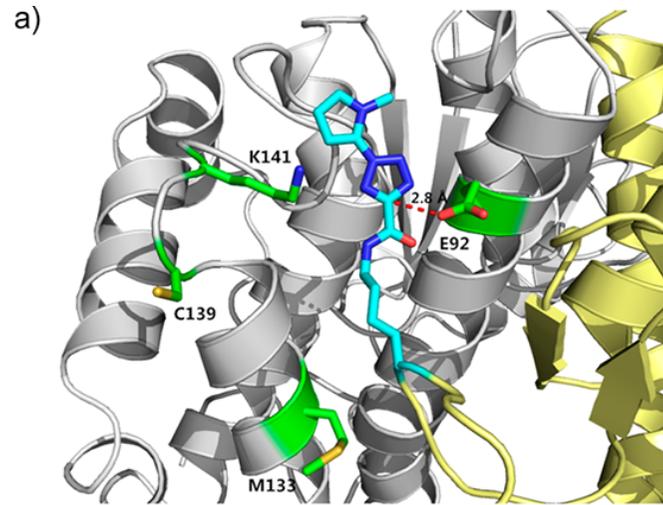
✓ model of the GST-E52mPyTK
  → Four nucleophilic residues (E92, M133, C139 and K141) were identified
✓ E92A mutation completely abolished the covalent dimer formation
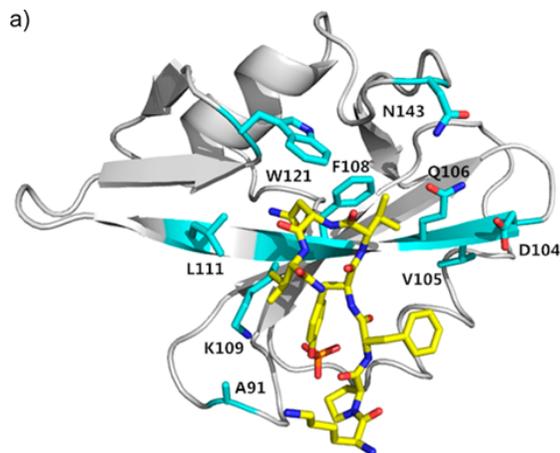✓ other mutations had no effect

Tian, Y., et al. *Journal of the American Chemical Society* **2017**, *139*(17), 6078–6081. 36
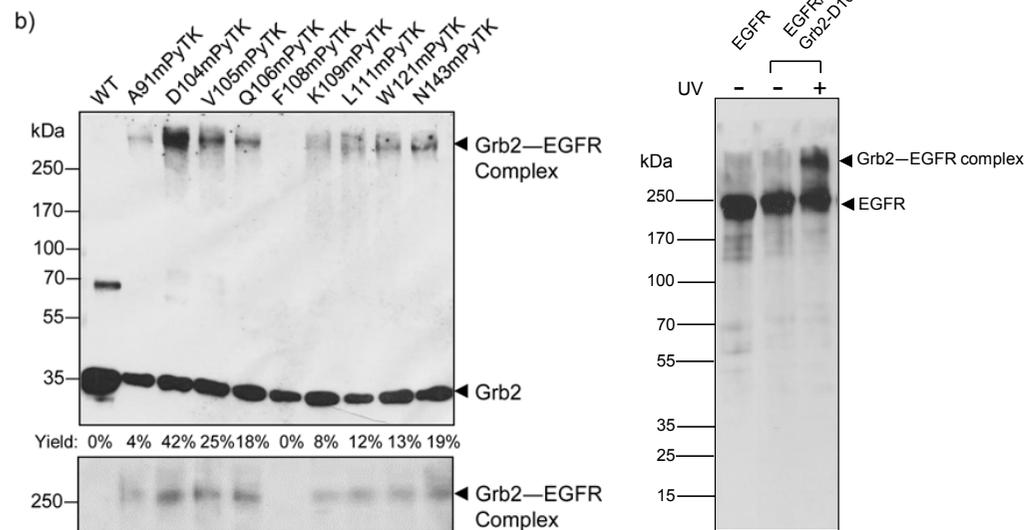
EGFR and Grb2
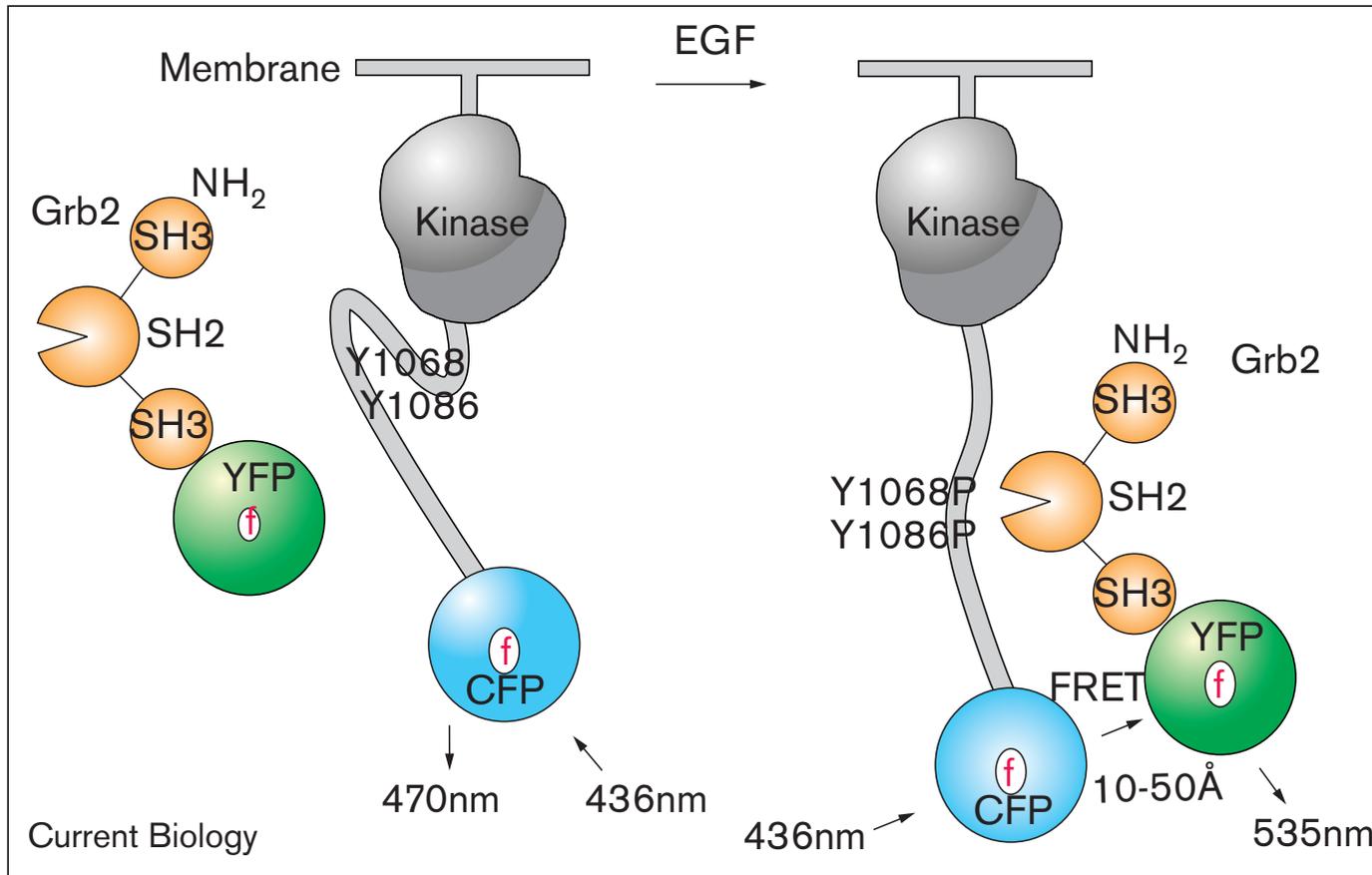
Sorkin, A., et al. *Current Biology* **2000**, *10*, 1395-1398.

Photo-crosslinking in mammalian cells
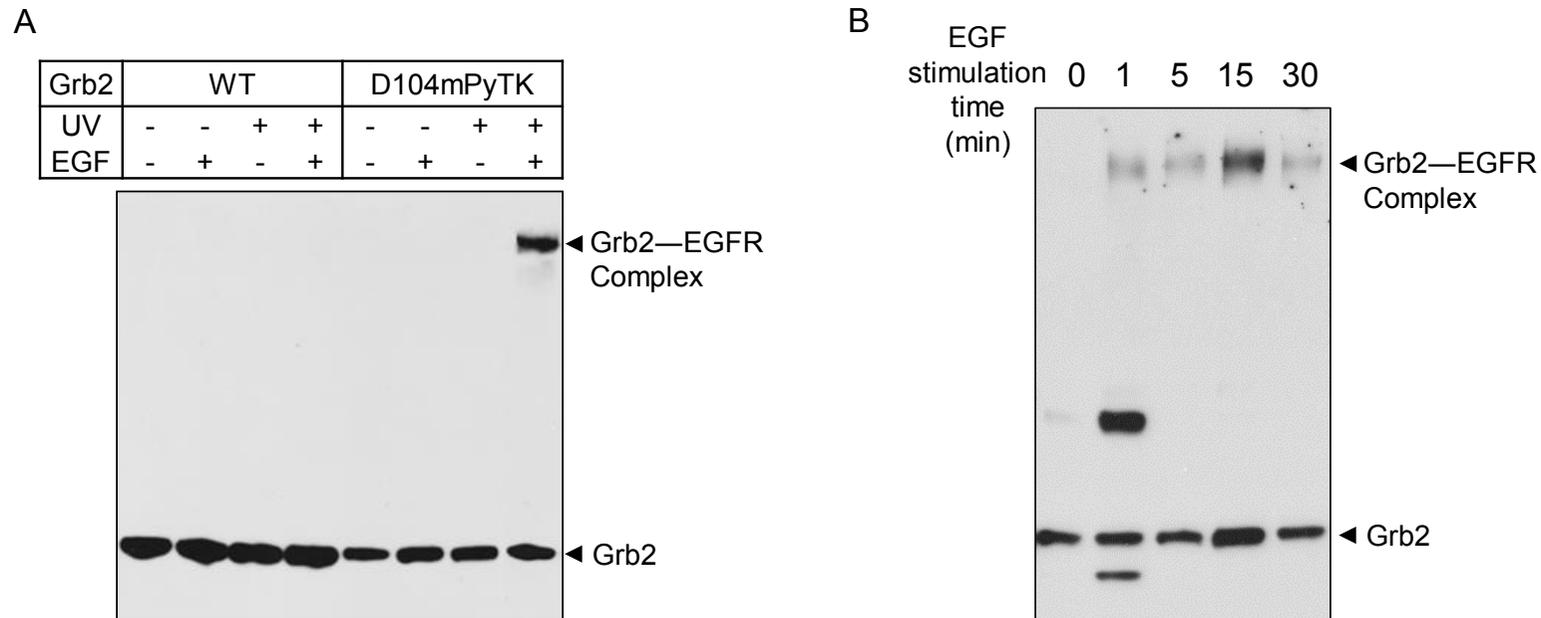
➤ crystal structure of
Grb2 SH2 domain

➤ Photo-crosslinking with EGFR
in HEK293T cells

✓ 9 residues have a potential to photo-crosslink with EGFR
✓ D104mPyTK mutant gave the highest photo-cross-linking yield followed by V105mPyTK, Q106mPyTK and N143mPyTK mutants
✓ may react with the same nucleophilic residue on EGFR across the interaction interface

Photo-crosslinking in mammalian cells

A

Grb2	WT				D104mPyTK			
UV	-	-	+	+	-	-	+	+
EGF	-	+	-	+	-	+	-	+

◄ Grb2—EGFR Complex

◄ Grb2

B

EGF stimulation time (min) 0 1 5 15 30

◄ Grb2—EGFR Complex

◄ Grb2

✓ the photo-crosslinking of EGFR is EGF stimulation and photoirradiation-dependent
✓ highest photo-cross-linking yield was when cells were stimulated with EGF for 15 min
    → Grb2–EGFR interaction is transient and dynamic

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>> PPI mapping by using ACT in living cells

> **Summary**

Summary

✗ conventional photo-crosslinkers (benzophenone, diazirine)
- very low target capturing yields
- react non-selectively (any proximal X-H bonds) → difficult for identifying target by MS
- high background

✓ Tetrazole has selectivity to amino acids (Asp, Glu, Cys, Lys)

✓ unique photo-crosslinking mechanism (non-radical)
→ lead to reduced background reactions with nonspecific targets
→ facile mapping of the ligand-binding site

Summary

✓ diaryltetrazole or ACT (2-aryl-5-carboxytetrazole) successfully profiling proteins in vitro and living cells

H. Meier and H. Heimgartner (**1985**), ref. 5

3a: R = Et, 72%
3b: R = ^{t}Bu, 53%
3c: R = CF$_3$, 21%
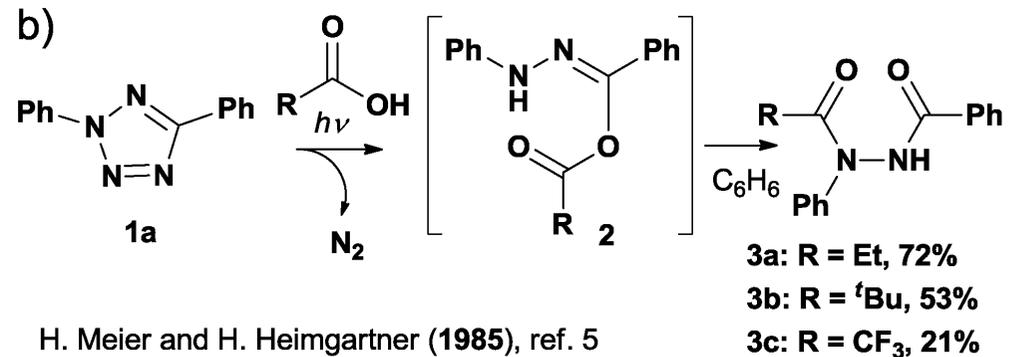
✓ Although more improvement is needed, these coupling reactions could be extensively applied to low-background protein labeling

✓ Other crosslinking methods without radical intermediate are also emerging

→ allow the identification of elusive **transient and dynamic protein-protein interactions** in vitro, in cell lysates, and in living cells

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Thank you
for your kind attention!