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RENOVATION OF GLYCOMOLECULES FOR MOLECULAR IMAGING STUDIES: LOW-AFFINITY GLYCAN LIGANDS CAN BE USED FOR SELECTIVE CELL IMAGING?

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Abstract – Since the interaction of single molecule of glycans is very weak to the lectins, in most cases they have been formed into clusters for binding studies. Then, a glycan molecule cannot be used for efficient cell imaging or targeting? We established a new strategy to selectively image the cells by using synergistic interaction of peptide and glycan ligands on cell surface. Namely, the peptide ligand with high affinity to the target surface receptor was pretargeted, and then the low-affinity glycan ligand with fluorescent label was interacted to the same cell; Bioorthogonal reaction between them can anchor the label on the target cells when both receptors are expressed and close in space on the surface. When the lectin is not expressed on the target or two receptors are separated, the weakly interacting glycan ligands are washed away the cells, hence the target cells are selectively imaged. While new idea and proof-of-concept data were exemplified based on our previous research, using this strategy, we would like to report the new data to visualize cell surface dynamics in response to the treatment with bioactive compounds.

CONTENTS

1. INTRODUCTION

One of the hurdles that prevent diagnosis and therapeutics from greater clinical usage is the lack of a general cell- and organ-selective biotargeting system, as well as greater control over excretion profiles. One approach to address this issue is to utilize glycans, which cover the cell surface and are responsible to selective interaction for biologically relevant systems,[1] to direct accumulation in specific cells and organs. It should be noted, however, that the individual interaction of a single glycan molecule is very weak.[2,3] Research therefore focuses on introducing multiple glycan structures onto specific templates, i.e., proteins, cells, nanoparticles, artificial dendrons, or microchips.[4-12] This is meant to bring out the multivalency and pattern recognition effects of "strong" and "selective" glycan interactions as mimic of natural glyco-bioenvironment. For instances, by chemically modifying albumin template with various asparagine-linked glycan (*N*-glycan) types and compositions,[13-16] these glycoclusters selectively interacted with the cancer and normal cell lines.[17] In addition, through the use of molecular imaging, our results have shown that glycan composition on protein, cell, or dendron templates can directly control accumulation towards specific organs and tumor cells, as well as affect their excretion profiles in mouse model studies.[16,18-21]

Despite this glycocluster strategy including our recent research met with success results, a question then arises "Can not we use the weak interaction of individual glycan for molecular imaging?" Working in parallel, we have also alternatively developed a novel concept for selective targeting in living systems by exploiting the "weak interaction" of single glycan molecules. More specifically, (1) "strong interactions" of peptide molecules, (2) "weak interactions" of glycans, and (3) selective bond-forming reactions, could simultaneously and synergistically be combined to achieve selective recognition in live cells. In this paper, we would like to report a new idea of "using weak interaction for molecular imaging" and proof-of-concept data were shown based on our previous research.[22] In addition, in this paper we applied this concept for the first time to visualize cell surface dynamics in response to the treatment with bioactive compounds.

2. CONCEPT

Generally, in order to achieve high imaging contrast for noninvasive diagnostics, e.g., cancer imaging, previous studies have largely focused on the development of ligands with high-affinity to receptors of interests (see schematic presentation in Figure 1a). For example, RGD peptides (represented by probe C

in Figure 1a) are widely used to image various cancers because of their high-affinity to $\alpha_V\beta_3$ integrins,[23,24] which are highly expressed cell adhesion proteins on tumor tissues (cell A).[25] However, RGD peptides often provide poor imaging contrast because integrins are also expressed on other endothelial cells (such as cell B). In addition, the strong interactions between RGD peptides and other integrins frequently leads to non-selective binding. These peptides can also be rapidly captured by cells via receptor-mediated endocytosis, resulting in a decrease of signal ratios for tumor to background.[26] These are all common issues in applications where ligands strongly interact with target cells. Alternatively, glycan molecules (probe D in Figure 1b) may also be used for imaging. Although glycans can selectively interact with target cells (cell A), glycan–lectin binding is known to be very weak, i.e., at K_D of mM level, which leads to insufficient probe binding to enable proper cell A visualization.

In our new concept, we combine strong and weak ligand/receptor interactions in a pre-targeting fashion, as frequently used in the field of molecular imaging (Figure 1c).[27-32]

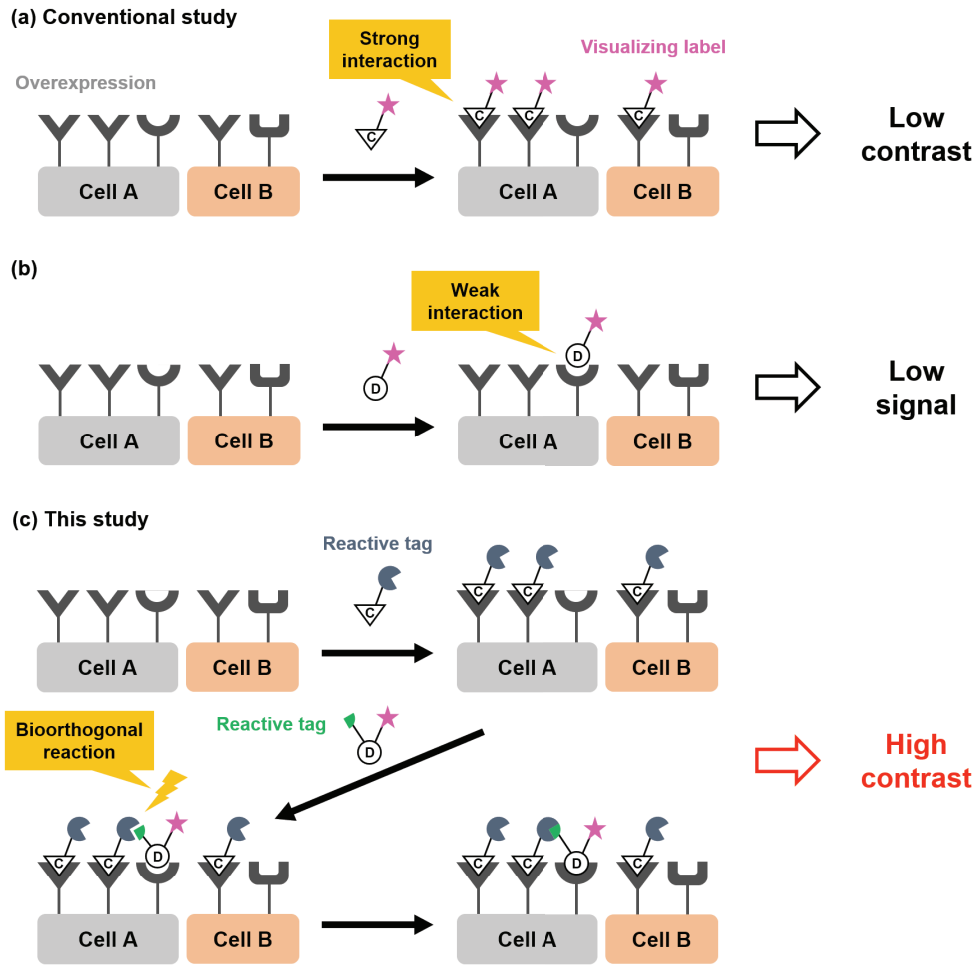

Figure 1. Schematic diagram of cell imaging methods involving high- and low-affinity ligands to cell surface receptors. (a) Conventional labeling method using high-affinity ligand C. (b) Labeling method using a low-affinity ligand D. (c) Labeling method using both high- and low-affinity ligands and a bioorthogonal reaction on the cell surface.

A high-affinity probe C is prepared where the imaging label is replaced with a reactive tag, followed by initial pre-targeting to a mixture of cells A and B. Subsequently, a low-affinity probe D, such as glycan, is prepared with both an imaging label and a bioorthogonal functional group that can react with the pre-targeted probe C. Because the low-affinity probe D is bound to the receptor very weakly, it can be washed away immediately from the cell surface. In contrast, binding of probe D to the pre-targeted cell selectively anchors an imaging probe C through bioorthogonal linkage, which is facilitated by the proximal effects between two surface receptors. The orchestration of strong and weak ligand/receptor interactions and the in situ click conjugation on the cell surface, thus, achieves a high selectivity and imaging contrast between cells A and B. In this strategy, non-specific binding can be avoided by choosing a probe D with low binding affinity that will minimize interactions with other cells expressing low levels of the same receptor. Therefore, the use of weak interactions for cell surface receptors is a key point to the success of this approach.

3. PROOF-OF-CONCEPT STUDY: SELECTIVE CELL IMAGING

Our concept (Figure 1) was successfully realized by selectively imaging human umbilical vein endothelial cells (HUVECs) that were expressed with $\alpha_V\beta_3$ integrins (wedge, Figure 2a) and platelet endothelial cell adhesion molecules (PECAM),[33,34] which is a sialic acid binding lectin (semi-circle). Since PECAM favors α(2,6)disialoglycans rather than α(2,3)disialoglycans,[34] α(2,6)disialoglycans were selected for our study. Azide-containing cyclic RGDyK peptides **1**, which serves as a high-affinity ligand for integrins, were initially pre-bound to cell surfaces. The cells were subsequently treated with a fluorescently labeled α(2,6)disialoglycan **2** bearing a strained acetylene unit. Under normal circumstances, these labeled sialoglycans only interact with PECAM weakly[35] and can be washed away readily from the cell surface. However, upon binding to the pre-targeted cells, the labeled glycan ligands can be selectively anchored to the cell surface through a bioorthogonal, strain-promoted click reaction[36-38] facilitated by the proximal effects of the two surface receptors. This perfect orchestration of strong and weak ligand/receptor interactions, as well as the in situ click ligation on the cell surface, forms the backbone of a highly selective imaging process and provides significantly enhanced contrast towards HUVEC imaging compared to imaging of other cells that lack the dual expression of $\alpha_V\beta_3$ integrins and PECAM in close proximity, such as HeLa cells (Figure 2b). To further highlight the power of this new pre-targeting concept, the use of the common imaging tracer, fluorescently labeled RGD peptide **1'**, was unable to replicate our results to discriminate between HUVEC and HeLa cell lines (Figure 2c, left and middle).

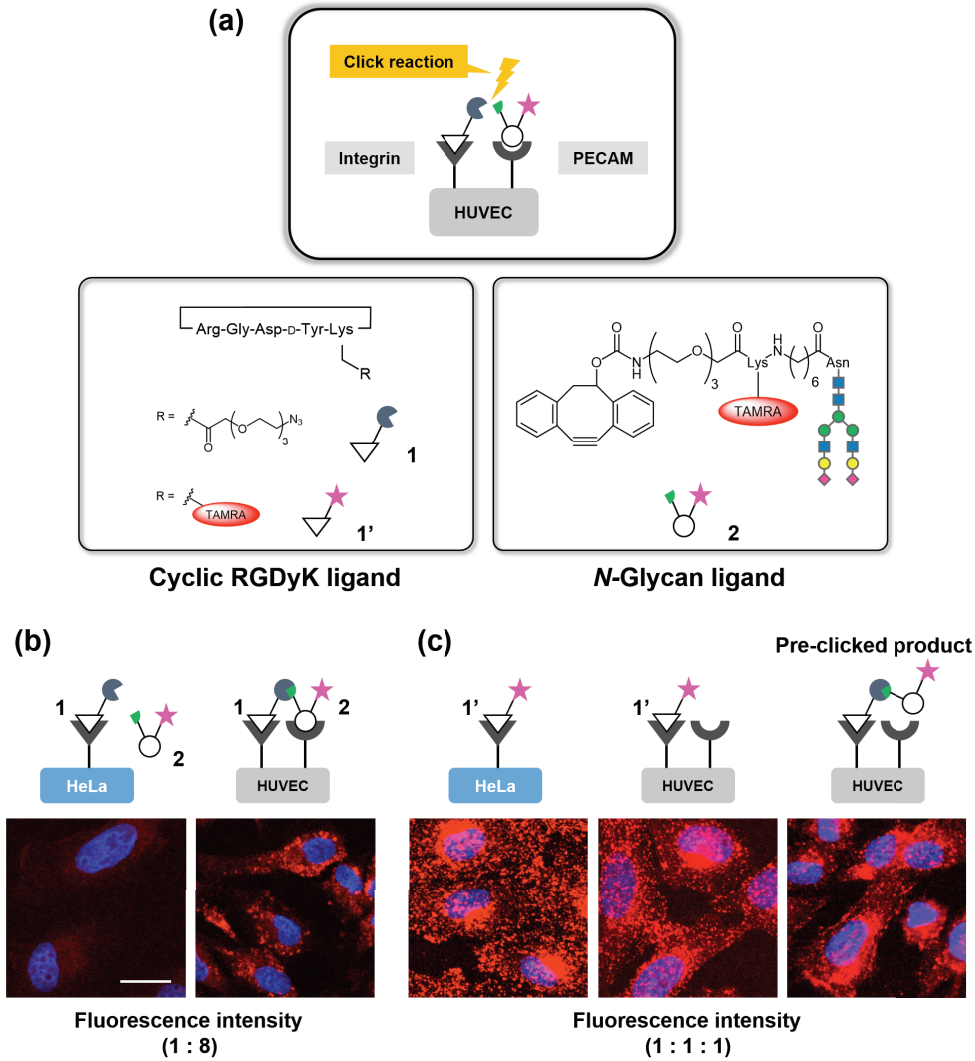

Figure 2. (a) Structures of the functionalized peptide and *N*-glycan ligand. (b) HeLa cells and HUVECs were treated with **1** followed by **2** (red). (c) HeLa cells and HUVECs were treated with **1'** (red) or with the pre-clicked product between **1** and **2** (red). Nuclei stained with DAPI (blue). Scale bar: 20 μm.

In the event where pre-linked glycan/peptide conjugates are used instead, they do not produce the same selectively offered by our imaging strategy. This is due to the strongly interacting peptides dominating and/or controlling the binding characteristics of the entire conjugate, which essentially negates the effects of the weakly interacting glycans (Figure 2c, right). With the in situ ligation of "weak" and "strong" interactions on target cells, our concept enables cell labeling with both high affinity and specificity.

4. IMAGING CELL SURFACE DYNAMICS

Our novel pre-targeted imaging concept also has the potential to be applied for a variety of in vivo molecular imaging. As expected from the data presented in Figure 2, another remarkable feature of our approach is that it allows direct analysis of the relative spatial arrangements between two different types of cell surface receptors[33,39] by comparing imaging efficiencies upon external stimuli. In Figure 2, the

HeLa cells could not be visualized by the new method, since either $\alpha_V\beta_3$ integrin or PECAM was not expressed or they were not close enough in space on cell surface. We therefore treated the HeLa cells with doxorubicin (anticancer drug), menadione (oxidative stress inducer),[40] Pitstop®[41] or MiTMAB™[42] (endocytosis inhibitors) to affect the membrane fluidity on the cells, before executing our method (Figure 3).

Although the treatment with menadione and endocytosis inhibitors did not show any TAMRA fluorescence from the cell surface (Figures 3c-e), a bright fluorescence was derived from the cells treated with doxorubicin (Figure 3b). Since the significant cell toxicity or internalization of the *N*-glycan ligand **2** did not observed under the conditions performed in Figure 3b, the anticancer drug may alter the spatial arrangement between $\alpha_V\beta_3$ integrin and PECAM on cell surface, hence the TAMRA fluorescence could be anchored on cell by click reaction. Based on the data shown here, cell surface dynamics, described as the characteristic arrangements of membrane proteins, and ligand-directed signaling pathways can be potentially monitored or even controlled.

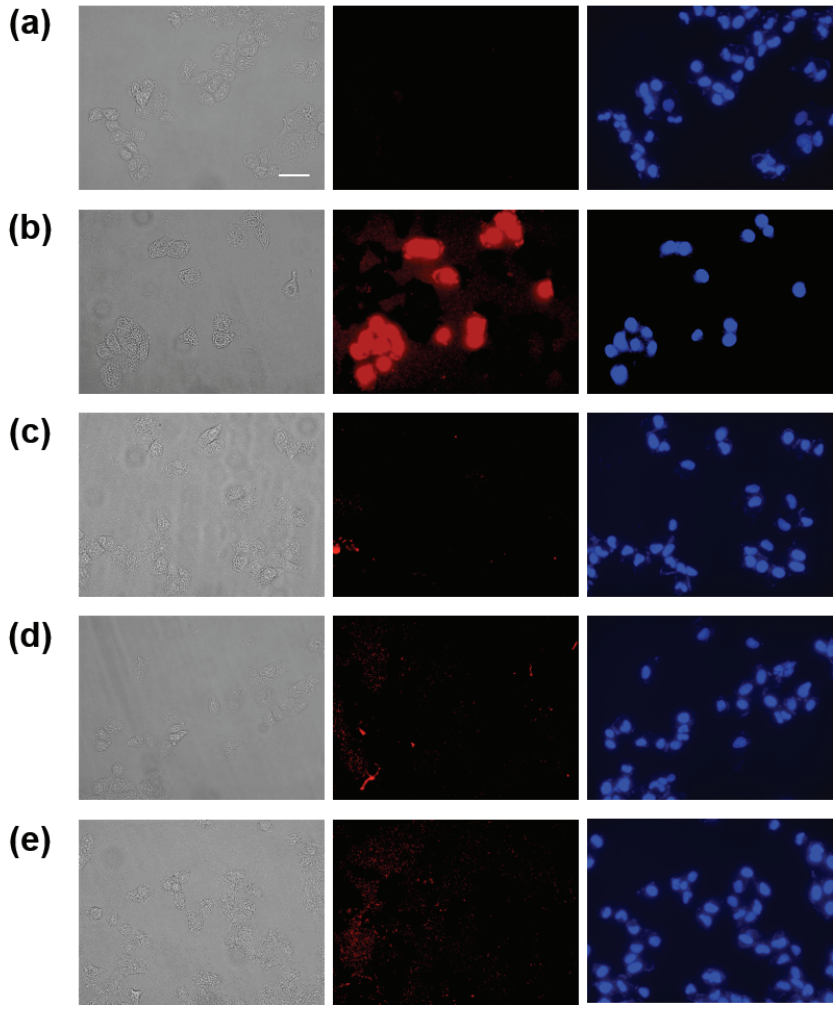

Figure 3. HeLa cells were treated with **1** followed by **2** (red) after incubation with (a) 0.5% of DMSO as control, (b) 10 μM of doxorubicin, (c) 10 μM of menadione, (d) 10 μM of Pitstop®, and (e) 5 μM of MiTMAB™ for 3 h. Nuclei stained with DAPI (blue). Scale bar: 50 μm.

5. SUMMARY

In summary, we developed a new concept to selectively image the cells using high and low-affinity ligands on two surface receptors. Normal and cancer cells, which could not be discriminated by using the conventional peptide ligand, could be successfully imaged, and the method was further applied to the surface dynamics analysis. A distinct advantage of using a low-affinity ligand, such as the glycans used in this study, is that any excess can easily be washed away from cells. However, for low-affinity ligands covalently linked to a pretargeted high-affinity ligand, they will be tightly anchored to the cell surface, even with binding affinities at the mM level. As a consequence, nonspecific fluorescence background can be minimized, which will lead to improved imaging contrast. Of significant note is that now notoriously weak glycan/lectin interactions can be utilized as highly selective ligands for cell targeting, which is a generally underdeveloped area in molecular imaging.

Based on this established method, we are now trying to discriminate the various integrin-overexpressing cancer cell lines. This might be accomplished by varying the combinations of bioactive peptides with different *N*-glycan ligands in a combinatorial approach (such as "microarray" analysis). Such data and further application to in vivo will be reported soon.

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REFERENCES

1. "Analysis of glycans, polysaccharide functional properties & biochemistry of glycoconjugate glycans, carbohydrate-mediated interactions", ed. by J. P. Kamerling, G.-J. Boons, Y. C. Lee, A. Suzuki, N. Taniguchi, and A. G. J. Voragen, *Comprehensive Glycoscience, From Chemistry to Systems Biology*, 2007, **Vol II & III**, Elsevier, UK.
2. S. P. Vyas, A. Singh, and V. Sihorkar, *Crit. Rev. Ther. Drug Carrier Syst*., 2001, **18**, 1.
3. M. Willis and E. Forssen, *Adv. Drug Deliv. Rev*., **29**, 249.
4. K. Fukase and K. Tanaka, *Curr. Opin. Chem. Biol*., 2012, **16**, 614.
5. K. Tanaka, E. R. O. Siwu, K. Minami, K. Hasegawa, S. Nozaki, Y. Kanayama, K. Koyama, W. C. Chen, J. C. Paulson, Y. Watanabe, and K. Fukase, *Angew. Chem. Int. Ed.*, 2010, **44**, 8195.
6. R. Roy and M.-G. Beak, *Rev. Mol. Biotechnol*., 2002, **90**, 291.

7. M. A. Leeuwenburg, G. A. van der Marel, and H. S. Overkleeft, *Curr. Opin. Chem. Biol*., 2003, **7**, 757.
8. O. Renaudet, *Mini-Rev. Org. Chem*., 2008, **5**, 274.
9. Y. M. Chabre and R. Roy, *Curr. Top. Med. Chem*., 2008, **8**, 1237.
10. Y. M. Chabre and R. Roy, *Adv. Carbohydr. Chem. Biochem.*, 2010, **63**, 165.
11. R. T. Lee and Y. C. Lee, *Glycoconj. J.*, 2000, **17**, 543.
12. J. J. Lundquist and E. J. Toone, *Chem. Rev*., 2002, **102**, 555.
13. A. Ogura, A. Kurbangalieva, and K. Tanaka, *Mini-Rev. Med. Chem.*, 2014, **14**, 1072.
14. M. Taichi, S. Kitazume, K. K. H. Vong, R. Imamaki, A. Kurbangalieva, N. Taniguchi, and K. Tanaka, *Glycoconj. J*., 2015, **32**, 497.
15. A. Ogura, A. Kurbangalieva, and K. Tanaka, *Glycobiology*, 2016, **26**, 804.
16. K. Tanaka, *Org. Biomol. Chem*., 2016, **14**, 7610.
17. A. Ogura, S. Urano, T. Tahara, S. Nozaki, R. Sibgatullina, K. Vong, T. Suzuki, N. Dohmae, A. Kurbangalieva, Y. Watanabe, and K. Tanaka, *Chem. Commun*., 2018, **54**, 8693.
18. K. Tanaka, T. Masuyama, K. Hasegawa, T. Tahara, H. Mizuma, Y. Wada, Y. Watanabe, and K. Fukase, *Angew. Chem. Int. Ed*., 2008, **47**, 102.
19. A. Ogura, T. Tahara, S. Nozaki, K. Morimoto, Y. Kizuka, S. Kitazume, M. Hara, S. Kojima, H. Onoe, A. Kurbangalieva, N. Taniguchi, Y. Watanabe, and K. Tanaka, *Sci. Rep*., 2016, **6**, 21797.
20. A. Ogura, T. Tahara, S. Nozaki, H. Onoe, A. Kurbangalieva, Y. Watanabe, and K. Tanaka, *Bioorg. Med. Chem. Lett*., 2016, **26**, 2251.
21. L. Latypova, R. Sibgatullina, A. Ogura, K. Fujiki, A. Khabibrakhmanova, T. Tahara, S. Nozaki, S. Urano, K. Tsubokura, H. Onoe, Y. Watanabe, A. Kurbangalieva, and K. Tanaka, *Adv. Sci*., 2017, **4**, 1600394.
22. M. Taichi, S. Nomura, I. Nakase, R. Imamaki, Y. Kizuka, F. Ota, N. Dohmae, S. Kitazume, N. Taniguchi, and K. Tanaka, *Adv. Sci*., 2017, **4**, 1700147.
23. Y. A. Zhou, S. Chakraborty, and S. Liu, *Theranostics*, 2011, **1**, 58.
24. M. D. Pierschbacher and E. Ruoslahti, *Nature*, 1984, **309**, 30.
25. K. Tanaka and K. Fukase, *Org. Biomol. Chem*., 2008, **6**, 815.
26. Y. Nakamoto, A. R. Pradipta, H. Mukai, M. Zouda, Y. Watanabe, A. Kurbangalieva, P. Ahmadi, Y. Manabe, K. Fukase, and K. Tanaka, *ChemBioChem*., 2018, **19**, 2055.
27. D. T. Reardan, C. F. Meares, D. A. Goodwin, M. McTigue, G. S. David, M. R. Stone, J. P. Leung, R. M. Bartholomew, and J. M. Frincke, *Nature*, 1985, **316**, 265.
28. D. M. Goldenberg, C-H. Chang, E. A. Rossi, W. J. McBride, and R. M. Sharkey, *Theranostics*, 2012, **2**, 523.

29. L. Carrol, H. L. Evans, E. O. Aboagye, and A. C. Spivey, *Org. Biomol. Chem.*, 2013, **11**, 5772.
30. S. B. Lee, H. L. Kim, H.-J. Jeong, S. T. Lim, M.-H. Sohn, and D. W. Kim, *Angew. Chem. Int. Ed.*, 2013, **52**, 10549.
31. R. Rossin, P. R. Verkerk, S. M. van den Bosch, R. C. M. Vulders, I. Verel, J. Lub, and M. S. Robillard, *Angew. Chem. Int. Ed*., 2010, **49**, 3375.
32. N. K. Devaraj, R. Upadhyay, J. B. Haun, S. A. Hilderbrand, and R. Weissleder, *Angew. Chem. Int. Ed.*, 2009, **48**, 7013.
33. C. W. Y. Wong, G. Wiedle, C. Ballestrem, B. Wehrle-Haller, S. Etteldorf, M. Bruckner, B. Engelhardt, T. H. Gisler, and B. A. Imhof, *Mol. Biol. Cell*, 2000, **11**, 3109.
34. S. Kitazume, R. Imamaki, A. Kurimoto, K. Ogawa, M. Kato, Y. Yamaguchi, K. Tanaka, H. Ishida, H. Ando, M. Kiso, N. Hashii, N. Kawasaki, and N. Taniguchi, *J. Biol. Chem.*, 2014, **289**, 27604.
35. M. Taichi, S. Kitazume, K. K. H. Vong, R. Imamaki, A. Kurbangalieva, N. Taniguchi, and K. Tanaka, *Glycoconj. J*., 2015, **32**, 497.
36. N. J. Agard, J. A. Prescher, and C. R. A. Bertozzi, *J. Am. Chem. Soc.*, 2004, **126**, 15046.
37. E. M. Sletten and C. R. Bertozzi, *Acc. Chem. Res.*, 2011, **44**, 666.
38. X. Ning, J. Guo, M. A. Wolfert, and G. J. Boons, *Angew. Chem. Int. Ed.*, 2008, **47**, 2253.
39. E. Tzima, M. Irani-Tehrani, W. B. Kiosses, E. Dejana, D. A. Schultz, B. Engelhardt, G. Cao, H. DeLisser, and M. A. Schwartz, *Nature*, 2005, **437**, 426.
40. D. N. Criddle, S. Gillies, H. K. B.-Wilson, M. Jaffar, E. C. Chinje, S. Passmore, M. Chvanov, S. Barrow, O. V. Gerasimenko, A. V. Tepikin, R. Sutton, and O. H. Petersen, *J. Biol. Chem*., 2006, **52**, 40485.
41. C. M. Smith, V. Haucke, A. McCluskey, P. J. Robinson, and M. Chircop, *Mol. Cancer*, 2013, **12**, 4.
42. V. F. Fiore, L. Ju, Y. Chen, C. Zhu, and T. H. Barker, *Nature Commun*., 2014, **5**, 4886.

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