MedImmune
A member of the AstraZeneca Group

Instability of thiol/maleimide conjugation and strategies for mitigation
(Linker design – Workshop F)

Changshou Gao, Ph.D.
Department of Antibody Discovery and Protein Engineering
MedImmune

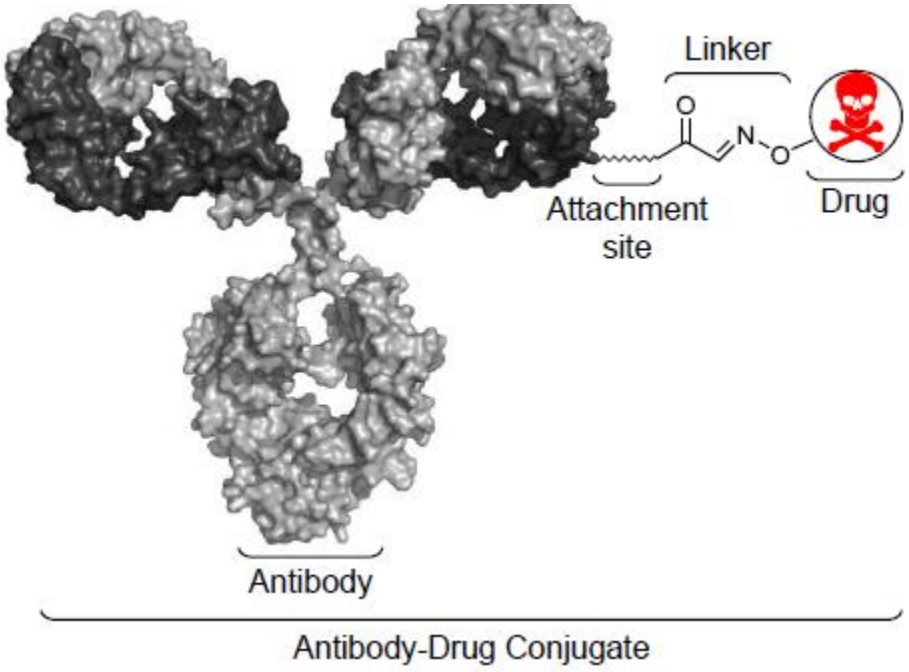
7th World ADC San Diego
Oct. 10, 2016

Overview

- Introduction - the instability associated with thiol-maleimide conjugation chemistry
- Optimization of maleimide chemistry for efficient and stable conjugation to antibodies
- Hydrolytically stable site-specific conjugation at the N-terminus of an engineered antibody
- Acknowledgements

MedImmune

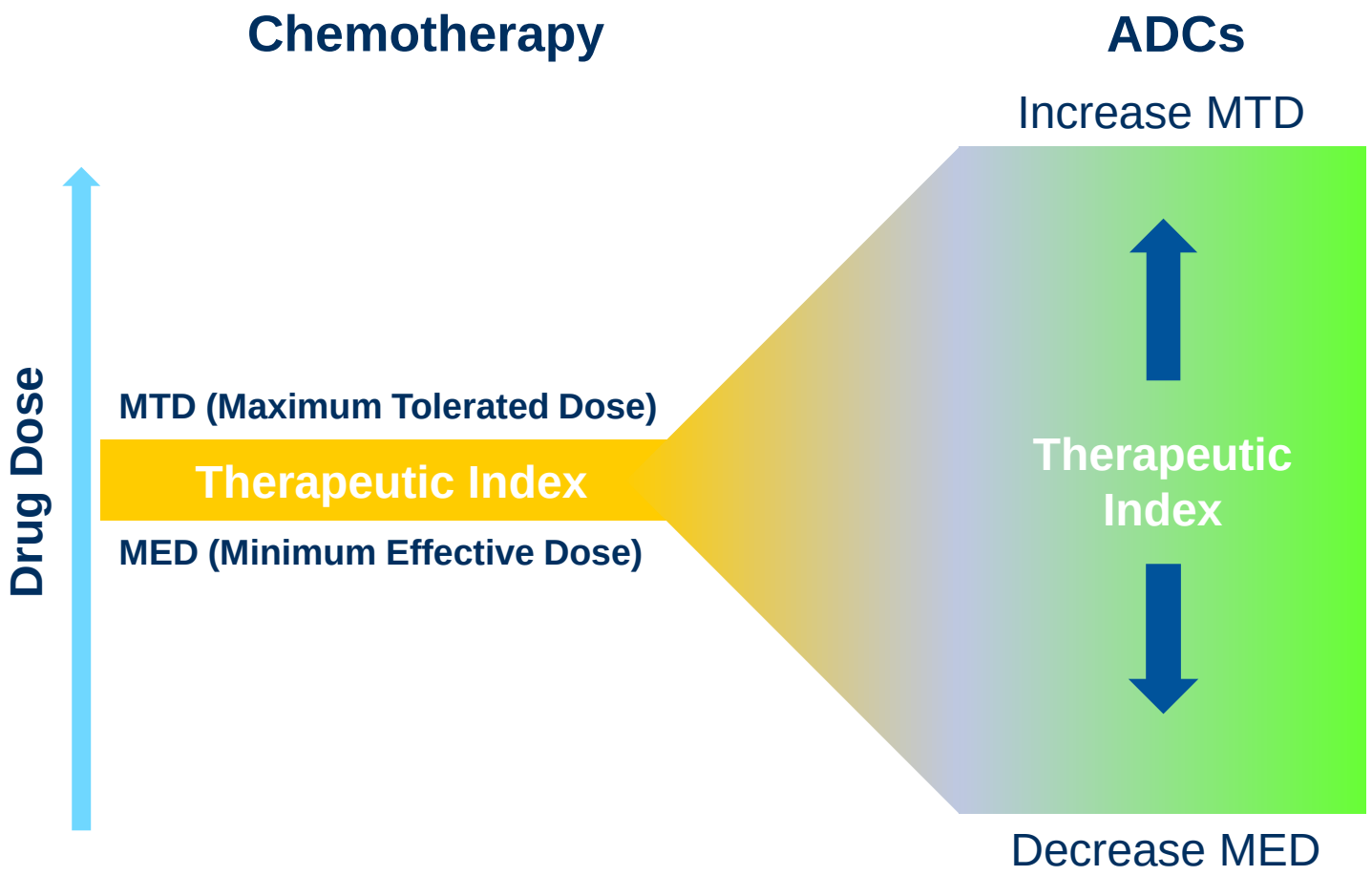
Antibody Drug Conjugate: Key Components



- **Antibody**
  - Target specific with high affinity
  - Internalization and traffic to lysosome for active drug release
  - Site specific conjugation for homogeneous product
  - Good PK
  - Retention of above properties after drug conjugation
- **Attachment site**
  - At native or engineered residues
- **Linker**
  - Stable in circulation and during process and storage
  - Selective release of active drug inside target cells
  - Cleavage or non-cleavable
- **Cytotoxic Drug (warhead)**
  - Highly potent and amenable to modifications (e.g., allow linker attachment)
  - Stable in circulation and lysosomes
  - Non-immunogenic
  - Bystander effect?

MedImmune

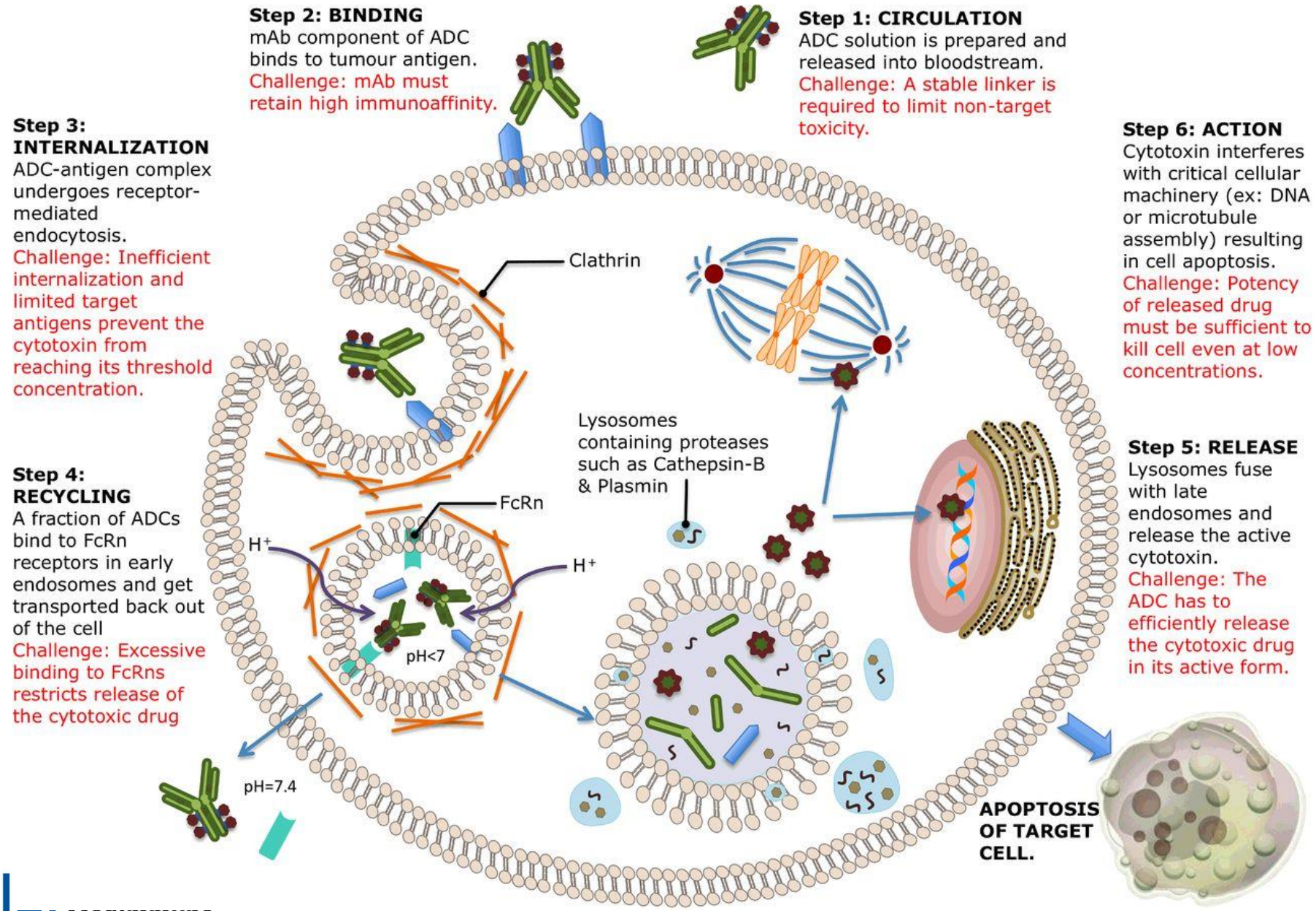
The promise of antibody drug conjugates – targeted delivery of potent cytotoxic drugs for improved therapeutic index



Therapeutic Index=MTD/MED

MedImmune

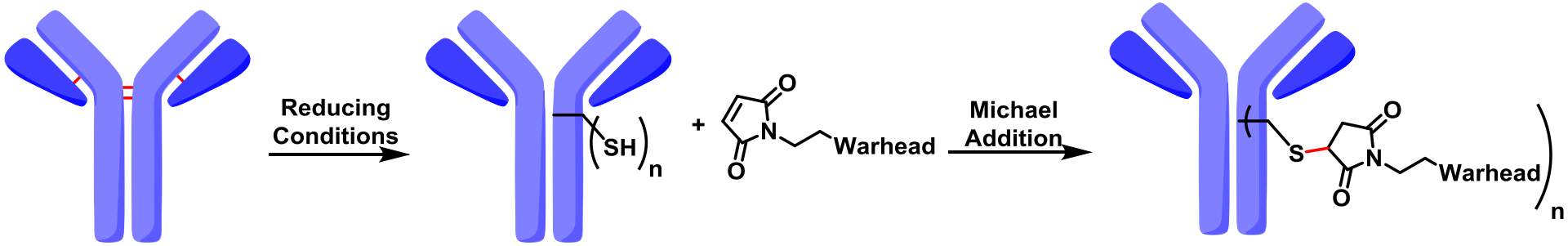
Mechanism of action of ADCs



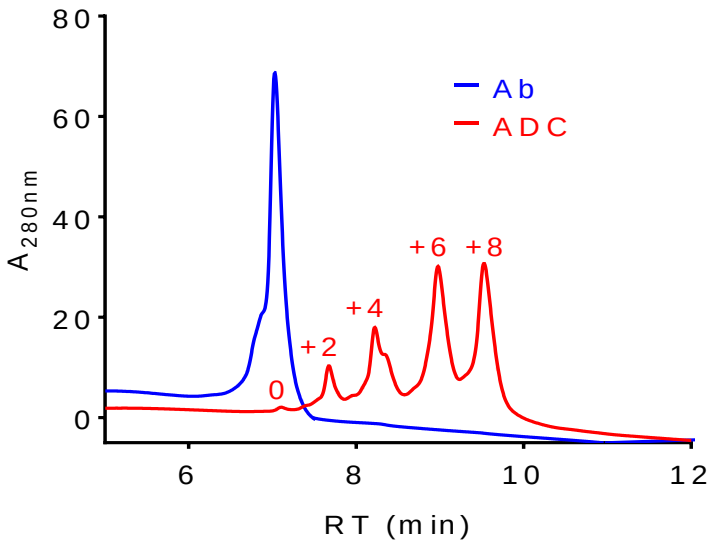
MedImmune

Christina Peters, and Stuart Brown Biosci. Rep. 2015;35:e00225

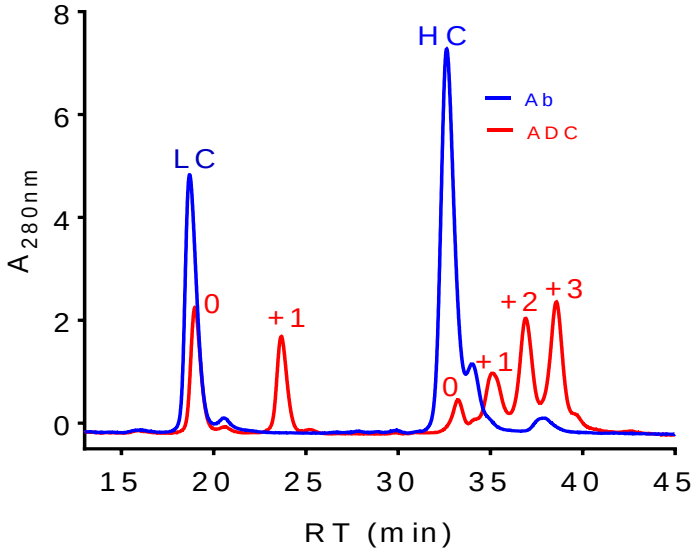
“Classically conjugated” ADCs are heterogeneous



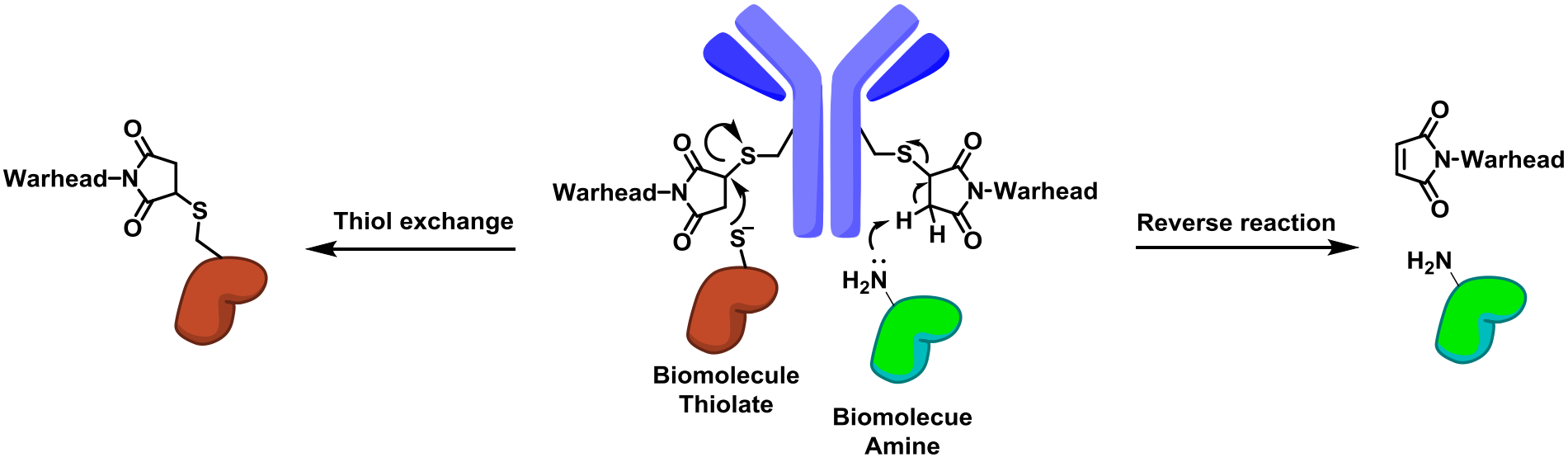
Hydrophobic Interaction Chromatography



Reduced Reverse-Phase Chromatography



“Classically Conjugated” ADCs are Suboptimal



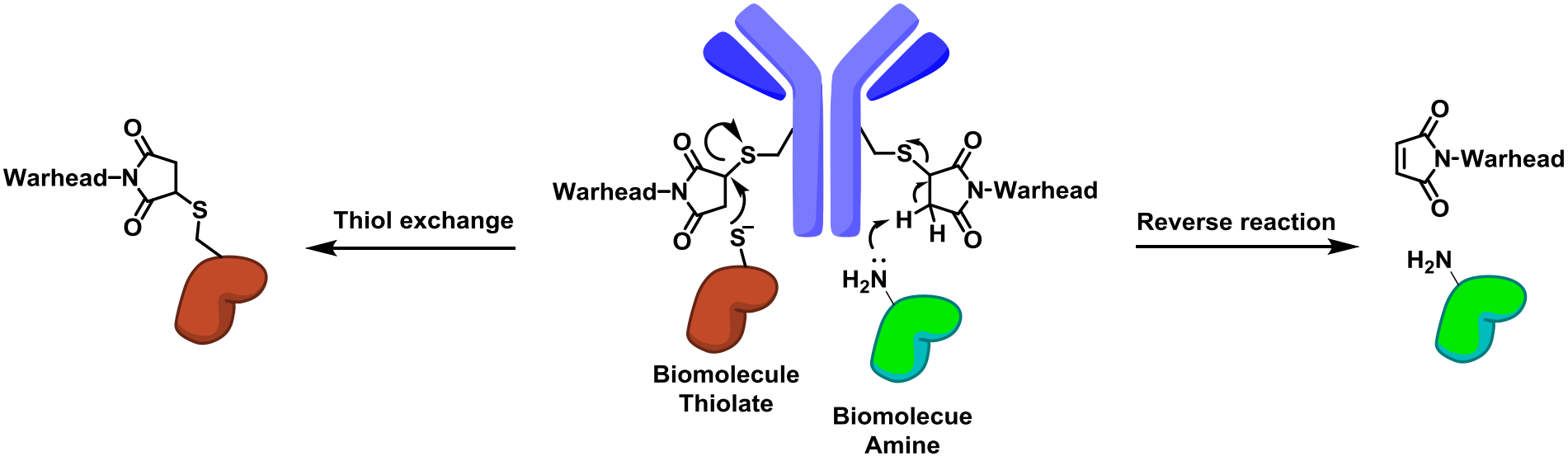
Payload deconjugation leads to:

- **Reduced on-target efficacy**
- **Increased off-target toxicity**
- **Uncontrolled distribution of drug**

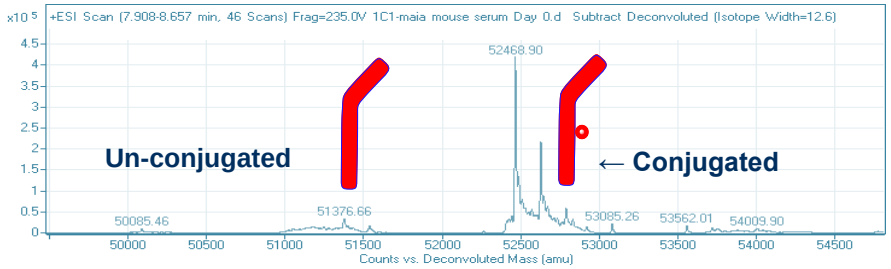
DAR change over time in mouse serum *in vitro*

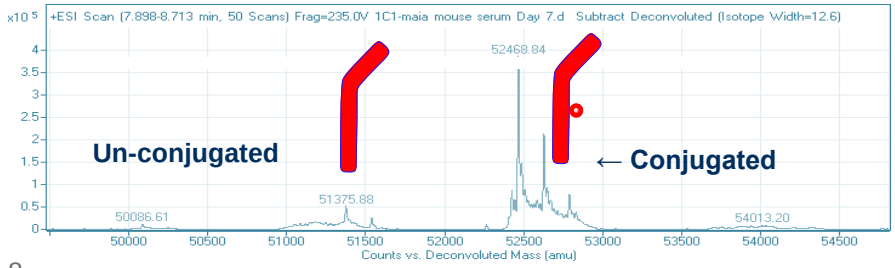
Name	Initial DAR	DAR at day 3 *(% drug loss)*	DAR at day 7 *(% drug loss)*
R347-FCC-DAR 4	3.88	3.81 *(1.8)*	3.76 *(3)*
1C1-CC-DAR 2	1.9	0.8 *(57)*	0.25 *(86.8)*
1C1-CC-DAR 4	4.0	1.7 *(57.5)*	0.21 *(94.75)*
1C1-FC-DAR 2	1.98	1.9 *(4)*	1.82 *(8)*
1C17-FCC-DAR 4	3.92	3.83 *(2.3)*	3.7 *(5.6)*

Site matters: the stability of site-specific ADCs using engineered cysteine is site dependent

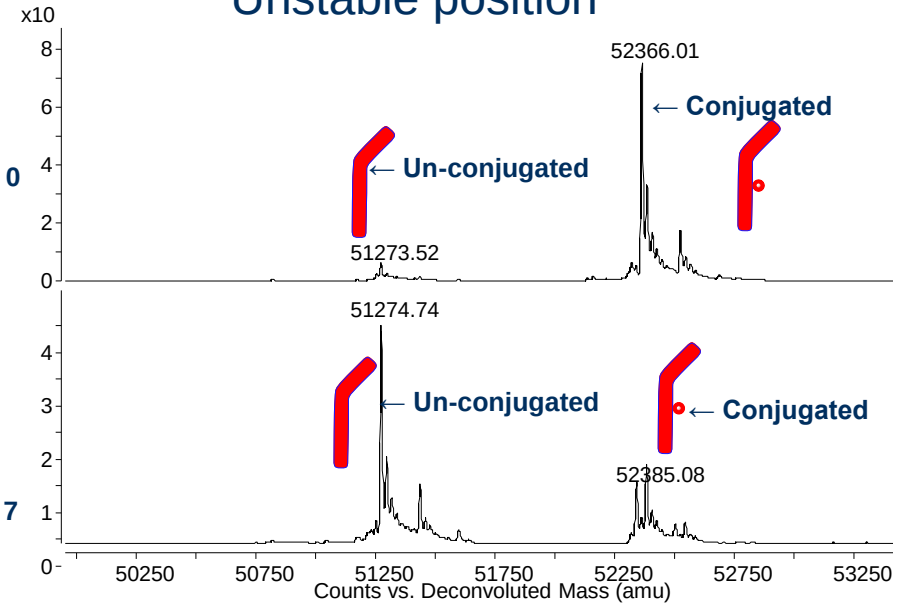


Stable position



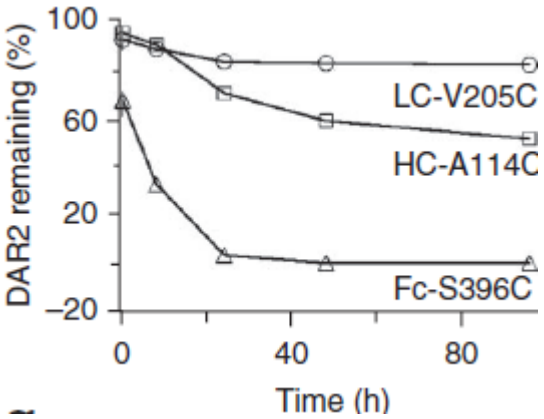


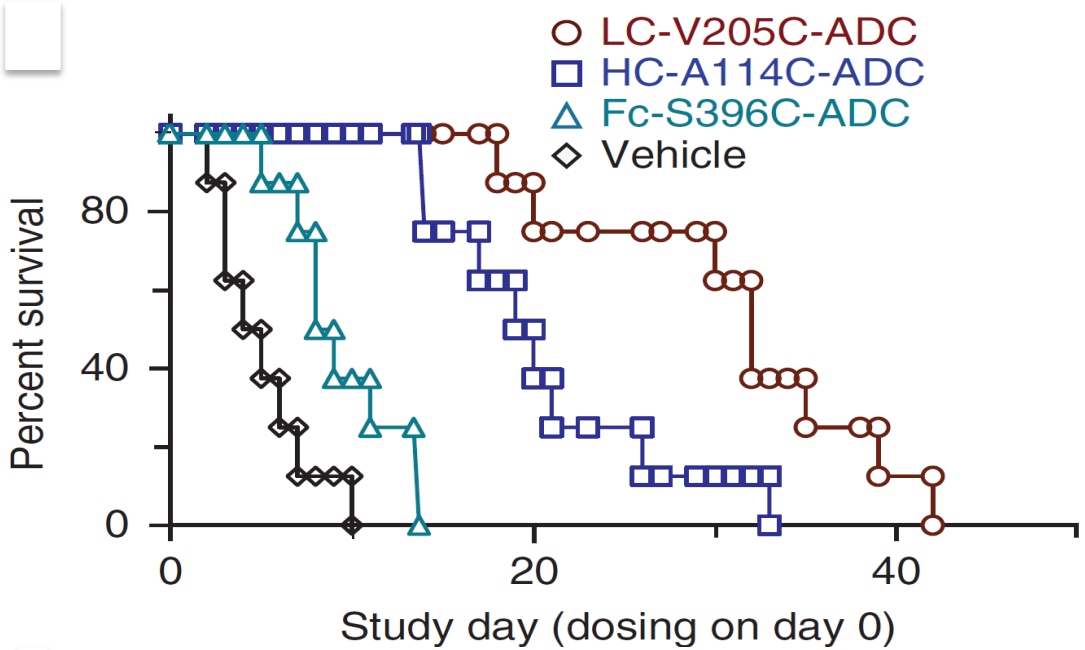
Unstable position

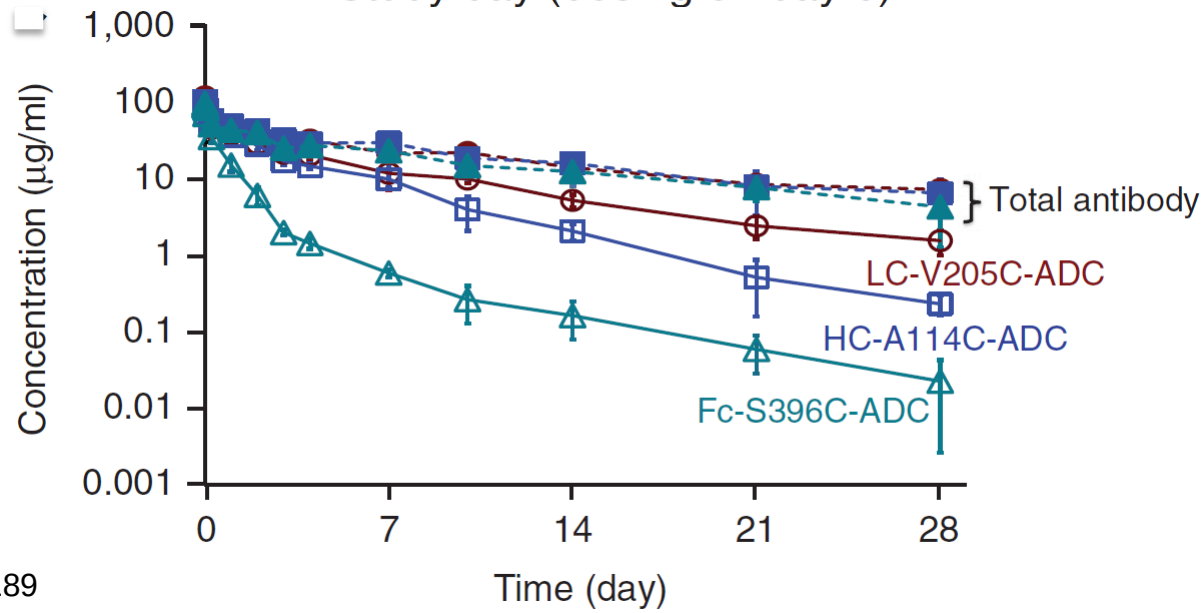


Site specific ADC DAR 2 in rat serum after 7 days

Site matters: drug conjugation site modulates the therapeutic activity of site-specific ADCs





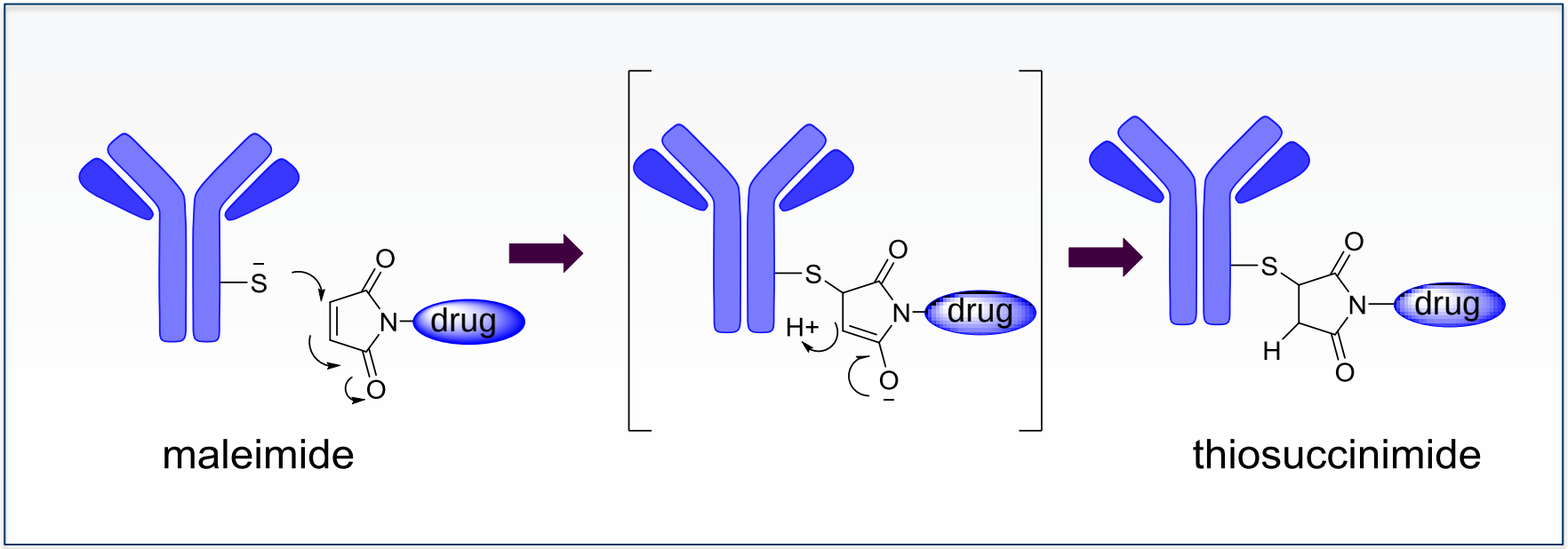


Shen B, et al., Nat. Biotech. 2012, 30: 184-189

Overview

- Introduction- the instability associated with thiol-maleimide conjugation chemistry
- Optimization of maleimide chemistry for efficient and stable conjugation to antibodies
- Hydrolytically stable site-specific conjugation at the N-terminus of an engineered antibody
- Acknowledgements

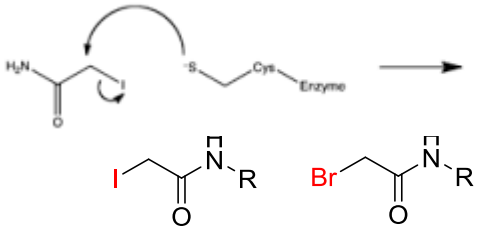
Conjugating drugs to cysteine using thiol/maleimide chemistry

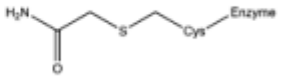


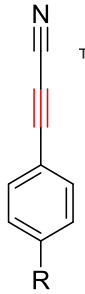
- 1,4-Michael addition reaction
- Fast and specific
  - $T_{1/2}$=minutes at pH 7.4
- Variable stability
  - Conjugation site
  - Linker/payload chemistry

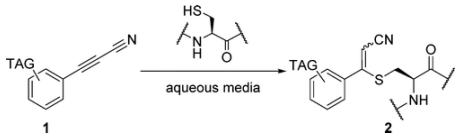
Chemical approaches to stabilize ssADCs

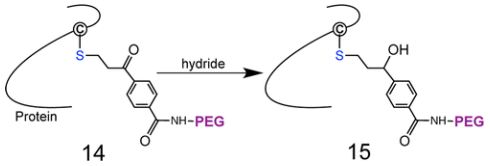
Change reaction chemistry

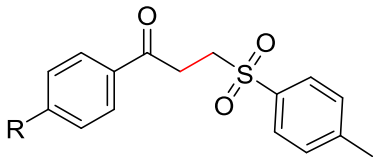












haloacetamides

Alley, S. C., et al. (**2008**) *Bioconjugate Chem. 19*, 759-765.

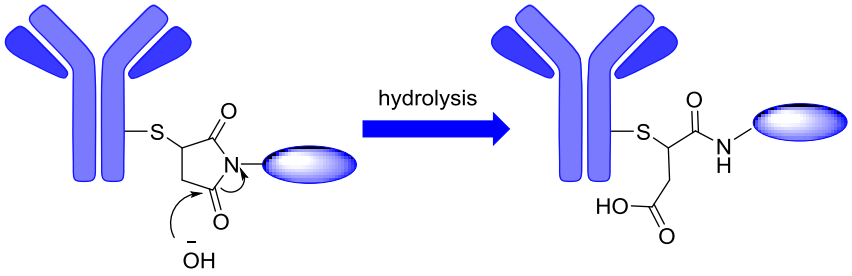
arylpropiolonitriles

Koniev, O., Leriche, et al.(**2014**) *Bioconjugate Chem. 25*, 202-6.

monosulphones

Badescu, G., et al. (**2014**) *Bioconjugate Chem. 25*, 460-9.

Hydrolyze thiosuccinimides after conjugation



thiosuccinimide

thioether

Lin, D., Saleh, S., and Liebler, D. C. (**2008**) *Chem. Res. Toxicol. 21*, 2361-2369

Palanki, M.S. et al. (**2012**) *Biorg. Med. Chem. Lett*. 22, 4249-4253.

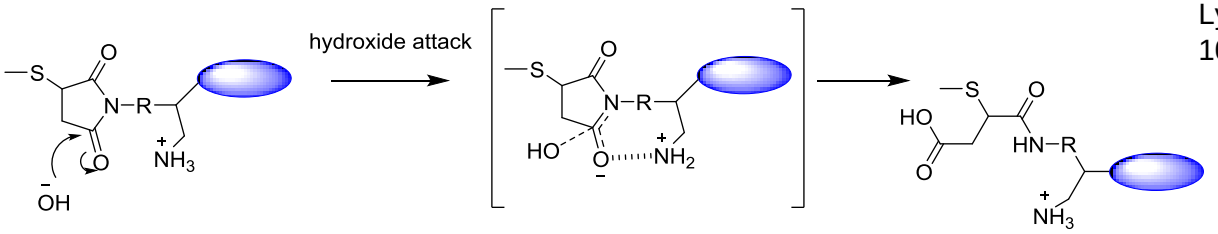
Fontaine, et al. (**2015**) *Bioconjugate Chem. 26*, 145-52.

Tumey, L. N., et al. (**2014**) *Bioconjugate Chem. 25*, 1871-80.

Lyon, R. P., et al. (**2014**) *Nat. Biotechnol. 32*, 1059-62.

Chemical approaches to stabilize ssADCs-thiosuccinimide hydrolysis

Maleimide design: proximal amine

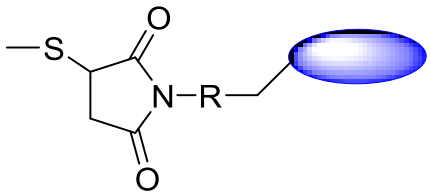


thiosuccinimide

thioether

Lyon, R. P., et al. (**2014**) *Nat. Biotechnol. 32*, 1059-62.

Maleimide design: R groups

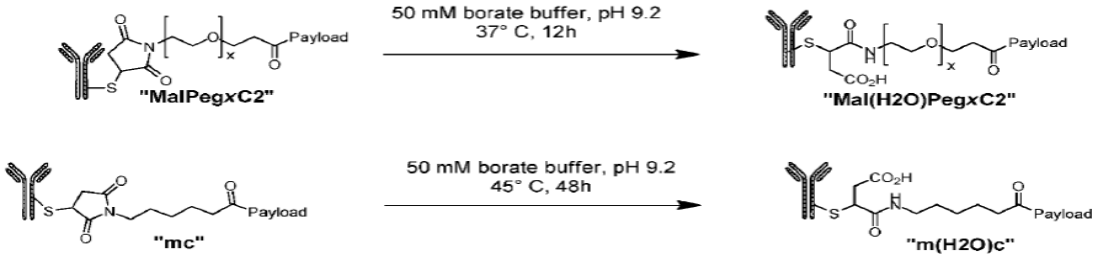


Fontaine, et al. (**2015**) *Bioconjugate Chem. 26*, 145-52.

Table 1. Rates of Ring-Opening and Retro-Michael Reactions of SITEs 2.1−2.19

	side chain[a]	$t_{1/2(Hyd+Exch)}$[b] hr	$t_{1/2,Hyd}$ hr	$t_{1/2,Exch}$ hr	$k_{Hyd}/(k_{Hyd}+k_{Exch})$
1	–Et	142	220	400	0.65
2	$-(CH_2)_5C(O)NH(CH_2)_2OCH_3$	180[c]	310	450	0.60
3	$-(CH_2)_2NH_2$	0.41	0.42	12	0.97
4	$-(CH_2)_2NC(O)OiPr$	45	55	260	0.83
5	$-(CH_2)_2N(CH_3)(CH_2)_2NHC(O)OiPr$	0.36	0.37 (1.2)[d,e]	36	0.99
6	$-(CH_2)_2N(-CH_2CH_2-)_2NBoc$	1.6	1.6 (1.7)[d]	61	0.97
7	$-(CH_2)_2CH_2N(-CH_2CH_2-)_2NC(O)OiPr$	12	12	180	0.93
8	$-(CH_2)_2N^+(CH_3)_2(CH_2)_2NHBoc$	1.5	1.6	29	0.95
9	$-(CH_2)_2N^+(CH_3)(-CH_2CH_2-)_2NBoc$	1.2	1.3	24	0.95
10	$-(CH_2)_2CH_2N^+(CH_3)(-CH_2CH_2-)_2NBoc$	5.7	6.2	67	0.92
11	$-CH_2C(O)NH(CH_2)_2NHBoc$	4.3	4.5	87	0.92
12	$-CH_2C(O)NH(CH_2)_2NHC(O)Ar$[f]	6.5	6.9	110	0.94
13	$-(CH_2)_2C(O)NH(CH_2)_2NHBoc$	26	30	220	0.88
14	$-(CH_2)_2SO_2(CH_2)_3C(O)NH(CH_2)_2OCH_3$	10	11	150	0.93
15	$-CH_2C{\equiv}CH$	5.4	5.7	94	0.94
16	$-CH(CF_3)CH_2C(O)NH(CH_2)_2NHBoc$	5.8	7.6	26	0.77
17	$-CH(CH_2SCH_3)C(O)NH(CH_2)_2OCH_3$	3.8	4.1	69	0.94
18	$-(CH_2)_2O(CH_2)_2OH$	35	40	280	0.87
19	$-CH(CO_2H)(CH_2)_2NHBoc$	170	220	690	0.77

Specific conditions: pH, temperature



Example of forced hydrolysis of maleimide-based conjugates

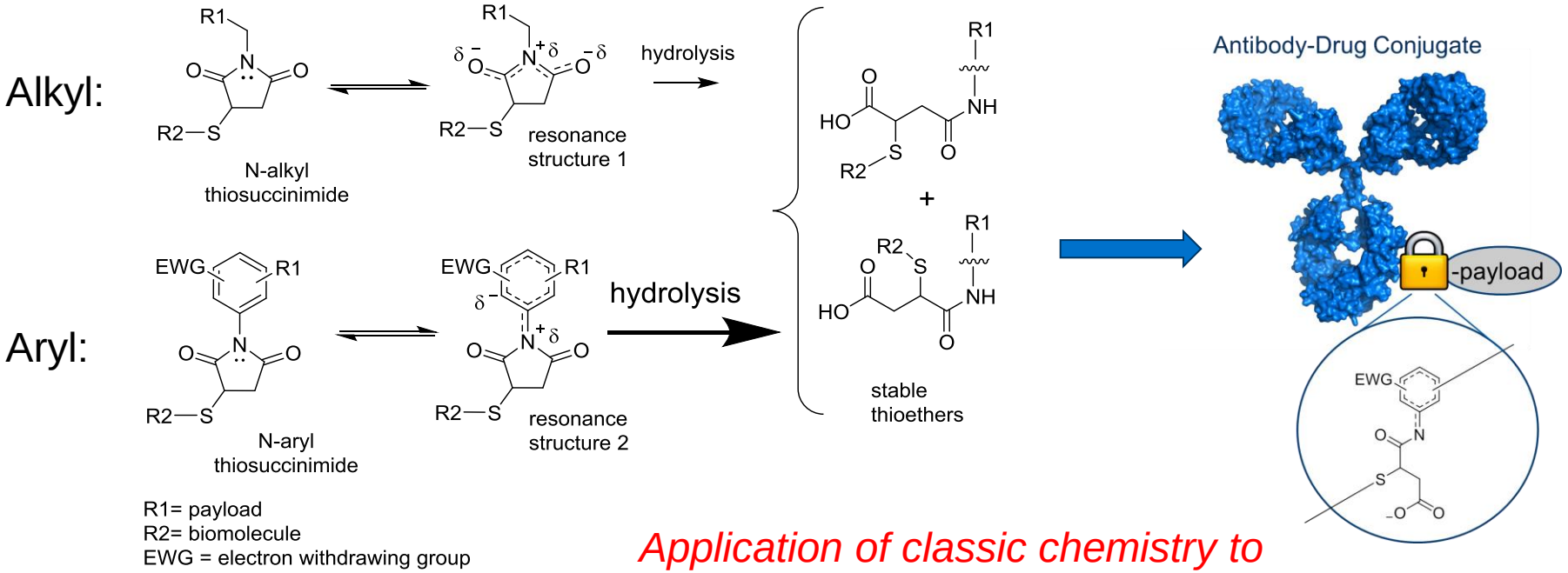
Tumey, L. N., et al. (**2014**) *Bioconjugate Chem. 25*, 1871-80.

Resonance-promoted thiosuccinimide hydrolysis

Hypothesis:

N-aryl maleimides will spontaneously produce stable thiol conjugates under mild conditions through resonance-driven thiosuccinimide hydrolysis.

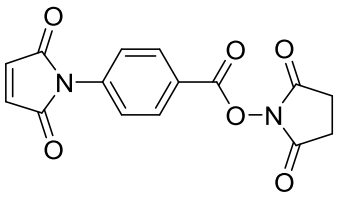
Concept:



Application of classic chemistry to solve a current problem

Machida, M., Machida, M. I., and Kanaoka, Y. (**1977**) *Chem. Pharm. Bull. 25*, 2739-2743.

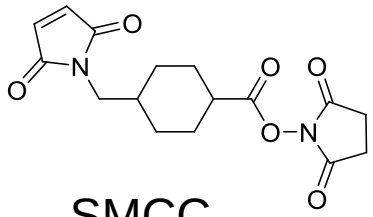
Why aren't N-aryl maleimide used for ADC preparation?



MBS

Kitagawa, T. and Aikawa, T.
J Biochem (Tokyo) (**1976)** 79:233-236

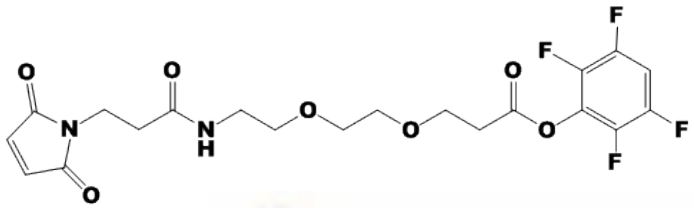
Maleimide hydrolysis + ester hydrolysis
= some crosslinking



SMCC

Partis, M.D., et al.
J Protein Chem (**1983**) 2:263-277

Reduced maleimide hydrolysis
= better crosslinking



QUANTA BIODESIGN LIMITED

Reduced maleimide hydrolysis +
reduced ester hydrolysis
= best crosslinking

crosslinker design has carried over to ADC payload design, i.e. hydrolytically stable maleimides

Molecules used to test hypothesis

Maleimides:

1 N-propionyl maleimido-PEG$_7$-biotin

2 N-(4-acetylphenyl) maleimido-PEG$_7$-biotin

3 N-(3-carboxy-4-fluorophenyl) maleimido-PEG$_7$-biotin

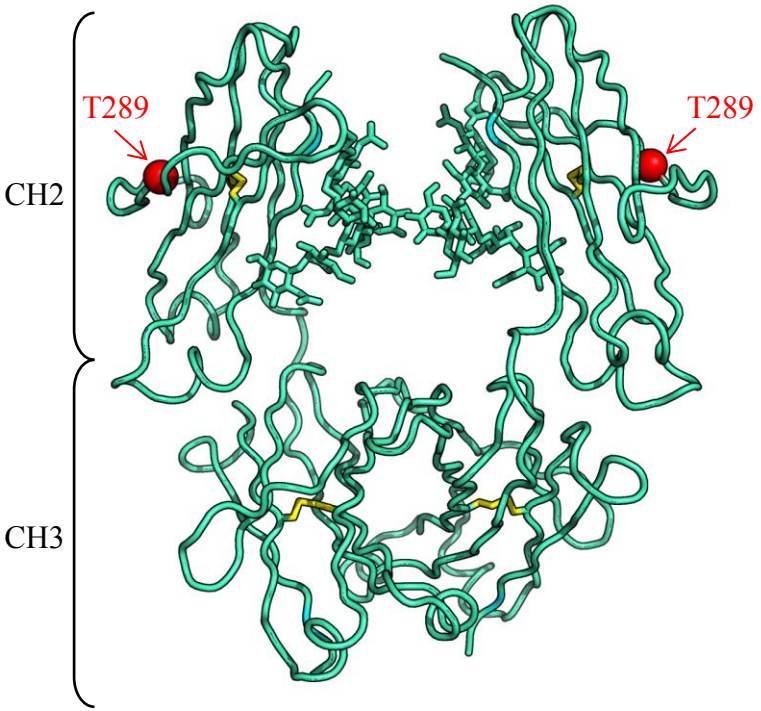
4 N-caproyl maleimido-Val-Cit-PAB-MMAE

5 N-(4-acetylphenyl) maleimido-Val-Cit-PAB-MMAE

- *Hydrophilic PEG-biotin payloads*
- *MMAE ADC payload*

Antibody:

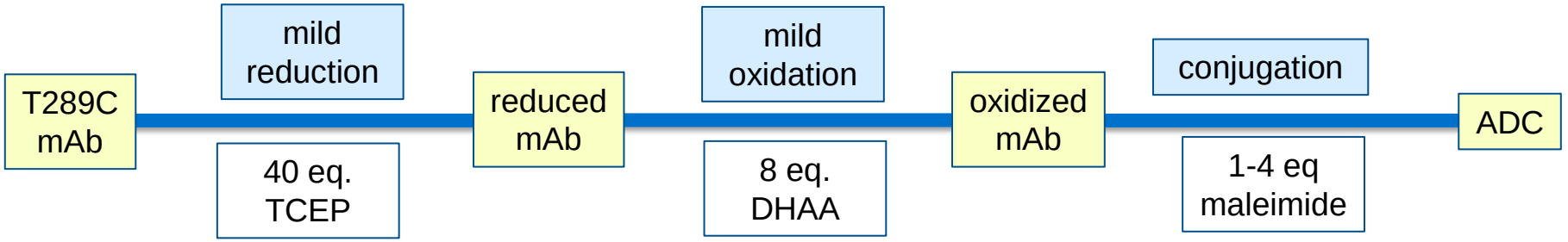
Fc domain:



- *solvent-exposed engineered cysteine for site-specific conjugation*

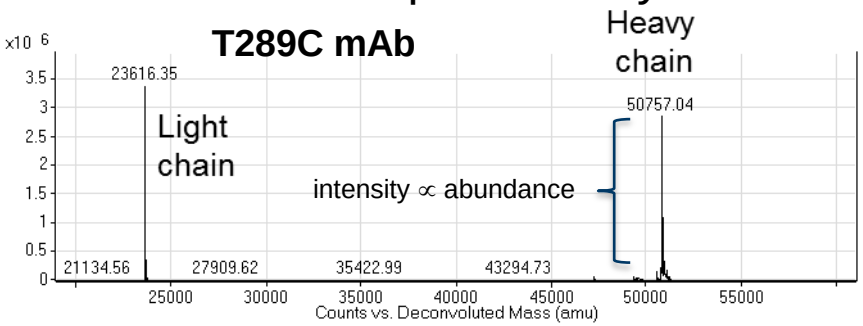
Payload Conjugation and Analysis

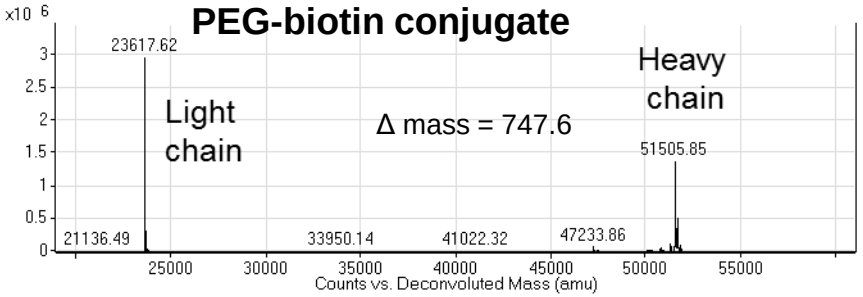
Conjugation Process:



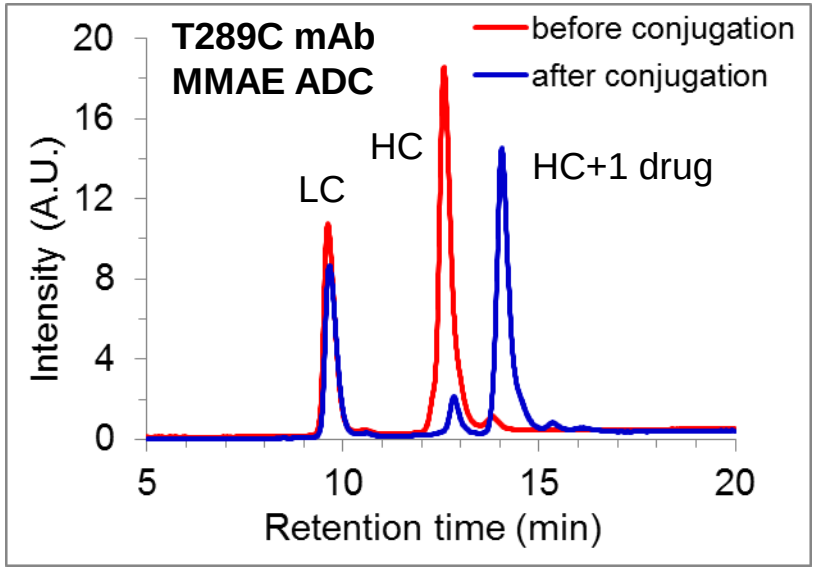
Analysis of Conjugates:

Reduced mass spectrometry





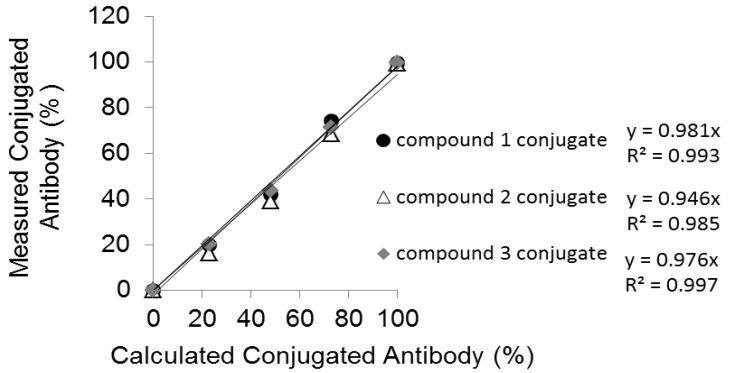
Reduced RP-HPLC



Valliere-Douglass, J. F., McFee, W. A., and Salas-Solano, O. (**2012**). *Anal. Chem. 84*, 2843-2849.

Quantification by Mass Spectrometry

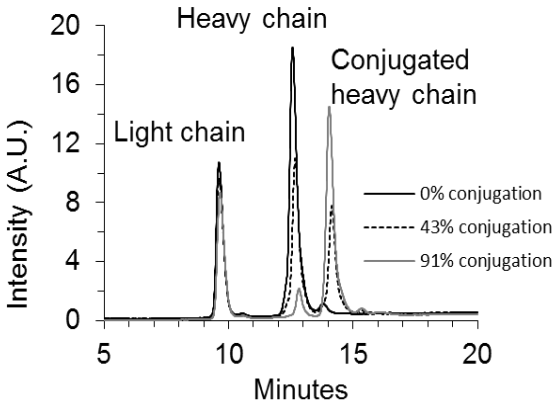
PEG-biotin conjugates



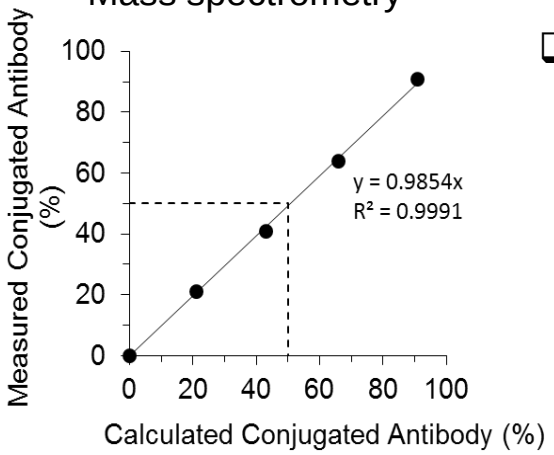
- Conjugated mAb mixed with unconjugated mAb at known ratios
- Deconvoluted mass spectrometry peak intensities used for calculations

MMAE conjugates

rRP-HPLC



Mass spectrometry

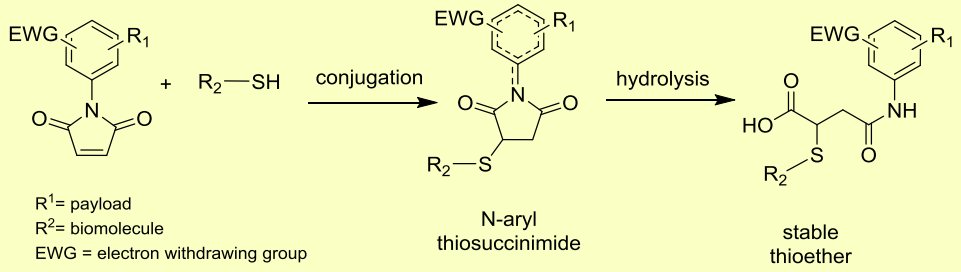


- rRP-HPLC heavy chain peak areas compared to deconvoluted mass spectrometry peak intensities

Mass spectrometry is quantitative for payloads used in this study

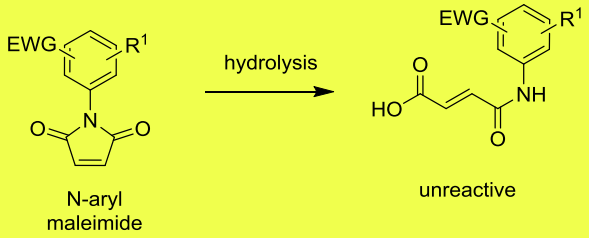
Maleimide Conjugation Efficiency

Desired reaction



vs.

Competing reaction



No thiol conjugation

Summary of Maleimide-PEG-Biotin Conjugation and Hydrolysis Kinetics

pH	5.5			7.4			8.6		
Maleimide type	**alkyl**	**phenyl**	**F-phenyl**	**alkyl**	**phenyl**	**F-phenyl**	**alkyl**	**phenyl**	**F-phenyl**
Conjugation @ 1hr (%) [a,c,e]	**47**	**57**	**78**	**>90**	**>90**	**>90**	**>90**	**>90**	**>90**
Conjugation $T_{1/2}$ (min)[a,e]	**74**	**40**	**17**	**1.7**	**0.72**	**0.21**	**~0.33[d]**	**~0.28[d]**	**~0.25[d]**
Maleimide hydrolysis $T_{1/2}$ (min)[a,b]	**2.8×10^4**	**1400**	**770**	**304**	**55**	**28**	**83**	**21**	**9**

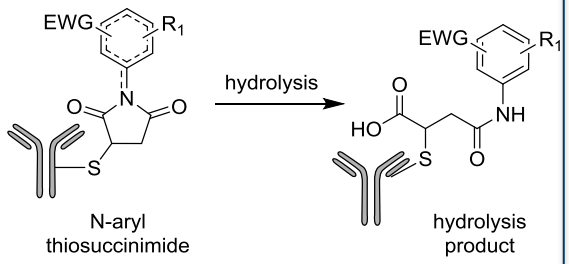
[a] Determined by mass spectrometry. [b] Determined by HPLC. [c] 1 h reaction. [d] Estimated from 2 measurements. [e] T289C mAb reaction performed at 1:1 thiol:maleimide stoichiometry, 1.3 mg/mL antibody and 17.3 µM maleimide.

Increased reactivity enabled efficient conjugation for N-aryl maleimides

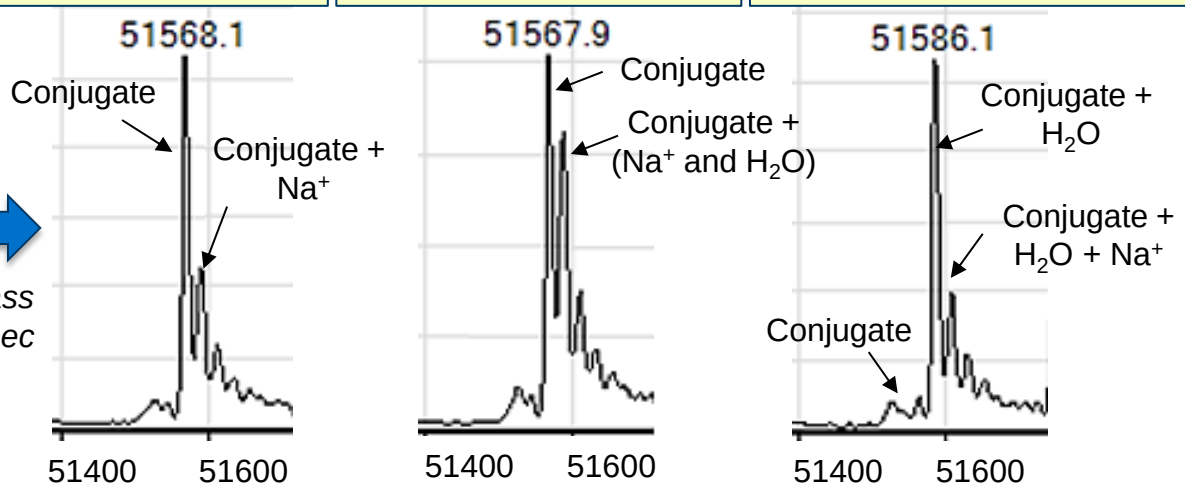
R. J. Christie, *et al*. *J. Controlled Release* **2015**, 220, 660-670.

Thiosuccinimide Hydrolysis

Observed reaction

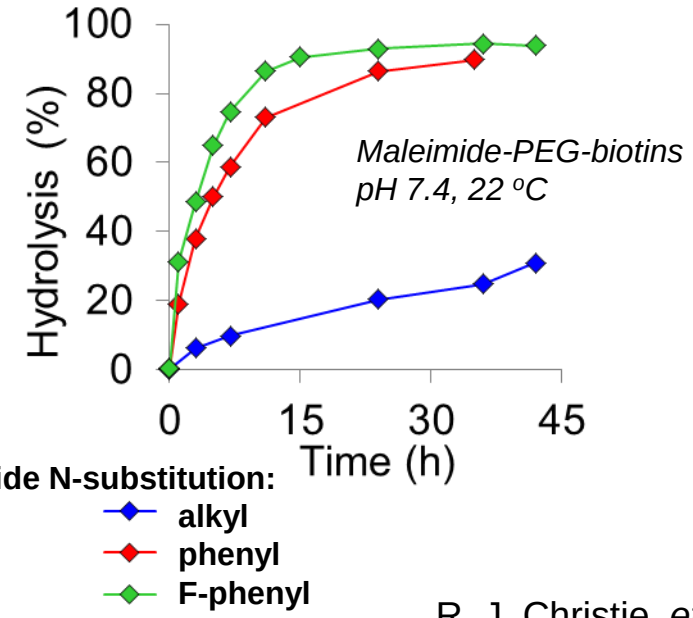


mass spec



reduced glycosylated mass spectrometry

Thiosuccinimide Hydrolysis



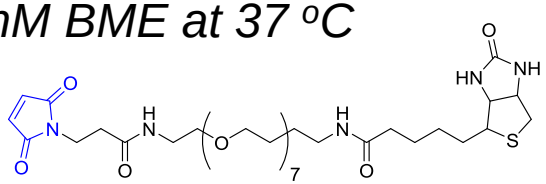
Hydrolysis half-lives

	Thiosuccinimide hydrolysis $T_{1/2}$ (hr)		
Functional group	pH 7.4, 22 °C	pH 7.4, 37 °C	pH 8.6, 22 °C
alkyl	96	32	10
phenyl	6.4	1.5	0.8
F-phenyl	4.3	0.7	0.4

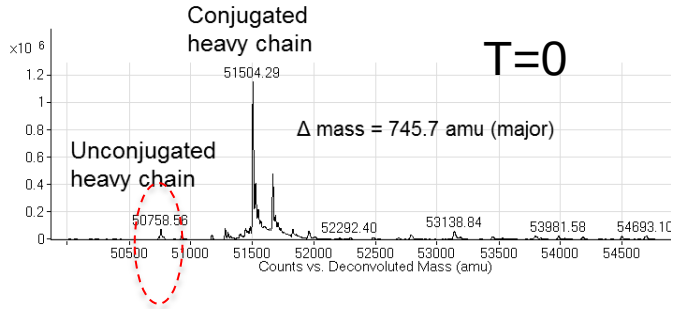
R. J. Christie, *et al*. *J. Controlled Release* **2015**, 220, 660-670.

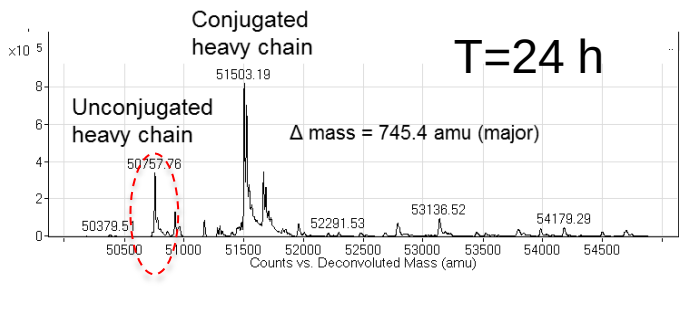
Conjugate Stability in Buffer

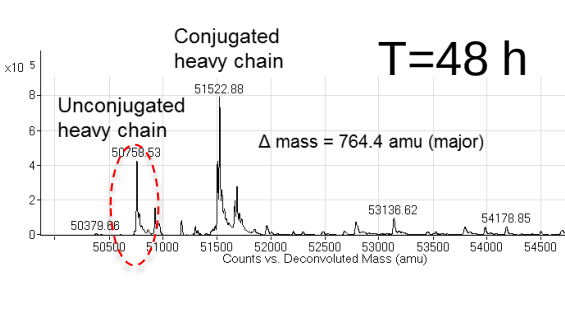
- *Stability assessed in PBS, pH 7.4 containing 143 mM BME at 37 °C*
- *Maleimide-PEG-biotin conjugates*
- *Mass spectrometry analysis*

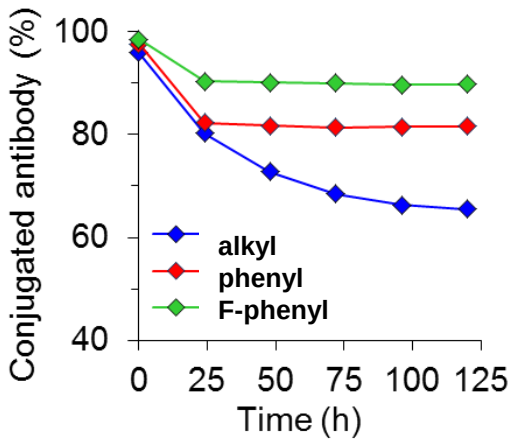


N-propionyl maleimido-PEG$_7$-biotin

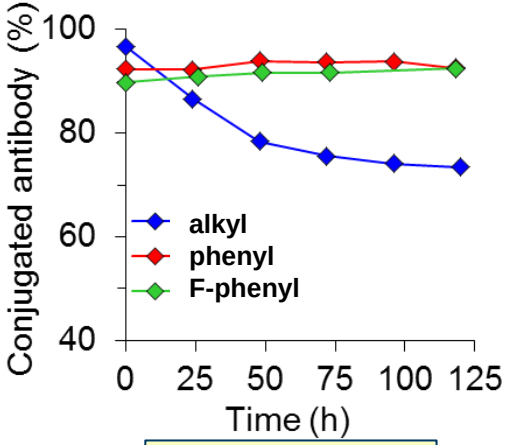




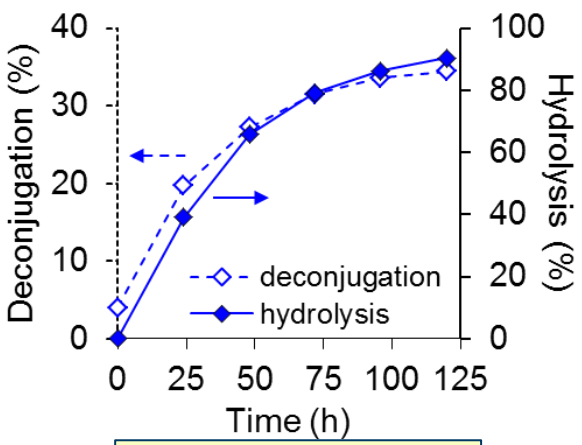




No forced hydrolysis



Forced hydrolysis
pH 8.6, 1hr, 37 °C



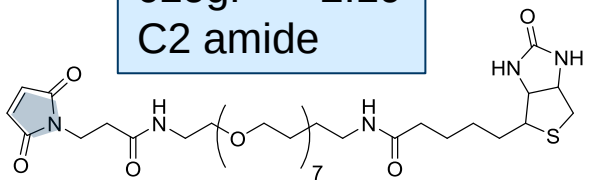
N-alkyl thiosuccinimide
No forced hydrolysis

- N-aryl maleimide conjugates are more stable
- Thiosuccinimide hydrolysis limits deconjugation

R. J. Christie, *et al*. *J. Controlled Release* **2015**, 220, 660-670.

Effect of Payload on Conjugate Stability

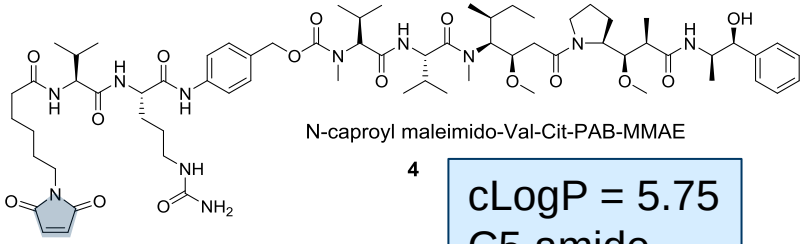
cLogP = -2.19
C2 amide



N-propionyl maleimido-PEG$_7$-biotin

1

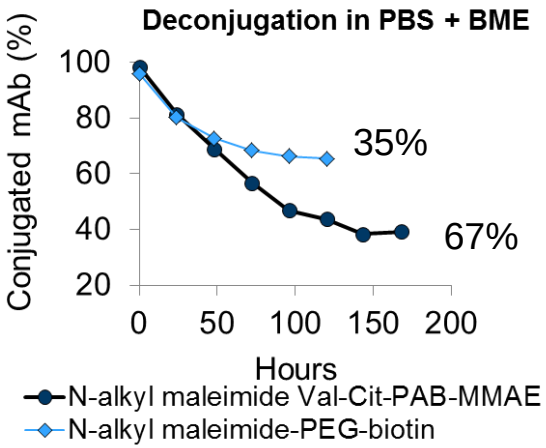
vs.

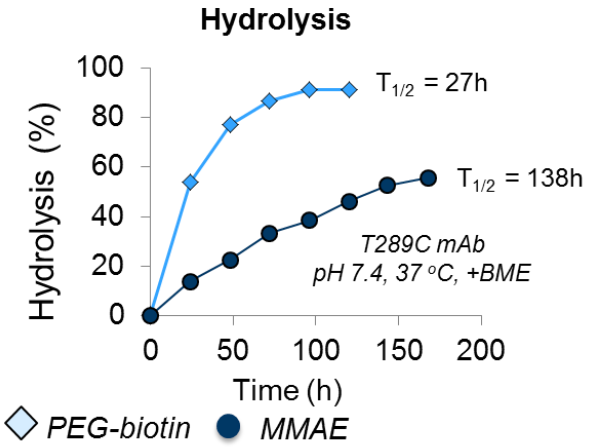


N-caproyl maleimido-Val-Cit-PAB-MMAE

4

cLogP = 5.75
C5 amide





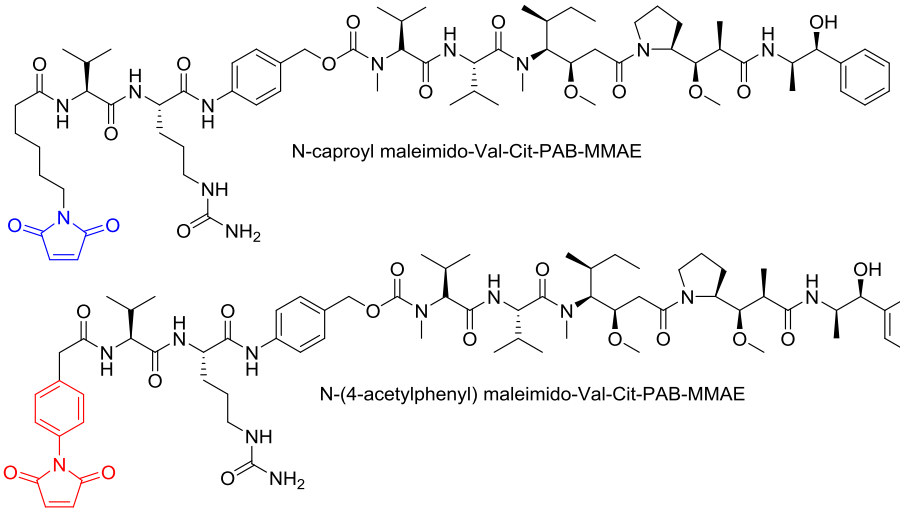
Payload deconjugation rate constants (37 °C)

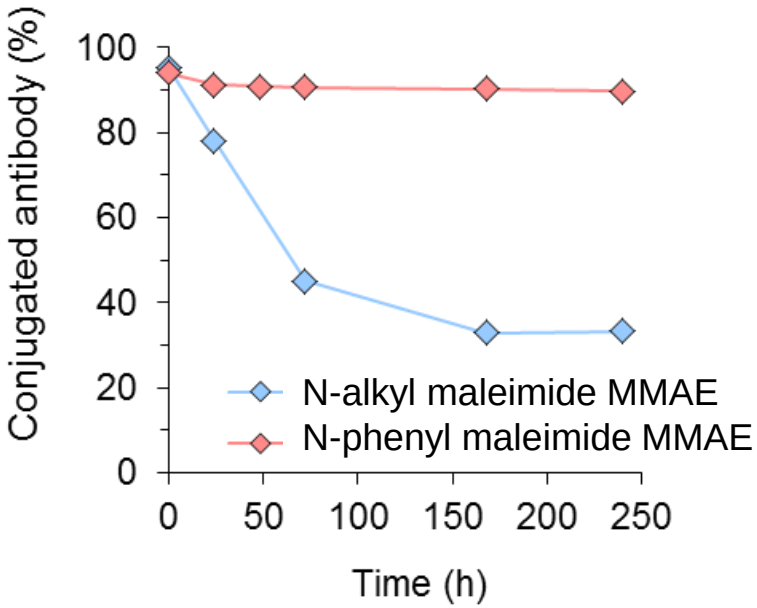
	pH 7.4 + BME (147 mM)		mouse serum
compound	PEG-biotin	MMAE	MMAE
Deconjugation ($K_{1, obs}$, s^{-1})	$1.3x10^{-6}$	$1.9x10^{-6}$	$1.8x10^{-6}$
Deconjugation $T_{1/2}$ (hr)	148	101	107
Maximum deconjugation (%)	35	60	67

- PEG and/or short amide linkage accelerate hydrolysis
- Payload hydrophobicity may also impact hydrolysis
- Slowly hydrolyzing thiosuccinimides exhibit more deconjugation.

Conjugate Stability in Serum

- *Whole mouse serum, 37 °C*
- *Maleimide-MMAE conjugates*
- *Immunocapture*
- *Mass spectrometry analysis*



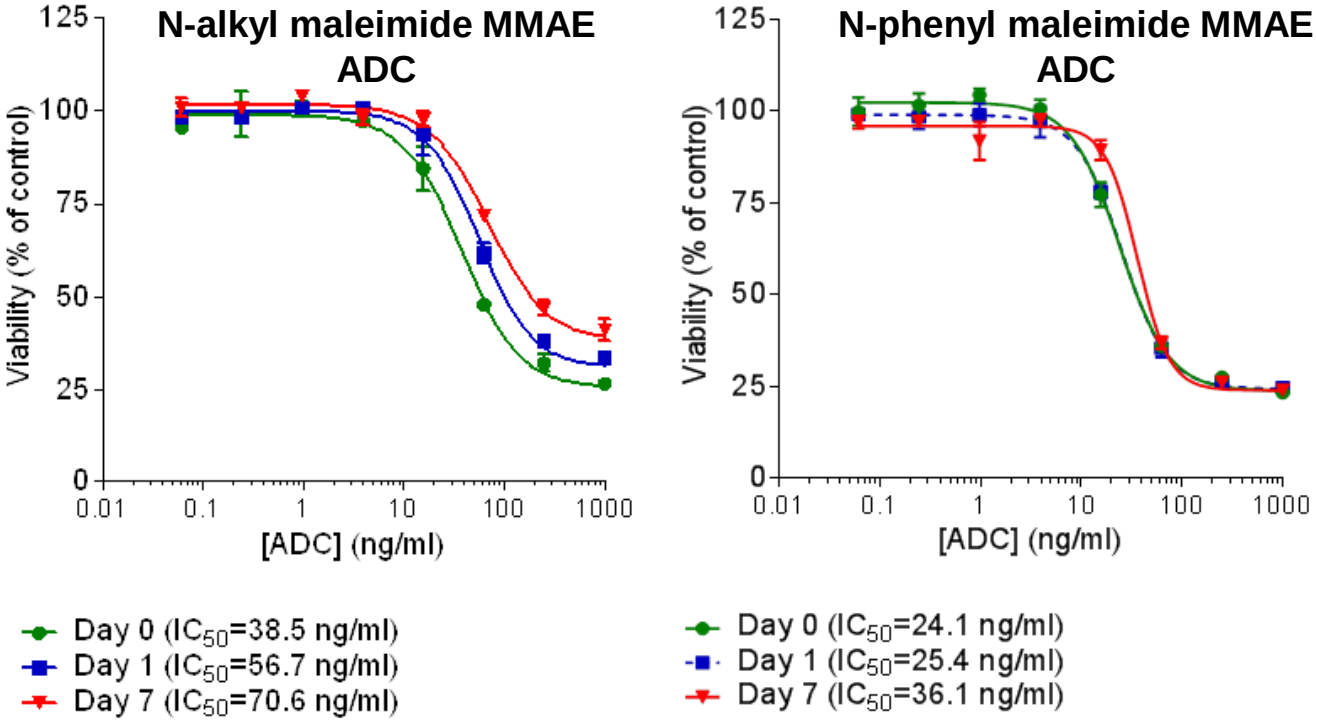


- N-phenyl maleimide ADC is stable in mouse serum for over 1 week.

R. J. Christie, *et al. J. Controlled Release* **2015**, 220, 660-670.

Activity of Serum-Incubated ADCs

- *Receptor-positive cancer cell line*
- *Maleimide-MMAE conjugates*



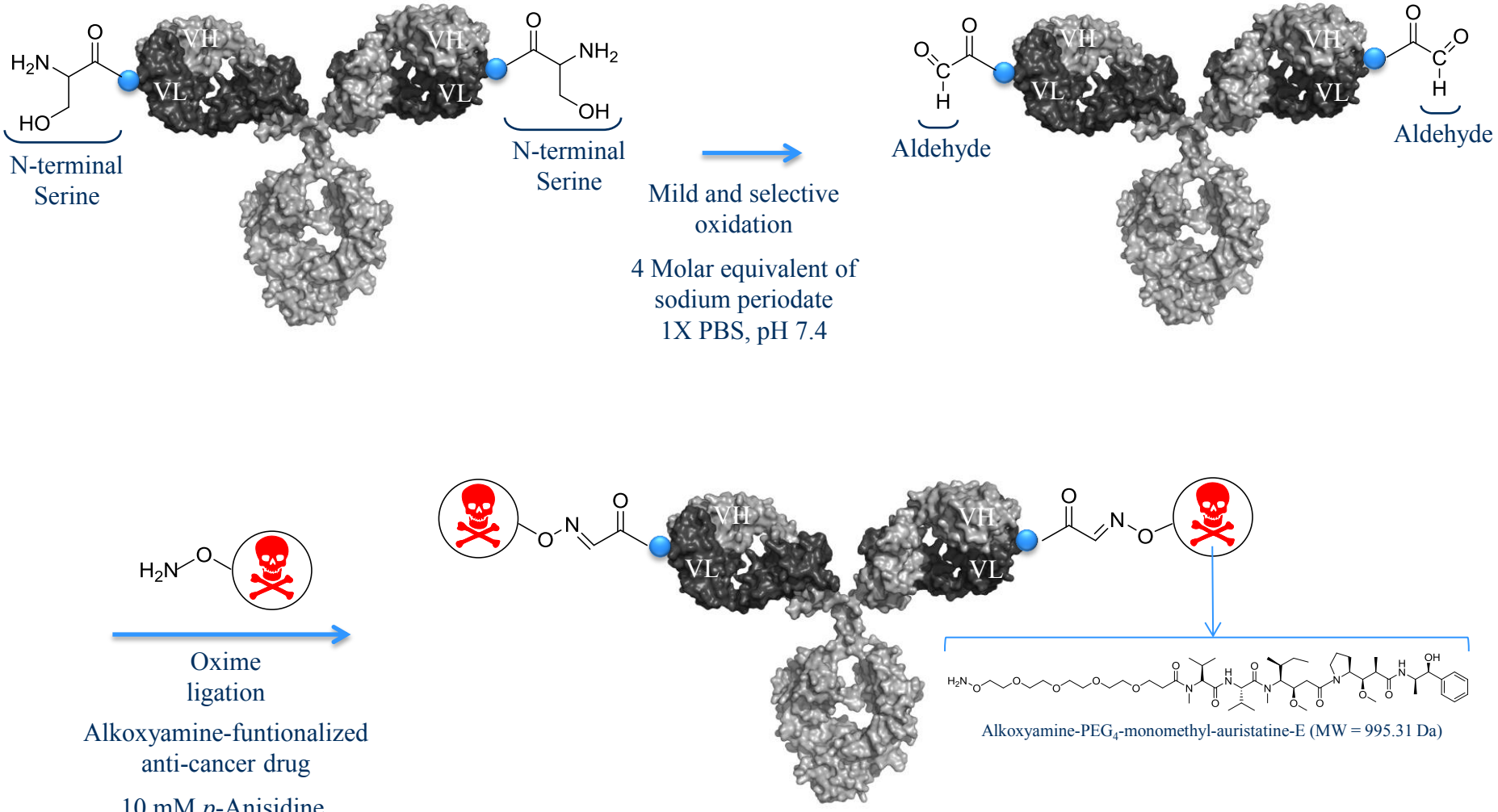
- N-alkyl maleimide ADC lost potency
  - IC_{50}
  - Max. kill
- N-phenyl maleimide ADC retained potency

R. J. Christie, *et al*. *J. Controlled Release* **2015**, 220, 660-670.

Overview

- Introduction - the instability associated with thiol-maleimide conjugation chemistry
- Optimization of maleimide chemistry for efficient and stable conjugation to antibodies
- Hydrolytically stable site-specific conjugation at the N-terminus of an engineered antibody
- Acknowledgements

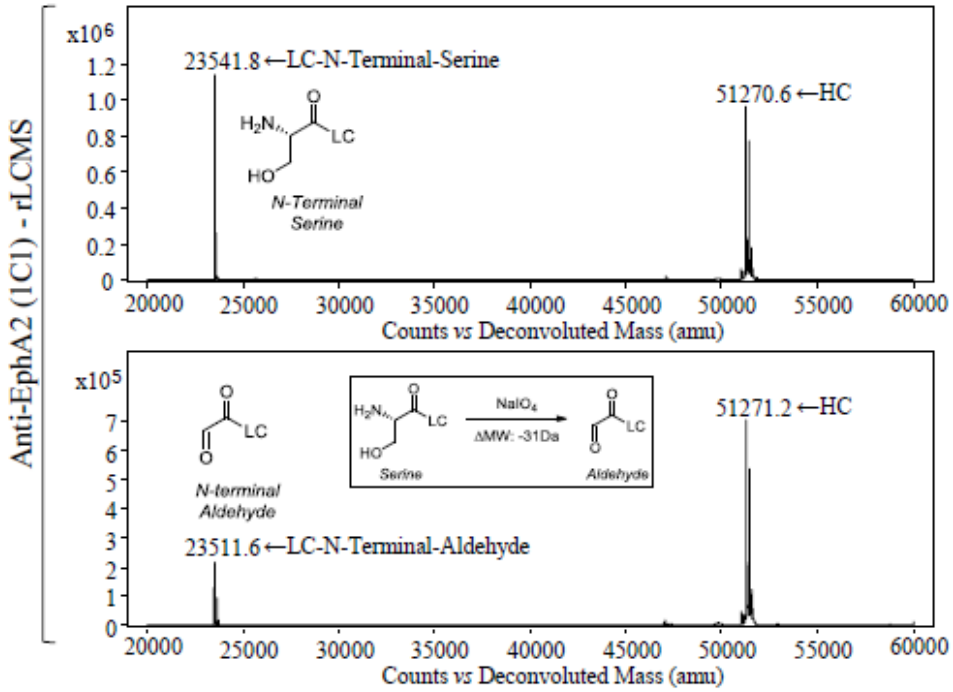
Site-specific ADCs based on *N*-terminal serine enables site-specific conjugation of a drug into any given mAbs

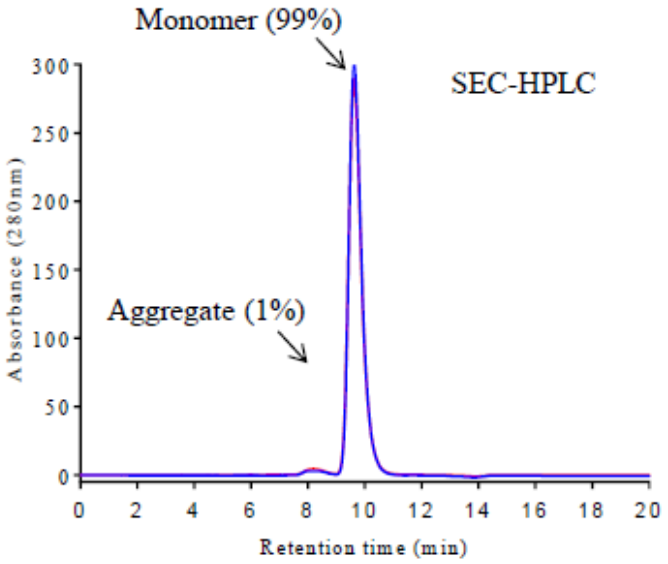


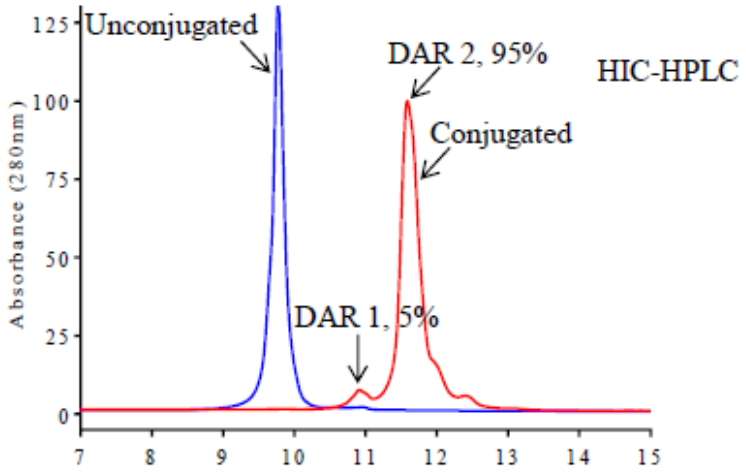
MedImmune

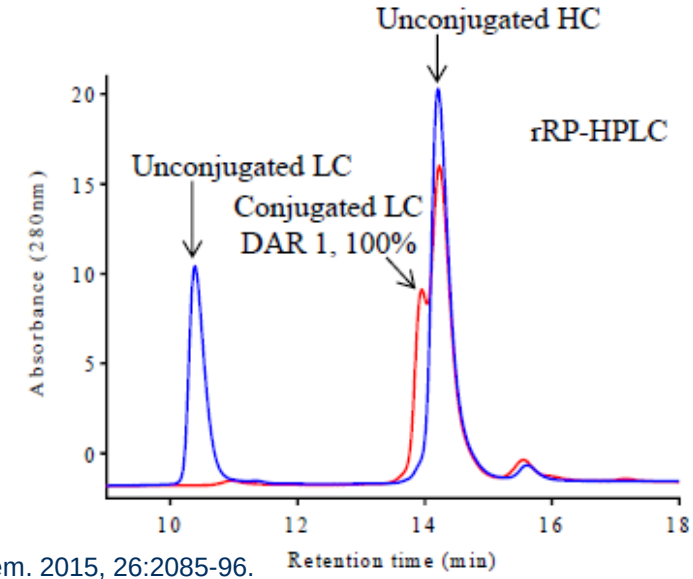
Thompson et al., Bioconjug Chem. 2015, 26:2085-96.

Quantitative oxidation of the N-terminal serine and analytical characterization of the Serine-ADC



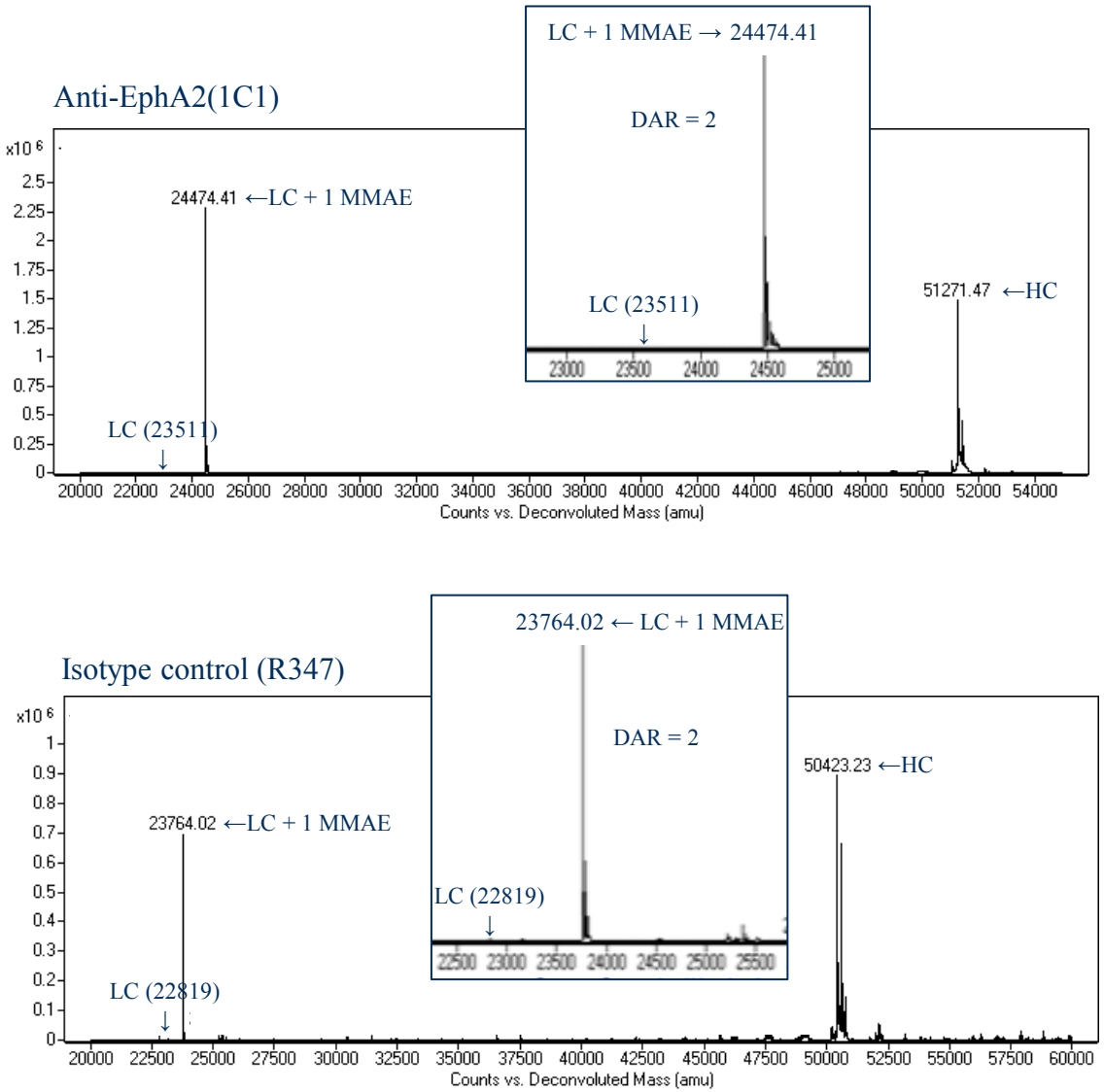






Thompson et al., Bioconjug Chem. 2015, 26:2085-96.

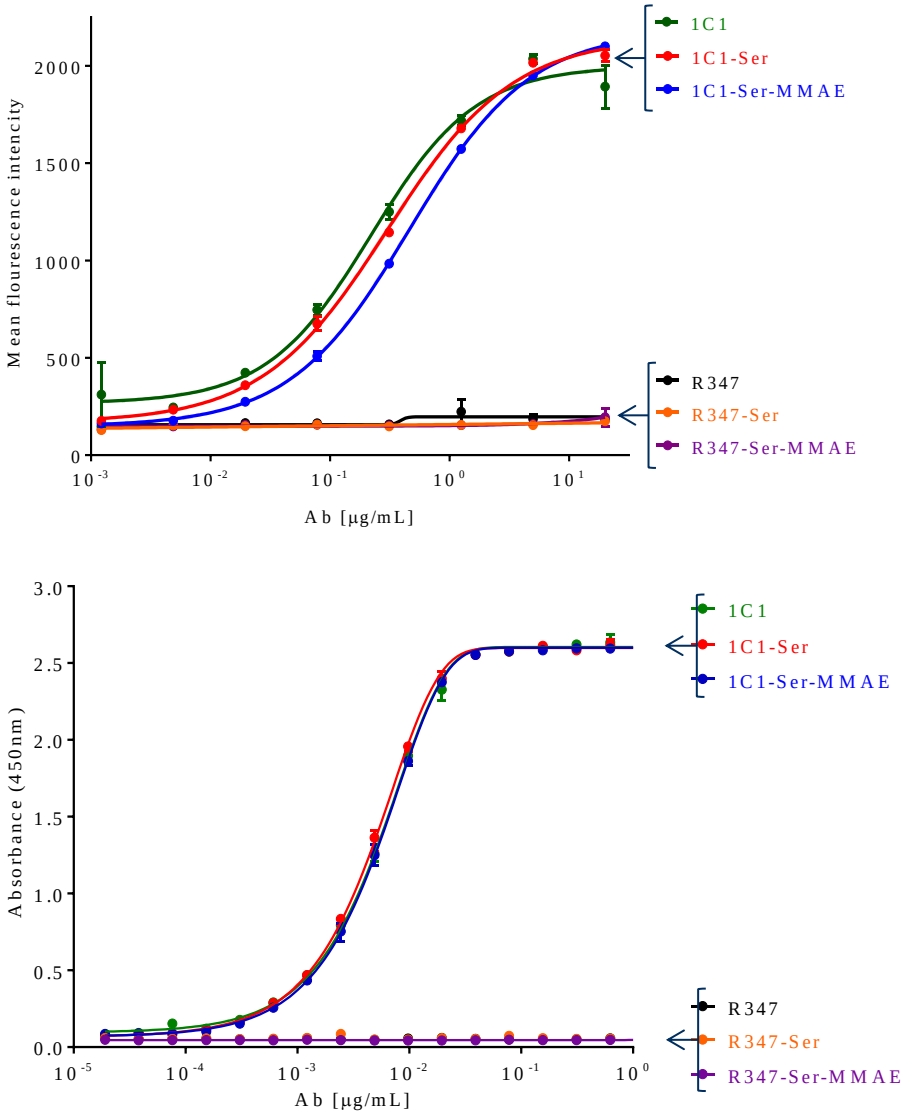
Conjugation of the alkoxyamine-PEG4-MMAE to the N-terminal aldehyde is highly efficient



MedImmune

Thompson et al., Bioconjug Chem. 2015, 26:2085-96.

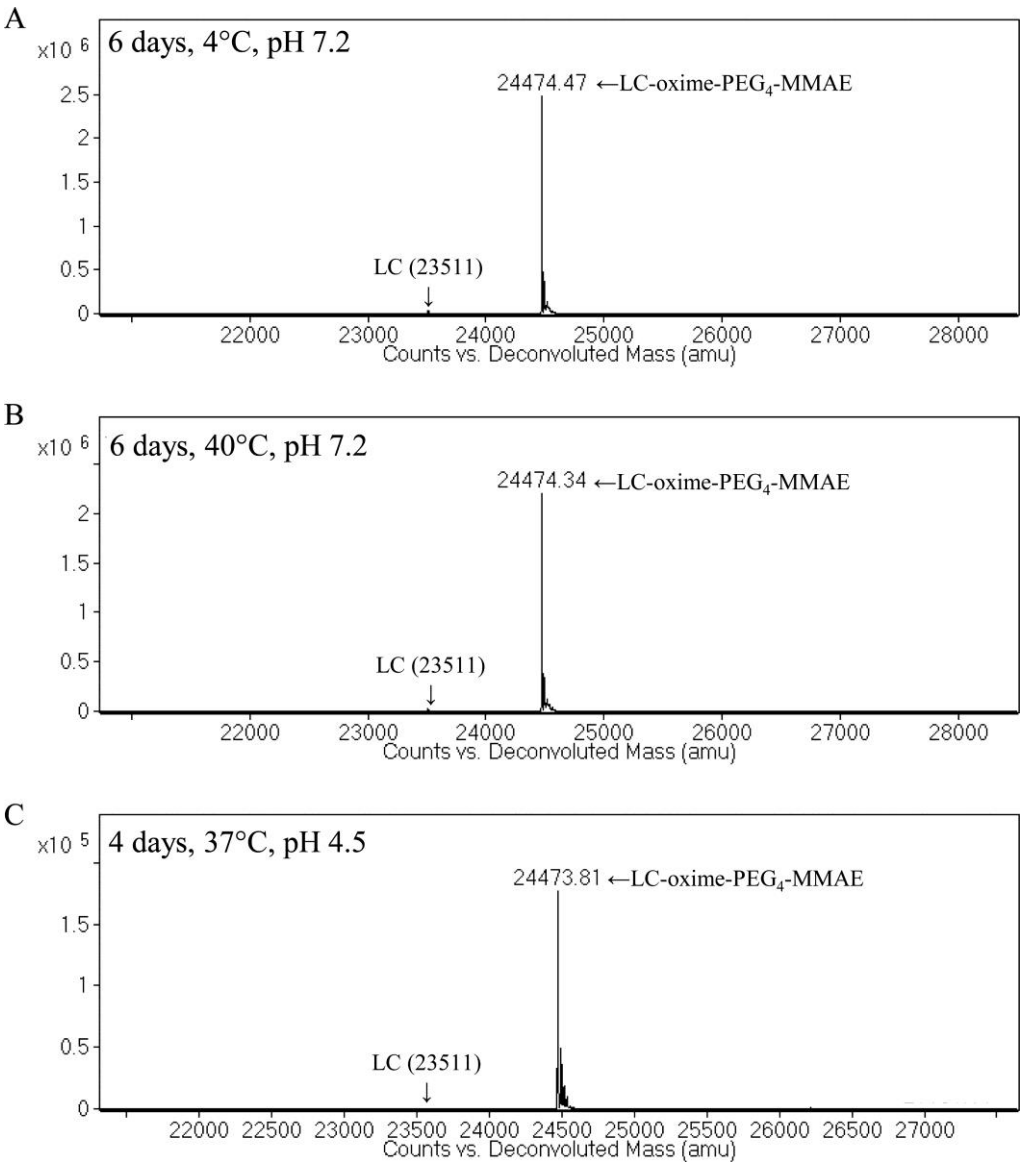
Drug conjugated to N-terminus of an antibodies doesn't have impact on antigen binding



MedImmune

Thompson et al., Bioconjug Chem. 2015, 26:2085-96.

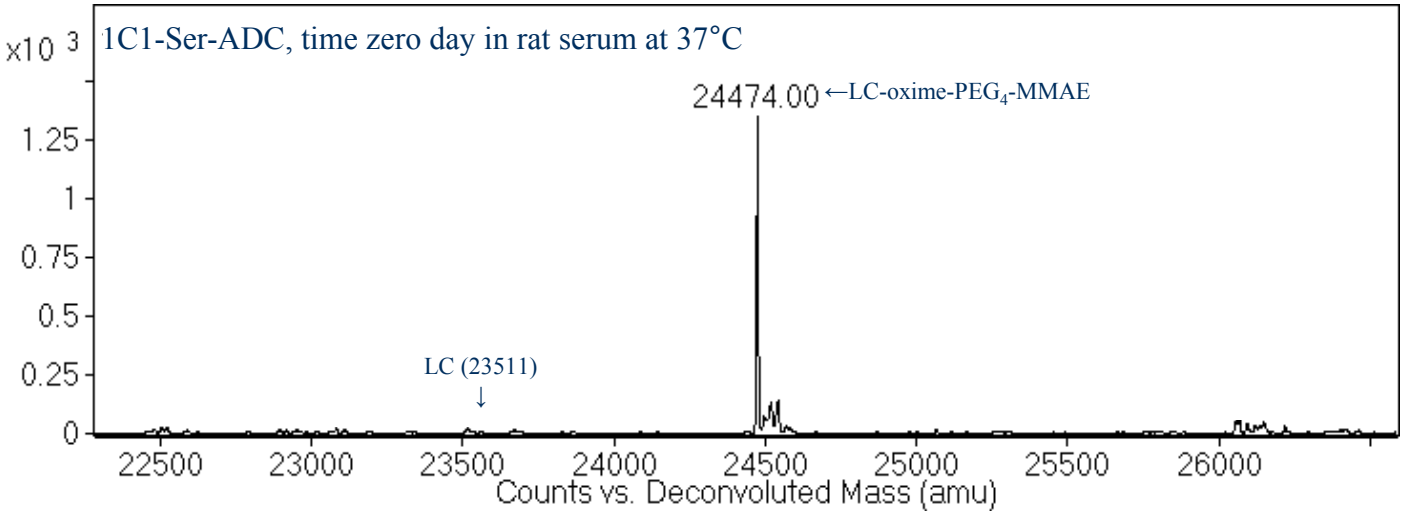
Hydrolytic stability of site-specific ADCs based on N-terminal serine

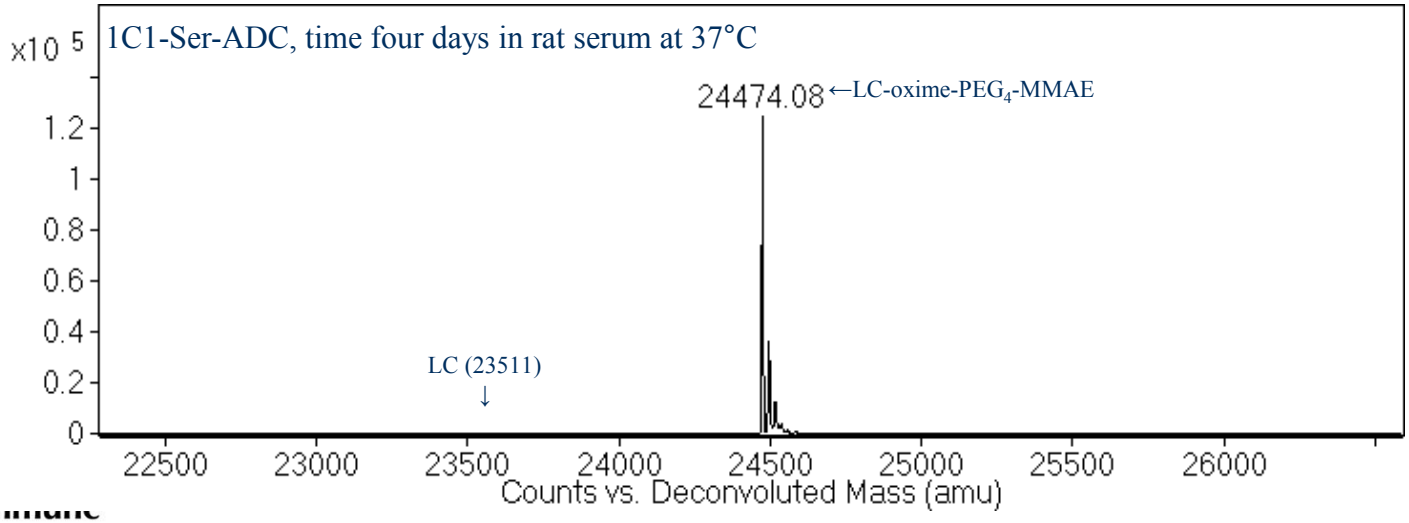


MedImmune

Thompson et al., Bioconjug Chem. 2015, 26:2085-96.

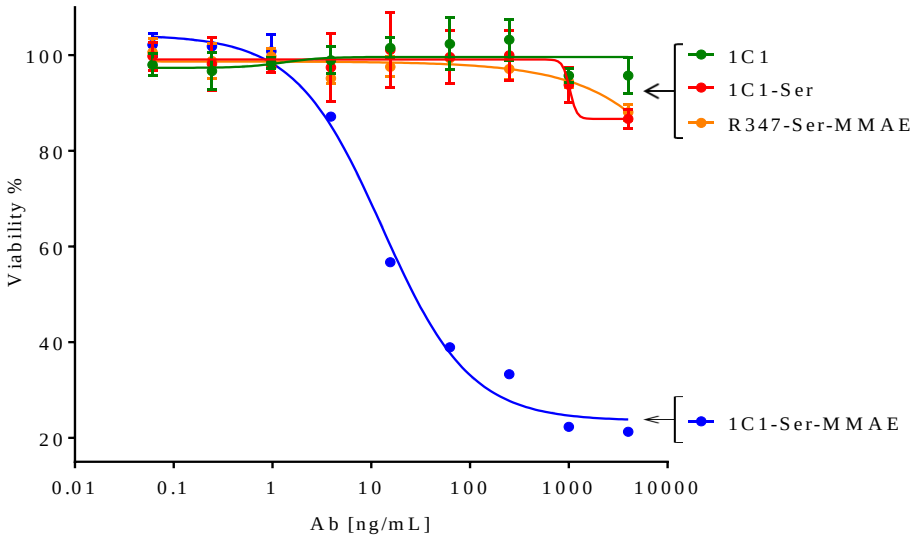
Site-specific ADCs based on N-terminal serine is serum stable

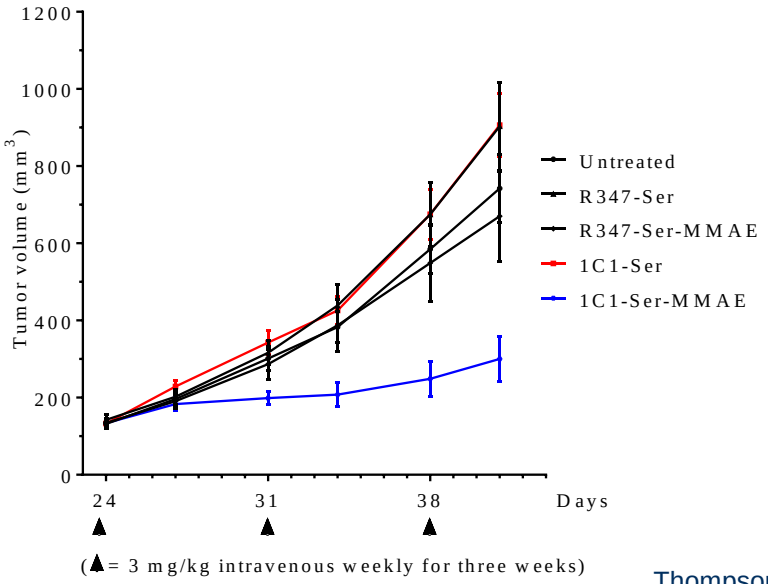




Thompson et al., Bioconjug Chem. 2015, 26:2085-96.

Site-specific ADCs based on N-terminal serine is highly active both in vitro and in vivo





MedImmune

Thompson et al., Bioconjug Chem. 2015, 26:2085-96.

Summary

- Thiol/maileimide conjugation chemistry for antibody drug conjugates
  - Thiol/maileimide conjugation can undergo drug exchange, resulting in drug premature release and decreased serum stability and in vivo efficacy
  - Position matters for ADCs using thiol/maileimide conjugation chemistry
- N-Aryl maleimides have been developed and applied to stabilize thiol-linked ADCs for increased potency and potentially improved therapeutic index.
  - Both efficient thiol conjugation and thiosuccinimide hydrolysis are achieved due to increased maleimide/thiosuccinimide reactivity.
  - Phenyl substitution allows tuning of both conjugation and hydrolysis kinetics.
  - N-Aryl maleimides are a simple and convenient platform to produce stable bioconjugates such as polymers, nanoparticles, biosensors, and more
- Site-specific ADC generated using the engineered N-terminal serine
  - Has high drug conjugation efficiency
  - Is serum stable
  - Is highly potent both in vitro and in vivo

Acknowledgments

Nazzareno Dimasi

Dorin Toader

Fengjiang Wang

Ryan Fleming

Binyam Bezabeh

Jim Christie

Pam Thompson

Stelios Florinas

Fengying Huang

Cuihua Gao

Srinath Kasturirangan

Jonah Rainey

Lena Shirinian

Jia Lin

Vineela Aleti

Linda Xu

Marcello Marelli

Mike Bowen

Herren Wu

Mary Jane Hinrichs

Shameen Afif-Rider

Rakesh Dixit

Chris Martin (Spirogen)

Philip Howard (Spirogen)

Francois D'Hooge (Spirogen)

Luke Masterson (Spirogen)

Song Cho

Shenlan Mao

Jay Harper

Chris Lloyd

Rob Woods

David Tice

Krista Kinneer

Chris Winter

Ellen O’Connor

Robert Hollingsworth

Ronald Herbst

Adeela Kamal

Cui Chen

Helen Zhong

Zhan Xiao

Xiangyang Wang

Chris Barton

Simon Thompson

David Jenkins

John Li

Parthiv Mahadevia

Steve Coats

Kris Sachsenmeier

Sue Spitz

Samuel Parry

Vanessa Muniz-Medina

Xinzhong Wang

Leslie Wetzel

Jane Osbourn

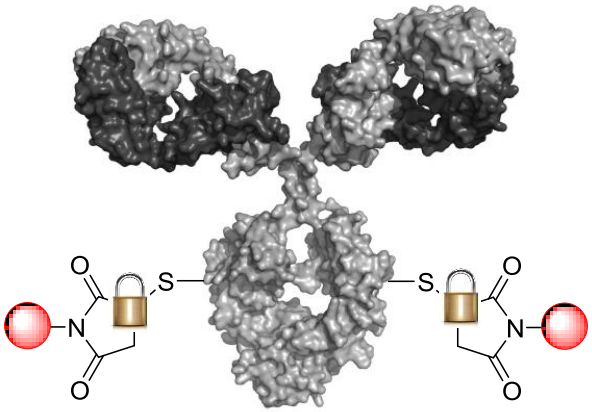
Melissa Vasbinder (AstraZeneca)

Lakshmaiah Gingipalli (AstraZeneca)

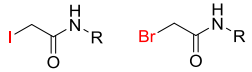
Many others

Thank You

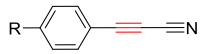
Chemical approaches to stabilize ssADCs



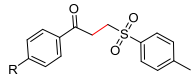
- Change conjugation chemistry
  - Haloacetamides; Arylpropiolonitriles; Monosulphones



Alley, S. C., et al. (**2008**) *Bioconjugate Chem. 19*, 759-5.

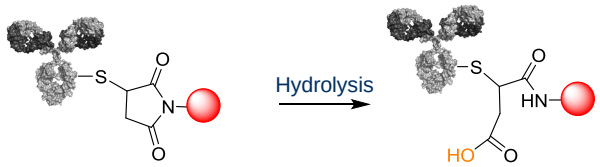


Koniev, O., et al.(**2014**) *Bioconjugate Chem. 25*, 202-6.



Badescu, G., et al. (**2014**) *Bioconjugate Chem. 25*, 460-9.

- Hydrolyze thiosuccinimides after conjugation
  -



Lin, D., et al. (**2008**) *Chem. Res. Toxicol. 21*, 2361-9

Palanki, M.S. et al. (**2012**) *Biorg. Med. Chem. Lett*. 22, 4249-4253.

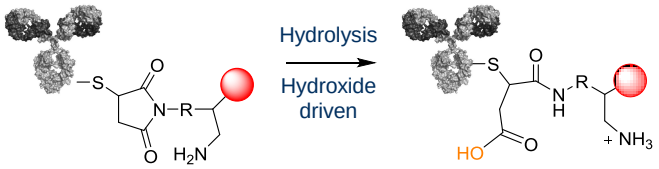
Fontaine, et al. (**2015**) *Bioconjugate Chem. 26*, 145-52.

Tumey, L. N., et al. (**2014**) *Bioconjugate Chem. 25*, 1871-80.

Lyon, R. P., et al. (**2014**) *Nat. Biotechnol. 32*, 1059-62.

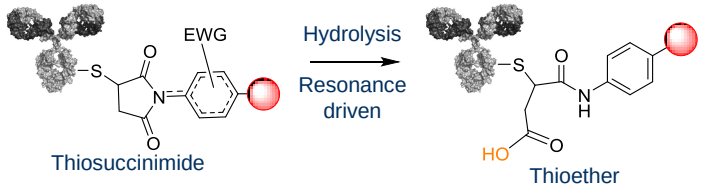
- Maleimide design

A. Proximal amine-promoted thiosuccinimide hydrolysis



Lyon, R. P., et al. (**2014**) *Nat. Biotechnol. 32*, 1059-62.

B. Resonance-promoted thiosuccinimide Hydrolysis (*N*-Aryl-Maleimide)



Christie et al., J Control Release. 2015, 220:660-70.

MedImmune