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Assessment of reagents for selenocysteine conjugation and the stability of selenocysteine adducts†

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Conventional antibody–drug conjugates (ADCs) are heterogeneous mixtures that have poor pharmacokinetic properties and decreased efficacy relative to homogenous ADCs. Furthermore, ADCs that are maleimide-based often have inadequate circulatory stability, which can result in premature drug release with consequent off-target toxicities. Selenocysteine-modified antibodies have been developed that allow site-specific antibody conjugation, yielding homogeneous ADCs. Herein, we survey several electrophilic functional groups that react with selenocystine with high efficiency. Several of these result in conjugates with stabilities that are superior to maleimide conjugates. Among these, the allenamide functional group reacts with notably high efficiency, leads to conjugates with remarkable stability, and shows exquisite selectivity for selenocysteine conjugation.

Introduction

Antibody-drug conjugates (ADCs) hold remarkable promise as cancer chemotherapeutic agents. The approval of brentuximab vedotin[1] and ado-trastuzumab emtansine[2] by the FDA for the treatment of relapsed Hodgkin's lymphoma and HER2$^+$ breast cancer, respectively, has renewed interest in ADCs.[3–5] While these two ADCs have been moderately successful, there remains room for improvement. First, the drugs are conjugated using hinge cysteine (Cys) and surface lysine residues, respectively, but owing to the abundance of these residues in antibody molecules, heterogeneous mixtures of ADCs result.[6] The heterogeneity convolutes pharmacokinetic studies and diminishes their therapeutic indices.[7] Secondly, the ADCs contain a thiol-succinimide unit known to hydrolyze in aqueous media, introducing more heterogeneity.[8,9] In addition, the currently approved conjugates may undergo a retro-Michael elimination resulting in premature release of the cytotoxic drugs; this also produces a maleimide-based drug that can react with plasma thiols.[10–12]

To address these issues, several groups are pursuing site-specific antibody conjugation. Published strategies toward the goal of site-specific conjugation fall into four main categories: introducing reactive cysteines,[7,13,14] incorporating unnatural amino acids,[10,15,16] enzymatic engineering of antibodies,[17–19] and incorporating natural amino acids with bio-orthogonal reactivities.[20–22] Using the fourth strategy, we have incorporated selenocysteine (Sec), the 21st natural amino acid, into antibodies.[23,24] Compared to its cysteine counterpart (pK_a = ~8), Sec has a lower pK_a (~5) and enhanced nucleophilicity, which permits selective Sec conjugation in the presence of cysteines and other natural amino acids.[25] Based on this property, we have introduced a unique class of selenocysteine (Sec)-modified antibodies (SELENOMABS) that allow site-specific antibody modification leading to homogeneous ADCs.[26,27] Furthermore, we demonstrated that SELENOMAB–oxadiazole conjugates had enhanced stability relative to maleimide counterparts.[26] We subsequently incorporated two selenocysteine residues into one antibody[28] for increased drug loading and also successfully labeled antibodies with two different reporter groups *via* engineered Cys and Sec residues.[29] In our efforts to expand the utility and applications of Sec-modified proteins in general, we surveyed the conjugation efficiencies of selenocysteine with a variety of electrophilic functional groups and determined the stability of the resulting conjugates. Herein, we identify allenamides as particularly interesting reagents for Sec conjugation as they efficiently react with Sec to produce stable adducts and permit the development of site-specific SELENOMAB conjugates.

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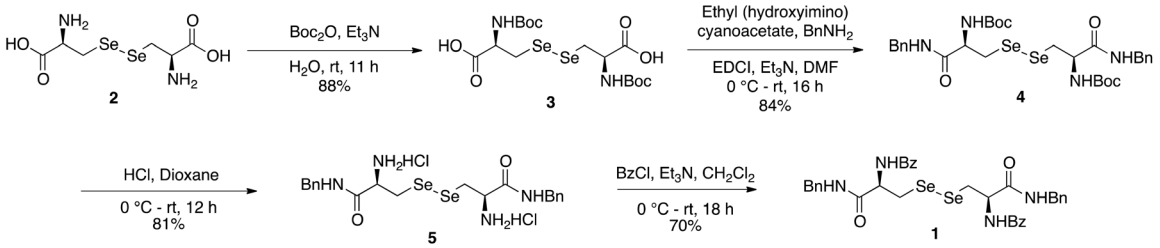
Results and discussion

In order to probe the stability profile of Sec adducts, we synthesized protected selenocystine **1** as a model substrate (Scheme 1).

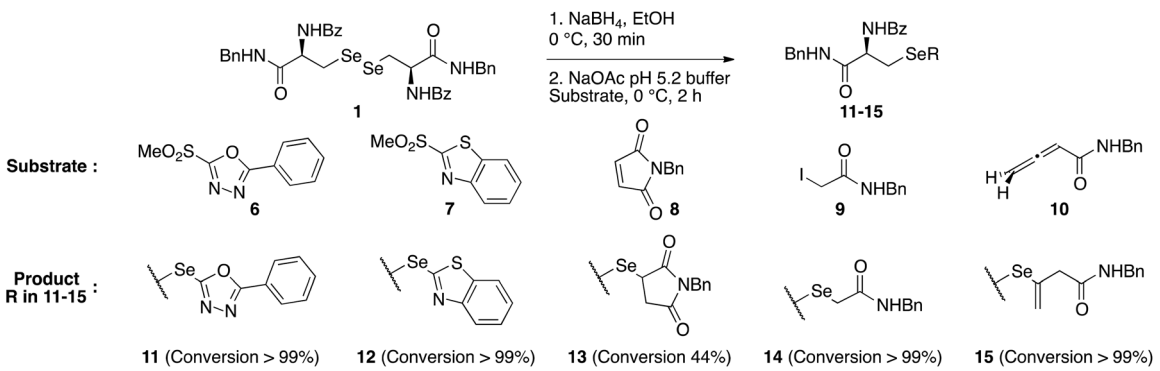
Commercially available L-selenocystine **2** was protected as bis *tert*-butyl carbamate **3**, which was coupled with benzyl amine to give bis-Boc-selenocystine-dibenzylamide **4**. The Boc protecting groups were removed with HCl in dioxane and the resulting hydrochloride salt **5** was coupled with benzoyl chloride to give model substrate **1**. We next examined conditions that would enable diselenide **1** to react with phenyloxadiazolyl sulfone (ODA) **6** and other electrophilic reagents **7–10** (Scheme 2). The optimal conditions deriving from this survey involved first reducing a solution of diselenide in EtOH with $NaBH_4$ at 0 °C, before adding pH 5.2 NaOAc buffer and substrate. The reaction with **6** was complete after 2 h at 0 °C as determined by ^1H NMR analysis of the crude product. Using these conditions, we surveyed the reaction of diselenide **1** with the other electrophilic groups summarized in Fig. S1 (ESI†) that have been used in cysteine conjugation.[30–32] Reactive probes that showed highest conversion are **6**, **7**, **9**, and **10** (Scheme 2). It is interesting to note that benzothiazolyl sulfone (BTA) **7** reacted with comparable efficiency to phenyloxadiazolyl sulfone (ODA) **6** with diselenide, unlike with cysteine as described by Toda *et al.*[30] This is presumably due to the increased nucleophilicity of Sec compared to Cys.

Next, we investigated the stability of the selenocysteine adducts **11–15** (Scheme 2) under several conditions (Fig. 1). Sec-MAL **13** was included for comparison. Under mildly acidic conditions, Sec-ODA **11**, Sec-BTA **12** and Sec-allene (Sec-ALL) **15** (Scheme 2) adducts were stable with minor degradation (Fig. 1, blue bars). However, Sec-MAL **13** and Sec-iodoacemide (Sec-IAM) **14** adducts degraded significantly at pH 5.2 under these conditions. Analogous trends were observed under basic and oxidizing conditions where ≥60% of Sec-ODA **11**, Sec-BTA **12**, and Sec-ALL **15** remained after 72 h while less than 60% Sec-MAL **13** and Sec-IAM **14** remained under these conditions (Fig. 1, red and green bars). In fact, Sec-MAL **13** and Sec-IAM **14** were completely degraded under basic conditions and in the presence of hydrogen peroxide, respectively. The main species observed from the treatment of Sec-MAL **13** with K_2CO_3 were hydrolyzed benzyl maleimide **16** (Scheme 3), diselenide **1**, and *N*-benzoyl dehydroalanine benzyl amide **17**. These compounds are consistent with the observed modes of chemical instability for maleimide-based conjugates (*i.e.* retro-Michael elimination and hydrolysis in aqueous media).[10–12]

Subsequently, we studied the stability of our conjugates under physiologically relevant conditions. The presence of thiol containing compounds in plasma has been implicated in *in vivo* degradation of maleimide-cysteine conjugates.[12] The source of this instability includes a retro-Michael reaction of the conjugate resulting in a reactive maleimide group. Human



Scheme 1 Synthesis of bis-*N*-benzoyl-selenocystine-dibenzamide.



Scheme 2 Conjugation of selenocysteine to select reactive probes.

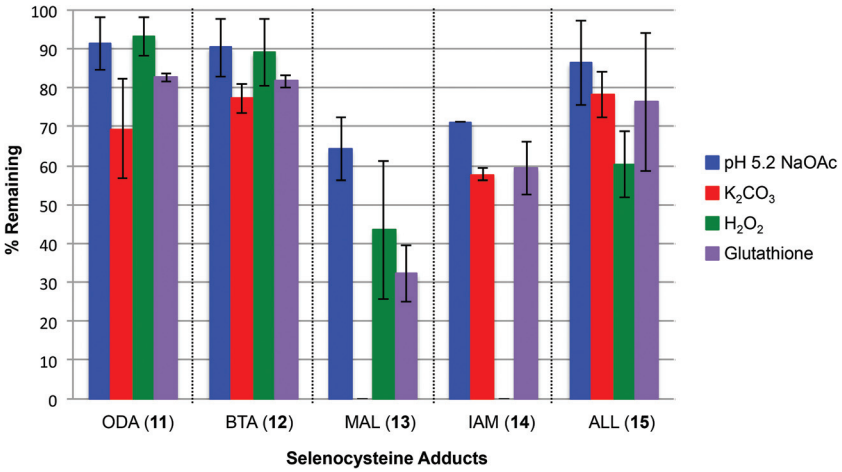
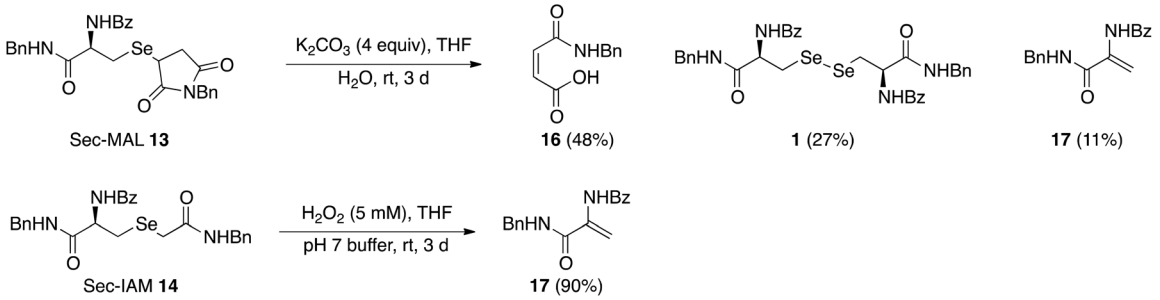


Fig. 1 Stability evaluation for selenocysteine conjugates. Blue: 1 mL THF, NaOAc buffer pH 5.2 (3 : 1), rt, 3 days. Red: K_2CO_3 (4 equiv.), 1 mL THF, H_2O (3 : 1), rt, 3 days. Green: H_2O_2 (5 mM), 1 mL THF, pH 7 buffer (3 : 1), rt, 3 days. Purple: Glutathione (20 μM), 1 mL THF, PBS buffer pH 7.4 (3 : 1), 37 °C, 3 days. % Remaining shown as mean ± standard deviation from three independent experiments.



Scheme 3 Stability of Sec-MAL **13** and Sec-IAM **14** in K_2CO_3 and H_2O_2, respectively. % Remaining determined by LC-MS.

serum albumin and glutathione are capable of reacting with reactive maleimides formed from this process.[10–12] It also seemed conceivable that adducts such as Sec-ODA **11** and Sec-BTA **12** could be unstable due to competing nucleophilic aromatic substitution reactions. As such, we wanted to examine the stability of our selenocysteine adducts in the presence of glutathione. We incubated Sec-adducts with reduced glutathione using the upper limit of extracellular concentration (20 μM),[33] in PBS buffer, at 37 °C, for 3 days and the results are shown in Fig. 1 (purple bars). Again, Sec-ODA **11**, Sec-BTA **12** and Sec-ALL **15** were the most stable with ≥70% remaining while Sec-MAL **13** (32% remaining) and Sec-IAM **14** (60% remaining) were considerably less stable under these conditions.

In addition to stability in the presence of glutathione, we also assessed the stability of Sec-adducts in human plasma at 37 °C. A comparison between the stability of a SELENOMAB–maleimide conjugate and a SELENOMAB–oxadiazole conjugate has been discussed elsewhere, where the latter was shown to be significantly more stable than the former.[26] As such, the Sec-MAL **13** conjugate was not included in this study. Of the remaining adducts, Sec-ODA **11** was the least stable ($t_{1/2}$ < 24 h) (Fig. 2, blue line). In a manner analogous to the cysteine adducts, Sec-BTA **12** was more stable in plasma than Sec-ODA **11** with a half-life of 33 h. Sec-IAM **14** was next most stable in plasma ($t_{1/2}$ = 77 h, calculated with Excel) and Sec-ALL **15** was the most stable of these adducts ($t_{1/2}$ = 139 h).

Due to the excellent robustness of Sec-ALL **15** from this stability profile, the allene carboxamide functional group may potentially be regarded as a compelling reactive handle for selenocysteine-based bioconjugation. While a fluorescein-oxadiazole sulfone probe (FL-ODA) **18**, based on a phenyl oxadiazole coupling partner (Scheme 4) has been shown to selectively conjugate selenocysteine in antibody applications, the selectivity for allene conjugation with Sec is as of yet unknown.[26,29] However, the allene electrophiles have been shown to conjugate cysteine with high efficiency.[32] In order to determine the selectivity of allene conjugation and establish whether allenes might be useful for SELENOMAB conjugation, we synthesized an allene-based fluorescein reactive probe **19** (Scheme 4, Fl-ALL). The synthesis commenced with the coupling of 3-butynoic acid (**20**) with *t*Boc-1,4-diaminobutane giving allene **21**. Trifluoroacetic acid-mediated removal of the Boc protecting group followed by coupling with 5-carboxyfluorescein provided the requisite allene probe **19**.

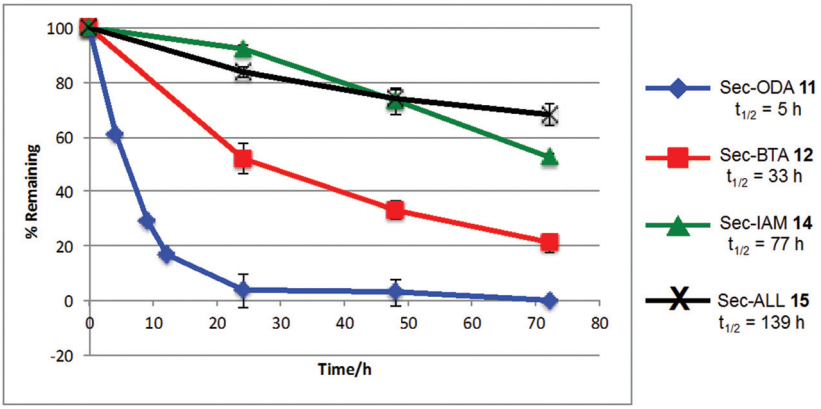
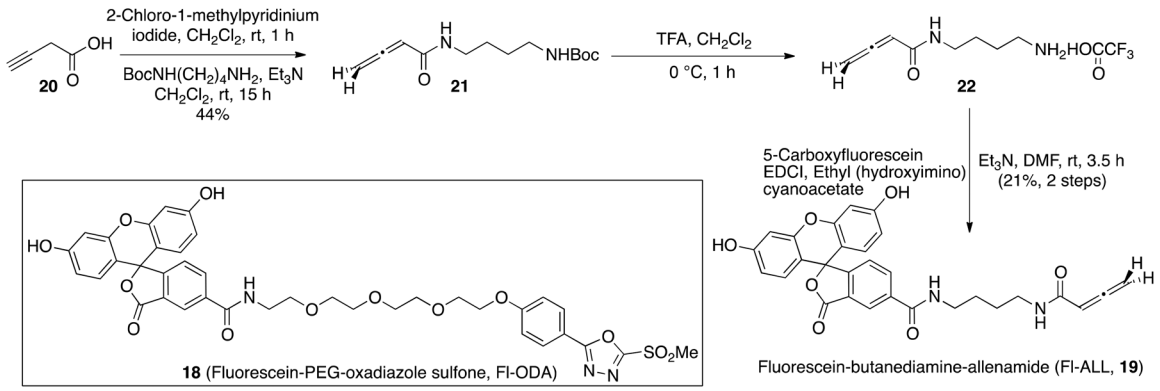


Fig. 2 Stability evaluation of Sec-conjugates in human plasma. 50 μL of 4 mM substrate in DMSO was added to human plasma (950 μL) and incubated at 37 °C for 72 h. Aliquots were analyzed by LC-MS with dibenzyl benzamide as the internal standard.



Scheme 4 Synthesis of allene-based fluorescent probe **19**.

To test the conjugation selectivity of fluorescein-allene probe **19**, selenocysteine-modified trastuzumab scFv-Fc-Sec (SELENOMAB) was reduced with DTT (0.1 mM) in pH 5.2 NaOAc buffer and the reduced antibody was allowed to react with Fl-ALL **19** for 2 h at room temperature (Fig. 3a). In parallel, trastuzumab scFv-Fc lacking selenocysteine (scFv-Fc) was also incubated with Fl-ALL **19**. For each reaction, the reaction mixture was purified using size-exclusion chromatography and analyzed by SDS-PAGE. The result showed that Fl-ALL **19** was selective for selenocysteine and did not react with any other

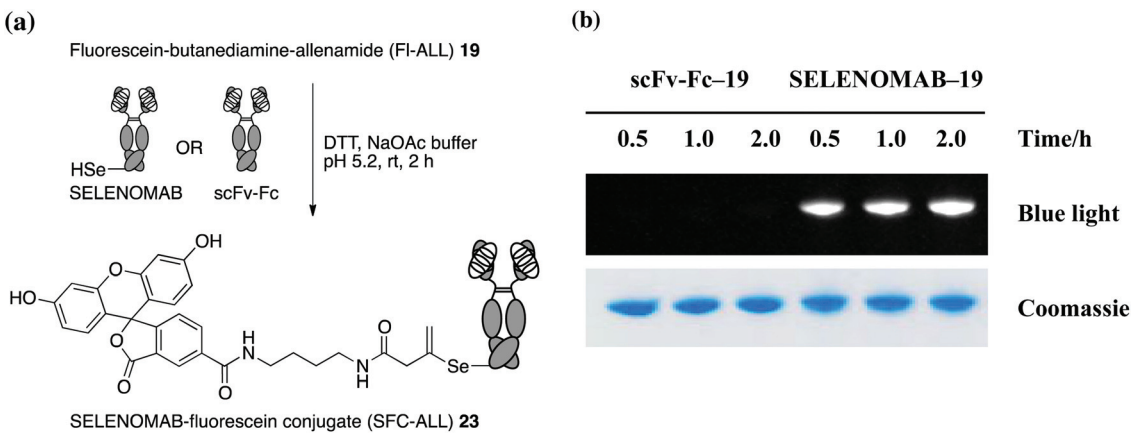


Fig. 3 (a) Conjugation of SELENOMAB or scFv-Fc to fluorescein-allene probe **19** (Fl-ALL). (b) Gel image of scFv-Fc and SELENOMAB reaction with Fl-ALL **19**.

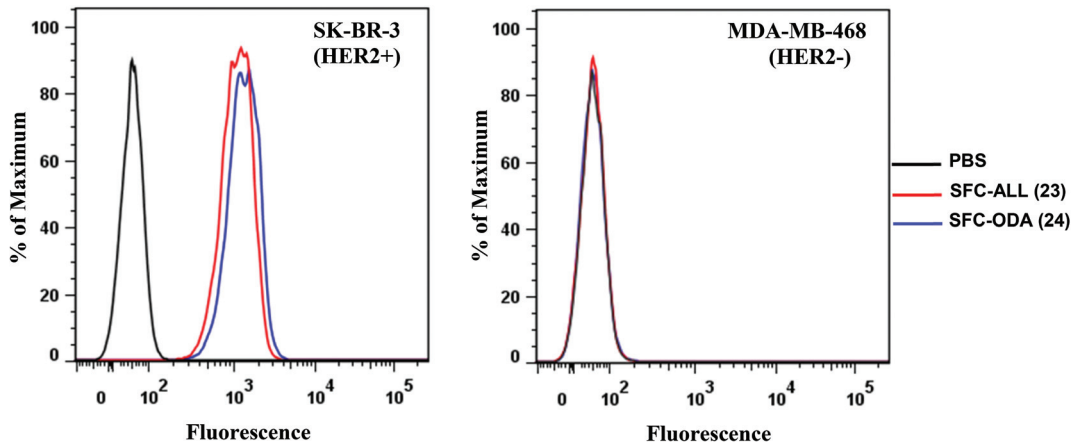


Fig. 4 Fluorescein conjugated SELENOMABs retain HER2-binding activity. SELENOMAB was incubated with HER2 expressing SK-BR-3 cells and bound antibody was detected by flow cytometry. No bound antibody was detected using HER2$^-$ MDA-MB-468 cells. SFC-ALL: allene-based SELENOMAB–fluorescein conjugate; SFC-ODA: oxadiazolyl sulfone-based SELENOMAB–fluorescein conjugate.

functional groups on the antibody (Fig. 3b). Furthermore, reduced SDS-PAGE highlighted the stability of SELENOMAB–fluorescein conjugate **23** (SFC-ALL, Fig. 3a) under reducing conditions (50 mM DTT) (data not shown). Interestingly, oxadiazole-based SELENOMAB–fluorescein conjugate **24** (SFC-ODA, Scheme S1, ESI†) is degraded in reduced SDS-PAGE (data not shown).

We next evaluated whether our SELENOMAB–fluorescein conjugates retained antigen binding activity. We investigated the ability of our SELENOMAB–fluorescein conjugates to bind to HER2 receptors on the surface of SK-BR-3 breast cancer cells using flow cytometry. As shown in Fig. 4, SK-BR-3 cells incubated with allene-based **23** (SFC-ALL) and those incubated with oxadiazole-based **24** (SFC-ODA) SELENOMAB–fluorescein conjugates had a shift in fluorescence relative to background (cells incubated in PBS buffer). Also, the SELENOMAB–fluorescein conjugates bound only to HER2 receptors since HER2-negative breast cancer MDA-MB-468 cells showed no difference in fluorescence between those incubated in PBS and SELENOMAB–fluorescein conjugates (Fig. 4).

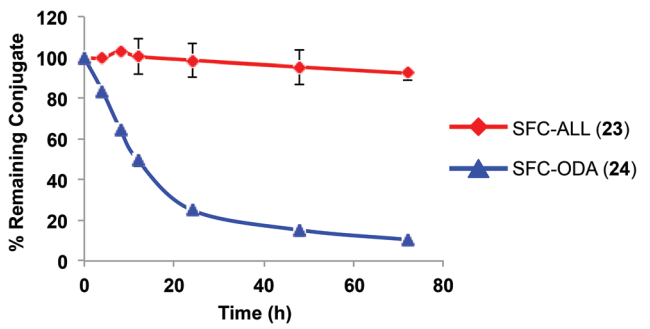


Fig. 5 Allene and sulfone-based SELENOMAB–fluorescein conjugate stability in human plasma. SFC-ALL and SFC-ODA: allene-based and oxadiazolyl sulfone-based SELENOMAB–fluorescein conjugate, respectively.

Finally, we wanted to examine the stability of SELENOMAB–fluorescein conjugates in plasma. The conjugates were incubated in human plasma at 37 °C for 3 days. Aliquots were collected at 0, 4, 8, 12, 24, 48, and 72 h and analyzed by SDS-PAGE. While the sulfone based conjugate **24** (SFC-ODA) was moderately stable with a half-life of about 12 h, the allene based conjugate **23** (SFC-ALL) was very stable with a half-life of over 28 days (Calculated with Excel using current data) (Fig. 5).

Conclusion

We demonstrated that heteroaryl sulfones, iodoacetamide, and allenamides are suitable for conjugation to selenocysteine with high conjugation efficiency. The heteroaryl sulfone and allenamide based conjugates were stable under a wide variety of conditions including physiologically relevant ones. The allenamide-based fluorescent probe was conjugated to SELENOMAB with remarkable chemo- and site selectivity. Furthermore, the resulting SELENOMAB–fluorescein conjugate was impressively stable in plasma. The allene functionality provides a way to generate stable, site-specific SELENOMAB conjugates with drugs, fluorophores, and contrast agents. Also, it offers another functional group that together with heteroaryl sulfones can be used for dual labeling of ADCs.

Conflict of interest

The authors declare no competing financial interests.

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References

1 S. O. Doronina, B. E. Toki, M. Y. Torgov, B. A. Mendelsohn, C. G. Cerveny, D. F. Chace, R. L. DeBlanc, R. P. Gearing, T. D. Bovee, C. B. Siegall, J. A. Francisco, A. F. Wahl, D. L. Meyer and P. D. Senter, *Nat. Biotechnol.*, 2003, **21**, 778–784.

2 G. D. L. Phillips, G. M. Li, D. L. Dugger, L. M. Crocker, K. L. Parsons, E. Mai, W. A. Blattler, J. M. Lambert, R. V. J. Chari, R. J. Lutz, W. L. T. Wong, F. S. Jacobson, H. Koeppen, R. H. Schwall, S. R. Kenkare-Mitra, S. D. Spencer and M. X. Sliwkowski, *Cancer Res.*, 2008, **68**, 9280–9290.

3 P. D. Senter and E. L. Sievers, *Nat. Biotechnol.*, 2012, **30**, 631–637.

4 J. M. Lambert and R. V. J. Chari, *J. Med. Chem.*, 2014, **57**, 6949–6964.

5 A. Younes, U. Yasothan and P. Kirkpatrick, *Nat. Rev. Drug Discovery*, 2012, **11**, 19–20.

6 K. J. Hamblett, P. D. Senter, D. F. Chace, M. M. C. Sun, J. Lenox, C. G. Cerveny, K. M. Kissler, S. X. Bernhardt, A. K. Kopcha, R. F. Zabinski, D. L. Meyer and J. A. Francisco, *Clin. Cancer Res.*, 2004, **10**, 7063–7070.

7 J. R. Junutula, H. Raab, S. Clark, S. Bhakta, D. D. Leipold, S. Weir, Y. Chen, M. Simpson, S. P. Tsai, M. S. Dennis, Y. M. Lu, Y. G. Meng, C. Ng, J. H. Yang, C. C. Lee, E. Duenas, J. Gorrell, V. Katta, A. Kim, K. McDorman, K. Flagella, R. Venook, S. Ross, S. D. Spencer, W. L. Wong, H. B. Lowman, R. Vandlen, M. X. Sliwkowski, R. H. Scheller, P. Polakis and W. Mallet, *Nat. Biotechnol.*, 2008, **26**, 925–932.

8 S. D. Fontaine, R. Reid, L. Robinson, G. W. Ashley and D. V. Santi, *Bioconjugate Chem.*, 2015, **26**, 145–152.

9 L. N. Tumey, M. Charati, T. He, E. Sousa, D. S. Ma, X. G. Han, T. Clark, J. Casavant, F. Loganzo, F. Barletta, J. Lucas and E. I. Graziani, *Bioconjugate Chem.*, 2014, **25**, 1871–1880.

10 D. Jackson, J. Atkinson, C. I. Guevara, C. Y. Zhang, V. Kery, S. J. Moon, C. Virata, P. Yang, C. Lowe, J. Pinkstaff, H. Cho, N. Knudsen, A. Manibusan, F. Tian, Y. Sun, Y. C. Lu, A. Sellers, X. C. Jia, I. Joseph, B. Anand, K. Morrison, D. S. Pereira and D. Stover, *PLoS One*, 2014, 9.

11 B. Q. Shen, K. Y. Xu, L. N. Liu, H. Raab, S. Bhakta, M. Kenrick, K. L. Parsons-Reponte, J. Tien, S. F. Yu, E. Mai, D. W. Li, J. Tibbitts, J. Baudys, O. M. Saadi, S. J. Scales, P. J. McDonald, P. E. Hass, C. Eigenbrot, T. Nguyen, W. A. Solis, R. N. Fuji, K. M. Flagella, D. Patel, S. D. Spencer, L. A. Khawlil, A. Ebens, W. L. Wong, R. Vandlen, S. Kaur, M. X. Sliwkowski, R. H. Scheller, P. Polakis and J. R. Junutula, *Nat. Biotechnol.*, 2012, **30**, 184–189.

12 S. C. Alley, D. R. Benjamin, S. C. Jeffrey, N. M. Okeley, D. L. Meyer, R. J. Sanderson and P. D. Senter, *Bioconjugate Chem.*, 2008, **19**, 759–765.

13 S. C. Jeffrey, P. J. Burke, R. P. Lyon, D. W. Meyer, D. Sussman, M. Anderson, J. H. Hunter, C. I. Leiske, J. B. Miyamoto, N. D. Nicholas, N. M. Okeley, R. J. Sanderson, I. J. Stone, W. P. Zeng, S. J. Gregson, L. Masterson, A. C. Tiberghien, P. W. Howard, D. E. Thurston, C. L. Law and P. D. Senter, *Bioconjugate Chem.*, 2013, **24**, 1256–1263.

14 J. R. Junutula, K. M. Flagella, R. A. Graham, K. L. Parsons, E. Ha, H. Raab, S. Bhakta, T. Nguyen, D. L. Dugger, G. M. Li, E. Mai, G. D. L. Phillips, H. Hiraragi, R. N. Fuji, J. Tibbitts, R. Vandlen, S. D. Spencer, R. H. Scheller, P. Polakis and M. X. Sliwkowski, *Clin. Cancer Res.*, 2010, **16**, 4769–4778.

15 F. Tian, Y. C. Lu, A. Manibusan, A. Sellers, H. Tran, Y. Sun, T. Phuong, R. Barnett, B. Hehli, F. Song, M. J. DeGuzman, S. Ensari, J. K. Pinkstaff, L. M. Sullivan, S. L. Biroc, H. Cho, P. G. Schultz, J. DiJoseph, M. Dougher, D. S. Ma, R. Dushin, M. Leal, L. Tchistiakova, E. Feyfant, H. P. Gerber and P. Sapra, *Proc. Natl. Acad. Sci. U. S. A.*, 2014, **111**, 1766–1771.

16 E. S. Zimmerman, T. H. Heibeck, A. Gill, X. F. Li, C. J. Murray, M. R. Madlansacay, C. Tran, N. T. Uter, G. Yin, P. J. Rivers, A. Y. Yam, W. D. Wang, A. R. Steiner, S. U. Bajad, K. Penta, W. J. Yang, T. J. Hallam, C. D. Thanos and A. K. Sato, *Bioconjugate Chem.*, 2014, **25**, 351–361.

17 P. Wu, W. Q. Shui, B. L. Carlson, N. Hu, D. Rabuka, J. Lee and C. R. Bertozzi, *Proc. Natl. Acad. Sci. U. S. A.*, 2009, **106**, 3000–3005.

18 P. Strop, S. H. Liu, M. Dorywalska, K. Delaria, R. G. Dushin, T. T. Tran, W. H. Ho, S. Farias, M. G. Casas, Y. Abdiche, D. H. Zhou, R. Chandrasekaran, C. Samain, C. Loo, A. Rossi, M. Rickert, S. Krimm, T. Wong, S. M. Chin, J. Yu, J. Dilley, J. Chaparro-Riggers, G. F. Filzen, C. J. O'Donnell, F. Wang, J. S. Myers, J. Pons, D. L. Shelton and A. Rajpal, *Chem. Biol.*, 2013, **20**, 161–167.

19 R. R. Beerli, T. Hell, A. S. Merkel and U. Grawunder, *PLoS One*, 2015, **10**, e0131177.

20 S. Panowski, S. Bhakta, H. Raab, P. Polakis and J. R. Junutula, *mAbs*, 2014, **6**, 34–45.

21 J. R. McCombs and S. C. Owen, *AAPS J.*, 2015, **17**, 339–351.

22 P. Agarwal and C. R. Bertozzi, *Bioconjugate Chem.*, 2015, **26**, 176–192.

23 T. Hofer, J. D. Thomas, T. R. Burke, Jr. and C. Rader, *Proc. Natl. Acad. Sci. U. S. A.*, 2008, **105**, 12451–12456.

24 T. Hofer, L. R. Skeffington, C. M. Chapman and C. Rader, *Biochemistry*, 2009, **48**, 12047–12057.

25 B. J. Byun and Y. K. Kang, *Biopolymers*, 2011, **95**, 345–353.

26 J. T. Patterson, S. Asano, X. Li, C. Rader and C. F. Barbas, III, *Bioconjugate Chem.*, 2014, **25**, 1402–1407.

27 B. Vire, M. Skarzynski, J. D. Thomas, C. G. Nelson, A. David, G. Aue, T. R. Burke, Jr., C. Rader and A. Wiestner, *Cancer Res.*, 2014, **74**, 7510–7520.

28 X. Li, J. Yang and C. Rader, *Methods*, 2014, **65**, 133–138.

29 X. Li, J. T. Patterson, M. Sarkar, L. Pedzisa, T. Kodadek, W. R. Roush and C. Rader, *Bioconjugate Chem.*, 2015, **26**, 2243–2248.

30 N. Toda, S. Asano and C. F. Barbas, III, *Angew. Chem., Int. Ed.*, 2013, **52**, 12592–12596.
31 P. M. Cal, G. J. Bernades and P. M. Gois, *Angew. Chem., Int. Ed.*, 2014, **53**, 10585–10587.
32 A. Abbas, B. G. Xing and T. P. Loh, *Angew. Chem., Int. Ed.*, 2014, **53**, 7491–7494.
33 G. Y. Wu, Y. Z. Fang, S. Yang, J. R. Lupton and N. D. Turner, *J. Nutr.*, 2004, **134**, 489–492.