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In vivo bioorthogonal chemistry for imaging and therapy

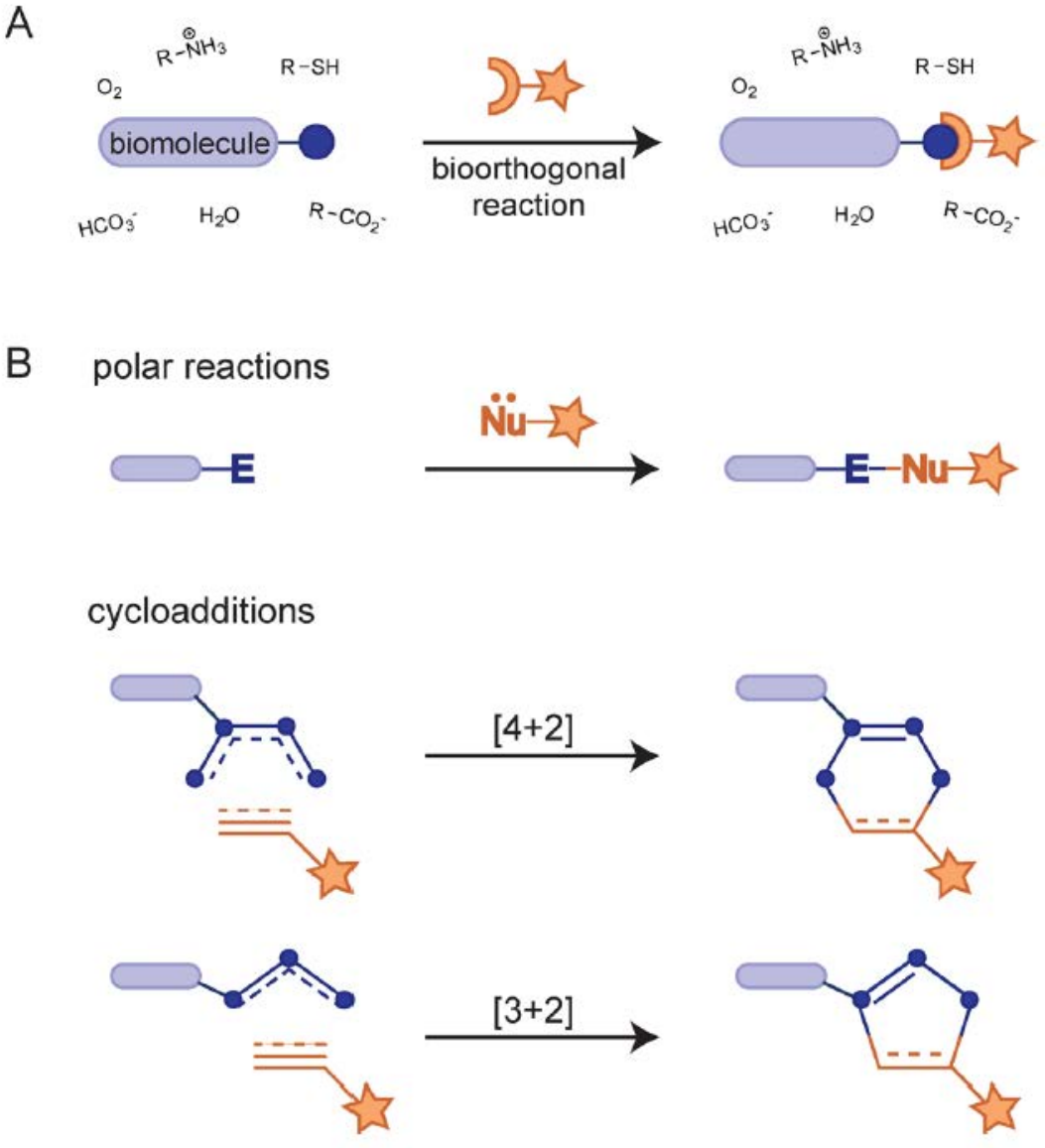
Marc Robillard

October 1, 2020

Outline

- **Part 1 - Click**
  - Bioorthogonal reactions recap
  - Quick introduction to nuclear imaging and “internal radiotherapy
  - Tumor pretargeting for radioimmuno-imaging and –therapy
  - Chemical approach to tumor pretargeting
  - Summary
- **Part 2 – Click-to-release**
  - “Click-to-release” ADC approach to cancer therapy
  - Designing a suitable activator for in vivo applications
  - Other applications of the IEDDA pyridazine elimination reaction

Bioorthogonal reactions recap



Examples:

Staudinger Ligation

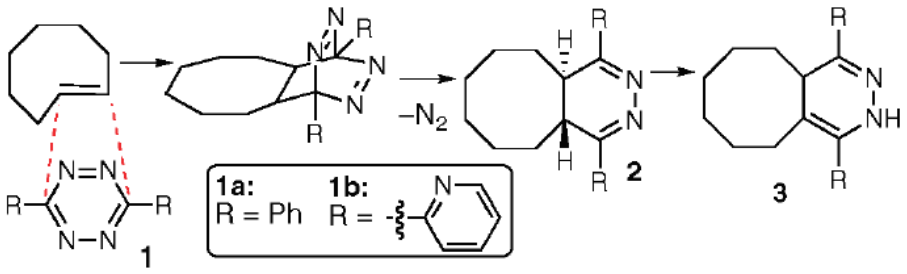
Inverse-electron demand Diels-Alder cycloaddition (IEDDA)

Strain-promoted azide-alkyn cycloaddition (SPAAC)

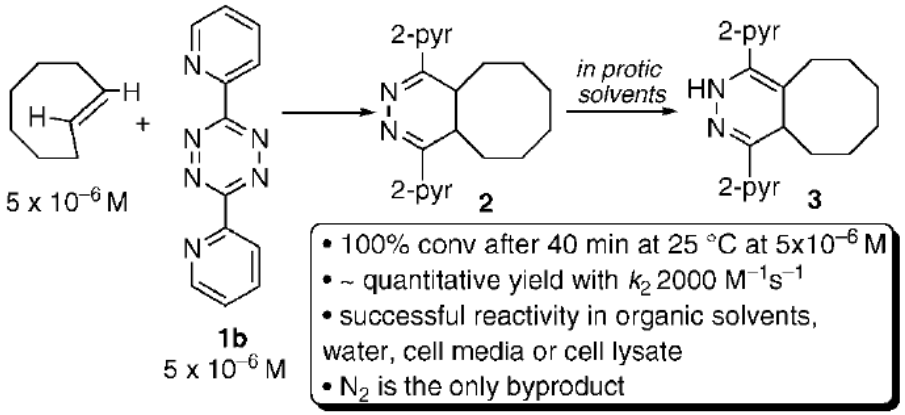
Patterson et al., *ACS Chem. Biol.* **2014,** 9, 592-605

Inverse-electron demand Diels-Alder cycloaddition (IEDDA)

Scheme 1. Diels–Alder Reactions of Tetrazines with *trans*-Cyclooctene

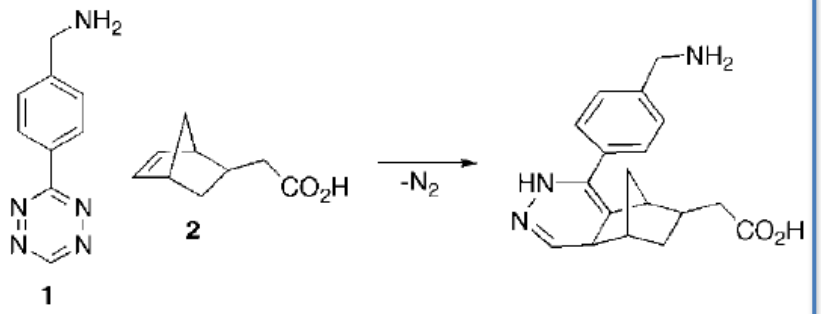


Scheme 2. Fast Reactivity at Low Micromolar Concentrations



tetrazine + *trans*-cyclooctene

Blackman et al., *JACS* **2008**, 130, 13518-13519



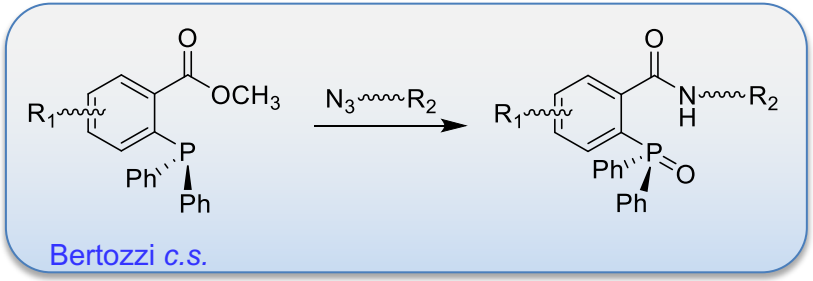
tetrazine + norbornadiene

Devaraj et al., *Bioconjug. Chem.* **2008**, 19, 2297-2299

Reactivity increases with increased strain, EWG-functionalized TZs and in protic solvents

Bioorthogonal chemistry for in vivo applications

Staudinger Ligation

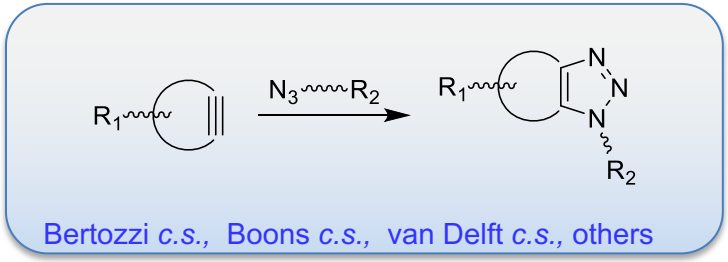


$K_2 = 2\times10^{-3}$ $M^{-1}s^{-1}$ (CD_3CN + 5% H_2O)

[A]=[B]= 1μM

15.9 yr

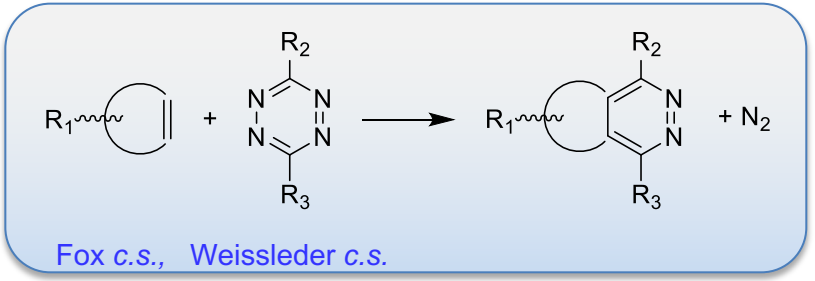
Strain-Promoted Azide-Alkyn Cycloaddition



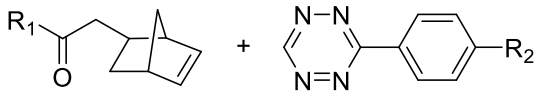
$K_2 = 4.2\times10^{-2}$ – 1.7 $M^{-1}s^{-1}$ (CD_3CN, MeOH or PBS)

6.8 d – 9.2 mo

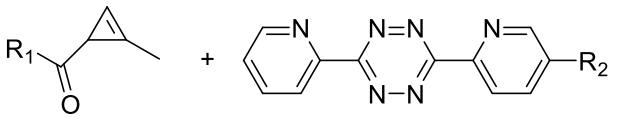
Inverse-Electron Demand Diels-Alder



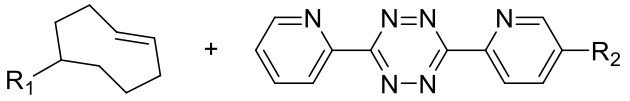
$K_2 = 1.9 – 2.3\times10^{6}$ $M^{-1}s^{-1}$ (PBS)



6.1 d



21.4 h



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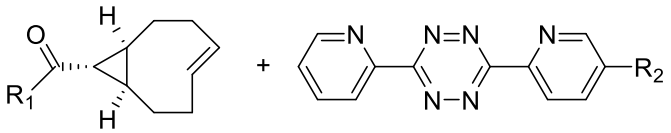

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Rossin & Robillard, *Curr. Opin. Chem. Biol.* **2014,** *21*, 161−169

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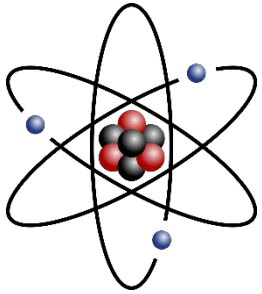
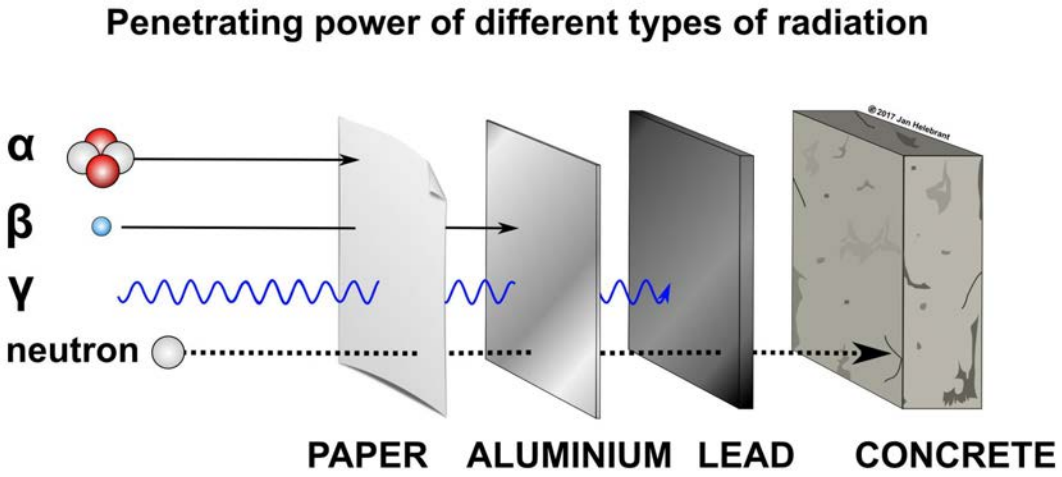
Quick introduction to nuclear imaging and “internal” radiotherapy

Types of radiations for nuclear imaging and “internal” radiotherapy



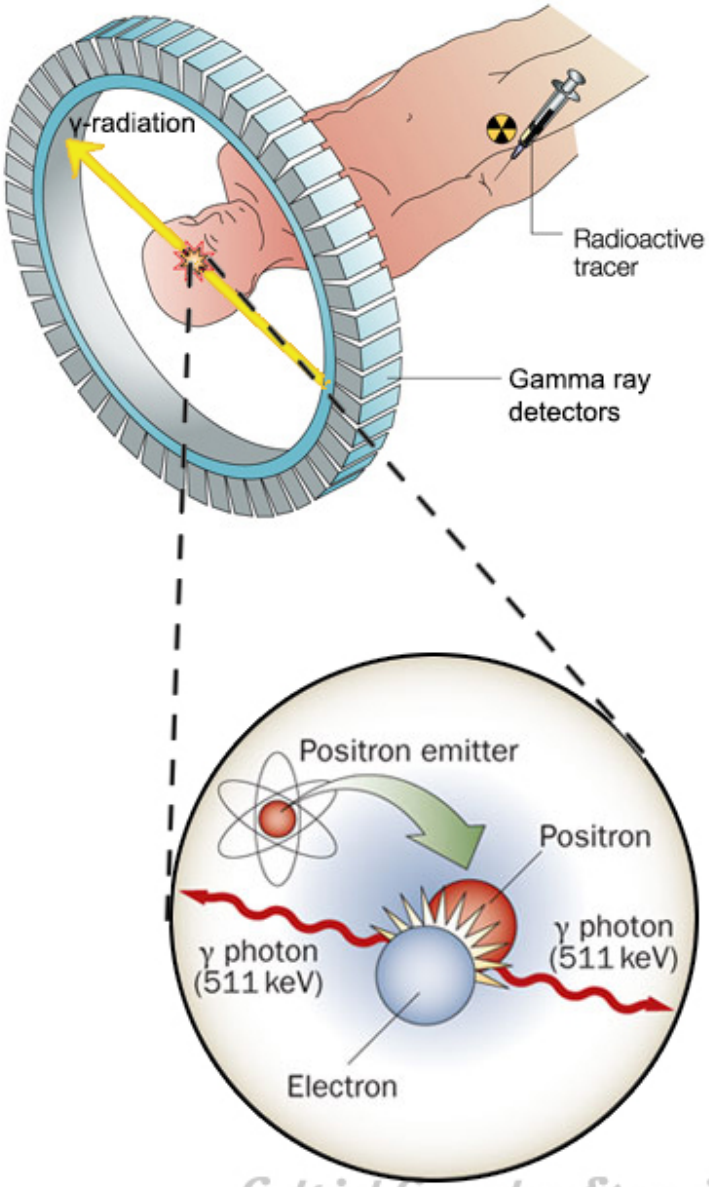


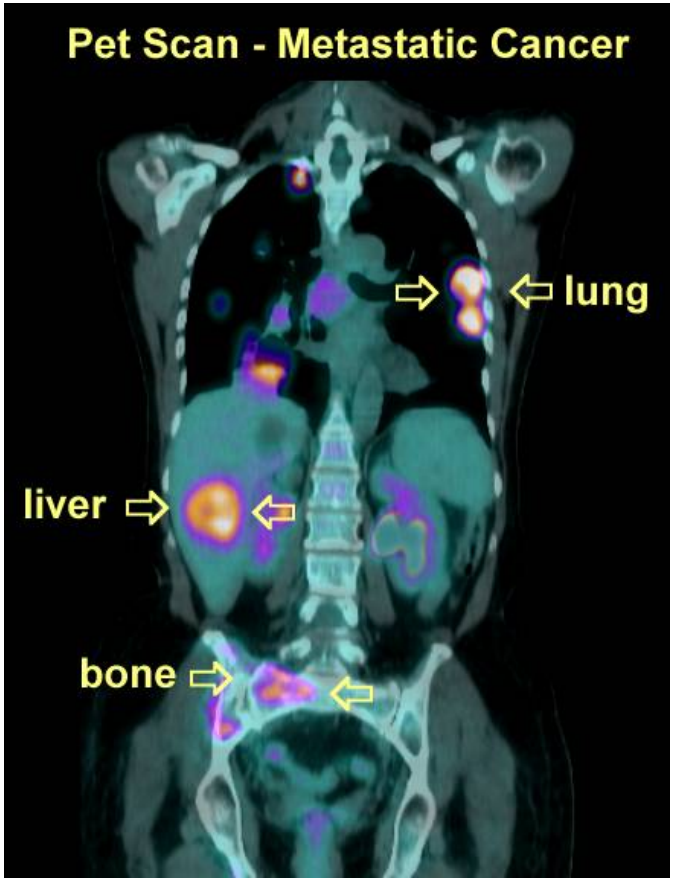
Radionuclides are unstable nuclei that decay to more stable states by emitting some sort of radiation

Radionuclides emitting γ (^{111}In, ^{99m}Tc, etc) and β^+ (^{18}F, ^{89}Zr, etc) can be used for **Single Photon Emission Computed Tomography** (**SPECT**) and **Positron Emission Tomography** (**PET**), respectively

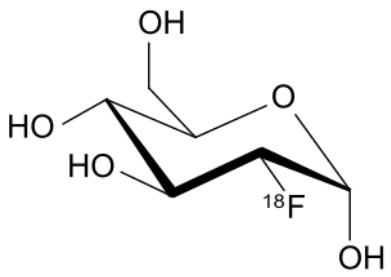
Radionuclides emitting β^- (^{177}Lu, ^{186}Re, etc) and α particles (^{225}Ac, ^{212}Pb, ^{223}Ra, etc) can be used for **radiotherapy** of cancer

Positron Emission Tomography

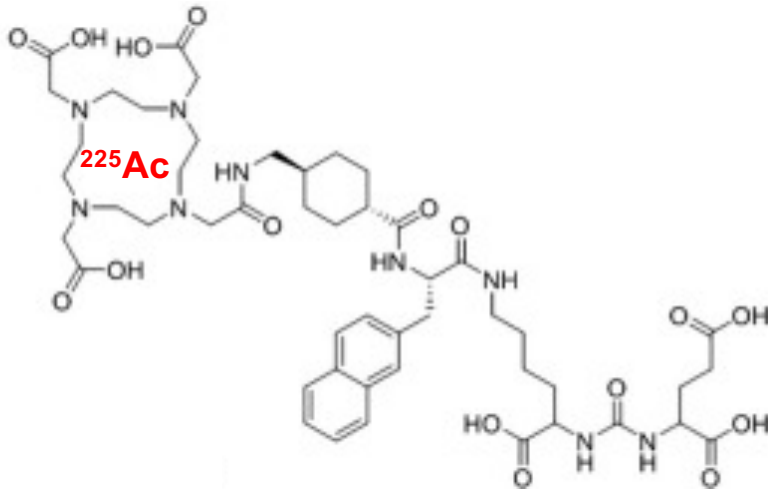




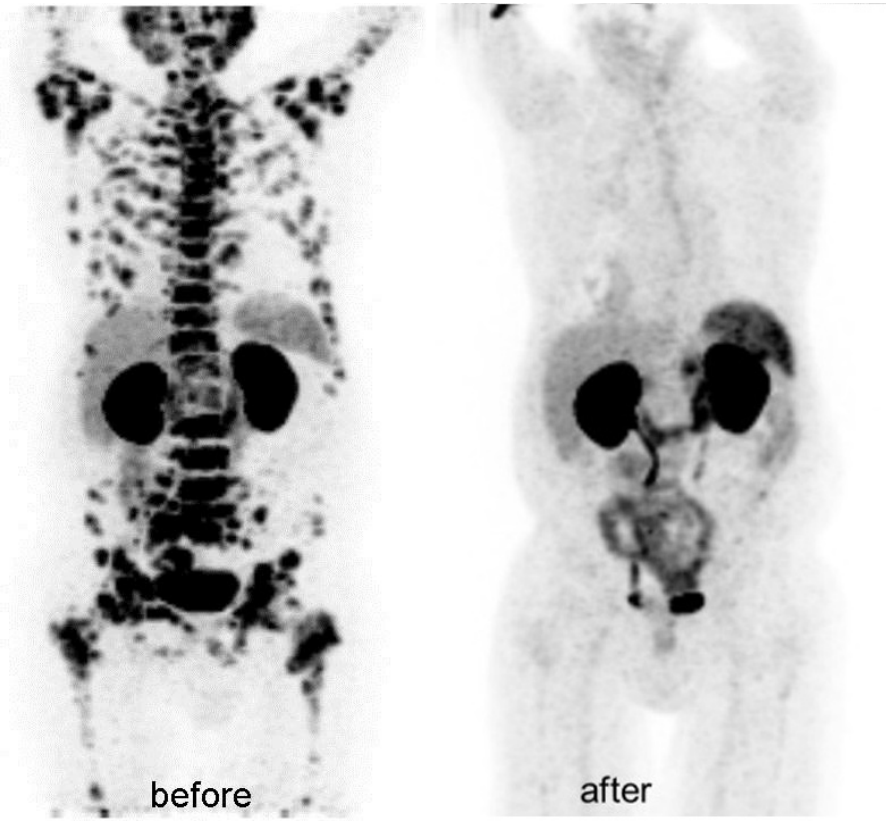
Cancer radiotherapy



[^{18}F]fluorodeoxyglucose (FDG) for PET imaging of tumor metabolism



^{225}Ac-PSMA-617 for targeted radiotherapy of PSMA expressing tumors



FDG-PET of a patient with metastatic prostate cancer before and after ^{225}At-PSMA-617 treatment

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Tumor pretargeting for radioimmuno-imaging and -therapy

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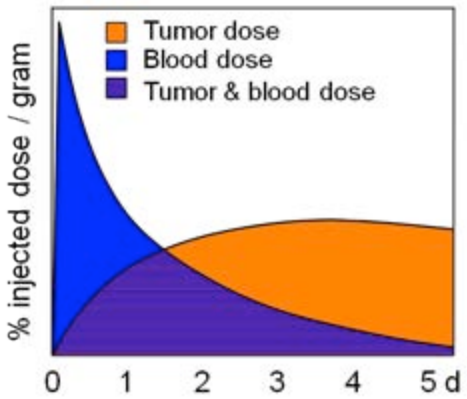
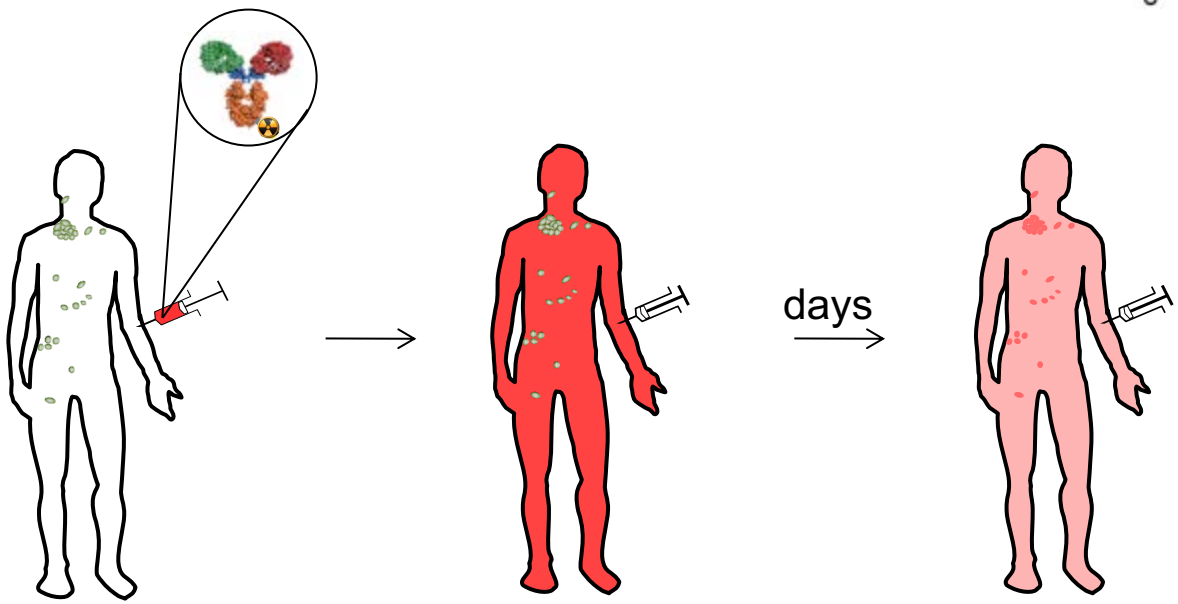
Directly labeled antibodies for imaging and therapy

Antibodies (mAbs) give superior target uptake compared to fragments **BUT**:

- They have slow target (e.g. tumor) uptake and slow blood clearance,
- hampering imaging applications
- and solid tumors remain out of reach of radioimmunotherapy (RIT) due to radiation dose-limiting side effects





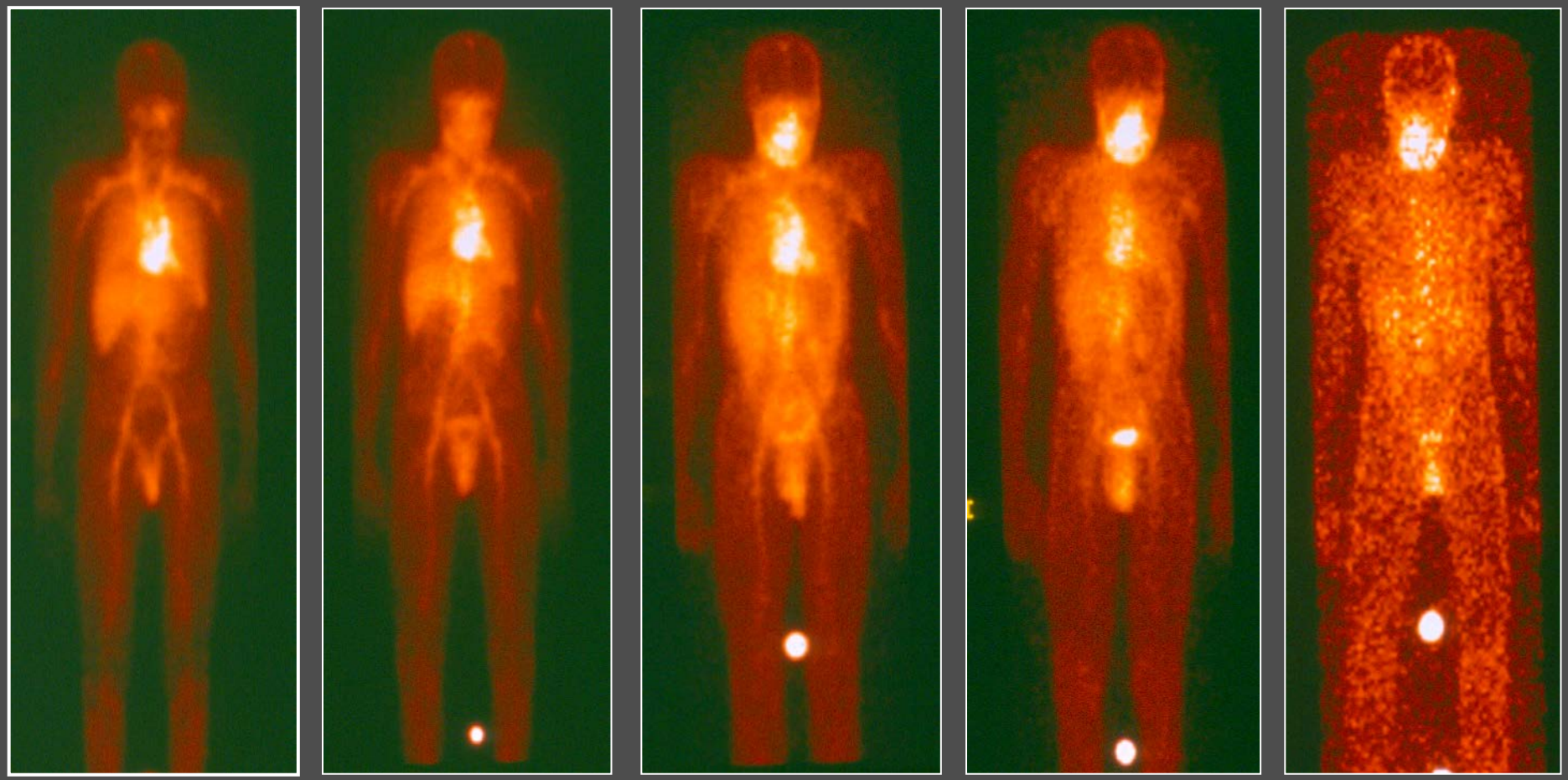
Injection of radioactive antibody

Systemic distribution, slow tumor uptake & clearance from non-tumor areas

Low tumor-blood ratios afford poor images and lead to high off-target toxicity limiting radioactive dose and therapeutic efficacy

Conventional radioimmunoimaging & –therapy: low target-blood ratios & high radiation dose to bone marrow

[^{186}Re]Re-U36 in head and neck cancer patient



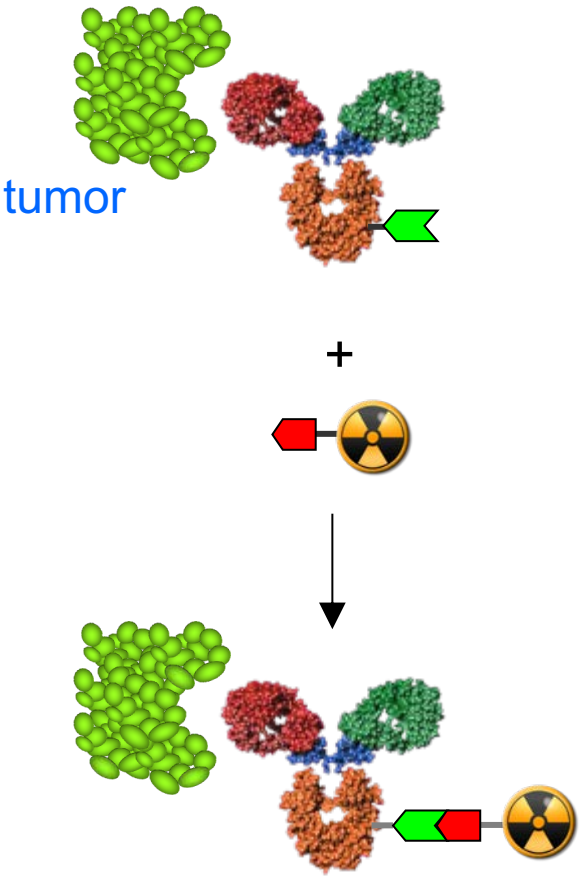
< 1 hour **24 hours** **48 hours** **72 hours** **312 hours**

Courtesy Prof. van Dongen, Vumc

Postema *et al*. *J. Nucl. Med.* **2003**, *44*,1690

Pretargeting..

..improves radioimmunotherapy and -imaging of tumors via a 2-step tumor targeting scheme



Step 1:
Slow tumor binding with antibody

Step 2:
Fast binding with small probe with

High tumor-background ratio &
increased efficacy

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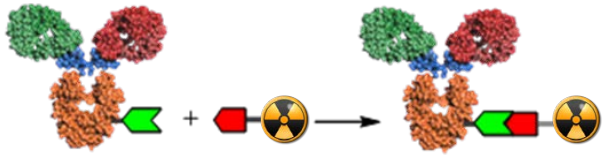
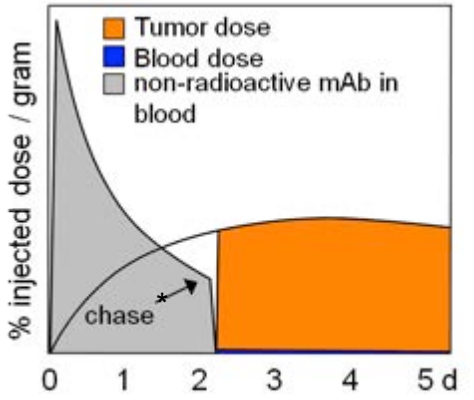
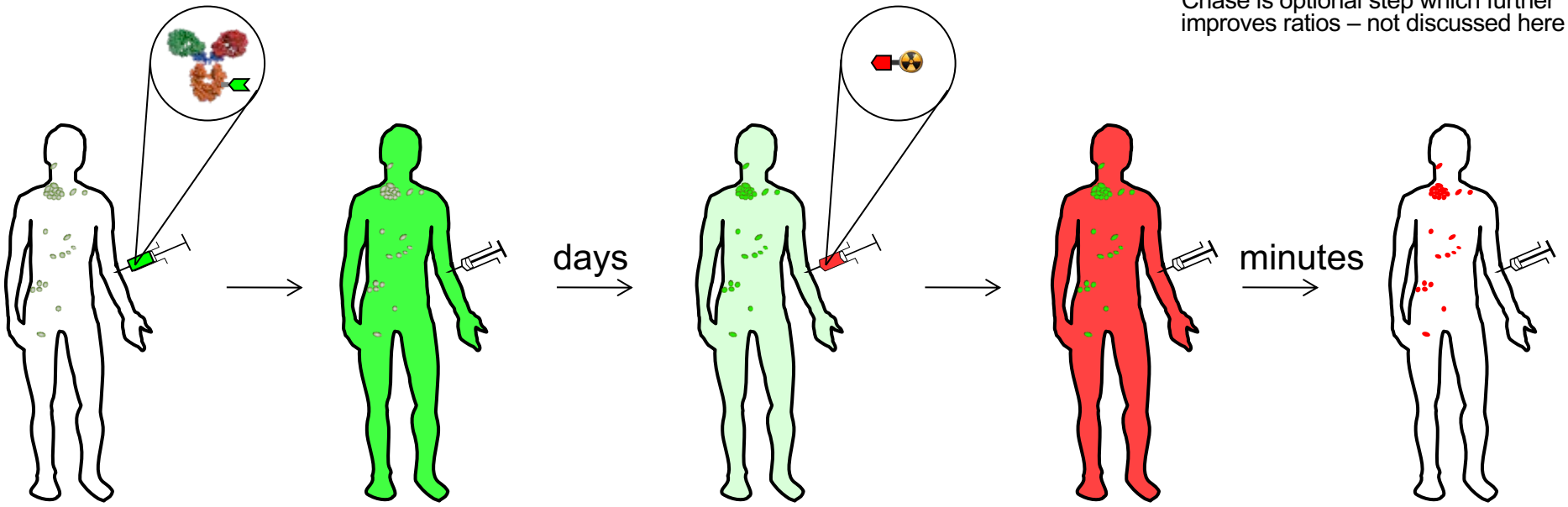


Pretargeting - general

- Overcomes limitations by separating tumor targeting from delivery of radioactive component
- 2 steps: tumor targeting of a “tagged” mAb followed by rapid binding of a small radiolabeled probe to the tumor-bound mAb in 2nd step
- Non-bound probe is excreted in a matter of minutes



* Chase is optional step which further improves ratios – not discussed here



Injection of tagged antibody

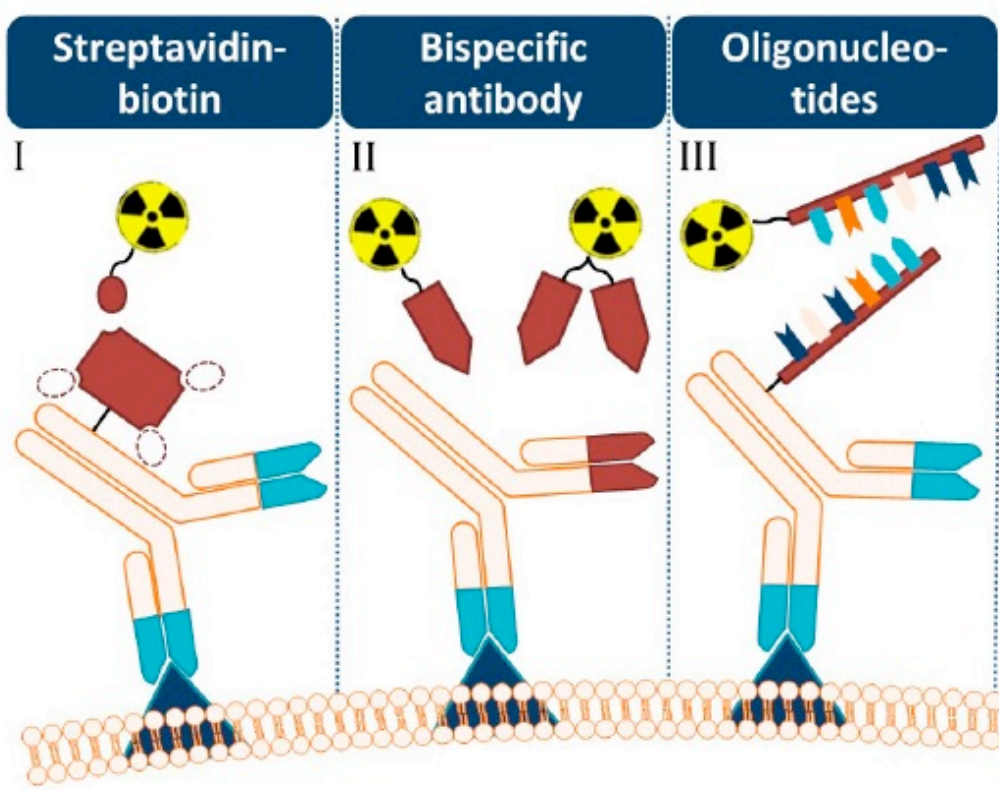
Systemic distribution, slow tumor uptake & clearance from non-tumor areas

At favorable tumor-blood ratio: injection of radioactive probe

Systemic distribution, fast tumor uptake & clearance from non-tumor areas

High tumor-blood ratios allow high quality images and therapeutic efficacy

Pretargeting pairs



Verhoeven et al., *Pharmaceutics* **2019,** *11*, E434

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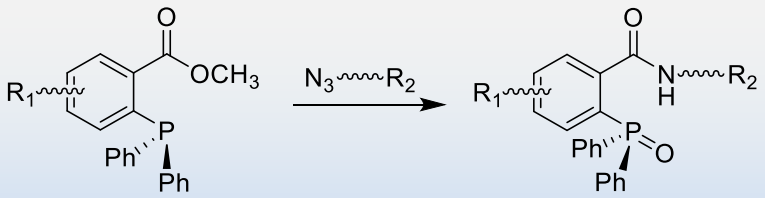
Chemical approach to tumor pretargeting

Bioorthogonal chemistry for in vivo applications: **Speed is key**

Staudinger Ligation



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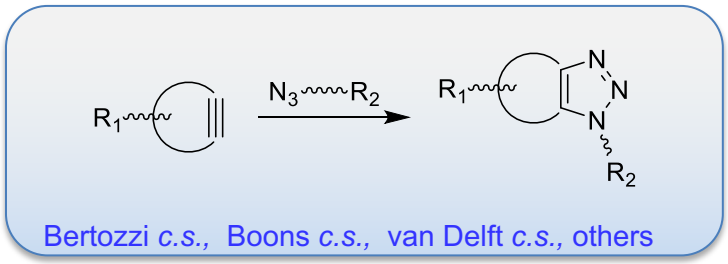

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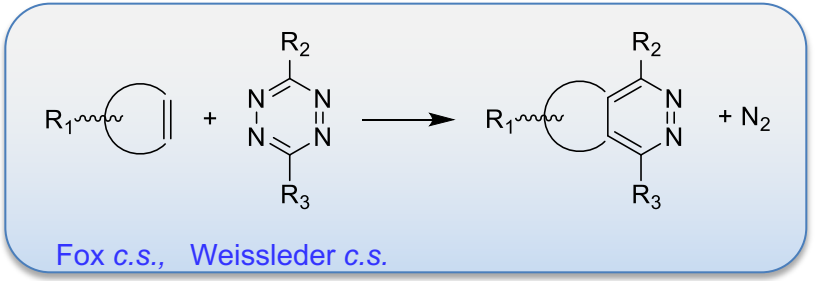
Inverse-Electron Demand Diels-Alder



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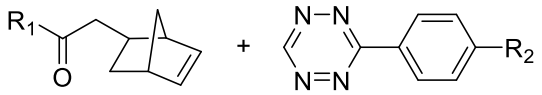
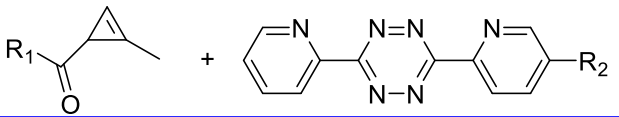
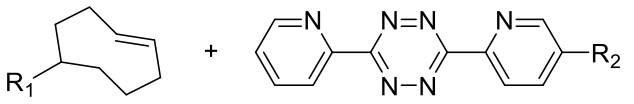


6.1 d



21.4 h



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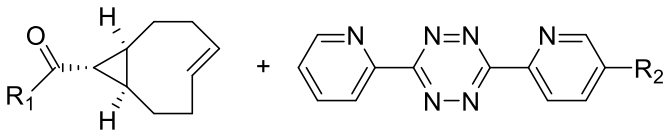

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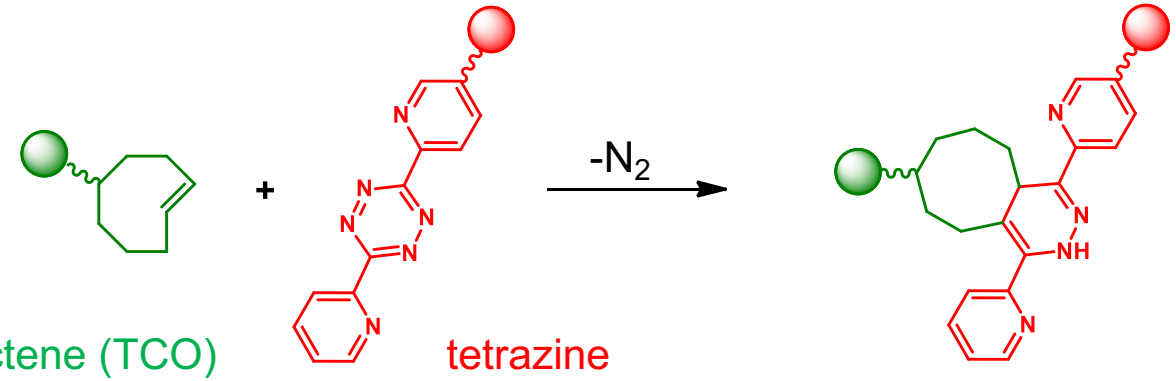
Rossin & Robillard, *Curr. Opin. Chem. Biol.* **2014,** *21*, 161−169

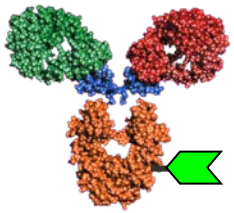
Bioorthogonal chemistry for pretargeting

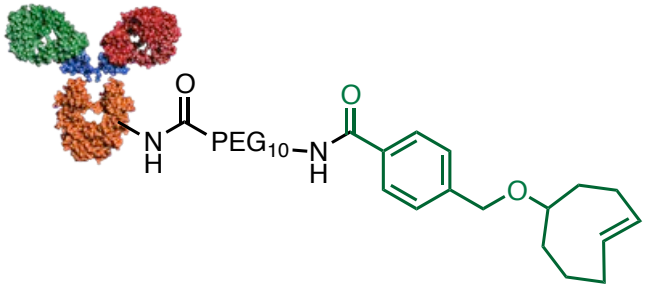
with CC49 antibody against TAG72, a pan-carcinoma marker (colon, breast, ovarian, lung, ..)

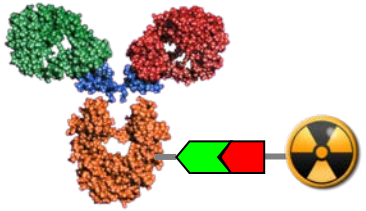


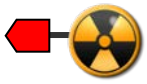
trans-cyclooctene (TCO) tetrazine

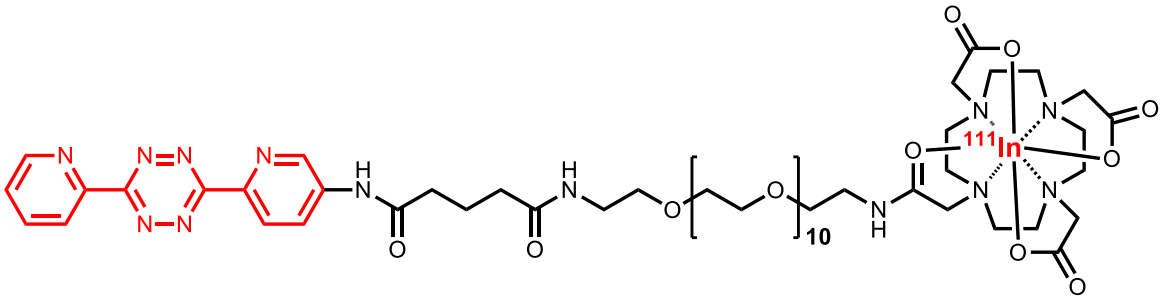
Fox *c.s. J. Am. Chem. Soc.* **2008**, *130*, 13518







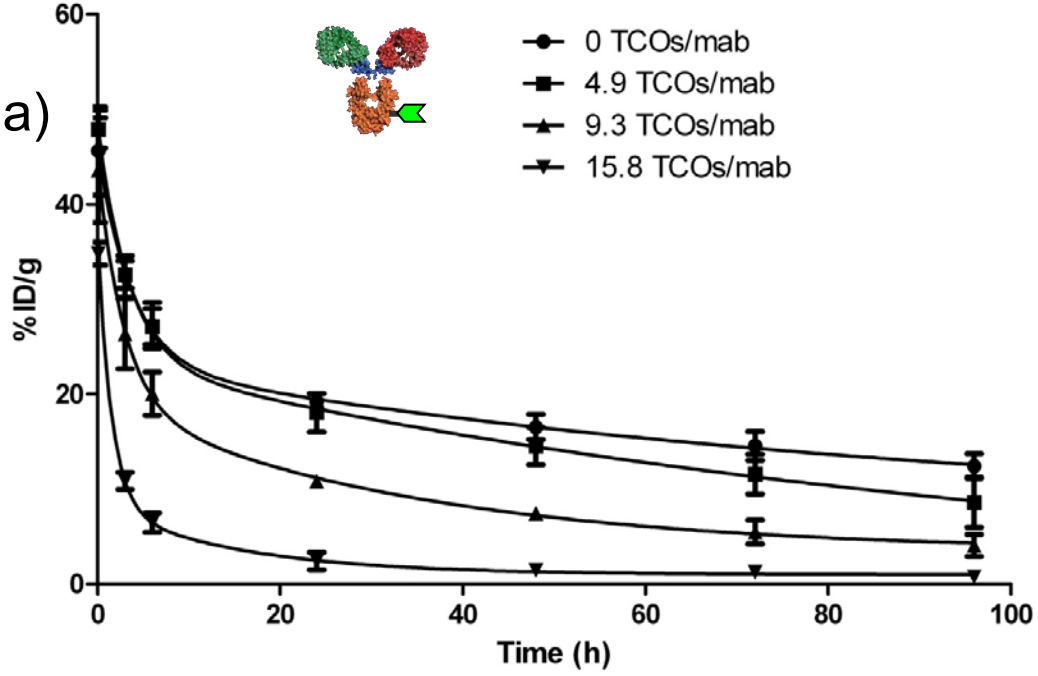




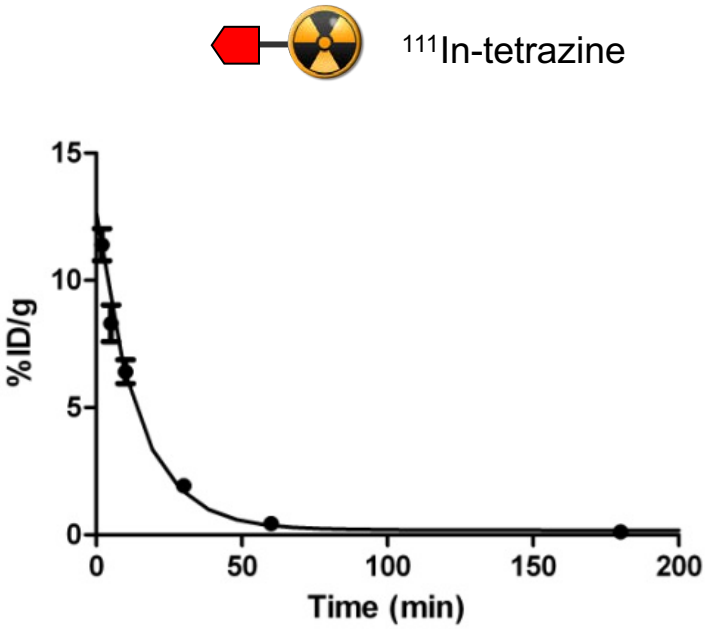
Blood clearance of both components

Nude mice (n=4) injected i.v. with a) ^{125}I-CC49 or ^{125}I-CC49-TCO tag (100 µg); or b) with ^{111}In-DOTA-tetrazine (21 µg)

a)



b)

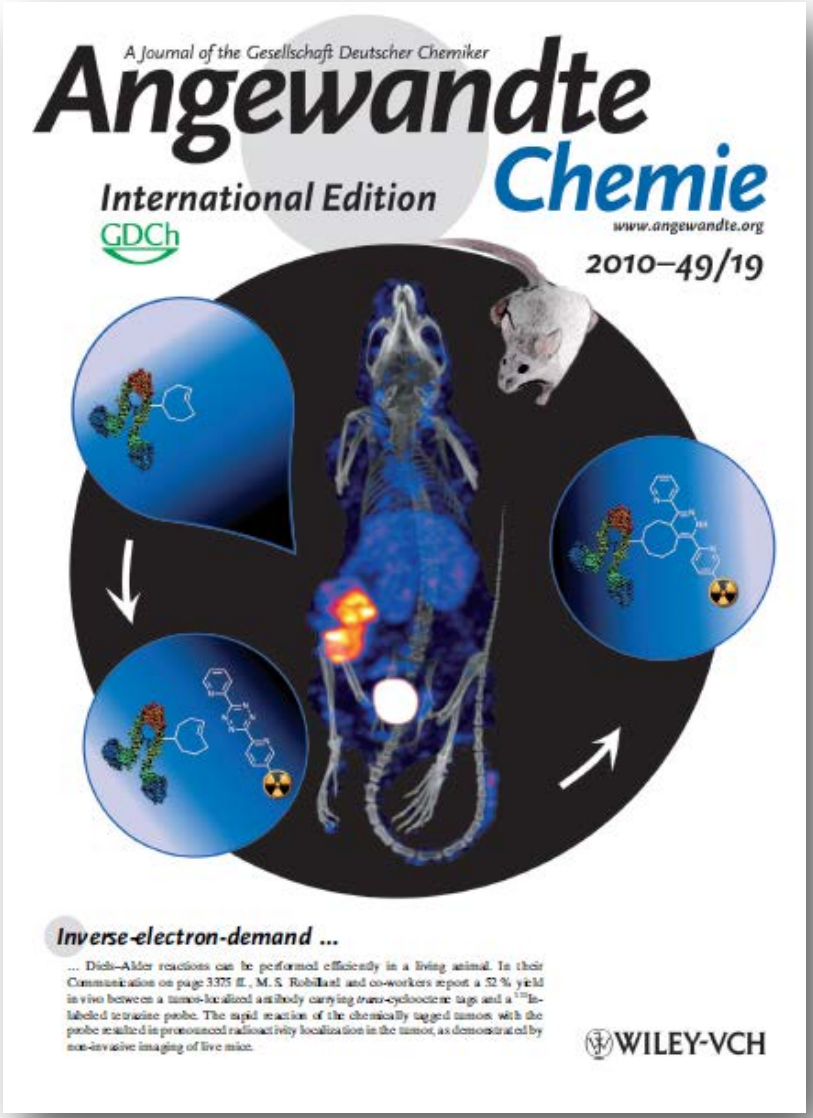


0 TCO	t_α = 2.58 h; t_β = 64.36 h
4.9 TCO	t_α = 2.39 h; t_β = 74.55 h
9.3 TCO	t_α = 1.97 h; t_β = 23.49 h
15.8 TCO	t_α = 1.05 h; t_β = 10.55 h

$t_{½}$ = 9.8 min

V_D = Dose/C_0 = 0.32 L/kg

Proof of concept:
in vivo reaction in equimolar conditions



Colorectal cancer mouse model (LS174T). Injection of CC49-TCO_7 followed 24h later by [^{111}In]In-tetrazine (25 eq)

- 52 % reaction yield on the tumor
- TCO in vivo stability: $t_{1/2}$ = 2.62 d
- TCO k_2 = 2.7 ×10^4 M^{-1} s^{-1}

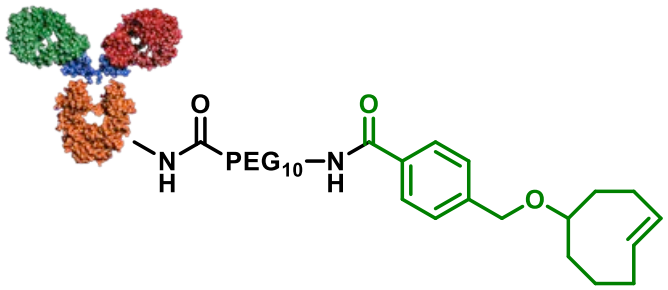
Next aims:

- Improve tag: stability, reactivity, PK
- Improve tumor-blood ratio
- Other targets and targeting agents

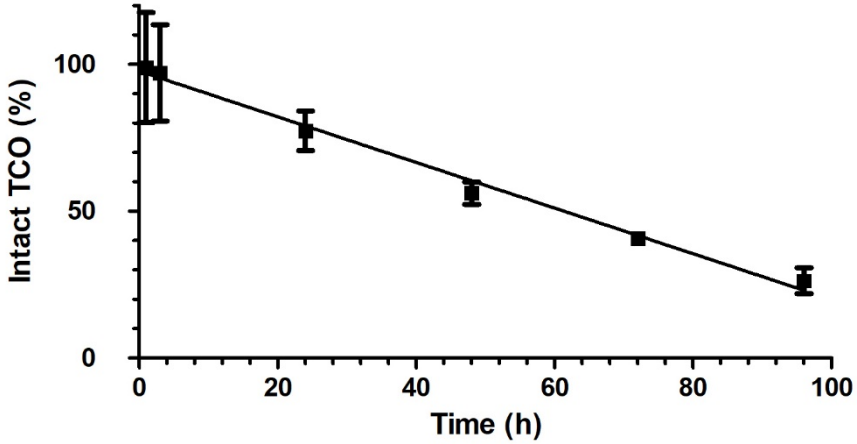
Rossin *et al.*, *Angew. Chem. Int. Ed.* **2010**, *49*, 3375–3378

Slow TCO deactivation in circulation

In vivo in mice *trans*-cyclooctene slowly becomes unreactive towards tetrazines

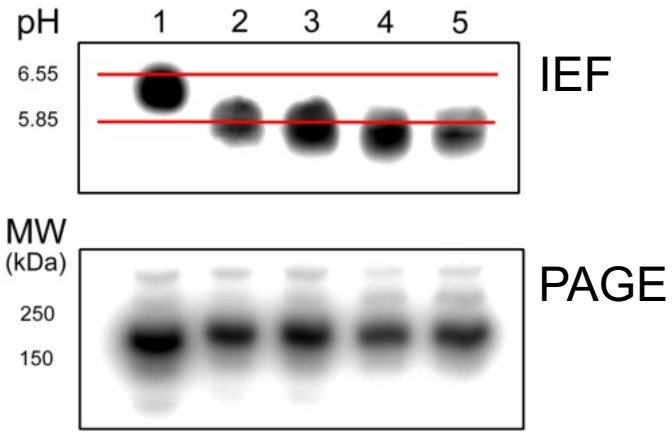


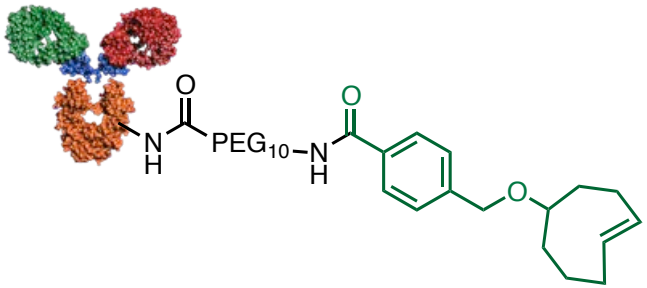
Half-life 2.62 days



In vivo TCO stability in blood in mice (n=3) assessed ex vivo with ^{177}Lu-DOTA-tetrazine, corrected for ^{125}I-ADC blood clearance

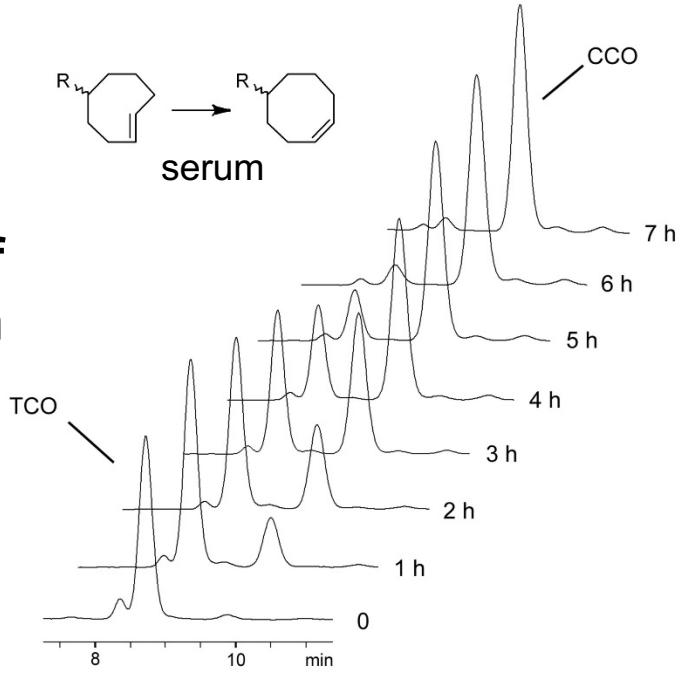
Mechanism of TCO deactivation





- ^{125}I-CC49 (lane 1, t = 0) and ^{125}I-CC49-TCO$_7$ after (lanes 2 to 5) 0, 1 h, 24 h and 48 h incubation at 37 °C in 50% mouse serum.
- Amide bonds in linker are stable

Instead, we found in vitro **isomerization of TCO** to the unreactive *cis*-cyclooctene (CCO) in serum, 37°C



Rossin *et al.*, *Bioconjugate Chem.* **2013**, *24*, 1210−1217

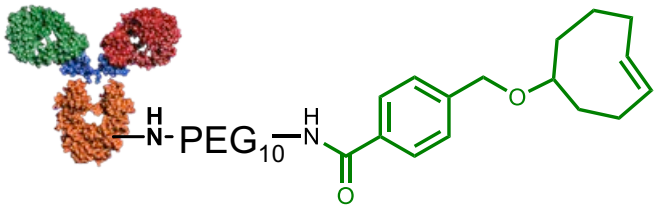
Stability of the TCO tag

In vitro isomerization TCO to *cis*-cyclooctene (CCO) in serum (components), 37 °C

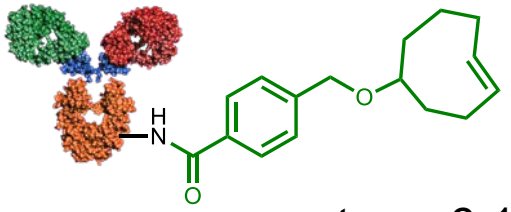
Medium	$t_{1/2}$
Fresh serum	3.26 h
PBS	>> 7 d
Vitamin B12	>> 7 d
Transferrin	>> 7 d
Ceruloplasmin	6.25 d
Transcuprein	1.39 h
Albumin (MSA)	0.65 h
Metal-depleted albumin (MD-MSA)	2.84 h

Albumin-bound Cu plays a role in TCO isomerization

By removing the linker TCO is less accessible and stability increases



$t_{1/2}$ = 2.62 d
k_2 = 2.7 ×10^4 M^{-1}s^{-1}

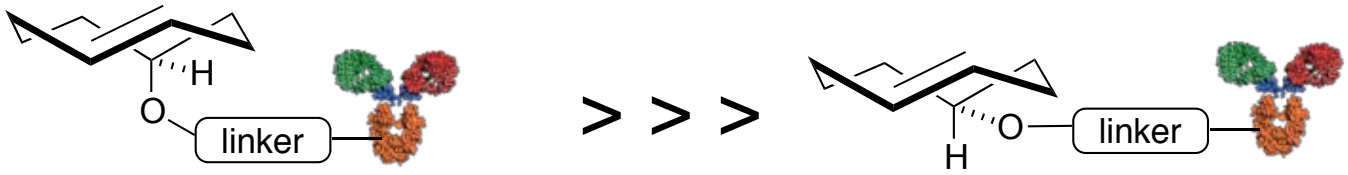


$t_{1/2}$ = 6.19 d
k_2 = 3.2 ×10^4 M^{-1}s^{-1}

Rossin *et al.*, *Bioconjugate Chem.* **2013**, *24*, 1210−1217

Reactivity of the TCO tag

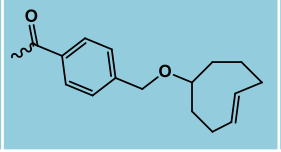
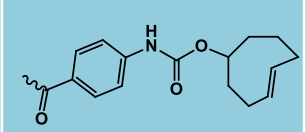
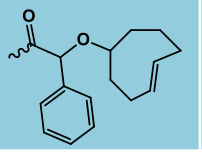
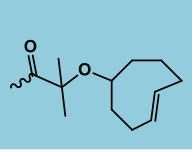
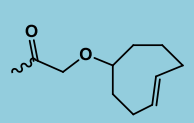
Axial TCOs are 10 times more reactive than the equatorial isomers due to higher energy (strain)



"axial" isomers

"equatorial" isomers

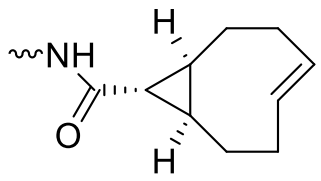
2nd order constants k_2 ×10^4 $M^{-1}s^{-1}$ between CC49-TCO's & ^{177}Lu-tetrazine, 37 °C, PBS

TCO structures / isomers					
Axial	27.3±0.5	10.0±1.3	16.3±0.3	14.8±1.2	13.5±0.1
Equatorial	3.2±0.2	1.3±0.1	2.9±0.2	2.5±0.4	2.1±0.0

Rossin *et al.*, *Bioconjugate Chem.* **2013**, *24*, 1210−1217

Reactivity of the TCO tag

Higher reactivity comes at the expenses of in vivo instability:
need for compromise

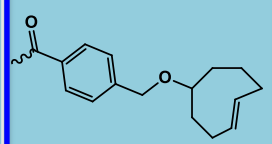
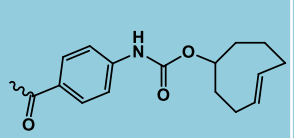
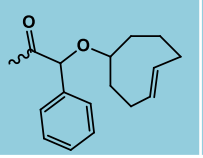
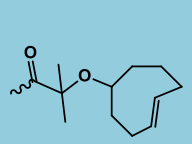
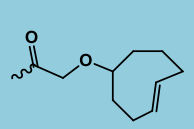


cyclopropyl-TCO
k_2 = 280 × 10^4 $M^{-1}s^{-1}$
$t_{1/2}$ = 0.67 days

$t_{1/2}$ = 3.94 days

$t_{1/2}$ = 10.3 days

2^{nd} order constants k_2 ×10^4 $M^{-1}s^{-1}$ between CC49-TCO's & ^{177}Lu-tetrazine, 37 °C, PBS

TCO structures / isomers					
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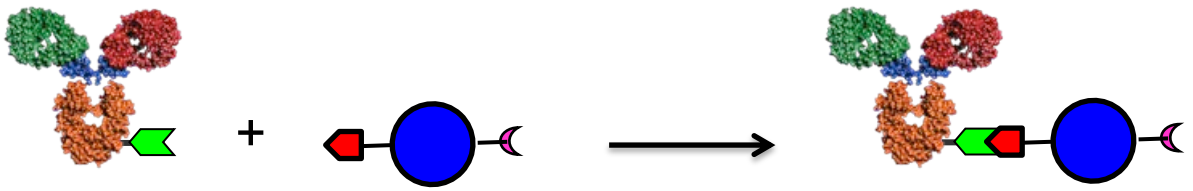
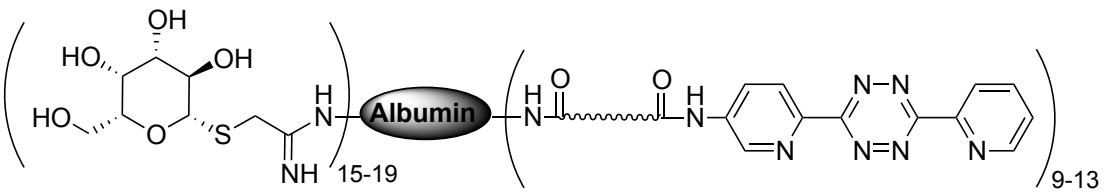
Rossin *et al.*, *Bioconjugate Chem.* **2013**, *24*, 1210−1217

Clearing agents

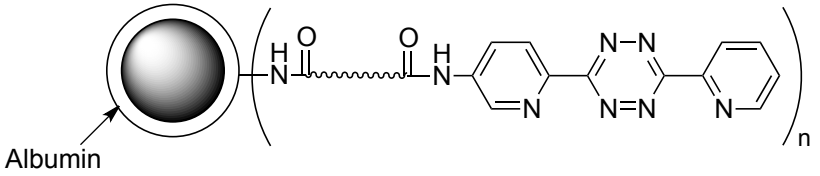
- Tetrazine-functionalized & liver-targeted albumin or microparticles enable instantaneous clearance of tagged constructs from blood
- Removal of residual mAb-tag from circulation before administration of the radioactive probe boosts the target-blood ratio



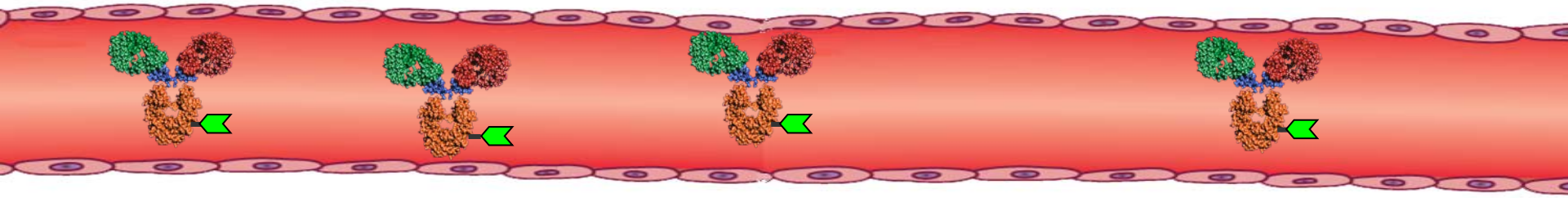
CA-1: Galactose/tetrazine-functionalized albumin; hepatocyte Ashwell receptor uptake

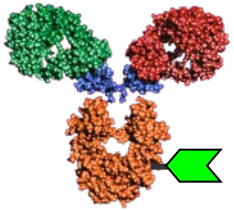


CA-2: Microparticles coated with tetrazine-functionalized albumin; size-related RES uptake

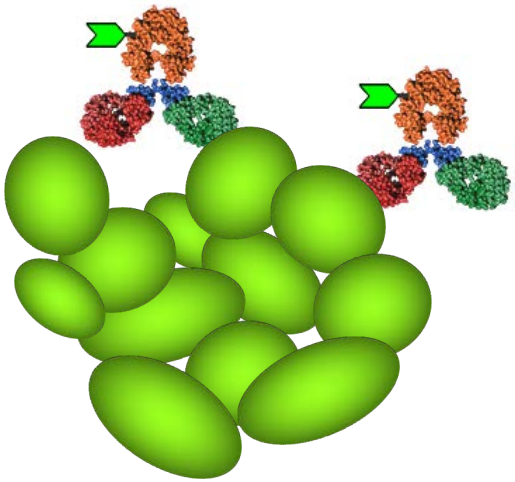


tagworks PHARMACEUTICALS

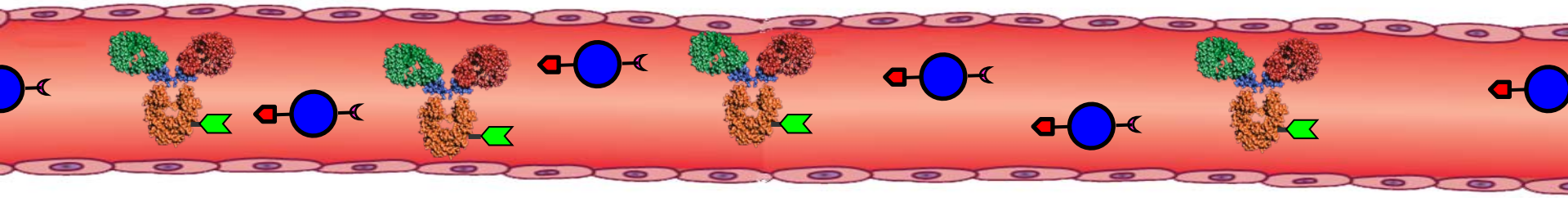


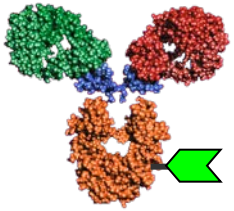


Step 1:
Slow tumor binding and clearance of antibody



tagworks PHARMACEUTICALS





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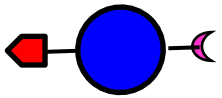
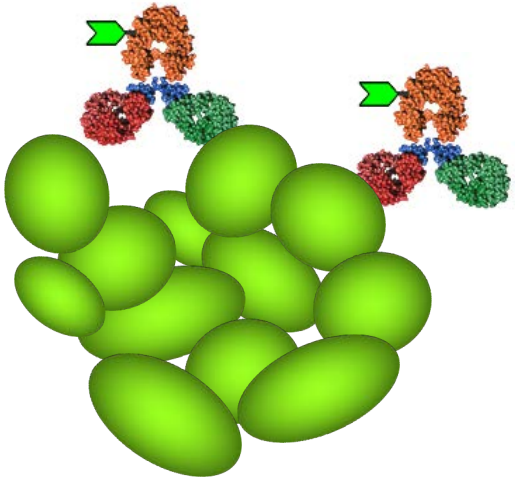


Step 2:
Administration of clearing agent; fast binding with circulating antibody



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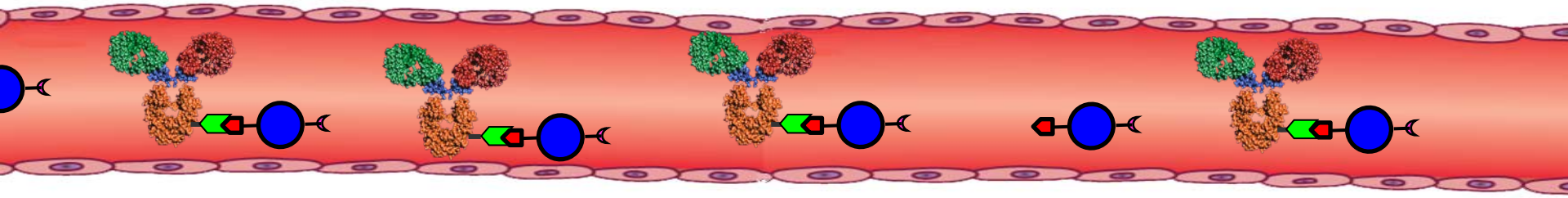

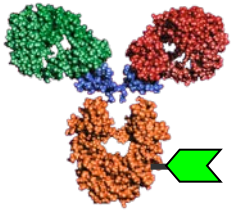

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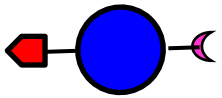

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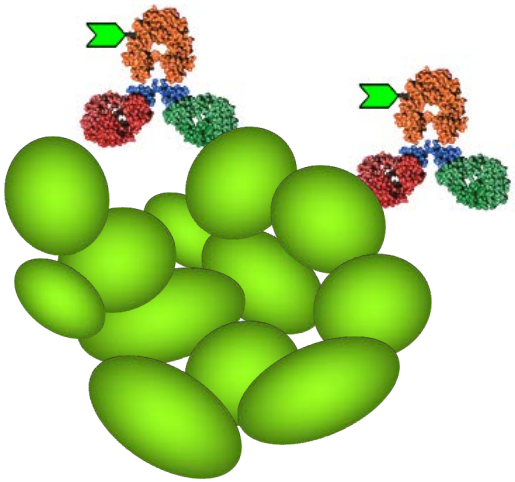

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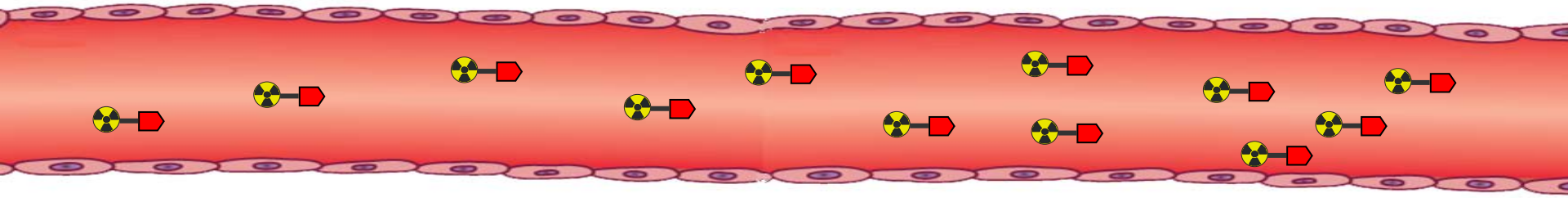

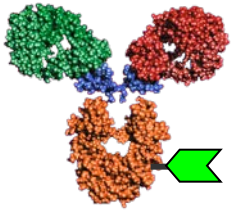

Step 1:
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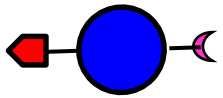
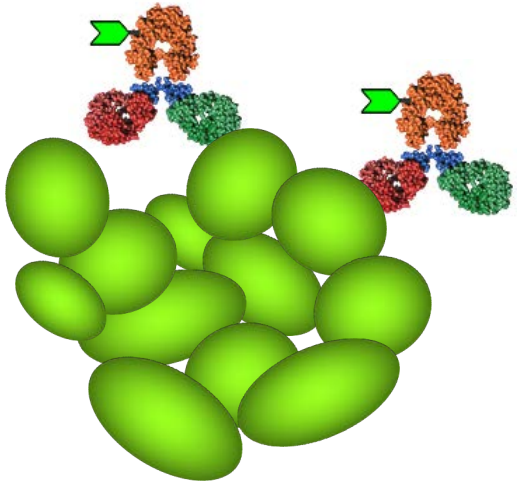
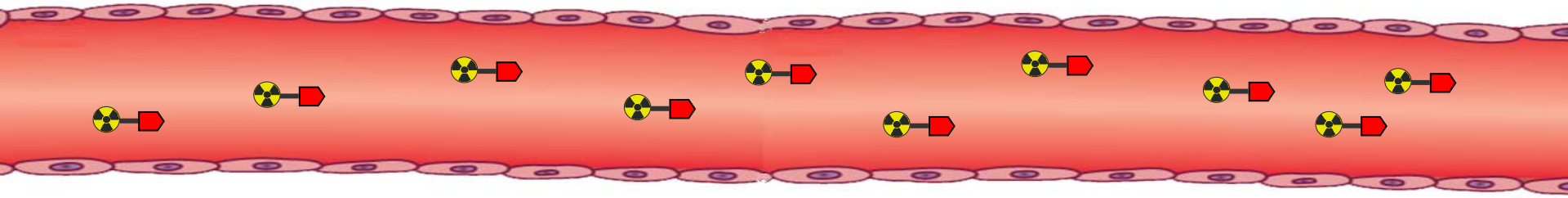


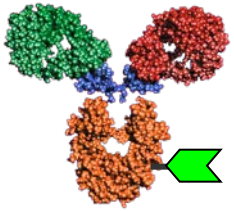
Step 2:
Administration of clearing agent; fast binding with circulating antibody

+

Step 3:
Fast binding with small radiolabeled probe







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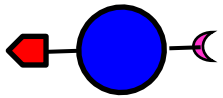

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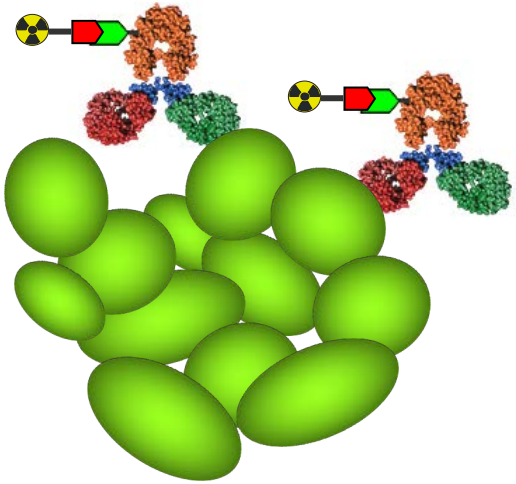
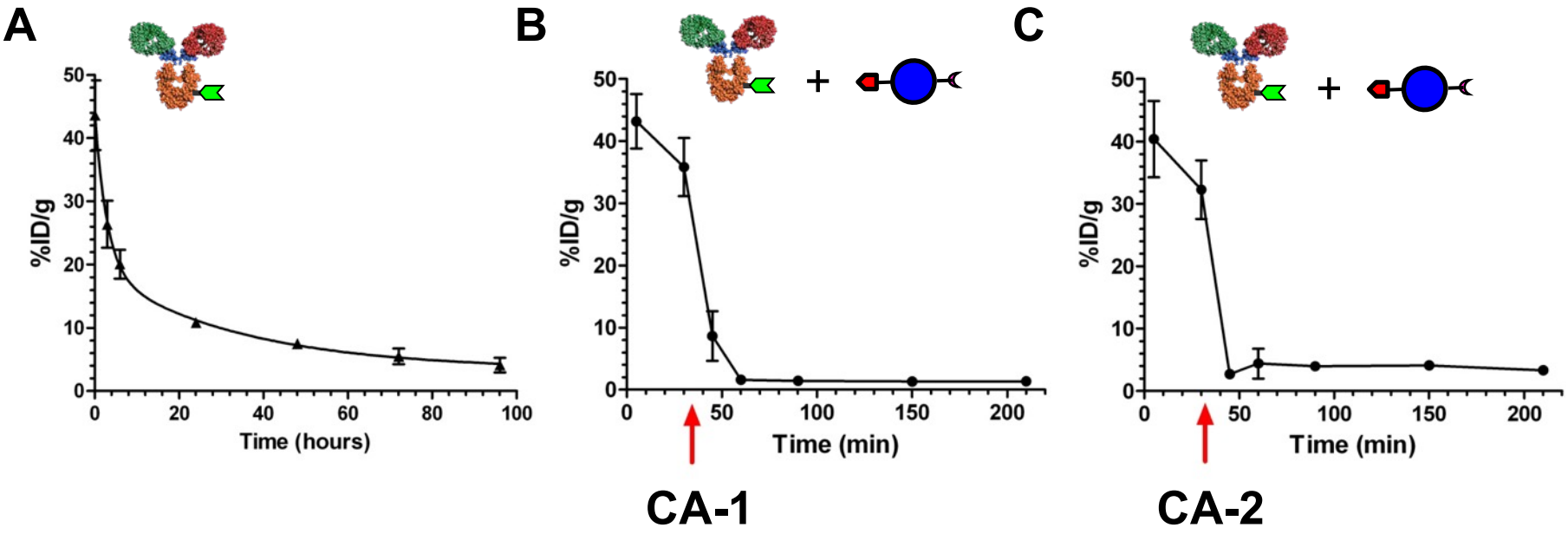


Blood clearance of ^{125}I-CC49-TCO

Tumor-free mice (n=3) injected with (A) 100 µg [^{125}I]I-CC49-TCO$_9$, (B) 20 µg [^{125}I]O-CC49-TCO$_9$ followed by 120 µg albumin CA-1, (C) 20 µg [^{125}I]I-CC49-TCO$_9$ followed by 4×10^7 particles CA-2

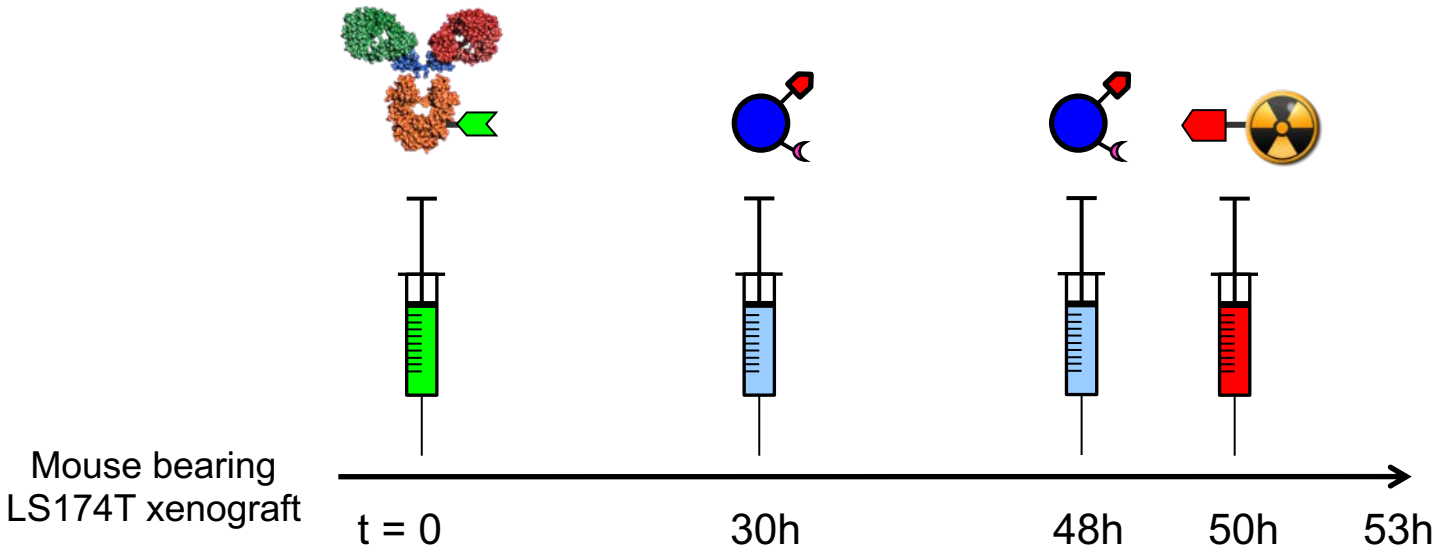


- Highly reactive towards mAb-tag in blood
- Efficient clearance of mAb-tag from blood through liver
- No extravasation into tumor
- No formation of small tag-reactive metabolites

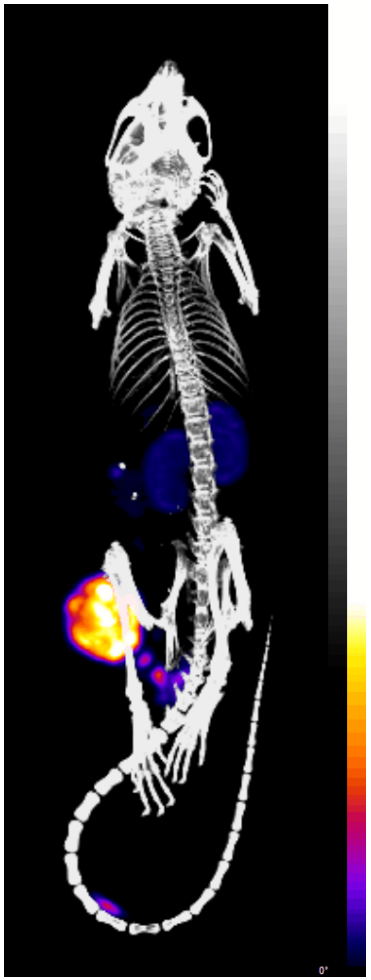
Rossin *et al., J. Nucl. Med.* **2013**, *54*,1989-1995

Optimized pretargeting protocol

Injection of 100 µg CC49-TCO_7, clearing agent (160 µg; 30 & 48 h), [^{111}In]In-tetrazine (10 eq to CC49; 50 h), image @ 53 h



SPECT/CT



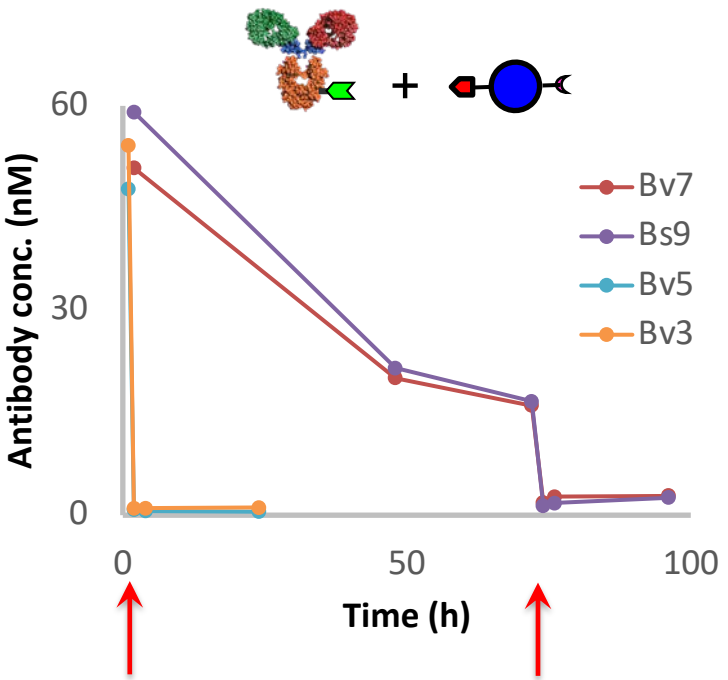
- 46 % yield; product stable in vivo > 1 week
- **7 day ^{177}Lu dosimetry: 8-fold higher tumor dose (171Gy) vs. [^{177}Lu]Lu-CC49**

T/B: 254 (ex vivo)
T/M:190
T/L: 55
T/K: 8

Rossin *et al., J. Nucl. Med.* **2013**, *54*,1989-1995

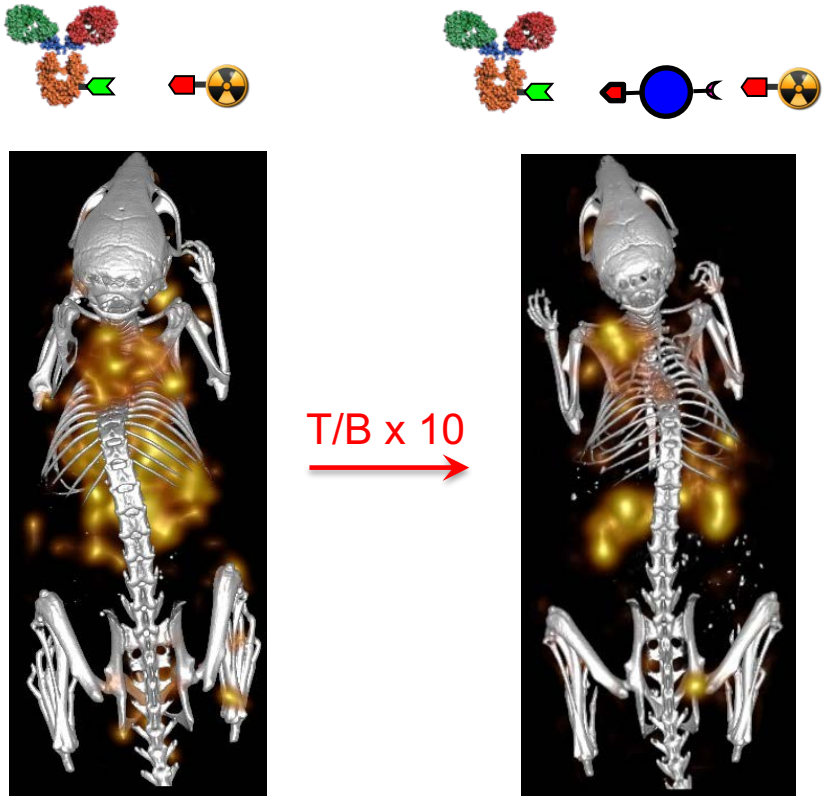
Triggered clearance of other mAbs

Amyloid-beta mAb for Alzheimer imaging



Collaboration with Syvänen, Uppsala Univ

Prostate cancer stem cells (PC3MA3) with anti-CD44v6-TCO & ^{68}Ga-NOTA-Tz



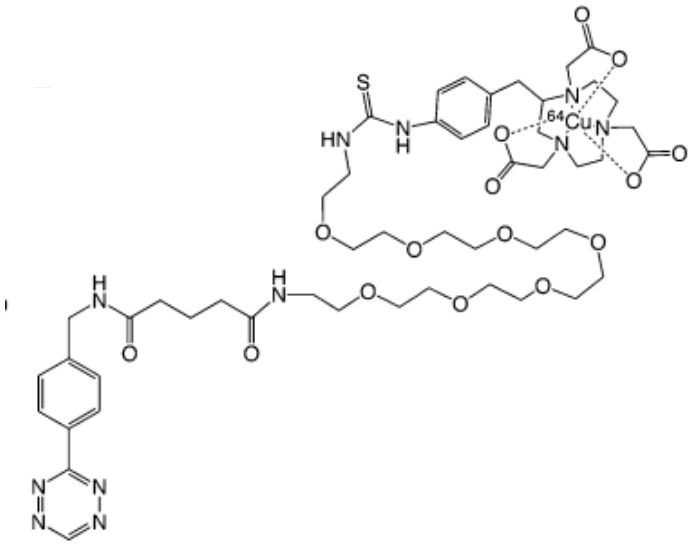
Clear 48h, probe 50h, image 53 h

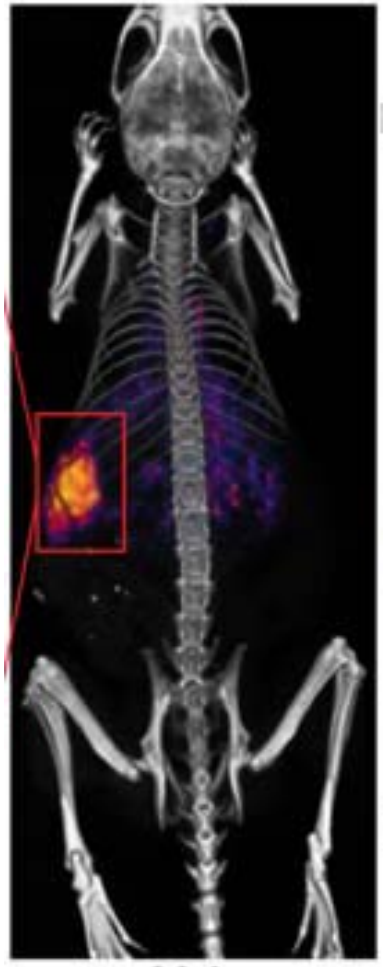
Collaboration with Quinn, Missouri Univ

Pretargeted mAb imaging without clearing agent

Orthotopic CA19.9+ Capan-2 pancreas xenograft; 200 µg 5B1-TCO + 1.1 eq ^{64}Cu-NOTA-PEG7-Tz 72h later.

- Rapid shedding and moderate internalization of target
- Tetrazine tumor uptake: 8.2 ± 1.7 %ID/g; T/B = 4
- Dosimetry: >25-fold reduction in total body radiation exposure relative to ^{89}Zr-DFO-5B1; >70-fold when using ^{68}Ga instead of ^{64}Cu

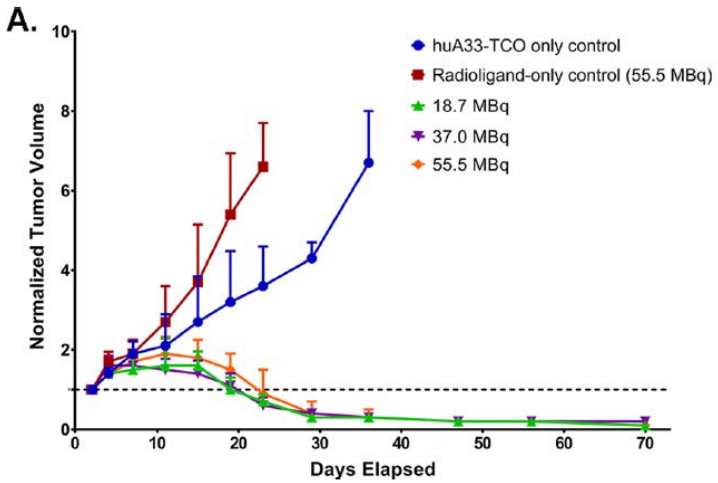


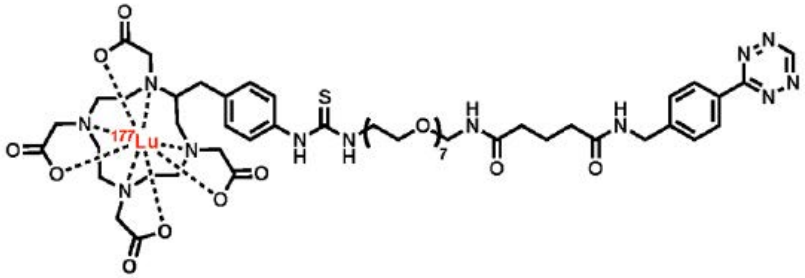


Houghton *et al.*, *J. Nucl. Med.* **2016**, 57, 453-459

Pretargeted β⁻ radioimmunotherapy

S.c. A33+ SW1222 xenograft; 100 µg huA33-TCO followed by 1.4 eq [^{177}Lu]Lu-DOTA-PEG7-Tz 24 h later.





Complete remission already at the lowest dose (18.5 MBq)

Controls:
Saline and [^{177}Lu]Lu-DOTA-Tz alone (55.5 MBq)

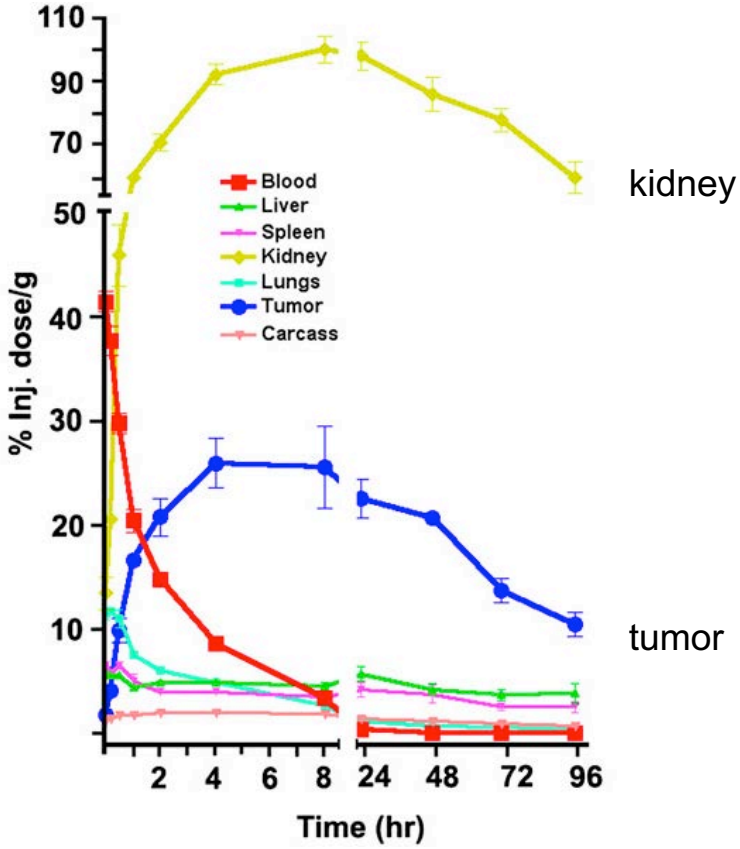
Directly labeled mAb is missing

Dosimetry: 2-to-3 lower dose to bone marrow and osteogenic cells

Membreno *et al.*, *Mol. Pharm.* **2018**, 15, 1729-1734

Tumor PT with a CC49 diabody (50 kDa)

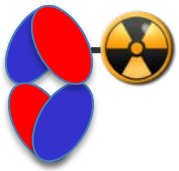
- Radiolabeled diabody-DOTA gives very poor tumor-to-kidney ratios
- Pretargeting may address this, enabling radioimaging and –therapy

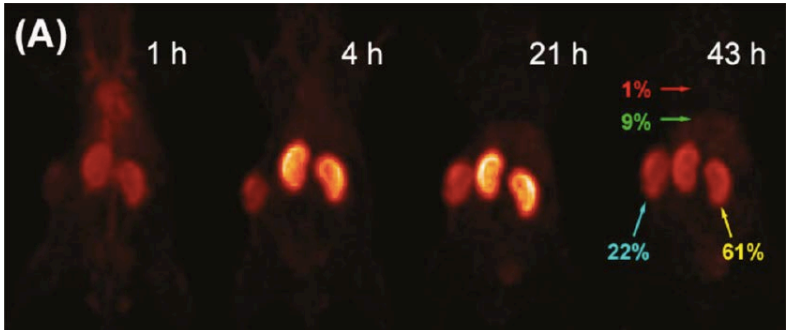


Courtesy Avipep

Pretargeting improves tumor-kidney ratios for radiolabeled proteins

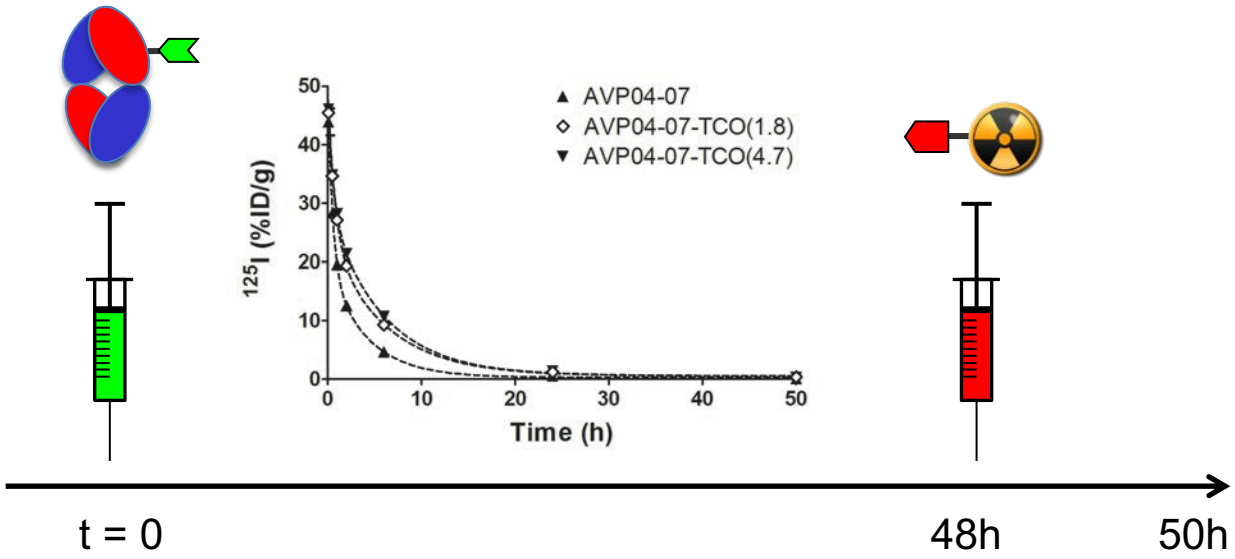
^{64}Cu-DOTA-CC49 diabody (55 kDa)



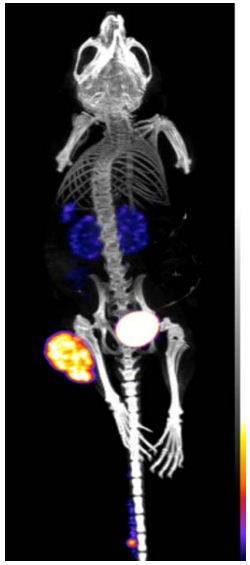


T/K: 0.3

TCO-CC49 diabody + ^{111}In-tetrazine



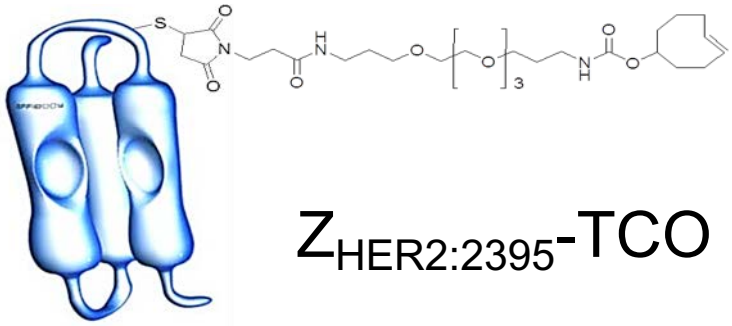
LS174T-tumored mice



T/K: 6

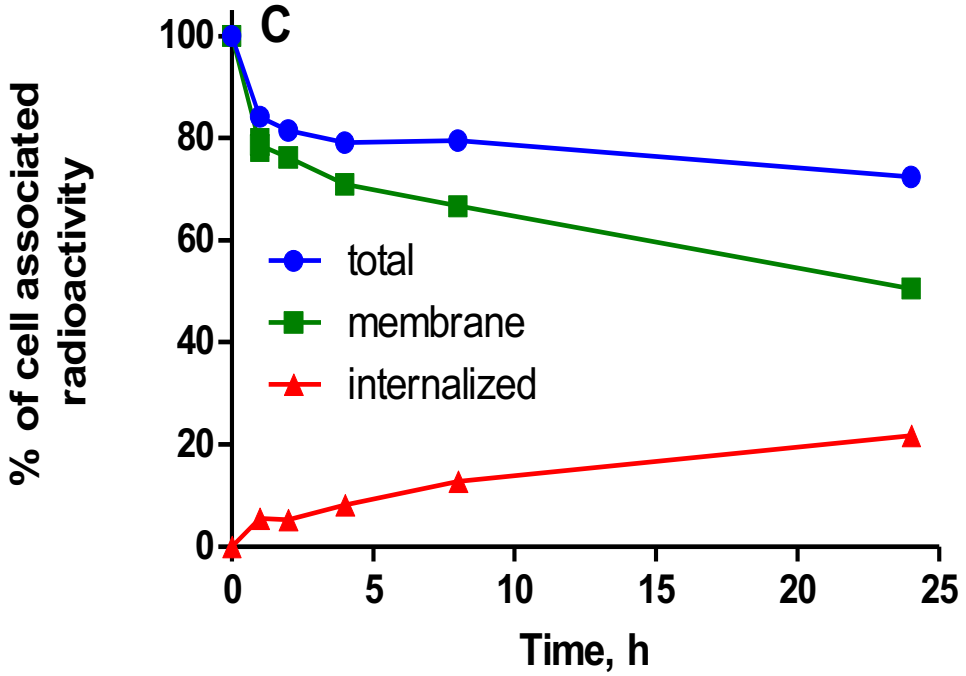
Tumor PT with anti-HER2 Affibody (7kDa)

- Radiolabeled Affibody-DOTA gives very poor tumor-to-kidney ratios
- Pretargeting may address this, enabling radioimaging and –therapy
- Affibodies internalize slower than full mAbs – pretargeting with dynamic targets ?



$Z_{HER2:2395}$-TCO

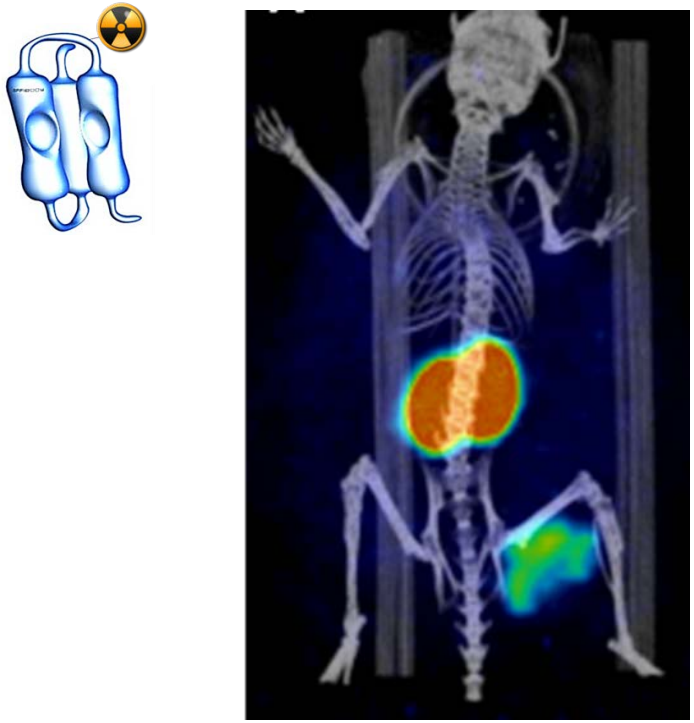
Cellular processing of Z_{2395}-TCO in SKOV-3 cells



Altai *et al., J. Nucl. Med.* **2016**, *57*, 431-6

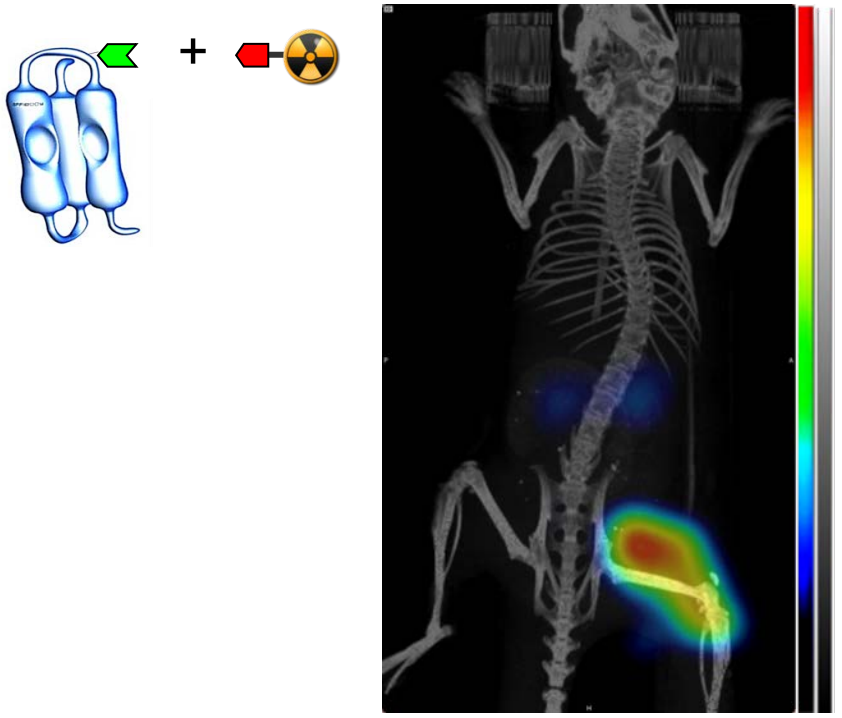
Pretargeting improves tumor-kidney ratios for radiolabeled peptide

anti-HER2 Affibody-DOTA-^{111}In (7kD)



Tumor/kidney: 0.06

Affibody-TCO + ^{111}In-Tz



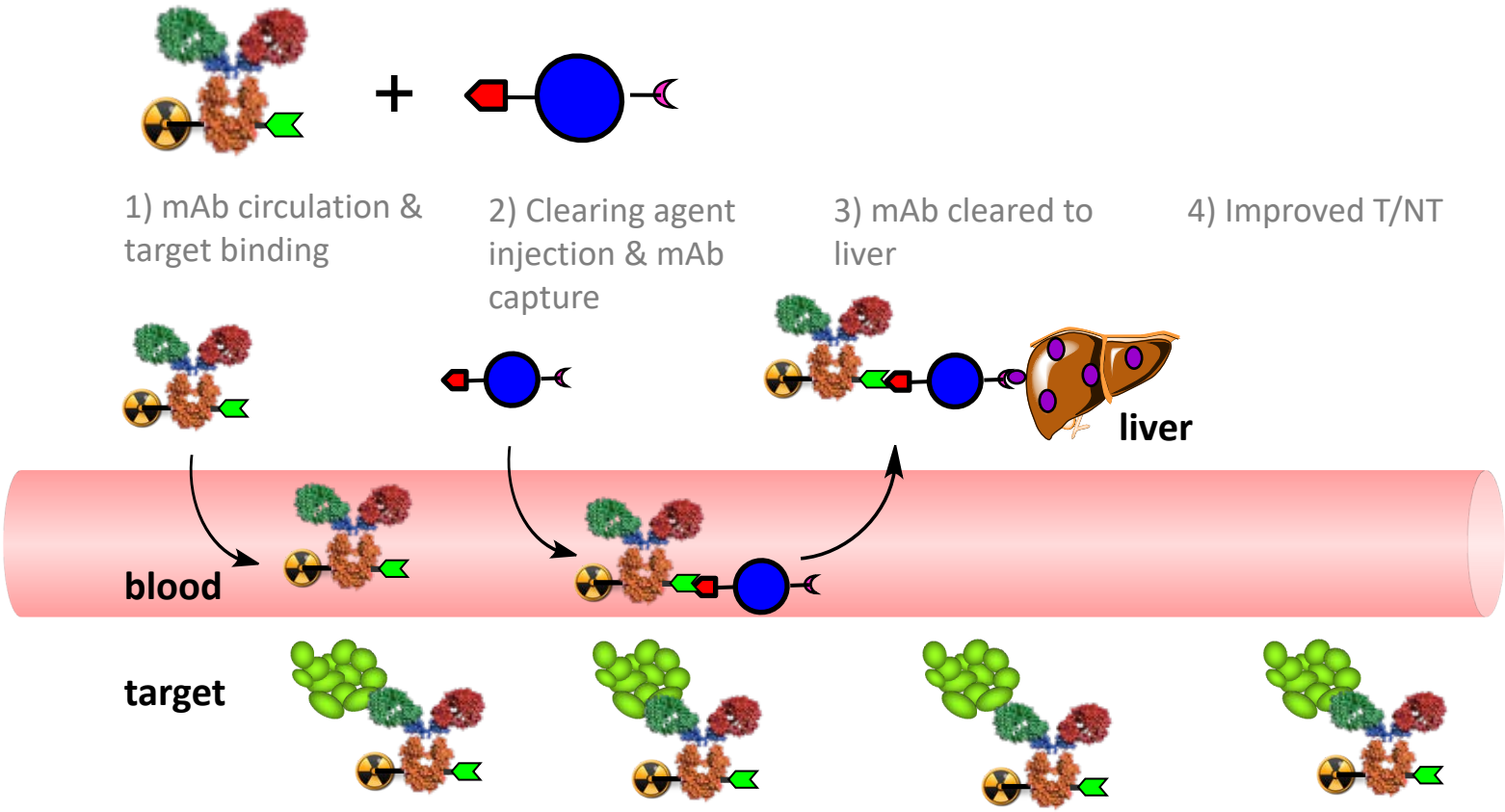
Tumor/kidney: 2

SKOV3 tumored mice: 30 µg Z_{2395}–TCO, 1 eq ^{111}In-DOTA-tetrazine (t = 4h); imaging @ 5 h

Altai *et al., J. Nucl. Med.* **2016**, *57*, 431-6

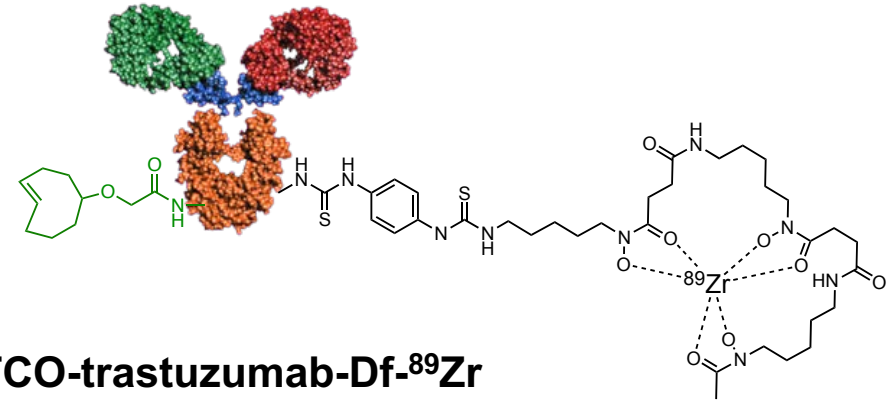
Clearing radiolabeled mAbs

- Removal of tagged & labeled (e.g. ^{89}Zr, ^{124}I) mAbs to boosts target-blood ratios, also for internalizing mAbs
- Well suited to improve brain-blood ratios of BBB-crossing mAbs, e.g. in (pre)clinical imaging & development of Alzheimer mAbs

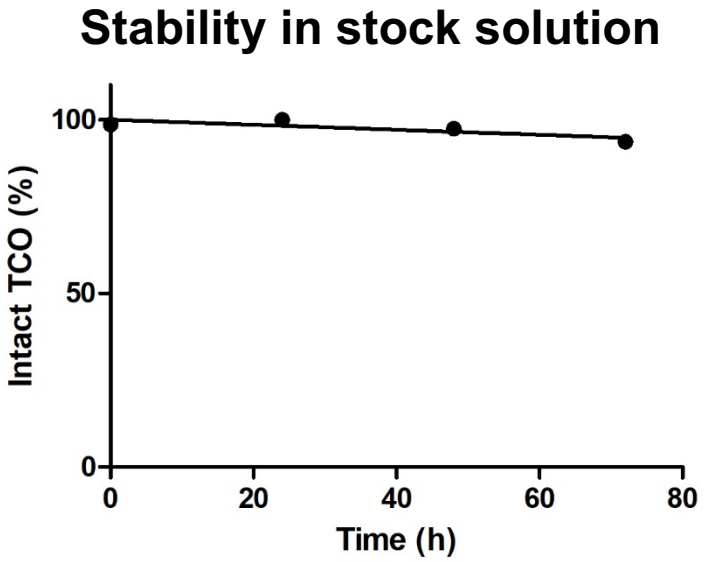


Clearing radiolabeled mAbs

- Removal of tagged & labeled (e.g. ^{89}Zr, ^{124}I) mAbs to boost target-blood ratios
- Partnerhip with biopharma on BBB-crossing mAbs



TCO-trastuzumab-Df-^{89}Zr



TCO is stable in ^{89}Zr-labeling conditions.

^{89}Zr-labeled TCO-mAb-Df solutions can be stored at +4°C for days (or can be shipped) without losses of TCO reactivity.

tagworks PHARMACEUTICALS

Summary - Click

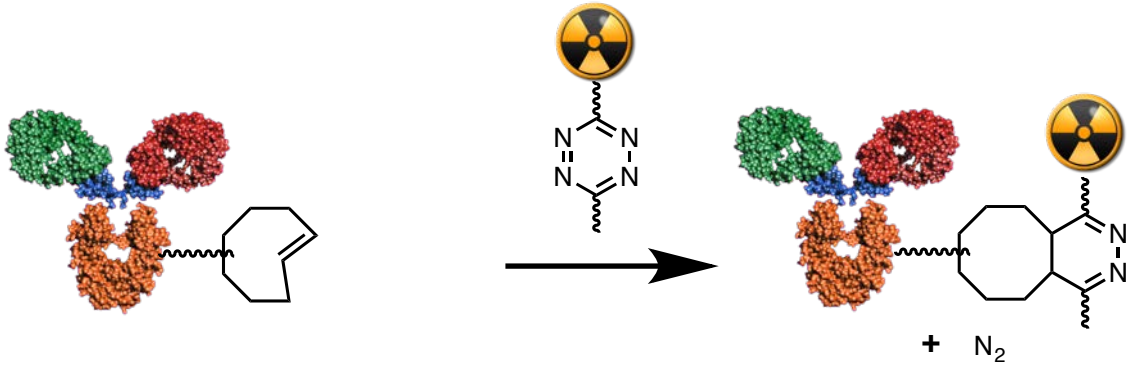
- Selective binding of radioactive probe to chemical tag of antibody via bioorthogonal reaction
- Very fast coupling system (up to k_2 ~ 3 x 10^5 $M^{-1}s^{-1}$) approaching streptavidin-biotin. High in vivo stability of tag ($t_{1/2}$ 10 days) and reaction product (>> 1 week)
- Boosts target-to-blood ratios: improved imaging for e.g. companion diagnostics (^{18}F-tetrazines), and increased tumor dose in radioimmunotherapy
- Low likelihood of immunogenicity compared to biological pretargeting components: repetition
- Universal & straightforward tag conjugation with minimal perturbation: antibodies, fragments, peptides, particles, ..

tagworks PHARMACEUTICALS

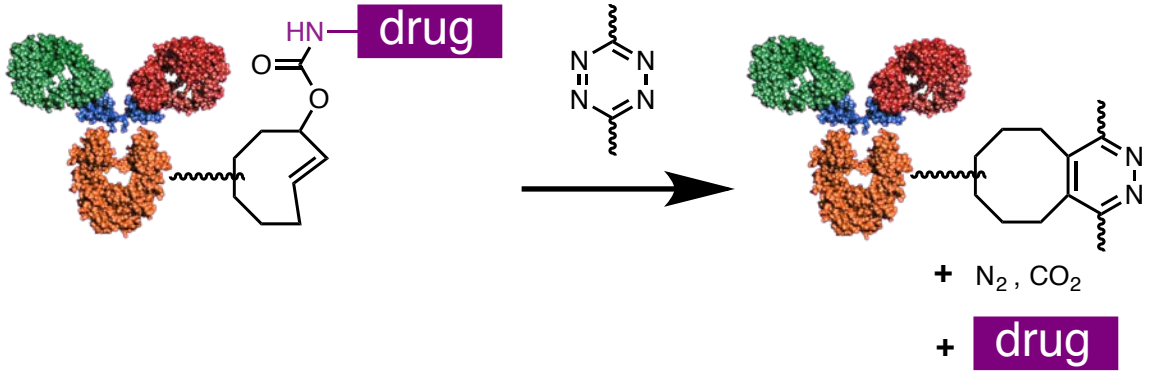
“Click-to-release” ADC approach to cancer therapy

Moving from Click to Click-to-Release

Click tags for Pretargeted Radioimmunoimaging & Radioimmunotherapy (RIT)



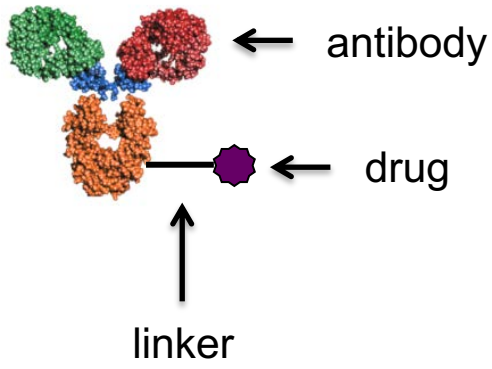
Click-cleavable linkers for non-internalizing Antibody-Drug Conjugates (ADC)

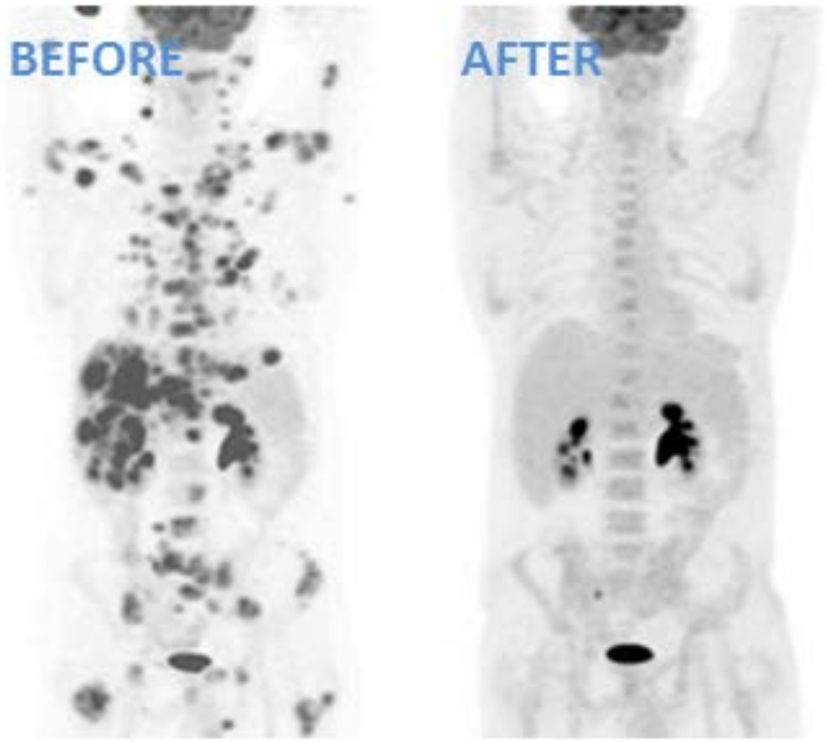


Versteegen *et al., Angew. Chem. Int. Ed.* **2013**, *52,* 14112–14116
Rossin *et al.*, *Bioconjugate Chem.* **2016**; *27*, 1697–1706

Antibody-Drug Conjugates (ADCs)

Highly potent biopharmaceuticals that use the targeting ability of antibodies to selectively bind to tumor cells where the conjugated cancer drug is released

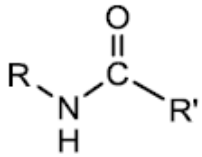




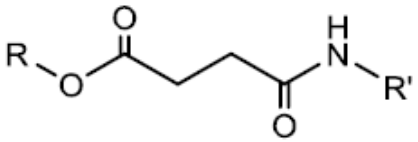
FDG PET scan before and 2 weeks after ADC treatment of patient with 70 Non-Hodgkin's Lymphoma tumors

Current ADC linkers

Non cleavable linkers

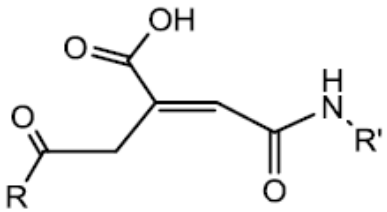


amide

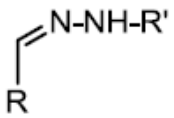


succinate spacer- amide and ester linkages

Acid labile linkers

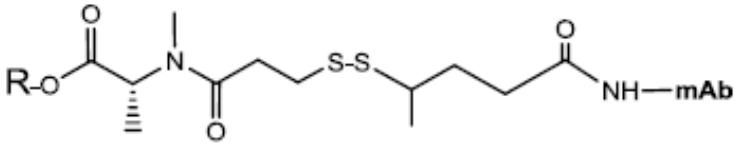


cis-aconitic

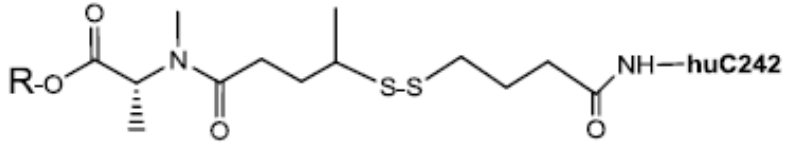


hydrazide

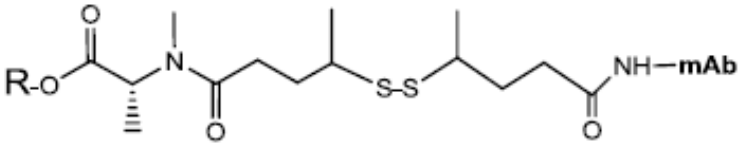
Thiol labile linkers



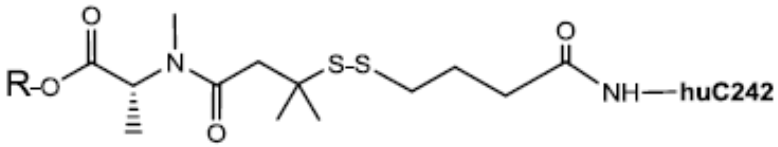
huC242-DM1 and TA.1-DM1



huC242-DM3



Tmab-DM3



huC242-DM4

Peptidase-labile linkers

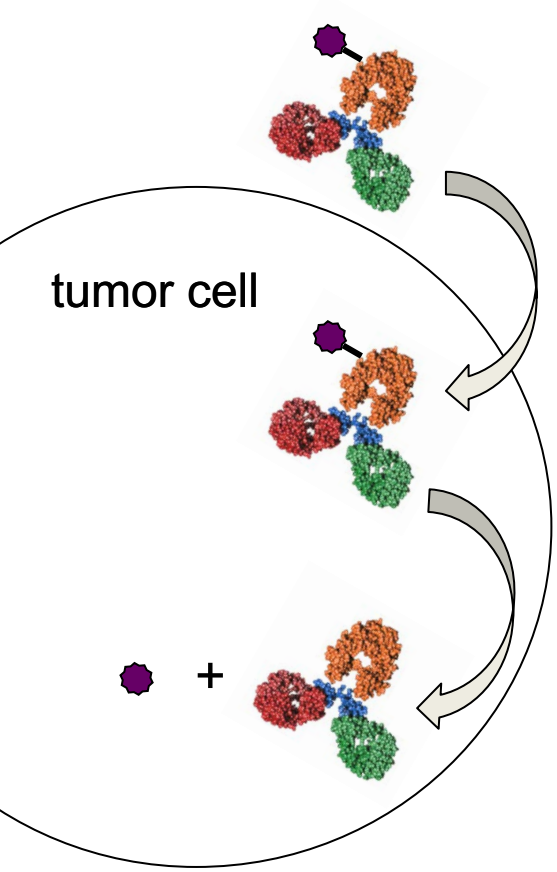
R-Ala-Leu-Ala-Ala-R'

R-Val-Cit-R'

Dosio *et al.*, *Toxins* **2011**, 3, 848-883

Antibody-Drug Conjugates (ADCs)

Current systems are based on **intracellular toxin release** by enzymes, thiols or pH

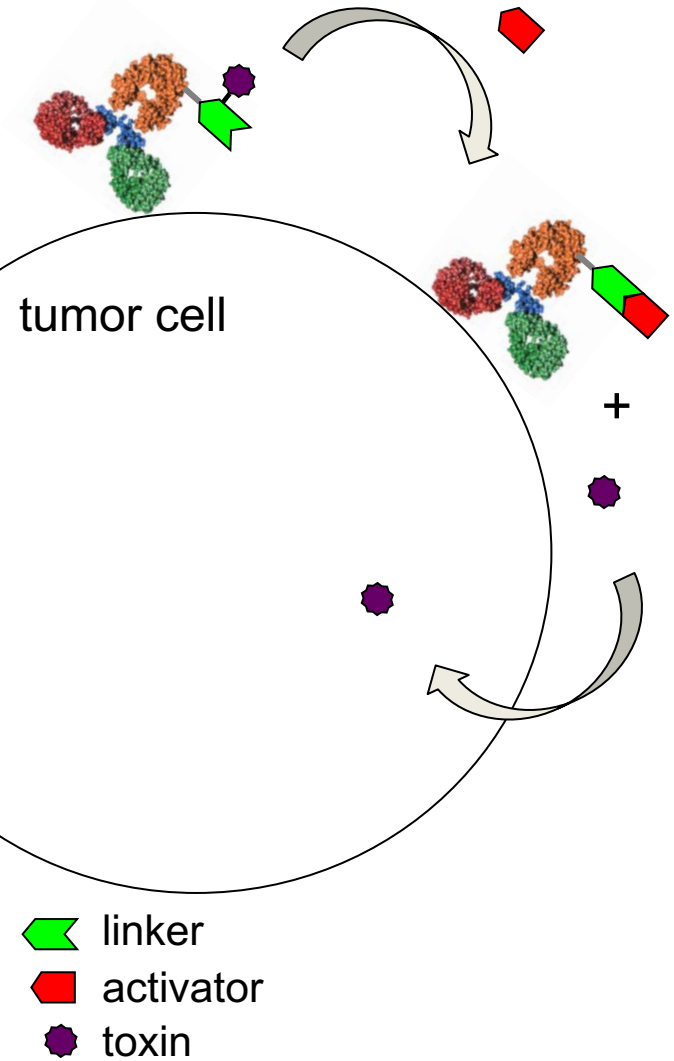


Issues

- Limited to efficiently internalizing receptors
- Shortage of suitable ADC targets in solid tumors
- Less effective in heterogeneous or poorly penetrated tumors, i.e. solid tumors

toxin

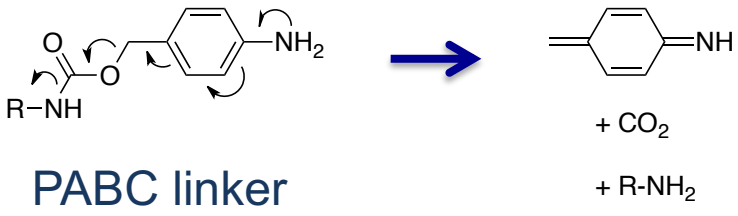
Click-to-Release ADCs



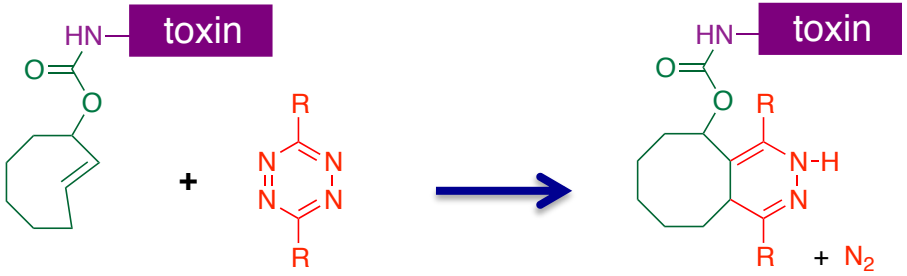
- Stable ADC linker, cleaved by a chemical probe in vivo
- 2 steps: after ADC has cleared from blood, probe is administered, triggering toxin release @ tumor
- Modification of in vivo validated pretargeting tech
- Expands the range of ADC targets: non-internalizing receptors, extracellular matrix constituents, stroma, etc
- Advantageous in heterogeneous or poorly penetrated tumors
- Universal & temporally controlled release independent from tumor biology
- Well suited for mAb fragments, or full mAbs in combination with a clearing agent

Moving from Click to Unclick

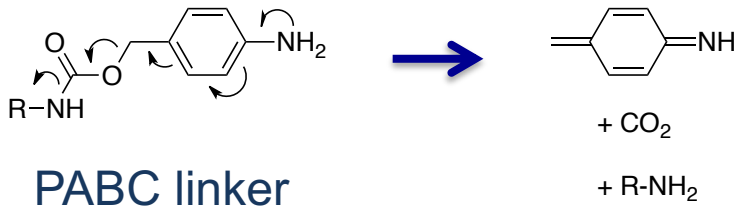
Moving from Click to Unclick



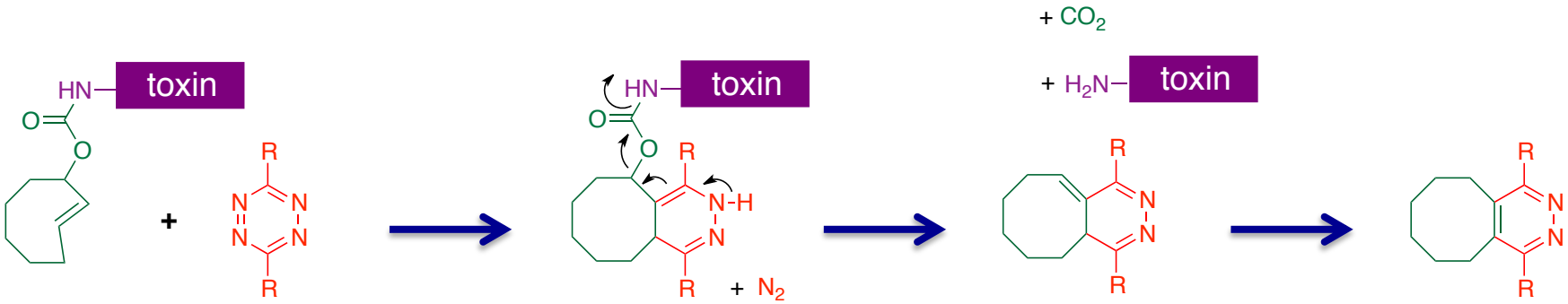
PABC linker



Moving from Click to Unclick

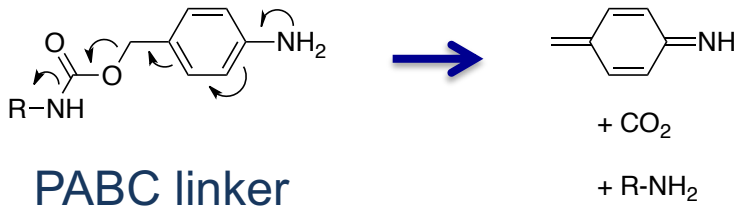


PABC linker

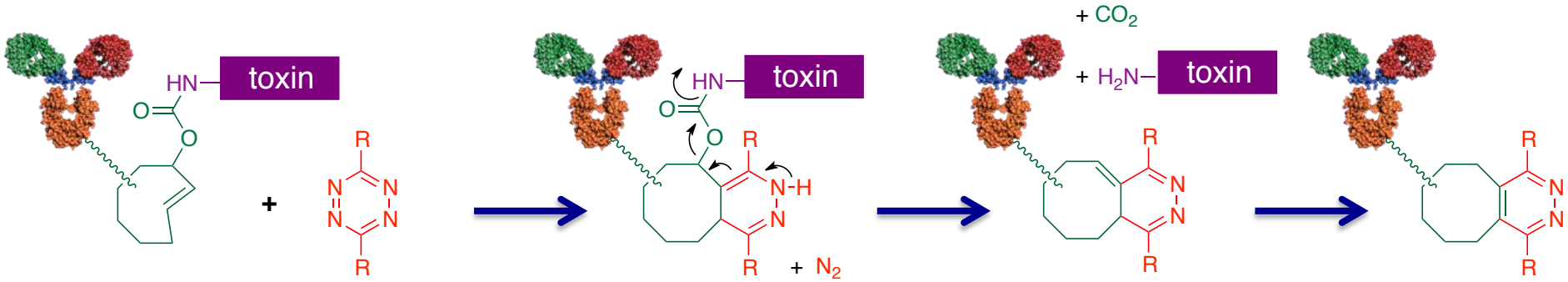


Versteegen *et al., Angew. Chem. Int. Ed.* **2013**, *52,* 14112–14116

Moving from Click to Unclick

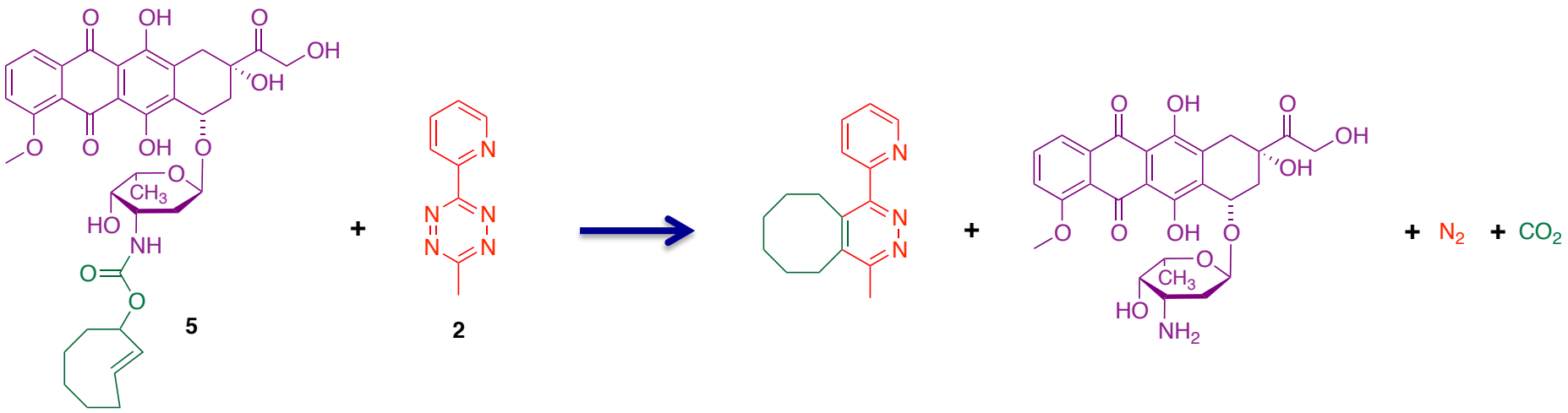


PABC linker



Versteegen et al., *Angew. Chem. Int. Ed.* **2013**, *52,* 14112–14116
Rossin et al., *Bioconjug. Chem.* **2016**, *27*, 1697–1706

Proof of concept: Dox release in vitro



- Doxorubicin can be deactivated by conjugation to *trans*-cyclooctene tag and…
- .. activated again by equimolar reaction with tetrazine probe in cell culture

IC50 values determined in A431 cell line, from concentration range incubated 72 h @ 37°C

Compound	IC50 (µM)
Doxorubicin	0.020 ± 0.002
Doxorubicin-tag	3.017 ± 0.486
Doxorubicin-tag + 1.5 eq. probe	0.137 ± 0.012
Probe	> 100

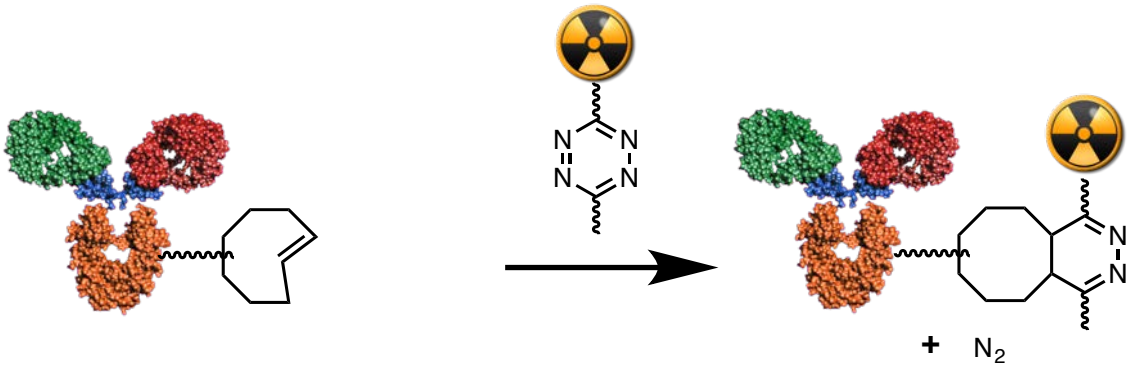
Versteegen *et al., Angew. Chem. Int. Ed.* **2013**, *52,* 14112–14116

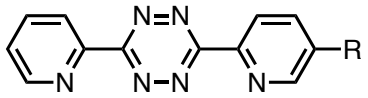
tagworks PHARMACEUTICALS

Designing a suitable activator for in vivo application

Click-to-Release - lower reactivities

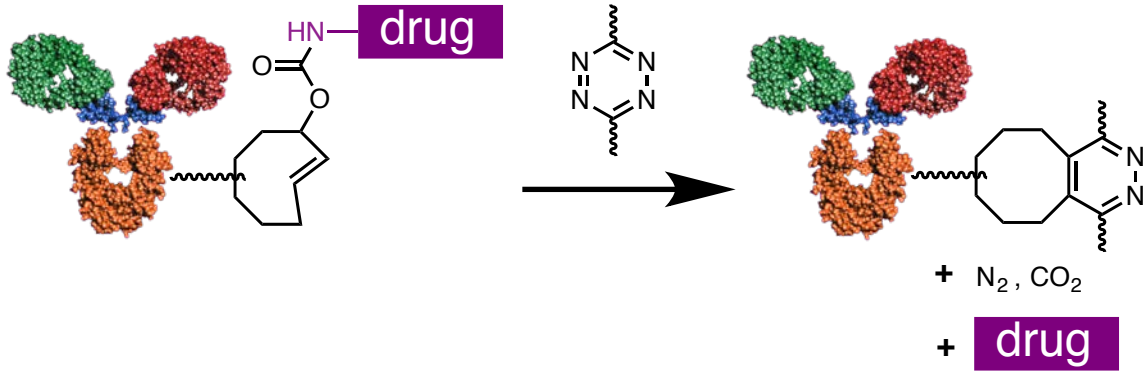
Click tags for Pretargeted Radioimmunoimaging & Radioimmunotherapy (RIT)

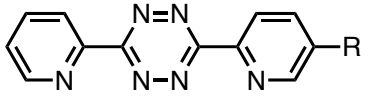




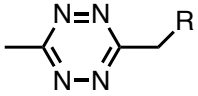
- $k_2 > 1.3 \times 10^5\ M^{-1}s^{-1}$

Click-cleavable linkers for non-internalizing Antibody-Drug Conjugates (ADC)





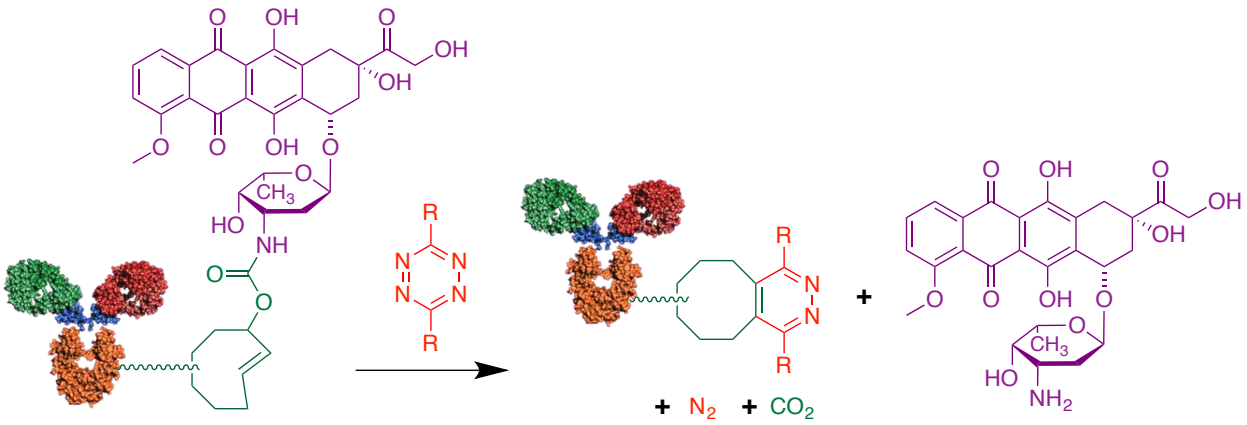
- $k_2 \sim 4 \times 10^3\ M^{-1}s^{-1}$
- release: 10 %

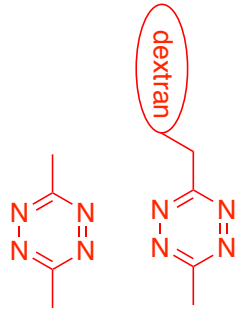


- $k_2 \sim 55\ M^{-1}s^{-1}$
- release: 85 %

(we can use a large excess of activator!)

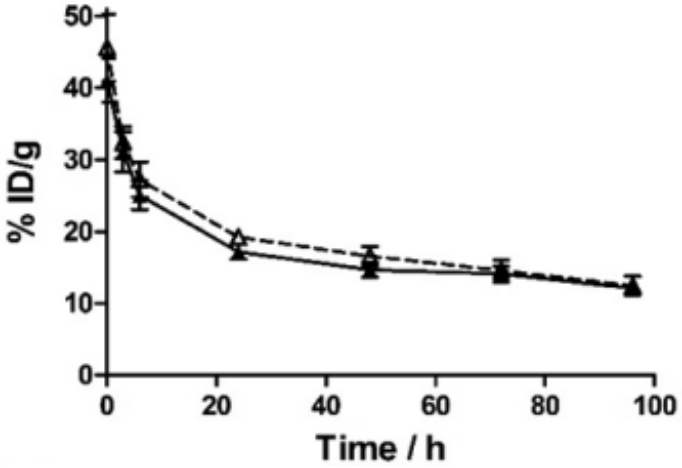
Proof of principle ADC: CC49-TCO-doxorubicin





In vitro release (%)	85	50
Clearance $t_{1/2}$ (min)	1	6
In vivo reaction (%)	0	100

ADC blood clearance

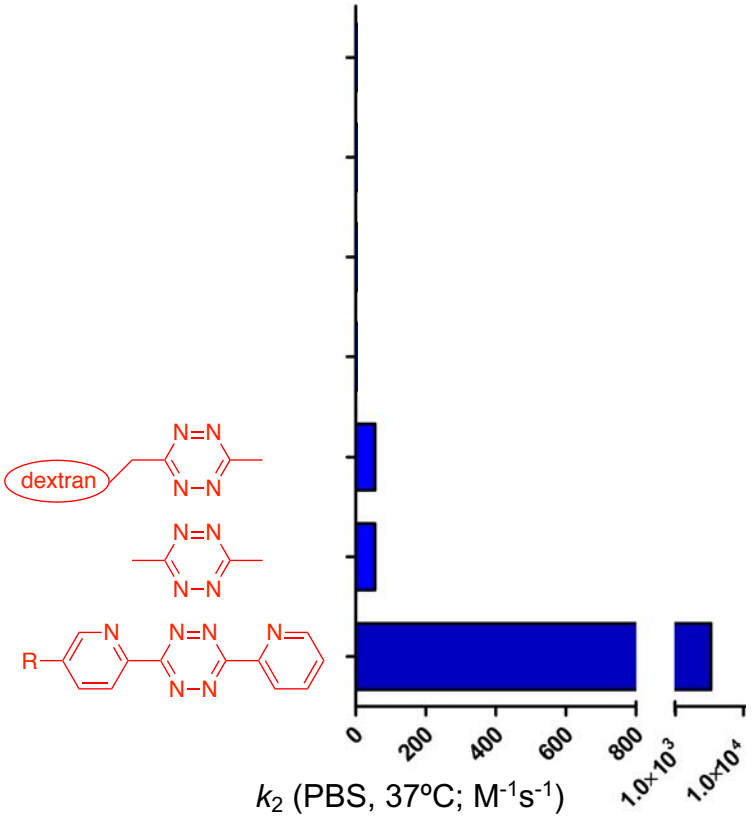


CC49 (dashed): $t_{1/2}$ 30.4 h
CC49-TCO-Dox (solid): $t_{1/2}$ 26.9 h

- High tumor uptake of CC49-TCO-dox, 100 % reaction with activator, good recovery of released dox, but..
- Slow mAb clearance requires use of clearing agent
- high dose of activator needed (30 mgs)
- Incomplete release

Rossin *et al.*, *Bioconjugate Chem.* **2016**; *27*, 1697–1706

Click-to-Release: reactivity *vs.* release



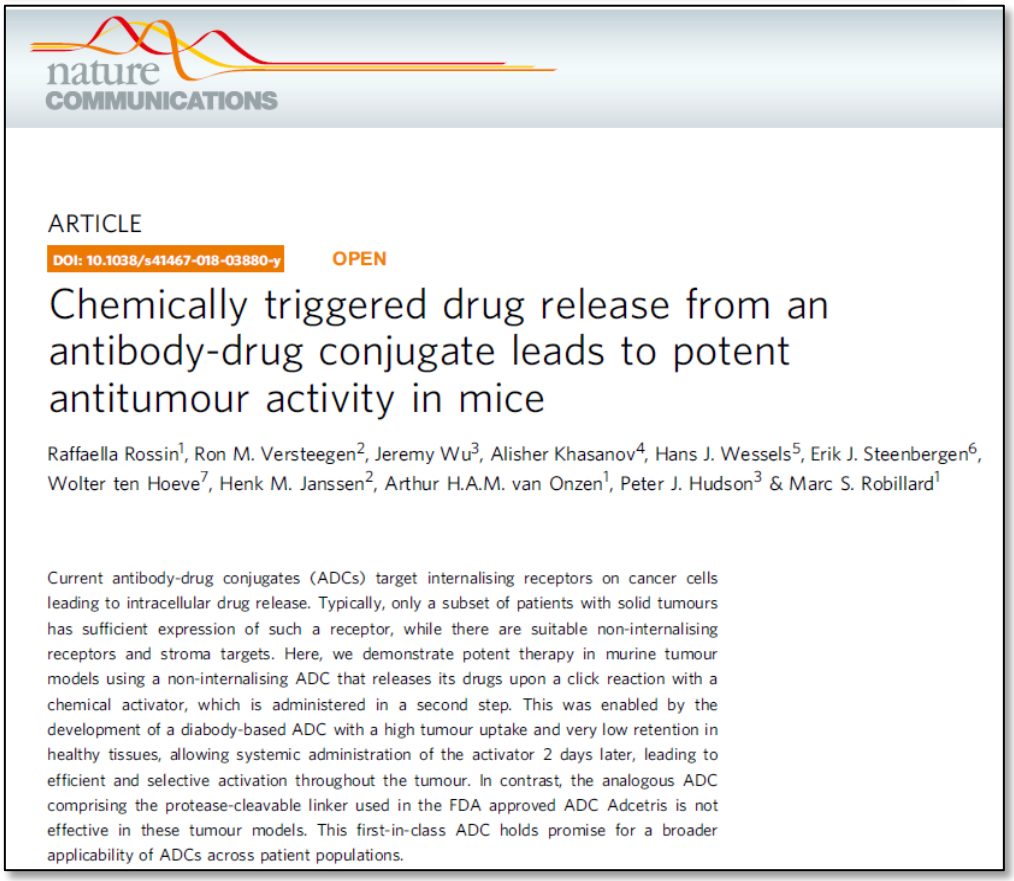
release: 50 % / dose: 10,000 eq, 268 mg/kg

release: 90 % / dose: >100,000 eq

release: 10 % / dose: 50 eq

tagworks PHARMACEUTICALS

In vivo proof-of-concept for Click-to-Release recently published in Nature Communications (2018)



nature COMMUNICATIONS

ARTICLE

DOI: 10.1038/s41467-018-03880-y OPEN

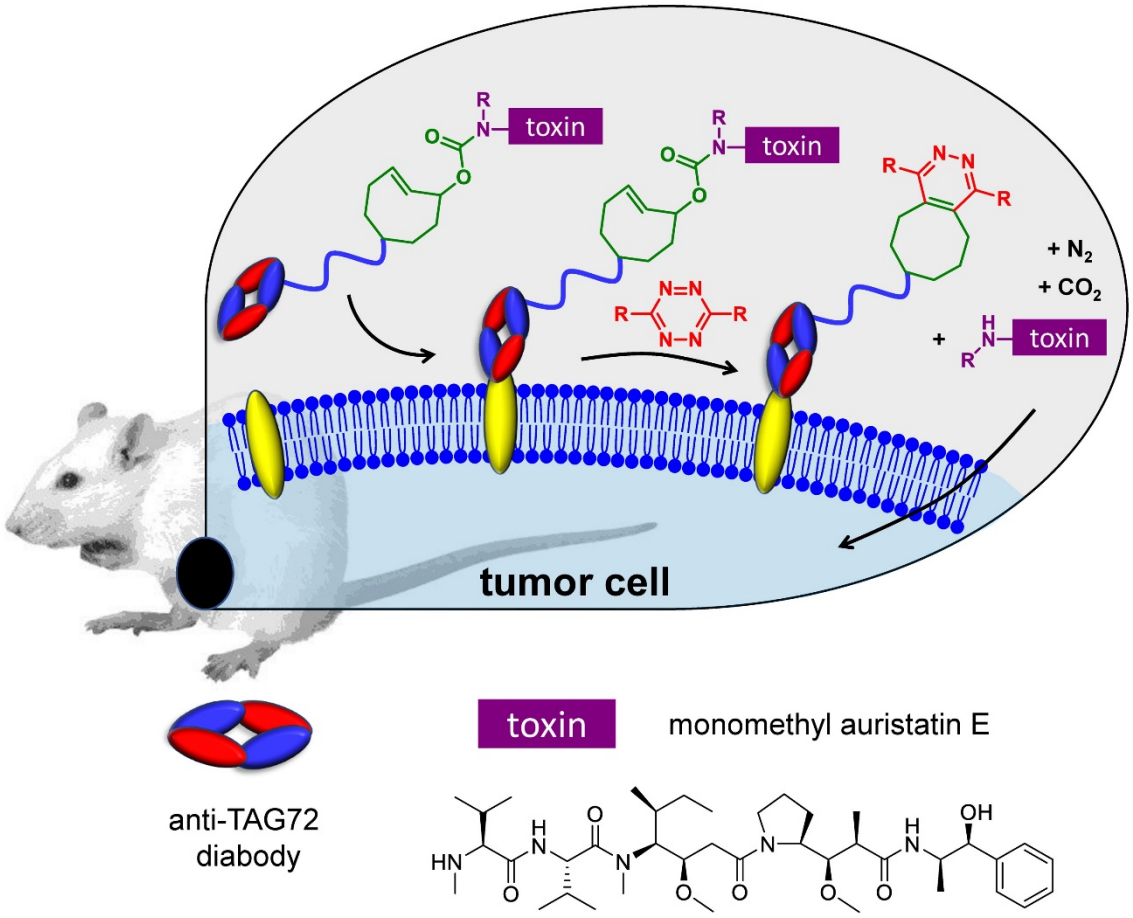
Chemically triggered drug release from an antibody-drug conjugate leads to potent antitumour activity in mice

Raffaella Rossin[1], Ron M. Versteegen[2], Jeremy Wu[3], Alisher Khasanov[4], Hans J. Wessels[5], Erik J. Steenbergen[6], Wolter ten Hoeve[7], Henk M. Janssen[2], Arthur H.A.M. van Onzen[1], Peter J. Hudson[3] & Marc S. Robillard[1]

Current antibody-drug conjugates (ADCs) target internalising receptors on cancer cells leading to intracellular drug release. Typically, only a subset of patients with solid tumours has sufficient expression of such a receptor, while there are suitable non-internalising receptors and stroma targets. Here, we demonstrate potent therapy in murine tumour models using a non-internalising ADC that releases its drugs upon a click reaction with a chemical activator, which is administered in a second step. This was enabled by the development of a diabody-based ADC with a high tumour uptake and very low retention in healthy tissues, allowing systemic administration of the activator 2 days later, leading to efficient and selective activation throughout the tumour. In contrast, the analogous ADC comprising the protease-cleavable linker used in the FDA approved ADC Adcetris is not effective in these tumour models. This first-in-class ADC holds promise for a broader applicability of ADCs across patient populations.

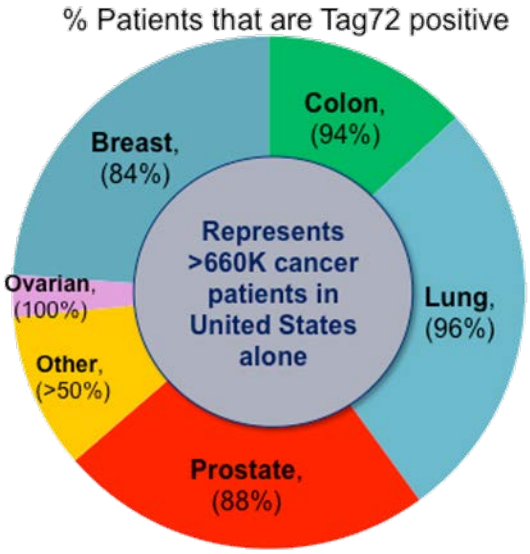
Click-to-Release of MMAE from TAG72-targeted diabody

Objectives: faster clearing ADC, improved activator, more potent drug



TAG72:

extracellular cell membrane target, non-internalizing, low shedding

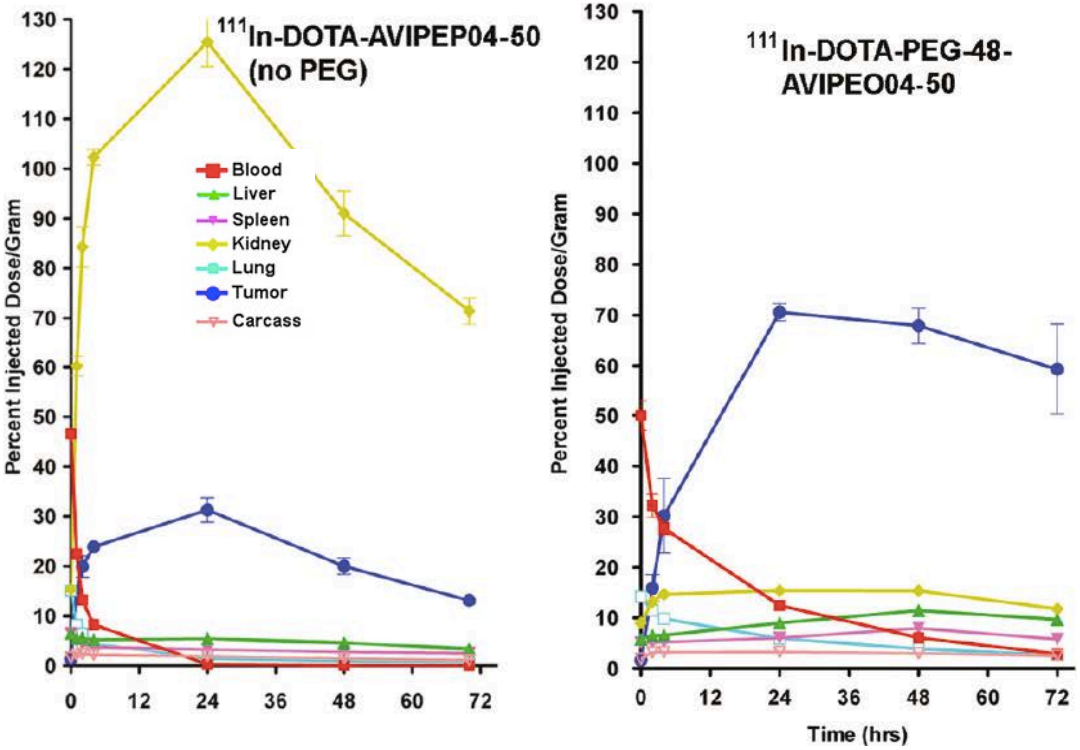


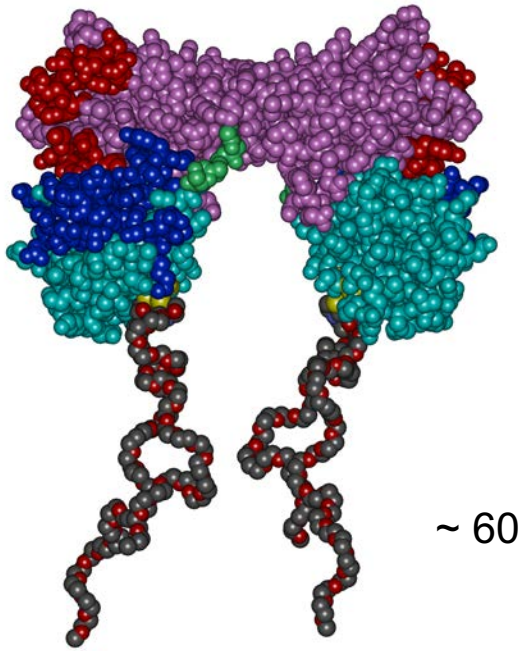
Rossin *et al.*, *Nat. Comm.* **2018**, 9, 1484

Anti-TAG72 diabody

no PEG

4 x PEG_{48}





~ 60 kD

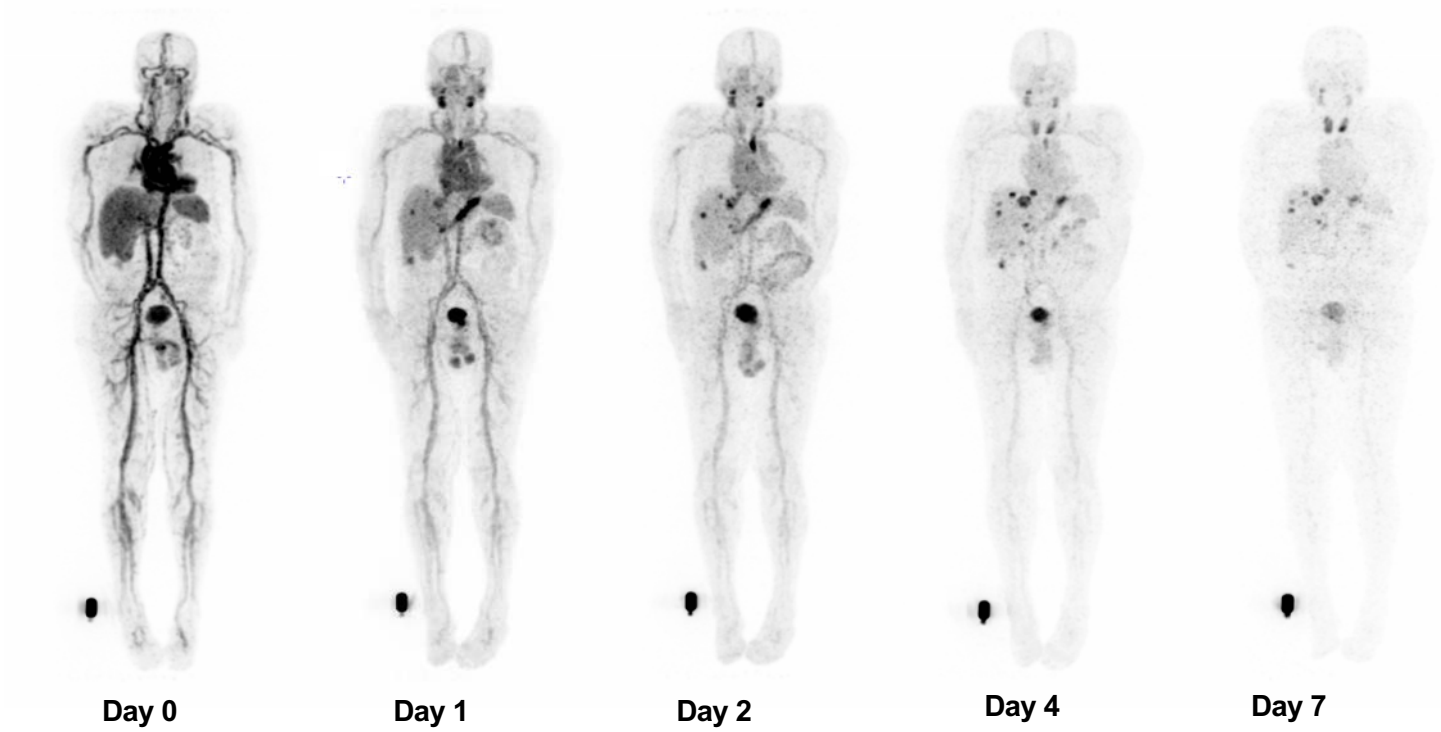
LS174T-tumor-bearing mice injected with ^{111}In-DOTA-AVP04-50 with / without 4 x PEG_{48} conjugated via cysteines

Li *et al.*, *Bioconjug. Chem.* **2011**, *22*, 709–716

Courtesy Avipep

Phase 1 imaging study with ^{124}I-PEG-AVP0458

prostate and colorectal cancer patients



- Tα 5hr from blood and Tβ from whole body 45hr
- High tumor:blood ratios - 22:1 at 7 days p.i.
- Reproducible pharmacokinetics across two dose levels (1 and 10 mg/m^2)

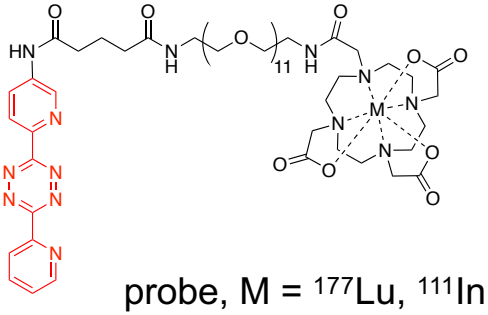
Courtesy of Andrew Scott (Ludwig Cancer Research; Avipep-sponsored study, disclosed at AACR and SNM

Slowing down the tetrazine clearance with a chelator

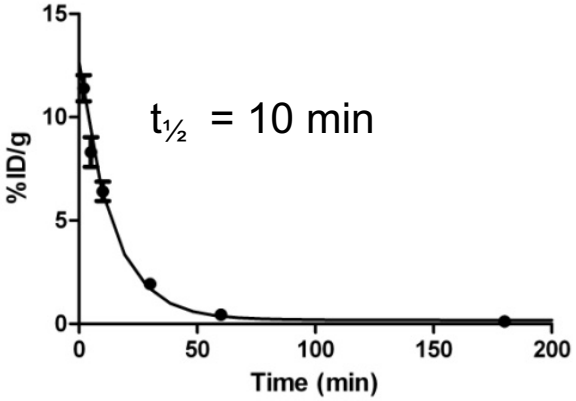
1st generation activators

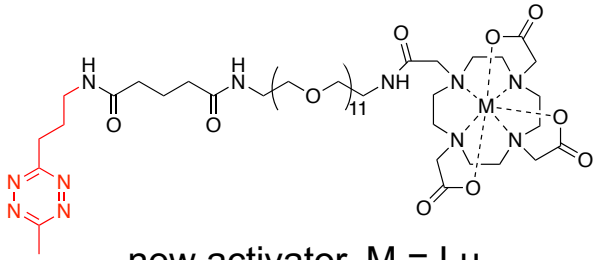
$t_{1/2}$ 1 min

$t_{1/2}$ 6 min

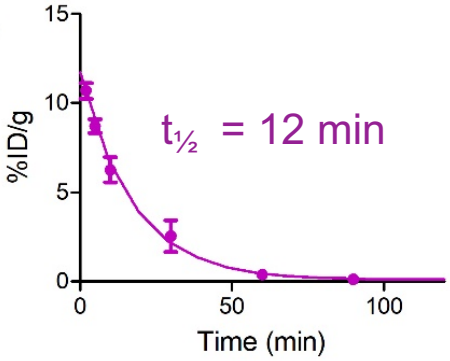


probe, M = ^{177}Lu, ^{111}In

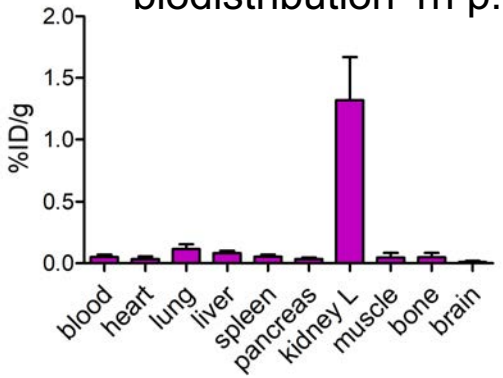




new activator, M = Lu

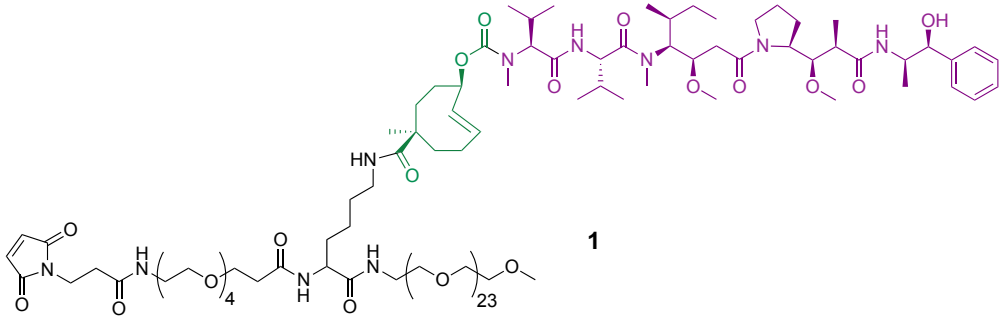


biodistribution 1h p.i.

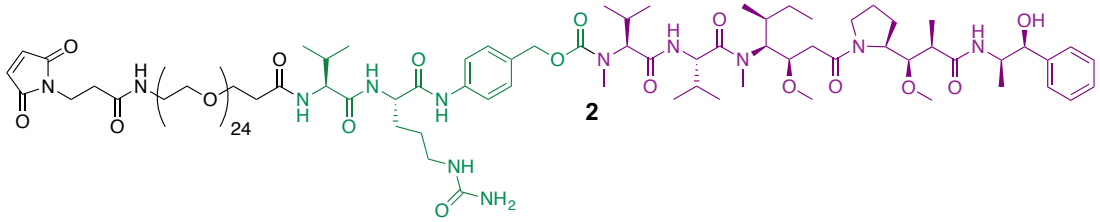


Rossin *et al.*, *Nat. Comm.* **2018**, 9, 1484

Anti-TAG72 diabody ADC and controls



1



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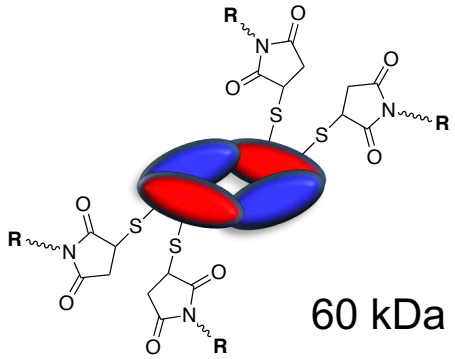
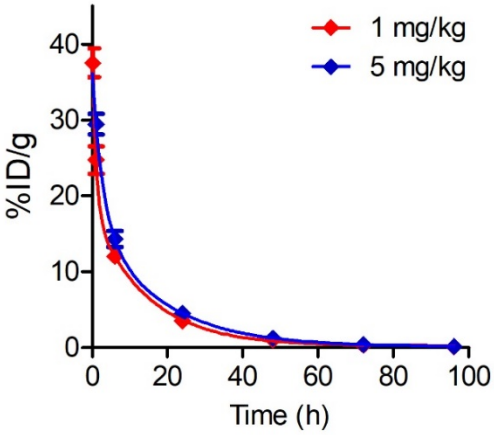
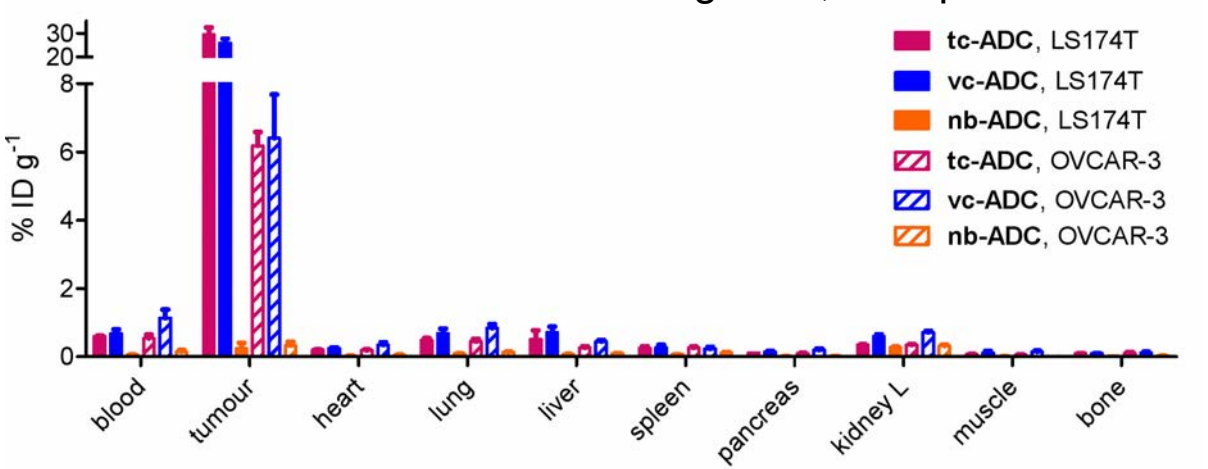


ADC	diabody	R
tc-ADC	anti-TAG72	-TCO-MMAE (**1**)
vc-ADC	anti-TAG72	-val-cit-MMAE (**2**)
nb-ADC	anti-PSMA	-TCO-MMAE (**1**)

Blood clearance



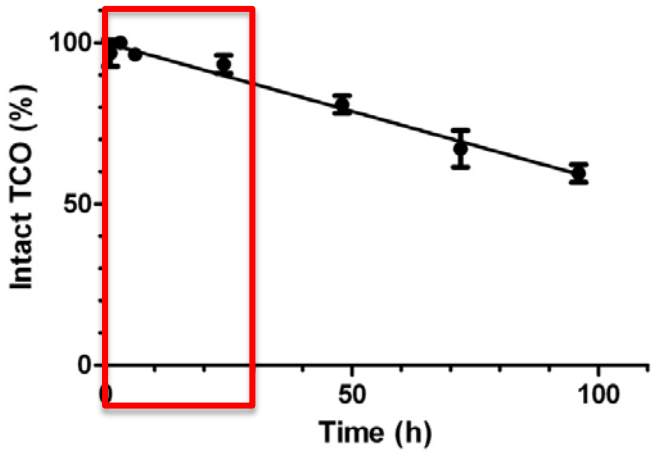
Biodistribution in tumor-bearing mice, 48 h p.i.

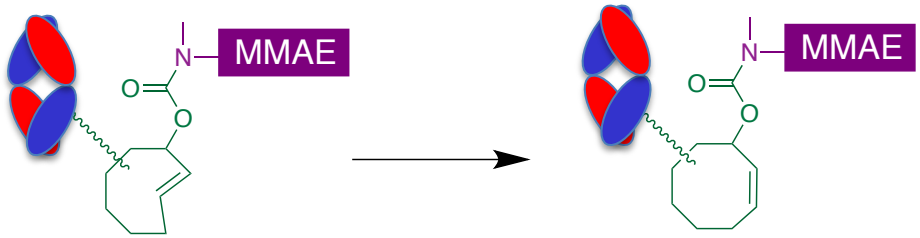


Fast clearance allows 2 day interval between diabody and activator

Slow linker deactivation of TCO ADC in circulation

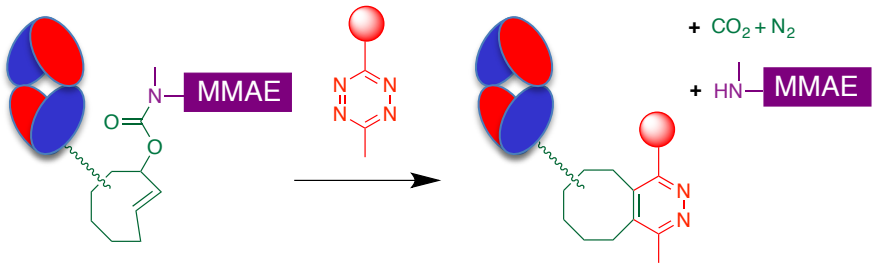
In vivo TCO stability in blood in mice (n=3) assessed ex vivo with ^{177}Lu-DOTA-tetrazine, corrected for ^{125}I-ADC blood clearance



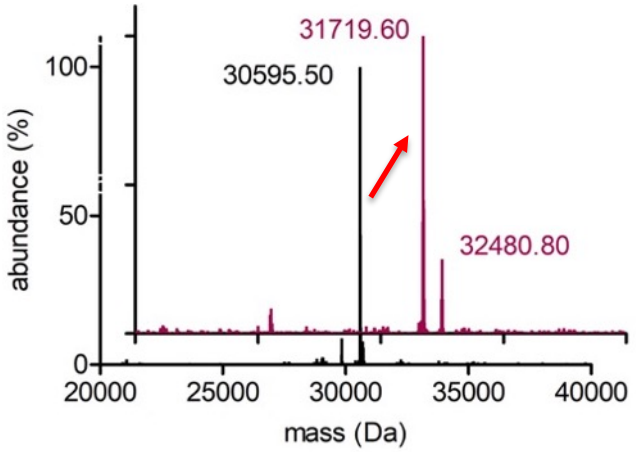


Stability $t_{1/2}$ = 5 days

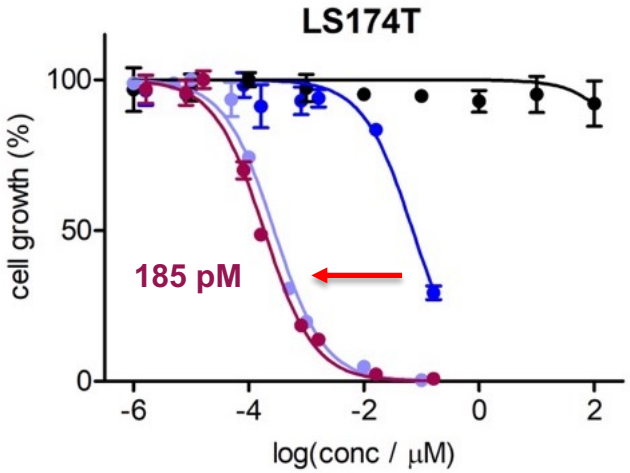
Reaction of ADC with activator leads to instantaneous drug release and 1000-fold increased cytotoxicity

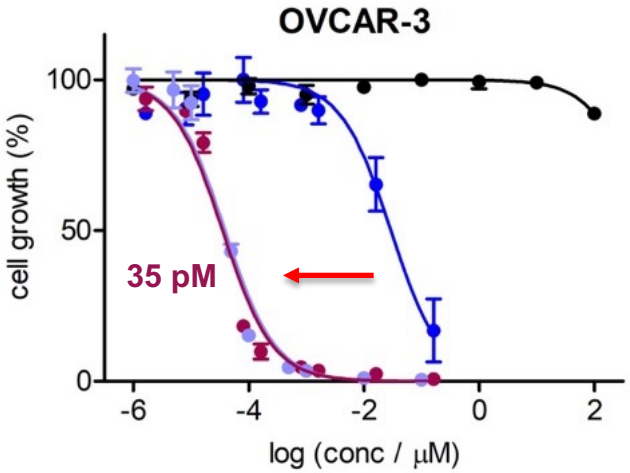


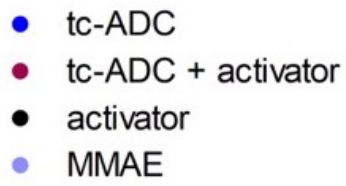
90 % release after 1h in PBS



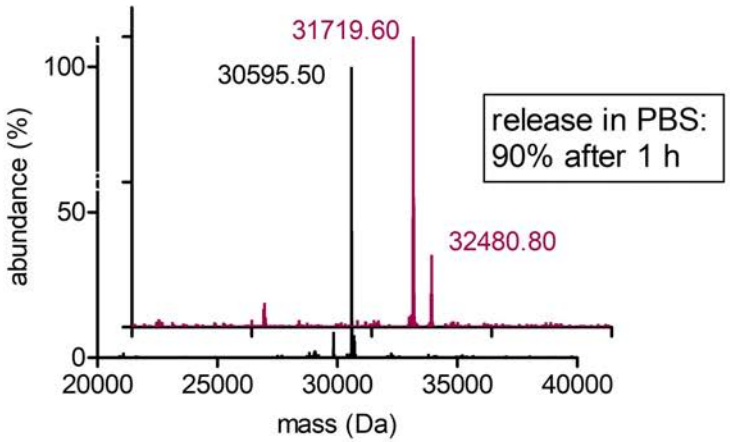
Cytotoxicity in colorectal (LS174T) and ovarian (OVCAR-3) cancer cell culture

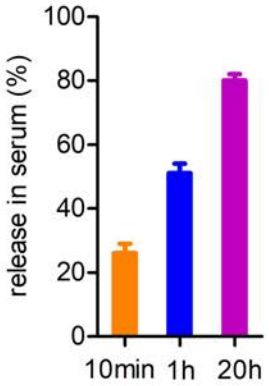


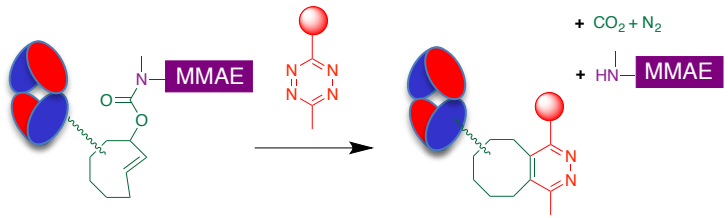


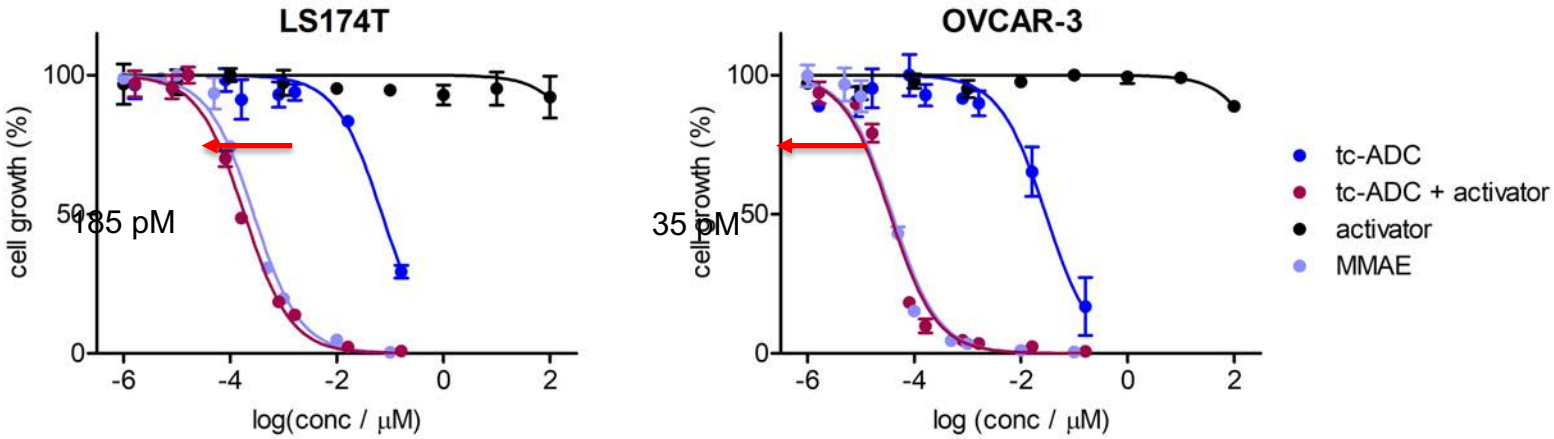


ADC activation: rapid drug release & 1000-fold higher cytotoxicity



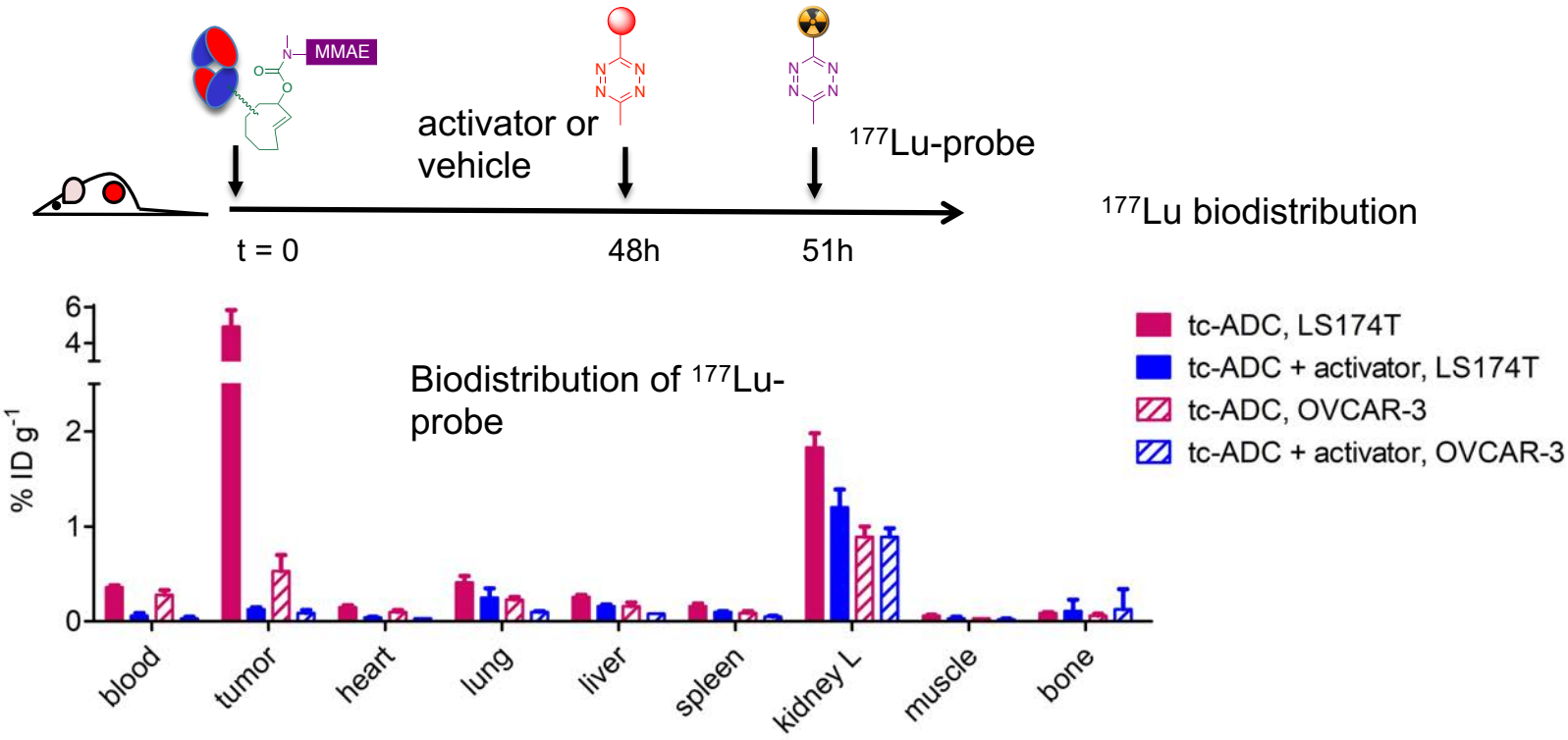






Rossin *et al.*, *Nature Commun.* **2018,** *9*, 1484

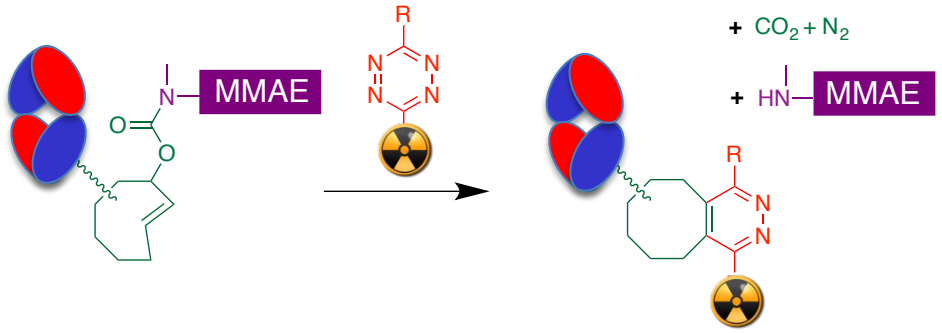
Complete reaction of tumor-bound ADC with excess tetrazine

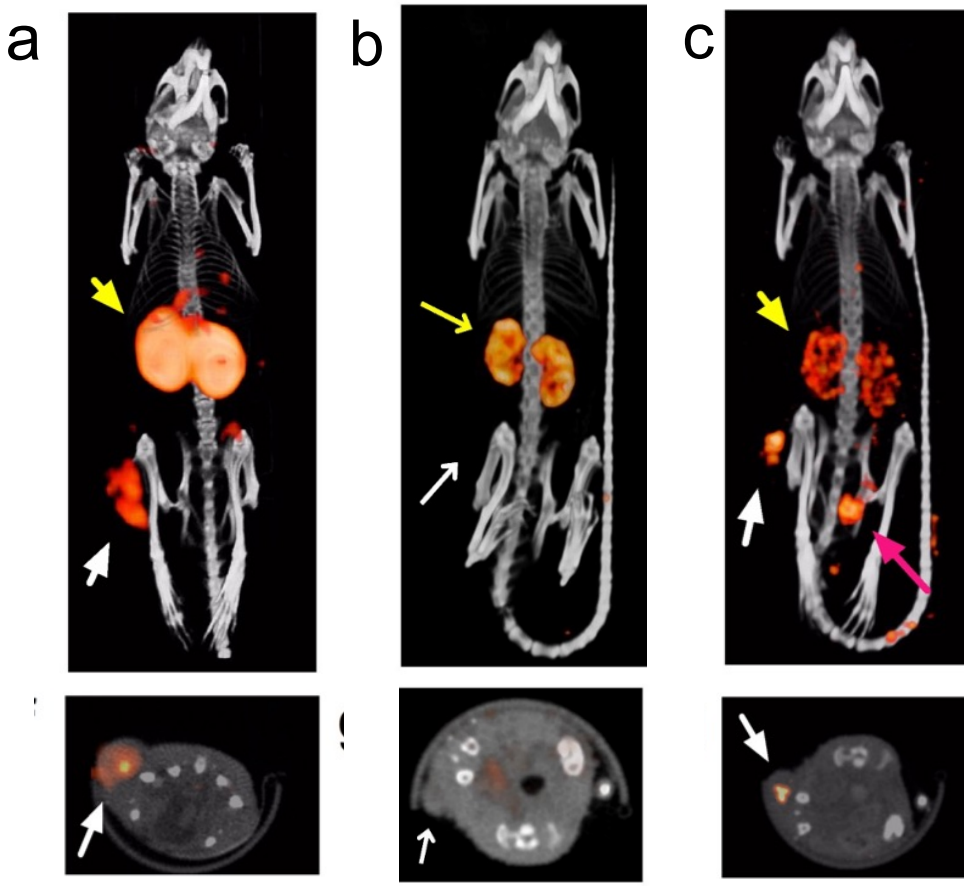


tumor-bearing mice (n=4) injected with 1) tc-ADC (0.033 µmol/kg) , 2) vehicle or activator (0.335 mmol/kg; 48h), 3) ^{177}Lu-probe (0.335 µmol/kg; 51 h), biodistribution @ 54 h.

Click-to-Image-Release

LS174T-mice inj. 1) tc-ADC; 2) 1 eq ^{111}In-Tz @ 48h; 3) imaging/biodistribution @ 51h



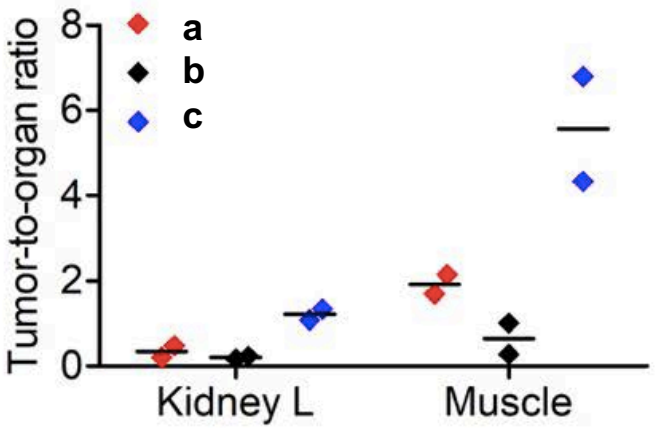


0.33 ± 0.07 %

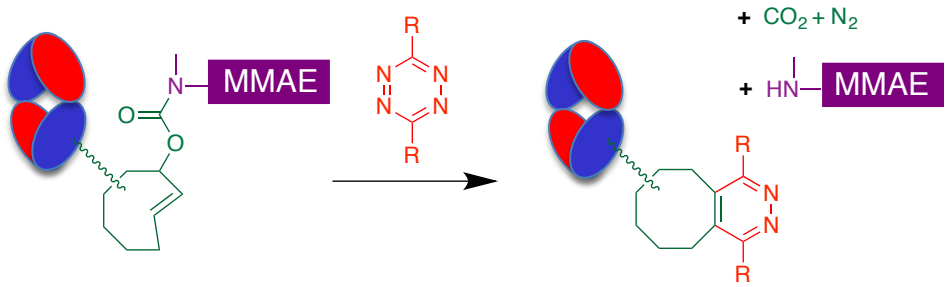
0.11±0.06 %

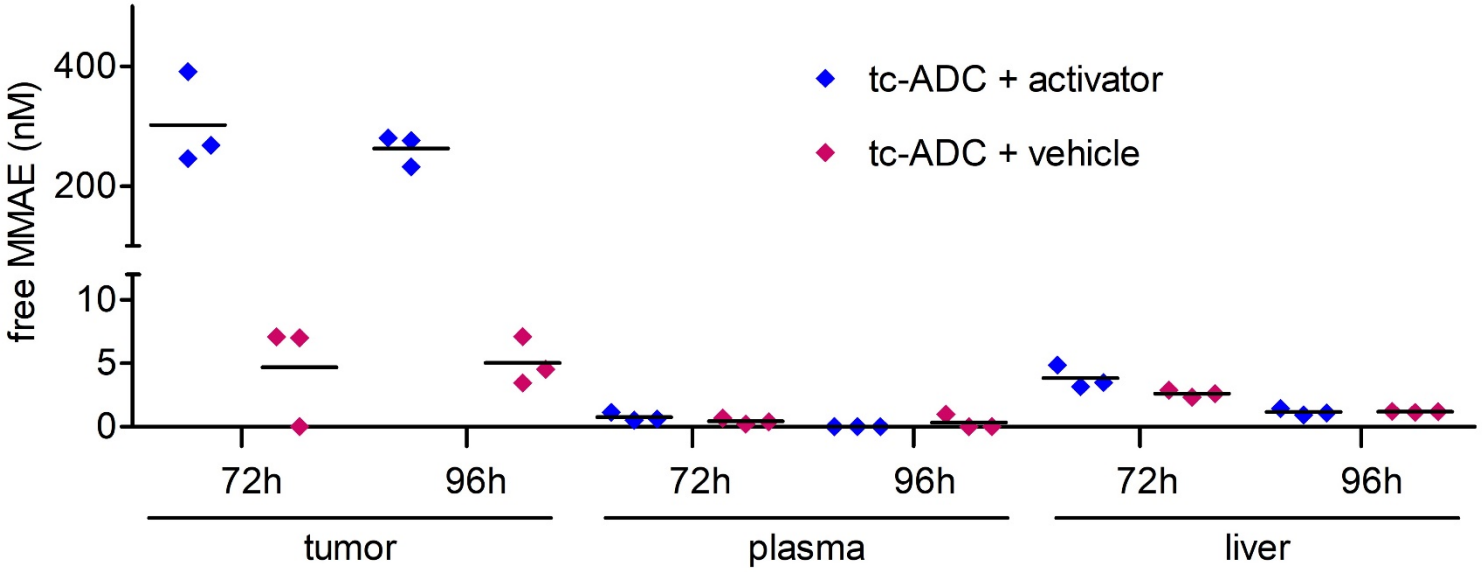
6 % ID/g

a) tc-ADC + ^{111}In-activator
b) ^{111}In-activator
c) tc-ADC + ^{111}In-probe



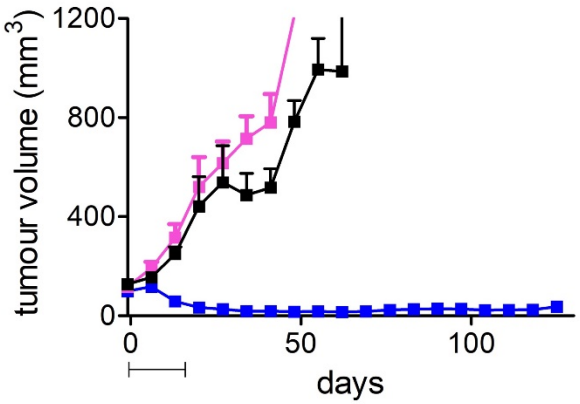
Free MMAE concentrations in vivo

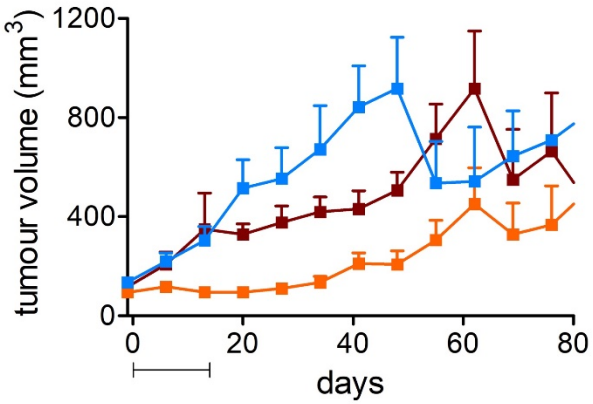


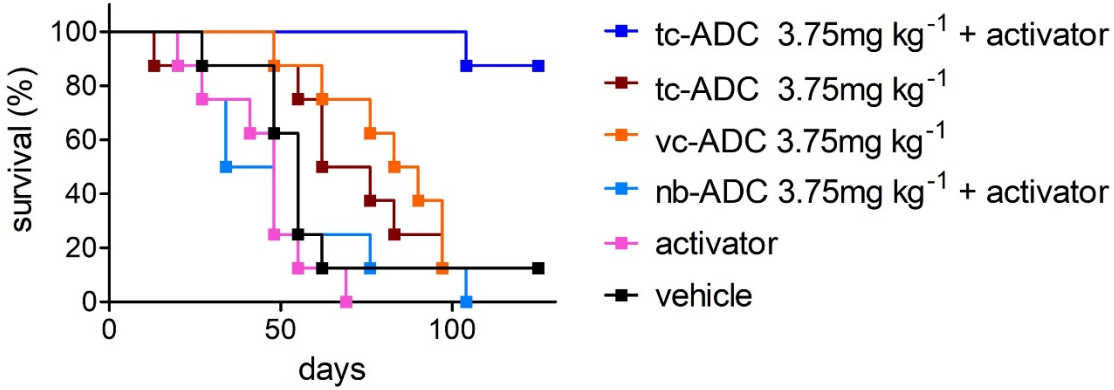


LS174T-mice inj. 1) tc-ADC (0.033 µmol/kg) , 2) activator (0.335 mmol/kg; 48h), biodistribution @ 72 and 96 h, MMAE extraction

Therapeutic efficacy in OVCAR-3 tumor bearing mice



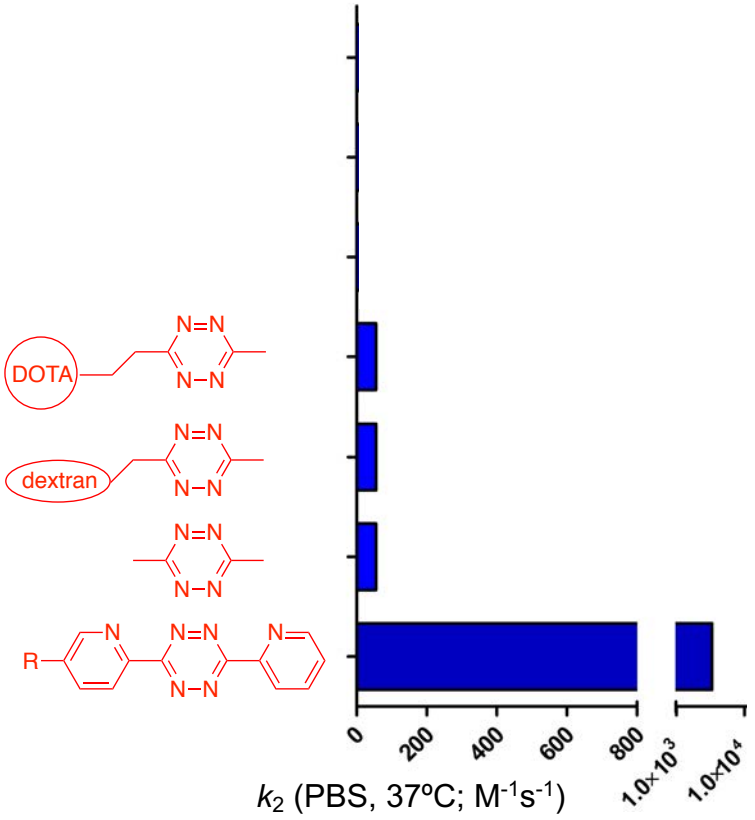




ADC	diabody	R
tc-ADC	anti-TAG72	-TCO-MMAE (**1**)
vc-ADC	anti-TAG72	-val-cit-MMAE (**2**)
nb-ADC	anti-PSMA	-TCO-MMAE (**1**)

Mice inj. within 2 weeks with 4 cycles of 1) tc-ADC, 2) activator (0.335 mmol/kg; 48h)

Click-to-Release: reactivity *vs.* release



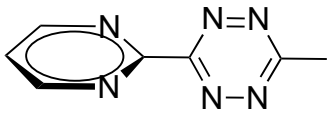
release: 90 % / dose: 10,000 eq, 36 mg/kg

release: 50 % / dose: 10,000 eq, 268 mg/kg

release: 90 % / dose: >100,000 eq

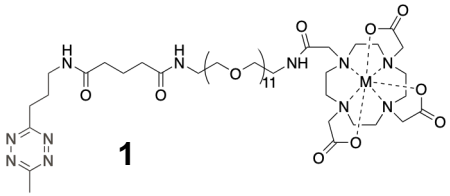
release: 10 % / dose: 50 eq

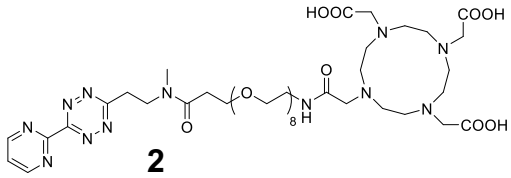
Pyrimidine tetrazines: boosted reactivity, slightly lower release

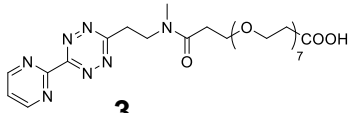


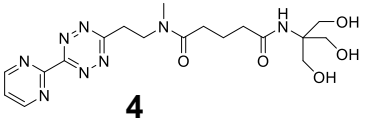
- k_2 ~ 1000 $M^{-1}s^{-1}$
- release: 65-75 %

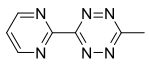
Fan *et al.*, *Angew. Chem. Int. Ed.* **2016,** 55, 14046

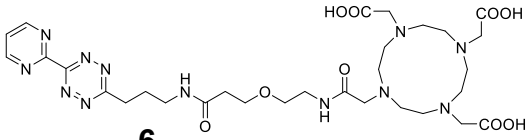


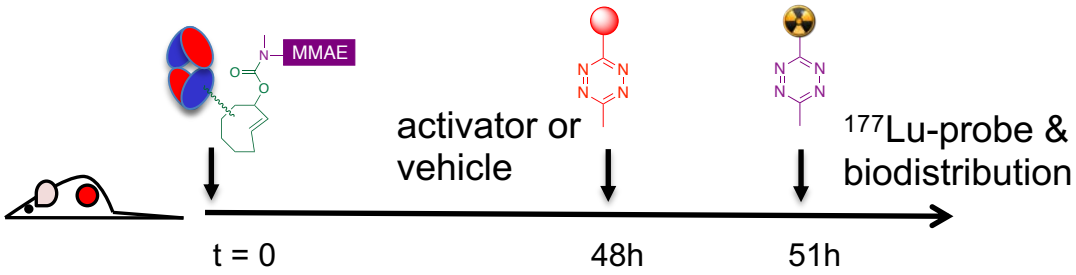


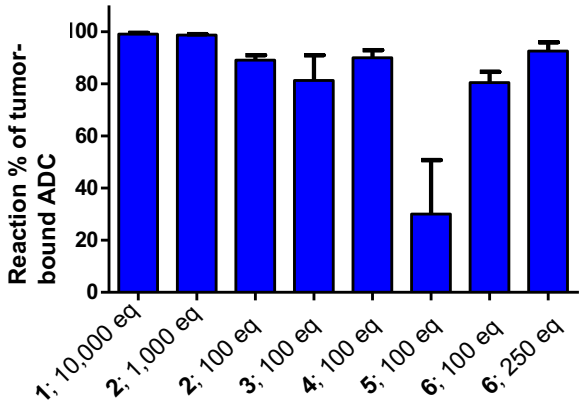




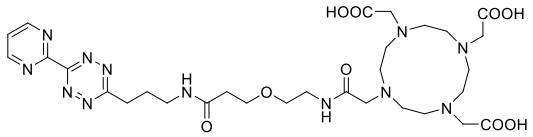




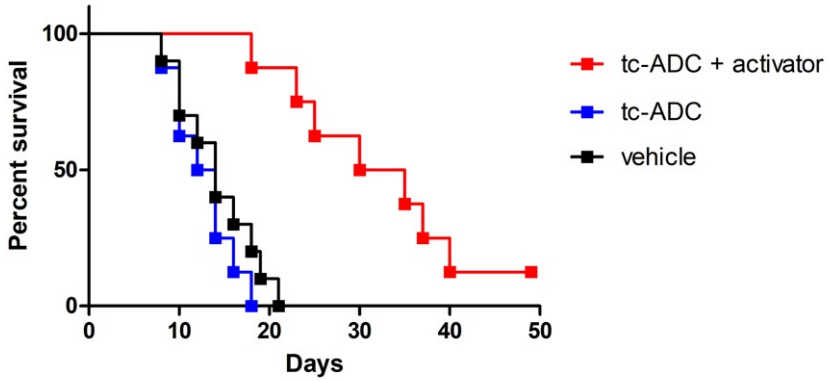


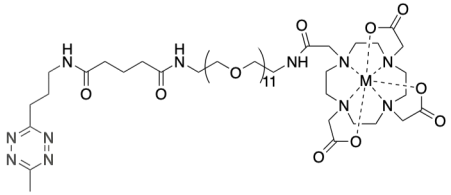


Same therapeutic efficacy of pyrimidine tetrazine at 20-fold lower dose

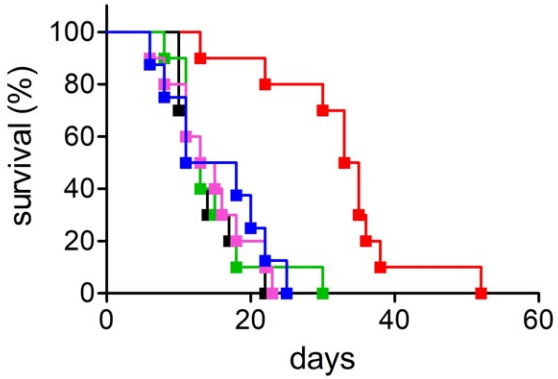


500 eq





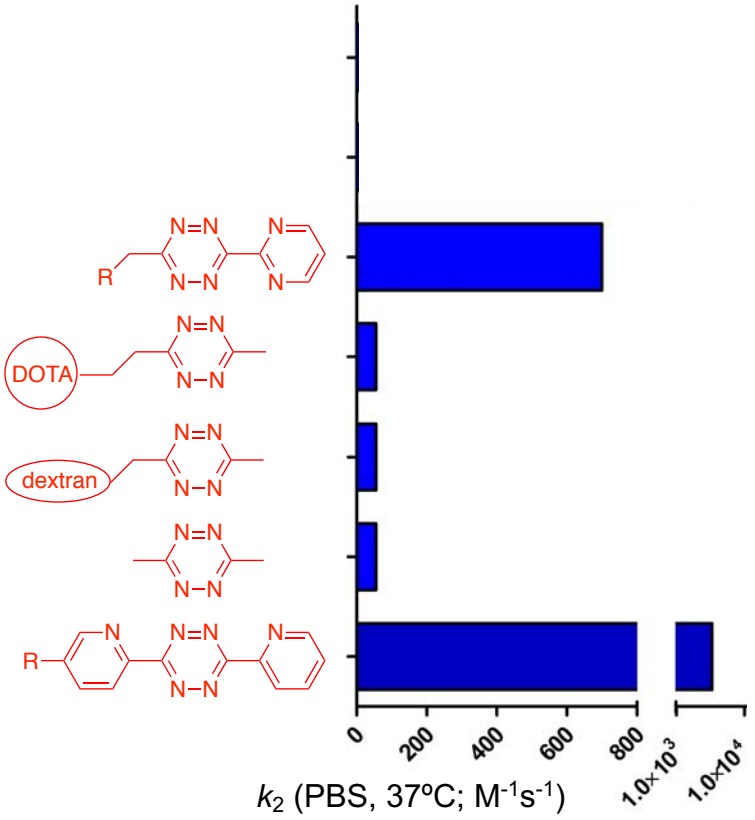
10,000 eq



Rossin *et al.*, *Nature Commun.* **2018,** *9*, 1484

LS174T tumored mice inj. i.v. within 2 weeks with 4 cycles of 1) tc-ADC (3 mg/Kg), 2) activator (17 umol/kg; 48h)

Click-to-Release: reactivity *vs.* release



release: 70 % / dose: 500 eq, 0.6 mg/kg

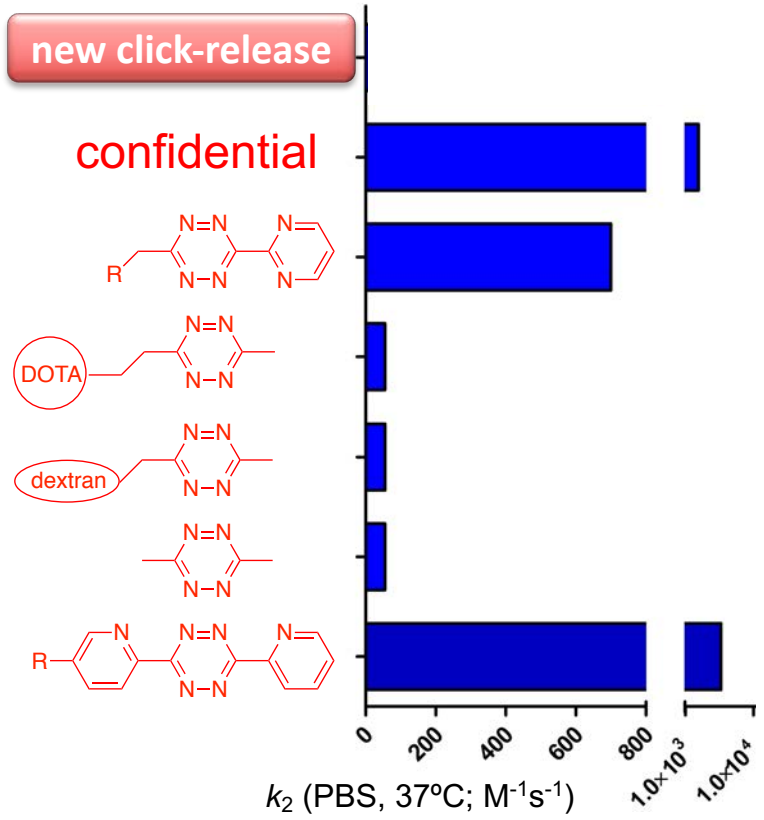
release: 90 % / dose: 10,000 eq, 36 mg/kg

release: 50 % / dose: 10,000 eq, 268 mg/kg

release: 90 % / dose: >100,000 eq

release: 10 % / dose: 50 eq

Click-to-Release: reactivity *vs.* release



release: 100 % / dose: 250 eq, 0.3 mg/kg

release: 70 % / dose: 500 eq, 0.6 mg/kg

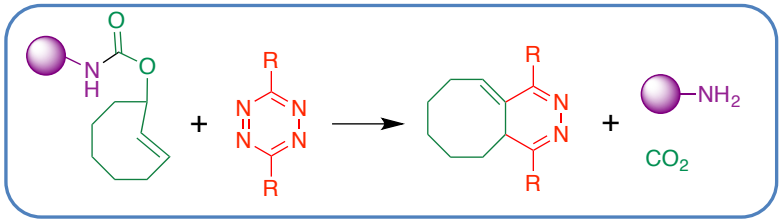
release: 90 % / dose: 10,000 eq, 36 mg/kg

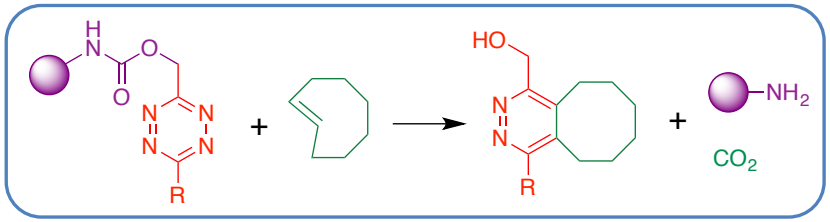
release: 50 % / dose: 10,000 eq, 268 mg/kg

release: 90 % / dose: >100,000 eq

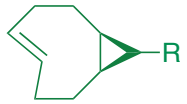
release: 10 % / dose: 50 eq

New click-to-release reaction: swapping TCO and tetrazine

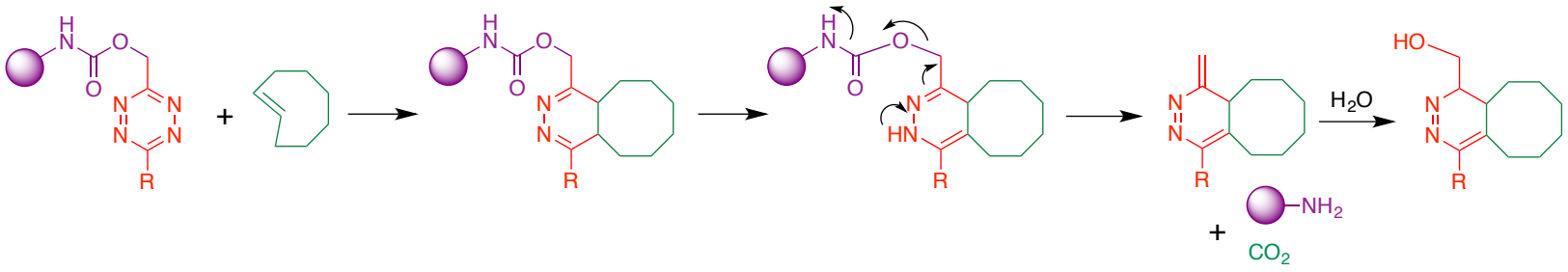




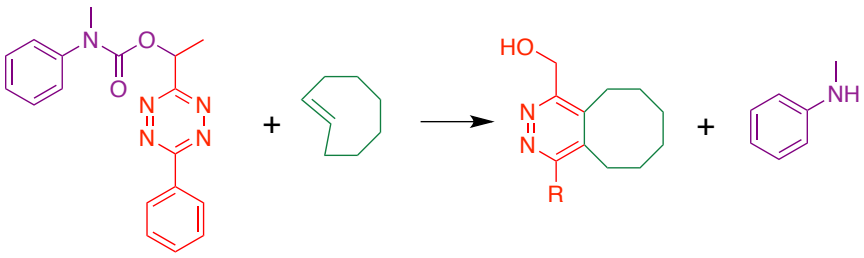
Allows use of highly reactive sTCO as activator to boost reactivity of system 100-fold



Envisioned mechanism

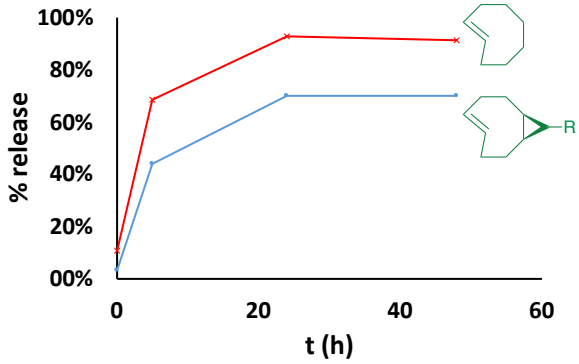


New click-to-release reaction: swapping TCO and tetrazine



20% MeCN/PBS@37°C:

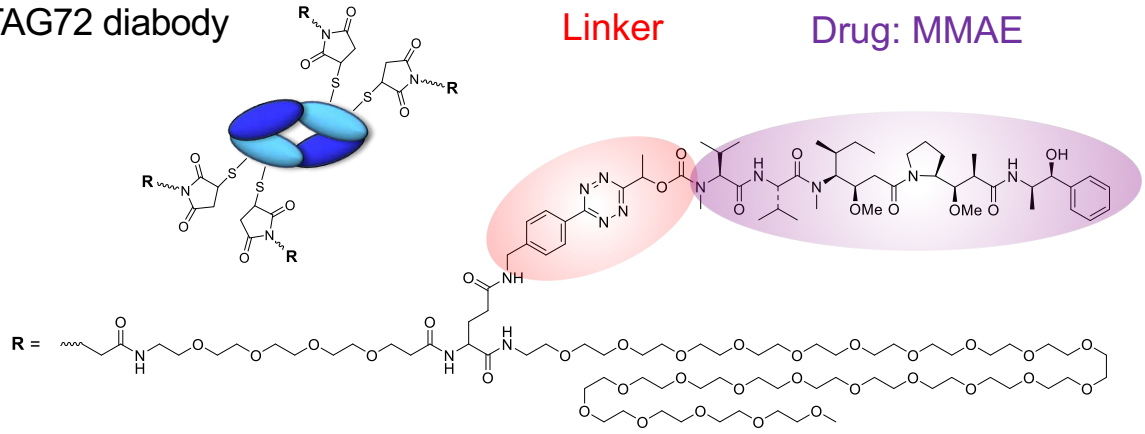
Stability $t_{1/2}$: 75 days
Release $t_{1/2}$: 10 h



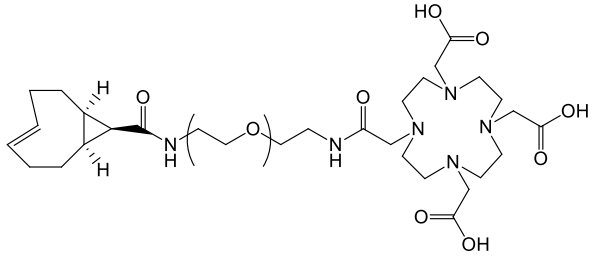
TAG72 diabody

Linker

Drug: MMAE

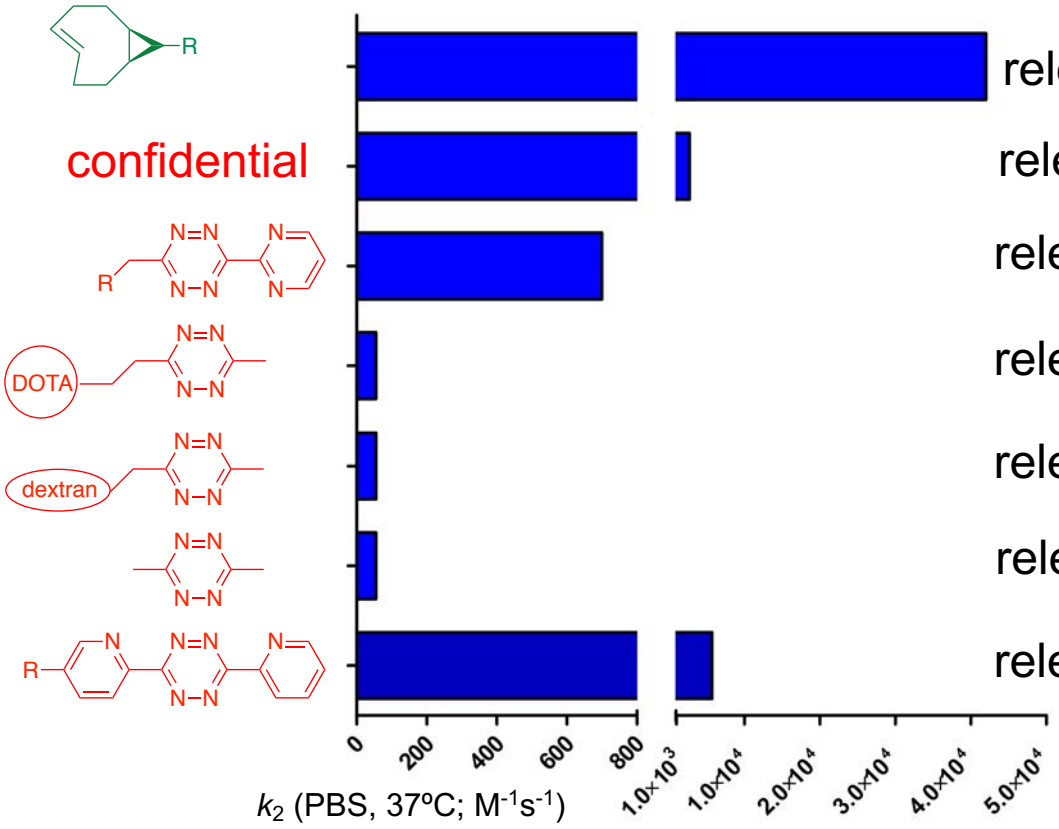


Activator



Reactivity (k_2): 42000 $M^{-1}s^{-1}$
Max release: 70 % (within 24 h)

Click-to-Release: reactivity *vs.* release



release: 70 % / dose: 10 eq, 0.012 mg/kg

release: 100 % / dose: 250 eq, 0.3 mg/kg

release: 70 % / dose: 500 eq, 0.6 mg/kg

release: 90 % / dose: 10,000 eq, 36 mg/kg

release: 50 % / dose: 10,000 eq, 268 mg/kg

release: 90 % / dose: >100,000 eq

release: 10 % / dose: 50 eq

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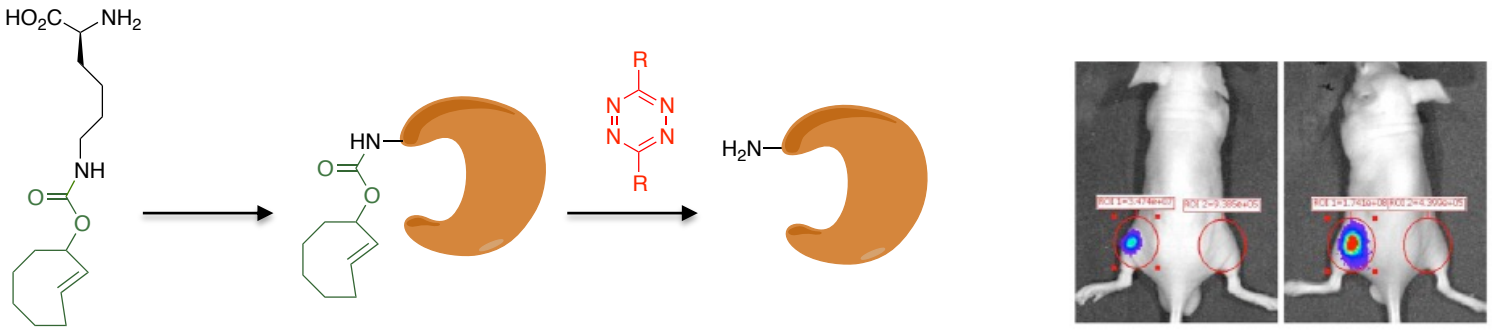
Other applications of the IEDDA pyridazine elimination reaction

Protein uncaging in vivo

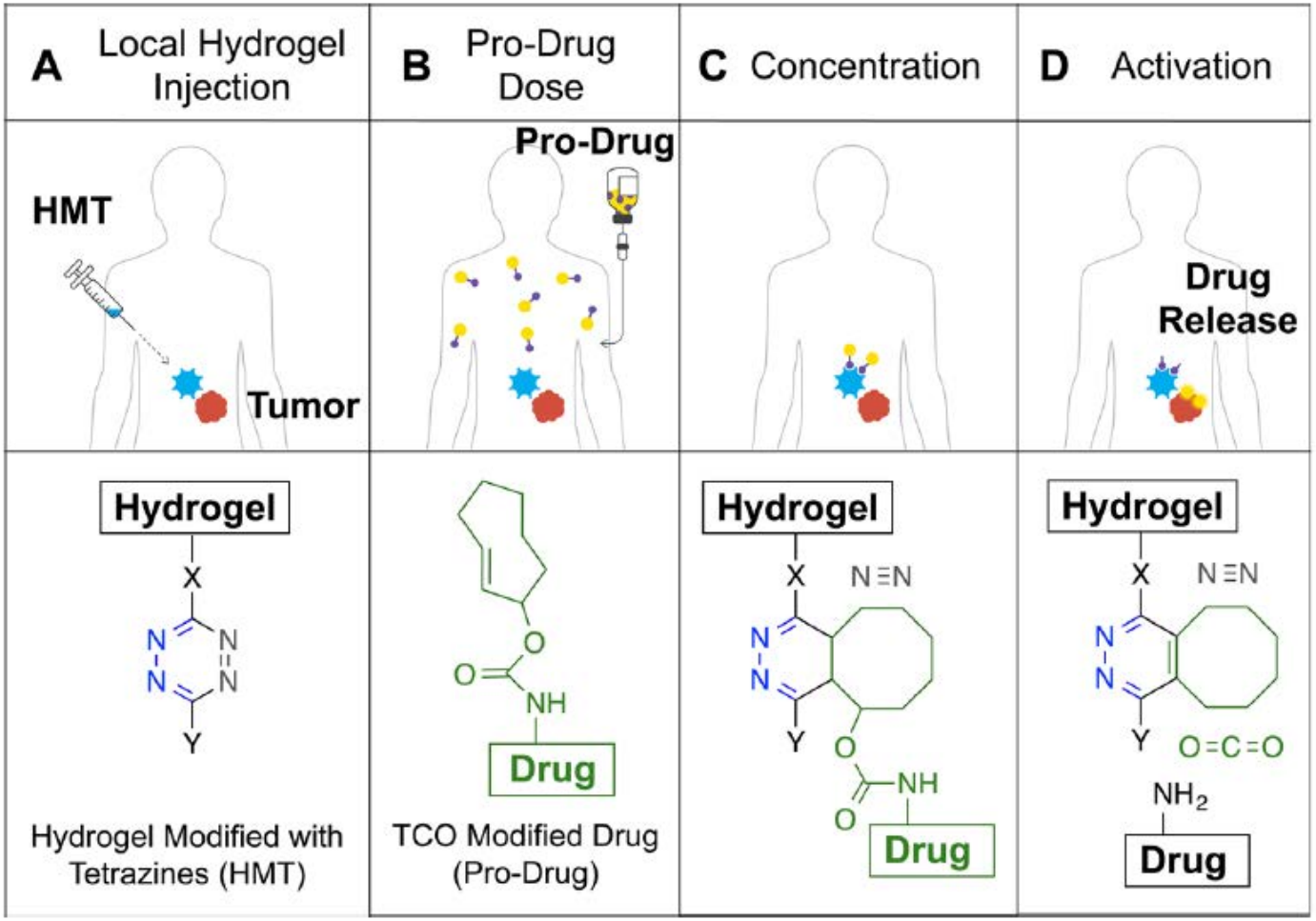
Genetic incorporation of masked lysine in luciferase and its in vivo intracellular unmasking with tetrazine



Incorporation of a single cleavable TCO at the right position can deactivate the protein. Protein activity can be restored on demand, also intracellularly, upon treatment with a tetrazine activator.

G. Zhang *et al.*, *ACS Central Science* **2016**, *2*, 325–331

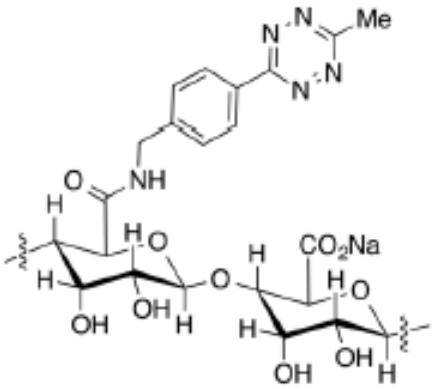
Click-to-release approach to local drug activation

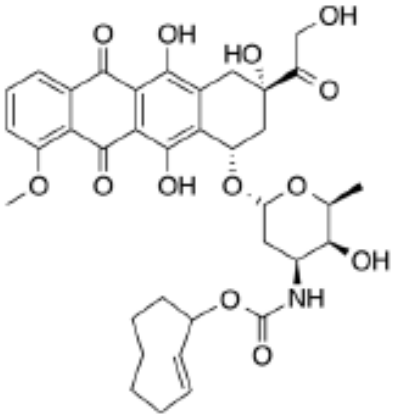


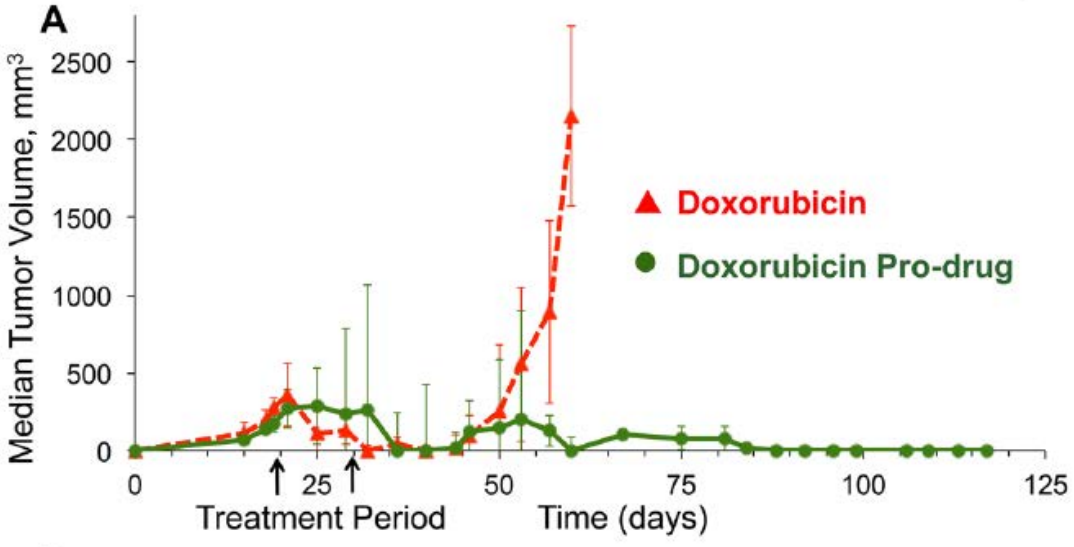
Meija-Oneto *et al.*, *ACS Centr. Sci.* **2016**, 2, 476-482

Click-to-release approach to local drug activation

Nude mice bearing HT-1080 soft tissue sarcoma locally injected with a Tz-alginate gel followed by TCO-Dox prodrug i.v.





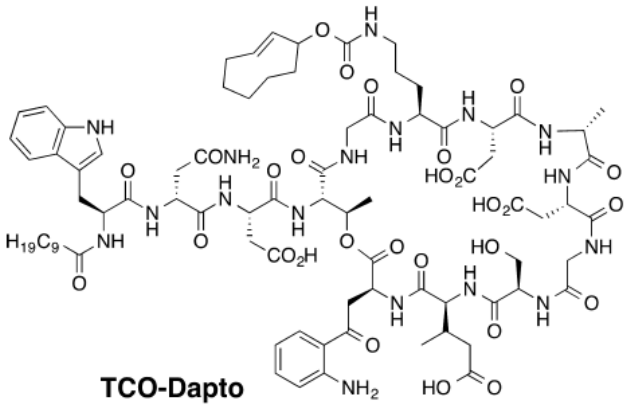


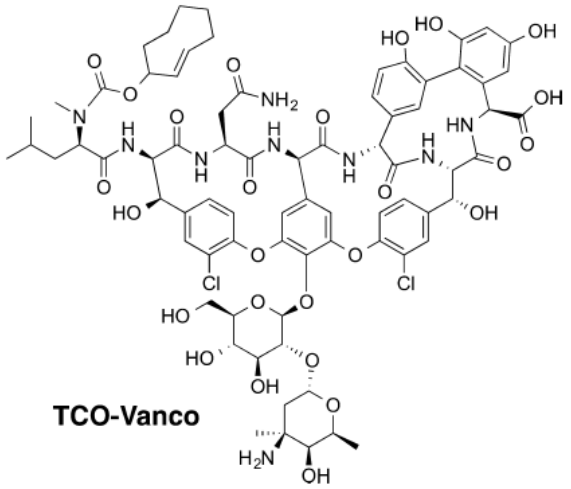
5/10 tumors treated (the remaining mice were euthanized with > 2 gr tumors)

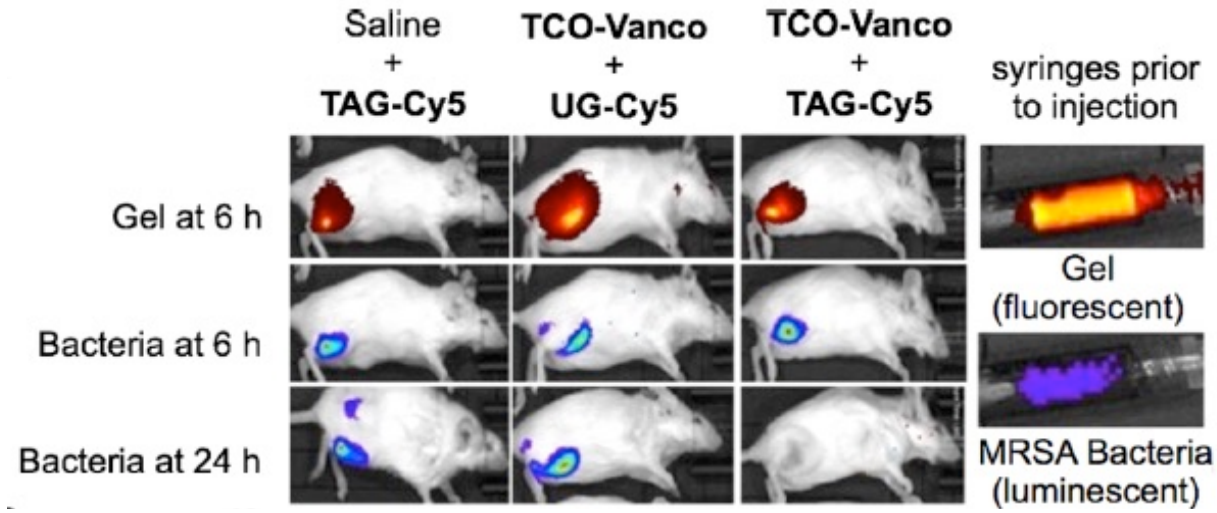
Promising approach for **treatment of tumor resection areas** (to eliminate residual tumor cells)

Meija-Oneto *et al.*, *ACS Centr. Sci.* **2016**, 2, 476-482

Click-to-release approach to local antibiotic activation





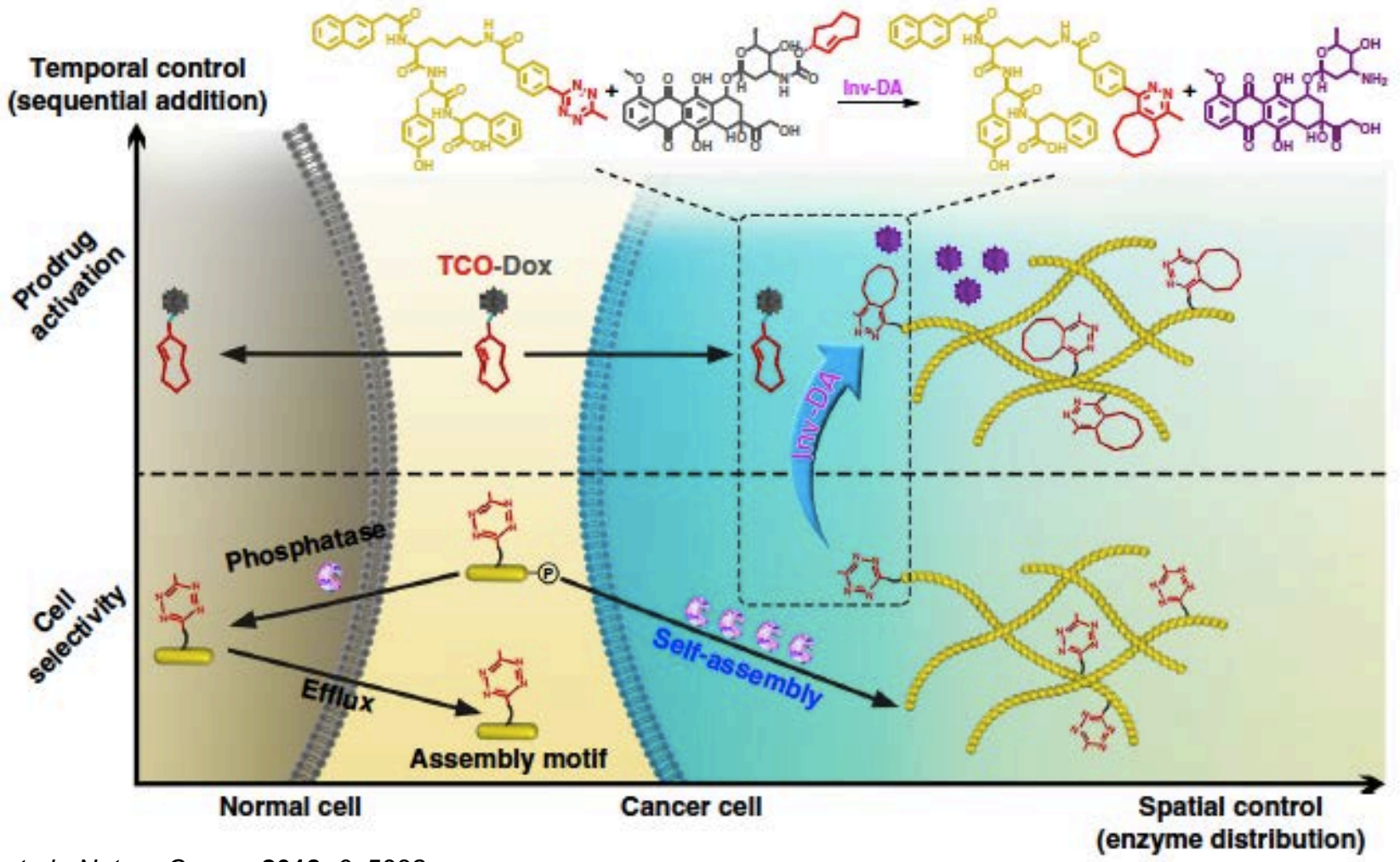


Tetrazine-loaded gel (TAG) in combination with TCO-Vancomycin eliminates bacterial infection in 24h

Czuban *et al.*, ACS *Centr Sci* **2018,** *4*, 1624

Enzymatic Tz accumulation followed by pro-drug uncaging

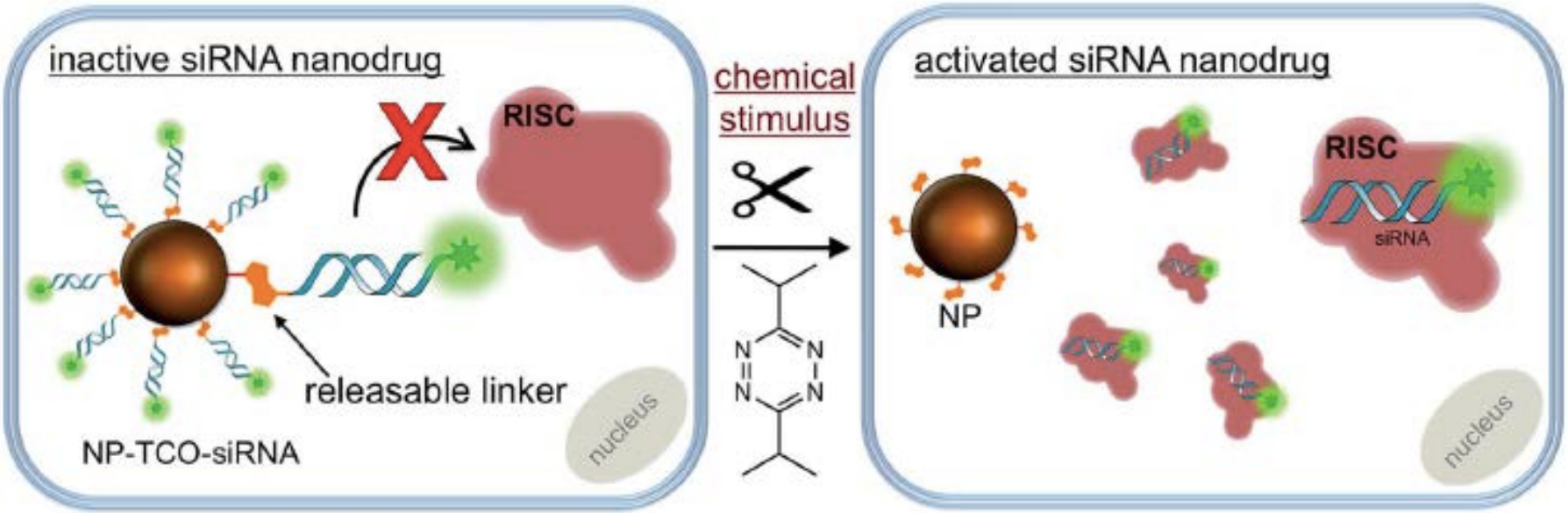
Tumor bearing mice injected with 3 cycles of 50 mg/kg Tz + 30mg/kg TCO-Dox



Yao *et al.*, *Nature Comm.* **2018,** *9*, 5032

Intracellular RNA delivery

Cleavage of siRNA from nanoparticle carrier

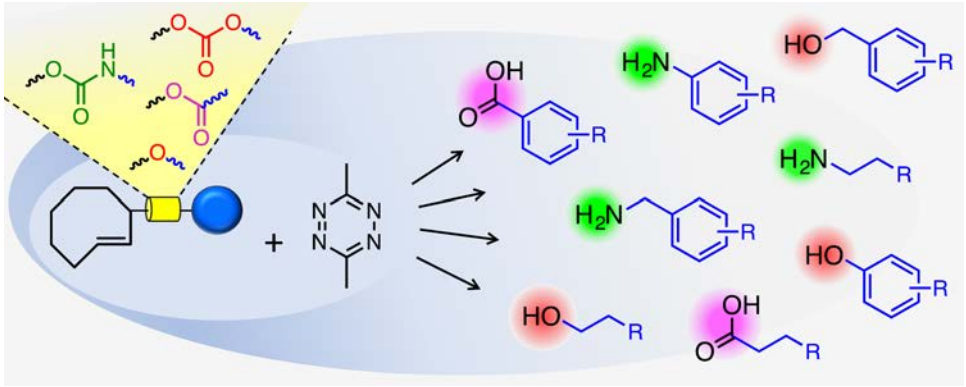


Khan *et al.*, *Chemical Science* **2017**, *8*, 5705

and many more…

Summary – Click-to-release

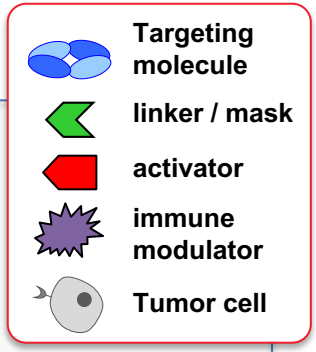
- Click-cleavable ADCs expand ADC scope to non-internalizing targets & platform technology for controlled payload delivery to TME
- Diabody ADC: high tumor uptake, fast clearance, low systemic exposure
- High intratumor MMAE levels, minimal washout, no toxicity observed
- Potential for homogenous drug distribution and activation of tumor-residing immune cells
- Platform technology with a variety of application, also in synthetic chemistry
- Broad chemical scope:



Versteegen *et al., Angew. Chem. Int. Ed.* **2018**, *57,* 10494–10499

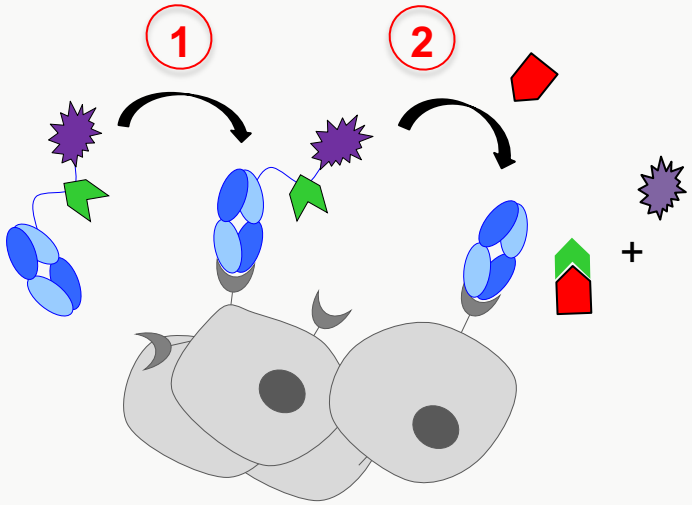
Tagworks' Click-to-Release

A platform technology that can be used with a broad array of immune modulators



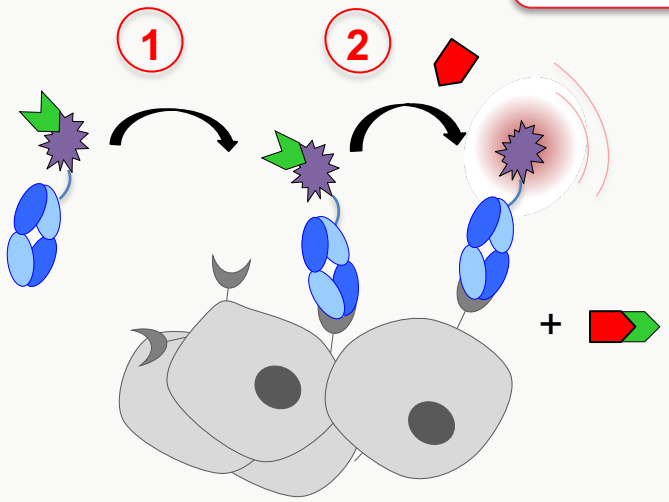
Applications:

Drug Release



Examples: TLR agonists, STING agonists, cytokines, etc

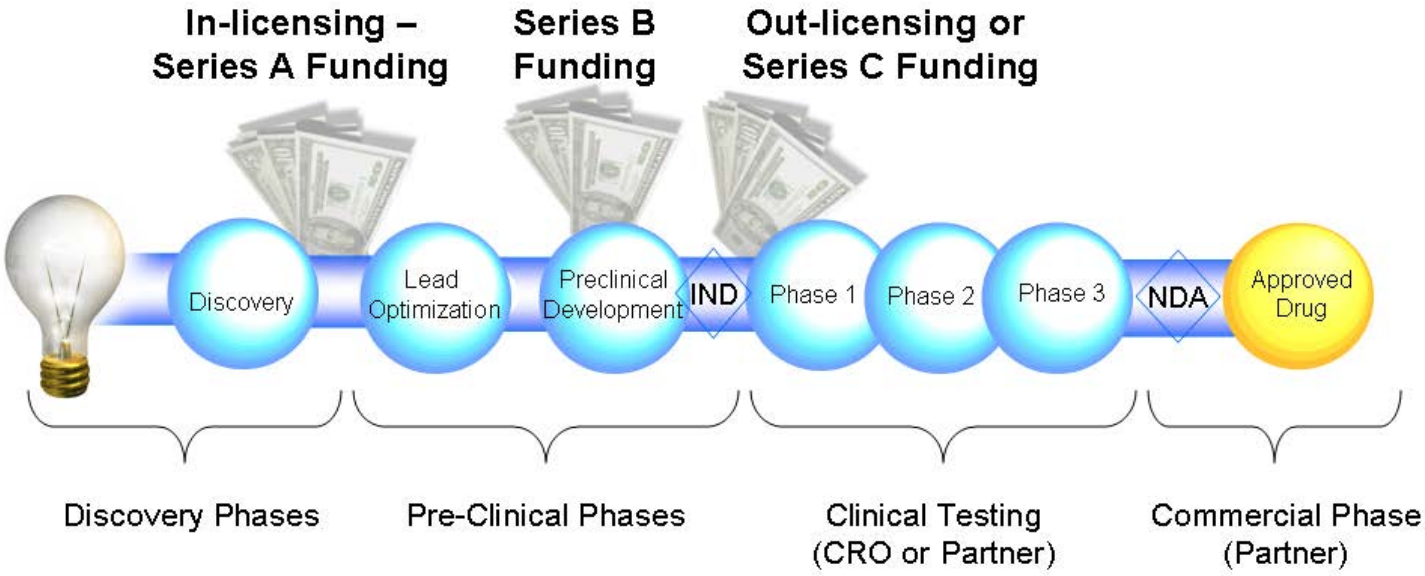
Drug unmasking



Examples: masked T-cell engagers, immunocytokines, etc

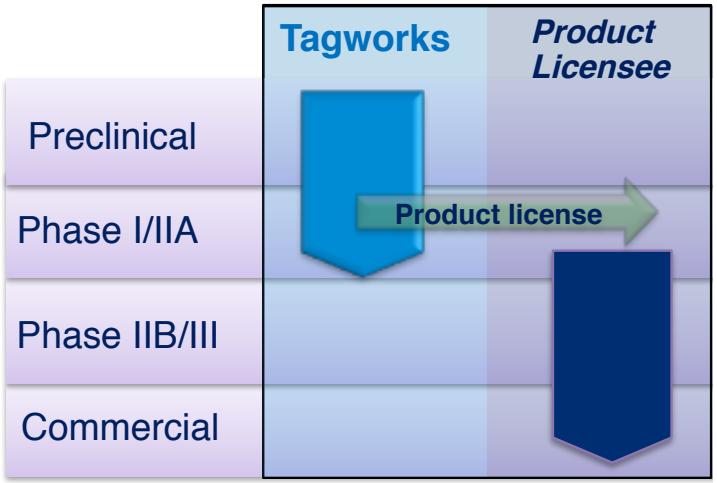
tagworks PHARMACEUTICALS

Drug discovery pipeline



Our business plan

Product Development and Licensing



Technology Licenses and Services