JACS
JOURNAL OF THE AMERICAN CHEMICAL SOCIETY
Communication
pubs.acs.org/JACS

A Small Molecule That Represses Translation of G-Quadruplex-Containing mRNA

Yousuke Katsuda,[†] Shin-ichi Sato,[*,†] Lisa Asano,[†,‡] Yoshitaka Morimura,[§] Tomoyuki Furuta,[†] Hiroshi Sugiyama,[†,∥] Masaki Hagihara,[*,⊥] and Motonari Uesugi[*,†,‡]

[†]Institute for Integrated Cell-Material Sciences (WPI-iCeMS) and [‡]Institute for Chemical Research, Kyoto University, Uji, Kyoto 611-0011, Japan

[§]Center for the Promotion of Excellence in Higher Education and [∥]Department of Chemistry, Graduate School of Science, Kyoto University, Sakyo-ku, Kyoto 606-8501, Japan

[⊥]Graduate School of Science and Technology, Hirosaki University, Hirosaki, Aomori 036-8561, Japan

Supporting Information

ABSTRACT: The G-quadruplexes form highly stable nucleic acid structures, which are implicated in various biological processes in both DNA and RNA. Although DNA G-quadruplexes have been studied in great detail, biological roles of RNA G-quadruplexes have received less attention. Here, a screening of a chemical library permitted identification of a small-molecule tool that binds selectively to RNA G-quadruplex structures. The polyaromatic molecule, RGB-1, stabilizes RNA G-quadruplex, but not DNA versions or other RNA structures. RGB-1 intensified the G-quadruplex-mediated inhibition of RNA translation in mammalian cells, decreased expression of the *NRAS* proto-oncogene in breast cancer cells, and permitted identification of a novel sequence that forms G-quadruplex in *NRAS* mRNA. RGB-1 may serve as a unique tool for understanding cellular roles of RNA G-quadruplex structures.

G-quadruplexes are stable nucleic acid structures involved in a variety of biological processes. For example, they are known to locate in the telomeric and promoter regions of DNA,[1,2] controlling genome stability and transcriptional regulation.[3,4] A number of DNA G-quadruplex binders have been developed as chemical tools for investigating the physical properties of G-quadruplexes[6,7] and for visualizing G-quadruplex dynamics in cells.[8−10] There is now increasing interest in RNA G-quadruplexes, which form more thermodynamically stable structures than DNA G-quadruplexes and which exist in various locations in untranslated regions (UTRs) of mRNA.[11,12] The best known endogenous RNA G-quadruplex is 5′UTR RNA G-quadruplex on oncogenic *NRAS* mRNA. Translation efficiency in cells increases when the 5′-terminal region of *NRAS*, containing the G-quadruplex-forming sequence, is deleted, indicating that RNA G-quadruplexes are involved in translational regulation, as well as other key biological processes.

Small molecules selective for RNA G-quadruplexes would accelerate further investigation of the biological functions of RNA G-quadruplexes. Several such molecules have been reported, and many of them were obtained from rational modification of pyridostatin.[10,13,14] Unbiased discovery of novel RNA G-quadruplex ligands from a chemical library might provide more diverse chemical scaffolds and physical properties than the pyridostatin analogs. In fact, a recent ^{19}F-NMR-based screening of a chemical library discovered several fluorinated compounds that bind TERRA.[15]

We designed *in vitro* screening to discover a new chemical scaffold that stabilizes RNA G-quadruplexes (Figure 1a,b). Because thermodynamically stable RNA G-quadruplex structures inhibit the elongation reaction of complementary DNA (cDNA) strands by reverse transcriptase (RTase),[5] we initially expected that an RNA G-quadruplex with its chemical stabilizer would more efficiently reduce production of full-length cDNA strands (Figure 1c,d). Therefore, evaluation of full-length cDNA production by quantitative PCR might identify potent RNA G-quadruplex stabilizers (Figure 1e). As a model RNA G-quadruplex structure, we chose the telomeric repeat-containing RNA (TERRA) sequence,[16] one of the best characterized RNA G-quadruplex structures. We screened 8000 diverse compounds from in-house chemical libraries and isolated 526 molecules that inhibited the elongation reaction of RTase ($\Delta Ct \geq 2$; Figure 1f). $\Delta Ct = 2$ theoretically indicates 75% inhibition of RT reaction. Counter-screening with a nonstructured RNA as a template in the RTase reaction was used to remove false positives. Twenty molecules inhibited the RTase elongation reaction in the TERRA-containing template more potently than in the nonstructured RNA template. The molecule that exhibited the most desirable profile (molecule **1**, RGB-1) was selected for further studies (Figure 1h).

We first evaluated the direct interaction of RGB-1 with TERRA G-quadruplex using size exclusion chromatography.[17,18] The apparent K_d value and its stoichiometry were estimated to be 5.9 μM and 1:1.2, respectively (Figure 2a). No detectable affinities of RGB-1 were observed with parallel, antiparallel, and mixed-type DNA G-quadruplex structures,[19,20] aside from a weak interaction with parallel DNA G-quadruplex at the highest concentration (100 μM) (Table S1 and Figure S1). To examine whether binding with RGB-1 stabilizes the RNA G-quadruplex structure, we measured the melting

Received: May 7, 2016
Published: July 13, 2016

DOI: 10.1021/jacs.6b04506
J. Am. Chem. Soc. 2016, 138, 9037−9040

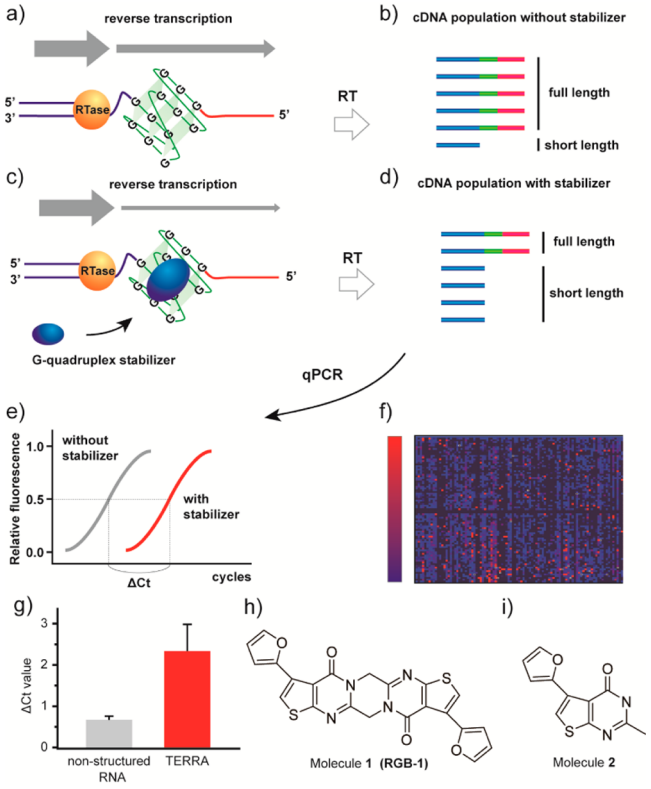

Figure 1. RTase-reaction-based screening for isolation of RNA G-quadruplex stabilizers. (a) RNA G-quadruplex blocks reverse transcriptase elongation, (b) resulting in the production of two cDNA populations. Previous RTase stop assays showed that two major cDNA species (full- and short-length) are produced from a G-quadruplex-containing RNA template.[5] (c) RNA G-quadruplex stabilizer enhances the arrest of reverse transcription at G-quadruplex, (d) resulting in reduced production of full-length cDNA. (e) Simulation of the full-length cDNA production by quantitative PCR. ΔCt values were determined by subtracting the Ct value of qPCR with a compound from the Ct value of qPCR without the compound. A high ΔCt value indicates strong inhibition of reverse transcription. (f) Heat map of screening results of the RNA G-quadruplex stabilizers. The colored spots indicate a gradient of ΔCt values in qPCR with or without library compounds at 10 μM: red (ΔCt ≥ 3) to blue (ΔCt ≤ 0). (g) Molecule **1** reduced the production of full-length cDNA from a TERRA-containing RNA template (red bar), but not from a nonstructured RNA template (gray bar). (h) The structure of molecule **1** (RGB-1). (i) The chemical structure of molecule **2**, a negative analog of RGB-1.

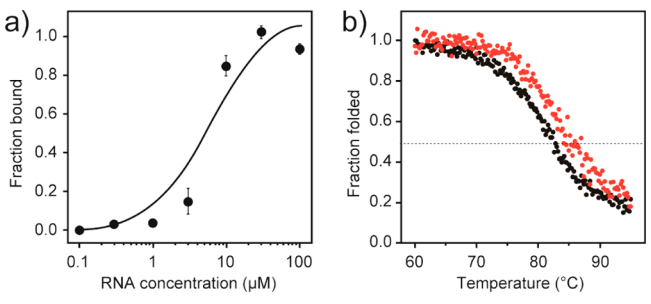

Figure 2. Determination of K_d and T_m values in 10 mM Tris-HCl buffer (pH 7.6) containing 100 mM KCl. (a) RNA G-quadruplex binding of RGB-1. Semilogarithmic plots showing the fraction of RGB-1 bound to TERRA at room temperature. (b) Melting temperature (T_m) of TERRA with (red circles) or without (black circles) RGB-1.

temperature (T_m) of RNA G-quadruplex in the presence or absence of RGB-1. As expected, RGB-1 increased the T_m of TERRA by ≥5 °C (Figures 2b and S2). The magnitude of the shift in T_m upon binding to varied concentrations of RGB-1 allowed us to estimate the K_d value to be 8.8 μM (Figure S3),[15] consistent with that from the size exclusion chromatography experiments. In contrast, RGB-1 induced no detectable T_m increase in the other nucleic acid structures, including stem-loop RNA, double-strand RNA, and DNA G-quadruplexes (Figures S4−S6). Molecule **2**, a half fragment of RGB-1, had no effect on the T_m of any of the tested nucleic acid structures, suggesting that the symmetric structure of RGB-1 is required for its activity (Figures 1 and S7). We also chemically synthesized RGB-1 and confirmed that the synthesized version retained its activity. These results indicated that RGB-1 binds to and stabilizes the RNA G-quadruplex with high structural selectivity.

The strict binding selectivity of RGB-1 raised the possibility that RGB-1 regulates translation of mRNA containing G-quadruplex at its 5′UTR. To test this hypothesis, we designed two mRNAs that encode firefly luciferase (FL), one with and one without TERRA G-quadruplex inserted at the 5′UTR (Figure S8a,b). In a cell-free translation system, RGB-1 reduced the translational efficiency of the mRNA with TERRA G-quadruplex, but had no detectable effect on translation from the mRNA without TERRA G-quadruplex (Figure S8c). In contrast, molecule **2**, a negative analog of RGB-1, had no detectable effects on the translational efficiency. TMPYP4, a well-known G-quadruplex stabilizer, reduced protein translation from both mRNAs, perhaps due to its low selectivity. These results confirm that RGB-1 down-regulates translation from mRNA that contains G-quadruplex.

Encouraged by the *in vitro* results, we examined the ability of RGB-1 to regulate protein translation in living HEK293 cells. For the initial cell-based assay, we prepared two dual reporter constructs that encode FL and Renilla luciferase (RL) genes. Although the two genes are encoded in a single mRNA, FL translation is initiated from the 5′ cap, while RL translation is mediated by an internal ribosome entry site.[21] This allowed us to quantify the effects of the 5′UTR on protein translation separately from the influence of transfection efficiency or mRNA stability. When we inserted a TERRA sequence at the 5′UTR in the reporter gene, RGB-1 reduced FL translation in a dose-dependent manner, but did not suppress RL translation (Figure S9). Without the TERRA sequence, FL translation was not affected. Thus, RGB-1 reduces translation of mRNA containing a TERRA G-quadruplex in HEK293 cells.

We used another cell-based assay to examine the effect of RGB-1 on translation of an endogenous mRNA. We chose an oncogenic *NRAS* gene as the target mRNA, because its 5′UTR region carries an RNA G-quadruplex that suppresses NRAS protein translation. The effect on NRAS expression level was estimated by Western blot analysis. Treatment of human MCF-7 cells with RGB-1 caused suppression of NRAS protein expression in a dose-dependent manner, whereas protein levels of actin and GAPDH had less effects (Figure 3a), suggesting that RGB-1 stabilized the G-quadruplex structure in the *NRAS* mRNA in cells. In contrast, molecule **2** failed to affect the protein levels even at a high concentration. We also used quantitative reverse transcription PCR (RT-qPCR) to examine the effect of RGB-1 on the amount of *NRAS* mRNA in treated cells and found no detectable effect (Figure 3b).

DOI: 10.1021/jacs.6b04506
J. Am. Chem. Soc. 2016, 138, 9037−9040

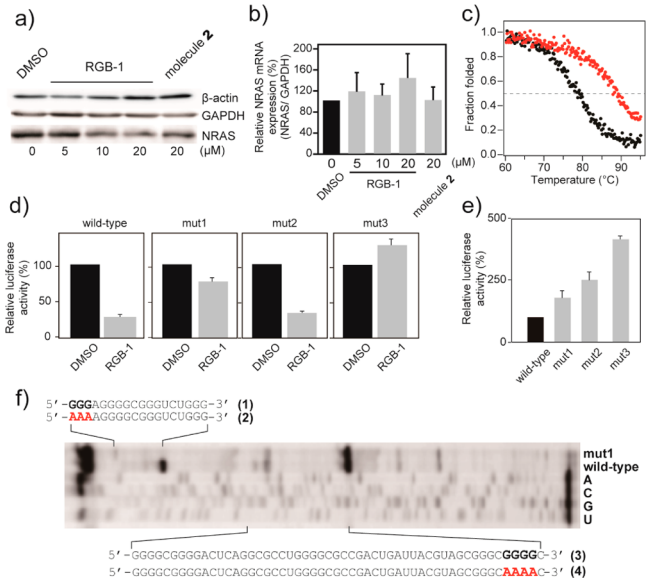

Figure 3. (a) Results of Western blot analysis showing that NRAS expression was down-regulated by RGB-1 in a dose-dependent manner, but was not affected by molecule **2**. β-actin (lower panel) was used as a loading control. (b) qPCR analysis of *NRAS* in MCF-7 cells treated with RGB-1 and molecule **2**. *NRAS* mRNA expression was normalized by GAPDH. (c) Melting temperature (T_m) measurements of an *NRAS* G-quadruplex with (red circles) or without (black circles) RGB-1 in 10 mM Tris-HCl buffer (pH 7.6) containing 10 mM KCl. (d) Effects of RGB-1 on translation of reporter RNA *in vitro*. FL-reporter mRNAs with native and mutated NRAS 5′UTR were translated in the presence (RGB-1; gray bars) or absence (DMSO; black bars) of 10 μM RGB-1. RGB-1 showed no detectable effect on translation from the mut3 RNA template, whereas RGB-1 notably reduced translation from wild-type, mut1, and mut2 RNA templates. (e) Relative translation efficiency of wild-type, mut1, mut2, and mut3 RNA templates, estimated as luciferase enzyme activity. Error bars represent SD of three independent experiments. (f) Interruption of RTase-mediated cDNA synthesis on wild-type and mut1 RNA template in the presence of 100 mM KCl. The lane markers U, G, C, and A indicate the bases on the template strand. Adenines replacing guanines in the mut1 RNA template are shown in red. Wild type: (1) + (3); mut1: (2) + (3); mut2: (1) + (4); mut3: (2) + (4).

The effects of RGB-1 on the stability of *NRAS* RNA G-quadruplex and on the translational efficiency of the *NRAS* 5′UTR were confirmed independently by T_m measurements and *in vitro* translation experiments, respectively. T_m measurements revealed that RGB-1 increased the melting temperature of *NRAS* G-quadruplex structures (Figure 3c), corroborating that the *NRAS* RNA G-quadruplex is stabilized by binding with RGB-1. For the *in vitro* translation experiments, we prepared two FL-reporter mRNAs: one with a native *NRAS* 5′UTR (wild-type) and the other with an *NRAS* 5′UTR mutated in the G-quadruplex region (mut1). RGB-1 unexpectedly suppressed translation in both mRNA models (Figure 3d).

To solve this mystery, we performed an *in vitro* RTase reaction-based stop assay that identifies internal G-quadruplex formation in RNA. The stop assay suggested the existence of a novel G-quadruplex formation site in the 5′UTR of *NRAS* mRNA (Figure 3f). Based on the previously reported computational prediction,[11,12] this particular region would be ruled out as a potential site of G-quadruplex formation. In fact, *NRAS* 5′UTR with double-site mutations (mut3) disrupting both G-quadruplexes was not responsive to RGB-1 in the FL-reporter translation assay, whereas the translation from *NRAS* 5′UTR with each single-site mutation (mut1 and mut2) was suppressed by RGB-1 (Figure 3d,e). These results indicate that the newly discovered G-quadruplex formation also affects NRAS protein translation from RNA. Thus, RGB-1 might serve as a potential tool for discovering RNA G-quadruplex sites that have not yet been identified, including those that are sterically formed.

To characterize the newly discovered G-quadruplex, we prepared the 53-base RNA that encompasses a potential G-quadruplex forming site containing five G tracts ((3) in Figure 3f). CD experiments revealed a positive Cotton effect at 265 nm, a typical signal for paralleled G-quadruplex structure. However, replacement of the first G tract with an A tract ((4) in Figure 3f) showed little, if any, alteration in the CD spectra (Figure S10). Systematic and detailed biophysical analyses including NMR experiments are needed for fully characterizing this long-range, sterically formed G-quadruplex.

In the present study, a screening of 8000 diverse molecules led to the discovery of RGB-1, a small molecule that binds selectively to RNA G-quadruplex structures. The binding affinity of RGB-1 to RNA G-quadruplex appears to be lower than the reported K_d value of the pyridostatin analog RR110[6] but higher than those of the fluorinated molecules discovered through an NMR-based screening.[15] We were unable to compare the degree of their selectivity between DNA and RNA G-quadruplexes because limited physicochemical data are available in literatures about their selectivity. Side-by-side comparison or biological use would clarify this point once the reagents become commercially available.

It remains unknown how the selective binding occurs. RGB-1 is structurally distinct from other G-quadruplex ligands, including two previously described RNA G-quadruplex ligands that are pyridostatin analogs. In both cases, the presence of a negatively charged carboxyl group is required for selective binding to RNA G-quadruplex, suggesting the possibility of hydrogen-bonding interactions between the carboxylate and the 2′-OH group of the RNA G-quadruplex, which is absent in the corresponding DNA.

Although not a small molecule, Takahama and Oyoshi developed an RNA G-quadruplex-binding protein by engineering the RGG (arginine-glycine-glycine) domain that is known to bind to both telomeric RNA and DNA G-quadruplexes. Interestingly, substitution of phenylalanines with tyrosines increased the affinity of this protein for RNA G-quadruplex and decreased its affinity for DNA G-quadruplex. Further analysis indicated that the acidic phenol group was important in recognizing the 2′-OH of the riboses located in the G-quadruplex loops.[22] Although RGB-1 is not equipped with any acidic functional groups, it contains a number of hydrogen-bonding acceptors, which might interact with the 2′-OH group of the RNA G-quadruplex. RGB-1 might also recognize the structural differences between G-quadruplex structures in RNA and DNA, such as twist or rise. Structural analysis of an RNA G-quadruplex complexed with RGB-1 would clarify the molecular basis of its selectivity and help in the design of the ligands that discern between DNA and RNA G-quadruplexes.

A key finding of the present study might be the use of a small molecule to discover a novel G-quadruplex formation site at the *NRAS* 5′UTR. A number of potential G-quadruplex formation sites on mRNAs have been computationally predicted, and some have been experimentally demonstrated.[12,23,24] However, identification of unpredicted, sterically formed G-quadruplexes

DOI: 10.1021/jacs.6b04506
J. Am. Chem. Soc. 2016, 138, 9037−9040

remains a significant challenge. Direct comparison of gene expression in protein and mRNA levels, in the presence or absence of RGB-1, might be useful for such identifications. Proteins whose levels are affected by RGB-1, but whose mRNA levels are unchanged, might represent genes controlled by RNA G-quadruplex formation. Thus, RGB-1 might serve as a tool for discovering and modulating RNA G-quadruplexes in cells and as a starting point for designing novel types of RNA G-quadruplex-specific ligands.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/jacs.6b04506.

Experimental details and data (PDF)

■ AUTHOR INFORMATION

Corresponding Authors

*ssato@scl.kyoto-u.ac.jp
*hagihara@hirosaki-u.ac.jp
*uesugi@scl.kyoto-u.ac.jp

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

This work was supported in part by JSPS (26220206 to M.U., 15K17883 to Y.K., 25350958 to M.H. and 26440005 to S.S.), ZE Research Program, IAE (ZE27C-6 to Y.K. and ZE27B-18 to S.S.), Asahi Glass Foundation, and an iCeMS research acceleration grant. iCeMS is supported by World Premier International Research Center Initiative (WPI), MEXT, Japan. We also thank M. Nakashima for manuscript preparation. This work was inspired by the international and interdisciplinary environments of the iCeMS and JSPS Asian CORE Program, "Asian Chemical Biology Initiative."

■ REFERENCES

(1) Petraccone, L.; Trent, J. O.; Chaires, J. B. *J. Am. Chem. Soc.* **2008**, *130*, 16530.
(2) Siddiqui-Jain, A.; Grand, C. L.; Bearss, D. J.; Hurley, L. H. *Proc. Natl. Acad. Sci. U. S. A.* **2002**, *99*, 11593.
(3) Qin, Y.; Hurley, L. H. *Biochimie* **2008**, *90*, 1149.
(4) Endoh, T.; Kawasaki, Y.; Sugimoto, N. *Angew. Chem., Int. Ed.* **2013**, *52*, 5522.
(5) Hagihara, M.; Yoneda, K.; Yabuuchi, H.; Okuno, Y.; Nakatani, K. *Bioorg. Med. Chem. Lett.* **2010**, *20*, 2350.
(6) Koirala, D.; Dhakal, S.; Ashbridge, B.; Sannohe, Y.; Rodriguez, R.; Sugiyama, H.; Balasubramanian, S.; Mao, H. B. *Nat. Chem.* **2011**, *3*, 782.
(7) Sannohe, Y.; Endo, M.; Katsuda, Y.; Hidaka, K.; Sugiyama, H. *J. Am. Chem. Soc.* **2010**, *132*, 16311.
(8) Shi, D. F.; Wheelhouse, R. T.; Sun, D. Y.; Hurley, L. H. *J. Med. Chem.* **2001**, *44*, 4509.
(9) Shin-ya, K.; Wierzba, K.; Matsuo, K.; Ohtani, T.; Yamada, Y.; Furihata, K.; Hayakawa, Y.; Seto, H. *J. Am. Chem. Soc.* **2001**, *123*, 1262.
(10) Biffi, G.; Tannahill, D.; McCafferty, J.; Balasubramanian, S. *Nat. Chem.* **2013**, *5*, 182.
(11) Bugaut, A.; Balasubramanian, S. *Nucleic Acids Res.* **2012**, *40*, 4727.
(12) Kumari, S.; Bugaut, A.; Huppert, J. L.; Balasubramanian, S. *Nat. Chem. Biol.* **2007**, *3*, 218.
(13) Bugaut, A.; Rodriguez, R.; Kumari, S.; Hsu, S. T. D.; Balasubramanian, S. *Org. Biomol. Chem.* **2010**, *8*, 2771.
(14) Di Antonio, M.; Biffi, G.; Mariani, A.; Raiber, E. A.; Rodriguez, R.; Balasubramanian, S. *Angew. Chem., Int. Ed.* **2012**, *51*, 11073.
(15) Garavis, M.; Lopez-Mendez, B.; Somoza, A.; Oyarzabal, J.; Dalvit, C.; Villasante, A.; Campos-Olivas, R.; Gonzalez, C. *ACS Chem. Biol.* **2014**, *9*, 1559.
(16) Martadinata, H.; Phan, A. T. *J. Am. Chem. Soc.* **2009**, *131*, 2570.
(17) Curtis, E. A.; Liu, D. R. *Chem. Biol.* **2013**, *20*, 521.
(18) Weill, L.; Louis, D.; Sargueil, B. *Nucleic Acids Res.* **2004**, *32*, 5045.
(19) Phan, A. T.; Mergny, J. L. *Nucleic Acids Res.* **2002**, *30*, 4618.
(20) Xu, Y.; Noguchi, Y.; Sugiyama, H. *Bioorg. Med. Chem.* **2006**, *14*, 5584.
(21) Jang, S. K.; Davies, M. V.; Kaufman, R. J.; Wimmer, E. *J. Virol* **1989**, *63*, 1651.
(22) Takahama, K.; Oyoshi, T. *J. Am. Chem. Soc.* **2013**, *135*, 18016.
(23) Chambers, V. S.; Marsico, G.; Boutell, J. M.; Di Antonio, M.; Smith, G. P.; Balasubramanian, S. *Nat. Biotechnol.* **2015**, *33*, 877.
(24) Kwok, C. K.; Balasubramanian, S. *Angew. Chem., Int. Ed.* **2015**, *54*, 6751.

DOI: 10.1021/jacs.6b04506
J. Am. Chem. Soc. 2016, 138, 9037−9040