Bioorganic & Medicinal Chemistry 22 (2014) 1268–1275

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry

journal homepage: www.elsevier.com/locate/bmc

Design and synthesis of peptide-MCA substrates for a novel assay of histone methyltransferases and their inhibitors

Hongfang Chi [a], Yasushi Takemoto [b], Tienabe K. Nsiama [a], Tamaki Kato [a,*], Norikazu Nishino [a], Akihiro Ito [b], Minoru Yoshida [b]

[a] Graduate School of Life Science and Systems Engineering, Kyushu Institute of Technology, Wakamatsu, Kitakyushu 808-0196, Japan
[b] Chemical Genomics Research Group, RIKEN Center for Sustainable Resource Science, Saitama 351-0198, Japan

ARTICLE INFO

Article history:
Received 20 November 2013
Revised 5 January 2014
Accepted 6 January 2014
Available online 21 January 2014

Keywords:
Peptide-MCA substrates
4-Methylcoumaryl-7-amides
Histone methyltransferases
Histone methyltransferase inhibitors

ABSTRACT

Histone methyltransferases (HMTs) play an important role in controlling gene expression through site-specific methylation of lysines in core and linker histones within chromatin. As the typical HMTs, G9a and Set7/9 have been intensively studied that G9a is specific to the methylation at H3K9 and H3K27 and represses transcription, while Set7/9 methylates at H3K4. In this report we prepared various peptide-MCAs (4-methylcoumaryl-7-amides) related to histone tail and protein-substrates such as p53 and estrogen receptor-α. The fluorogenic substrates are applied for the assay of HMTs and an inhibitor, for example. The most sensitive and specific MCA-substrates to G9a and Set7/9 are discovered. The peptide-MCAs corresponding to the methylation sequences are promising for screening of HMT inhibitors.

1. Introduction

Chromatin, the complexes between eukaryotic DNA and proteins, distributes in the nucleus that condenses to form chromosomes during cell division. Histones are the major protein of chromatin which contains a high proportion of basic amino acids that facilitate binding to the DNA molecule. Five types of histones are known which are H1, H2A, H2B, H3, and H4 consisting of a globular carboxyl terminus and linear amino terminus that is called histone tails.[1–3] These tails are controlled by the post-translational modifications such as acetylation, methylation, phosphorylation, ubiquitination, sumoylation and so on[4,5] (Fig. 1). The functional characterization of histone modifications are believed to affect cellular processes like gene transcription or expression, apoptosis, DNA repair, and the regulation of cell cycle.[6–10]

The histone methylation of lysine side chain on histone tails has important roles in regulating chromatin dynamics and transcription. Methylation is mediated by various histone methyltransferases (HMTs). Also it is believed that these enzymes are significant to unrestricted expression that associates with diseases such as cancer.[10–13] By recent studies, the relation between methylation marks and etiological role in human disorders had been known.[14–21] As the typical HMTs, G9a (specific to H3K9 and H3K27) and Set7/9 (specific to H3K4) have been extensively studied so far.[22–26] Thus, the HMT inhibitor is expected as a therapeutic agent for diseases and to be applied to regenerative medicine using iPS cells. Accordingly, the convenient assay method of HMTs and their inhibitors is of an urgent need.

Based on the above understanding, the screening of HMT inhibitor has been extensively challenged, though the assay method is limited by the conventional methods such as radioisotope (RI) detection and ELISA.[27,28] Recently, the Immuno-Bead based assays were developed, which used AlhpaLISA technology for measuring the catalytic activity of epigenetic enzymes and their inhibitors.[29,30] However, the RI method is limited in safety due to the use of radioisotope and requiring many experimental procedures including the absorption of reaction products to filter paper. The immunoassay methods have the drawbacks such as a number of experimental procedures like washing procedure, which takes much time. Since the AlhpaLISA assay depends on the methylation antibody, the assay system is hard to work well without good antibody. Also, it is costly and it needs special device for measurements. In the present study, we attempted to solve above problems and construct a simple and sensitive evaluation system

Abbreviations: AcOH, acetic acid; Ac_2O, acetic anhydride; AcOEt, ethyl acetate; AMC, 7-amino-4-methylcoumarin; DCM, dichloromethane; DCC, dicyclohexylcarbodiimide; DIEA, *N*,*N*-diisopropylethylamine; DMF, *N*,*N*-dimethylformamide; Fmoc, fluorenylmethyloxycarbonyl; HOBt·H_2O, 1-hydroxybenzotriazole hydrate; HBTU, *O*-(benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate; MCA, 4-methylcoumaryl-7-amide; HPLC, high performance liquid chromategraphy; HRMS (FAB), high resolution fast atom bombardment mass spectroscopy; TFA, trifluoroacetic acid; TLC, thin-layer chromatography; SAM, S-adenosyl-L-methionine.

* Corresponding author. Tel./fax: +81 093 695 6063.
E-mail address: tmkato@life.kyutech.ac.jp (T. Kato).

0968-0896/$ - see front matter © 2014 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.bmc.2014.01.011

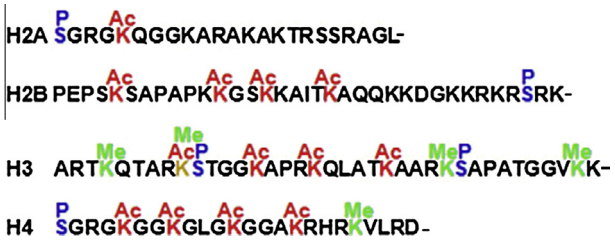

Figure 1. Post-translational modifications of histone tails. Ac: Acetylation; Me: Methylation and P: Phosphorylation.

that is different from the past. We designed and synthesized a number of peptide-4-methylcoumaryl-7-amides (peptide-MCAs) with lysine at the C-terminal as an index to measure the activities of HMTs and their inhibitors. Not only that the peptide-Lys-MCA releases fluorescent 7-amino-4-methylcoumarin (AMC) upon the action of peptidases such as trypsin and lysyl endopeptidase (LEP), but also Lys residue could be the methylation substrate of HMTs. Therefore, we attempted to utilize these actions of enzymes by combination into a system for convenient assay of HMTs and their inhibitors.

2. Results and discussion

At our earliest stage, in order to confirm the difference of methylated and unmethylated peptide-MCAs. We prepared Boc-Lys(Me)$_n$-MCA, where n = 0, 1, 2, and 3 as model compounds (preparation will be reported elsewhere). They were subjected to the action of trypsin and LEP (Fig. 2). We used excess amount of trypsin and LEP to digest for 15 min as described in Section 4.3.1. These results showed that the addition of trypsin or LEP to Boc-Lys-MCA increased the fluorescence intensity of AMC (λ_{ex} 390 nm/λ_{em} 460 nm) in a manner dependent on the concentration of Boc-Lys-MCA. In details, when trypsin was applied to 1000 μM Boc-Lys(Me)$_n$-MCA, the fluorescence intensities of AMC released from Boc-Lys(Me)-MCA, Boc-Lys(Me)$_2$-MCA, and Boc-Lys(Me)$_3$-MCA (when the fluorescence intensity from Boc-Lys-MCA was set to 100%) were only 2.7%, 0.12%, and 0.24%, respectively (Fig. 2A). When LEP was used to 1000 μM Boc-Lys(Me)$_n$-MCA, the fluorescence intensities of AMC released from Boc-Lys(Me)-MCA, Boc-Lys(Me)$_2$-MCA, and Boc-Lys(Me)$_3$-MCA (when the fluorescence intensity from Boc-Lys-MCA was set to 100%) were only 0.096%, 0.053%, and 0.25%, respectively (Fig. 2B).

Furthermore, we successfully detected the fluorescence from Boc-Lys(Me)$_n$-MCA (n = 0, 1, 2, and 3) by λ_{ex} 330 nm/λ_{em} 380 nm without interruption by fluorescence of AMC as shown in Figure 3A1 and A2. In addition, Boc-Lys(Me)$_n$-MCA and AMC also could be discriminated (Fig. 3B1 and B2) (Section 4.3.2 gives the details).

According to the facts above, though we at first focused on the decrease of fluorescence intensity of AMC (λ_{ex} 390 nm/λ_{em} 460 nm) as an index of measuring HMT activity, we found out that the increase of fluorescence intensity of methylated peptide-MCA (λ_{ex} 330 nm/λ_{em} 380 nm) can be also measured as HMT activity. Thus, we designed our assay strategy as shown in Figure 4.

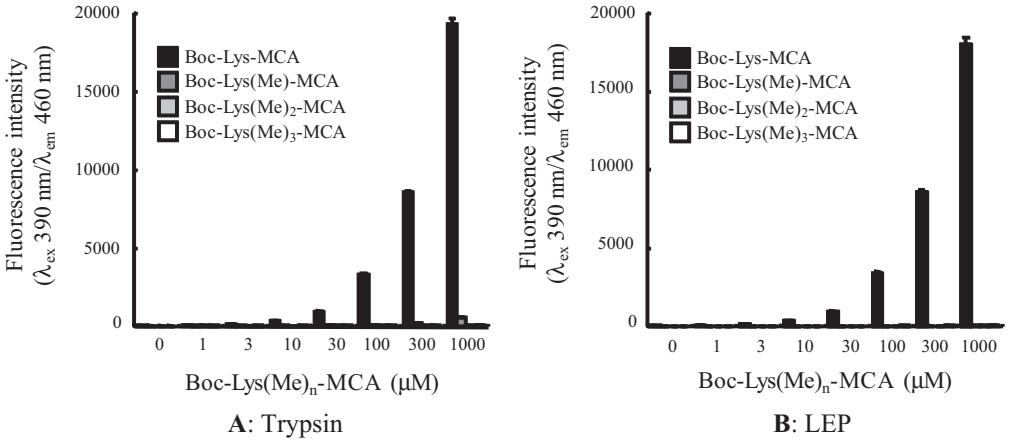

Figure 2. Susceptibilities of Boc-Lys-MCA and methylated Boc-Lys-MCA to trypsin and LEP. (A) Trypsin. (B) LEP.

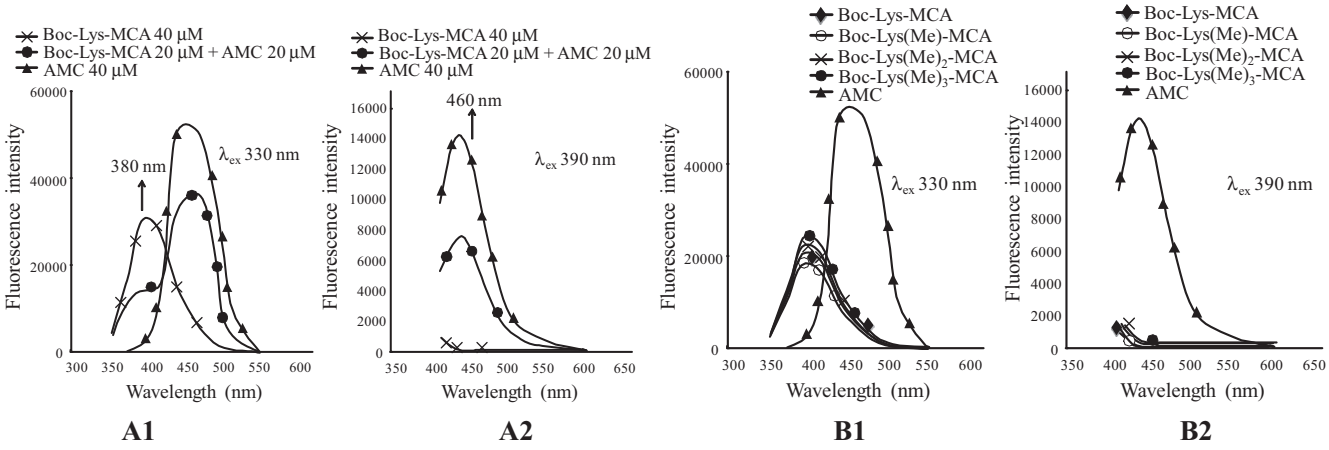

Figure 3. Fluorescence spectra of Boc-Lys(Me)$_n$-MCA (n = 0, 1, 2, 3) and AMC. (A1 and B1) exited at 330 nm. (A2 and B2) Exited at 390 nm.

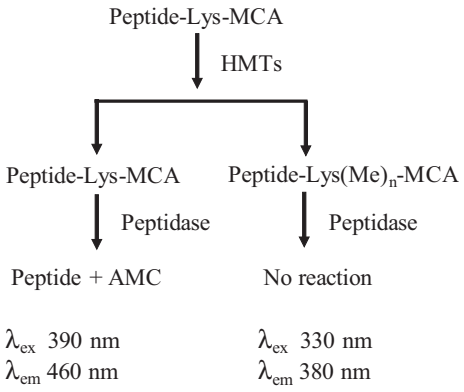

Figure 4. Strategy of HMTs assay using peptide-MCA containing Lys. Note that the fluorescence of AMC and MCA could be detected by different sets of λ_{ex} /λ_{em}.

In the next stage, we applied Boc-Lys-MCA for HMT assay using GST-mG9a and trypsin. S-Adenosyl-L-methionine (SAM)[31] was also employed (Fig. 5A). Figure 5B shows that the fluorescence intensities of AMC released by trypsin decreased in order of the GST-mG9a concentrations. Though the increasing concentration of Boc-Lys-MCA, from 0.009 to 0.09 mM, reflected fluorescence intensities, there observed was almost no difference in relative intensities according to the GST-mG9a concentrations. The concentration of SAM was examined for the effect in the methylation reaction. 1 mM concentration gave the lowest intensity, which means the highest activity of GST-mG9a (Fig. 5C).

Table 1
Peptide-MCA substrates of HMTs based on histone tail[13]

Peptide-MCA	Amino acid sequence
Ac-histone H3 (1–4)-MCA	Ac-ARTK-MCA
Ac-histone H3 (5–9)-MCA	Ac-QTARK-MCA
Ac-histone H3 (1–9)-MCA	Ac-ARTKQTARK-MCA
Ac-histone H3 (7–9/25–27)-MCA	Ac-ARK-MCA
Ac-histone H3 (23–27)-MCA	Ac-KAARK-MCA
Ac-histone H3 (19–27)-MCA	Ac-QLATKAARK-MCA

Table 2
Peptide-MCA substrates of HMTs based on methylation sequence in proteins[32–35]

Peptide-MCA	Amino acid sequence
Ac-p53 (369–372)-MCA	Ac-LKSK-MCA
Ac-p53 (367–372)-MCA	Ac-SHLKSK-MCA
Ac-ERα (299–302)-MCA	Ac-KRSK-MCA
Ac-ERα (297–302)-MCA	Ac-MIKRSK-MCA
Ac-AR (630–633)-MCA	Ac-RKLK-MCA
Ac-AR (628–633)-MCA	Ac-GARKLK-MCA
Ac-GR (491–494)-MCA	Ac-RKTK-MCA
Ac-GR (489–494)-MCA	Ac-EARKTK-MCA

ERα, estrogen receptor α; AR, androgen receptor; GR, glucocorticoid receptor.

In the third stage, we designed and synthesized a series of peptide-MCAs containing Lys at the C-terminal that were chosen from the amino acid sequences of histone tail and some methylated proteins such as p53 (Tables 1 and 2). We adopted G9a and Set7/9 as HMTs (Figs. 6 and 7). The results given in Figure 6A show that most

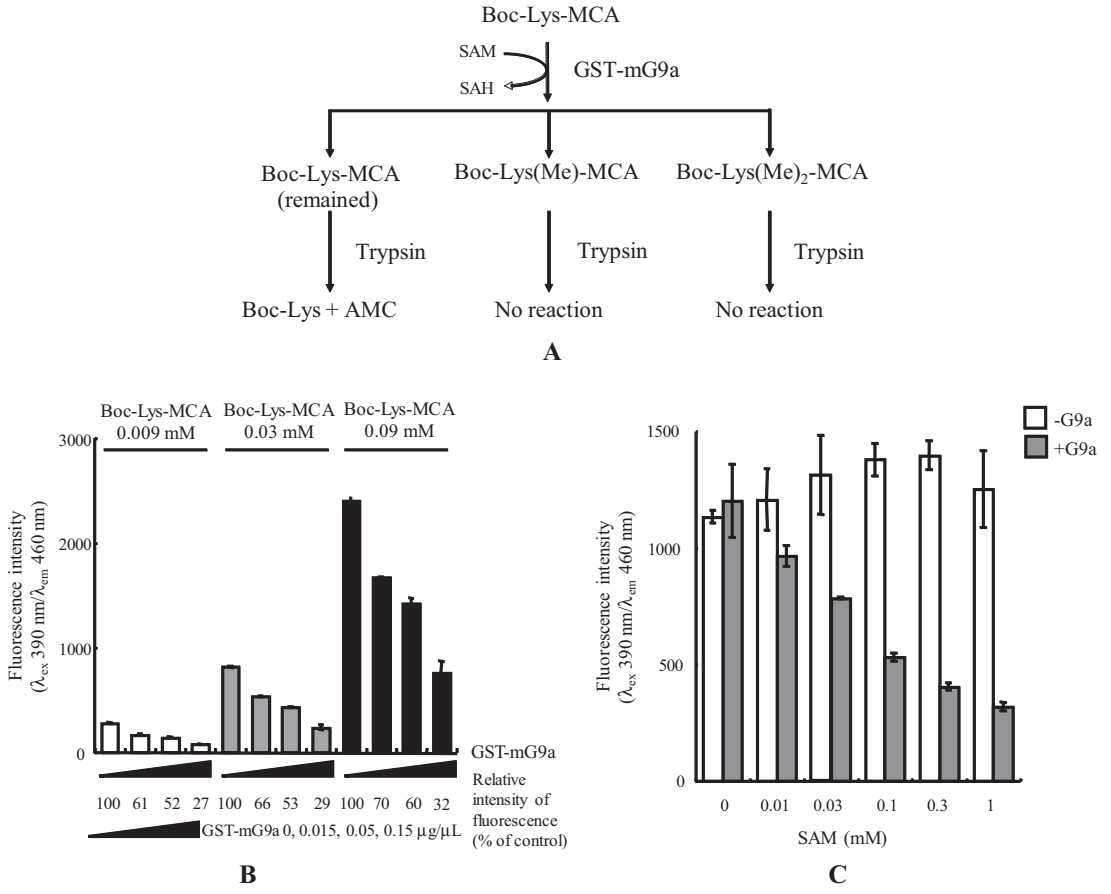

Figure 5. Measurements of G9a activity using Boc-Lys-MCA. (A) Outline of measuring G9a activity. (B) Dependences of concentrations of Boc-Lys-MCA and G9a. (C) Dependence of SAM concentration.

of peptide-MCA based on histone tail except Ac-histone H3 (1-4)-MCA are susceptible to G9a. On the other hand, Set7/9 methylates Ac-histone H3 (1–4)-MCA selectively (Fig. 6 gives data of Ac-p53 (369–372)-MCA for comparison). Figure 7 shows the results of action of G9a and Set7/9 to peptide-MCA based on sequences of methylated proteins. The G9a exhibited no methylation of

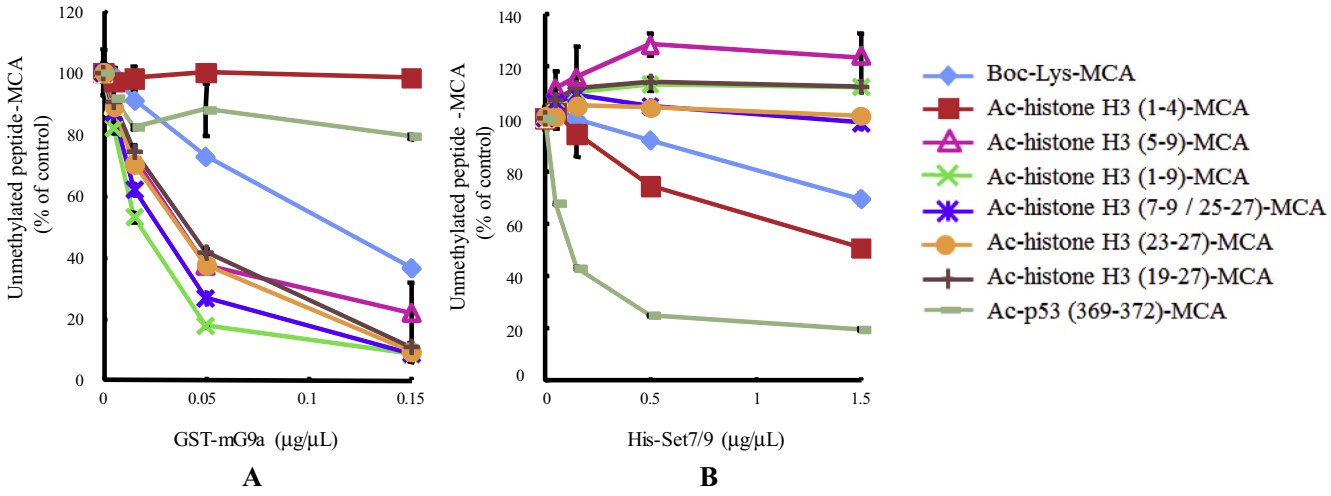

Figure 6. Susceptibilities of peptide-MCAs based on histone tail sequences to HMTs. (A) G9a. (B) Set7/9.

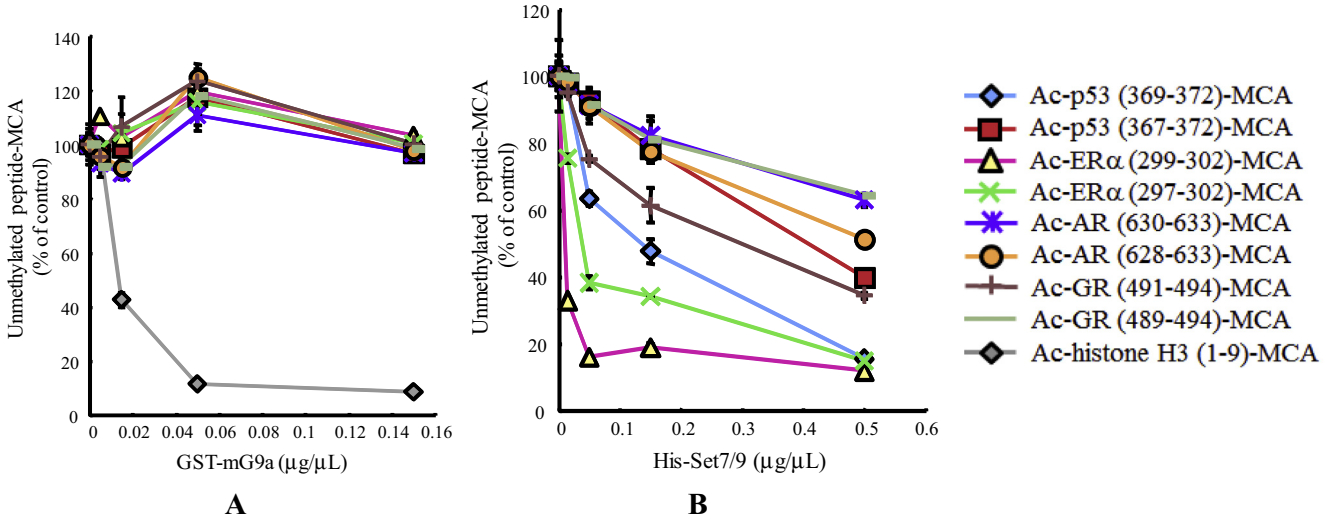

Figure 7. Susceptibilities of peptide-MCAs based on methylation sequence in proteins to HMTs. (A) G9a. (B) Set7/9.

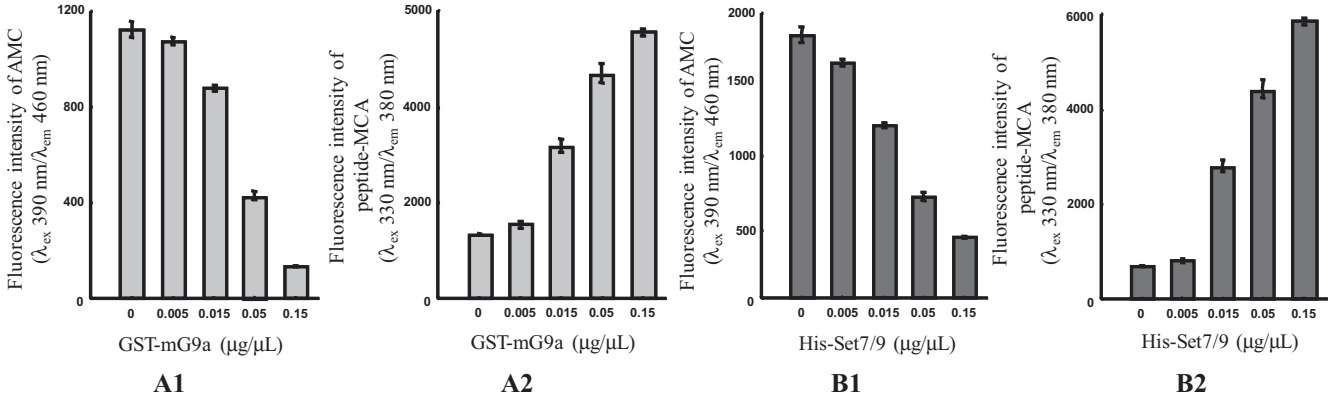

Figure 8. Measurements of HMTs activities in different sets of $\lambda_{ex}/\lambda_{em}$. (A1 and A2) G9a activity (Ac-ARTKQTARK-MCA) by measuring AMC ($\lambda_{ex}/\lambda_{em}$: 390 nm/460 nm) and methylated peptide-MCA ($\lambda_{ex}/\lambda_{em}$: 330 nm/380 nm), respectively. (B1 and B2) Set7/9 activity (Ac-KRSK-MCA) by measuring AMC ($\lambda_{ex}/\lambda_{em}$: 390 nm/460 nm) and methylated peptide-MCA ($\lambda_{ex}/\lambda_{em}$: 330 nm/380 nm), respectively.

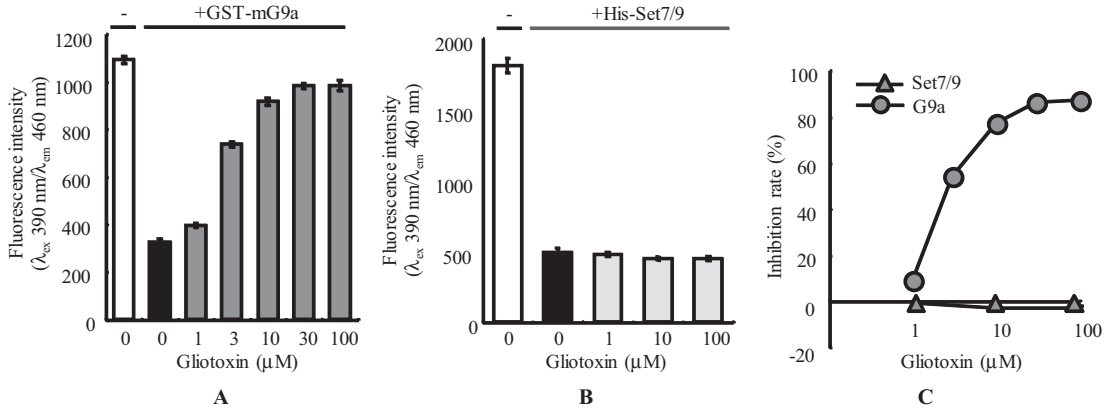

Figure 9. Evaluation of an HMT inhibitor (gliotoxin) with peptide-MCAs. (A) G9a with Ac-ARTKQTARK-MCA. (B) Set7/9 with Ac-LKSK-MCA. (C) The rates (%) of methylation inhibition by gliotoxin.

peptide-MCA based on proteins. The data of Ac-histone H3 (1–9)-MCA is shown as reference in Figure 7A. On the contrary, most of peptide-MCA from proteins are susceptible to Set7/9 (Fig. 7B). Among them, the most sensitive one is Ac-ERα (299–302)-MCA. According to the above results, the peptide-MCA derived from histone H3 (1–9) is specific for G9a and ERα (299–302) is somewhat selective for Set7/9. Thus, we could figure out the substrate specificities by using peptide-MCAs. (Section 4.3.4 gives the details.)

As the strategy described previously in Figure 4, the decrease of fluorescence intensity of AMC could be an index at λ_{ex} 390 nm/λ_{em} 460 nm, and the increase of fluorescence intensity of methylated peptide-MCA could be reference at λ_{ex} 330 nm/λ_{em} 380 nm. Actually Figure 8 shows the results of the assays of G9a and Set7/9 using peptide-MCAs (Ac-histone H3 (1–9)-MCA and Ac-ERα (299–302)-MCA) at λ_{ex} 390 nm/λ_{em} 460 nm to measure AMC, λ_{ex} 330 nm/λ_{em} 380 nm to measure peptide-MCA, respectively. (The experimental and evaluation details were not shown.) This could eliminate some misunderstandings, such as the unclearly distinguish of fluorescence signal whether HMTs works well or the fluorescence just quenched accidentally.

In summary, we confirmed the substrate specificities of HMTs regarding the amino acid sequences. We discovered that Ac-ARTKQTARK-MCA (Ac-histone H3 (1–9)-MCA), the longest peptide is the most specific and susceptible substrate of G9a. However, Set7/9 methylated moderately histone tail-related peptide-MCA, but showed higher activity to p53 and estrogen receptor α sequences; Ac-KRSK-MCA (Ac-ER α (299–302)-MCA) from ER α was the best substrate of Set7/9.

At the final stage, we carried out the evaluation of gliotoxin,[36] a known G9a inhibitor by this new assay method using Ac-ARTKQTARK-MCA and obtained IC_{50} as 2.8 μM, close to the reported value[37] (Fig. 9). When Ac-LKSK-MCA was used to examine the Set7/9 inhibition by gliotoxin, this compound showed no inhibitory activity (>100 μM) as expected to be specific to G9a.

3. Conclusion

We have revisited the action of trypsin and LEP to Boc-Lys(Me)$_n$-MCA, where $n = 0$, 1, 2, and 3, and confirmed that methylated Boc-Lys(Me)$_n$-MCA ($n > 1$) show very poor to null susceptibility to the key hydrolytic enzymes. As sensitive fluorogenic substrates of HMTs we have designed a series of peptide-MCAs whose sequences are selected from the known methylation points in histone and other proteins. The most sensitive and specific substrates to G9a (Ac-ARTKQTARK-MCA) and Set7/9 (Ac-KRSK-MCA) are discovered. The usefulness of the new assay method was demonstrated by the measurement of inhibitory activity of gliotoxin. These substrates are convenient indicators for screening of HMTs inhibitors.

4. Experimental

4.1. General methods

Unless otherwise noted, all solvents and reagents were reagent grade obtained from WAKO, Japan. Flash chromatography was performed using silica gel 60 (230–400 mesh) eluting with solvents as indicated. All compounds were routinely checked by thin layer chromatography (TLC) and/or high performance liquid chromatography (HPLC). TLC was performed on aluminum-backed silica gel plates (Merck DC-Alufolien Kieselgel 60 F_{254}) with spots visualized by UV light or charring.

Analytical HPLC was performed on a Hitachi L-7100 instrument equipped with a chromolith performance RP-18e column (4.6 × 100 mm, Merck). The mobile phases used were A: H_2O with 0.1% TFA, B: CH_3CN with 0.1% TFA using a solvent gradient of A–B over 15 min with a flow rate of 2 mL/min, with detection at 220 nm. HRMS (FAB) were measured on a JEOL JMS-SX 102A instrument.

Peptide-MCAs were synthesized by conventional solution method or automated solid phase peptide synthesis (SPPS). Some protected peptide fragments were assembled on Barlos resin (substitution 0.7 mmol/g) by peptide synthesizer (Applied Biosystems 433A) using Fmoc/piperidine strategies in FastMoc Chemistry at 0.25 mmol scale. HBTU and HOBt·H_2O were used as activating agent. After capping the N-terminal with acetic anhydride, the protected peptides were cleaved from the resin in DCM containing TFE and AcOH for 2 h. The protected peptides were condensed with H-Lys(Boc)-MCA. All protecting groups were removed by the treatment with TFA containing phenol (5%), thioanisole (5%), ethanedithiol (2.5%) and water (5%). The peptides were precipitated by *tert*-butyl methyl ether. Crude peptides were purified by size exclusion chromatography performed on a column of Sephadex G-25 with 10% AcOH. The purified peptide-MCAs were analyzed by HRMS (FAB). HPLC profiles and HRMS (FAB) data are provided in Supplementary data.

4.2. Synthesis of peptide-MCAs

4.2.1. Ac-ARTK-MCA

To a chilled solution of Fmoc-Lys(Boc)-OH (1.86 g, 4 mmol) in DCM (10 mL) was added DCC (0.42 g, 2 mmol). After 1 h on ice

bath, AMC (0.36 g, 2 mmol) was added to the mixture. The suspension was stirred at room temperature overnight, then the product was filtered and washed with methanol. Fmoc-Lys(Boc)-MCA was obtained as crystalline solid (1.13 g, 90%). Fmoc-Lys(Boc)-MCA (1.13 g, 1.8 mmol) was dissolved in 20% piperidine/DMF (5 mL) at room temperature. After 30 min the reagent and solvent were removed by evaporation. The residues were extracted into AcOEt, washed with sat. Na_2CO_3, and dried over anhydrous Na_2CO_3. The filtrate was concentrated to yield H-Lys(Boc)-MCA as free amine (0.73 g, 100%). Fmoc-Thr(^{t}Bu)-OH (119 mg, 0.3 mmol) and H-Lys(-Boc)-MCA (121 mg, 0.3 mmol) were condensed by the aid of HBTU (125 mg, 0.33 mmol), HOBt·H_2O (50 mg, 0.33 mmol) and DIEA (0.06 mL, 0.33 mmol) in DMF for 2 h. The product was extracted into AcOEt, washed with 10% citric acid, 4% $NaHCO_3$, and brine. After dried over $MgSO_4$, the AcOEt solution was filtrated and concentrated to give Fmoc-Thr(^{t}Bu)-Lys(Boc)-MCA as white solid (384 mg, 87%). The protected dipeptide-MCA was treated with 20% piperidine/DMF as described above. The free amine H-Thr (^{t}Bu)-Lys(Boc)-MCA was obtained (140 mg, 100%). Fmoc-Arg(Pmc)-OH (165 mg, 0.25 mmol) and H-Thr(^{t}Bu)-Lys(Boc)-MCA (140 mg, 0.25 mmol) were condensed by the same procedure described above. After purification with a silica gel column Fmoc-Arg(Pmc)-Thr(^{t}Bu)-Lys(Boc)-MCA was obtained as white solid (238 mg, 82%). The protected tripeptide-MCA was treated with 20% piperidine/DMF as described above. The free amine H-Arg(Pmc)-Thr(^{t}Bu)-Lys(Boc)-MCA was obtained (197 mg, 100%). Fmoc-Ala-OH (62 mg, 0.2 mmol) and H-Arg(Pmc)-Thr (^{t}Bu)-Lys(Boc)-MCA (197 mg, 0.2 mmol) were condensed by the same procedure described above. After silica gel column purification, Fmoc-Ala-Arg(Pmc)-Thr(^{t}Bu)-Lys(Boc)-MCA was obtained as white solid (168 mg, 70%). Fmoc-Ala-Arg(Pmc)-Thr(^{t}Bu)-Lys(Boc)-MCA (84 mg, 0.07 mmol) was treated with 20% piperidine/DMF as described above. The free amine H-Ala-Arg(Pmc)-Thr(^{t}Bu)-Lys(-Boc)-MCA was obtained (75 mg, 100%), which was reacted with Ac_2O (0.08 mmol) and NEt_3 (0.08 mmol) in DMF for 2 h. In the work-up step, the product was crystallized in AcOEt. Finally the deprotection of Ac-Ala-Arg(Pmc)-Thr(^{t}Bu)-Lys(Boc)-MCA (76 mg) was carried out with TFA reagent containing phenol, thioanisole, ethanedithiol, and water as described before for 2 h. Concentration and addition of *tert*-butyl methyl ether (20 mL) gave white precipitate, which was collected by centrifugation and washed with same solvent. The crude material was passed through a column of Sephadex G-25 with 10% AcOH. Lyophilization gave 42 mg of Ac-ARTK-MCA_$(AcOH)_2$. HRMS (FAB) *m*/*z* found: 674.3607, calcd for $[M+H]^+$ $C_{31}H_{48}N_9O_8$: 674.3626.

4.2.2. Ac-QTARK-MCA

This compound was synthesized starting from Fmoc-Lys(Boc)-MCA by solution method as described above. Deprotection of Ac-Gln(Trt)-Thr(^{t}Bu)-Ala-Arg(Pmc)-Lys(Boc)-MCA (57 mg), purification, and lyophilization gave 26 mg of Ac-QTARK-MCA_$(AcOH)_2$. HRMS (FAB) *m*/*z* found: 802.4196, calcd for $[M+H]^+$ $C_{36}H_{56}N_{11}O_{10}$: 802.4212.

4.2.3. Ac-ARTKQTARK-MCA

The protected peptide Ac-Ala-Arg(Pmc)-Thr(^{t}Bu)-Lys(Boc)-Gln(Trt)-Thr(^{t}Bu)-Ala-Arg(Pmc)-OH was prepared by assembling on Barlos resin by peptide synthesizer, acetylation at the N-terminal with Ac_2O, and subsequent cleavage from the resin. This fragment (428 mg, 0.22 mmol) and H-Lys(Boc)-MCA (88 mg, 0.22 mmol) were condensed by the aid of HBTU (90 mg, 0.24 mmol), HOBt·H_2O (36 mg, 0.24 mmol) and DIEA (0.04 mL, 0.24 mmol) in DMF for 2 h. The product was extracted into AcOEt. The work-up afforded Ac-Ala-Arg(Pmc)-Thr(^{t}Bu)-Lys(Boc)-Gln(Trt)-Thr(^{t}Bu)-Ala-Arg(Pmc)-Lys(Boc)-MCA as white solid (470 mg, 90%). Deprotection of this final precursor (50 mg) and purification by Sephadex G-25 column with 10% AcOH, and lyophilization gave 20 mg of Ac-ARTKQTARK-MCA_$(AcOH)_4$. HRMS (FAB) *m*/*z* found: 1258.6858, calcd for $[M+H]^+$ $C_{55}H_{92}N_{19}O_{15}$: 1258.7020.

4.2.4. Ac-ARK-MCA

This compound was synthesized starting from Fmoc-Lys (Boc)-MCA by solution method as described above. Deprotection of Ac-Ala-Arg(Pmc)-Lys(Boc)-MCA (66 mg), purification, and lyophilization gave 32 mg of Ac-ARK-MCA_$(AcOH)_2$. HRMS (FAB) *m*/*z* found: 573.3113, calcd for $[M+H]^+$ $C_{27}H_{41}N_8O_6$: 573.3149.

4.2.5. Ac-KAARK-MCA

The protected peptide Ac-Lys(Boc)-Ala-Ala-Arg(Pmc)-OH was prepared by assembling on Barlos resin, capping with Ac_2O, and cleavage as described above. This fragment was condensed with H-Lys(Boc)-MCA to obtain Ac-Lys(Boc)-Ala-Ala-Arg(Pmc)-Lys (Boc)-MCA. Deprotection of fully protected peptide (60 mg), purification, and lyophilization gave 15 mg of Ac-KAARK-MCA_$(AcOH)_3$. HRMS (FAB) *m*/*z* found: 772.4436, calcd for $[M+H]^+$ $C_{36}H_{58}N_{11}O_8$: 772.7740.

4.2.6. Ac-QLATKAARK-MCA

The protected peptide Ac-Gln(Trt)-Leu-Ala-Thr(^{t}Bu)-Lys(Boc)-Ala-Ala-Arg(Pmc)-OH was prepared by assembling on Barlos resin, capping with Ac_2O, and cleavage as described above. This fragment was condensed with H-Lys(Boc)-MCA to obtain Ac-Gln(Trt)-Leu-Ala-Thr(^{t}Bu)-Lys(Boc)-Ala-Ala-Arg(Pmc)-Lys(Boc)-MCA. Deprotection of fully protected peptide (136 mg), purification, and lyophilization gave 28 mg of Ac-QLATKAARK-MCA_$(AcOH)_3$. HRMS (FAB) *m*/*z* found: 1185.6716, calcd for $[M+H]^+$ $C_{54}H_{89}N_{16}O_{14}$: 1185.6744.

4.2.7. Ac-LKSK-MCA

This compound was synthesized starting from Fmoc-Lys(Boc)-MCA by solution method as described above. Deprotection of Ac-Leu-Lys(Boc)-Ser(^{t}Bu)-Lys(Boc)-MCA (87 mg), purification, and lyophilization gave 25 mg of Ac-LKSK-MCA_$(AcOH)_2$. HRMS (FAB) *m*/*z* found: 674.3658, calcd for $[M+H]^+$ $C_{31}H_{48}N_9O_8$: 674.3626.

4.2.8. Ac-SHLKSK-MCA

The protected peptide Ac-Ser(^{t}Bu)-His-Leu-Lys(Boc)-Ser(^{t}Bu)-OH was prepared by assembling on Barlos resin, capping with Ac_2O, and cleavage as described above. This fragment was condensed with H-Lys(Boc)-MCA to obtain Ac-Ser(^{t}Bu)-His-Leu-Lys(Boc)-Ser(^{t}Bu)-Lys(Boc)-MCA. Deprotection of fully protected peptide (120 mg), purification, and lyophilization gave 32 mg of Ac-SHLKSK-MCA_$(AcOH)_2$. HRMS (FAB) *m*/*z* found: 898.4748, calcd for $[M+H]^+$ $C_{42}H_{64}N_{11}O_{11}$: 898.4709.

4.2.9. Ac-KRSK-MCA

This compound was synthesized starting from Fmoc-Lys(Boc)-MCA by solution method as described above. Deprotection of Ac-Lys(Boc)-Arg(Pmc)-Ser(^{t}Bu)-Lys(Boc)-MCA (930 mg), purification, and lyophilization gave 500 mg of Ac-KRSK-MCA_$(AcOH)_3$. HRMS (FAB) *m*/*z* found: 717.4039, calcd for $[M+H]^+$ $C_{33}H_{53}N_{10}O_8$: 717.3770.

4.2.10. Ac-MIKRSK-MCA

The protected peptide Ac-Met-Ile-Lys(Boc)-Arg(Pmc)-Ser(^{t}Bu)-OH was prepared by assembling on Barlos resin, capping with Ac_2O, and cleavage as described above. This fragment was condensed with H-Lys(Boc)-MCA to obtain Ac-Met-Ile-Lys(Boc)-Arg(Pmc)-Ser(^{t}Bu)-Lys(Boc)-MCA. Deprotection of fully protected peptide (97 mg), purification, and lyophilization gave 28 mg of Ac-MIKRSK-MCA_$(AcOH)_3$. HRMS (FAB) *m*/*z* found: 961.5269, calcd for $[M+H]^+$ $C_{44}H_{73}N_{12}O_{10}S$: 961.5215.

4.2.11. Ac-RKLK-MCA

This compound was synthesized starting from Fmoc-Lys(Boc)-MCA by solution method as described above. Deprotection of Ac-Arg(Pmc)-Lys(Boc)-Leu-Lys(Boc)-MCA (80 mg), purification, and lyophilization gave 30 mg of Ac-RKLK-MCA_$(AcOH)_3$. HRMS (FAB) m/z found: 744.4380, calcd for $[M+H]^+$ $C_{36}H_{58}N_9O_8$: 744.4408.

4.2.12. Ac-GARKLK-MCA

The protected peptide Ac-Gly-Ala-Arg(Pmc)-Lys(Boc)-Leu-OH was prepared by assembling on Barlos resin, capping with Ac_2O, and cleavage as described above. This fragment was condensed with H-Lys(Boc)-MCA to obtain Ac-Gly-Ala-Arg(Pmc)-Lys(Boc)-Leu-Lys(Boc)-MCA. Deprotection of fully protected peptide (107 mg), purification, and lyophilization gave 42 mg of Ac-GARKLK-MCA_$(AcOH)_3$. HRMS (FAB) m/z found: 871.5152, calcd for $[M+H]^+$ $C_{41}H_{67}N_{12}O_9$: 871.5076.

4.2.13. Ac-RKTK-MCA

The protected peptide Ac-Arg(Pmc)-Lys(Boc)-Thr(^{t}Bu)-OH was prepared by assembling on Barlos resin, capping with Ac_2O, and cleavage as described above. This fragment was condensed with H-Lys(Boc)-MCA to obtain Ac-Arg(Pmc)-Lys(Boc)-Thr(^{t}Bu)-Lys(Boc)-MCA. Deprotection of fully protected peptide (100 mg), purification, and lyophilization gave 44 mg of Ac-RKTK-MCA_$(AcOH)_3$. HRMS (FAB) m/z found: 731.4197, calcd. for $[M+H]^+$ $C_{34}H_{55}N_{10}O_8$: 731.4126.

4.2.14. Ac-EARKTK-MCA

The protected peptide Ac-Glu(O^{t}Bu)-Ala-Arg(Pmc)-Lys(Boc)-Thr(Trt)-OH was prepared by assembling on Barlos resin, capping with Ac_2O, and cleavage as described above. This fragment was condensed with H-Lys(Boc)-MCA to obtain Ac-Glu(O^{t}Bu)-Ala-Arg(Pmc)-Lys(Boc)-Thr(Trt)-Lys(Boc)-MCA. Deprotection of fully protected peptide (98 mg), purification, and lyophilization gave 37 mg of Ac-EARKTK-MCA_$(AcOH)_3$. HRMS (FAB) m/z found: 931.5050, calcd for $[M+H]^+$ $C_{42}H_{67}N_{12}O_{12}$: 931.4923.

4.3. Assays of enzymes

4.3.1. Loss of susceptibility to peptidases by methylation of Boc-Lys-MCA

1 μL of a Boc-Lys-MCA solution was mixed with 10 μL of 2 × HMT buffer (100 mM Tris–HCl (pH 8.5), 20 mM $MgCl_2$, 40 mM KCl, 20 mM 2-mercaptoethanol, 500 mM sucrose), and distilled water was added to adjust the total volume to 20 μL. To the resultant solution was added 30 μL of a peptidase solution (20 mg/mL trypsin or 20 mAU/mL LEP), which was then mixed and incubated at 37 °C for 15 min. Then, the fluorescence intensity of the solution was measured at λ_{ex} 390 nm/λ_{em} 460 nm with a SpectraMax M2^e microplate reader (Molecular Devices).

4.3.2. Fluorescence spectra of Boc-Lys-MCA and AMC

2 μL of Boc-Lys-MCA (2 mM) and 48 μL of distilled water were mixed to prepare 50 μL of a solution (Boc-Lys-MCA 40 μM). 1 μL of Boc-Lys-MCA (2 mM), 1 μL of AMC (2 mM), and 48 μL of distilled water were also mixed to prepare 50 μL of a solution (Boc-Lys-MCA 20 μM + AMC 20 μM). 2 μL of AMC (2 mM) and 48 μL of distilled water were mixed to prepare 50 μL of a solution (AMC 40 μM). 1 μL of Boc-Lys(Me)-MCA (2 mM) and 49 μL of distilled water were mixed to prepare a mixed solution. Using Boc-Lys(Me)$_2$-MCA, Boc-Lys(Me)$_3$-MCA, or AMC in place of Boc-Lys-MCA, a mixed solution was similarly prepared. The fluorescence spectrum of these solutions was measured with a SpectraMax M2^e microplate reader.

4.3.3. Measurement of G9a activity with Boc-Lys-MCA

GST-fused mG9a (706-stop a.a.) and $(His)_6$-fused Set7/9 were obtained as previously described.[37] 10 μL of 2 × HMT buffer, 2 μL of a BSA solution (30 μg/mL), and a predetermined concentration (final concentration: 0, 0.015, 0.05, or 0.15 μg/μL) of the G9a solution (total volume to 16 μL) to adjust the total volume to 16 μL. Then the resultant solution was incubated at room temperature for 1 h. Thereafter, 2 μL of SAM (10 mM) and 2 μL of Boc-Lys-MCA (concentration: 0.009 mM, 0.03 mM, or 0.09 mM) were added thereto, which was mixed and then incubated at 37 °C for 15 min. Subsequently, 30 μL of a trypsin solution (20 mg/mL) was added thereto, followed by incubation at 37 °C for 15 min. The fluorescence intensity of the solution was measured at λ_{ex} 390 nm/λ_{em} 460 nm.

4.3.4. Substrate specificity of HMTs

Susceptibilities of peptide-MCAs to G9a were examined in the following conditions. 10 μL of 2 × HMT buffer, 0.4 μL of a BSA solution (150 μg/mL), 4.5 μL of GST-mG9a (0, 0.022–0.66 μg/μL), and 1.1 μL of distilled water were mixed and then incubated at room temperature for 1 h. Then, 2 μL of SAM (10 mM) and 2 μL of a peptide-MCA solution (0.6 mM) were added thereto, which was then incubated at 37 °C for 15 min. Thereafter, 30 μL of a trypsin solution (20 mg/mL) was added thereto, followed by incubation at 37 °C for 15 min. Then, the fluorescence intensity of the solution was measured at λ_{ex} 390 nm/λ_{em} 460 nm. The final concentration of GST-mG9a during the measurement of fluorescence intensity was 0, 0.005, 0.015, 0.05, or 0.15 μg/μL.

Susceptibilities of peptide-MCAs to Set7/9 were examined in the following conditions. 10 μL of 2 × HMT buffer, 2 μL of a BSA solution (30 μg/mL), 3 μL of His-Set7/9 (0, 0.33–10 μg/μL), and 1 μL of distilled water were mixed and then incubated at room temperature for 1 h. Then, 2 μL of SAM (10 mM) and 2 μL of a peptide-MCA solution (0.6 mM) were added thereto, which was then incubated at 37 °C for 15 min. Thereafter, 30 μL of a trypsin solution (20 mg/mL) was added thereto, followed by incubation at 37 °C for 15 min. Then, the fluorescence intensity of the solution was measured at λ_{ex} 390 nm/λ_{em} 460 nm. The final concentration of His-Set7/9 during the measurement of fluorescence intensity was 0, 0.005, 0.015, 0.05, or 0.15 μg/μL.

4.3.5. Inhibition of HMTs by gliotoxin

10 μL of 2 × HMT buffer, 0.4 μL of a BSA solution (150 μg/mL), 2 μL of GST-mG9a (0, 0.5 μg/μL) or 4.5 μL of His-Set7/9 (0, 2.25 μg/μL), 1 μL of gliotoxin (any various concentrations) and 2.6 μL of distilled water were mixed and then incubated at room temperature for 1 h. Then, 2 μL of SAM (10 mM) and 2 μL of Ac-ARTKQTARK-MCA solution (0.6 mM) for G9a or Ac-LKSK-MCA solution (0.6 mM) for Set7/9 were added thereto, which was then incubated at 37 °C for 1 h. Thereafter, 30 μL of a trypsin solution (20 mg/mL) was added thereto, followed by incubation at 37 °C for 15 min. Then, the fluorescence intensity of the solution was measured at λ_{ex} 390 nm/λ_{em} 460 nm. The final concentrations of GST-mG9a and His-Set7/9 during the measurement of fluorescence intensity were 0.05 and 0.5 μg/μL, respectively.

Acknowledgments

This work was supported in part by the Chemical Genomics Research Project, RIKEN Center for Sustainable Resource Science, the CREST Research Project, the Japan Science and Technology Corporation, and by Grants-in-Aid for Innovative Area 'Cancer' from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

Supplementary data

Supplementary data (HPLC profiles and HRMS (FAB) data of peptide-MCAs) associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmc.2014.01.011.

References and notes

1. Kornberg, R. D. *Science* **1974**, *184*, 868.
2. Luger, K.; Mader, A. W.; Richmond, R. K.; Sargent, D. F.; Richmond, T. J. *Nature* **1997**, *389*, 251.
3. Lachner, M.; Jenuwein, T. *Curr. Opin. Cell Biol.* **2002**, *14*, 286.
4. Schäfer, S.; Jung, M. *Arch. Pharm. Chem. Life Sci.* **2005**, *338*, 347.
5. Berger, S. L. *Nature* **2007**, *447*, 407.
6. Villeneuve, L. M.; Natarajan, R. *Contrib. Nephrol.* **2011**, *170*, 57.
7. Villeneuve, L. M.; Natarajan, R. *Am. J. Physiol. Renal Physiol.* **2010**, *299*, F14.
8. Kumar, A.; Choi, K. H.; Renthal, W.; Tsankova, N. M.; Theobald, D. E.; Truong, H. T.; Russo, S. J.; Laplant, Q.; Sasaki, T. S.; Whistler, K. N.; Neve, R. L.; Self, D. W.; Nestler, E. J. *Neuron* **2005**, *48*, 303.
9. Bayarsaihan, D. *J. Dent. Res.* **2011**, *90*, 9.
10. Maze, I.; Covington, H. E.; Dietz, D. M.; LaPlant, Q.; Renthal, W.; Russo, S. J.; Mechanic, M.; Mouzon, E.; Neve, R. L.; Haggarty, S. J.; Ren, Y.; Sampath, S. C.; Hurd, Y. L.; Greengard, P.; Tarakhovsky, A.; Schaefer, A.; Nestler, E. J. *Science* **2010**, *327*, 213.
11. Martin, C.; Zhang, Y. *Nat. Rev. Mol. Cell Biol.* **2005**, *6*, 838.
12. Rando, O. J.; Chang, H. Y. *Annu. Rev. Biochem.* **2009**, *78*, 245.
13. Spannhoff, A.; Hauser, A. T.; Heinke, R.; Sippl, W.; Jung, M. *ChemMedChem* **2009**, *4*, 1568.
14. Magerl, C.; Ellinger, J.; Braunschweig, T.; Kremmer, E.; Koch, L. K.; Höller, T.; Büttner, R.; Lüscher, B.; Gütgemann, I. *Hum. Pathol.* **2010**, *41*, 181.
15. Chi, P.; Allis, C. D.; Wang, G. G. *Nat. Rev. Cancer* **2010**, *10*, 457.
16. Barlési, F.; Giaccone, G.; Gallegos-Ruiz, M. I.; Loundou, A.; Span, S. W.; Lefesvre, P.; Kruyt, F. A.; Rodrigues, J. A. *J. Clin. Oncol.* **2007**, *25*, 4358.
17. Ruthenburg, A. J.; Li, H.; Patel, D. J.; Allis, C. D. *Nat. Rev. Mol. Cell Biol.* **2007**, *8*, 983.
18. Manuyakorn, A.; Paulus, R.; Farrell, J.; Dawson, N. A.; Tze, S.; Cheung-Lau, G.; Hines, O. J.; Reber, H.; Seligson, D. B.; Horvath, S.; Kurdistani, S. K.; Guha, C.; Dawson, D. W. *J. Clin. Oncol.* **2010**, *28*, 1358.
19. Seligson, D. B.; Horvath, S.; McBrian, M. A.; Mah, V.; Yu, H.; Tze, S.; Wang, Q.; Chia, D.; Goodglick, L.; Kurdistani, S. K. *Am. J. Pathol.* **2009**, *174*, 1619.
20. Seligson, D. B.; Horvath, S.; Shi, T.; Yu, H.; Tze, S.; Grunstein, M.; Kurdistani, S. K. *Nature* **2005**, *435*, 1262.
21. Fraga, M. F.; Ballestar, E.; Villar-Garea, A.; Boix-Chornet, M.; Espada, J.; Schotta, G.; Bonaldi, T.; Haydon, C.; Ropero, S.; Petrie, K.; Iyer, N. G.; Pérez-Rosado, A.; Calvo, E.; Lopez, J. A.; Cano, A.; Calasanz, M. J.; Colomer, D.; Piris, M. A.; Ahn, N.; Imhof, A.; Caldas, C.; Jenuwein, T.; Esteller, M. *Nat. Genet.* **2005**, *37*, 391.
22. Lee, S. H.; Kim, J.; Kim, W. H.; Lee, Y. M. *Oncogene* **2009**, *28*, 184.
23. Wang, H.; Cao, R.; Xia, L. *Mol. Cell* **2001**, *8*, 1207.
24. Zegerman, P.; Canas, B.; Pappin, D.; Kouzarides, T. *J. Biol. Chem.* **2002**, *277*, 11621.
25. Kondo, Y.; Shen, L.; Suzuki, S.; Kurokawa, T.; Masuko, K.; Tanaka, Y.; Kato, H.; Mizuno, Y.; Yokoe, M.; Sugauchi, F.; Hirashima, N.; Orito, E.; Osada, H.; Ueda, R.; Guo, Y.; Chen, X.; Issa, J. P.; Sekido, Y. *Hepatol. Res.* **2007**, *37*, 974.
26. Nishioka, K.; Chuikov, S.; Sarma, K.; Erdjument-Bromage, H.; Allis, C. D.; Tempst, P.; Reinberg, D. *Genes Dev.* **2002**, *16*, 479.
27. Kubicek, S.; O'Sullivan, R. J.; August, E. M.; Hickey, E. R.; Zhang, Q.; Teodoro, M. L.; Rea, S.; Mechtler, K.; Kowalski, J. A.; Homon, C. A.; Kelly, T. A.; Jenuwein, T. *Mol. Cell* **2007**, *25*, 473.
28. Dhayalan, A.; Dimitrova, E.; Rathert, P.; Jeltsch, A. *J. Biomol. Screen.* **2009**, *14*, 1129.
29. Simard, J. R.; Plant, M.; Emkey, R.; Yu, V. *Assay Drug Dev. Technol.* **2013**, *11*, 152.
30. Gauthier, N.; Caron, M.; Pedro, L.; Arcand, M.; Blouin, J.; Labonté, A.; Normand, C.; Paquet, V.; Rodenbrock, A.; Roy, M.; Rouleau, N.; Beaudet, L.; Padrós, J.; Rodriguez-Suarez, R. *J. Biomol. Screen.* **2012**, *17*, 49.
31. Smith, B. C.; Denu, J. M. *Biochem. Biophys. Acta* **2009**, *1789*, 45.
32. Dahlman-Wright, K.; Cavailles, V.; Fuqua, S. A.; Jordan, V. C.; Katzenellenbogen, J. A.; Korach, K. S.; Maggi, A.; Muramatsu, M.; Parker, M. G.; Gustafsson, J. A. *Pharmacol. Rev.* **2006**, *58*, 773.
33. Lu, N. Z.; Wardell, S. E.; Bernstein, K. L.; Defranco, D.; Fuller, P. J.; Giguere, V.; Hochberg, R. B.; McKay, L.; Renoir, J. M.; Weigel, N. L.; Wilson, E. M.; McDonnell, D. P.; Cidlowski, J. A. *Pharmacol. Rev.* **2006**, *58*, 782.
34. Roy, A. K.; Lavrovsky, Y.; Song, C. S.; Chen, S.; Jung, M. H.; Velu, N. K.; Bi, B. Y.; Chatterjee, B. *Vitam. Horm.* **1999**, *55*, 309.
35. Pratt, W. B.; Morishima, Y.; Murphy, M.; Harrell, M. *Handb. Exp. Pharmacol.* **2006**, *117*, 111.
36. Scharf, D. H.; Heinekamp, T.; Remme, N.; Hortschansky, P.; Brakhage, A. A.; Hertweck, C. *Appl. Microbiol. Biotechnol.* **2012**, *93*, 467.
37. Takahashi, M.; Takemoto, Y.; Shimazu, T.; Kawasaki, H.; Tachibana, M.; Shinkai, Y.; Takagi, M.; Shin-ya, K.; Igarashi, Y.; Ito, A.; Yoshida, M. *J. Antibiot.* **2012**, *65*, 263.