RESEARCH ARTICLE

View Article Online
View Journal | View Issue Cite this: *Med. Chem. Commun.*,
2017, 8, 390A histone H1-binding-aptide-based apoptosis
imaging probe for monitoring tumor responses to
cancer therapy†‡Yonghyun Lee,^a Sunghyun Kim,^b Daejin Kim^a and Sangyong Jon^{*a}Received 15th December 2016,
Accepted 16th December 2016

DOI: 10.1039/c6md00696e

rsc.li/medchemcomm

Here we report the feasibility of using a high-affinity histone H1-binding peptide as a potential molecular apoptosis imaging probe. The Cy5.5-labeled peptide showed high selectivity for drug-treated apoptotic tumors compared with non-apoptotic tumors in various *in vivo* settings, enabling detection of tumor responses to both docetaxel chemotherapy and Herceptin antibody therapy as early as 12 hours after treatment with a high signal-to-background ratio, even at extremely low doses.

Abnormally high levels of apoptosis are closely related to various pathological conditions, including neurodegenerative disorders (e.g., Parkinson's disease), cardiovascular diseases (e.g., myocardial infarction and atherosclerosis), cancers, and others.^{1–5} Thus, considerable effort has been devoted to developing molecular probes that can target apoptosis-associated biomarkers for imaging and diagnosing such diseases or monitoring tumor responses to cancer treatments such as chemotherapy.^{6–12} Typically, molecular probes targeting either intracellular apoptosis biomarkers such as caspases^{6,7} or extracellular biomarkers such as phosphatidylserine (PS)^{1,2,8–12} have demonstrated their feasibility as apoptosis-imaging agents in various *in vitro* and *in vivo* settings.^{1–3} For example, fluorophore- or radionuclide-labeled annexin V,^{10–12} a PS-binding protein, is a representative probe for detecting apoptosis. [¹⁸F]-labeled isatin, a small-molecule covalent inhibitor of activated caspase-3 and -7, can be used to monitor tumor responses to chemotherapy by positron emission tomography (PET) imaging.⁹ However, the performance of these probes *in vivo* still needs improvement in terms of their dose requirements, signal-to-background ratio, and specificity for apoptotic cells *versus* non-apoptotic cells.^{1–3}

Among the various apoptosis-associated biomarkers, histone H1, a nuclear protein that binds to the linker DNA between nucleosomes, has emerged as a novel extracellular biomarker of apoptosis because, during apoptosis, it is released into the cytoplasm and plasma membrane from the nu-

cleus.^{13,14} Thus, histone H1 is a promising apoptotic marker in terms of its higher specificity for apoptotic cells.^{13–15} In this context, the histone H1-binding hexapeptide, CQRPPR (ApoPep), has been shown to detect apoptosis and necrosis of cells in tissues *in vivo*.^{15,16} However, despite the considerable potential of ApoPep as an apoptosis-imaging probe, its low-affinity (dissociation constant [K_d] $\approx$ 803 μ M) for the target limits further translation to the clinic.¹⁷ Very recently, we used a β -hairpin-scaffold-assisted approach to successfully convert the original ApoPep to a high-affinity histone H1-binding aptide, designated as ATP_{HH1}, that exhibited a K_d of $\sim$ 80 nM—a binding affinity four orders of magnitude stronger than that of the parental peptide.¹⁷ Cy5.5-labeled ATP_{HH1} has a high selectivity for the target protein over other non-relevant proteins, and thus is able to detect apoptotic cells *in vitro* at a much lower concentration than ApoPep.

In this study, we examined the feasibility of using ATP_{HH1} as an apoptosis-imaging probe for monitoring tumor responses to cancer treatment in tumor-bearing mouse models.¹⁷

ATP_{HH1}, shown schematically in Fig. 1a, consists of 28 amino acids containing ApoPep at the N-terminus. For fluorescence-based optical imaging, the near-infrared dye, Cy5.5, was attached to the lysine residue of the β -hairpin scaffold at the center of the aptide, yielding Cy5.5-ATP_{HH1}. We first explored the ability of Cy5.5-ATP_{HH1} to detect apoptotic cells compared with that of ApoPep in A549 lung adenocarcinoma cells treated with the apoptosis-inducing chemotherapeutic agent, etoposide. As shown in Fig. 1b, a negligible fluorescence signal was observed in untreated A549 cells, whereas a high fluorescence intensity was clearly detected in the cell membrane and cytoplasm of etoposide-treated, apoptotic cells after incubation with 100 nM Cy5.5-ATP_{HH1}. Cy5.5-ApoPep at the same concentration failed to detect apoptotic cells, and required a 100-fold higher

^a KAIST Institute for the BioCentury, Department of Biological Sciences, Korea Advanced Institute of Science and Technology, 291 Daehak-ro, Daejeon 34141, Republic of Korea

^b Korea Institute of Ceramic Engineering and Technology, Jinju 660-031, Korea

† The authors declare no competing interests.

‡ Electronic supplementary information (ESI) available: Details for experimental procedure and further IVIS images of Cy5.5-ATP_{HH1} (25 pmol) and Cy5.5-ApoPep (5 nmol, 50 pmol). See DOI: 10.1039/c6md00696e

Fig. 1 ATP_{HH1}, a matured histone H1-binding aptide for the detection of apoptosis and necrosis. (a) Schematic representation of ATP_{HH1}, a β-hairpin-scaffold-assisted heterodimeric peptide for the target protein, histone H1. (b) A549 cells were treated with 150 μM etoposide for 2 h (to induce apoptosis), glucose-depleted medium for 8 h (to induce necrosis) or control medium, after which the cells were incubated with Cy5.5-ApoPep (10 μM) or Cy5.5-ATP_{HH1} (100 nM). For competition studies, the cells were pre-treated with excess free ATP_{HH1} (1 mM). (c) Flow cytometry analyses were performed after treatment with etoposide or control medium for 2 h, followed by incubation with Cy5.5-ApoPep (10 μM) or Cy5.5-ATP_{HH1} (100 nM or 1 μM).

concentration (10 μM) to reveal a similar pattern of apoptosis. We further confirmed that Cy5.5-ATP_{HH1}, as expected, was also a suitable probe for detecting necrotic cancer cells under glucose-depleted conditions (Fig. 1b). Binding of Cy5.5-ATP_{HH1} to both apoptotic and necrotic cancer cells was significantly compromised by pre-treatment with free ATP_{HH1}. Moreover, a non-relevant, scrambled control aptide (Cy5.5-ATP_{SCR}) did not show any appreciable binding to both apoptotic and necrotic cancer cells, suggesting that the imaging results reflected specific binding to histone H1. A similar apoptotic pattern was obtained in flow cytometry experiments (Fig. 1c), which confirmed the specificity of ATP_{HH1} for apo-

ptotic cells and greater sensitivity compared with ApoPep for apoptosis imaging.

We next examined the feasibility of using Cy5.5-ATP_{HH1} as an imaging probe for the detection of apoptosis/necrosis after cancer therapy. As a proof-of-concept study, we intravenously injected Cy5.5-ATP_{HH1} (25 pmol) *via* the tail vein into xenograft mice bearing A549 human lung adenocarcinoma tumors before and after treatment with the anticancer drug, docetaxel. To minimize possible necrosis in the core region of tumors, we chose experimental conditions that limited the size of tumors to less than ~500 mm³. As shown in Fig. 2a, no appreciable fluorescence signal was observed in tumors before docetaxel treatment (signal to background ratio = 1.05 for 4 h and 1.18 for 6 h, respectively). In contrast, the Cy5.5 fluorescence intensity increased significantly with time in tumors treated with docetaxel 12 hours previously, reaching the maximum intensity at approximately 4–6 h post

Fig. 2 ATP_{HH1} can selectively detect apoptotic, anticancer-agent-treated tumors relative to non-apoptotic tumors with a high signal-to-background ratio, even at extremely low doses. (a and b) A549 tumor xenograft mice were treated intratumorally with docetaxel (10 mg kg⁻¹) for 12 h, after which 25 pmol of Cy5.5-ATP_{HH1} was intravenously injected *via* the tail vein. (a) The dorsal side of each mouse was visualized using a Xenogen IVIS Lumina *in vivo* imaging system, with a Cy5.5 filter and an exposure time of 5 s. (b) After the mice were sacrificed, tumor tissues were collected and assayed for apoptosis by TUNEL staining. Fluorescence images were acquired by confocal microscopy. (c) Mice treated with Cy5.5-ApoPep instead of Cy5.5-ATP_{HH1}, imaged as described in (a).

administration (signal to background ratio = 7.13 for 4 h and 7.45 for 6 h, respectively). *In vivo* optical imaging of the lateral and ventral sides of mice clearly showed the difference in tumor localization of Cy5.5-ATP_{HH1} before and after docetaxel treatment (Fig. S1†). We also found that the fluorescence signal of Cy5.5-ATP_{HH1} was highly co-localized in the apoptotic regions of dissected tumors, identified by TUNEL (terminal deoxynucleotidyl transferase dUTP nick end labeling) staining. This result clearly indicates that ATP_{HH1} can effectively and specifically recognize apoptotic cells resulting from chemotherapy *in vivo*. For comparison, Cy5.5-ApoPep at a dose similar to that of Cy5.5-ATP_{HH1} (50 pmol) did not produce any fluorescence signal in docetaxel-treated tumors (Fig. S2†), and only yielded clear apoptosis imaging of the tumor at 200-fold higher concentrations (5 nmol; Fig. 2c). These results further indicate that the high-affinity ATP_{HH1} is greatly superior to the original ApoPep for molecular imaging of apoptosis.

We further examined the ability of the ATP_{HH1}-based probe to detect apoptosis in other types of tumors and cancer therapies. First, we created a xenograft model with tumors on both left and right dorsal flanks of the same mouse by subcutaneously injecting MDA-MB-231-derived breast cancer cells, as shown in Fig. 3a, and then intratumorally injected docetaxel and phosphate buffered saline (PBS) into the left and right tumors, respectively. After 12 h, Cy5.5-ATP_{HH1} (25 pmol) was intravenously injected *via* the tail vein. Both dorsal

and lateral imaging of the mouse clearly showed a high fluorescence intensity in the left tumor (signal to background ratio = 6.21 for 3 h and 5.50 for 6 h, respectively) that received docetaxel, whereas no appreciable signal was detected in the right (PBS-treated) tumor (signal to background ratio = 0.84 for 3 h, 0.80 for 6 h, respectively) (Fig. 3a). This distinct difference in probe accumulation in each tumor in the same mouse clearly demonstrates the ability of the ATP_{HH1}-based probe to detect tumor responses to chemotherapy.

Next, we examined whether the Cy5.5-ATP_{HH1} probe could detect tumor responses to antibody therapeutics. Two subcutaneous xenograft mouse models—an MDA-MB-231 breast tumor (HER-2 negative) and an SK-OV-3 ovarian tumor (HER-2 positive)—were prepared and then intravenously injected with Herceptin, an anti-HER-2 monoclonal antibody therapeutic used in the clinic. As shown in whole-body optical images and *ex vivo* images of the major organs and tissues, Cy5.5-ATP_{HH1} showed much higher localization in the HER-2-positive ovarian tumor (signal to background ratio = 1.96 for tumor/liver and 2.12 for tumor/kidney) than in the HER-2-negative breast tumor (signal to background ratio = 0.59 for tumor/liver and 0.81 for tumor/kidney), where the signal intensity was marginal (Fig. 3b). Taken together, these results demonstrate that the ATP_{HH1}-based imaging probe can effectively monitor or detect tumor responses to not only chemotherapy, but also to biological treatment, exhibiting a high signal-to-background ratio even at an extremely low concentration (25 pmol).

In conclusion, we have demonstrated that ATP_{HH1}, a high-affinity histone H1-binding aptide developed using our scaffold-assisted affinity conversion method, can be used as an imaging probe for detecting apoptosis *in vitro* and *in vivo* by specifically recognizing the apoptosis-associated extracellular biomarker, histone H1. The Cy5.5-labeled aptide showed high selectivity for drug-treated apoptotic tumors relative to non-apoptotic tumors in *in vivo* settings, enabling monitoring or detection of tumor responses to both docetaxel chemotherapy and antibody therapy after as little as 12 hours with high signal-to-background ratios at doses dramatically lower than those required for ApoPep. Current strategies for assessing the efficacy of cancer therapy are based on measurements of tumor size using conventional imaging modalities, such as computed tomography and magnetic resonance imaging.¹⁸ However, these approaches are time consuming and do not provide an accurate indication of tumor responses.¹⁸ Because our histone H1-specific aptide can detect apoptotic cancer cells in less than a full day after drug treatment, it has promise as an apoptosis-imaging probe that could be used to monitor tumor responses to therapy and help clinicians make better decisions about appropriate drug treatment regimens. Moreover, the imaging probe described here could be utilized for the detection of apoptosis/necrosis in other pathological conditions, including neuronal disorders, cardiovascular diseases, and autoimmune diseases.

All animal procedures were reviewed and approved (approval number: KA2013-17) by the Korea Advanced Institute

Fig. 3 ATP_{HH1} can monitor selective tumor responses to the diverse anticancer agents, docetaxel and Herceptin. (a) Xenograft mice bearing MDA-MB-231 tumors on both right and left flanks were injected (i.v.) with 25 pmol of Cy5.5-ATP_{HH1} *via* the tail vein 12 h after intratumoral treatment of the left tumor with 5 mg kg⁻¹ of docetaxel. Time course of *in vivo* fluorescence images were acquired using a Xenogen IVIS Lumina *in vivo* imaging system. (b) MDA-MB-231 (Her-2 negative) and SK-OV-3 (Her-2 positive) tumor xenograft mice were injected (i.v.) with 25 pmol Cy5.5-ATP_{HH1} *via* the tail vein 12 h after intratumoral treatment with 20 mg kg⁻¹ of Herceptin. After 8 h, the mice were sacrificed and each organ was collected and imaged using a Xenogen IVIS Lumina system to detect Cy5.5-ATP_{HH1} fluorescence signals.

of Science and Technology Institutional Animal Care and Use Committee (KAIST-IACUC) for compliance with ethical procedures and standards of scientific care.

This work was supported by the Global Research Laboratory grant (2015045887) and the Radiation Technology R&D program (2014033231) through the National Research Foundation of Korea (NRF), funded by the Ministry of Science, ICT & Future Planning.

Notes and references

- 1 B. A. Smith and B. D. Smith, *Bioconjugate Chem.*, 2012, **23**, 1989–2006.
- 2 A. Phinikaridou, M. E. Andia, A. M. Shah and R. M. Botnar, *Am. J. Physiol.*, 2012, **303**, H1397–H1410.
- 3 F. G. Blankenberg, *J. Nucl. Med.*, 2008, **49**(Suppl 2), 81S–95S.
- 4 C. B. Thompson, *Science*, 1995, **267**, 1456–1462.
- 5 J. M. Brown and L. D. Attardi, *Nat. Rev. Cancer*, 2005, **5**, 231–237.
- 6 J. Li and J. Yuan, *Oncogene*, 2008, **27**, 6194–6206.
- 7 C. Pop and G. S. Salvesen, *J. Biol. Chem.*, 2009, **284**, 21777–21781.
- 8 K. Emoto, N. Toyama-Sorimachi, H. Karasuyama, K. Inoue and M. Umeda, *Exp. Cell Res.*, 1997, **232**, 430–434.
- 9 D. L. Chen, J. T. Engle, E. A. Griffin, J. P. Miller, W. Chu, D. Zhou and R. H. Mach, *Mol. Imaging Biol.*, 2015, **17**, 384–393.
- 10 V. Ntziachristos, E. A. Schellenberger, J. Ripoll, D. Yessayan, E. Graves, A. Bogdanov Jr., L. Josephson and R. Weissleder, *Proc. Natl. Acad. Sci. U. S. A.*, 2004, **101**, 12294–12299.
- 11 J. Toretzky, A. Levenson, I. N. Weinberg, J. F. Tait, A. Uren and R. C. Mease, *Nucl. Med. Biol.*, 2004, **31**, 747–752.
- 12 C. Vangestel, C. Van de Wiele, N. Van Damme, S. Staelens, P. Pauwels, C. P. Reutelingsperger and M. Peeters, *J. Nucl. Med.*, 2011, **52**, 1786–1794.
- 13 A. Konishi, S. Shimizu, J. Hirota, T. Takao, Y. Fan, Y. Matsuoka, L. Zhang, Y. Yoneda, Y. Fujii, A. I. Skoultschi and Y. Tsujimoto, *Cell*, 2003, **114**, 673–688.
- 14 N. Yan and Y. Shi, *Nat. Struct. Biol.*, 2003, **10**, 983–985.
- 15 K. Wang, S. Purushotham, J. Y. Lee, M. H. Na, H. Park, S. J. Oh, R. W. Park, J. Y. Park, E. Lee, B. C. Cho, M. N. Song, M. C. Baek, W. Kwak, J. Yoo, A. S. Hoffman, Y. K. Oh, I. S. Kim and B. H. Lee, *J. Controlled Release*, 2010, **148**, 283–291.
- 16 H. K. Jung, K. Wang, M. K. Jung, I. S. Kim and B. H. Lee, *PLoS One*, 2014, **9**, e100341.
- 17 S. Kim, D. Kim, Y. Lee, H. Jeon, B. H. Lee and S. Jon, *ChemBioChem*, 2015, **16**, 43–46.
- 18 W. A. Weber, *J. Nucl. Med.*, 2009, **50**(Suppl 1), 1S–10S.