Targeted Cell Ablation

DOI: 10.1002/anie.201403221

Selective Ablation of β-Galactosidase-Expressing Cells with a Rationally Designed Activatable Photosensitizer**

*Yuki Ichikawa, Mako Kamiya, Fumiaki Obata, Masayuki Miura, Takuya Terai, Toru Komatsu, Tasuku Ueno, Kenjiro Hanaoka, Tetsuo Nagano, and Yasuteru Urano**

Abstract: *We have developed an activatable photosensitizer capable of specifically inducing the death of β-galactosidase-expressing cells in response to photoirradiation. By using a selenium-substituted rhodol scaffold bearing β-galactoside as a targeting substituent, we designed and synthesized HMDESeR-βGal, which has a non-phototoxic spirocyclic structure owing to the presence of the galactoside moiety. However, β-galactosidase efficiently converted HMDESeR-βGal into phototoxic HMDESeR, which exists predominantly in the open xanthene form. This structural change resulted in drastic recovery of visible-wavelength absorption and the ability to generate singlet oxygen ($^1O_2$). When HMDESeR-βGal was applied to larval Drosophila melanogaster wing disks, which express β-galactosidase only in the posterior region, photoirradiation induced cell death in the β-galactosidase-expressing region with high specificity.*

To understand the function of a specific cell type within a complex network, such as the nervous system, or during biological development, an efficient method of ablating cells of interest is needed. Several methods for conditional cell ablation in a spatiotemporally controlled manner have been reported. One approach is to use genetically encodable photosensitizer proteins, such as KillerRed and its variants.[1] Upon light irradiation, these photosensitizer proteins generate reactive oxygen species (ROS), such as singlet oxygen ($^1O_2$), which cause oxidative stress and induce cell death. Furthermore, the selective ablation of target cells is relatively straightforward, since photosensitizer proteins can be genetically expressed only in the cells of interest by the use of suitable promoters. However, these methods have several limitations. For example, the induction of phototoxicity is inefficient as compared to that with small-molecule-based photosensitizers,[2] so that a high dose of light is needed to induce cell death. Also, the protein expression level is generally limited owing to the large protein size. To overcome these limitations, we are interested in developing small-molecule-based photosensitizers as an alternative tool for conditional cell ablation. However, it is quite difficult to control the distribution of small-molecular photosensitizers, so nonspecific phototoxicity is an issue. Furthermore, it is technically difficult to localize photoirradiation only on target cells. Therefore, to minimize nonspecific photoinduced damage and to obtain the maximum effect in the region of interest, we focused on an "enzyme-activation" strategy. In other words, we required a probe that would initially have very low phototoxicity, but that would become strongly phototoxic after activation by a target enzyme. An expected advantage of this approach was that the phototoxicity would be strongly amplified by enzymatic turnover.

We have previously attempted to develop activatable photosensitizers whose photosensitizing ability can be switched by β-galactosidase,[3] but we found that they were not able to induce cell death specifically in regions of interest in living tissues, probably owing at least in part to their relatively low activation ratio. Therefore, to design an activatable photosensitizer that shows drastic activation of photosensitizing ability, we focused on the spirocyclization of xanthene-based dyes. We have already reported several fluorescent probes for detecting the activity of various hydrolases on the basis of precise control of the spirocyclization reaction of fluorescent xanthene dyes.[4] These probes are mainly present in their colorless nonfluorescent spirocyclic form, but are converted by the enzyme into the colored fluorescent open form. Since the initial process of light absorption (to form the excited-state molecule) is common to fluorophores and photosensitizers, and the critical difference is the major relaxation process from the singlet excited state S_1, the spirocyclization-based approach should also be applicable to the design of photosensitizers. Furthermore, on the basis of the report that a xanthene-based fluorophore can be converted into a photosensitizer simply by replacing the oxygen atom at the 10-position with a selenium atom,[5] we decided to develop an activatable photosensitizer by introducing selenium at the 10-position of our previously reported rhodol derivative HMDER, which is a suitable scaffold for

[*] Dr. Y. Ichikawa, Dr. F. Obata, Prof. M. Miura, Dr. T. Terai, Dr. T. Komatsu, Dr. T. Ueno, Dr. K. Hanaoka, Prof. T. Nagano
Graduate School of Pharmaceutical Sciences
The University of Tokyo
7-3-1 Hongo, Bunkyo-ku, Tokyo 113-0033 (Japan)
Dr. M. Kamiya, Prof. Y. Urano
Graduate School of Medicine, The University of Tokyo
7-3-1 Hongo, Bunkyo-ku, Tokyo 113-0033 (Japan)
E-mail: uranokun@m.u-tokyo.ac.jp

[**] This research was supported in part by the Ministry of Education, Culture, Sports, Science and Technology of Japan (Grant-in-Aid for Scientific Research (KAKENHI), grants 20117003 and 23249004 to Y.U., grants 23113504, 25113707, and 25870180 to M.K., and grant 25104506 to T.T.), by a Specially Promoted Research Grant (22000006 to T.N.), by the Basic Research Program of the Japan Science and Technology Agency (grants to Y.U. and to M.M.), by The Daiichi-Sankyo Foundation of Life Science and a Naito Foundation Natural Science Scholarship (grants to Y.U.), by The Mochida Memorial Foundation for Medical and Pharmaceutical Research (grant to M.K.), by The Tokyo Society of Medical Sciences (grant to M.K.), and by a JSPS stipend to Y.I.

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/anie.201403221.

a β-galactosidase fluorescent probe. We chose a hydroxymethyl group as an intramolecular nucleophile to stabilize the spirocyclized structure.[4,6] Accordingly, we synthesized HMDESeR and its methylated derivative (HMDESeR-Me) as pilot compounds (Figure 1a), and examined their photochemical properties.

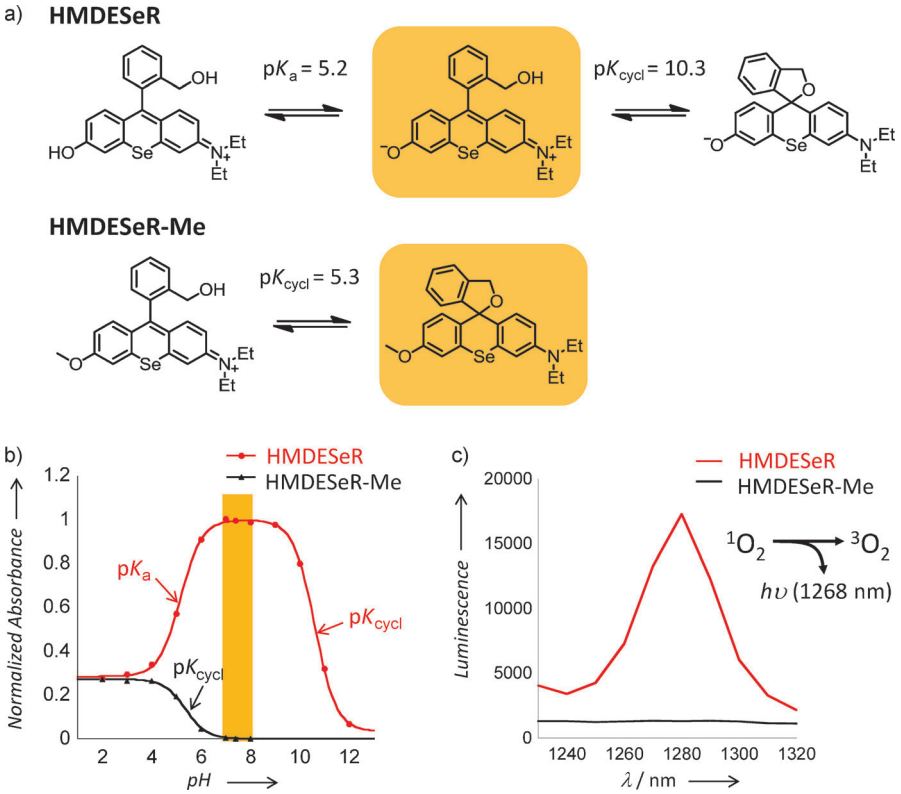

Figure 1. a) Structures of HMDESeR and HMDESeR-Me at various pH values. The highlighted structures are the putative forms predominant at physiological pH values. b) pH dependence of the absorbance of the dyes. c) Luminescence spectra of $^1O_2$ generated by excitation of the dyes (3 μM in phosphate buffered saline, pH 7.4) with a 532 nm laser.

By measuring absorption at various pH values (pH 2–12), we confirmed that HMDESeR has three possible forms, depending on the pH value (Figure 1a; see also Figure S1 in the Supporting Information), as was the case for the parent compound HMDER. The calculated pK_a value of the phenolic hydroxy group was 5.2, and the pK_{cycl} value (pH value at which the extent of spirocyclization is sufficient to reduce the absorbance of the compound to one-half of the maximum absorbance) was 10.3 (Figure 1b), thus indicating that HMDESeR exists mainly in the open xanthene form at the physiological pH value of 7.4. The open form of HMDESeR exhibits an absorption in the visible region (maximum at 558 nm), however, the fluorescence quantum yield is suppressed ($\Phi_{fl} = 0.006$, Table 1). Furthermore, it was shown (by measurement of the luminescence of produced $^1O_2$ in the infrared region) to have a high ability to produce $^1O_2$ upon laser irradiation at 532 nm ($\Phi_\Delta = 0.99$), which was superior to that observed for the original compound HMDER (Figure 1c; see also Figure S2). This result indicates that the replacement of the oxygen atom in the fluorescent rhodol scaffold with a selenium atom indeed makes this compound an effective photosensitizer. On the other hand, the methylated derivative, HMDESeR-Me, exists as an equilibrium mixture of two forms. Its pK_{cycl} value was determined to be 5.3, thus indicating that HMDESeR-Me is present predominantly as the colorless spirocyclic form under physiological conditions (Figure 1b). Therefore, HMDESeR-Me shows little visible-light absorption at pH 7.4 and therefore does not function as a photosensitizer, since it cannot be excited readily to the S_1 state by visible light to generate $^1O_2$ (Figure 1c, Table 1; see also Figure S2).

These differences in pH dependency between HMDESeR and HMDESeR-Me suggest that the alkylation of the phenolic hydroxy group stabilizes the spirocyclic form, as was also the case for the original rhodol derivative HMDER.[4a] Interestingly, replacement of the oxygen atom at the 10-position with selenium shifted the pK_{cycl} value to the acidic side. As a consequence, HMDESeR-Me is completely in the closed form at the physiological pH value of approximately 7.4 (Figure 1b; see also Figure S1). This difference in configuration seems consistent with the shift in the calculated LUMO energy levels, since the fluorophores act as electrophiles in the spirocyclization reaction: HMDESeR-Me (−0.10126 hartree) has a lower LUMO energy level than HMDER-Me (−0.09674 hartree).

To apply this finding to the selective photoablation of specific cell types, we selected β-galactosidase, a widely used reporter enzyme, as the activating enzyme.[7] It has been shown that the

Table 1: Photochemical properties of rhodol derivatives and selenium-substituted rhodol derivatives.

	λ_{abs} [nm]	λ_{em} [nm]	ε_{max} at pH 7.4[a]	Φ_{fl}	pK_a	pK_{cycl}
HMDESeR	558[b]	590[b]	92000	0.006[c]	5.2	10.3
HMDESeR-Me	537, 574[d]	n.d.	120	n.d.	–	5.3
HMDESeR-βGal	543, 580[d]	n.d.	420	n.d.	–	5.8
HMDER[e]	525	543	79000	0.141	5.4	11.3
HMDER-βGal[e]	493, 525	536	8300	0.009	–	6.9

[a] The ε value was calculated from the absorbance at pH 7.4 or from the maximum absorbance and the pK_{cycl} value. [b] The λ_{abs} and λ_{em} values were measured in 100 mM sodium phosphate buffer (pH 7.4). [c] The absolute fluorescence quantum yield was measured in 100 mM sodium phosphate buffer (pH 7.4). [d] The λ_{abs} values were measured in 100 mM sodium phosphate buffer (pH 2.0). [e] The values for HMDER and HMDER-βGal were determined in a previous study.[4a] n.d. = not detectable.

expression of β-galactosidase in many organisms, including *Drosophila, Caenorhabditis elegans*, zebrafish, and mice, can be restricted to a specific region, organ, or cell line by genetically placing it under specific promoters/enhancers. Thus, the selective ablation of β-galactosidase-expressing cells with a β-galactosidase-activatable photosensitizer is expected to have a great impact in biological research, since it would be directly applicable to well-established lines. To develop a photosensitizer activatable by β-galactosidase, we replaced the methyl group of HMDESeR-Me with a β-galactoside group, which should be cleaved by β-galactosidase. The incorporation of such an enzyme-reactive moiety into HMDESeR should result in the suppression of background phototoxicity owing to the spirocyclic, non-phototoxic form of the unactivated molecule, whereas reaction with the enzyme should release phototoxic HMDESeR.

Indeed, the visible-light absorption and $^1O_2$ productivity of the β-galactoside-substituted analogue, HMDESeR-βGal, were suppressed (Figure 2a–c; see also Figure S2), as we had expected. The pH dependency resembled that of HMDESeR-Me, and the pK_{cycl} value was calculated to be 5.8 (Table 1; see also Figure S1). We also found that HMDESeR-βGal reacted rapidly with β-galactosidase to produce HMDESeR (see Figure S3).

Next, we examined whether HMDESeR-βGal could induce the cell death of only β-galactosidase-expressing cells. We applied HMDESeR and HMDESeR-βGal to two cultured cell lines of HEK293 cells with or without β-galactosidase expression (HEK/*lacZ* cells and HEK cells), and exposed the cells to light irradiation. Whereas HMDESeR induced cell death equally in both cell lines, HMDESeR-βGal induced dose-dependent cell death only in HEK/*lacZ* cells (Figure 2d; see also Figure S4). Although HMDESeR tends to accumulate mainly in mitochondria and partially in lysosomes (see Figure S5), we consider that the activation site of HMDESeR-βGal is predominantly the cytosol, since β-galactosidase is expressed in the cytosol of HEK/*lacZ* cells[4a] and we did not observe any phototoxic effect in cells without *lacZ* expression. Furthermore, as compared to our previously reported activatable photosensitizer based on the photoinduced intramolecular electron transfer (PeT) mechanism, which showed only 20-fold activation of phototoxicity,[3a] spirocyclization-controlled HMDESeR-βGal showed much greater activation (>100-fold). We believe that this activatability is the main reason why it is able to effectively induce cell death specifically in cells expressing β-galactosidase.

We further tested our photosensitizer in cultured tissue of *Drosophila melanogaster*, which is one of the most widely used model organisms for genetic studies.[8] For this purpose, we used wing disks, a precursor structure of a part of the adult thorax, including the wing. In the *en-lacZ* reporter line, β-galactosidase is expressed only in the posterior region of the wing disks. Our photosensitizer was applied to a culture of wing disks from third instar larvae, and whole wing disks were then exposed to light with a wavelength of 561 nm under a confocal microscope equipped with a diode-pumped solid-state (DPSS) laser. After incubation for 3 h at room temperature, we observed morphological changes, such as loss of

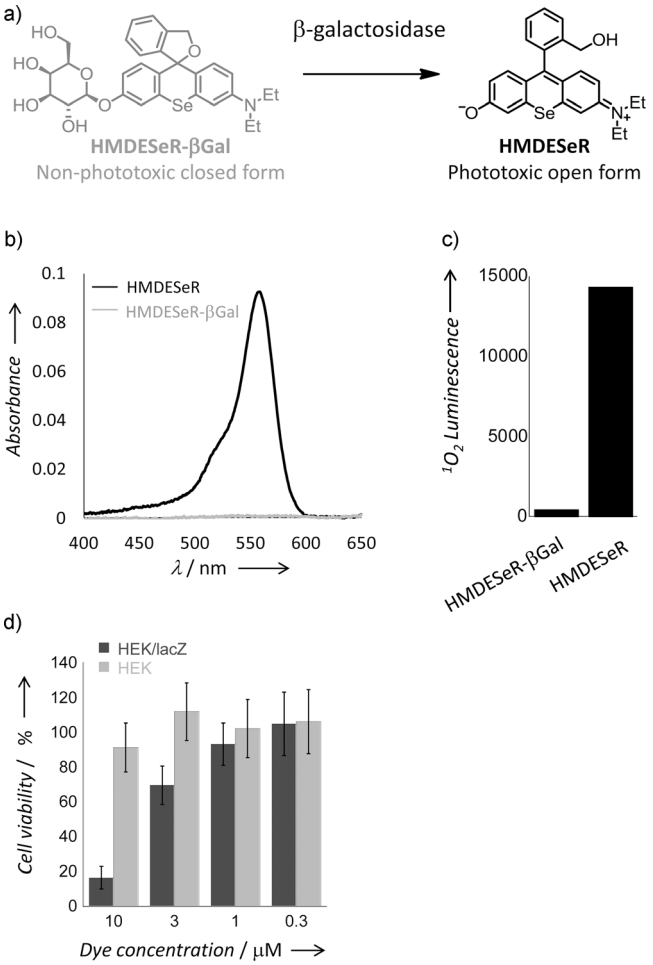

Figure 2. a) Enzymatic reaction of HMDESeR-βGal with β-galactosidase. b) Absorption spectra of 1 μM HMDESeR-βGal and HMDESeR in 100 mM sodium phosphate buffer (pH 7.4). c) $^1O_2$ production upon laser irradiation at 532 nm. d) Photoinduced cytotoxicity to HEK/*lacZ* cells and HEK cells. Cells were incubated with HMDESeR-βGal for 4 h and then irradiated with light (510–555 nm, 6 J cm^{-2}). Cell viability was measured by using a CCK-8 assay at 24 h postirradiation. Bars indicate the viability of cells as compared with untreated cells; means ± standard deviation ($n=4$).

asperity and obscured cellular boundaries, only in the posterior region, where β-galactosidase is expressed (Figure 3b; see also Figure S6). When we examined cell viability by live/dead fluorescence staining (CalceinAM for live cells, EthD-1 for dead cells), the pattern of dead cells was similar to that for β-galactosidase immunostaining; that is, dead cells were observed only in the posterior region (Figure 3a,c).

In the middle of the tissue, there was a region where no fluorescence signal was observed, although the cells were clearly stressed in terms of morphological changes (see Figure S6). The lack of a fluorescence signal may be explained by insufficient distribution of EthD-1 to the middle region. Furthermore, dead cells were not observed in either of the control samples without irradiation with light or incubation with HMDESeR-βGal (Figure 3c), and HMDESeR induced cell death in all regions of the wing disk, regardless of β-galactosidase expression (see Figure S7). These results

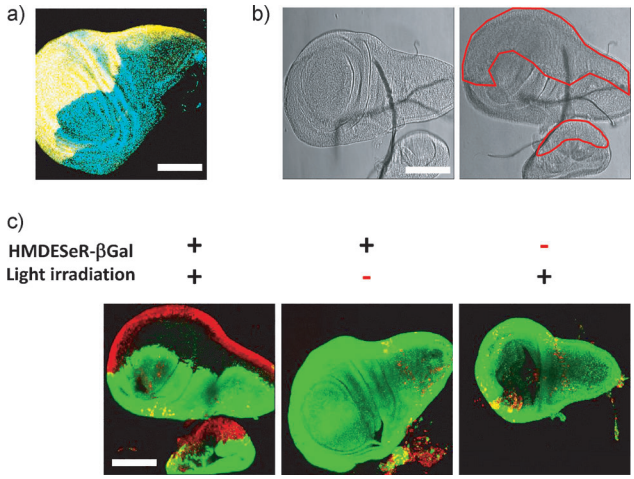

Figure 3. a) Immunostaining of β-galactosidase (yellow) in an *en-lacZ Drosophila* larval wing disk. b) Bright-field image of a wing disk pretreated with HMDESeR-βGal before (left) and after irradiation with a DPSS laser (561 nm, 100%, 3 min; right). After irradiation with light, a morphological change was observed in the posterior region highlighted in red (see Figure S6 for magnified images). c) Live/dead fluorescence staining of the wing disks with CalceinAM (for live cells, green) and EthD-1 (for dead cells, red) 3 h after treatment. A similar pattern of living and dead cells was observed in the haltere disk (the smaller area of tissue seen below the wing disk), in which β-galactosidase is expressed in the posterior region. Scale bars: 100 μm.

strongly indicate that HMDESeR-βGal can efficiently trigger cell death/stress only in β-galactosidase-expressing regions upon exposure to photoirradiation.

In conclusion, we have designed, synthesized, and evaluated HMDESeR-βGal as a novel activatable photosensitizer on the basis of the precise control of spirocyclization. This simple strategy has several advantages: 1) HMDESeR-βGal can be activated to induce cell death only in cells of interest, that is, those expressing β-galactosidase, 2) undesired phototoxicity is suppressed, since HMDESeR-βGal is not activated until it has reacted with the target enzyme. An ex vivo analysis of a larval *Drosophila* wing disk confirmed that HMDESeR-βGal induced cell death specifically in the β-galactosidase-expressing region. To our knowledge, cell death has not previously been induced specifically in target cells of living tissue with a small-molecular photosensitizer by the use of nonlocalized photoirradiation. Finally, we expect that this design strategy can be extended to the development of activatable photosensitizers directed at other types of cells. For example, it should be possible to develop a new strategy for tumor-selective photodynamic therapy (PDT)[9] by targeting enzymes that are overexpressed in tumors. We anticipate that a range of activatable photosensitizers based on the present strategy will be versatile tools for cellular manipulation (cell ablation) and also for tumor therapy.

Received: March 11, 2014
Published online: May 21, 2014

Keywords: activatable photosensitizers · cell ablation · fluorescent probes · intramolecular spirocyclization · selenium

[1] a) M. Bulina, K. Lukyanov, O. Britanova, D. Onichtchouk, S. Lukyanov, D. Chudakov, *Nat. Protoc.* **2006**, *1*, 947; b) C. Teh, D. Chudakov, K.-L. Poon, I. Mamedov, J.-Y. Sek, K. Shidlovsky, S. Lukyanov, V. Korzh, *BMC Dev. Biol.* **2010**, *10*, 110; c) Y. B. Qi, E. J. Garren, X. Shu, R. Y. Tsien, Y. Jin, *Proc. Natl. Acad. Sci. USA* **2012**, *109*, 7499.

[2] K. Takemoto, T. Matsuda, M. McDougall, D. H. Klaubert, A. Hasegawa, G. V. Los, K. V. Wood, A. Miyawaki, T. Nagai, *ACS Chem. Biol.* **2011**, *6*, 401.

[3] a) T. Yogo, Y. Urano, M. Kamiya, K. Sano, T. Nagano, *Bioorg. Med. Chem. Lett.* **2010**, *20*, 4320; b) Y. Koide, Y. Urano, A. Yatsushige, K. Hanaoka, T. Terai, T. Nagano, *J. Am. Chem. Soc.* **2009**, *131*, 6058.

[4] a) M. Kamiya, D. Asanuma, E. Kuranaga, A. Takeishi, M. Sakabe, M. Miura, T. Nagano, Y. Urano, *J. Am. Chem. Soc.* **2011**, *133*, 12960; b) M. Sakabe, D. Asanuma, M. Kamiya, R. J. Iwatate, K. Hanaoka, T. Terai, T. Nagano, Y. Urano, *J. Am. Chem. Soc.* **2013**, *135*, 409.

[5] M. R. Detty, P. N. Prasad, D. J. Donnelly, T. Ohulchanskyy, S. L. Gibson, R. Hilf, *Bioorg. Med. Chem.* **2004**, *12*, 2537.

[6] S. Kenmoku, Y. Urano, H. Kojima, T. Nagano, *J. Am. Chem. Soc.* **2007**, *129*, 7313.

[7] a) J. Alam, J. L. Cook, *Anal. Biochem.* **1990**, *188*, 245; b) D. J. Spergel, U. Kruth, D. R. Shimshek, R. Sprengel, P. H. Seeburg, *Prog. Neurobiol.* **2001**, *63*, 673.

[8] a) S. Ohsawa, K. Sugimura, K. Takino, T. Xu, A. Miyawaki, T. Igaki, *Dev. Cell* **2011**, *20*, 315; b) S. Ohsawa, K. Sugimura, K. Takino, T. Igaki, *Methods Enzymol.* **2012**, *506*, 407; c) S. Aldaz, L. M. Escudero, M. Freeman, *Proc. Natl. Acad. Sci. USA* **2010**, *107*, 14217.

[9] a) G. Zheng, J. Chen, K. Stefflova, M. Jarvi, H. Li, B. C. Wilson, *Proc. Natl. Acad. Sci. USA* **2007**, *104*, 8989; b) S.-M. Shon, Y. Choi, J.-Y. Kim, D. K. Lee, J.-Y. Park, D. Schellingerhout, D.-E. Kim, *Arterioscler. Thromb. Vasc. Biol.* **2013**, *33*, 1360; c) Y. Choi, R. Weissleder, C.-H. Tung, *Cancer Res.* **2006**, *66*, 7225.

