Lozano-Torres, B.; Galiana, I.; Rovira, M.; Garrido-García, EM.; Chaib, S.; Bernardos Bau, A.; Muñoz-Espín, D.... (2017). An OFF-ON Two-Photon Fluorescent Probe for Tracking Cell Senescence in Vivo. Journal of the American Chemical Society. 139(26):8808-8811. doi:10.1021/jacs.7b04985

# An OFF-ON two-photon fluorescent probe for tracking cell senescence *in vivo.*

Beatriz Lozano-Torres,[†,‡,§,#] Irene Galiana,[†,‡,§,#] Miguel Rovira,[Δ] Eva Garrido, [†,‡,§,#] Selim Chaib,[Δ] Andrea Bernardos,[†,‡,§, #] Daniel Muñoz-Espín, [Δ, ¥] Manuel Serrano,[Δ,*] Ramón Martínez-Máñez[†,‡, §,#,*] and Félix Sancenón[†,‡,§,#]

[†] Instituto Interuniversitario de Investigación de Reconocimiento Molecular y Desarrollo Tecnológico (IDM), Universitat Politècnica de València, Universitat de València. Spain.

[‡] Unidad Mixta UPV-CIPF de Investigación en Mecanismos de Enfermedades y Nanomedicina, Universitat Politècnica de València, Centro de Investigación Príncipe Felipe, Valencia, Spain.

[§] CIBER de Bioingeniería, Biomateriales y Nanomedicina (CIBER-BBN).

[#] Unidad Mixta de Investigación en Nanomedicina y Sensores. Universitat Politècnica de València, IIS La Fe, Valencia, Spain

[Δ] Tumor Suppression Group. Spanish National Cancer Research Centre (CNIO). Madrid, Spain

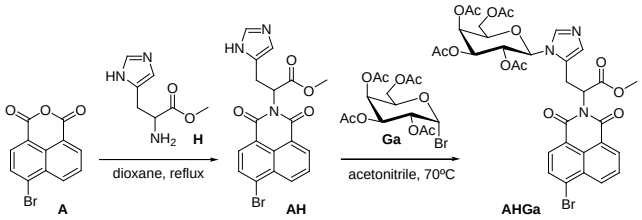
[¥] CRUK Cancer Centre Early Detection Programme, Department of Oncology. University of Cambridge. Hutchison/MRC Research Centre. Cambridge, UK

**ABSTRACT:** A naphthalimide-based two-photon probe (**AHGa**) for the detection of cell senescence is designed. The probe contains a naphthalimide core, an L-histidine methyl ester linker and an acetylated galactose bonded to one of the aromatic nitrogen atoms of the L-histidine through a hydrolysable *N*-glycosidic bond. Probe **AHGa** is transformed into **AH** in senescent cells resulting in an enhanced fluorescent emission intensity. *In vivo* detection of senescence is validated in mice bearing tumor xenografts treated with senescence-inducing chemotherapy.

The main purpose of cellular senescence is to prevent the proliferation of damaged or stressed cells and to trigger tissue repair.[1] However, upon persistent damage or during aging, the dynamic process of tissue repair becomes inefficient and senescent cells tend to accumulate. This accumulation in tissues is believed to impair tissue functions and accelerate aging.[1] Several markers and phenotypic characteristics have been described to define senescence: (i) a complex paracrine response termed as senescence-associated secretory phenotype (SASP),[2] (ii) the expression of cell cycle inhibitors and/or tumor suppressors (p16, p21 or p53)[3] and (iii) high levels of lysosomal β-galactosidase (βGal) activity[4] and other hydrolases.[5]

It has been demonstrated that genetic ablation of senescent cells ameliorates a variety of ageing-associated diseases, reverts long-term degenerative processes and extends longevity.[6] Inspired by these findings, strategies to prevent, replace or remove senescent cells have become of interest. For instance, there is an increasing interest in the development of senolytic molecules able to induce apoptosis preferentially in senescent cells.[7,8] A related key issue in this field, is the design of probes to accurately detect senescent cells in aged or damaged tissues.[1] However, one of the major obstacles limiting progress in this research area, is the nearly lack of real-time methods to selectively track senescence in *in vivo* systems.

**Scheme 1**. Synthetic route used for the preparation of **AHGa** probe.




Detection of senescent cells usually relies on the detection of senescence-associated βgal (SAβgal), and several fluorescent or chromogenic probes have been reported for the visualization of this enzymatic activity.[9] However, these first-generation probes are usually unsuitable for *in vivo* imaging as they rely on chromogenic changes or on the use of classical one-photon fluorescence excitation. As an alternative, recent stimulating studies developing two-photon fluorescent probes for the visualization of βgal activity have been described (Table S1).[10] However, some of the reported probes are synthesized by using tedious multistep protocols. Another common drawback is the fact that probes are tested in cultured cells or in animal models that were not directly related to senescence. The methodology used in these studies, is based on a *lacZ* gene transfection, which results in high levels of bacterial βgal expression in the cell cytoplasm (unlike the lysosomal βgal activity associated with senescent cells).[11] A limited number of works describe the use of human cell lines overexpressing endogenous βgal *per se*, as it is the case of ovarian cancer cell lines.[12] However, these cancer cell lines are obviously not senescent because senescent cells do not proliferate. As regards *in vivo*

studies with mice, probes are tested in models that (i) labeled tumors with avidin-βgal[13] or (ii) transfected tumor cells with pCMV-*lacZ* plasmid.[14] In both cases a bacterial βgal overexpression is achieved. In this scenario, the development reliable two-photon fluorescence-based probes for *in vivo* detection of senescence is still a challenge.

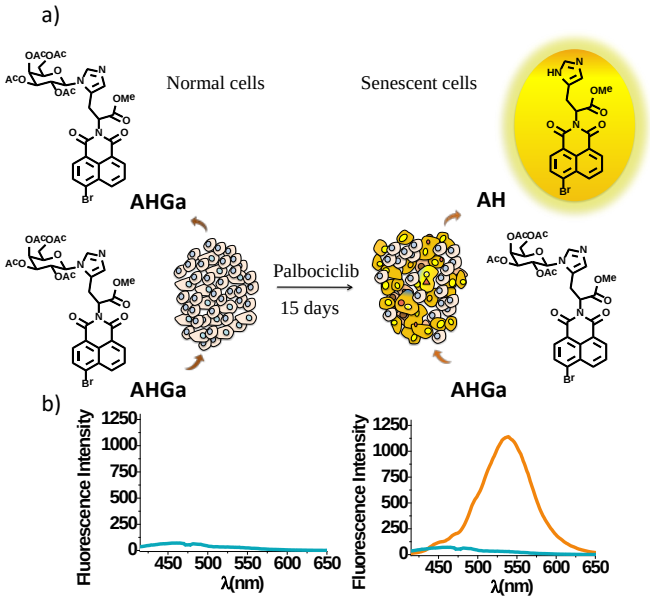



**Figure 1**. a) Schematic representation of **AHGa** activation in senescent cells. b) Emission spectra of PBS (pH 4.5)-DMSO (0.01%) solutions of $10^{-5}$ M **AHGa** (blue) and **AH** (orange) when excited at 405 nm.

In view of the aspects mentioned above, we report herein a novel molecular probe for the two-photon fluorogenic *in vivo* detection of senescence. The probe (**AHGa**) is based on a naphthalimide fluorophore as signaling unit containing an L-histidine methyl ester linker and an acetylated galactose attached to one of the aromatic nitrogen atoms of the L-histidine through a hydrolysable *N*-glycosidic bond. Probe **AHGa** is transformed into **AH** in senescent cells (Figure 1a) resulting in an enhanced fluorescent emission intensity. *In vivo* detection of senescence was demonstrated in mice bearing tumor xenografts treated with senescence-inducing chemotherapy.

**AHGa** was easily prepared using a two-step protocol (Scheme 1). 4-bromo-1,8-naphtalic anhydride (**A**) and L-histidine methyl ester (**H**) were reacted in refluxing dioxane yielding fluorophore **AH**. Then, a nucleophilic substitution reaction between **AH** and 2,3,4,6-tetra-O-acetyl-α-D-galactopyranosyl bromide (**Ga**) yielded the **AHGa** probe. Both, **AH** and **AHGa**, were fully characterized (Figures S1-S6.). Figure 1b summarizes the fluorogenic behavior of **AHGa** and **AH**. Excitation at 405 nm of **AHGa** in PBS (pH 4.5)-DMSO (0.01%) did not show any emission band ($\phi_{\mathbf{AHGa}}$ = 0.002), whereas under the same experimental conditions **AH** displayed an intense fluorescence ($\phi_{\mathbf{AH}}$ = 0.458, 286-fold enhancement) at 540 nm (Figure 1b and S7). Moreover, the emission intensity of **AH** remained unchanged in the 4-8 pH range (Figure S8).

Targeting of senescent cells *in vitro* with **AHGa** was validated with the SK-MEL-103 (human melanoma) cell line treated with palbociclib (Figure 2).

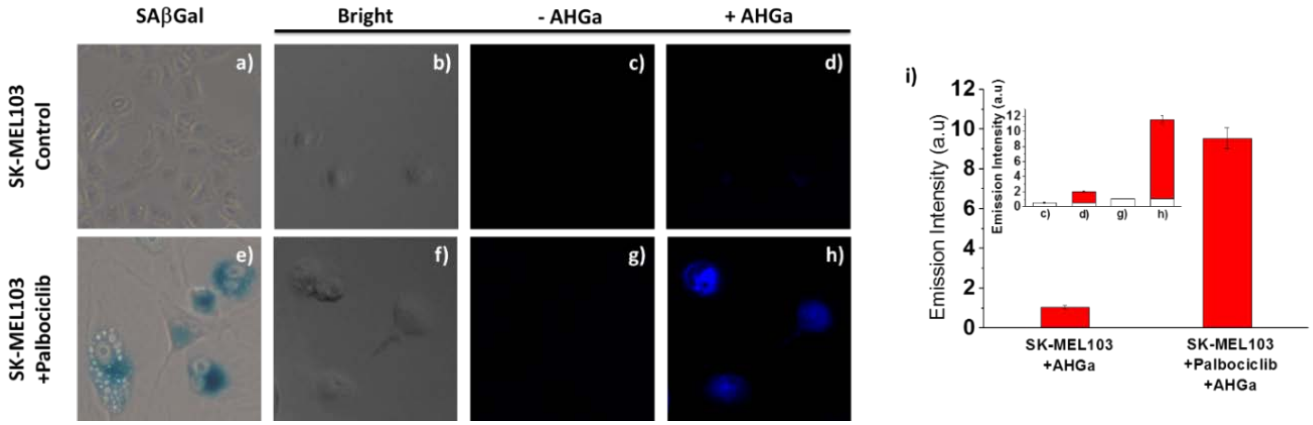



**Figure 2**. (a, e) Conventional X-Gal chemical assay for SAβGal expression of control (a) and palbociclib-treated (senescent) (e) SK-MEL-103 cells. (b, f) Bright channel for control SK-MEL-103 (b) and senescent SK-MEL-103 (f) cells. (c, d, g, h) Two-photon images of (c, d) control SK-MEL-103 and (g, h) senescent SK-MEL-103 cells in the absence (c, g) and in the presence (d, h) of **AHGa** probe. Cells were incubated with **AHGa** (10 μM) with DMEM (10% FBS, 0.1% DMSO) in 20% $O_2$ and 5% $CO_2$ at 37°C for 2 h, and images were acquired by using a femtosecond pulse laser (excitation at 750 nm). Representative images from repeated experiments (n = 3) are shown. (i) Quantification of the fluorescence emission intensity of control and palbociclib-treated SK-MEL-103 cells incubated with **AHGa**. Inset: Basal fluorescence is represented in white bars and **AHGa** ascribed fluorescence is represented in red. Error bars represent s.d.

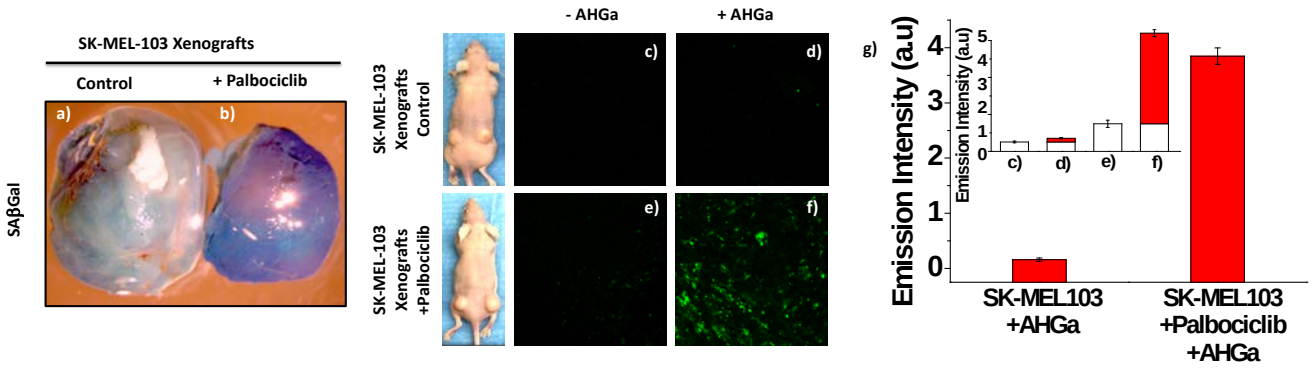



**Figure 3.** (a,b) Tissue portions of SK-MEL-103 control tumors (a) or SK-MEL-103 senescent tumors after whole-mount SAβGal staining. (c-f) Confocal images of representative portions of SK-MEL-103 control tumors (c,d) and SK-MEL-103 senescent tumors (e,f) vehicle (c,e) and after intravenous tail injection (d,f) of **AHGa** probe (10 mg/ml, 200 µl). Mice were sacrificed at 3 hours post-treatment. (g) Quantification of the fluorescence emission intensity of SK-MEL-103 (control) and SK-MEL-103 treated with palbociclib (senescent) in the absence or in the presence of the **AHGa** probe. Inset: Basal fluorescence is represented in white bars and **AH** ascribed fluorescence is represented in red. Error bars represent s.d. (n = 6 tumors for each condition).

Palbociclib is a CDK4/6 inhibitor which has been displayed to induce cell cycle arrest in melanoma cells.[15] In a common experiment, SK-MEL-103 cells were treated with 5 µM palbociclib during two weeks in order to induce senescence (Figure 2b and 2f). After this treatment, cellular senescence in SK-MEL-103 cells was assessed by SAβGal activity assay[16] (Figure 2a and 2e). Moreover it was found that **AH** and **AHGa** proved to be not toxic for both control and palbociclib-treated SK-MEL-103 cells after 48 h at concentrations of up to 20 µM (Figure S9).

Both, control and palbociclib-treated SK-MEL-103 cells were seeded in flat-bottom-clear 96 well plates and incubated with a solution containing **AHGa** (10 µM in DMEM, 0.1% DMSO) for 2 h. Cells were then analyzed by two-photon confocal microscopy using 750 nm as excitation wavelength. Control SK-MEL-103 cells in the absence (Figure 2c) or presence (Figure 2d) of **AHGa** did not exhibit any noticeable fluorescence signal. Similarly, in the absence of the probe, senescent SK-MEL-103 cells did not show significant auto-fluorescence (Figure 2g). In contrast, senescent cells treated with **AHGa** showed a clear bright emission (Figure 2h) due to the presence of an emission band at ca. 540 nm, and attributed to the hydrolysis of **AHGa** into **AH** that selectively occurred in senescent cells. The quantification of the fluorescence associated to **AH** was also determined for each treatment from confocal images (Figure 2i). A remarkable fluorescence enhancement (ca. 10-fold) for palbociclib-treated SK-MEL-103 cells in the presence of **AHGa** when compared with control SK-MEL-103 cells was observed.

Once assessed the activation of **AHGa** in senescent cultured cells, the probe was validated in mice bearing tumor xenografts treated with senescence-inducing chemotherapy. In a typical experiment, tumor xenografts were generated by injecting subcutaneously SK-MEL-103 melanoma cells. Upon tumor formation, mice were treated daily with palbociclib for 10 days in order to induce senescence and arrest tumor growth. Finally, **AHGa** probe was injected intravenously in the tail vain and mice were sacrificed at 3 h after the treatment. Tumors were extracted and cellular senescence was assessed by a SAβGal staining (Figure 3b). The absence of the proliferative marker Ki67, and the reduction in phosphorylated Rb in tumors were also indicative of senescence.[17] Then, fresh tumor sections were analyzed using confocal microscopy (Figures 3c-3f). Control tumors showed negligible fluorescence either in the absence or in the presence of **AHGa** (Figures 3c and 3d). Tumors from mice treated with palbociclib in the absence of the probe were used as a control for tissue autofluorescence, and showed a weak emission (Figure 3e). In sharp contrast, isolated tumors from mice treated with palbociclib and intravenously injected with **AHGa** showed a strong fluorescent signal (Figure 3f). Quantification of the associated **AH** fluorescence was determined for each condition (Figure 3g). A marked emission enhancement (ca. 15-fold) in tumors treated with palbociclib compared to control tumors was observed. In addition, *ex vivo* imaging of fresh sections from different organs showed that emission from **AH** was only observed in senescent tumors (Figure S10).

Altogether, we describe a two-photon fluorescent probe (**AHGa**) for the *in vivo* detection of cell senescence. Targeting of senescent cells *in vitro* with **AHGa** was validated with the SK-MEL-103 cancer cell line treated with palbociclib. A remarkable fluorescence emission enhancement (ca. 10-fold) in the presence of **AHGa** for palbociclib-treated SK-MEL-103 (senescent) cells was observed when compared with control SK-MEL-103 cells, due to the formation of **AH**. The ability of tracking senescence of probe **AHGa** was also studied *in vivo* by employing mice bearing subcutaneous tumor xenografts generated with SK-MEL-103 melanoma cells and treated with palbociclib. Tumors in palbociclib-untreated mice showed negligible fluorescence emission both in the absence or in the presence of **AHGa**, whereas tumors in mice treated with palbociclib and intravenously injected with **AHGa** showed a clear fluorescent signal. A marked emission enhancement (ca. 15-fold) in tumors treated with palbociclib compared to non-treated tumors was observed. **AH** fluorescence was only found in senescent tumors but not in other organs. The combination of selectivity, sensitivity and straightforward synthesis make **AHGa** and efficient OFF-ON two-photon probe for the *in vivo* signaling of senescence.

## ASSOCIATED CONTENT

The Supporting Information is available free of charge on the ACS Publications website at DOI:

Materials and method, synthesis and characterization of probe, cell assays and *in vivo* xenograft assays.

## AUTHOR INFORMATION

### Corresponding Author

*rmaez@qim.upv.es
*manuel.serrano@irbbarcelona.org
The author declare no competing financial interest

**ACKNOWLEDGMENTS**

The authors thank the financial support from the Spanish Government (projects MAT2015-64139-C4-1-R) and the Generalitat Valenciana (project PROMETEOII/2014/047). B.L-T. is grateful to the Spanish Ministry of Economy for her PhD grant. I.G. thanks the Generalitat Valenciana for her PhD grant. A.B. thanks the Spanish Government for the financial support "Juan de la Cierva-Incorporación". M.R. is grateful to "La Caixa" foundation for his PhD scholar-ship. Work in M. S.´s group was funded by the CNIO and by grants from Spanish Ministry of Economy, the European Regional Development Fund (SAF project) the European Research Council (ERC Advanced Grant), and the Botin Foundation and Banco Santander (Santander Universities Global Division). D.M.-E. was funded by a "Ramón y Cajal" Programme Senior Grant and the "RETOS" National Programme for Research (MINECO), Cancer Research UK, and the CRUK Cambridge Centre Early Detection Programme.

**REFERENCES**

(1) Muñoz-Espin, D.; Serrano, M. *Nat. Rev. Mol. Cell Biol*. **2014**, *15*, 482-496.

(2) (a) Yun, M. H.; Davaapil, H.; Brockes *J. P. eLife* **2015**, *4*, e05505. (b) Chiche, A.; Le Roux, I.; von Joest, M.; Sakai, H.; Aguin, S. B.; Czin, C.; Salam, R.; Fiette, L.; Alegria, O.; Flamant, P.; Tajbahksh, S.; Li, H. *Cell Stem Cell* **2017**, *20*, 407-414. (c) Demaria, M.; Ohtani, N.; Youssef, S. A.; Rodier, F.; Touissant, W.; Mitchell, J. R.; Laberge, R. M.; Vijg, J.; Van Steeg, H.; Dollé, M. E. T.; Hoeijmakers, J. H. J.; de Bruin, A.; Hara, E.; Campisi, J. *Dev. Cell* **2014**, *31*, 722-733.

(3) (a) Haber, D. A. *Cell* **1997**, 91, 555-558. (b) Jung, Y. –S.; Qian, Y.; Chen, X. *Cell. Signal*. **2010**, *22*, 1003-1012.

(4) Dimri, G. P.; Lee, X.; Basile, G.; Acosta, M.; Scott, G.; Roskelley, C; Medrano, E. E.; Linskens, M.; Rubelj, I.; Pereira-Smith, O.; Peacocke, M.; Campisi, J. *Proc. Natl. Acad. Sci. USA* **1995**, *92*, 9363-9367.

(5) Knás, M.; Zalewska, A.; Kretowski, R.; Niczyporuk, M.; Waszkiewicz, N.; Cechowska-Pasko, M.; Waszkiel, D.; Zwierz, K. *Folia Histochem. Cytobiol*. **2012**, *50*, 220-227.

(6) (a) Baker, D. J.; Childs, B. J.; Durik, M.; Wijers, M. E.; Sieben, C. J.; Zhong, J.; Saltness, R. A.; Jeganathan, K. B.; Verzosa, G. C.; Pezeshki, A.; Khazaie, K.; Miller, J. D.; van Deursen, J. M. *Nature* **2016**, *530*, 184-189. (b) Childs, B. G.; Baker, D. J.; Wijshake, T.; Conover, C. A.; Campisi, J.; van Deursen, J. M. *Science* **2016**, *354*, 472-477.

(7) Zhu, Y.; Tchkonia, T.; Pirtskhalava, T.; Gower, A. C.; Ding, H.; Giorgadze, N.; Palmer, A. K.; Ikeno, Y.; Hubbard, G. B.; Lengurg, M.; O´Hara, S. P.; LaRusso, N. F.; Miller, J. D.; Roos, C. M.; Verzosa, G. C.; LeBrasseur, N. K.; Wren, J. D.; Farr, J. N.; Khosla, S.; Stout, M. B.; McGowan, S. J.; Fuhrmann-Stroissnigg, H.; Gurkar, A. U.; Zhao, J.; Colangelo, D.; Dorronsoro, A.; Ling, Y. Y.; Barghouthy, A. S.; Navarro, D. C.; Sano, T.; Robbins, P. D.; Niedernhofer, L. J.; Kirkland, J. L. *Aging Cell* **2015**, *14*, 644-658.

(8) (a) Chang, J.; Wang, Y.; Shao, L.; Laberge, R. M.; Demaria M.; Campisi J.; Janakiraman, K.; Sharpless, N. E.; Ding, S.; Feng, W.; Luo, Y.; Wang, X.; Aykin-Burns, N.; Krager, K.; Ponnappan, U.; Hauer-Jensen, M.; Meng, A.; Zhou, D. *Nat. Med*. **2016**, *22*, 78-83. (b) Yosef, R.; Pilpel, N.; Tokarsky-Amiel, R.; Biran, A.; Ovayda, Y.; Cohen, S.; Vadai, E.; Dassa, L.; Shahar, E.; Condiotti, R.; Ben-Porath, I.; Krizhanovsky, V. *Nat. Commun*. **2016**, *7*, 11190. (c) Baar, M. P.; Brandt, R. M.; Putavet, D. A.; Klein, J. D.; Derks, K. W.; Bourgeois, B. R.; Stryeck, S.; Rijksen, Y.; van Willigenburg, H.; Feijtel, D. A.; van der Pluijm, I.; Essers, J.; van Cappellen, W. A.; van IJcken, W. F.; Houtsmuller, A. B.; Pothof, J.; de Bruin, R. W.; Madl, T.; Hoeijmakers, J. H.; Campisi, J.; de Keizer, P. L. *Cell* **2017**, *169*, 132-147.

(9) (a) Wang, P.; Du, J.; Liu, H.; Bi, G.; Zhang, G. *Analyst* **2016**, *141*, 1483-1487. (b) Otsubo, T.; Minami, A.; Fujii, H.; Taguchi, R.; Takahashi, T.; Suzuki, T.; Teraoka, F.; Ikeda, K. *Bioorg. Med. Chem. Lett*. **2013**, *23*, 2245-2249. (c) Mandal, P. K.; Cattiaux, L.; Bensimon, D.; Mallet, J. M. *Carbohyd. Res.* **2012**, *358*, 40-46. (d) Na, S. Y.; Kim, H. J. *Dyes Pigm*. **2016**, *134*, 526-530. (e) Miranda, O. R.; Cehn, H. T.; You, C. C.; Mortenson, D. E.; Yang, X. C.; Bunz, U. H. F.; Rotello, V. M. *J. Am. Chem. Soc.* **2010**, *132*, 5285-5289. (f) Zhang, C.; Wang, D.; Zhang, L.; Guo, J. F.; Rem, A. M. *RSC Adv.* **2016**, *6*, 70960-70961.

(10) Kim, H. M.; Cho, B. R. *Chem. Rev.* **2015**, *115*, 5014-5055.

(11) (a) Tung, C. H.; Zeug, Q.; Shah, K.; Kim, D. E.; Schellingerhout, D.; Weissleder, R. *Cancer Res*. **2004**, *64*, 1579-1583. (b) Urano, Y.; Kamiya, M.; Kanda, K.; Ueno, T.; Hirose, K.; Nagano, T. *J. Am. Chem. Soc.* **2005**, *127*, 4888-4894. (c) Lee, H. W.; Heo, C. H.; Sen, D.; Byun, H. O.; Kwak, I. H.; Yoon, G.; Kim, H. M. *Anal. Chem.* **2014**, *86*, 10001-10005.(d) Egawa, T.; Koide, Y.; Hanaoka, K.; Komatsu, T.; Terai, T.; Nagano, T. *Chem. Commun*. **2011**, *47*, 4162-4164. (e) Kamiya, M.; Asanuma, D.; Kuranaga, E.; Takeishi, A.; Sakabe, M.; Miura, M.; Nagano, T.; Urano, Y. *J. Am. Chem. Soc.* **2011**, *133*, 12960-12963. (f) Sakabe, M.; Asanuma, D.; Kamiya, M.; Iwatate, R. J.; Hanaoka, K.; Terai, T.; Nagano, T.; Urano, Y. *J. Am. Chem. Soc.* **2013**, *135*, 409-414. (g) Peng, L.; Gao, M.; Cai, X.; Zhang, R.; Li, K.; Feng, G.; Tong, A.; Liu, B. *J. Mater. Chem. B* **2015**, *3*, 9168-9172. (h) Han, J.; Han, M. S.; Tung, C. H. *Mol. BioSyst.* **2013**, *9*, 3001-3008. (i) Zhang, X. X.; Wu, H.; Li, P.; Qu, Z. J.; Tan, M. Q.; Han K. L. *Chem. Commun*. **2016**, *52*, 8283-8286.

(12) (a) Asanuma, D.; Sakabe, M.; Kamiya, M.; Yamamoto, K.; Hiratake, J.; Ogawa, M.; Kosaka, N.; Choyke, P. L.; Nagano, T.; Kobayashi, H.; Urano, Y. *Nat. Commun*. **2015**, *6*, 6463-6470. (b) Gu, K.; Xu, Y.; Li, H.; Guo, Z.; Zhu, S.; Shi, P.; James, T. D.; Tian, H.; Zhu, W. H. *J. Am. Chem. Soc.* **2016**, *138*, 5334-5340.

(13) (a) Kamiya, M.; Kobayashi, H.; Hama, Y.; Koyama, Y.; Bernardo, M.; Nagano, T.; Choyke, P. L. Urano, Y. *J. Am. Chem. Soc.* **2007**, *129*, 3918-3929. (b) Gu, K.; Xu, Y.; Li, H.; Guo, Z.; Zhu, S.; Zhu, S.; Shi, P.; James T. D.; Tian, H.; Zhu, W. H. *J. Am. Chem. Soc.* **2016**, *138*, 5334-5340.

(14) Oushiki, D.; Kojima, H.; Takahashi, Y.; Komatsu, T.; Terai, T.; Hanaoka, K.; Nishikawa, M.; Takakura, Y.; Nagano, T. *Anal. Chem*. **2012**, *84*, 4404-4410.

(15) Whittaker, S. R.; Mallinger, A.; Workman, P.; Clarke, P. A. *Pharmacol. Ther.* **2017**, *173*, 83-105.

(16) Horwitz, J. P., Chua, J., Curby, R. J., Thomsom, A. J., Da Rooge, M. A., Fisher, B. E., Mauricio, J., Klundt, I. *J. Med. Chem.* **1964**, *7*, 574–575.

(17) Takahashi, A., Ohtani, N., Hara, E. *Cell Div*. **2007**, *2*, 10-15.